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Vitamin D and Skeletal Muscle: Novel Effects on Morphology and Morphogenesis

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This thesis was submitted as part of the requirement for admission to the degree of Doctor of Philosophy in the Faculty of Medicine, the University of Sydney, NSW, Australia.

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List of Chapters and Sections

This thesis consists of a series of published articles.

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Dedication

I dedicate this work to my family:

my wife Christina,

my daughters Madeleine and Hannah,

my parents Maged and Suzan.
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Firstly, I would like to acknowledge the University of Sydney for supporting me with an Australian Postgraduate Award (2011-2014), the Endocrine Society of Australia for awarding me the Ipsen Travel Grant (2013) and the Royal Australian College of Physicians for awarding me the Joseph Thornton Tweddle Research Scholarship (2014). By this generous support, I was able to conduct this PhD and travel twice to the Salk Institute where I was immersed in the fascinating world of nuclear receptors.

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Declaration

The studies presented in this thesis are the results of original studies carried out while the author was enrolled as a full-time candidate for the degree of Doctor of Philosophy in the Faculty of Medicine, University of Sydney. Any contribution made to the research by others, whom the author worked with at University of Sydney or elsewhere, is explicitly acknowledged in the thesis.

These studies were conducted at the Garvan Institute of Medical Research between February 2011 and October 2014. Ethics approval for all studies was granted by the Garvan Animal Ethics Committee.

The work presented in this thesis has not been submitted for a degree or diploma in any other university. The work fulfills criteria for thesis by publication as set out by the University of Sydney (Chapter 4, clause 4.22 of the University of Sydney Higher Degree by Research Rule 2011).
Abstract

In prehistoric times, the sun was revered as a source of physical strength and vitality. Following the discovery of vitamin D in the 1920’s and the role of UV-light in its production, research has focused predominantly on its effects on bone and mineral homeostasis. However, emerging evidence supports broader effects of vitamin D in modulating the cell cycle, tissue development and the response to injury. At a tissue level, skeletal muscle underpins the emerging concept that vitamin D exerts functions beyond bone. This is unsurprising as muscle and bone arise together from embryonic mesoderm and interact via complex biomechanical and humoural factors during post-natal life. In the clinical setting, vitamin D deficiency leads to concurrent defects in bone and muscle. In addition to an increased risk of fracture, patients with vitamin D deficiency report muscle weakness, myalgia and have a greater risk of falls. Precise, bio-molecular effects of vitamin D on muscle are unclear and hampered by contradictory reports on the presence of the vitamin D receptor (VDR) in this tissue. This thesis sought to address this question by examining four different models of murine muscle and by employing a range of established and novel techniques. VDR was clearly detected in skeletal muscle in vivo but this depended on specific experimental conditions. In vitro, VDR activation led to an intriguing anabolic effect with doubling in the diameter of C2C12 myotubes and an anti-proliferative effect in C2C12 myocytes. Myogenic precursor cells, isolated using a novel technique, also expressed VDR and functional CYP27B1 on the basis of luciferase reporter studies. In vivo, mice lacking VDR displayed significantly lighter muscles, smaller muscle fibres and increased nuclei to fibre ratio compared to wild-type (WT) mice. These data, together with higher VDR levels found in muscle of
young mice, support a predominantly developmental and anabolic role for VDR in muscle. In this thesis, vitamin D effects were not confined to development. Adult VDR knockout (VDRKO) mice and mice with diet-induced vitamin D deficiency displayed reduced grip strength which progressed with age. They also displayed activation of atrophy genes, suggesting links between vitamin D and muscle ageing. In the absence of VDR, muscle fibres displayed reduced uptake and storage of $^{3}$H-25(OH)D$_{3}$. In summary, this thesis reports the presence of VDR in murine muscle and diverse effects of vitamin D/VDR signals on muscle development, anabolism, strength and 25OHD uptake. These findings in muscle are consistent with emerging roles of VDR at other sites in tissue pleiotropy, cardiac regeneration and response to tissue fibrosis. To confirm these direct effects and settle the controversy of VDR’s expression in skeletal muscle, the generation of muscle-specific VDR knockout mice is an essential, next step. The use of chromatin immunoprecipitation (ChIP) assays to define the VDR cistrome in muscle cells will further clarify potential interactions of VDR with TGF-B and genomic effects at this site. On the basis of findings presented in this thesis, the vitamin D pathway holds promise in the treatment of muscle disorders and in enhancing muscle repair.
Chapter 1 – Introduction, Hypothesis and Aims

References for this chapter are listed at the end of the Chapter 10. Abbreviations in Chapters 1 and 10 are defined in the text in order of appearance.

Introduction

The association between vitamin D deficiency and muscle disease is long-standing. More than three hundred years ago, the English physician Daniel Whistler reported the combination of “flabby, toneless muscles” and “flexible, waxy bones” in young children with nutritional rickets (1). More recently, adults with vitamin D deficiency have been reported to display muscle wasting and weakness in association with defects in bone mineralisation (2, 3). Whilst vitamin D’s effects on bone are well characterised, its effects on muscle remain elusive and subject to debate. This takes place within the broader controversy of whether vitamin D exerts extra-skeletal effects at all (4, 5). Although the vitamin D receptor (VDR) is expressed in a range of tissues not involved in bone/mineral homeostasis, its precise role at these sites is not fully understood. Observations made in the laboratory – particularly relating to its anti-cancer and immune-modulatory effects – have not always translated into robust clinical findings (5).

In its comprehensive report, the Institute of Medicine concluded that clinical evidence for an extra-skeletal role of vitamin D was “not yet compelling” (6). In the controversy that ensued, meta-analyses with different perspectives on the clinical data and conflicting guidelines on the optimal daily intakes of vitamin D and serum targets were published (7-10). This debate shows no sign of abating and highlights the need for greater understanding of vitamin D biology, serving as a basis upon which its purported, non-classical roles may be pursued in the clinic.
Beyond bone, there is biologic plausibility for a role of vitamin D in skeletal muscle. Muscle and bone share their ancestral origin in the common mesenchymal stem cell and arise together from embryonic mesoderm. Following the formation of bone by endochondral or intramembranous ossification, skeletal modeling and remodeling relies continually on mechanical signals from muscle (11). Myogenesis relies on its master regulator Pax3 that drives differentiation of mesenchymal precursor cells into myoblasts, which subsequently fuse to form multinucleated syncytia (12).

In post-natal life, bone and muscle function in close proximity and are regulated by overlapping genes, growth factors and mechanical cues (13). Muscle is dependent upon calcium for contraction, insulin sensitivity, and cellular plasticity (2). Since vitamin D is essential for calcium homeostasis, this at least indirectly links vitamin D activity to muscle function. Therefore, for these reasons, muscle stands at the frontier of the emerging, debated concept of vitamin D’s extra-skeletal functions.

The aim of this thesis was to elucidate mechanisms by which vitamin D affects skeletal muscle, in particular how it may regulate muscle development, morphology and gene expression. One particular difficulty is that changes in serum calcium and phosphate levels – under the systemic control of vitamin D – independently lead to muscle changes (2, 14). This has been a major confounding factor in human and animal studies assessing muscle effects of vitamin D (2, 14, 15). In this thesis, cell and mouse models were employed in which serum calcium and phosphate levels were controlled thereby avoiding confounding observed effects of vitamin D on muscle. Parameters with direct relevance to a clinical setting – strength, muscle fibre size and
atrophy – were examined in response to changes in vitamin D, thereby adopting a translational approach to this important question.

In the published literature review, a number of unifying themes and controversies arising from human clinical, animal and cell studies on vitamin D and muscle were presented (The Roles of Vitamin D in Skeletal Muscle: Form, Function, and Metabolism, Endocrine Reviews 2013; 34(1):33–83) (2). This review – cited > 80 times since publication – outlined the central controversy in this field, namely whether skeletal muscle responds in a direct fashion to vitamin D via the local expression and activity of the vitamin D receptor (VDR) in muscle.

The first original research article in this thesis employed a cell model of skeletal muscle, namely C2C12 cells, to examine the presence of vitamin D signalling components and direct effects of vitamin D on muscle cells (Vitamin D Signaling Regulates Proliferation, Differentiation, and Myotube Size in C2C12 Skeletal Muscle Cells, Endocrinology 2014; 155(2):347–357) (16). Using a luciferase reporter system, C2C12 muscle cells were shown to express functional CYP27B1. CYP27B1 is the enzyme responsible for conversion of 25-hydroxyvitamin D (25OHD) into the bioactive hormone, 1,25-dihydroxyvitamin D (1,25(OH)2D). C2C12 muscle cells also expressed VDR and this protein increased in a dose-dependent fashion in response to its natural ligand, 1,25(OH)2D (1- 100 nM). Treatment of developing C2C12 muscle cells with 25OHD or 1,25(OH)2D resulted in anti-proliferative effects due to changes in cell cycle genes. In later stages of differentiation (i.e. following fusion of myoblasts into myotubes), an intriguing growth-promoting effect was consistently found. C2C12 myotubes treated with
25OHD or 1,25(OH)\(_2\)D doubled in association with profound down-regulation of myostatin, a member of the TGF-\(\beta\) superfamily that negatively regulates muscle mass. These novel findings provided prima facie evidence that vitamin D directly affects muscle and exerts effects on muscle development and anabolism in a cell line. However, an important limitation of this study is the uncertain correlation between in vitro responses to vitamin D and in vivo physiology.

In the second original research article, the in vivo expression of VDR in skeletal muscle was examined by RT-PCR, western blot and immunohistochemistry (The Vitamin D Receptor (VDR) is Expressed in Skeletal Muscle of Male Mice and Modulates 25-Hydroxyvitamin D (25OHD) Uptake in Myofibers, *Endocrinology* 2014; 155(9):3227-37) (17). Using the specific VDR-D6 antibody, particular methodological factors (i.e. hyperosmolar lysis buffer) and appropriate experimental controls, VDR was successfully detected in mouse muscle, albeit at low levels. In vivo, VDR was localised within muscle fibres in both the cytoplasm and nucleus. Intriguingly, substantially higher levels of VDR were found in muscle of younger mice suggesting a developmental role of VDR in muscle. As conclusive proof of functional presence, a novel physiological role of VDR was demonstrated in modulating the uptake of 25OHD within muscle fibres. Therefore, this work clearly demonstrated the presence of VDR in muscle and was published with accompanying editorial articles (i.e. News/Views and Counterpoint articles) (18, 19). These articles and the invited response to the Counterpoint article have been included in this thesis (20).
The third manuscript, submitted for publication, examines functional effects of vitamin D and VDR on muscle strength and morphology (Vitamin D Receptor Ablation and Vitamin D Deficiency Result in Reduced Grip Strength, Altered Muscle Fibers and Increased Myostatin in Mice, Calcified Tissue International, Submitted). Diet-induced vitamin D deficiency led to progressive reductions in grip strength in mice and there was a significant dose-dependent effect of VDR on grip strength (i.e. VDR +/+ mice > VDR +/- mice > VDR -/- mice). In the absence of altered serum calcium and phosphate levels, these findings supported a direct in vivo effect of vitamin D signalling on muscle strength. Developmental defects in skeletal muscle from VDR knockout (VDRKO) mice were also demonstrated. These included a reduction in muscle mass, reduction in muscle fibre size and increased nuclei to fibre ratio. To explain these changes, VDRKO mice showed altered expression of myogenic regulatory factors and increased myostatin. Consistent with changes reported in C2C12 cells, muscle fibre hyper-nuclearity may relate to altered cell cycle regulation of muscle precursors during embryogenesis in the absence of VDR. This manuscript therefore supports developmental effects of VDR on muscle and the influence of vitamin D and VDR on muscle strength.

A research letter published in Endocrinology reported differences in the skeletal phenotype of VDRKO mice (“Direct Effects of VDR Ablation on Bone Mass”) (21). In response to a publication describing increased bone mineral density/mass in VDR heterozygote mice (VDR +/-) (22), no such difference was found on DXA scan in a group of 7 VDR heterozygote (VDR+/-) males and 14 WT littermates. Possible reasons for this discrepancy included the different genetic bases for these models (i.e. ablation of exon 2 vs. exon 3) and the presence of a biologically active mutant VDR
protein in the former model (23). This highlights the complexity of VDR’s effects on bone and suggests that an integrated approach in examining musculoskeletal roles of VDR may yield further information.

During this period of research, a contribution was made to another published work that described a novel technique to isolate muscle stem cells (i.e. satellite cells) from whole muscle (Grb10 deletion enhances muscle cell proliferation, differentiation and GLUT4 plasma membrane translocation. Journal of Cell Physiology 2014 Nov;229(11):1753-6) (24). My contribution was in examining alterations in the cell cycle associated with ablation of Grb10 (growth-factor receptor-bound protein 10), in muscle stem cells. Techniques described in the study were also used in isolating muscle stem cells from VDRKO and WT mice, as described in this thesis (17).

Three published review articles have been inserted in this thesis (Chapter 8). The first two articles present recent clinical data on vitamin D’s role in various aspects of muscle function, including insulin sensitivity, physical/athletic performance and age-related muscle changes (Effects of vitamin D in skeletal muscle: falls, strength, athletic performance and insulin sensitivity, Clinical Endocrinology 2014; 80:169–181; and Vitamin D and muscle function in the elderly: the elixir of youth? Current Opinion in Clinical Nutrition and Metabolic Care 2014 Nov;17(6):546-50) (3, 25). The third of these articles discusses the integrated biology of muscle and bone and novel therapies for musculoskeletal diseases, including a section on vitamin D (Therapies for Musculoskeletal Disease: Can we Treat Two Birds with One Stone? Current Osteoporosis Reports 2014;12(2):142-53) (26).
Three additional review articles have been included in this thesis (Chapter 9). One, published in *Molecular and Cellular Endocrinology*, discusses the role of vitamin D in integrating musculoskeletal biology, specifically in development, ageing and injury. Another review on which I am co-author was published in *Bone* and covers integrated effects of vitamin D on bone-muscle interactions. Another review on which I am co-author was published in *Nutrition and Dietary Supplements* and discusses metabolic effects of vitamin C and D, particularly on the prevention of diabetes.

Over the course of this thesis, these published and submitted works have addressed the question “does vitamin D directly affect skeletal muscle?” Using a basic model of muscle – C2C12 cells – the presence of an innate vitamin D endocrine system in these cells with distinct developmental and anabolic effects was found (16). To address the physiological relevance of these *in vitro* findings, the presence of VDR was examined in whole muscle *in vivo*. Here VDR was found to be present at low levels and its detection depended on a range of methodological factors (17). A novel role for VDR in the uptake of 25OHD in muscle fibres confirmed its presence and physiologic relevance at this site. Finally, in experiments examining muscle morphology, VDR exerted direct effects on muscle mass, fibre size and strength. Taken together, these findings indicate that vitamin D does *directly* affect skeletal muscle, supporting a predominantly developmental/pleiotropic function of VDR at this site and bringing closure to this long-standing controversy.
Hypothesis

To explain the phenomenon of impaired muscle function in subjects with vitamin D deficiency and VDR mutations, vitamin D plays a vital role in skeletal muscle morphology and physiology via local expression and activity of components of its signalling pathway.

Aims

The aims of this thesis were to:

- investigate effects of vitamin D on skeletal muscle using a range of in vitro and in vivo mouse models.
- determine whether the vitamin D receptor (VDR) is expressed in skeletal muscle and consider technical and methodological reasons for this controversy.
- examine specific roles of vitamin D signalling, independent of calcium and phosphate, in skeletal muscle morphology, strength and development.
- ascertain whether the vitamin D endocrine system holds promise in the management of debilitating muscle conditions, in particular age-related sarcopaenia and acquired myopathies.
Chapter 2 – Literature Review

This chapter consists of a review of human clinical, animal and molecular studies examining effects of vitamin D in skeletal muscle. This was published in the February 2013 edition of Endocrine Reviews. The PhD candidate and primary author of this article, Christian Girgis, conducted the literature review, drew conclusions from the existing evidence-base and prepared the manuscript and figures for publication. This has been verified by co-authors (see Appendix).

This review is presented in the current format (i.e. Times New Roman, font size 12) for ease of reading and following this, in published pdf format. Figures, tables and references may be found within the published format.

Abbreviations and spelling may differ between chapters, according to the specific guidelines of journals where these articles were published.
The Roles of Vitamin D in Skeletal Muscle: Form, Function and Metabolism (Endocrine Reviews 2013; 34: 33–83)

Abstract

Beyond its established role in bone and mineral homeostasis, there is emerging evidence that vitamin D exerts a range of effects in skeletal muscle. Reports of profound muscle weakness and changes in the muscle morphology of adults with vitamin D deficiency have long been described. These reports have been supplemented by numerous trials assessing the impact of vitamin D on muscle strength and mass and falls in predominantly elderly and deficient populations. At a basic level, animal models have confirmed that vitamin D deficiency and congenital aberrations in the vitamin D endocrine system may result in muscle weakness. To explain these effects, some molecular mechanisms by which vitamin D impacts on muscle cell differentiation, intracellular calcium handling, and genomic activity have been elucidated. There are also suggestions that vitamin D alters muscle metabolism, specifically its sensitivity to insulin, which is a pertinent feature in the pathophysiology of insulin resistance and type 2 diabetes. We will review the range of human clinical, animal, and cell studies that address the impact of vitamin D in skeletal muscle, and discuss the controversial issues. This is a vibrant field of research and one that continues to extend the frontiers of knowledge of vitamin D’s broad functional repertoire.
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I. Introduction

In recent times, there has been a great deal of interest in vitamin D, with over 1000 publications in PubMed in 2011 alone. A remarkable number of studies dealing with novel aspects of its biological activity and its potential to exert broad-ranging effects beyond calcium and mineral homeostasis have emerged (1–4). Vitamin D deficiency is a highly prevalent condition in the developed world and in the populous regions of Asia, India, and the Middle East (5, 6). Significant downward trends in vitamin D status in U.S. population-based studies suggest that vitamin D deficiency/insufficiency is increasing in frequency (7, 8). Accordingly, health agencies including the International Osteoporosis Foundation, The Endocrine Society, and Institute of Medicine have recently outlined recommendations for the prevention of vitamin D deficiency and have called for further research to guide the field (9–11).

Beyond the classic effects on bone and calcium health, the effects of vitamin D are a matter of considerable debate and have been recently reviewed in detail (12). A recent Institute of Medicine report contended that the evidence in support of an extraskeletal role for vitamin D was “not yet compelling” (11). However, there is a large and expanding body of observational data about associations between vitamin D deficiency and diverse medical conditions, ranging from multiple sclerosis to malignancy (3). Reports of the presence of the vitamin D receptor (VDR) in almost every tissue strengthen the case in favor of direct extraskeletal functions (13). The effective use of active vitamin D and vitamin D analogs in the treatment of the skin disorder, psoriasis, demonstrate that skin is an extraskeletal target tissue for vitamin D. Long before the recognition of UV
radiation as an essential component in the synthesis of vitamin D, the sun’s rays were considered a source of physical strength and vitality. Ancient Egyptians revered the Sun-God, Amon-Rah, whose rays could make “a single man stronger than a crowd” (14). Herodotus recommended solaria in Ancient Greece as a cure for “weak and flabby muscles,” and ancient Olympians were instructed to lie exposed and train under the sun’s rays (15). In 1952, Spellberg (16), a German sports physiologist, conducted an extensive study examining the effects of UV irradiation on elite athletes. He informed the German Olympic Committee that UV irradiation had a “convincing effect” on physical performance. This was consistent with earlier studies that reported improvements in speed and endurance among students after treatment with sunlamps (17, 18).

We have known for more than 30 yr that vitamin D exerts effects on muscle cells at a molecular level. In this journal in 1986, Ricardo Boland reviewed the effects of vitamin D on calcium handling, mineral homeostasis, and signaling pathways in muscle cells (19). Since that time, we have gained further insight in its effects on the regulation of cell survival (20), differentiation (21), and calcium handling (22). In more recent times, clinical studies have examined the effects of vitamin D supplementation on muscle function and falls in various populations (23, 24). However, the field is challenged by controversy. A recent report suggesting that VDR was not detectable in muscle has fueled the debate as to whether vitamin D effects on muscle are direct or indirect (25–27). The creation of the VDR knockout (VDRKO) mouse in 1997 gave a new focus to this question, which we will discuss (28). A continuing area of uncertainty stems from conflicting meta-analyses of clinical studies examining the effect of vitamin D supplementation on
muscle strength and falls in older individuals (23, 29–31). In this review, molecular, animal, and human studies examining the various roles of vitamin D in muscle will be presented. We will discuss contentious issues that have made this a vibrant field of research and one that continues to extend the frontiers of our knowledge of vitamin D’s broad functional repertoire.

II. Background Physiology

A. The vitamin D pathway

The family of molecules known collectively as vitamin D are not true vitamins, which are defined as essential substances obtained exclusively from the diet. The misnomer is a remnant of the early work of a number of scientific pioneers from the 1900s. After inducing rickets in a group of dogs by keeping them indoors for prolonged periods, the British physician Sir Edward Mellanby (32) discovered that feeding them cod-liver oil cured them and attributed this to the recently identified vitamin (33). However, in 1922, McCollum et al. (34) showed that after heating and aerating cod-liver oil to destroy the vitamin A, it remained effective in the treatment of rickets but no longer cured night blindness. McCollum followed the sequential alphabetical designations and labeled the new substance “vitamine D.” In the 1920s, it was recognized that children with rickets had profound muscle weakness, and Alfred F. Hess (35) reported that exposing rachitic children to direct sunlight led to the “rapid disappearance” of their illness and improved “general vigor and nutrition.” This finding was the direct extension of earlier work by Huldschinsky (36), who achieved the same outcome with artificially produced UV light. After these seminal studies, Harry Goldblatt and Katherine Soames (37) reported in 1923 that the irradiation of
certain foodstuffs rendered them antirachitic. In 1926, Adolf Windaus et al. (38) identified the chemical structure of cholecalciferol (vitamin D3) as found in irradiated pig skin as well as the structure of its parent molecule, 7-dehydrocholesterol. Windaus also isolated vitamins D1 and D2 and was awarded the 1928 Nobel prize for his work on sterols and vitamins. The nomenclature in the field is often confusing. Names, alternate names, and molecular structures of vitamin D and related molecules are shown in Fig. 1. The metabolic pathway of vitamin D, including the various steps in its activation and degradation, are depicted in Fig. 2. It was assumed that exposure of the skin to UV radiation drove the conversion of 7-dehydrocholesterol to cholecalciferol (step 1, Fig. 2). However, proof of this emerged more than 30 yr later with independent discoveries by two groups: Holick et al. (39) and Esvelt et al. (40). The photo-production is subject to a variety of factors including amount of UV exposure (latitude, season, and use of sunscreen and clothing), ethnicity (skin pigmentation), and age (41–43).

After the photochemical conversion of 7-dehydrocholesterol to pre-vitamin D3 and its thermal isomerization to vitamin D3, it binds to the vitamin D-binding protein (DBP), and is transported to the liver where a hydroxyl group is attached at the carbon-25 atom (i.e. C-25) to generate 25-hydroxyvitamin D (25D) (step 2, Fig. 2). The importance of the liver in this first phase of hydroxylation was reported in 1969 by DeLuca and colleagues (44). A number of 25-hydroxylases have been reported including cytochrome P450 CYP27A1 and CYP2R1 (45, 46). CYP2R1 is probably the major enzyme required for 25-hydroxylation of vitamin D3, at least in humans (47, 48). A patient with classic rickets and low circulating
levels of 25D was found to have a homozygous mutation of the CYP2R1 gene, implying that other enzymes were unable to compensate (49). Much remains unknown about the 25-hydroxylase enzymes including the significance of their reported presence in skin, kidney, and intestine (50). These enzymes are generally considered to be constitutively expressed with little feedback regulation; however, this is unusual for the CYP family (50). In the absence of severe hepatic dysfunction, 25-hydroxylation of vitamin D is not usually rate limiting. However, in mild to moderate liver impairment, the associated fat malabsorption can cause vitamin D deficiency. In contrast, 1α-hydroxylation is the major rate-limiting step in synthesis of 1,25-dihydroxyvitamin D (1,25D) (step 3, Fig. 2). Synthesis of 1,25D is tightly regulated (51) and is mediated by the enzyme 1α-hydroxylase. Factors regulating 1α-hydroxylase in kidney are shown in Fig. 2. Due to its sequence similarity to CYP27A1, the gene encoding 1α-hydroxylase was called CYP27B1 (52). CYP27B1 encodes the enzyme responsible for conversion of 25-hydroxyvitamin D (25OHD) into the bioactive hormone, 1,25-dihydroxyvitamin D (1,25(OH)₂D). Its role was demonstrated in 1998 by the development of rickets and reduced circulating 1,25D levels in four patients with gene mutations for this enzyme (53). Cyp27B1-null mice also develop rickets with reduced levels of circulating 1,25D (54). CYP27B1 mRNA is expressed in a number of vitamin D target tissues including kidney, skin, intestine, macrophages, and bone. Although its expression is relatively high in skin, the kidney is thought to be primarily responsible for circulating levels of 1,25D (50). This is supported by 1,25D deficiency in people with renal failure (55, 56). However, this has not been conclusively proven with renal-specific CYP27B1 deletion. The presence of CYP27B1 in other cell types, especially
macrophages, is demonstrated by the fact that people with granulomatous diseases can have elevated 1,25D levels (57).

Entry of 25D into the proximal renal tubular cells requires receptor-mediated uptake of DBP plus 25D at the brush border, degradation of DBP by legumain, and endocytic internalization and translocation of 25D to mitochondria (58). Megalin, a member of the low-density lipoprotein receptor family, is required for reabsorption of filtered DBP-bound 25D (59). It is in the mitochondria that 1α-hydroxylation of 25D into its biologically active form, 1,25D, occurs (50). A number of factors contribute to the tight regulation of 1α-hydroxylase enzyme expression and activity in the kidney (Fig. 2). These include calcium, PTH, calcitonin, GH, IGF-I, and fibroblast growth factor 23 (FGF23). In addition, 1,25D negatively regulates its own synthesis by suppressing 1α-hydroxylase expression in kidney and bone (60). There is also evidence to suggest that estrogen, progesterone, and prolactin may regulate 1α-hydroxylase activity (61, 62). In macrophages, regulation of CYP27B1 is primarily cytokine mediated (63).

The final important enzyme in the vitamin D endocrine system is 24-hydroxylase (CYP24A1). Found in nearly all cells and highly expressed by the kidney, CYP24A1 limits the amount of 1,25D in target tissues by converting 1,25D to inactive metabolites, including 1,24,25-(OH)3D and calcitroic acid and by converting 25D to 24,25(OH)2D (step 4, Fig. 2). In addition to 24-hydroxylation, this multicatalytic enzyme is able to catalyze side-chain hydroxylations at the C23 and C26 positions (64). Recently, mutations in CYP24A1 were reported in
six children with infantile hypercalcemia, thereby providing conclusive evidence of the importance of this enzyme in the in vivo regulation of vitamin D metabolism (65).

The VDR, to which 1,25D binds to exert its biological effects, was described in 1974 by Brumbaugh and Haussler (66). This is depicted in Fig. 3. Insights into the structure and function of this protein have been gained via the cloning and subsequent analysis of the recombinant protein (67), by x-ray crystallography (68) and molecular modeling using atomic coordinates of the protein x-ray structure (69). The protein comprises three distinct regions: an N-terminal dual zinc finger domain that binds to DNA (a characteristic feature of the steroid receptor family), a C-terminal domain that binds to 1,25D, and an extensive, unstructured region that links these two functional domains. Binding of 1,25D to VDR leads to conformational changes within the receptor that allows it to interact with its heterodimeric partner, retinoid X receptor (RXR) (Fig. 3) (70). VDR also forms homodimers that bind DNA and regulate gene expression (71). The liganded complex (i.e. 1,25D-VDR-RXR) binds to vitamin D response elements (VDRE) in the DNA (72). Classic VDRE are direct repeats of two hexameric core binding sites with a three-nucleotide separation (73, 74). However, numerous nonclassic sites have been proven to act as VDRE. VDR-containing dimers interact with large coregulatory complexes required for gene modulation (70, 75). Although a number of coregulatory molecules have been characterized including the VDR interacting protein and the steroid receptor coactivator complex (SRC), the precise mechanisms by which these molecules operate are just beginning to emerge (76). The system is more complex, because
VDR is one of the few nuclear hormone receptors that has been clearly demonstrated to be able to regulate gene expression in the absence of ligand. Unliganded VDR dimers can bind to and regulate some genes, mostly to repress their expression. This is thought to be the mechanism for spontaneous hair loss in mice with mutations in VDR (77). Expression of the VDR in virtually every tissue and the diverse phenotypic changes in the VDRKO mouse are consistent with the wide spectrum of activity of the 1,25D-VDR endocrine system (13). As well as regulation of VDR, CYP27B1, and CYP24A1 (78, 79), the 1,25D-VDR-RXR complex is involved in regulation of a variety of cellular functions including DNA repair, cell differentiation, apoptosis, metabolism, and oxidative stress (13). Its effects on calcium and mineral homeostasis are well established and, in brief, result from the transcriptional regulation of specific proteins within the intestine (calcium-binding proteins, calbindin D28k, and epithelial calcium channels), bone (osteocalcin, osteopontin, and receptor activator of nuclear factor-κ B ligand), and parathyroid glands (PTH) (80). These effects provide potential indirect routes for regulation of muscle function in addition to direct effects (Fig. 4). This review will focus on diverse effects of the vitamin D endocrine system on the functional and metabolic capacity of skeletal muscle as reported by a range of clinical and translational studies. We will also discuss the central role of skeletal muscle in our emerging understanding of the nongenomic capabilities of the VDR.

B. Skeletal muscle physiology

Skeletal muscle is estimated to account for 42% of total body mass in males and 35% in females (81). Its primary function is to generate force and to provide
locomotion. The functional units of skeletal muscle are the muscle fibers, themselves comprised of many myofibrils. Myofibrils are long cylindrical multinucleated cells that vary considerably in their morphological, biochemical, and physiological properties, thereby forming the basis of the well-known structural and functional diversity of skeletal muscle (82). This complexity causes difficulty in classifying muscle fibers. At one stage, one author described fiber classification as “showing an alarming trend toward the incomprehensible” (83). At this time, the most widely used classification is based on histochemical methods that determine the pH lability of myofibrillar ATPase activity and divides fibers into type I (low activity) and type II (high activity) with further subdivision into IIA, IIX, and IIB (84) depending on the expression of different myosin heavy chain (MHC) forms (summarized in Table 1). The reliance on oxidative or glycolytic metabolic pathways determines the contractile speed of these various fiber types. There is substantial evidence that muscle fibers are dynamic in their response to a variety of contractile and metabolic stimuli and are able to convert from one fiber type to another or undergo atrophy (84). Both vitamin D deficiency and age-related sarcopenia have been associated with preferential atrophy of type II (fast-twitch) fibers (84, 85). On a macroscopic scale, the generation of force by a muscle is dependent on several factors including size, fiber composition, and individual fiber functional capacity. The cross-sectional area is the sum of the individual, parallel fibers, themselves made up of thousands of individual myofibrils and other cell types (86). The sarcomere is the basic unit of contraction and is defined as the portion of the myofibril that lies between two bands, known as Z bands. Between successive Z bands, an array of myosin and actin molecules are intricately arranged to form alternating
filaments, suspended in the sarcoplasm and lying in close proximity to mitochondria, indicative of the significance of ATP in contraction. The role of calcium, also fundamentally important in the tight regulation of muscle contraction (87), will be discussed below.

Apart from the generation of force, skeletal muscle is a highly metabolic tissue that produces and responds to a variety of hormones and factors, leading one author to describe it as a true endocrine organ (88). Exercise leads to the increased expression and secretion of a family of myokines including IL-6 and brain-derived neurotrophic factor that can stimulate glucose uptake and fat oxidation within muscle, lipolysis in adipocytes, and gluconeogenesis in hepatocytes via various autocrine, paracrine, and endocrine pathways (87, 89). Skeletal muscle is also responsive to a range of hormones including, but not limited to, insulin, IGF, glucocorticoids, thyroid hormones, and 1,25D, all of which exert influences on the differentiation, metabolism, and function of muscle via a number of established and evolving mechanisms.

C. Calcium and muscle contraction

As well as regulating whole-body calcium homeostasis, there is also evidence that 1,25D increases calcium influx in muscle cells and thus may have both direct and indirect calcium-related effects on muscle (Fig. 4) (90). The sliding filament theory, first proposed in 1954 (91), describes the highly complex movement of actin and myosin filaments over each other. This and potential effects by which vitamin D may affect this model, based on data to be discussed, are depicted in Fig. 5. It is primarily the influx of calcium from the sarcoplasmic
reticulum (SR) and binding to the troponin-tropomyosin complex that results in the exposure of active binding sites on the actin filament and their engagement with the myosin heads (82). In the presence of ATP, contraction ensues as the myosin head tilts from an obtuse angle to the perpendicular, causing movement of myosin over actin filaments in a process named the power stroke, after which ADP and inorganic phosphate are released (86). The binding of new ATP to the myosin head causes its detachment from the active site of the actin filament and movement back into the obtuse position.

D. Calcium and exercise-related glucose uptake

Calcium plays a vital role in exercise-related glucose uptake by skeletal muscle. Vitamin D regulates calcium homeostasis, giving potential for indirect regulation. Exercise increases glucose transporter 4 (GLUT4) expression; after contraction, increased cytosolic calcium activates Ca2+/calmodulin-dependent protein kinase (CAMK) pathways and causes transcriptional up-regulation of myocyte enhancer factors 2A and 2D, which increase GLUT4 expression (92–94). Exercise also increases GLUT4 translocation to the muscle cell membrane, independently of insulin. Activation of the AMP-kinase pathway contributes to this process (95). GLUT4 vesicle translocation and insertion into the cell membrane is a calcium- and ATP-dependent process (96). A putative mechanism for this is the synaptotagmins. They are calcium-sensitive proteins required for insertion of the GLUT4 proteins into the cell membrane, as demonstrated in adipocytes (97). Synaptotagmins in turn regulate Myo1c, an actin-filament-attached protein that binds to and transports GLUT4 vesicles (98). Contraction-induced calcium influx stimulates a range of signaling pathways that regulate
muscle differentiation and function (99, 100). These include myogenic transcription factors, myostatin, peroxisome proliferator-activated receptor-γ and utrophin A, mainly via CAMK and calcineurin-mediated pathways (82, 99, 101). These processes, generally referred to as excitation-transcription coupling, give rise to the plasticity and unique adaptive ability of muscle to alter vital components in its function, fiber type, and contractile force on demand.

E. Calcium and insulin-stimulated glucose uptake

Skeletal muscle is responsible for approximately 85% of insulin-mediated glucose uptake in lean individuals (102). Insulin induces translocation of GLUT4 to the cell surface, facilitating glucose uptake and clearance of circulating glucose. Insulin binds to the α-subunits of its receptor, activating a signaling cascade. This has been covered in many elegant reviews (103, 104). The mechanisms by which the activation of these proteins then leads to the insertion of GLUT4 protein into the cell membrane remain incompletely understood but have also been the subject of recent review (105). As with exercise, GLUT4 vesicle translocation is ATP and calcium dependent. Pharmacological inhibition of calcium influx in muscle reduces insulin-mediated glucose uptake, independent of effects on Akt (96). Calcium regulates components of the proximal insulin signaling pathway such as the binding of calmodulin to insulin receptor substrate (IRS)-2 (106). Increases in calcium influx improved insulin-mediated glucose uptake in isolated muscle fibers of both normal and insulin-resistant mice (96). Calcium regulates cytoskeletal components involved in GLUT4 translocation (98). Studies on L6 myotubes reported significant increases in GLUT4 expression in response to caffeine-related increases in
intracellular calcium. The effects were negated by dantrolene, an inhibitor of SR calcium release (92). However, additional research examining the role of calcium in insulin sensitivity is needed.

III. Vitamin D and Muscle: Cell Models

On a cellular level, a variety of mechanisms by which vitamin D impacts upon the function of skeletal muscle have been elucidated. These can be broadly divided into 1) genomic effects that arise from the binding of the 1,25D-VDR-RXR heterodimer at specific nuclear receptors to influence gene transcription and 2) nongenomic effects that arise from a host of complex intracellular signal transduction pathways after binding of 1,25D to nonnuclear receptor. Over the past 30 yr, the majority of research in this area has mainly focused on the nongenomic effects of vitamin D on skeletal muscle, in particular its regulation of protein kinaseA(PKA)/cAMP, protein kinase B, protein kinase C (PKC), CAMK, and multiple MAPK pathways (90).

A. VDR in muscle

After the discovery of the VDR in 1969 (107), the isolation of unoccupied 1,25D receptors partitioned between the cytosol and cell nucleus in intestinal cells in 1980 raised the possibility of rapid, non-transcriptional pathways associated with this receptor (108). The rapidity, over minutes, with which 1,25D treatment resulted in changes in intracellular calcium transport in vascularized duodenal cells supported this possibility (109). Furthermore, it became apparent from studies that examined the binding properties of VDR isolated from the caveolae-enriched membrane fraction of chick intestinal cells that the cytosolic
receptors were identical to nuclear VDR (110). To confirm this, significant reductions in the capacity of [3-H]1,25D to bind to isolated caveolae-membrane fractions were reported in tissues obtained from VDRKO mice (110). Studies examining the Tokyo strain of VDRKO mice, in which the second zinc finger of the DNA-binding domain of VDR is ablated, reported some residual binding of 1,25D in kidney cells (111). Treatment with 1,25D elicits rapid uptake of calcium within muscle cells in vitro and in vivo (112). However, after transfection of muscle cells with anti-VDR antisense oligodeoxynucleotides (ODN), 1,25D-dependent mechanisms by which rapid calcium entry occurs, namely store operated calcium entry (SOCE) or capacitative calcium entry, are inhibited (113, 114), therefore implying a direct nongenomic role for VDR in calcium handling. The presence of the VDR has been reported in avian, murine, and human muscle cells on the basis of immunohistochemistry (20, 115), equilibrium binding studies (25), and detection of VDR mRNA by RT-PCR (real-time PCR) (21). However, these findings are subject to challenge due to the nonspecificity of many of the VDR antibodies (116). A recent paper that used a validated antibody (which did not show bands in VDRKO mice) did not find VDR expression in skeletal, cardiac, or smooth muscle by Western blot and immunohistochemistry (27). Differences in experimental conditions and the possibility of tight protein binding of VDR to DNA may have accounted for this finding. Moreover, low levels may be sufficient for significant function in muscle. Major studies examining the presence of VDR in muscle and the various techniques used have been summarized in Table 2.

Another possibility is that there may be differences in the expression of VDR in
muscle in different species and throughout the various stages of muscle differentiation. In support of the latter, in vitro studies predominantly examine the activity of VDR within myoblasts and myotubes rather than fully differentiated adult cells. In vivo, VDR mRNA was reported in the muscle of 3-wk-old wild-type mice but not in their 8-wk-old counterparts (21). The authors suggested a primary role for VDR in early muscle development (21). Thus, the expression of VDR within muscle over time requires further clarification. Within cultured chick myoblasts, VDR translocates from the nucleus to the cytoplasm quite rapidly (i.e. 1–10 min) after exposure to 1,25D (117). Intact microtubular transport and caveolae structure were essential as demonstrated by the disruption of VDR translocation by inhibition of these individual components in C2C12 cells (i.e. with colchicine and methyl-β-cyclodextrin, respectively) (20). After a longer period of exposure to 1,25D, the VDR appears to translocate back to the nucleus to presumably carry out its role in transcriptional regulation. This shuttling of the VDR between cytoplasm and nucleus indicates its versatility in both rapid and genomic actions, depending on location (118). Coimmunoprecipitation analyses have also demonstrated direct binding of the VDR with a component of the tyrosine phosphorylation cascade, namely c-Src, under the influence of 1,25D (119). Transfection of muscle cells with three different anti-VDR antisense ODN inhibited 1,25D dependent dephosphorylation and subsequent activation of c-Src as assessed 15 min after treatment with 1,25D or vehicle (120). More recently, treatment of muscle cells with 1,25D resulted in VDR binding with c-Src, time-dependent increases in c-Src activity, and the redistribution of c-Src from the periplasma membrane zone, where it resides under basal conditions, to the cytoplasm and nucleus as seen on confocal
microscopy (20). There is also evidence that 1,25D induces the association between c-Src and c-myc, a transcription factor involved in cell growth and apoptosis. This evidence includes coimmunoprecipitation analyses that demonstrate 1,25D-mediated formation of complexes between c-Src and c-myc (20) and significant inhibition (i.e. 94%) of 1,25D-mediated c-myc tyrosine phosphorylation on immunoblot after transfection of muscle cells with anti-VDR antisense ODN (120) or treatment with PP1, a c-Src-specific inhibitor (120). Mechanistically, c-Src probably interacts with VDR and c-myc, both of which are hormone-dependent phosphotyrosine proteins, via its Src homology 2 domain, but this requires further evaluation (121).

A role for caveolin-1 in 1,25D-mediated activation of c-Src has also been suggested. Caveolin-1 belongs to a family of membrane-scaffolding proteins with potential roles in different disease phenotypes and binds to c-Src under basal conditions near the cell membrane. After treatment of C2C12 myoblasts with 1,25D, colocalization of caveolin-1 and c-Src was disturbed, and they were redistributed into cytoplasm and nucleus (20). Interestingly, when the caveolae structure was disrupted by methyl-β-cyclodextrin, 1,25D was not able to separate caveolin-1 from c-Src, preventing its activation and also preventing the nuclear translocation of VDR. Therefore, the caveolae and associated proteins appear to play an upstream role in the activation of c-Src. It also appears that 1,25D-mediated activation of c-Src by VDR is a downstream regulator of several nongenomic effects of 1,25D in muscle, specifically involving differentiation and calcium homeostasis, which we will discuss. Apart from the specific ability of the VDR to translocate from nucleus to cytosol in response to 1,25D,
conformational changes within the highly flexible 1,25D molecule also determine the mediation of genomic and nongenomic actions via its receptor. It has been shown that the relatively planar 6-s-cis locked JN [1,25-(OH)2-lumisterol3] displays nongenomic activity and that the 6-s-trans locked JB [1,25-(OH)2-dihydrotachysterosol3] possesses predominantly genomic activity on binding the VDR (69). By molecular modeling of the VDR using atomic coordinates of the protein x-ray structure and computer docking, specific binding sites on the VDR that determine its functional activity have been reported (122, 123). Interestingly, the pockets that, respectively, mediate the regulation of nongenomic and genomic responses overlap and therefore result in mutually exclusive conformational forms of the VDR. It has been suggested that the unbound VDR may possibly exist in the cytoplasm as multiple, equilibrating receptor conformations according to standard statistical distribution (69). Also intriguing, a potential role for 25D in binding the alternative pocket and initiating intracellular calcium flux has recently been reported (123).

Apart from the VDR, it is also possible that other cytosol receptors may be responsible for the rapid actions of 1,25D in muscle. Contrary to an earlier report, it does not appear that annexin II binds to 1,25D in a physiologically relevant manner (124, 125). Recent data have suggested a role for membrane-associated rapid response steroid binding in potentially inducing rapid effects of 1,25D in muscle, but further assessment is required (126).

B. Calcium homeostasis

Although the nongenomic regulation of intracellular calcium by 1,25D has been
well characterized in cultured myoblasts and myotubes, it has also been confirmed by in vivo studies on chicks and in vitro assessment of differentiated soleus muscle samples. Studies report a time- and dose-dependent increase in intracellular muscle calcium uptake in response to 1,25D. The use of particular agents and antisense oligonucleotides to block components of the signal transduction pathway has led to the elucidation of specific step-wise mechanisms by which vitamin D influences intracellular calcium homeostasis (127–129). These have been depicted in Fig. 6.

First, the rapid mobilization of calcium from the SR into the cytosol relies on 1,25D-dependent activation of two components of the signal transduction pathway, namely c-Src and phosphoinositide-3 kinase. These in turn lead to the activation of phospholipase C-γ, the rapid production of inositol triphosphate (IP3) and the first of two phases of diacylglycerol (DAG) synthesis from the membrane phosphoinositides (22, 130). It is IP3 that then mediates the rapid movement of calcium from the SR into the cytosol (130). In the continued presence of 1,25D, two additional processes are then involved in the more sustained phase of calcium entry from the extracellular compartment, namely SOCE and L-type voltage-dependent calcium channel (VDCC) entry mechanisms. SOCE relies on several factors including IP3-dependent calcium release from the SR that activates calmodulin, CAMKII, and PKC, the latter also being activated by a by-product of a previous reaction, namely DAG (131). The particular channels responsible for SOCE have been identified as the transient receptor potential-canonical-like proteins. Interestingly, a direct role of the VDR in activating these channels has been suggested by the coimmunoprecipitation of
both molecules after treatment with 1,25D in chick skeletal muscle cells (113).

Apart from its reported role in SOCE, evidence supports vital additional roles for PKC in 1,25D-dependent calcium homeostasis in muscle. Rapid translocation of PKC-α from the cytosol to the cell membrane after the in vitro treatment of chick soleus muscle and cultured rat and chick myoblasts with 1,25D (132, 133) and marked reduction in intracellular calcium influx after selective knockout of PKC-α by the use of specific antisense oligonucleotides have been described (128). Furthermore, PKC also activates VDCC-mediated calcium entry as evidenced by the rapid stimulation of 45-Ca2+ uptake by cultured myoblasts after treatment with PKC activators, namely DAG and phorbol 12-myristate 13-acetate, inhibited by the addition of nifedipine, an L-type VDCC blocker (133). PKC-α may also have a role in the 1,25D-dependent activation of the ERK1/2 signaling pathway as will be discussed in Section III.D on proliferation and differentiation.

VDCC-mediated calcium entry also relies on 1,25D-dependent activation of the cAMP/PKA pathway. Very rapid increases (within 30 sec) in the levels of adenylyl cyclase (AC) and cAMP levels, together with increased PKA activity, occur in differentiated muscle cells and cultured myotubes after 1,25D treatment (127, 134). These studies also report that 1,25D stimulation of VDCC- 45-Ca2+ entry can be mimicked by treatment with dibutyryl cAMP and abolished by specific inhibitors of AC and PKA. Another mechanism is emerging by which PKC and cAMP/PKA pathways may cross talk in the regulation of VDCC-mediated calcium flux (90). The preliminary data indicates an increase in the cAMP content of myoblasts after treatment with a PKC activator, phorbol 12-
myristate 13-acetate, which may stem from the phosphorylation of Gαi that is mediated by PKC in other cells (133). Furthermore, the phosphorylation of Gαi in myoblast membranes after treatment with 1,25D is likely to be essential in the stimulation of AC activity as evidenced by the effects of abolishing G protein regulatory pathways on 1,25D mediated AC activity (134).

In summary, 1,25D induces changes in intracellular calcium levels in cultured muscle cells initially via rapid IP3-dependent calcium shifts from the SR to the cytosol followed by processes resulting in extracellular calcium influx via the activation of SOCE and VDCC activity. These mechanisms are evident in both immature myoblasts and differentiated myotubes, suggesting a potential role for rapid calcium influx in muscle cell differentiation and contraction, respectively (19). In support of this inference, an interesting report from 1974 described a direct correlation between in vivo skeletal muscle dysfunction and demonstrable defects in intracellular calcium handling (136). A group of rabbits, rendered vitamin D deficient by dietary methods, were found to be substantially weaker and hypotonic compared with their vitamin D-replete counterparts and in vitro, displayed significant reductions in calcium uptake in the SR on isolation from psoas muscle.

C. Phosphate homeostasis

Phosphate is an essential substrate in the production of ATP and in protein synthesis. There is early evidence demonstrating that phosphate uptake in muscle may be influenced by 25D (19, 137). The administration of 25D to vitamin D-deficient, phosphate-deplete rats resulted in a significant increase in the in vitro
concentration of [32-P]phosphate in muscle cells, followed by the stimulation of phosphate-dependent metabolic processes including ATP synthesis in these cells (137). This effect was not reproduced by the repletion of phosphate in these rats. The abolition of 1α-hydroxylase activity by nephrectomy had no effect on 25D-mediated phosphate uptake, implying a direct role of the prehormone in this process. Another study reported the specificity of 25D on in vitro phosphate uptake of differentiated muscle cells with the absence of such effects after 1,25D and 24,25-(OH)2D treatment (138–140). Another study demonstrated the specific transport of [32-P]phosphate across muscle plasma membranes after in vivo vitamin D repletion and 32-P labeling of vitamin D deficient chicks (141). This finding was later confirmed by an increase in vesicle phosphate transport in muscle cells via the isolation of highly purified sarcolemma vesicles in vitamin D-deficient chicks treated with vitamin D (19). The direct effect on phosphate uptake may be mediated via a sodium-dependent mechanism as reported by studies on cultured muscle cells (138, 139).

D. Proliferation and differentiation

There is evidence that 1,25D activates components of the MAPK family in cultured myoblasts, thereby influencing the expression of genes involved in cellular proliferation and differentiation. The majority of research in this area has focused on the effects of 1,25D on the ERK1/2 signaling pathway. The initial activation of c-Src by 1,25D, previously described as an apparent gateway to the nongenomic effects of 1,25D in muscle, results in the rapid activation of Raf-1 by the phosphorylation of its serine residue, which relies on the involvement of Ras and PKC-α (142, 143). Raf-1 then leads to the activation of MAPK kinase,
which activates ERK1/2, after which the phosphorylation of a range of proteins and transcription factors including cAMP response element-binding protein and Elk-1 and the increased expression of other proteins relevant to cell proliferation and differentiation, namely c-myc and c-fos, take place (144, 145). Another MAPK activated by 1,25D in cultured myoblasts is p38. Rapid c-Src-dependent stimulation of MAPK kinases MKK3 and 6 and p38 was demonstrated in C2C12 myoblasts after treatment with 1,25D (146). After this, p38-dependent activation of MAPK2 and subsequent phosphorylation of heat-shock protein 27 was demonstrated. Heat shock protein 27 has an important role through its association with the actin microfilament system and cytoskeletal remodeling of muscle cells (146, 147). More recently, 1,25D-mediated AKT phosphorylation in differentiating C2C12 cells was also shown to occur via c-Src, p38, MAPK and phosphoinositide-3 kinase pathways (148). Although little is known about its exact activity in this context, a third member of the MAPK family, namely c-Jun N-terminal kinase-1/2, is also phosphorylatively activated by 1,25D in C2C12 myoblasts (146). Therefore, an intricate system of nongenomic regulatory responses to 1,25D may control cellular proliferation and differentiation of muscle cells, although the relative role of each component remains unclear.

A variety of genomic responses to 1,25D have also recently been elucidated. Apart from being the first to describe the presence of VDR within muscle cells, Simpson et al. (25) also demonstrated dose-dependent reduction in the proliferation of G-8 myoblast cells that were treated with 1,25D and a commensurate reduction in DNA synthesis, suggesting that genomic responses to
1,25D gave rise to the down-regulation in myoblast proliferation and enhanced differentiation into myotubes.

In a recent study, treatment of C2C12 myoblasts with 1,25D for 7 d as opposed to vehicle resulted in increased mRNA and protein expression of transcription factors known to enhance myogenesis, namely myogenic differentiation antigen, desmin, myogenin, and IGF-II and the reduced expression of proliferating cell nuclear antigen and myostatin, which, respectively, enhance cell proliferation and negatively regulate muscle mass (118). Morphologically, cells treated with 1,25D for 10 d displayed significantly increased muscle fiber size and diameter, as indicated by staining for MHC type II a late myogenic marker. These changes were associated with VDR-induced genomic mechanisms as evidenced by significant increases in the expression of the receptor by 1 d of 1,25D treatment and its nuclear translocation at 4d as opposed to its persistent location in the cytoplasm of cells treated with vehicle. However, these findings stand in contrast to an earlier report that described the down-regulation of myogenin and myogenic transcription factor 5 (myf5) at an mRNA level in C2C12 myoblasts treated with 1,25D for 48 and 96 h compared with those treated with vehicle (21). Furthermore, this coincided with the reduced expression of neonatal forms of MHC, suggesting myoblast maturation in cells treated with 1,25D. It is possible that differences in study design may have accounted for these contradictory findings, specifically pertaining to myogenin expression. Different durations of treatment, and daily vs. single treatment regimens were employed in these studies (21, 118).

In another study Artaza and Norris (390), vitamin D treatment of mesenchymal
stem cells resulted in increased expression of follistatin, an antagonist of myostatin, and caused down-regulation of TGF-β. These vitamin D-mediated changes in gene expression imply that it has a potential role in the inhibition of fibrosis and, perhaps, the promotion of myogenesis and osteogenesis in mesenchymal stem cells.

In a recent study, European sea bass treated with various doses of dietary cholecalciferol from 9–44 d after hatching demonstrated dose-dependent effects in the gene expression of a number of myogenic transcription factors and dose-dependent increases in white muscle fiber size and number (149).

Although vitamin D has clear effects on muscle differentiation, more research is needed to elucidate the nature of these mechanisms. It is also important to note that the overlapping functions and complex regulatory pathways determining the activity of myogenic transcription factors are themselves something of a mystery although recently reviewed in detail (150).

**E. Muscle contractile proteins**

There is evidence that vitamin D may play a role in the regulation of key components in the cytoskeletal structure of muscle cells. As discussed in Section II.B, the complex interaction between actin and myosin, two cytoskeletal proteins, forms the basis of understanding muscle contraction (Fig. 5). Similar proteins play a potential role in intracellular trafficking and, potentially, GLUT4 translocation. Significant reductions in components of the actomyosin-troponin complex in the skeletal muscle of vitamin D-deficient rats and rabbits have been
reported in two separate studies, although in the latter, this may not have been directly related to 1,25D because in vivo administration of ethane-1-hydroxy-1,1-diphosphinate in doses known to inhibit 1,25D had no effect on these components of the cytoskeleton (151, 152). A study also reported an increase in the muscle concentrations of actin and troponin C in chicks replete with vitamin D as opposed to their vitamin D-deficient counterparts (141). Taken together, these reports suggest a direct role for 25D in the up-regulation of these muscle contractile proteins (141, 151). Furthermore, the direct role of 25D in phosphate homeostasis, as discussed, and the recent demonstration of direct binding between 25D and the alternative pocket of the VDR with subsequent biological effects in COS-1 kidney cells suggest that reconsideration of the ability of 25D to generate biological responses in vivo may be in order (123).

F. Phospholipid composition

Phospholipids, a class of lipids that reside within the cell membrane, have been implicated in a variety of signal transduction pathways, including insulin signaling and calcium handling, and play a role in cell membrane function including caveolae. Alterations in phospholipid composition have been associated with insulin-resistant states (153). There is also evidence of a direct role of 1,25D in the regulation of the phospholipid metabolic pathway. A study reported higher relative concentrations of phospholipids in the muscle SR membranes of vitamin D-replete vs. -deficient animals (141). There were also significant changes in the levels of particular phospholipids, specifically an increased concentration of phosphatidylcholine and decreases in phosphatidylethanolamine, in the sarcolemma vesicles of vitamin D-replete vs.
deficient chicks (141). Another study suggested that 1,25D treatment led to the activation of specific methylation pathways that leads to the conversion of phosphatidylethanolamine to phosphatidylcholine in muscle cells (154). This is likely to represent a genomic effect as 1,25D-mediated binding of [3-H]glycerol and [14-C]ethanolamine to phosphatidylcholine in cultured myoblasts was inhibited by the action of actinomycin D, an inhibitor of DNA synthesis. Although the precise significance of genomic 1,25D-mediated influences on phospholipid composition remains uncertain, potential influences on calcium handling, cell proliferation, and insulin signaling merit further consideration.

G. Bone-muscle cross talk and vitamin D

It is well established that muscle strength and muscle mass are important determinants of bone density, bone geometry, and fracture risk. Vitamin D therefore plays a key role in bone metabolism not only through its direct effects mediated by the VDR in osteoblasts and its effects on calcium absorption by the intestines but also through its effects on muscle fiber size and muscle function noted above. Interestingly, during growth, serum 25D levels have been found to be negatively associated with the accrual of bone mineral content in girls (155), and 25D levels decrease as lean mass increases (156). These observations raise the possibility that muscle tissue may require additional vitamin D during growth and that an important function of vitamin D on bone mass accrual may be mediated by the effects of vitamin D on accumulation of lean mass, which has been documented to precede gains in bone (157). Another pertinent consideration is the role of FGF23, a protein whose regulation is closely linked to phosphate homeostasis and the activation of vitamin D (step 3, Fig. 2). Although 1,25D up-
regulates the expression of FGF23 by osteocytes and osteoblasts, FGF23 inhibits 1,25D synthesis and stimulates its breakdown (158). Therefore, FGF23 excess, as seen in those with oncogenic or X-linked hypophosphatemic osteomalacia, leads to reduced 1,25D and phosphate levels and is also associated with muscle weakness. In Hyp mice, a model of X-linked hypophosphatemic rickets, the administration of neutralizing FGF23 antibodies increased 1,25D and phosphate levels as well as leading to improvements in grip strength and spontaneous movement (159). Therefore, the FGF23-vitamin D feedback loop presents another layer of complexity when assessing effects on muscle function.

H. Cell models and molecular pathways for insulin signaling and diabetes

1. Insulin signaling in cell models

Treatment of U-937 human promonocytic cells with 1,25D leads to time- and dose-dependent increases in the mRNA expression of insulin receptors, as shown in two separate studies (160, 161). In the second study, 1,25D treatment also resulted in an increase in VDR expression, suggesting that the accompanying increases in insulin-mediated [14-C]2-deoxyglucose uptake and [125-I]insulin binding in these cells resulted from the activation of genomic pathways. The authors also reported a putative VDRE in the human insulin receptor gene promoter on the basis of luciferase assays on transfected plasmid constructs (162). However, differences in insulin signaling mechanisms between skeletal muscle and immune cells, with the reliance of the latter on GLUT1 rather than GLUT4 transporters for glucose uptake, may limit extrapolation of these results. In a recent study, differentiated C2C12 muscle cells were rendered insulin resistant and atrophic by treatment with free fatty acids (FFA) (163). However,
coadministration of 1,25D with FFA resulted in significant dose- and time-dependent increases in the insulin-mediated uptake of [3-H]2-deoxyglucose compared with cells that had received FFA alone. This effect of 1,25D was initially observed at 12 h and reached stability at 36 h, at which point complete reversal of the FFA-mediated insulin resistance was observed (using 10 nM 1,25D). In addition, 1,25D treatment prevented muscle atrophy as demonstrated by significantly increased myotube diameter in cells that had been cotreated with 1,25D and FFA as opposed to those receiving FFA alone. To account for these effects on insulin resistance, the authors reported that 1,25D treatment reversed a number of FFA-induced abnormalities in the insulin-signaling pathway. At a protein level, 1,25D significantly inhibited FFA-induced serine phosphorylation of IRS-1 and increased the tyrosine phosphorylation of IRS-1 and the phosphorylation of Akt. In addition, the FFA-mediated activation of c-Jun N-terminal kinase, a protein with a significant role in insulin resistance, was significantly reversed by 1,25D treatment. Thus, this report provided the first demonstration of a direct effect of 1,25D in the restoration of key components of the insulin-signaling pathway in an established cellular model of insulin resistance.

2. Arachidonic acid (AA) release

Apart from direct effects on the composition of membrane phospholipids as discussed earlier, there is evidence that 1,25D leads indirectly to the release of AA by a process involving deacylation of membrane-bound phosphatidylcholine (164). This is relevant because levels of AA, a polyunsaturated fatty acid, in skeletal muscle correlate inversely with insulin resistance in humans. AA may
also modulate the function of membrane insulin receptors and glucose transporters and may influence the action of insulin by acting as a precursor for the generation of second messengers such as DAG (90). AA also plays a central role in inflammation, with the production of both pro- and antiinflammatory metabolites that may also have potential effects on insulin signaling. In chick myoblasts, 1,25D treatment resulted in dose-dependent increases in the release of [3-H]AA, but the effect appeared to be dependent on the influx of extracellular calcium and the indirect activation of phospholipaseA2 by PKC (164). More research is needed to clarify the possibilities that arise from an effect of 1,25D on AA release and its availability for intracellular processes.

3. Caveolin-I mediated insulin sensitivity

Some data suggests that vitamin D may play a role in caveolin-I mediated insulin sensitivity (165). Caveolin-I, a scaffolding protein within the caveolar membrane, has recently been shown to play an important role in insulin sensitivity. Its selective down-regulation in the skeletal muscle of wild-type mice and its reduced expression in JYD mice, an age-dependent type 2 diabetes model, were associated with significant impairments in insulin sensitivity (166). VDR is present within the caveolae, appears in close proximity to caveolin-I on confocal microscopy after treatment of ROS 17/2.8 cells with 1,25D (110) and relies on caveolin-I for the mediation of nongenomic effects within skeletal muscle cells (20), all supporting a close association between vitamin D and caveolin-I and raising the question as to whether vitamin D might also have an impact on caveolin-I mediated insulin sensitivity. Perhaps the strongest evidence to support this possibility is the combination of marked insulin resistance and
vitamin D resistance in humans with homozygous nonsense caveolin-1 mutations, otherwise known as Berardinelli-Seip congenital lipodystrophy (167). Future research may address this intriguing conceptual link.

IV. Vitamin D and Muscle: Studies in Animal Models

A. VDRKO mice

The VDRKO mouse model has provided valuable insights into the biological function of the vitamin D endocrine system and, specifically, the genomic activity of the transcription factor VDR (13). The role of vitamin D in the development, morphology, and function of skeletal muscle has been made clearer by studies on this mouse model. The earliest form of the VDRKO mouse, generated by targeted ablation of the DNA encoding the second zinc finger of the DNA-binding domain, was described in 1997 (28). Mice appeared phenotypically normal at birth, despite known expression of the VDR in fetal life in wildtype mice, but became hypocalcemic with secondary hyperparathyroidism around weaning (21 d) and developed alopecia associated with large dermal cysts by 4 wk of age and rickets and growth retardation by d 35. The initial lack of hypocalcemia is considered to be due to the early presence of nonsaturable 1,25D-independent mechanisms of intestinal calcium absorption. However, when 1,25D-dependent mechanisms take over, mice require a high calcium (2%), phosphorus (1.25%), and lactose (20%) rescue diet for survival. The alopecia is interesting, because people with mutations in VDR (vitamin D-resistant rickets) also develop alopecia (168, 169).

1. Muscle morphology and development
A study in 2003 described histological changes in the muscles of VDRKO mice directly before and after the development of hypocalcemia. At 3wk of age, samples taken from the quadriceps muscle of VDRKO mice displayed a wider degree of variability in fiber diameter in addition to significant reductions in the size of both type I and II fibers compared with those in wild-type mice (21). By 8 wk of age, generalized atrophy of type I and II muscle fibers had worsened in the VDRKO mice, suggesting progression due to the absence of the VDR or the additional effect of systemic biochemical changes that had not been present at 3 wk. These changes were also reported in VDRKO mice on a high-calcium, -phosphorus, and -lactose rescue diet, suggesting that the absence of VDR was the predominant cause rather than systemic biochemical changes. Neither degenerative nor necrotic changes were observed in VDRKO skeletal muscle, and similar results were obtained in a range of other muscle groups in this model, suggesting a diffuse effect of the VDR on skeletal muscle morphology. Impaired regulation in the expression of particular myogenic transcription factors, known to control muscle phenotype, was reported as an explanation for these findings. On immunohistochemistry, Northern blot, and RTPCR analyses, the expression of myf5, myogenin, and E2A was significantly higher in quadriceps samples of 3- and 8-wk-old VDRKO mice compared with age-matched wild-type mice. Persistent expression of immature forms of MHC was also found in the small muscle fibers of VDRKO mice but not in their type II muscle fibers. The expression of two other myogenic transcription factors, namely MyoD and MRF4, were not particularly affected in VDRKO mice. Although these data support a role for vitamin D in the regulation of muscle development, precise genomic mechanisms by which the VDR influences myogenesis are unclear, and
the issue is further complicated by the failure to identify negative VDRE in the promoter region of the genes encoding myf5 and myogenin (170, 171).

2. Muscle strength and functional assessment

A number of studies have examined muscle strength and performance in VDRKO mice. These investigations rarely assess muscle function in isolation, with results being potentially influenced by a range of other factors including cardiovascular endurance, balance, and the ability to learn new skills. Furthermore, behavioral differences between VDRKO and wild-type mice have been reported, which may confound the assessment of some tests (172). Nevertheless, these studies provide an indication of the functional motor deficits associated with loss of VDR by testing swimming ability and motor coordination.

a. Forced swim test analysis. Three studies have described differences in the swimming behavior of VDRKO mice. In one study comparing six VDRKO mice with 10 wildtype and heterozygote mice that were shaved to account for alopecia-related alterations in buoyancy, severe impairment of swimming was seen in the knockout mice (173). Baseline levels of locomotor, sensory, and vestibular activity were similar. On swim testing, they were observed to swim in a predominantly vertical position, had a significantly greater number of sinking episodes, and displayed stereotypic rotations and catatonic-like upper limb spasms. In another study, a significantly greater number of sinking episodes were seen among eight VDRKO mice compared with six wild-type controls (174). However, on being given a stimulus, they showed no impairment in ability to
move down a 1-m laneway or in reaching a visible platform. However, VDRKO mice did display greater fatigue after the swim based upon differences in rearing and grooming behavior. The authors hypothesized that this was due to the reduced availability of calcium in VDRKO mice after exercise. A third study reported that abnormal patterns of swimming behavior among VDRKO mice were more marked in older (5–13 months) rather than younger knockouts (175). Interestingly, no such impairment in swimming ability was seen among a group of 11 1α-hydroxylase (Cyp27B1)-knockout mice (175). That suggests the intriguing possibility that there might be vitamin D-independent effects of VDR upon muscle function (175).

b. Tests of motor coordination. The time taken for a mouse to fall off a device that rotates at fixed speeds and in acceleration is considered to indicate its degree of motor coordination (174). In one study, VDRKO mice stayed on a rotating device for a significantly shorter time than wild-type mice on both the accelerating and fixed-speed rotarod tests (174). On gait assessment, VDRKO mice took significantly shorter steps and traveled a shorter distance than wild-type mice when placed in an open field for 5 min. Significantly shorter retention times on accelerated rotarod testing were confirmed in another group of 12-month-old VDRKO mice compared with age-matched wild-type mice (175). These 12-month-old mice demonstrated similar impairments in muscle coordination in tilting box and tilting tube tests, which measure the latency and the angle at which the animal displaces off a device that, respectively, tilts or rotates at different angles. However, no differences were found in 6-month-old VDRKO mice.
In another study, the vertical screen test that measures the time taken for a mouse to fall from a screen that becomes suddenly vertical while the mouse rests on it in a horizontal position (i.e. retention time) was employed (173). VDRKO mice demonstrated markedly shorter retention times compared with both wild-type and heterozygous groups, implying impaired motor coordination or strength.

In summary, VDRKO mice demonstrate notable defects in their overall motor performance with significant impairments in their ability to remain afloat while swimming and shorter stride length and impaired motor coordination and balance on rotarod, rotating tube, and tilting box tests. Although the effect of alopecia was accounted for in one study, other biochemical and behavioral changes in VDRKO mice together with the widespread expression of the VDR in the central nervous system, vestibular system, and spinal cord under normal conditions make direct assessment of its role in muscle function difficult in this setting (175). Nevertheless, it is clear that the overall motor function of these mice is impaired and that further work is needed to clarify the individual components that may account for this.

B. Other animal models

Although the VDRKO mouse model has provided valuable insights into the biological activity of vitamin D, it is more accurate to consider this as a model of type II vitamin D-dependent rickets rather than vitamin D deficiency. Studies have examined muscle function in animals rendered vitamin D deficient by a range of dietary and other methods (176). In a study from 1978, vitamin D-
deficient male Sprague-Dawley rats demonstrated significantly prolonged times
to peak tension and recovery times in electrically stimulated soleus muscle
contraction compared with vitamin D-replete rats on normal chow (177). These
changes normalized after vitamin D repletion. In contrast, no impairments were
noted in rats rendered phosphate deficient or calcium deficient 10 d before
experimentation. A study from 1979 examined muscle function in chicks raised
from hatching on a vitamin D-deficient diet (178). Significant reductions in the
tension generated by the triceps surae during stimulation of the posterior tibial
nerve compared with that in vitamin D-replete chicks were found. This occurred
independently of calcium and phosphate levels. The authors found no difference
in the histological appearance of muscle samples but did isolate reduced muscle
mitochondrial calcium levels. This was independent of serum calcium levels and
was proposed as the mechanism by which vitamin D deficiency affected muscle
contraction force. Four weeks after the commencement of a vitamin D deficient
diet, rats displayed marked skeletal muscle hypersensitivity on calf compression
compared with those fed a normal diet (179). This finding was not related to
hypocalcemia but was rather accelerated by increased dietary calcium and was
accompanied by early impairments in balance on a beam-walk test to assess the
frequency of foot slips. Histologically, vitamin D-deficient rats displayed
increased numbers of presumptive nocioceptor axons in skeletal muscle,
providing an explanation for their hyperalgesic phenotype. In contrast to these
studies, a recent report questioned the primary role of vitamin D, rather than
biochemical changes associated with vitamin D deficiency, in resulting in
myopathy (176). In 58 male Wistar rats that were rendered vitamin D,
phosphorus, and calcium deficient by dietary methods and housing under
incandescent lighting, muscle strength of the soleus as assessed by a force transducer detecting isometric contraction was significantly reduced compared with that in replete controls (176). The authors concluded that phosphorus deficiency was the primary culprit on the basis of several findings. These included a direct independent correlation between phosphorus levels and the reduction in soleus muscle force in these animals, complete restoration in slow-twitch muscle force after dietary repletion with phosphorus despite persistent vitamin D deficiency and similar measures of muscle function among phosphorus-replete rats that were either vitamin D deficient or replete. Similarly, there was no difference in muscle contraction among rats that were calcium deficient and replete, in which vitamin D and phosphorus levels were within the reference range. The central importance of phosphorus in the production of ATP, essential for muscle contraction, was considered as the explanation for these findings.

Therefore, the difficulty in differentiating the effects of severe vitamin D deficiency from those of hypocalcemia and hypophosphatemia in the development of muscle pathology is common to animal and human clinical studies. However, cell lines and tissue culture enable study of direct effects, and these studies date back to the 1970s.

C. Animal studies on insulin sensitivity and diabetes

Although vitamin D deficiency has been associated with more aggressive disease among nonobese diabetic mice (180), a model for type 1 diabetes, few animal trials have addressed the role of vitamin D in insulin resistance. In one study of
ob/ob mice, an obese type 2 diabetes model, significant improvements in hyperglycemia and hyperinsulinemia in response to treatment with 1α-hydroxyvitaminD3 (1α-OHD3) were observed (181). In another study of aged rats with type 2 diabetes and insulin resistance that received 25D, 1α-OHD3, or no treatment, serum 1,25D levels correlated positively with the glucose infusion rate on euglycemic clamp studies at the end of the 12-wk treatment period (182), suggesting a potential role in insulin sensitivity. However, the main focus of this study was the demonstration of bone loss among insulin-resistant rats attributed to a reduction in their renal 1α-hydroxylase activity.

D. Summary: vitamin D and muscle function in animal studies

Congenital absence of the VDR in VDRKO mice is associated with atrophy of both type I and type II fibers, poor musculoskeletal performance in behavioral tests, and marked changes in gait. Vitamin D deficiency induced in rats has also been found to alter muscle function when compared with vitamin D-replete animals. Together, these studies using animal models provide additional evidence that vitamin D plays an important role in muscle physiology. However, they do not separate the effects of muscle vs. whole-body deletion of VDR. Biochemical abnormalities in these mouse models may once again confound these findings (Fig. 4). A similar question has been recently answered in relation to cardiac muscle; mice with specific deletion of VDR in cardiac muscle displayed significant cardiac hypertrophy, similar to that seen in whole-body VDRKO mice (183). Specific deletion of VDR in skeletal muscle using Cre-Lox technology will be required to clarify this question.
V. VDR Polymorphisms and Muscle Function

Several single-nucleotide polymorphisms (SNP) in the gene encoding the VDR have been associated with a range of phenotypic characteristics including muscle strength. These have been summarized in Table 3.

A. FokI polymorphism

The FokI polymorphism of the VDR gene is a T/C transition in exon 2 of the VDR gene that results in a shorter 424-amino-acid protein with enhanced transactivation capacity using a reporter gene assay (184). Although this greater VDR activity might suggest improved muscle strength in light of some of the clinical data, the FokI polymorphism is associated with reduced muscle strength in two studies in men (185, 186). It is interesting to speculate that increased VDR function could increase CYP24A1 expression and thereby degradation of 1,25D.

Among 302 Caucasian men (aged 58–93 yr), those who were homozygous for the FokI polymorphism displayed significantly lower fat-free mass and appendicular muscle mass on dual-energy x-ray absorptiometry (DXA) scanning (185). Furthermore, men with this polymorphism demonstrated a 2.17-fold higher risk of sarcopenia (defined by appendicular fat-free mass < 7.26 kg/m²), independent of age. Lower fat-free mass was associated with significantly lower quadriceps muscle strength. Differences in quadriceps strength were not significant after adjusting for fat-free mass, suggesting that the difference was mediated by altered muscle mass. In another study that included 107 patients with stable chronic obstructive pulmonary disease and 104 healthy, age-matched controls, homozygosity for the FokI polymorphism was associated with reduced quadriceps strength compared with heterozygosity or control subjects (186). The
difference became more significant in a model that adjusted for age, sex, forced expiratory volume in 1 sec (FEV1), fat-free mass, and angiotensin-converting enzyme genotypes. There was no evidence that the presence or absence of lung disease affected the relation between FokI genotype and quadriceps strength. Among 240 women (41.5 ± 13.2 yr), those with the FokI polymorphism (FF) had weaker isometric knee extensor strength (P<0.05 for both 90° and 120° incline) vs. those who were heterozygous (Ff) or lacking the polymorphism (ff) (187). However, on adjusting for age, height, and total fat-free mass, the differences were no longer significant, again suggesting that the polymorphism may be affecting muscle mass.

**B. BsmI polymorphism**

The data are less consistent for the BsmI polymorphism. This SNP is located in the 3’ region of the VDR gene, known to play an important role in the regulation of gene expression (188). In a group of 121 nonobese, healthy women over 70 yr of age, the bb genotype was associated with 23% higher quadriceps strength and 7% higher wrist strength compared with those with the BB genotype (189). However, among the 380 obese women over the age of 70 in this same study, no effect was found. Conversely, a study of 175 young healthy women (age range 20–39 yr) in Belgium found that those with the bb genotype had lower hamstring strength on dynamometer and lower fat-free mass on DXA compared with the BB genotype (190). The difference in hamstring strength became of borderline significance after adjustment for age, fat mass, and lean mass. Interestingly, the significance of another polymorphism known to be in linkage disequilibrium with the BsmI B allele, namely the polyadenosine repeat (ss genotype), was
associated with higher hamstring strength and greater body weight and fat mass compared with those with LL genotype in this study. Among 109 healthy female university students in China (age around 19–21 yr), those with the bb genotype of the BsmI polymorphism displayed significantly lower peak torque in concentric knee flexors at a specific setting on the isokinetic dynamometer than the combined BB and Bb group (P<0.03) (191). However, when this parameter was tested at other settings, namely 120°/sec and 30°/sec, the differences were not statistically significant. When examining another polymorphism at the ApaI site, peak torque in eccentric knee extensors at 120°/sec was significantly lower in the AA homozygous group compared with the aa and Aa groups. In a study of 253 men (54.9 ± 10.2 yr), Bt homozygotes (i.e. those with BB genotype at the Bsm I site and tt genotype at an associated polymorphism site, namely Taq I) had higher isometric quadriceps strength at 150° on isokinetic dynamometer than b or T allele carriers without and with adjustment for confounding factors (P<0.01 after adjustment) (187). However, no such association was found when this same parameter was tested at 90° or 120° or when assessing knee flexor strength. Among 120 Turkish men (>65 yr), knee extensor strength on dynamometer was significantly higher in those with BB homozygosity at the Bsm I site than in the Bb/bb group, but no significant association between muscle mass and strength was found (192).

Two population studies examining the rate of falls suggest that the bb genotype of the Bsm I polymorphism may be protective against falls. In a study from Italy that included 259 community-dwelling older patients (>80 yr of age), the rate of falls differed according to Bsm I genotype with more seen among those with BB
or bb genotype on multivariate analysis (193). Data collected from two separate population cohorts of older women, namely the Aberdeen Prospective Osteoporosis Screening Study (APOSST) and Osteoporosis and Ultrasound study (OPUS), also identified a greater incidence of self-reported recurrent falls among those with the BB genotype of the Bsm I polymorphism compared with those with the bb genotype (194). Significant differences in function with greater ease in rising from a chair were seen in bb homozygotes compared with carriers of the B allele. These studies failed to demonstrate an association between Fok I polymorphisms and balance or muscle power measurements. Apart from VDR polymorphisms, a recent population based study that involved 153 men and 596 women (65–101 yr) reported an association between SNP in the CYP27B1 gene (i.e. −1260 and +2838) and the risk of fracture over a 2.2-yr follow-up period (195). There was no difference in the risk of falls among subjects, and muscle strength was not examined. There are limitations in the interpretation of these data. Larger studies to assess the association between muscle strength and genetic polymorphisms are needed, and more functional studies of effects on VDR function are required.

C. VDR polymorphisms and insulin resistance/type 2 diabetes

Particular polymorphisms in the gene encoding the VDR may be associated with the development of insulin resistance and type 2 diabetes among certain populations. However, once again, the data are inconclusive due to the generally small sample size in these studies and the variability in the populations and examined endpoints. These studies have been summarized in Table 3.
VI. Vitamin D and Muscle: Human Studies

A. Myopathy

Rickets and osteomalacia have been associated with muscle weakness and hypotonia for centuries (196, 197). Weakness affecting the proximal lower limb musculature was reported in a group of adults with osteomalacia who responded to high-dose vitamin D therapy in the 1960s (198). The paper did not report whether all patients responded or whether responses were complete. In addition to general weakness, more specific proximal muscle deficits are commonly described, including difficulty rising from a seated or squat position, ascending a flight of stairs, or lifting objects (199–201). Changes in gait, often described as waddling or penguin-like in appearance, are widely reported and are possibly a combined result of bone pain, muscle pain, and proximal weakness (199, 202). However, the classic pattern of proximal weakness seen in vitamin D deficiency is not specific. Many endocrine and metabolic disorders including renal failure, hyperparathyroidism, hypophosphatemia, Cushing’s syndrome, and hyperthyroidism as well as glucocorticoid therapy may display similar clinical features (203, 204). Electromyographic changes seen in vitamin D-deficient subjects with muscle weakness confirm myopathy, but without specific features (201). In the reports of myopathy with vitamin D deficiency, many subjects had multiple biochemical abnormalities involving calcium, phosphate, and PTH that co-corrected with vitamin D repletion, making it difficult to assess the individual role of each component in the development of osteomalacic myopathy (205). These observations formed the basis for the belief that myopathy in these
subjects was not directly related to vitamin D deficiency but rather a general result of osteomalacia and its associated biochemical abnormalities (206).

**Observational and uncontrolled treatment studies**

The reversibility of myopathy with vitamin D supplementation has been described in some case series (200, 207, 208). A recent series described the presence of progressive muscle weakness among young vitamin D-deficient veiled women from Saudi Arabia [90% had 25D<8 ng/ml (20 nmol/liter)] (205).

Some women required a wheelchair. Substantial improvements followed 3 months of vitamin D and calcium supplementation (800 IU and 1200 mg daily, respectively). Wheelchair-bound patients walked independently by the end of the study. In another case series, five patients with myopathy resulting in wheelchair use were treated with vitamin D2 (50,000 IU weekly) (209). At baseline, they were deficient [25D 5–13 ng/ml (12–32 nmol/liter)] with secondary hyperparathyroidism (intact PTH range 13–89 pmol/liter). Treatment resulted in marked improvements in strength, pain, and mobility within 4–6 wk, despite persisting hyperparathyroidism. The authors suggested that this indicated a role for vitamin D independent of PTH. A study assessing vitamin D-deficient women [25D<7 ng/ml [17 nmol/liter]] vs. controls with higher levels [25D>19 ng/ml (47 nmol/liter)] reported an independent association between 25D levels and maximal voluntary knee extension force (208). No correlations were found for PTH or total or bone-specific alkaline phosphatase.

A common theme in these case series that describe patients with muscle pain and weakness is the high rate of initial misdiagnosis. The diagnoses of diabetic
neuropathy, general debility, motor neuron disease, orthopedic disorders, psychiatric conditions, or inherited myopathy were described before the recognition of vitamin D deficiency (205, 209, 210). The nonspecific clinical features of vitamin D-deficiency myopathy, the wide range of severity from mild weakness to debilitating pain and immobility, and low index of suspicion may contribute to the frequent delay in the diagnosis. We are not aware of any reports of muscle biopsy or muscle function studies in people with mutations in VDR.

B. Myalgia and vitamin D deficiency

People with vitamin D deficiency and proximal myopathy (i.e. weakness of the proximal musculature arising from muscle pathology) often have associated proximal myalgia (i.e. muscle pain) (198). Many authors have also proposed that low vitamin D is associated with more diffuse muscle pain (211–213). However, this is controversial, and other studies do not support this (214–216). The issue is made more difficult because osteomalacia is associated with bone pain and microfractures, making causal discrimination of the source of pain challenging. Because it is conceptually obvious that people with muscle pain may be less likely to exercise, go out, and carry out normal outside activities of daily living, establishing cause and effect is important. Ideally, demonstrating a therapeutic response would clarify the issue.

1. Observational studies

A cross-sectional study of 3075 men from eight European centers found that those who reported chronic widespread pain (8.6%) were more likely to have low 25D levels [<15 ng/ml (37.5 nmol/liter)] (217). However, the relationship was
attenuated by adjusting for age, season, activity, and other factors. Ninety-three percent of 153 patients who were being assessed for persistent, nonspecific musculoskeletal pain in Minneapolis were vitamin D deficient [mean 25D 12 ng/ml [30 nmol/liter]] (211). All African-American, East African, Hispanic, and American Indian patients had 25D below 20 ng/ml (50 nmol/liter) as did 82% of Caucasian patients. None had fibromyalgia or medical conditions known to decrease production, absorption, or hydroxylation of vitamin D. This study did not include a control group of similar ethnicity. The propensity for particular ethnic groups, including those migrating from Asia and the Indian subcontinent to Western countries, to develop myalgia and bone pain as the primary manifestation of vitamin D deficiency has been reported since the 1970s (218, 219). A study describing 33 mostly Somalian female asylum seekers with musculoskeletal pain and low 25D [<8.5 ng/ml [21 nmol/liter]] found that vitamin D and calcium supplementation led to the symptom resolution in 22 (66.7%) by 3 months (220). The authors noted a 2.5-yr mean lag time between symptom onset and diagnosis. There was no control group. A study from the United Arab Emirates found that 86% of patients who were initially diagnosed with fibromyalgia or nonspecific muscle pain were vitamin D deficient. The majority reported improvement in response to supplementation (221). Another study compared cultural differences in the reporting of muscle pain among South Asians (i.e. Indian, Pakistani, and Bangladeshi) and white Europeans living in England (212). Reporting of widespread pain was significantly more common in the 1945 South Asians. However, in the 137 South Asians in whom 25D was measured, there was no association between deficiency and pain.
A report describing myalgia in six women who had migrated to the Netherlands considered vitamin D deficiency [<8 ng/ml (20 nmol/liter) in 5 patients] to be the cause. There was a lengthy lag period (7–103 months) from the onset of symptoms to diagnosis (222). In three cases, misdiagnosis led to treatment with prednisone, estrogen, or cholecystectomy. Vitamin D and calcium supplementation was effective in reversing the myalgia in each case.

2. Case-control studies

Muscle pain was recently proposed as a marker for vitamin D deficiency among Aboriginal Australians (223). A case-control study of eight urban Aboriginal patients with muscle pain and eight matched Aboriginal controls without pain reported significantly lower vitamin D levels among those with pain [17 vs. 23 ng/ml (41 vs. 58 nmol/liter), P<0.017]. Wide cultural and gender differences in the reporting of pain, subjective features in the diagnosis of fibromyalgia, and the presence of other features known to affect vitamin D status among patients with persistent pain syndromes confound the assessment of the role of vitamin D deficiency in muscle pain. Also, the observational nature of these studies does not equip them to address the question of causality.

3. Randomized controlled trials (RCT) for myalgia

We identified only one randomized placebo-controlled trial of supplementation where treatment of muscle pain appeared to be the primary endpoint. The study examined people with diffuse muscle pain (214). Fifty subjects with 25D below 20 ng/ml (50 nmol/liter) at baseline were randomized to placebo or vitamin D2 50,000 IU weekly for 3 months. There was no benefit of treatment. The authors
note that 50% of the placebo group achieved normal vitamin D levels during the study; however, the improvement in pain scores was not substantial for either group. Using the PowerStat program (224), we calculate that the study had 80% power with $\alpha$ of 0.05 to detect a difference of $\pm 19$ in pain score. The baseline visual analog pain score in the treatment group was high at $67 \pm 23$, so if there is any benefit, it is probably smaller than this.

4. Summary: myalgia
Vitamin D deficiency with osteomalacia is associated with muscle pain that in most cases resolves with treatment. The pain is more commonly located in large proximal muscle groups rather than displaying diffuse distribution (198, 222, 225, 226) and is often associated with bone pain and other features of osteomalacia and myopathy (205, 208). Thus, for pain (proximal or diffuse) in patients without osteomalacia, other etiologies should also be considered. In patients with diffuse pain, without obvious osteomalacia, the data remain inconclusive. There are strong associations, but the only RCT found no convincing benefit of supplementation. The trial was adequately powered to detect a clinically meaningful change in pain score. There are no RCT examining specific treatment of proximal muscle pain. Larger randomized placebo-controlled trials should be carried out, preferably with stratification by baseline vitamin D status, in people with fibromyalgia and in people with proximal myalgia. There is no evidence to support supplementation for myalgia in people with normal levels.

C. Fibromyalgia
Fibromyalgia is not purely a muscle disorder, but there are a number of studies examining the potential association between it and vitamin D. Myalgia was examined in a cross-sectional study of 6824 white, middle-aged subjects living in the United Kingdom. A significant association between fibromyalgia, defined by the American College of Rheumatology criteria, and 25D was found among women (227). However, no such association was found in men, although they reported similar rates of pain (11.4% for men, 12.5% for women). No association with myalgia was found. In two separate case-control studies of patients with fibromyalgia from the United States and Brazil, no statistically significant differences were found in 25D levels (214, 228). In contrast, another case-control study that examined 40 premenopausal women with fibromyalgia and 37 controls found a significantly higher proportion of vitamin D deficiency [25D<8 ng/ml (20 nmol/liter)] among women with fibromyalgia (229). However, this was not adjusted for physical activity, smoking, or body mass index (BMI). Fibromyalgia symptoms did not differ depending on the vitamin D status. A subset of deficient patients who received supplementation (eight of 18) reported subjective improvement that persisted at 3 months; however, 10 of 18 did not. One placebo-controlled study examining the effect of vitamin D on fibromyalgia was identified (230). In that study of 138 patients with fibromyalgia, the subset of 100 patients with mild to moderate vitamin D deficiency and insufficiency [25D 10–25 ng/ml (25–62.5 nmol/liter)] who were randomized to receive vitamin D3 (50,000 IU weekly) showed significant improvement over 8 wk vs. placebo-treated controls. However, this did not persist at 1 yr, and in the same study, the subset of 38 people with severe deficiency [25D <10 ng/ml (25 nmol/liter)] who received vitamin D in an unblinded fashion did not report any
improvement at either 8 wk or 1 yr (230).

Overall, there is conflicting evidence regarding the possibility of an association between low vitamin D and fibromyalgia, and clearly, having fibromyalgia may have an impact on time spent outdoors/exercising. The data from the one randomized placebo-controlled trial do not provide a clear answer, because the most deficient subjects did not benefit, but less deficient subjects did. More research is needed in this area.

D. Drug-related myopathy and vitamin D

1. Aromatase inhibitors

The effect of vitamin D supplementation on myalgia due to a drug class, namely aromatase inhibitors, has been recently reported. For this class, there is both observational and randomized controlled data, and myalgia is a very common side effect. An observational study found significantly less musculoskeletal pain among 60 women on letrozole who had achieved median25D levels over 66 ng/ml (165 nmol/liter) compared with women with levels below 66 ng/ml (165 nmol/liter) (19 vs. 52%) after weekly supplementation with 50,000 IU vitamin D3 for 12 wk (231). In a double-blind RCT, 60 patients with early-stage breast cancer with new or worsening musculoskeletal pain on aromatase inhibitor therapy were randomized to receive either high-dose vitamin D supplementation at a regimen that depended on their baseline 25D or placebo (232). Those with 25D of 10–19 ng/ml (25–47 nmol/liter) received 50,000 IU vitamin D2 for 16 wk, whereas those with 25D of 20–29 ng/ml (50–72 nmol/liter) received 50,000 IU for 8 wk (232). There were significant improvements in pain at 2 months in
the vitamin D group vs. placebo on the basis of several indices. Therapy was decreased from weekly to monthly after 2 months in most subjects. The beneficial effects did not remain at the 4- and 6-month visits. There was evidence of a dose-response effect; women who were more deficient had greater benefits, and in that subgroup, the beneficial effect was seen across the whole study period.

2. 3-Hydroxy-3-methyl-glutaryl-coenzyme A reductase inhibitors (statins)

A number of reports suggest that vitamin D deficiency may potentiate myopathy in patients on lipid lowering statin therapy (3-hydroxy-3-methyl-glutarylcoenzyme A reductase inhibitors) (233–242). These reports are both anecdotal and based on case series and cross-sectional studies. Not all studies have confirmed this relationship (237).

Cross-sectional and cohort studies.

Among a group of 621 statin-treated patients, the 128 who reported myalgia had lower serum 25D (238). Levels were low [defined as <32 ng/ml (80 nmol/liter) in that study] in 64% of myalgic patients vs. 43% of the others. A subset of 38 of the 82 25D-deficient myalgic patients were treated with 50,000 IU/wk of vitamin D2 for 12wk (unblinded) while continuing statins, and 35 had their myalgia resolve. The three nonresponders achieved similar vitamin D levels. A prospective study of 150 statin-intolerant patients with 25D below 32 ng/ml (80 nmol/liter) reported that 83% were free of myalgia and no longer statin intolerant after approximately 8 months of vitamin D2 treatment (50,000 IU twice weekly for 3 wk and then 50,000 IU weekly) (240). However, treatment had little effect
on elevated creatine phosphokinase (CPK). However, not all studies find an
association. A retrospective study of 6808 statin users found no correlation
between 25D levels, myalgia, or CPK levels (242). A smaller study of 129
patients on statins also found no difference in the 25D levels of those with and
without myalgia (237).

Some hypotheses have been proposed regarding mechanisms by which vitamin
D deficiency may potentiate statin-induced myalgia. The suggestion that statins
may reduce vitamin D synthesis by inhibition of synthesis of its cholesterol
precursor has not been supported by clinical studies (243, 244). The enzyme
CYP34 demonstrates in vitro 25-hydroxylase activity (245), and it has been
speculated that vitamin D deficiency may lead to preferential shunting or use of
this enzyme for vitamin D hydroxylation, reducing its availability to metabolize
(deactivate) statins (239). This hypothesis could be tested by measuring statin
levels in vitamin D-deficient and vitamin D-sufficient individuals matched for
the presence or absence of myopathy and/or CPK levels. Although an association
between a polymorphism of the solute carrier organic anion transporter family
member 1B1 gene (SLCO1B1) and statin-induced myalgia was reported, no
interaction between vitamin D deficiency and genotype was found in a group of
46 patients on statin therapy (241).

These studies of statin myalgia/myopathy are interesting; however, their
unblinded nature and lack of control groups are particular limitations, especially
given the well known placebo effect in pain studies. At this time, the evidence of
an association between vitamin D deficiency and statin-related myalgia is
derived from observational studies and small series. Obviously, people who experience pain with muscle movement may be less likely to be participating in outdoor activities and may therefore make less vitamin D than their pain-free counterparts. Randomized placebo-controlled studies are needed. In the meantime, we suggest treating deficient patients because of the known bone and calcium benefits. There is at present no evidence to support treating people on statins who have normal vitamin D status.

3. Summary: drug-related myopathy and vitamin D

Myalgia has been observed in patients receiving statins or aromatase inhibitors. A number of these studies reveal that patients on statins who suffer from myalgia are also vitamin D deficient, and vitamin D supplementation might improve symptoms of myalgia in these patients. These findings suggest that vitamin D deficiency may potentiate statin-induced myalgia, but the molecular mechanisms underlying the interaction between vitamin D, statin treatment, and muscle function have not been elucidated. Based on the limited data, we recommend treating all deficient subjects who are receiving statins or aromatase inhibitors. The only RCT in patients taking aromatase inhibitor used high-dose therapy. If this is used, patients should be monitored for hypercalcemia and hypercalciuria.

E. Falls and vitamin D

Approximately 30% of community-dwelling people over the age of 65 fall each year, and approximately 20% of these require medical attention (246). Falls pose a substantial risk to an aging population, are a major risk factor for fracture and other injury, and impact negatively on quality of life (247). Therefore,
identifying reversible factors is important. In older individuals, falls are closely related to sarcopenia (loss of muscle mass), loss of muscle tone, and a range of conditions that contribute to the complex syndrome of frailty (248). This issue has also recently been discussed in an Endocrine Society statement on extraskeletal roles of vitamin D (12).

1. Falls: observational studies

There is a seasonal variation in the incidence of falls, with more occurring in winter among older women. This raises the suggestion of a putative role for vitamin D in the occurrence of falls (249), although other factors such as decreased daylight and increased likelihood of slipping on wet or icy surfaces could also explain the differences. The possibility that vitamin D deficiency, which is highly prevalent in frail and older individuals, may contribute to falls has been examined by a number of studies. These are summarized in Table 4. In 1619 women in low-level and high-level residential care (mean age 84 yr), a significant inverse association between serum 25D levels and the incidence of falls over approximately 5 months was reported. The authors estimated a 20% reduction in falls risk with doubling vitamin D status on analysis of log[25D] levels (250). A similar sized study of 1231 community-dwelling individuals identified baseline 25D as a predictor for falls over a 1-yr period, particularly among those 65–75 yr of age (251). Those with 25D levels below 10 ng/ml (25 nmol/liter) displayed the highest risk of recurrent falls. However, in a smaller study, although older individuals who fell had significantly lower 25D levels vs. the overall group of 83 subjects, this was not significant on multivariate analysis (252).
The possibility that 25D does not directly contribute to falls but is rather an associative marker of frailty remains a potential limitation in the interpretation of these observational data. As in the situation with myalgia, it is obvious that people who fall may spend less time outdoors and thereby have lower exposure to solar UV radiation. The other issue is that malnutrition is very common in elderly persons, affecting up to 40% of those living in institutions, and may contribute simultaneously to vitamin D deficiency and frailty.

2. Falls: interventional studies

Interventional studies seeking to examine the effects of vitamin D supplementation or UV exposure on the risk of falls among older individuals have been performed and are summarized in Table 5.

a. Sunlight therapy

In a study of 602 residents of aged-care facilities, those who complied with a daily regimen of increased sunlight exposure had fewer falls than those randomized to the control group (253). Compliance was surprisingly poor despite the use of sunlight officers who visited the facilities (median adherence was 26%). Thus, on intention-to-treat analysis, the study was underpowered, and no effects were seen. The authors concluded that vitamin D supplementation was a more practical approach to reduce falls in residential care. It should also be noted that sunlight exposure during the early morning hours rather than at midday when UV levels are highest might not have been sufficiently effective in the synthesis of vitamin D.
b. Intervention studies: non-placebo controlled

The combination of daily 400 IU vitamin D3 plus 1000 mg calcium reduced severe falls requiring admission in 5063 community-dwelling city residents above the age of 66 yr in Denmark (254). The number needed to treat was nine.

c. Intervention studies: placebo controlled

A reduced incidence of falls, regardless of baseline 25D, was seen in a randomized trial of vitamin D supplementation (255). Among 625 older residents of assisted-living facilities, those who received calcium (600mg daily) and vitamin D2 (initially 10,000 IU once weekly and then 1,000 IU daily) for 2 yr had a lower rate of falls compared with those receiving calcium alone. However, the rate of ever falling was not different, implying that the benefits were among repeat fallers. On subgroup analysis of compliant participants (defined as taking >50% of the doses, n=540) the risk of ever falling was also significantly lower. The number needed to treat to prevent one fall per year was eight, similar to the above open-label study. A 3-yr double-blinded randomized study examined combined supplementation with vitamin D3 (700 IU) and calcium citrate malate (500 mg) vs. placebo in 445 community-dwelling older individuals (256). By intention-to treat analysis, combined therapy was not effective. A subgroup analysis of the women found a significant reduction in the incidence of falls that was most pronounced in those who were less active. Inter-gender differences in muscle mass were postulated to lead to greater susceptibility for falls among this particular group. The trial was not originally powered to detect effect
modification by sex and activity levels, and falls were also a secondary outcome. The primary outcome examined bone density, which was significantly improved (257). In another study, 242 community-dwelling older individuals were randomized to receive calcium (1000 mg daily) with or without vitamin D3 (800 IU daily) for 1 yr and then followed 8 months without treatment but continuing blinding to treatment (258). On intention-to-treat analysis, subjects on dual supplementation reported 27% fewer falls at 1 yr and 39% fewer falls at 20 months. Among 122 older women in long-stay geriatric care who received calcium (1200 mg/d) with or without vitamin D3 (800 IU/d), those on dual supplementation had significantly fewer falls during the 12-wk treatment period compared with the preceding 6-wk observation period. There was no difference in the proportion who fell at all, but people who fell the most appeared to obtain the greatest benefit (259). In a particularly vulnerable group, namely older individuals with poststroke hemiplegia, a significant reduction in the number of falls per person and the total number of repeat fallers was seen among 48 women receiving vitamin D2 (1000 IU daily) vs. controls who received placebo for 2 yr (85). The study included biopsies of the unaffected side and reported that the baseline proportion and diameter of type II muscle fibers were significant independent predictors for falls over the 2 yr. In demonstrating significant improvements in these parameters in a subset of individuals who received vitamin D supplementation for 2 yr, they offered a mechanistic explanation for the reduction in falls. By contrast, recurrent fallers were the least likely to benefit from supplementation in another study (260). Community dwelling ambulant older women were randomized to receive calcium citrate (1000 mg daily) and either vitamin D2 (1000 IU) or placebo for 1 yr. After adjusting for height,
which was not equal at baseline, the vitamin D group had a 19% lower risk of a single fall compared with controls. The effect was most pronounced in winter/spring rather than in summer/autumn. It was calculated that 25D levels over 22 ng/ml (54 nmol/liter) were adequate for the benefit. The results were not statistically significant without height correction. Another study that specifically targeted vitamin D insufficient older individuals [25D < 20 ng/ml (50 nmol/liter)] reported that 800 IU vitamin D3 and 1200 mg calcium daily decreased falls per subject compared with the 148 controls who received calcium alone (261). One noncompliant individual was excluded from analysis. The authors propose that the mechanism relates to a significant improvement in body sway at 8 wk (261, 262). Sagittal body sway improved with treatment, but frontal sway improved in both groups (261).

d. Negative randomized studies and falls.

A number of randomized studies have also failed to demonstrate an effect of vitamin D supplementation upon falls. Among 354 older persons in The Netherlands who were randomized to receive vitamin D3 (400 IU) or placebo for 28 wk, impaired mobility was the major predictor of falls, and vitamin D treatment had no significant effect (263). This study had marked patient heterogeneity with inclusion of both institutionalized and community-dwelling persons. A positive effect of vitamin D supplementation on falls risk was reported by Broe et al. (264) among the subgroup of 124 nursing home residents receiving high-dose vitamin D (800 IU/d) but not in the 600-, 400-, or 200-IU groups. The risk of falling was lower if those receiving 800 IU vitamin D2 were compared with the combined group of smaller doses (200, 400, or 600 IU) and
placebo. There was no suggestion of a normal dose-response curve with the lower doses actually having nonsignificantly higher fall rates compared with placebo (0 IU, 44%; 200 IU, 58%; 400 IU, 60%; 600 IU, 60%; 800 IU, 20%). A large study of 5292 subjects over 70 yr of age with a recent minimal-trauma fracture did not find a beneficial effect of 800 IU of vitaminD3 on falls over 26–62 months (265). Falls were a secondary endpoint with information collected only for the week before each 4-monthly questionnaire. Compliance (patients taking 80% or more of tablets) was poor (<45% at 2 yr); however, there was also no benefit if only compliant patients were examined. Baseline levels were measured in approximately 1% of patients (n=60) and were 15.2 ng/ml (38 nmol/liter). Another study in 3314 older women living in nursing homes did not find fewer falls among those randomized to receive a combination of vitamin D3 (800 IU), calcium (1000 mg), and a falls prevention leaflet over 25 months compared with those receiving the leaflet alone (266). Falls were also a secondary outcome in this study.

In reaction to generally poor adherence to daily regimens of vitamin D supplementation as described by a number of these reports, other studies have examined the efficacy of infrequent high-dose vitamin D supplementation. In one widely reported study of 2256 community dwelling women over 70 yr of age, an annual oral dose of 500,000 IU of vitamin D3 for 3–5 yr appeared to increase the risk of falls, particularly in the first 3 months after the vitamin D dose (267). The median baseline 25D concentration was 21.2 ng/ml (53 nmol/liter), and 1 month after treatment, the median was 48 ng/ml (120 nmol/liter). Another study using 300,000 IU, given annually by im injection, reported no effect on falls among
9440 older individuals (268). No benefit was observed in any subgroup.

3. Meta-analyses and falls

Substantial heterogeneity among these randomized trials with regard to differences in study populations, variable treatment durations, and regimens of supplemental vitamin D and whether or not calcium was coadministered together with inconsistencies in the identification and analysis of falls among studies (e.g. falls per subject, number of falls, and number of fallers) makes collective assessment of these data difficult. Nevertheless, several meta-analyses have been published and are the subject of debate (23, 29, 30, 269–272). Most recently, a meta-analysis reported on 26 randomized trials that enrolled 45,782 participants, mainly elderly females (23). Studies were not excluded for lack of double randomization or strict definition of falls; hence, there were a substantially larger number of included individuals than in earlier meta-analyses. Vitamin D use was associated with statistically significant reduction in the risk of falls. However, there was no difference among those receiving higher doses (>800 IU) vs. lower doses. Vitamin D appeared to be effective in both community dwelling and institutionalized people and in those receiving vitamin D2 or D3. Perhaps reassuringly, the reduction in falls was most prominent in patients who were vitamin D deficient at baseline. Studies in which calcium was coadministered with vitamin D showed a greater effect than those where vitamin D alone was given. Falls reduction in studies without calcium coadministration did not reach statistical significance. Calcium alone was the placebo in most of the combination studies. Therefore, despite substantial heterogeneity among studies, vitamin D supplementation is probably effective in conjunction with calcium in
the prevention of falls among older individuals. The positive impact of vitamin D and calcium supplementation on fall prevention appears to be best among those who are vitamin D deficient at baseline. We recommend consideration of vitamin D plus calcium therapy in people over 65 yr of age with baseline deficiency. However, appropriate supplemental dose of vitamin D and target serum levels required to prevent falls remain hotly debated issues.

F. Muscle strength and physical performance

Many studies have examined the specific effects of vitamin D on measures of muscle function and physical performance. Comparing these studies is made very difficult by the variety of outcome measures used to assess muscle function.

1. Observational studies

A number of cross-sectional studies have reported associations between 25D levels and various parameters related to muscle function including handgrip, lower limb strength, balance, 6-min walk distance, and gait speed (273–275), although not all such studies have supported the association after multivariate adjustment (276, 277). These are summarized in Table 6. In a population-based study of 4100 ambulatory adults aged over 60 yr, an association between serum 25D and lower-extremity function assessed by 8-ft. walk and the repeated sit-to-stand test (STST) was reported (278). The improvements were modest: 3.9% worsening in STST and 5.6% in 8-ft. walk comparing the lowest vs. the highest quintiles of 25D. Interestingly, a trend toward impaired function with longer STST was seen in patients with high normal 25D [>48 ng/ml (120 nmol/liter)] vs. lower levels [16–38 ng/ml (40–94 nmol/liter)].
Significantly poorer physical performance was seen in 1234 older individuals among those with 25D below 10 ng/ml (25 nmol/liter) and 10–20 ng/ml (25–50 nmol/liter) vs. those higher than 30 ng/ml (75 nmol/liter) (279). In a subgroup of 979 participants followed over 3 yr, those with lower 25D levels had significantly higher risk of decline in physical performance compared with those with levels above 30 ng/ml (75 nmol/liter). Those in the intermediate 25D group of 20–30 ng/ml (50–75 nmol/liter) did not display significantly greater rates of decline. In a prospective study of 1008 older individuals in the Netherlands, baseline 25D below 10 ng/ml (25 nmol/liter) was associated with poorer grip strength and reduced muscle mass on DXA after 3 yr compared with people with normal levels [>20 ng/ml (50 nmol/liter)] (280). Those in the intermediate 25D group of 10–20 ng/ml (25–50 nmol/liter) demonstrated significantly greater losses of muscle mass without difference in grip strength vs. those with 25D over 20 ng/ml (50 nmol/liter). An accelerated rate of decline in physical performance over 2.5 yr on timed up and go tests (TUAG) and timed STST were seen among 769 older women with lower baseline 25D (281). However, low 25D levels are associated with frailty (273), so a range of factors not included in adjustment models may confound data interpretation. A recent study of 714 Chinese men (>65 yr) found no association between baseline 25D levels and changes in performance measures or appendicular skeletal muscle mass over 4 yr (282). These men were not vitamin D deficient [mean baseline 25D 31 ng/ml (78 nmol/liter)].

2. Nonrandomized studies
Few studies have examined the effect of vitamin D supplementation on muscle function in younger people. Glerup et al. (208) reported a case-control study in which 55 veiled Arabic women with a mean age of 32 and severe vitamin D deficiency [mean 25D 3 ng/ml (7 nmol/liter)] were compared with 22 Danish women of similar age but higher 25D levels [19 ng/ml (47 nmol/liter)]. At baseline, all parameters of muscle function were significantly lower in the Arabic women. Baseline 25D levels were independently associated with maximal voluntary knee extension. After vitamin D repletion, the Arabic women displayed significant improvements in parameters of muscle function at 3 and 6 months. At 6 months, a subgroup that was retested showed no difference in electrically stimulated muscle function vs. Danish controls. Subjective improvements in muscle and deep bone pain were reported by the treated Arabic women.

3. Randomized controlled studies

The randomized controlled studies are summarized in Table 7. Two studies discussed in Section VI.E and Table 6 [Bischoff et al. (259) and Pfeifer et al. (258)] reported improvements in muscle function as well as decreased falls in those randomized to receive supplemental vitamin D. Over a 12-wk treatment period, those on calcium and vitamin D demonstrated significant improvements in the summed score of knee flexor and extensor strength, grip strength, and TUAG compared with those receiving calcium alone (259). Significant improvements were reported in quadriceps strength and TUAG in another study with reduced falls after 12 months of dual supplements compared with calcium alone (258).
A two by two randomized study of patients treated with calcium 800 mg with or without resistance training, with or without vitamin D 400 IU daily for 9 months found that vitamin D improved physical performance (283). Quadriceps strength, physical performance test, and TUAG were significantly improved by vitamin D and by resistance training, with an additive benefit in the group that received both (283). In a study of 139 ambulatory older subjects with a history of falls and vitamin D deficiency [25D <12 ng/ml (30 nmol/liter)], treatment with vitamin D2 (600,000 IU im) had a significant effect on aggregate functional performance time but no effect on either falls or quadriceps strength at 6 months follow-up vs. placebo (284). Among 56 institutionalized persons over 60 yr of age, those randomized to receive calcium and vitamin D (two doses of 150,000 IU then 90,000 IU monthly) demonstrated significant improvements in maximal isometric strength of hip flexors and knee extensors after 6 months (285). Subgroup analysis demonstrated greater improvements in muscle function among subjects with lower baseline 25D levels [<20 ng/ml (50 nmol/liter)]. Similarly, among 302 older, community-dwelling women, those in the lowest tertile of 25D levels who received daily calcium (1000 mg) and vitamin D2 (1000 IU) displayed the most pronounced improvements in lower limb muscle function over 1 yr as opposed to those on calcium alone (286). Among 69 postmenarcheal adolescent females, those randomized to receive 150,000 IU vitamin D2 orally every 3 months for 1 yr demonstrated significant improvements in movement efficiency, a composite of jump height and velocity measured by mechanography, compared with baseline (287). Additionally, at baseline, higher 25D levels correlated with greater jumping velocity. In a short
study of 42 postmenopausal women, 12 wk treatment with 1α-OHD3 (0.5 µg) and calcium led to significant improvements in quadriceps strength compared with those receiving calcium alone (288).

4. Muscle strength and function: negative studies

In a study of 70 vitamin D-deficient women [25D 8–20 ng/ml (20–50 nmol/liter)], 6 months of vitamin D and calcium (400 IU and 500 mg) had no effect on grip or knee strength vs. calcium alone. Baseline 25D levels showed an inverse correlation with these parameters of muscle function (289). In a study of 686 community-dwelling women over 70 yr, treatment with oral vitamin D3 (150,000 IU every 3 months) for a 9-month period had no significant effect on falls or hand grip strength compared with placebo (290). However, a randomly selected subgroup of 40 participants had a mean baseline 25D of 26 ng/ml (66 nmol/liter), suggesting that the group at large were vitamin D sufficient. Other studies have failed to demonstrate improvements in muscle strength after vitamin D supplementation, regardless of baseline vitamin D status (291–295), as summarized in Table 7. A multicenter study of 243 frail, older patients reported no difference in parameters of physical performance between those randomized to receive a single dose of 300,000 IU of vitamin D vs. placebo (291). There was no effect in the subset with low baseline 25D levels [<12 ng/ml (30 nmol/liter)] despite significant improvements in 25D levels. Some studies have examined other forms of vitamin D, namely 1,25D and 1α-OHD3, with variable effects. Among 98 older subjects with mild renal impairment, 1,25D (0.5 µg daily) resulted in no improvement over a 6-month period vs. placebo, and some subjects on 1,25D developed hypercalciuria and required dose reduction (294).
One negative study examined 179 vitamin D-deficient adolescent females in Lebanon. Those randomized to receive vitamin D3 (doses of 1,400 or 14,000 IU/wk) did not demonstrate improved grip strength but did have greater increases in lean mass, bone area, and total hip bone mineral content vs. placebo after 1 yr (295). Two studies have examined the effects of combining vitamin D supplementation with high-resistance training on muscle strength in older individuals (283, 293). In one study of 180 community-dwelling males (50–79 yr), those randomized to receive an intensive program of resistance training three times per week demonstrated improvements in strength. Those randomized to receive fortified milk alone (containing vitamin D 800IU, calcium1000mg, and protein 13.2 g daily) demonstrated no additional improvements (293). Those receiving high-resistance training with vitamin D (400 IU) and calcium (800 mg) over a 9-month period showed improvements in TUAG but not in quadriceps strength compared with those who received resistance training and calcium (283).

5. Meta-analyses of muscle function

In one meta-analysis, the substantial variability in the parameters of muscle function among studies, use of measures without established validity or reliability, and lack of blinded outcome assessments were cited as reasons for inability to pool data (30).

On assessing 17 RCT involving 5072 participants, there was no significant effect of vitamin D supplementation on grip strength or proximal lower limb strength in adults with 25D levels over 10 ng/ml (25 nmol/liter) at baseline (24). However,
for adults with deficiency [25D <10 ng/ml (25 nmol/liter)], a beneficial effect on hip muscle strength was found. In another meta-analysis of 16 RCT, in which baseline 25D levels were below 20 ng/ml (50 nmol/liter) in 11 studies, the authors noted the publication of a greater number of studies that showed no effect rather than a beneficial effect of vitamin D supplementation on muscle function and that there were no obvious characteristics to differentiate studies with positive and negative findings (296).

In a more recent meta-analysis of 13 RCT involving elderly subjects who were predominantly vitamin D deficient or insufficient, vitamin D supplementation with 800–1000 IU daily was associated with improvements in lower extremity strength and balance (297). The metaanalysis included only randomized trials of older individuals in whom baseline and posttreatment parameters of muscle function were assessed. Trials in younger individuals (295) or those that included muscle training as part of the treatment were not included (293). This meta-analysis found no effect on gait. 6.

**Summary: vitamin D, muscle strength, and physical performance**

Although some data suggest a beneficial effect of vitamin D supplementation on muscle function, particularly in vulnerable populations and those with low baseline vitamin D levels (208, 286), the evidence base is limited by highly heterogeneous studies that assess muscle function by different methods. Hence, larger studies that use standardized, reproducible assessments of muscle strength and double-blinded treatment regimens are necessary to clarify this important issue and guide recommendations. Such studies should ideally consider baseline
vitamin D status and confirm adequate replacement is achieved by a rise in 25D to the normal range.

G. Muscle morphology and electromyography (EMG)

Several reports, dating back to the 1970s, have characterized the morphological appearance of skeletal muscle among vitamin D-deficient subjects and thus provided some evidence in support of a direct role for vitamin D in the morphology and development of muscle.

1. Open-label studies

In 1974, a case series of 13 patients with various degrees of proximal myopathy in the context of chronic renal failure was described (203). Ten of the 13 patients displayed significantly shorter mean action potential durations of the deltoid and quadriceps muscles on EMG. They also displayed moderate atrophy of type II (i.e. fast twitch) muscle fibers on the basis of myofibrillar ATPase staining and degenerative changes on electron microscopy with small foci of fiber necrosis, lytic vacuoles, and Z-band degeneration in four patients. Although vitamin D levels were not determined, because this was before the era of the standardized assay, substantial improvement in muscle strength after im vitamin D treatment in a proportion of the patients was reported. In another series of four uremic patients, the finding of type II muscle fiber atrophy was linked to vitamin D deficiency on the basis of significantly elevated PTH levels (298).

In 1975, gluteal muscle biopsies of 12 patients with laboratory evidence of osteomalacia displayed nonspecific muscle fiber atrophy (299). A distinction was made between patients with isolated nutritional deficiency in whom biopsy
changes were mild compared with those with an additional condition including hyperparathyroidism, hyperthyroidism, or uremia who also demonstrated myofibrillar degeneration and infiltration with amorphous material. One year later, Irani (201) reported a case series of 15 women with nutritional osteomalacia who demonstrated significantly shorter motor unit action potentials and a greater proportion of polyphasic potentials on EMG compared with controls. Muscle biopsies from those with osteomalacia demonstrated nonspecific muscle fiber atrophy. Complete resolution in EMG changes after a 5-wk course of high-dose vitamin D supplementation (600,000 IU of vitamin D2 weekly or fortnightly) was noted in the three patients who were retested. In 1979, two patients with osteomalacia demonstrated type II muscle fiber atrophy in addition to scattered necrosis and derangement of the intermyofibrillar network on muscle biopsy (300). Eleven patients with a condition described as bone loss of aging had muscle biopsies from the vastus lateralis before and after treatment with 1α-OHD3 and calcium for 3–6 months (301). The predominant finding was an increase in the proportion and cross-sectional size of fasttwitch type IIa fibers. Measures of the oxidative capacity of muscle, succinate dehydrogenase, and total phosphorylase activity were low at baseline and increased with treatment. Lactate dehydrogenase activity, a measure of anaerobic metabolism, did not change. Interestingly, the proportion of type IIb fibers (very-fast-twitch fibers) decreased significantly with treatment. This was the first report to demonstrate changes in muscle morphology and oxidative capacity after treatment of presumably vitamin D-deficient subjects with a vitamin D analog.

Three years later, Young et al. (302) confirmed these findings by demonstrating
significant increases in the proportion of type II muscle fibers in biopsies of the vastus lateralis muscle after 3 months of vitamin D supplementation among 12 patients with osteomalacia. In association with these findings, quadriceps muscle strength also improved significantly using an isodynamic dynamometer. However, this body of evidence dating back to the 1970s may be confounded by the many biochemical abnormalities associated with renal failure and osteomalacia such as hyperparathyroidism and disturbances in calcium and phosphate levels. These provide indirect mechanisms that may independently alter muscle function (Fig. 4). Nevertheless, the changes in muscle morphology and performance after vitamin D supplementation in these subjects are important preliminary observations. Recent studies have reported an association between significantly higher skeletal muscle fat content and vitamin D deficiency. In one study of 90 postpubertal females in California, the proportion of muscle fat, assessed by comparing the attenuation signal of a 2-cm² section of the rectus femoris with the adjacent sc fat on computed tomography, was found to strongly correlate in an inverse fashion with serum 25D levels (303). This was independent of body mass or computed tomography measures of sc and visceral fat. The percentage of muscle fat was significantly lower in women with normal vs. subnormal serum 25D levels. In another study of 366 older patients receiving magnetic resonance imaging (MRI) of one shoulder for the investigation of potential rotator cuff injury, a correlation between higher fatty infiltration of rotator cuff muscles and lower serum levels of 25D was reported (304). After multivariate linear regression analysis, this association remained statistically significant in two muscle groups (i.e. supraspinatus and infraspinatus muscles) but only among those whose MRI also demonstrated a full-thickness rotator cuff
tear (228 patients). A third study using MRI of the thigh in 20 older subjects also reported an inverse correlation between muscular fatty degeneration and 25D (305). Interestingly, selective and near-total fatty degeneration of at least one muscle was observed among 11 vitamin D-deficient patients [25D <20 ng/ml (50 nmol/liter)]. A recent cross-sectional study demonstrated a positive correlation between 1,25D levels and total skeletal muscle mass as measured on DXA among subjects younger than 65 yr (306). This was supported by greater isometric knee extension moment in women with higher 1,25D levels. However, no association was found between 25D levels and muscle mass or strength or in those over 65 yr of age. Among 26 subjects with chronic kidney disease, thigh muscle cross-sectional area on MRI correlated significantly with a model including 1,25D levels, calcium levels, and daily physical activity (307). Functional parameters assessing gait and proximal musculature also independently correlated with 1,25D.

Although a majority of highly trained adolescent male ballet dancers had low vitamin D levels [25D <20 ng/ml (50 nmol/liter) in nine of 16 study participants], there was no correlation between 25D, body composition on DXA, or reports of muscle injury in this study (308).

2. EMG and muscle biopsies: randomized study

A study that randomized 96 elderly women with poststroke hemiplegia and severe vitamin D deficiency [<10 ng/ml (25 nmol/liter)] to vitamin D2 (1000 IU daily) or placebo for 2 yr reported significant and dramatic increases in the proportion and diameter of type II muscle fibers (85). These parameters
deteriorated significantly in the placebo group.

3. EMG and muscle biopsies: summary

In summary, it appears that vitamin D deficiency results in significant and reversible changes in EMG and type II muscle fiber atrophy, the latter being an independent predictor for falls in one study (85). However, the changes are nonspecific, being similar to those seen in other conditions. Although fatty infiltration in skeletal muscle has been suggested by three recent studies, these are cross-sectional and based on imaging modalities that may not be validated for the assessment of muscle fat. Muscle becomes fatty with disuse, and thus this measure may be confounded by decreased exercise associated with both increased muscle lipid and lower vitamin D. These modalities are not equipped to identify intracellular fat, perhaps of greater pathophysiological significance.

H. Insulin sensitivity and glucose handling

A broad range of epidemiological and randomized clinical studies together with specific research on molecular pathways and animal models have drawn links between vitamin D and insulin sensitivity. This is relevant to the topic of vitamin D and muscle because under normal physiological conditions, skeletal muscle is responsible for approximately 85% of whole-body insulin-mediated glucose uptake (102). Insulin resistance, a highly prevalent condition that contributes to the pathogenesis of type 2 diabetes, is primarily due to defective insulin-stimulated glucose uptake in skeletal muscle resulting from the production of various inflammatory mediators, adipokines, and FFA by adipocytes in predominantly overnourished and obese individuals (309). However, in
recognizing the complex processes involved in insulin resistance, a number of reversible factors with potential etiological relevance to this condition are being considered. One of these factors is vitamin D deficiency. In this section, we will review human clinical studies that examine the association between vitamin D status and insulin sensitivity.

1. Cross-sectional studies: vitamin D and insulin sensitivity

Studies have examined the association between parameters of insulin resistance and vitamin D status in nondiabetic individuals. In one report, 25D was inversely correlated with the homeostasis model assessment of insulin resistance (HOMA-IR) among 214 Arab-American men, but no such association was found among the 317 women of the same ethnicity included in this study (310). Among 808 nondiabetic participants of the Framingham Offspring Study, plasma 25D concentrations were inversely associated with fasting insulin concentrations and HOMA-IR after adjustment for age, sex, and BMI (311).

A similar association between 25D levels and HOMA-IR was found in a group of 712 subjects at risk of diabetes (312). Among 1941 adolescents who participated in the National Health and Nutrition Examination Survey from 2001–2006, adjusted concentrations of insulin were significantly higher among male subjects who were vitamin D deficient [<20 ng/ml (50 nmol/liter)] compared with those with higher vitamin D levels [>30 ng/ml (75 nmol/liter)], suggesting a potential role vitamin D status in insulin sensitivity (313).

However, a recent study that employed the gold-standard technique in the
assessment of insulin sensitivity, namely the hyperinsulinemic-euglycemic clamp, found that the association between insulin sensitivity in 39 nondiabetic subjects and 25D levels become nonsignificant after adjustment for other factors including BMI (314). Similarly, among 381 nondiabetic university students in Lebanon and 510 nondiabetic subjects from a largely obese ethnic minority in Canada (i.e. Canadian Cree), the inverse association between 25D levels and HOMA-IR also became nonsignificant after adjustment for BMI in addition to other factors (315, 316). In another study of 126 healthy young adults, there was a significant association between 25D levels and insulin sensitivity on a hyperglycemic clamp study that remained after adjustment for a range of factors (317). Therefore, it is clear that the inverse correlation between 25D and BMI, as reported in a number of studies (314, 318), may particularly confound the assessment of these observational data. In fact, a recent study suggests that it may be more accurate to consider adiposity rather than BMI per se as the particular confounding factor (319). In a study of 1882 nondiabetic individuals, it was the inclusion of a computed tomography measure of visceral adiposity rather than BMI and waist circumference in the multivariate analysis that caused the inverse association between vitamin D status and markers of insulin resistance, namely HOMA-IR and log insulin levels, to be insignificant (319). Several mechanisms associating vitamin D deficiency with obesity have been proposed, including the great capacity of adipose tissue to store vitamin D (80, 320) and the avoidance of sunlight exposure and outdoor activity among potentially self-conscious, obese individuals (320).

In confirmation of the former mechanism, a study of 116 obese women reported
that fat mass measured by isotope dilution method was a strong predictor of serum 25D levels both 5 yr before and 10 yr after bilio-pancreatic diversion surgery and that vitamin D levels did not correlate with insulin sensitivity at either time on the basis of the euglycemic-hyperinsulinemic clamp studies (321). The impact of PTH, which has an inverse relation to vitamin D status and is also associated with diabetes (322), has been addressed in a small number of studies. A study including 15 subjects with secondary hyperparathyroidism (serum PTH 6.4 pmol/liter) and 15 controls found that after adjustment for BMI, age, and sex, serum 25D levels were significantly associated with the insulin sensitivity index on a 3-h hyperglycemic clamp, but PTH levels were not (323). Similarly, a significant adjusted association between 25D and fasting insulin was reported in a study of 654 adult subjects from Canada, but PTH was not associated with this parameter after multivariate adjustment (324).

Apart from insulin resistance of skeletal muscle, the pathophysiology of type 2 diabetes comprises a range of other factors. Vitamin D may play a role in these other processes with cross-sectional studies and meta-analyses reporting an association between vitamin D deficiency/insufficiency and the incidence of diabetes in various populations (325–328). However, not all studies confirm this observation, and there is substantial heterogeneity between studies in their design and adjustment for confounders (329–331).

2. Prospective studies: vitamin D and insulin sensitivity
Observational studies have examined the relationship between 25D and the prospective risk of developing insulin resistance. In the prospective Ely study
(1990–2000), baseline 25D levels of 524 nondiabetic men and women were inversely associated with the 10-yr risk of insulin resistance, on the basis of HOMA-IR and fasting insulin after adjustment for a range of factors including age, sex, BMI, and calcium and PTH levels (332). Each 10-ng/ml (25 nmol/liter) increase in baseline 25D was associated with a significant decrease in HOMA-IR score (i.e. 0.16 U) at 10 yr.

In a recent study that assessed 5200 participants of the Australian Diabetes, Obesity, and Lifestyle (AusDiab) study, lower baseline 25D levels were associated with a higher risk of developing diabetes over the 5-yr follow-up period (333). After adjustment for a range of factors, the authors reported that each 10-ng/ml (25 nmol/liter) increment in serum 25D was associated with a 24% reduced 5-yr risk of diabetes. Regarding insulin resistance, a positive and independent association with HOMA-IR at 5 yr was also reported. In contrast to an earlier report (334), no association between dietary calcium intake and diabetes risk or the follow-up homeostasis model assessment of insulin sensitivity score was found (333). Apart from insulin resistance, a number of studies have also reported an association between baseline 25D levels and the long-term risk of diabetes (334–336). However, not all such studies have supported this association (337–339).

3. Interventional studies: vitamin D and insulin sensitivity
Mixed results have emerged from a number of interventional studies that have sought to address the impact of vitamin D supplementation on glucose homeostasis and parameters of insulin sensitivity. There was no difference in
parameters of glucose homeostasis among 238 postmenopausal women who were randomized to receive 2 yr of treatment with vitamin D3 (2000 IU daily) or \( \Delta \alpha - \text{OHD3} \) (0.25 µg daily) or 1 yr of treatment with 1,25D (0.25–0.50 µg) daily vs. placebo (340). Similarly, no differences in insulin-mediated glucose uptake on the euglycemic clamp study were found in 18 healthy males randomized to receive either 1,25D 1.5 µg daily or placebo; however, treatment in this study was only 7 d (341). Studies examining subjects at risk of diabetes have suggested improvements in glucose homeostasis with vitamin D supplementation. In three studies, significant improvements were reported in insulin sensitivity, insulin secretion, and/or the disposition index in subjects at risk of diabetes who were randomized to receive supplemental vitamin D3 and calcium (2000 IU and 500 mg, respectively) vs. calcium alone for 16 wk, high-dose vitamin D3 (120,000 IU) every 2 wk vs. placebo for 6 wk, and vitamin D3 and calcium (700 IU and 500 mg daily, respectively) vs. placebo for 3 yr (342–344).

A double-blind randomized trial of 81 South Asian women living in New Zealand who were found to be both insulin resistant on HOMA-IR and vitamin D deficient [25D 20 ng/ml (50 nmol/liter)] reported significant reductions in insulin resistance and fasting insulin levels among those randomized to receive vitamin D3 (4000 IU daily) vs. placebo for 6 months (345). In a recent randomized trial including 90 diabetic subjects, those randomized to receive vitamin D-fortified yogurt twice daily (each containing 500 IU) or vitamin D- and calcium fortified yogurt demonstrated improved glycemic control on glycated hemoglobin (HbA1c) and improved insulin resistance on HOMA-IR compared with those receiving plain yogurt for 12 wk (346). Importantly, an inverse correlation was
observed between changes in serum 25D and HOMA-IR in this study. In another study, 10 females with type 2 diabetes who were predominantly vitamin D deficient reported a significant reduction in a marker of peripheral insulin resistance after 1 month of vitamin D3 supplementation (1332 IU daily) (347). However, this study had no control group. Conversely, a number of small studies have failed to demonstrate any benefits in association with vitamin D supplementation in patients with type 2 diabetes. In 20 diabetic subjects, a randomized trial reported no improvements in fasting or stimulated glucose, insulin, C-peptide, or glucagon concentrations among those receiving 1,25D (1 µg daily) for 4 d vs. placebo (348). Among 28 Asian Indian patients with type 2 diabetes, those randomized to receive vitamin D supplementation for 4 wk did not demonstrate a significant difference in markers of insulin resistance (i.e. fasting insulin, post-oral glucose tolerance test serum insulin levels, and HOMA-IR) compared with those receiving placebo (349). Similarly, in 32 diabetic subjects, 6 months of supplemental vitamin D (40,000 IU/wk) had no effect on fasting insulin, C-peptide, or HbA1c levels compared with baseline or those receiving placebo (350). It is probably reasonable to conclude that these studies were underpowered to answer the question in either direction. In one case series of three British Asians with diabetes and vitamin D deficiency [25D <6 ng/ml (15 nmol/liter)], high-dose vitamin D supplementation (300,000 IU im) was associated with subsequent deterioration in glycemic control on HbA1c and progression of insulin resistance on fasting insulin resistance index (351). Recent post hoc analyses of eight trials including participants with normal glucose tolerance at baseline and three small trials of patients with established type 2 diabetes demonstrated no effect of vitamin D supplementation on glycemic
outcomes (352). However, two trials examining patients with baseline glucose intolerance reported improvements in insulin resistance among those receiving vitamin D supplementation (349, 350). A recent study of 95 adults also demonstrated an improvement in insulin sensitivity amongst patients with pre-diabetes who were randomized to receive calcium carbonate (1200 mg d) and vitamin D supplementation (2000 to 6000 IU d) versus placebo (Gagnon C, Daly RM, Carpentier A et al. PLoS One. 2014 Oct 9;9(10):e109607).

4. Summary: vitamin D and insulin sensitivity

Substantial differences in study design, duration, and type of vitamin D supplementation and the particular populations studied in these trials make collective assessment of these results difficult. Although some large trials suggest a beneficial effect of vitamin D supplementation in the reduction of insulin resistance, others do not. Furthermore, to ascertain whether the potential glycemic benefits of supplemental vitamin D are more pronounced in those with vitamin D deficiency or poor glycemic control at baseline, larger trials of longer duration are necessary. More than 20 trials are currently under way to address this question (www.clinicaltrials.gov).

VII. Conclusions

See Table 8 for conclusions. In his 1922 publication on the cure of rickets by sunlight, Alfred Hess (35) remarked that “although we have realized the importance of light in the growth of plant life, we have [until now] accorded it too little significance in the development of animal life.” Since that time, we have come a long way in recognizing the role of UV radiation in the
photochemical synthesis of vitamin D, the role of vitamin D in calcium and mineral homeostasis, and the similarity of 1,25D to members of the steroid family with their cholesterol precursor, carbon-ringed structures, and ability to bind to specific nuclear receptors in the genomic mediation of developmental and functional effects. Although steroid hormones are known to exert diverse effects in multiple organs and tissues, a role for vitamin D beyond its predominant effects on bone and mineral homeostasis has been hotly contested. Muscle stands at the frontier of the emerging concept of vitamin D’s extraskeletal role because it shares its ancestral origin with bone in the common mesenchymal stem cell and relies heavily on intracellular calcium handling for contraction, insulin sensitivity, and cellular plasticity (99). In this review, we have adopted a multilayered approach in examining evidence from human clinical studies as well as reports on animal and cell models to piece together the current knowledge of vitamin D’s effects in skeletal muscle. The broad evidence base is generally in favor of a role for vitamin D in the development and function of skeletal muscle. The strongest evidence comes from studies that report distinct morphological changes in the muscle of vitamin D-deficient subjects, others that describe significant impairments in the muscle function of VDRKO mice, and molecular studies that have mapped out the various intracellular responses of cultured muscle cells to vitamin D (129, 173, 174). As a result, we have come closer to answering the perennial question as to whether vitamin D’s influence on muscle is direct or indirect, and the answer appears to be both.

Outstanding questions remain, including the precise role of vitamin D in muscle differentiation, the possibility of specific biological activity of 25D in muscle,
and the current controversy regarding the in vivo presence of the VDR in muscle tissue.

In the clinical domain, observations of reversible myopathy in subjects with severe vitamin D deficiency have been reported for some time. Cross-sectional data reporting a high prevalence of vitamin D deficiency among subjects with falls, muscle weakness, and insulin resistance are also present (211, 274, 327). However, confounding variables are a caveat in the interpretation of this circumstantial evidence. Furthermore, the demonstration of unequivocal improvements in muscle function among subjects with mild to moderate degrees of vitamin D deficiency randomized to receive vitamin D supplementation has been elusive. Possible reasons for this include heterogeneity in study design and supplemental regimens and the general lack of large-scale trials to address this issue. These challenges and others remain to be addressed.

There is reasonable evidence from cellular, animal, and at least some human studies that muscle responds to vitamin D. Although molecular pathways by which vitamin D acts on the myocyte have been identified, there is scope for more clarification. Studies are also needed to clarify the therapeutic potential of vitamin D in the treatment of age-related sarcopenia and perhaps other myopathies. In the meantime, it would be prudent for clinicians to seek and manage vitamin D deficiency in individuals at risk of these conditions.
The Roles of Vitamin D in Skeletal Muscle: Form, Function, and Metabolism

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Beyond its established role in bone and mineral homeostasis, there is emerging evidence that vitamin D exerts a range of effects in skeletal muscle. Reports of profound muscle weakness and changes in the muscle morphology of adults with vitamin D deficiency have long been described. These reports have been supplemented by numerous trials assessing the impact of vitamin D on muscle strength and mass and falls in predominantly elderly and deficient populations. At a basic level, animal models have confirmed that vitamin D deficiency and congenital aberrations in the vitamin D endocrine system may result in muscle weakness. To explain these effects, some molecular mechanisms by which vitamin D impacts on muscle cell differentiation, intracellular calcium handling, and genomic activity have been elucidated. There are also suggestions that vitamin D alters muscle metabolism, specifically its sensitivity to insulin, which is a pertinent feature in the pathophysiology of insulin resistance and type 2 diabetes. We will review the range of human clinical, animal, and cell studies that address the impact of vitamin D in skeletal muscle, and discuss the controversial issues. This is a vibrant field of research and one that continues to extend the frontiers of knowledge of vitamin D’s broad functional repertoire. (Endocrine Reviews 34: 33–83, 2013)

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Abbreviations: AA, Arachidonic acid; AC, adenylyl cyclase; BMI, body mass index; CAMK, Ca\(^{2+}\)/calmodulin-dependent protein kinase; CPK, creatine phosphokinase; CYP, cytochrome P450; 1,25D, 1,25-dihydroxyvitamin D; 25D, 25-hydroxyvitamin D; DAG, diacylglycerol; DBP, vitamin D-binding protein; DEXA, dual-energy x-ray absorptiometry; EMG, electromyography; FFA, free fatty acid; FGF23, fibroblast growth factor 23; GLUT4, glucose transporter 4; HbA1c, glycated hemoglobin; HOMA-IR, homeostasis model assessment of insulin resistance; IP3, inositol triphosphate; IRS, insulin receptor substrate; MHC, myosin heavy chain; MRI, magnetic resonance imaging; myf5, myogenic transcription factor 5; ODN, oligodeoxynucleotides; 1a-OHD\(_3\), 1a-hydroxyvitamin D\(_3\); RXR, retinoid X receptor; SNP, single-nucleotide polymorphism; SOCE, store-operated calcium entry; SR, sarcoplasmic reticulum; SRC, steroid receptor coactivator complex; STST, sit-to-stand test; TUAG, timed up and go test; VDCC, voltage-dependent calcium-channel; VDR, vitamin D receptor; VDRE, vitamin D response element; VDRKO, VDR knockout.
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VII. Conclusions

I. Introduction

In recent times, there has been a great deal of interest in vitamin D, with over 1000 publications in PubMed in 2011 alone. A remarkable number of studies dealing with novel aspects of its biological activity and its potential to exert broad-ranging effects beyond calcium and mineral homeostasis have emerged (1–4). Vitamin D deficiency is a highly prevalent condition in the developed world and in the populous regions of Asia, India, and the Middle East (5, 6). Significant downward trends in vitamin D status in U.S. population-based studies suggest that vitamin D deficiency/insufficiency is increasing in frequency (7, 8). Accordingly, health agencies including the International Osteoporosis Foundation, The Endocrine Society, and Institute of Medicine have recently outlined recommendations for the prevention of vitamin D deficiency and have called for further research to guide the field (9–11).

Beyond the classic effects on bone and calcium health, the effects of vitamin D are a matter of considerable debate and have been recently reviewed in detail (12). A recent Institute of Medicine report contended that the evidence in support of an extraskeletal role for vitamin D was “not yet compelling” (11). However, there is a large and expanding body of observational data about associations between vitamin D deficiency and diverse medical conditions, ranging from multiple sclerosis to malignancy (3). Reports of the presence of the vitamin D receptor (VDR) in almost every tissue strengthen the case in favor of direct extraskeletal functions (13). The effective use of active vitamin D and vitamin D analogs in the treatment of the skin disorder, psoriasis, demonstrate that skin is an extraskeletal target tissue for vitamin D.

Long before the recognition of UV radiation as an essential component in the synthesis of vitamin D, the sun’s rays were considered a source of physical strength and vitality. Ancient Egyptians revered the Sun-God, Amon-Rah, whose rays could make “a single man stronger than a crowd” (14). Herodotus recommended solaria in Ancient Greece as a cure for “weak and flabby muscles,” and ancient Olympians were instructed to lie exposed and train under the sun’s rays (15).

In 1952, Spellberg (16), a German sports physiologist, conducted an extensive study examining the effects of UV irradiation on elite athletes. He informed the German Olympic Committee that UV irradiation had a “convincing effect” on physical performance. This was consistent with earlier studies that reported improvements in speed and endurance among students after treatment with sunlamps (17, 18).

We have known for more than 30 yr that vitamin D exerts effects on muscle cells at a molecular level. In this journal in 1986, Ricardo Boland reviewed the effects of vitamin D on calcium handling, mineral homeostasis, and signaling pathways in muscle cells (19). Since that time, we have gained further insight in its effects on the regulation of cell survival (20), differentiation (21), and calcium handling (22). In more recent times, clinical studies have examined the effects of vitamin D supplementation on muscle function and falls in various populations (23, 24).

However, the field is challenged by controversy. A recent report suggesting that VDR was not detectable in muscle has fueled the debate as to whether vitamin D effects on muscle are direct or indirect (25–27). The creation of the VDR knockout (VDRKO) mouse in 1997 gave a new focus to this question, which we will discuss (28). A continuing area of uncertainty stems from conflicting meta-analyses of clinical studies examining the effect of vitamin D supplementation on muscle strength and falls in older individuals (23, 29–31).

In this review, molecular, animal, and human studies examining the various roles of vitamin D in muscle will be presented. We will discuss contentious issues that have made this a vibrant field of research and one that continues to extend the frontiers of our knowledge of vitamin D’s broad functional repertoire.

II. Background Physiology

A. The vitamin D pathway

The family of molecules known collectively as vitamin D are not true vitamins, which are defined as essential substances obtained exclusively from the diet. The misnomer is a remnant of the early work of a number of scientific pioneers from the 1900s.

After inducing rickets in a group of dogs by keeping them indoors for prolonged periods, the British physician Sir Edward Mellanby (32) discovered that feeding them cod-liver oil cured them and attributed this to the recently identified vitamin A (33). However, in 1922, McCollum et al. (34) showed that after heating and aerating cod-liver oil to destroy the vitamin A, it remained effective in the treatment of rickets but no longer cured night blindness. McCollum followed the sequential alphabetical designations and labeled the new substance “vitamine D.” In the 1920s, it was recognized that children with rickets had profound muscle weakness, and Alfred F. Hess (35) reported that exposing rachitic children to direct sunlight led to the
“rapid disappearance” of their illness and improved “general vigor and nutrition.” This finding was the direct extension of earlier work by Huldschinsky (36), who achieved the same outcome with artificially produced UV light.

After these seminal studies, Harry Goldblatt and Katherine Soames (37) reported in 1923 that the irradiation of certain foodstuffs rendered them antirachitic. In 1926, Adolf Windaus et al. (38) identified the chemical structure of cholecalciferol (vitamin D₃) as found in irradiated pig skin as well as the structure of its parent molecule, 7-dehydrocholesterol. Windaus also isolated vitamins D₁ and D₂ and was awarded the 1928 Nobel prize for his work on sterols and vitamins. The nomenclature in the field is often confusing. Names, alternate names, and molecular structures of vitamin D and related molecules are shown in Fig. 1. The metabolic pathway of vitamin D and related molecules are shown in Fig. 2.

It was assumed that exposure of the skin to UV radiation drove the conversion of 7-dehydrocholesterol to cholecalciferol (step 1, Fig. 2). However, proof of this
emerged more than 30 yr later with independent discoveries by two groups: Holick et al. (39) and Esvelt et al. (40). The photo-production is subject to a variety of factors including amount of UV exposure (latitude, season, and use of sunscreen and clothing), ethnicity (skin pigmentation), and age (41–43). After the photochemical conversion of 7-dehydrocholesterol to pre-vitamin D₃ and its thermal isomerization to vitamin D₃, it binds to the vitamin D-binding protein (DBP), and is transported to the liver where a hydroxyl group is attached at the carbon-25 atom (i.e. C-25) to generate 25-hydroxyvitamin D (25D) (step 2, Fig. 2). The importance of the liver in this first phase of hydroxylation was reported in 1969 by DeLuca and colleagues (44). A number of 25-hydroxylases have been reported including cytochrome P450 CYP27A1 and CYP2R1 (45, 46). CYP2R1 is probably the major enzyme required for 25-hydroxylation of vitamin D₃, at least in humans (47, 48). A patient with classic rickets and low circulating levels of 25D was found to have a homozygous mutation of the CYP2R1 gene, implying that other enzymes were unable to compensate (49).

Much remains unknown about the 25-hydroxylase enzymes including the significance of their reported presence in skin, kidney, and intestine (50). These enzymes are generally considered to be constitutively expressed with little feedback regulation; however, this is unusual for the CYP family (50). In the absence of severe hepatic dysfunction, 25-hydroxylation of vitamin D is not usually rate limiting. However, in mild to moderate liver impairment, the associated fat malabsorption can cause vitamin D deficiency.

In contrast, 1α-hydroxylation is the major rate-limiting step in synthesis of 1,25-dihydroxyvitamin D (1,25D) (step 3, Fig. 2). Synthesis of 1,25D is tightly regulated (51) and is mediated by the enzyme 1α-hydroxylase. Factors regulating 1α-hydroxylase in kidney are shown in Fig. 2. Due to its sequence similarity to CYP27A1, the gene encoding 1α-hydroxylase was called CYP27B1 (52). Its role was demonstrated in 1998 by the development of rickets and reduced circulating 1,25D levels in four patients with gene mutations for this enzyme (53). Cyp27B1-null mice also develop rickets with reduced levels of circulating 1,25D (54).

CYP27B1 mRNA is expressed in a number of vitamin D target tissues including kidney, skin, intestine, macrophages, and bone. Although its expression is relatively high in skin, the kidney is thought to be primarily responsible for circulating levels of 1,25D (50). This is supported by 1,25D deficiency in people with renal failure (55, 56). However, this has not been conclusively proven with renal-specific CYP27B1 deletion. The presence of CYP27B1 in other cell types, especially macrophages, is demon-

Figure 2. Summary of the vitamin D pathway. Steps are discussed in the text. Molecular structures and alternate names for each of the compounds are found in Fig. 1.
strated by the fact that people with granulomatous diseases can have elevated 1,25D levels (57).

Entry of 25D into the proximal renal tubular cells requires receptor-mediated uptake of DBP plus 25D at the brush border, degradation of DBP by legumain, and endocytic internalization and translocation of 25D to mitochondria (58). Megalin, a member of the low-density lipoprotein receptor family, is required for reabsorption of filtered DBP-bound 25D (59). It is in the mitochondria that 1α-hydroxylation of 25D into its biologically active form, 1,25D, occurs (50). A number of factors contribute to the tight regulation of 1α-hydroxylase enzyme expression and activity in the kidney (Fig. 2). These include calcium, PTH, calcitonin, GH, IGF-I, and fibroblast growth factor 23 (FGF23). In addition, 1,25D negatively regulates its own synthesis by suppressing 1α-hydroxylase expression in kidney and bone (60). There is also evidence to suggest that estrogen, progesterone, and prolactin may regulate 1α-hydroxylase activity (61, 62). In macrophages, regulation of CYP27B1 is primarily cytokine mediated (63).

The final important enzyme in the vitamin D endocrine system is 24-hydroxylase (CYP24A1). Found in nearly all cells and highly expressed by the kidney, CYP24A1 limits the amount of 1,25D in target tissues by converting 1,25D to inactive metabolites, including 1,24,25-(OH)3D and calcitroic acid and by converting 25D to 24,25(OH)2D (step 4, Fig. 2). In addition to 24-hydroxylation, this multicatalytic enzyme is able to catalyze side-chain hydroxylations at the C23 and C26 positions (64). Recently, mutations in CYP24A1 were reported in six children with infantile hypercalcemia, thereby providing conclusive evidence of the importance of this enzyme in the in vivo regulation of vitamin D metabolism (65).

The VDR, to which 1,25D binds to exert its biological effects, was described in 1974 by Brumbaugh and Haussler (66). This is depicted in Fig. 3. Insights into the structure and function of this protein have been gained via

Figure 3.

Figure 3. Classic vitamin D signaling pathway. Vitamin D binds to its receptor, which dimerizes, preferentially with RXR. This complex binds to VDRE in the DNA to regulate transcription.
the cloning and subsequent analysis of the recombinant protein (67), by x-ray crystallography (68) and molecular modeling using atomic coordinates of the protein x-ray structure (69). The protein comprises three distinct regions: an N-terminal dual zinc finger domain that binds to DNA (a characteristic feature of the steroid receptor family), a C-terminal domain that binds to 1,25D, and an extensive, unstructured region that links these two functional domains.

Binding of 1,25D to VDR leads to conformational changes within the receptor that allows it to interact with its heterodimeric partner, retinoid X receptor (RXR) (Fig. 3) (70). VDR also forms homodimers that bind DNA and regulate gene expression (71). The liganded complex (i.e., 1,25D-VDR-RXR) binds to vitamin D response elements (VDRE) in the DNA (72). Classic VDRE are direct repeats of two hexameric core binding sites with a three-nucleotide separation (73, 74). However, numerous nonclassic sites have been proven to act as VDRE.

VDR-containing dimers interact with large coregulatory complexes required for gene modulation (70, 75). Although a number of coregulatory molecules have been characterized including the VDR interacting protein (67), by x-ray crystallography (68) and molecular modeling (69), the VDR-RXR complexes are best characterized as a true endocrine organ (88). Exercise leads to spontaneous hair loss in mice with mutations in VDR (77).

Expression of the VDR in virtually every tissue and the diverse phenotypic changes in the VDRKO mouse are consistent with the wide spectrum of activity of the 1,25D-VDR endocrine system (13). As well as regulation of VDR, CYP27B1, and CYP24A1 (78, 79), the 1,25D-VDR-RXR complex is involved in regulation of a variety of cellular functions including DNA repair, cell differentiation, apoptosis, metabolism, and oxidative stress (13). Its effects on calcium and mineral homeostasis are well established and, in brief, result from the transcriptional regulation of specific proteins within the intestine (calcium-binding proteins, calbindin D28k, and epithelial calcium channels), bone (osteocalcin, osteopontin, and receptor activator of nuclear factor-κB ligand), and parathyroid glands (PTH) (80). These effects provide potential indirect routes for regulation of muscle function in addition to direct effects (Fig. 4).

This review will focus on diverse effects of the vitamin D endocrine system on the functional and metabolic capacity of skeletal muscle as reported by a range of clinical and translational studies. We will also discuss the central role of skeletal muscle in our emerging understanding of the nongenomic capabilities of the VDR.

**B. Skeletal muscle physiology**

Skeletal muscle is estimated to account for 42% of total body mass in males and 35% in females (81). Its primary function is to generate force and to provide locomotion. The functional units of skeletal muscle are the muscle fibers, themselves comprised of many myofibrils. Myofibrils are long cylindrical multinucleated cells that vary considerably in their morphological, biochemical, and physiological properties, thereby forming the basis of the well-known structural and functional diversity of skeletal muscle (82). This complexity causes difficulty in classifying muscle fibers. At one stage, one author described fiber classification as “showing an alarming trend toward the incomprehensible” (83).

At this time, the most widely used classification is based on histochemical methods that determine the pH lability of myofibrillar ATPase activity and divides fibers into type I (low activity) and type II (high activity) with further subdivision into IIA, IIX, and IIB (84) depending on the expression of different myosin heavy chain (MHC) forms (summarized in Table 1). The reliance on oxidative or glycolytic metabolic pathways determines the contractile speed of these various fiber types. There is substantial evidence that muscle fibers are dynamic in their response to a variety of contractile and metabolic stimuli and are able to convert from one fiber type to another or undergo atrophy (84). Although vitamin D deficiency and age-related sarcopenia have been associated with preferential atrophy of type II (fast-twitch) fibers (84, 85).

On a macroscopic scale, the generation of force by a muscle is dependent on several factors including size, fiber composition, and individual fiber functional capacity. The cross-sectional area is the sum of the individual, parallel fibers, themselves made up of thousands of individual myofibrils and other cell types (86). The sarcomere is the basic unit of contraction and is defined as the portion of the myofibril that lies between two bands, known as Z bands. Between successive Z bands, an array of myosin and actin molecules are intricately arranged to form alternating filaments, suspended in the sarcoplasm and lying in close proximity to mitochondria, indicative of the significance of ATP in contraction. The role of calcium, also fundamentally important in the tight regulation of muscle contraction (87), will be discussed below.

Apart from the generation of force, skeletal muscle is a highly metabolic tissue that produces and responds to a variety of hormones and factors, leading one author to describe it as a true endocrine organ (88). Exercise leads to...
the increased expression and secretion of a family of myokines including IL-6 and brain-derived neurotrophic factor that can stimulate glucose uptake and fat oxidation within muscle, lipolysis in adipocytes, and gluconeogenesis in hepatocytes via various autocrine, paracrine, and endocrine pathways (87, 89). Skeletal muscle is also responsive to a range of hormones including, but not limited to, insulin, IGF, glucocorticoids, thyroid hormones, and 1,25D, all of which exert influences on the differentiation, metabolism, and function of muscle via a number of established and evolving mechanisms.

**C. Calcium and muscle contraction**

As well as regulating whole-body calcium homeostasis, there is also evidence that 1,25D increases calcium influx in muscle cells and thus may have both direct and indirect calcium-related effects on muscle (Fig. 4) (90). The sliding filament theory, first proposed in 1954 (91), describes the highly complex movement of actin and myosin filaments over each other. This and potential effects by which vitamin D may affect this model, based on data to be discussed, are depicted in Fig. 5. It is primarily the influx of calcium from the sarcoplasmic reticulum (SR) and binding to the troponin-tropomyosin complex that results in the exposure of active binding sites on the actin filament and their engagement with the myosin heads (82). In the presence of ATP, contraction ensues as the myosin head tilts from an obtuse angle to the perpendicular, causing movement of myosin over actin filaments in a process named the power

**TABLE 1.** Respective characteristics of muscle fiber types based on the current classification

<table>
<thead>
<tr>
<th></th>
<th>Type I</th>
<th>Type IIA</th>
<th>Type IIX</th>
<th>Type IIB</th>
</tr>
</thead>
<tbody>
<tr>
<td>mATPase activity</td>
<td>Low</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>MHC type</td>
<td>I-MHC</td>
<td>IIA-MHC</td>
<td>IIx-MHC (rodents), Ilb-MHC (humans)</td>
<td>Ilb-MHC</td>
</tr>
<tr>
<td>Mitochondrial density</td>
<td>High</td>
<td>High</td>
<td>Medium</td>
<td>Low</td>
</tr>
<tr>
<td>Anatomic color</td>
<td>Red</td>
<td>Red</td>
<td>White</td>
<td>White</td>
</tr>
<tr>
<td>Energy source</td>
<td>Oxidative</td>
<td>Mainly oxidative</td>
<td>Oxidative and glycolytic</td>
<td>Mainly glycolytic</td>
</tr>
<tr>
<td>Activity</td>
<td>Aerobic</td>
<td>Anaerobic (long-term)</td>
<td>Aerobic (short-term)</td>
<td>Aerobic (short-term)</td>
</tr>
<tr>
<td>Contractile speed</td>
<td>Slow</td>
<td>Moderately fast</td>
<td>Fast</td>
<td>Very Fast</td>
</tr>
<tr>
<td>Resistance to fatigue</td>
<td>High</td>
<td>High</td>
<td>Moderate</td>
<td>Low</td>
</tr>
</tbody>
</table>

References are Schiffiano (353), Spagenburg and Booth (354), Berchtold et al. (82), and Brooke and Kaiser (83). mATPase, Myofibrillar ATPase.
stroke, after which ADP and inorganic phosphate are released (86). The binding of new ATP to the myosin head causes its detachment from the active site of the actin filament and movement back into the obtuse position.

D. Calcium and exercise-related glucose uptake

Calcium plays a vital role in exercise-related glucose uptake by skeletal muscle. Vitamin D regulates calcium homeostasis, giving potential for indirect regulation. Exercise increases glucose transporter 4 (GLUT4) expression; after contraction, increased cytosolic calcium activates Ca\(^{2+}\)/calmodulin-dependent protein kinase (CAMK) pathways and causes transcriptional up-regulation of myocyte enhancer factors 2A and 2D, which increase GLUT4 expression (92–94). Exercise also increases GLUT4 translocation to the muscle cell membrane, independently of insulin. Activation of the AMP-kinase pathway contributes to this process (95). GLUT4 vesicle translocation and insertion into the cell membrane is a calcium- and ATP-dependent process (96). A putative mechanism for this is the synaptotagmins. They are calcium-sensitive proteins required for insertion of the GLUT4 proteins into the cell membrane, as demonstrated in adipocytes (97). Synaptotagmins in turn regulate Myo1c, an actin-filament-attached protein that binds to and transports GLUT4 vesicles (98). Contraction-induced calcium influx stimulates a range of signaling pathways that regulate muscle differentiation and function (99, 100). These include myogenic transcription factors, myostatin, peroxisome proliferator-activated receptor δ and utrophin A, mainly via CAMK and calcineurin-mediated pathways (82, 99, 101). These processes, generally referred to as excitation-transcription coupling, give rise to the plasticity and unique adaptive ability of muscle to alter vital components in its function, fiber type, and contractile force on demand.

E. Calcium and insulin-stimulated glucose uptake

Skeletal muscle is responsible for approximately 85% of insulin-mediated glucose uptake in lean individuals (102). Insulin induces translocation of GLUT4 to the cell surface, facilitating glucose uptake and clearance of circulating glucose. Insulin binds to the α-subunits of its receptor, activating a signaling cascade. This has been covered in many elegant reviews (103, 104). The mechanisms by which the activation of these proteins then leads to the insertion of GLUT4 protein into the cell membrane remain incompletely understood but have also been the subject of recent review (105). As with exercise, GLUT4 vesicle translocation is ATP and calcium dependent.

Pharmacological inhibition of calcium influx in muscle reduces insulin-mediated glucose uptake, independent of effects on Akt (96). Calcium regulates components of the proximal insulin signaling pathway such as the binding of calmodulin to insulin receptor substrate (IRS)-2 (106). Increases in calcium influx improved insulin-mediated glucose uptake in isolated muscle fibers of both normal and
insulin-resistant mice (96). Calcium regulates cytoskeletal components involved in GLUT4 translocation (98). Studies on L6 myotubes reported significant increases in GLUT4 expression in response to caffeine-related increases in intracellular calcium. The effects were negated by dantrolene, an inhibitor of SR calcium release (92). However, additional research examining the role of calcium in insulin sensitivity is needed.

III. Vitamin D and Muscle: Cell Models

On a cellular level, a variety of mechanisms by which vitamin D impacts upon the function of skeletal muscle have been elucidated. These can be broadly divided into 1) genomic effects that arise from the binding of the 1,25D-VDR-RXR heterodimer at specific nuclear receptors to influence gene transcription and 2) nongenomic effects that arise from a host of complex intracellular signal transduction pathways after binding of 1,25D to nonnuclear receptor. Over the past 30 yr, the majority of research in this area has mainly focused on the nongenomic effects of vitamin D on skeletal muscle, in particular its regulation of protein kinase A (PKA)/cAMP, protein kinase B, protein kinase C (PKC), CAMK, and multiple MAPK pathways (90).

A. VDR in muscle

After the discovery of the VDR in 1969 (107), the isolation of unoccupied 1,25D receptors partitioned between the cytosol and cell nucleus in intestinal cells in 1980 raised the possibility of rapid, nontranscriptional pathways associated with this receptor (108). The rapidity, over minutes, with which 1,25D treatment resulted in changes in intracellular calcium transport in vascularized duodenal cells supported this possibility (109). Furthermore, it became apparent from studies that examined the binding properties of VDR isolated from the caveolae-enriched membrane fraction of chick intestinal cells that the cytosolic receptors were identical to nuclear VDR (110). To confirm this, significant reductions in the capacity of [3H]1,25D to bind to isolated caveolae-membrane fractions were reported in tissues obtained from VDRKO mice (110). Studies examining the Tokyo strain of VDRKO mice, in which the second zinc finger of the DNA-binding domain of VDR is ablated, reported some residual binding of 1,25D in kidney cells (111).

Coimmunoprecipitation analyses have also demonstrated direct binding of the VDR with a component of the tyrosine phosphorylation cascade, namely c-Src, under the influence of 1,25D (119). Transfection of muscle cells with three different anti-VDR antisense oligodeoxynucleotides (ODN), 1,25D-dependent mechanisms by which rapid calcium entry occurs, namely store-operated calcium entry (SOCE) or capacitative calcium entry, are inhibited (113, 114), therefore implying a direct nongenomic role for VDR in calcium handling.

The presence of the VDR has been reported in avian, murine, and human muscle cells on the basis of immunohistochemistry (20, 115), equilibrium binding studies (25), and detection of VDR mRNA by RT-PCR (21). However, these findings are subject to challenge due to the nonspecificity of many of the VDR antibodies (116). A recent paper that used a validated antibody (which did not show bands in VDRKO mice) did not find VDR expression in skeletal, cardiac, or smooth muscle by Western blot and immunohistochemistry (27). Differences in experimental conditions and the possibility of tight protein binding of VDR to DNA may have accounted for this finding. Moreover, low levels may be sufficient for significant function in muscle. Major studies examining the presence of VDR in muscle and the various techniques used have been summarized in Table 2.

Another possibility is that there may be differences in the expression of VDR in muscle in different species and throughout the various stages of muscle differentiation. In support of the latter, in vitro studies predominantly examine the activity of VDR within myoblasts and myotubes rather than fully differentiated adult cells. In vivo, VDR mRNA was reported in the muscle of 3-wk-old wild-type mice but not in their 8-wk-old counterparts (21). The authors suggested a primary role for VDR in early muscle development (21). Thus, the expression of VDR within muscle over time requires further clarification.

Within cultured chick myoblasts, VDR translocates from the nucleus to the cytoplasm quite rapidly (i.e., 1–10 min) after exposure to 1,25D (117). Intact microtubular transport and caveolae structure were essential as demonstrated by the disruption of VDR translocation by inhibition of these individual components in C2C12 cells (i.e., with colchicine and methyl-β-cyclodextrin, respectively) (20). After a longer period of exposure to 1,25D, the VDR appears to translocate back to the nucleus to presumably carry out its role in transcriptional regulation. This shuttling of the VDR between cytoplasm and nucleus indicates its versatility in both rapid and genomic actions, depending on location (118).

Com Immunoprecipitation analyses have also demonstrated direct binding of the VDR with a component of the tyrosine phosphorylation cascade, namely c-Src, under the influence of 1,25D (119). Transfection of muscle cells with three different anti-VDR antisense ODN inhibited 1,25D-dependent dephosphorylation and subsequent activation of c-Src as assessed 15 min after treatment with 1,25D or vehicle (120). More recently, treatment of muscle cells with 1,25D resulted in VDR binding with c-Src, time-dependent increases in c-Src activity, and the redistribution
of c-Src from the periplasmic membrane zone, where it resides under basal conditions, to the cytoplasm and nucleus as seen on confocal microscopy (20). There is also evidence that 1,25D induces the association between c-Src and c-myc, a transcription factor involved in cell growth and apoptosis. This evidence includes coimmunoprecipitation analyses that demonstrate 1,25D-mediated formation of complexes between c-Src and c-myc (20) and significant inhibition (i.e., 94%) of 1,25D-mediated c-myc tyrosine phosphorylation on immunoblot after transfection of muscle cells with anti-VDR antisense ODN (120) or treatment with PP1, a c-Src-specific inhibitor (120). Mechanistically, c-Src probably interacts with VDR and c-myc, both of which are hormone-dependent phosphotyrosine proteins, via its Src homology 2 domain, but this requires further evaluation (121). A role for caveolin-1 in 1,25D-mediated activation of c-Src has also been suggested. Caveolin-1 belongs to a family of membrane-scaffolding proteins with potential roles in different disease phenotypes and binds to c-Src under basal conditions near the cell membrane. After treatment of C2C12 myoblasts with 1,25D, colocalization of caveolin-1 and c-Src was disturbed, and they were redistributed into cytoplasm and nucleus (20). Interestingly, when the caveolae structure was disrupted by methyl-β-cyclodextrin, 1,25D was not able to separate caveolin-1 from c-Src, preventing its activation and also preventing the nuclear translocation of VDR. Therefore, the caveolae and associated proteins appear to play an upstream role in the activation of c-Src. It also appears that 1,25D-mediated activation of c-Src by VDR is a downstream regulator of several nongenomic effects of 1,25D in muscle, specifically involving differentiation and calcium homeostasis, which we will discuss.

Apart from the specific ability of the VDR to translocate from nucleus to cytosol in response to 1,25D, conformational changes within the highly flexible 1,25D molecule also determine the mediation of genomic and nongenomic actions via its receptor. It has been shown that the relatively planar 6-s-cis locked JN [1,25-(OH)2-lumisterol] displays nongenomic activity and that the 6-s-trans locked JB [1,25-(OH)2-dihydrotachysterol] possesses predominantly genomic activity on binding the VDR (69). By molecular modeling of the VDR using atomic coordinates of the protein x-ray structure and computer docking, specific binding sites on the VDR that determine its functional activity have been reported (122, 123). Interestingly, the pockets that, respectively, mediate the regulation of nongenomic and genomic responses overlap and therefore result in mutually exclusive conformational forms of the VDR. It has been suggested that the unbound VDR may possibly exist in the cytoplasm as multiple, equilibrating receptor conformations according to standard statistical distribution (69). Also intriguing, a potential role for 25D in binding the alternative pocket and initiating intracellular calcium flux has recently been reported (123).

Apart from the VDR, it is also possible that other cytosol receptors may be responsible for the rapid actions of 1,25D in muscle. Contrary to an earlier report, it does not appear that annexin II binds to 1,25D in a physiologically relevant manner (124, 125). Recent data have suggested a role for membrane-associated rapid response steroid bind-
B. Calcium homeostasis

Although the nongenomic regulation of intracellular calcium by 1,25D has been well characterized in cultured myoblasts and myotubes, it has also been confirmed by \textit{in vivo} studies on chicks and \textit{in vitro} assessment of differentiated soleus muscle samples. Studies report a time- and dose-dependent increase in intracellular muscle calcium uptake in response to 1,25D. The use of particular agents and antisense oligonucleotides to block components of the signal transduction pathway has led to the elucidation of specific step-wise mechanisms by which vitamin D influences intracellular calcium homeostasis (127–129). These have been depicted in Fig. 6.

First, the rapid mobilization of calcium from the SR into the cytosol relies on 1,25D-dependent activation of two components of the signal transduction pathway, namely c-Src and phosphoinositide-3 kinase. These in turn lead to the activation of phospholipase C\(_\gamma\), the rapid production of inositol triphosphate (IP\(_3\)) and the first of two phases of diacylglycerol (DAG) synthesis from the membrane phosphoinositides (22, 130). It is IP\(_3\) that then mediates the rapid movement of calcium from the SR into the cytosol (130).

In the continued presence of 1,25D, two additional processes are then involved in the more sustained phase of calcium entry from the extracellular compartment, namely SOCE and L-type voltage-dependent calcium-channel (VDCC) entry mechanisms. SOCE relies on several factors including IP\(_3\)-dependent calcium release from the SR that activates calmodulin, CAMKII, and PKC, the latter also being activated by a by-product of a previous reaction, namely DAG (131). The particular channels responsible for SOCE have been identified as the transient receptor potential-canonical-like proteins. Interestingly, a
direct role of the VDR in activating these channels has been suggested by the coimmunoprecipitation of both molecules after treatment with 1,25D in chick skeletal muscle cells (113).

Apart from its reported role in SOCE, evidence supports vital additional roles for PKC in 1,25D-dependent calcium homeostasis in muscle. Rapid translocation of PKC-α from the cytosol to the cell membrane after the in vitro treatment of chick soleus muscle and cultured rat and chick myoblasts with 1,25D (132, 133) and marked reduction in intracellular calcium influx after selective knockout of PKC-α by the use of specific antisense oligonucleotides have been described (128). Furthermore, PKC also activates VDCC-mediated calcium entry as evidenced by the rapid stimulation of 45Ca2+ uptake by cultured myoblasts after treatment with PKC activators, namely DAG and phorbol 12-myristate 13-acetate, inhibited by the addition of nifedipine, an L-type VDCC blocker (133). PKC-α may also have a role in the 1,25D-dependent activation of the ERK1/2 signaling pathway as will be discussed in Section III.D on proliferation and differentiation.

VDCC-mediated calcium entry also relies on 1,25D-dependent activation of the cAMP/PKA pathway. Very rapid increases (within 30 sec) in the levels of adenyl cyclase (AC) and cAMP levels, together with increased PKA activity, occur in differentiated muscle cells and cultured myotubes after 1,25D treatment (127, 134). These studies also report that 1,25D stimulation of VDCC-45Ca2+ entry can be mimicked by treatment with dibutyryl cAMP and abolished by specific inhibitors of AC and PKA.

Another mechanism is emerging by which PKC and cAMP/PKA pathways may cross talk in the regulation of VDCC-mediated calcium flux (90). The preliminary data indicates an increase in the cAMP content of myoblasts after treatment with a PKC activator, phorbol 12-myristate 13-acetate, which may stem from the phosphorylation of Goi that is mediated by PKC in other cells (133). Furthermore, the phosphorylation of Goi in myoblast membranes after treatment with 1,25D is likely to be essential in the stimulation of AC activity as evidenced by the effects of abolishing G protein regulatory pathways on 1,25D-mediated AC activity (134).

In summary, 1,25D induces changes in intracellular calcium levels in cultured muscle cells initially via rapid IP3-dependent calcium shifts from the SR to the cytosol followed by processes resulting in extracellular calcium influx via the activation of SOCE and VDCC activity. These mechanisms are evident in both immature myoblasts and differentiated myotubes, suggesting a potential role for rapid calcium influx in muscle cell differentiation and contraction, respectively (19). In support of this inference, an interesting report from 1974 described a direct correlation between in vitro skeletal muscle dysfunction and demonstrable defects in intracellular calcium handling (136). A group of rabbits, rendered vitamin D deficient by dietary methods, were found to be substantially weaker and hypotonic compared with their vitamin D-replete counterparts and in vitro, displayed significant reductions in calcium uptake in the SR on isolation from psoas muscle.

C. Phosphate homeostasis

Phosphate is an essential substrate in the production of ATP and in protein synthesis. There is early evidence demonstrating that phosphate uptake in muscle may be influenced by 25D (19, 137). The administration of 25D to vitamin D-deficient, phosphate-deplete rats resulted in a significant increase in the in vitro concentration of [32P]phosphate in muscle cells, followed by the stimulation of phosphate-dependent metabolic processes including ATP synthesis in these cells (137). This effect was not reproduced by the repletion of phosphate in these rats. The abolition of 1α-hydroxylase activity by nephrectomy had no effect on 25D-mediated phosphate uptake, implying a direct role of the prehormone in this process. Another study reported the specificity of 25D on in vitro phosphate uptake of differentiated muscle cells with the absence of such effects after 1,25D and 24,25-(OH)2D treatment (138–140).

Another study demonstrated the specific transport of [32P]phosphate across muscle plasma membranes after in vitro vitamin D repletion and 32P labeling of vitamin D-deficient chicks (141). This finding was later confirmed by an increase in vesicle phosphate transport in muscle cells via the isolation of highly purified sarcolemma vesicles in vitamin D-deficient chicks treated with vitamin D (19). The direct effect on phosphate uptake may be mediated via a sodium-dependent mechanism as reported by studies on cultured muscle cells (138, 139).

D. Proliferation and differentiation

There is evidence that 1,25D activates components of the MAPK family in cultured myoblasts, thereby influencing the expression of genes involved in cellular proliferation and differentiation. The majority of research in this area has focused on the effects of 1,25D on the ERK1/2 signaling pathway. The initial activation of c-Src by 1,25D, previously described as an apparent gateway to the nongenomic effects of 1,25D in muscle, results in the rapid activation of Raf-1 by the phosphorylation of its serine residue, which relies on the involvement of Ras and PKC-α (142, 143).

Raf-1 then leads to the activation of MAPK kinase, which activates ERK1/2, after which the phosphorylation...
of a range of proteins and transcription factors including cAMP response element-binding protein and Elk-1 and the increased expression of other proteins relevant to cell proliferation and differentiation, namely c-myc and c-fos, take place (144, 145).

Another MAPK activated by 1,25D in cultured myoblasts is p38. Rapid c-Src-dependent stimulation of MAPK kinases MKK3 and 6 and p38 was demonstrated in C2C12 myoblasts after treatment with 1,25D (146). After this, p38-dependent activation of MAPK2 and subsequent phosphorylation of heat-shock protein 27 was demonstrated. Heat-shock protein 27 has an important role through its association with the actin microfilament system and cytoskeletal remodeling of muscle cells (146, 147). More recently, 1,25D-mediated AKT phosphorylation in differentiating C2C12 cells was also shown to occur via c-Src, p38, MAPK and phosphoinositide-3 kinase pathways (148).

Although little is known about its exact activity in this context, a third member of the MAPK family, namely c-Jun N-terminal kinase-1/2, is also phosphorylatively activated by 1,25D in C2C12 myoblasts (146). Therefore, an intricate system of nongenomic regulatory responses to 1,25D may control cellular proliferation and differentiation of muscle cells, although the relative role of each component remains unclear.

A variety of genomic responses to 1,25D have also recently been elucidated. Apart from being the first to describe the presence of VDR within muscle cells, Simpson et al. (25) also demonstrated dose-dependent reduction in the proliferation of G-8 myoblast cells that were treated with 1,25D and a commensurate reduction in DNA synthesis, suggesting that genomic responses to 1,25D gave rise to the down-regulation in myoblast proliferation and enhanced differentiation into myotubes.

In a recent study, treatment of C2C12 myoblasts with 1,25D for 7 d as opposed to vehicle resulted in increased mRNA and protein expression of transcription factors known to enhance myogenesis, namely myogenic differentiation antigen, desmin, myogenin, and IGF-II and the reduced expression of proliferating cell nuclear antigen and myostatin, which, respectively, enhance cell proliferation and negatively regulate muscle mass (118). Morphologically, cells treated with 1,25D for 10 d displayed significantly increased muscle fiber size and diameter, as indicated by staining for MHC type II a late myogenic marker. These changes were associated with VDR-induced genomic mechanisms as evidenced by significant increases in the expression of the receptor by 4 d of 1,25D treatment and its nuclear translocation at 4 d as opposed to its persistent location in the cytoplasm of cells treated with vehicle.

However, these findings stand in contrast to an earlier report that described the down-regulation of myogenin and myogenic transcription factor 5 (myf5) at an mRNA level in C2C12 myoblasts treated with 1,25D for 48 and 96 h compared with those treated with vehicle (21). Furthermore, this coincided with the reduced expression of neonatal forms of MHC, suggesting myoblast maturation in cells treated with 1,25D. It is possible that differences in study design may have accounted for these contradictory findings, specifically pertaining to myogenin expression. Different durations of treatment, and daily vs. single treatment regimens were employed in these studies (21, 118).

In another study Artaza and Norris (390), vitamin D treatment of mesenchymal stem cells resulted in increased expression of follistatin, an antagonist of myostatin, and caused down-regulation of TGF-β. These vitamin D-mediated changes in gene expression imply that it has a potential role in the inhibition of fibrosis and, perhaps, the promotion of myogenesis and osteogenesis in mesenchymal stem cells.

In a recent study, European sea bass treated with various doses of dietary cholecalciferol from 9—44 d after hatching demonstrated dose-dependent effects in the gene expression of a number of myogenic transcription factors and dose-dependent increases in white muscle fiber size and number (149).

Although vitamin D has clear effects on muscle differentiation, more research is needed to elucidate the nature of these mechanisms. It is also important to note that the overlapping functions and complex regulatory pathways determining the activity of myogenic transcription factors are themselves something of a mystery although recently reviewed in detail (150).

E. Muscle contractile proteins

There is evidence that vitamin D may play a role in the regulation of key components in the cytoskeletal structure of muscle cells. As discussed in Section II.B, the complex interaction between actin and myosin, two cytoskeletal proteins, forms the basis of understanding muscle contraction (Fig. 5). Similar proteins play a potential role in intracellular trafficking and, potentially, GLUT4 translocation.

Significant reductions in components of the actomyosin-troponin complex in the skeletal muscle of vitamin D-deficient rats and rabbits have been reported in two separate studies, although in the latter, this may not have been directly related to 1,25D because in vivo administration of ethane-1-hydroxy-1,1-diphosphate in doses known to inhibit 1,25D had no effect on these components of the cytoskeleton (151, 152). A study also reported an increase in the muscle concentrations of actin and troponin C in chicks replete with vitamin D as opposed to their vitamin D-deficient counterparts (141). Taken to-
gether, these reports suggest a direct role for 25D in the up-regulation of these muscle contractile proteins (141, 151). Furthermore, the direct role of 25D in phosphate homeostasis, as discussed, and the recent demonstration of direct binding between 25D and the alternative pocket of the VDR with subsequent biological effects in COS-1 kidney cells suggest that reconsideration of the ability of 25D to generate biological responses in vivo may be in order (123).

F. Phospholipid composition

Phospholipids, a class of lipids that reside within the cell membrane, have been implicated in a variety of signal transduction pathways, including insulin signaling and calcium handling, and play a role in cell membrane function including caveolae. Alterations in phospholipid composition have been associated with insulin-resistant states (153). There is also evidence of a direct role of 1,25D in the regulation of the phospholipid metabolic pathway.

A study reported higher relative concentrations of phospholipids in the muscle SR membranes of vitamin D-replete vs. -deficient animals (141). There were also significant changes in the levels of particular phospholipids, specifically an increased concentration of phosphatidylcholine and decreases in phosphatidylethanolamine, in the sarcolemma vesicles of vitamin D-replete vs. -deficient chicks (141). Another study suggested that 1,25D treatment led to the activation of specific methylation pathways that leads to the conversion of phosphatidylethanolamine to phosphatidylcholine in muscle cells (154). This is likely to represent a genomic effect as 1,25D-mediated binding of [3H]glycerol and [14C]ethanolamine to phosphatidylcholine in cultured myoblasts was inhibited by the action of actinomycin D, an inhibitor of DNA synthesis. Although the precise significance of genomic 1,25D-mediated influences on phospholipid composition remains uncertain, potential influences on calcium handling, cell proliferation, and insulin signaling merit further consideration.

G. Bone-muscle cross talk and vitamin D

It is well established that muscle strength and muscle mass are important determinants of bone density, bone geometry, and fracture risk. Vitamin D therefore plays a key role in bone metabolism not only through its direct effects mediated by the VDR in osteoblasts and its effects on calcium absorption by the intestines but also through its effects on muscle fiber size and muscle function noted above. Interestingly, during growth, serum 25D levels have been found to be negatively associated with the accrual of bone mineral content in girls (155), and 25D levels decrease as lean mass increases (156). These observations raise the possibility that muscle tissue may require additional vitamin D during growth and that an important function of vitamin D on bone mass accrual may be mediated by the effects of vitamin D on accumulation of lean mass, which has been documented to precede gains in bone (157).

Another pertinent consideration is the role of FGF23, a protein whose regulation is closely linked to phosphate homeostasis and the activation of vitamin D (step 3, Fig. 2). Although 1,25D up-regulates the expression of FGF23 by osteocytes and osteoblasts, FGF23 inhibits 1,25D synthesis and stimulates its breakdown (158). Therefore, FGF23 excess, as seen in those with oncogenic or X-linked hypophosphatemic osteomalacia, leads to reduced 1,25D and phosphate levels and is also associated with muscle weakness. In Hyp mice, a model of X-linked hypophosphatemic rickets, the administration of neutralizing FGF23 antibodies increased 1,25D and phosphate levels as well as leading to improvements in grip strength and spontaneous movement (159). Therefore, the FGF23-vitamin D feedback loop presents another layer of complexity when assessing effects on muscle function.

H. Cell models and molecular pathways for insulin signaling and diabetes

1. Insulin signaling in cell models

Treatment of U-937 human promonocytic cells with 1,25D leads to time- and dose-dependent increases in the mRNA expression of insulin receptors, as shown in two separate studies (160, 161). In the second study, 1,25D treatment also resulted in an increase in VDR expression, suggesting that the accompanying increases in insulin-mediated [14C]2-deoxyglucose uptake and [125]Iinsulin binding in these cells resulted from the activation of genomic pathways. The authors also reported a putative VDRE in the human insulin receptor gene promoter on the basis of luciferase assays on transfected plasmid constructs (162). However, differences in insulin signaling mechanisms between skeletal muscle and immune cells, with the reliance of the latter on GLUT1 rather than GLUT4 transporters for glucose uptake, may limit extrapolation of these results.

In a recent study, differentiated C2C12 muscle cells were rendered insulin resistant and atrophic by treatment with free fatty acids (FFA) (163). However, coadministration of 1,25D with FFA resulted in significant dose- and time-dependent increases in the insulin-mediated uptake of [3H]2-deoxyglucose compared with cells that had received FFA alone. This effect of 1,25D was initially observed at 12 h and reached stability at 36 h, at which point complete reversal of the FFA-mediated insulin resistance was observed (using 10 nM 1,25D). In addition, 1,25D treatment prevented muscle atrophy as demonstrated by significantly increased myotube diameter in cells that had
been cotreated with 1,25D and FFA as opposed to those receiving FFA alone.

To account for these effects on insulin resistance, the authors reported that 1,25D treatment reversed a number of FFA-induced abnormalities in the insulin-signaling pathway. At a protein level, 1,25D significantly inhibited FFA-induced serine phosphorylation of IRS-1 and increased the tyrosine phosphorylation of IRS-1 and the phosphorylation of Akt. In addition, the FFA-mediated activation of c-Jun N-terminal kinase, a protein with a significant role in insulin resistance, was significantly reversed by 1,25D treatment. Thus, this report provided the first demonstration of a direct effect of 1,25D in the restoration of key components of the insulin-signaling pathway in an established cellular model of insulin resistance.

2. Arachidonic acid (AA) release

Apart from direct effects on the composition of membrane phospholipids as discussed earlier, there is evidence that 1,25D leads indirectly to the release of AA by a process involving deacylation of membrane-bound phosphatidylcholine (164). This is relevant because levels of AA, a polyunsaturated fatty acid, in skeletal muscle correlate inversely with insulin resistance in humans. AA may also modulate the function of membrane insulin receptors and glucose transporters and may influence the action of insulin by acting as a precursor for the generation of second messengers such as DAG (90). AA also plays a central role in inflammation, with the production of both pro- and antiinflammatory metabolites that may also have potential effects on insulin signaling.

In chick myoblasts, 1,25D treatment resulted in dose-dependent increases in the release of [3H]AA, but the effect appeared to be dependent on the influx of extracellular calcium and the indirect activation of phospholipase A_2 by PKC (164). More research is needed to clarify the possibilities that arise from an effect of 1,25D on AA release and its availability for intracellular processes.

3. Caveolin-I mediated insulin sensitivity

Some data suggests that vitamin D may play a role in caveolin-I mediated insulin sensitivity (165). Caveolin-I, a scaffolding protein within the caveolar membrane, has recently been shown to play an important role in insulin sensitivity. Its selective down-regulation in the skeletal muscle of wild-type mice and its reduced expression in JYD mice, an age-dependent type 2 diabetes model, were associated with significant impairments in insulin sensitivity (166). VDR is present within the caveolae, appear in close proximity to caveolin-1 on confocal microscopy after treatment of ROS 17/2.8 cells with 1,25D (110) and relies on caveolin-I for the mediation of nongenomic effects within skeletal muscle cells (20), all supporting a close association between vitamin D and caveolin-I and raising the question as to whether vitamin D might also have an impact on caveolin-I mediated insulin sensitivity.

Perhaps the strongest evidence to support this possibility is the combination of marked insulin resistance and vitamin D resistance in humans with homozygous nonsense caveolin-I mutations, otherwise known as Berardinelli-Seip congenital lipodystrophy (167). Future research may address this intriguing conceptual link.

IV. Vitamin D and Muscle: Studies in Animal Models

A. VDRKO mice

The VDRKO mouse model has provided valuable insights into the biological function of the vitamin D endocrine system and, specifically, the genomic activity of the transcription factor VDR (13). The role of vitamin D in the development, morphology, and function of skeletal muscle has been made clearer by studies on this mouse model.

The earliest form of the VDRKO mouse, generated by targeted ablation of the DNA encoding the second zinc finger of the DNA-binding domain, was described in 1997 (28). Mice appeared phenotypically normal at birth, despite known expression of the VDR in fetal life in wild-type mice, but became hypocalcemic with secondary hyperparathyroidism around weaning (21 d) and developed alopecia associated with large dermal cysts by 4 wk of age and rickets and growth retardation by d 35. The initial lack of hypocalcemia is considered to be due to the early presence of nonsaturable 1,25D-independent mechanisms of intestinal calcium absorption. However, when 1,25D-dependent mechanisms take over, mice require a high calcium (2%), phosphorus (1.25%), and lactose (20%) rescue diet for survival. The alopecia is interesting, because people with mutations in VDR (vitamin D-resistant rickets) also develop alopecia (168, 169).

1. Muscle morphology and development

A study in 2003 described histological changes in the muscles of VDRKO mice directly before and after the development of hypocalcemia. At 3 wk of age, samples taken from the quadriceps muscle of VDRKO mice displayed a wider degree of variability in fiber diameter in addition to significant reductions in the size of both type I and II fibers compared with those in wild-type mice (21). By 8 wk of age, generalized atrophy of type I and II muscle fibers had worsened in the VDRKO mice, suggesting progression due to the absence of the VDR or the additional effect of systemic biochemical changes that had not been present at 3
wk. These changes were also reported in VDRKO mice on a high-calcium, -phosphorus, and -lactose rescue diet, suggesting that the absence of VDR was the predominant cause rather than systemic biochemical changes. Neither degenerative nor necrotic changes were observed in VDRKO skeletal muscle, and similar results were obtained in a range of other muscle groups in this model, suggesting a diffuse effect of the VDR on skeletal muscle morphology.

Impaired regulation in the expression of particular myogenic transcription factors, known to control muscle phenotype, was reported as an explanation for these findings. On immunohistochemistry, Northern blot, and RT-PCR analyses, the expression of myf5, myogenin, and E2A was significantly higher in quadriceps samples of 3- and 8-wk-old VDRKO mice compared with age-matched wild-type mice. Persistent expression of immature forms of MHC was also found in the small muscle fibers of VDRKO mice but not in their type II muscle fibers. The expression of two other myogenic transcription factors, namely MyoD and MRF4, were not particularly affected in VDRKO mice.

Although these data support a role for vitamin D in the regulation of muscle development, precise genomic mechanisms by which the VDR influences myogenesis are unclear, and the issue is further complicated by the failure to identify negative VDRE in the promoter region of the genes encoding myf5 and myogenin (170, 171).

2. Muscle strength and functional assessment

A number of studies have examined muscle strength and performance in VDRKO mice. These investigations rarely assess muscle function in isolation, with results being potentially influenced by a range of other factors including cardiovascular endurance, balance, and the ability to learn new skills. Furthermore, behavioral differences between VDRKO and wild-type mice have been reported, which may confound the assessment of some tests (172). Nevertheless, these studies provide an indication of the functional motor deficits associated with loss of VDR by testing swimming ability and motor coordination.

a. Forced swim test analysis. Three studies have described differences in the swimming behavior of VDRKO mice. In one study comparing six VDRKO mice with 10 wild-type and heterozygote mice that were shaved to account for alopecia-related alterations in buoyancy, severe impairment of swimming was seen in the knockout mice (173). Baseline levels of locomotor, sensory, and vestibular activity were similar. On swim testing, they were observed to swim in a predominantly vertical position, had a significantly greater number of sinking episodes, and displayed stereotypic rotations and catatonic-like upper limb spasms.

In another study, a significantly greater number of sinking episodes were seen among eight VDRKO mice compared with six wild-type controls (174). However, on being given a stimulus, they showed no impairment in ability to move down a 1-m laneway or in reaching a visible platform. However, VDRKO mice did display greater fatigue after the swim based upon differences in rearing and grooming behavior. The authors hypothesized that this was due to the reduced availability of calcium in VDRKO mice after exercise.

A third study reported that abnormal patterns of swimming behavior among VDRKO mice were more marked in older (5–13 months) rather than younger knockouts (175). Interestingly, no such impairment in swimming ability was seen among a group of 11 1α-hydroxylase (Cyp27B1)-knockout mice (175). That suggests the intriguing possibility that there might be vitamin D-independent effects of VDR upon muscle function (175).

b. Tests of motor coordination. The time taken for a mouse to fall off a device that rotates at fixed speeds and in acceleration is considered to indicate its degree of motor coordination (174). In one study, VDRKO mice stayed on a rotating device for a significantly shorter time than wild-type mice on both the accelerating and fixed-speed rotarod tests (174). On gait assessment, VDRKO mice took significantly shorter steps and traveled a shorter distance than wild-type mice when placed in an open field for 5 min.

Significantly shorter retention times on accelerated rotarod testing were confirmed in another group of 12-month-old VDRKO mice compared with age-matched wild-type mice (175). These 12-month-old mice demonstrated similar impairments in muscle coordination in tilting box and tilting tube tests, which measure the latency and the angle at which the animal displaces off a device that, respectively, tilts or rotates at different angles. However, no differences were found in 6-month-old VDRKO mice.

In another study, the vertical screen test that measures the time taken for a mouse to fall from a screen that becomes suddenly vertical while the mouse rests on it in a horizontal position (i.e. retention time) was employed (173). VDRKO mice demonstrated markedly shorter retention times compared with both wild-type and heterozygous groups, implying impaired motor coordination or strength.

In summary, VDRKO mice demonstrate notable defects in their overall motor performance with significant impairments in their ability to remain afloat while swim-
ming and shorter stride length and impaired motor coordination and balance on rotarod, rotating tube, and tilting box tests. Although the effect of alopecia was accounted for in one study, other biochemical and behavioral changes in VDRKO mice together with the widespread expression of the VDR in the central nervous system, vestibular system, and spinal cord under normal conditions make direct assessment of its role in muscle function difficult in this setting (175). Nevertheless, it is clear that the overall motor function of these mice is impaired and that further work is needed to clarify the individual components that may account for this.

B. Other animal models

Although the VDRKO mouse model has provided valuable insights into the biological activity of vitamin D, it is more accurate to consider this as a model of type II vitamin D-dependent rickets rather than vitamin D deficiency. Studies have examined muscle function in animals rendered vitamin D deficient by a range of dietary and other methods (176). In a study from 1978, vitamin D-deficient male Sprague-Dawley rats demonstrated significantly prolonged times to peak tension and recovery times in electrically stimulated soleus muscle contraction compared with vitamin D-replete rats on normal chow (177). These changes normalized after vitamin D repletion. In contrast, no impairments were noted in rats rendered phosphate deficient or calcium deficient 10 d before experimentation.

A study from 1979 examined muscle function in chicks raised from hatching on a vitamin D-deficient diet (178). Significant reductions in the tension generated by the triceps surae during stimulation of the posterior tibial nerve compared with that in vitamin D-replete chicks were found. This occurred independently of calcium and phosphate levels. The authors found no difference in the histological appearance of muscle samples but did isolate reduced muscle mitochondrial calcium levels. This was independent of serum calcium levels and was proposed as the mechanism by which vitamin D deficiency affected muscle contraction force.

Four weeks after the commencement of a vitamin D-deficient diet, rats displayed marked skeletal muscle hypersensitivity on calf compression compared with those fed a normal diet (179). This finding was not related to hypocalcemia but was rather accelerated by increased dietary calcium and was accompanied by early impairments in balance on a beam-walk test to assess the frequency of foot slips. Histologically, vitamin D-deficient rats displayed increased numbers of presumptive nociceptor axons in skeletal muscle, providing an explanation for their hyperalgesic phenotype.

In contrast to these studies, a recent report questioned the primary role of vitamin D, rather than biochemical changes associated with vitamin D deficiency, in resulting in myopathy (176). In 58 male Wistar rats that were rendered vitamin D, phosphorus, and calcium deficient by dietary methods and housing under incandescent lighting, muscle strength of the soleus as assessed by a force transducer detecting isometric contraction was significantly reduced compared with that in replete controls (176). The authors concluded that phosphorus deficiency was the primary culprit on the basis of several findings. These included a direct independent correlation between phosphorus levels and the reduction in soleus muscle force in these animals, complete restoration in slow-twitch muscle force after dietary repletion with phosphorus despite persistent vitamin D deficiency and similar measures of muscle function among phosphorus-replete rats that were either vitamin D deficient or replete. Similarly, there was no difference in muscle contraction among rats that were calcium deficient and replete, in which vitamin D and phosphorus levels were within the reference range. The central importance of phosphorus in the production of ATP, essential for muscle contraction, was considered as the explanation for these findings.

Therefore, the difficulty in differentiating the effects of severe vitamin D deficiency from those of hypocalcemia and hypophosphatemia in the development of muscle pathology is common to animal and human clinical studies. However, cell lines and tissue culture enable study of direct effects, and these studies date back to the 1970s.

C. Animal studies on insulin sensitivity and diabetes

Although vitamin D deficiency has been associated with more aggressive disease among nonobese diabetic mice (180), a model for type 1 diabetes, few animal trials have addressed the role of vitamin D in insulin resistance. In one study of ob/ob mice, an obese type 2 diabetes model, significant improvements in hyperglycemia and hyperinsulinemia in response to treatment with 1α-hydroxyvitamin D$_3$ (1α-OHD$_3$) were observed (181). In another study of aged rats with type 2 diabetes and insulin resistance that received 25D, 1α-OHD$_3$, or no treatment, serum 1,25D levels correlated positively with the glucose infusion rate on euglycemic clamp studies at the end of the 12-wk treatment period (182), suggesting a potential role in insulin sensitivity. However, the main focus of this study was the demonstration of bone loss among insulin-resistant rats attributed to a reduction in their renal 1α-hydroxylase activity.
V. VDR Polymorphisms and Muscle Function

Several single-nucleotide polymorphisms (SNP) in the gene encoding the VDR have been associated with a range of phenotypic characteristics including muscle strength. These have been summarized in Table 3.

A. FokI polymorphism

The FokI polymorphism of the VDR gene is a T/C transition in exon 2 of the VDR gene that results in a shorter 424-amino-acid protein with enhanced transactivation capacity using a reporter gene assay (184). Although this greater VDR activity might suggest improved muscle strength in light of some of the clinical data, the FokI polymorphism is associated with reduced muscle strength in two studies in men (185, 186). It is interesting to speculate that increased VDR function could increase CYP24A1 expression and thereby degradation of 1,25D.

Among 302 Caucasian men (aged 58–93 yr), those who were homozygous for the FokI polymorphism displayed significantly lower fat-free mass and appendicular muscle mass on dual-energy x-ray absorptiometry (DEXA) scanning (185). Furthermore, men with this polymorphism demonstrated a 2.17-fold higher risk of sarcopenia (defined by appendicular fat-free mass <7.26 kg/m²), independent of age. Lower fat-free mass was associated with significantly lower quadriceps muscle strength. Differences in quadriceps strength were not significant after adjusting for fat-free mass, suggesting that the difference was mediated by altered muscle mass.

B. BsmI polymorphism

The data are less consistent for the BsmI polymorphism. This SNP is located in the 3’ region of the VDR gene, known to play an important role in the regulation of gene expression (188). In a group of 121 nonobese, healthy women over 70 yr of age, the bb genotype was associated with 23% higher quadriceps strength and 7% higher wrist strength compared with those with the BB genotype (189). However, among the 380 obese women over the age of 70 in this same study, no effect was found.

Conversely, a study of 175 young healthy women (age range 20–39 yr) in Belgium found that those with the bb genotype had lower hamstring strength on dynamometer and lower fat-free mass on DEXA compared with the BB genotype (190). The difference in hamstring strength became of borderline significance after adjustment for age, fat mass, and lean mass. Interestingly, the significance of another polymorphism known to be in linkage disequilibrium with the BsmI B allele, namely the polyadenosine repeat (ss genotype), was associated with higher hamstring strength and greater body weight and fat mass compared with those with LL genotype in this study.

In another study that included 107 patients with stable chronic obstructive pulmonary disease and 104 healthy, age-matched controls, homozygosity for the FokI polymorphism was associated with reduced quadriceps strength compared with heterozygosity or control subjects (186). The difference became more significant in a model that adjusted for age, sex, forced expiratory volume in 1 sec (FEV₁), fat-free mass, and angiotensin-converting enzyme genotypes. There was no evidence that the presence or absence of lung disease affected the relation between FokI genotype and quadriceps strength.

Among 240 women (41.5 ± 13.2 yr), those with the FokI polymorphism (FF) had weaker isometric knee extensor strength (P < 0.05 for both 90° and 120° incline) vs. those who were heterozygous (Ff) or lacking the polymorphism (ff) (187). However, on adjusting for age, height, and total fat-free mass, the differences were no longer significant, again suggesting that the polymorphism may be affecting muscle mass.
<table>
<thead>
<tr>
<th>Study (Ref.) other</th>
<th>n, Sex, Age</th>
<th>Polymorphisms and groups</th>
<th>Outcome and adjustments</th>
<th>Findings and effects</th>
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<tr>
<td>Barr et al. (359)</td>
<td>3145</td>
<td>BsmI</td>
<td>Falls (self-reported)</td>
<td>More falls OR 1.5 (1.01–2.3)</td>
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<td>BB, Bb vs. bb</td>
<td>Adj: age, Ht, Wt, 25D, month</td>
<td>More falls, ↓ STST</td>
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<td>OPUS</td>
<td>2374</td>
<td>BsmI</td>
<td>Falls, leg force, STST</td>
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<td>121</td>
<td>BsmI</td>
<td>Quadriceps strength</td>
<td>Stronger by 23%</td>
</tr>
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<td>bb vs. BB</td>
<td>Adj: age, calcium, BMD</td>
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<td>bb vs. BB</td>
<td>Adj: age, calcium, BMD</td>
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<td>FokI</td>
<td>Quadriceps strength</td>
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<td>240</td>
<td>FokI</td>
<td>Quadriceps strength</td>
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<td>F, 42</td>
<td>FF, Ff vs. Ff vs. ff</td>
<td>Adj: age, Ht, FFM</td>
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<td>302, M 58–93</td>
<td>FokI</td>
<td>Quadriceps strength</td>
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<td>vs. b/T</td>
<td>Adj: age, Ht, FFM FPG</td>
<td></td>
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<tr>
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<td>259</td>
<td>BsmI</td>
<td>Quadriceps strength</td>
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<tr>
<td>CommD</td>
<td>M, F, &gt;80</td>
<td>BB/Bb vs. bb</td>
<td>Adj: age, sex, ADL, co-morbidities</td>
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<td>107 COPD,</td>
<td>M, F</td>
<td>FF vs. Ff and ff</td>
<td>Adj: age, sex, FFM, ACE</td>
<td>Weaker in all (41 vs. 46 kg)</td>
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<td>104 Normal</td>
<td>M, 55</td>
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<td>Hamstring strength, FFM</td>
<td>Both lower H5: r = −0.18</td>
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<td>Polyadenosine repeat LL vs. ss</td>
<td>Hamstring strength, FFM</td>
<td>Trend lower</td>
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<td></td>
<td></td>
<td>Adj: nil</td>
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<tr>
<td>Bahat et al. (192)</td>
<td>120</td>
<td>BsmI BB vs. bb</td>
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<td>Turkish</td>
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<td>Wang et al. (191)</td>
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<td>BsmI: BB vs. bb</td>
<td>Hamstring strength</td>
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<td>Chinese</td>
<td>F, 21</td>
<td>AplI: AA vs. aa/Aa</td>
<td>Adj: nil</td>
<td>Lower: 29%</td>
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<td>Oh and Barrett-Connor (360)</td>
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<td>BsmI: bb vs. BB</td>
<td>HOMA-IR</td>
<td>↑ HOMA, normal</td>
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<td>242 DM</td>
<td>M, F, 72</td>
<td>AplI: aa vs. AA</td>
<td>T2D incidence. Adj: nil</td>
<td>↑ T2D</td>
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<td>BsmI</td>
<td>FPG</td>
<td>↑ FPG, ↑ IGT</td>
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<tr>
<td>Aircrew</td>
<td>M, F, 33</td>
<td>BB vs. Bb or bb</td>
<td>Adj: nil</td>
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<tr>
<td>Malecki et al. (362)</td>
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<td>FokI, AplI, BsmI, TaqI</td>
<td>T2D</td>
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<td>M, F</td>
<td>TaqI TT vs. Ta/tt</td>
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<td>Ye et al. (363)</td>
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<td>BMI at onset T2D</td>
<td>BMI 29 vs. 32</td>
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<td>Foki, BsmI, TaqI</td>
<td>T2D Adj: nil</td>
<td>No association</td>
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<td>Indian 100 T2D</td>
<td>M, F, 49</td>
<td>Combinations</td>
<td>T2D Adj: nil</td>
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<td>Speer et al. (365)</td>
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<td>BsmI</td>
<td>Postprandial C-peptide</td>
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<td>18–83</td>
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<td>Fasting insulin. Adj: nil</td>
<td>↑ insulin</td>
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<td>BsmI: BB vs. bb</td>
<td>BMI Adj: nil</td>
<td>↑ BMI</td>
</tr>
</tbody>
</table>

Each section is listed in order of number of participants, highest to lowest. ACE, Angiotensin converting enzyme genotype; Adj, adjustments; AplI, appendicular muscle mass; ADL, activities of daily living; APOSS, Aberdeen Prospective Osteoporosis Screening Study; COPD, chronic obstructive pulmonary disease; F, female; FFM, fat-free mass; FPG, fasting plasma glucose; Ht, height; IGT, impaired glucose tolerance; M, male; N, normal group; OPUS, Osteoporosis and Ultrasound Study; OR, odds ratio; PP, postprandial; T2D, type 2 diabetes mellitus; Wt, weight.
extensors at 120°/sec was significantly lower in the AA homozygous group compared with the aa and Aa groups.

In a study of 253 men (54.9 ± 10.2 yr), Bt homozygotes (i.e., those with BB genotype at the BsmI site and tt genotype at an associated polymorphism site, namely TaqI) had higher isometric quadriceps strength at 150° on isokinetic dynamometer than b or T allele carriers without and with adjustment for confounding factors (P = 0.01 after adjustment) (187). However, no such association was found when this same parameter was tested at 90° or 120° or when assessing knee flexor strength.

Among 120 Turkish men (>65 yr), knee extensor strength on dynamometer was significantly higher in those with BB homozygosity at the BsmI site than in the Bb/bb group, but no significant association between muscle mass and strength was found (192).

Two population studies examining the rate of falls suggest that the bb genotype of the BsmI polymorphism may be protective against falls. In a study from Italy that included 259 community-dwelling older patients (>80 yr of age), the rate of falls differed according to BsmI genotype with more seen among those with BB or bb genotype on multivariate analysis (193).

Data collected from two separate population cohorts of older women, namely the Aberdeen Prospective Osteoporosis Screening Study (APOSS) and Osteoporosis and Ultrasound study (OPUS), also identified a greater incidence of self-reported recurrent falls among those with the BB genotype of the BsmI polymorphism compared with those with the bb genotype (194). Significant differences in function with greater ease in rising from a chair were seen in bb homozygotes compared with carriers of the B allele. These studies failed to demonstrate an association between FokI polymorphisms and balance or muscle power measurements.

Apart from VDR polymorphisms, a recent population-based study that involved 153 men and 596 women (65–101 yr) reported an association between SNP in the CYP27B1 gene (i.e., +1260 and +2838) and the risk of fracture over a 2.2-yr follow-up period (195). There was no difference in the risk of falls among subjects, and muscle strength was not examined. There are limitations in the interpretation of these data. Larger studies to assess the association between muscle strength and genetic polymorphisms are needed, and more functional studies of effects on VDR function are required.

C. VDR polymorphisms and insulin resistance/type 2 diabetes

Particular polymorphisms in the gene encoding the VDR may be associated with the development of insulin resistance and type 2 diabetes among certain populations. However, once again, the data are inconclusive due to the generally small sample size in these studies and the variability in the populations and examined endpoints. These studies have been summarized in Table 3.

VI. Vitamin D and Muscle: Human Studies

A. Myopathy

Rickets and osteomalacia have been associated with muscle weakness and hypotonia for centuries (196, 197). Weakness affecting the proximal lower limb musculature was reported in a group of adults with osteomalacia who responded to high-dose vitamin D therapy in the 1960s (198). The paper did not report whether all patients responded or whether responses were complete.

In addition to general weakness, more specific proximal muscle deficits are commonly described, including difficulty rising from a seated or squat position, ascending a flight of stairs, or lifting objects (199–201). Changes in gait, often described as waddling or penguin-like in appearance, are widely reported and are possibly a combined result of bone pain, muscle pain, and proximal weakness (199, 202). Pictures of a child with rickets demonstrates the potentially profound effects of vitamin D deficiency (Fig. 7). However, the classic pattern of proximal weakness seen in vitamin D deficiency is not specific. Many endocrine and metabolic disorders including renal failure, hyperparathyroidism, hypophosphatemia, Cushing’s syndrome, and hyperthyroidism as well as glucocorticoid therapy may display similar clinical features (203, 204). Electromyographic changes seen in vitamin D-deficient subjects with muscle weakness confirm myopathy, but without specific features (201). In the reports of myopathy with vitamin D deficiency, many subjects had multiple biochemical abnormalities involving calcium, phosphate, and PTH that co-corrected with vitamin D repletion, making it difficult to assess the individual role of each component in the development of osteomalacic myopathy (205). These observations formed the basis for the belief that myopathy in these subjects was not directly related to vitamin D deficiency but rather a general result of osteomalacia and its associated biochemical abnormalities (206).

Observational and uncontrolled treatment studies

The reversibility of myopathy with vitamin D supplementation has been described in some case series (200, 207, 208). A recent series described the presence of progressive muscle weakness among young vitamin D-deficient veiled women from Saudi Arabia [90% had 25D < 8 ng/ml (20 nmol/liter)] (205). Some women required a wheelchair. Substantial improvements followed 3 months
of vitamin D and calcium supplementation (800 IU and 1200 mg daily, respectively). Wheelchair-bound patients walked independently by the end of the study.

In another case series, five patients with myopathy resulting in wheelchair use were treated with vitamin D$_2$ (50,000 IU weekly) (209). At baseline, they were deficient (25D = 5–13 ng/ml (12–32 nmol/liter)) with secondary hyperparathyroidism (intact PTH range = 13–89 pmol/liter). Treatment resulted in marked improvements in strength, pain, and mobility within 4–6 wk, despite persisting hyperparathyroidism. The authors suggested that this indicated a role for vitamin D independent of PTH.

A study assessing vitamin D-deficient women (25D = 7 ng/ml [17 nmol/liter]) vs. controls with higher levels (25D = 19 ng/ml [47 nmol/liter]) reported an independent association between 25D levels and maximal voluntary knee extension force (208). No correlations were found for PTH or total or bone-specific alkaline phosphatase.

A common theme in these case series that describe patients with muscle pain and weakness is the high rate of initial misdiagnosis. The diagnoses of diabetic neuropathy, general debility, motor neuron disease, orthopedic disorders, psychiatric conditions, or inherited myopathy were described before the recognition of vitamin D deficiency (205, 209, 210). The nonspecific clinical features of vitamin D-deficiency myopathy, the wide range of severity from mild weakness to debilitating pain and immobility, and low index of suspicion may contribute to the frequent delay in the diagnosis.

We are not aware of any reports of muscle biopsy or muscle function studies in people with mutations in VDR.

B. Myalgia and vitamin D deficiency

People with vitamin D deficiency and proximal myopathy (i.e., weakness of the proximal musculature arising from muscle pathology) often have associated proximal myalgia (i.e., muscle pain) (198). Many authors have also proposed that low vitamin D is associated with more diffuse muscle pain (211–213). However, this is controversial, and other studies do not support this (214–216). The issue is made more difficult because osteomalacia is associated with bone pain and microfractures, making causal discrimination of the source of pain challenging. Because it is conceptually obvious that people with muscle pain may be less likely to exercise, go out, and carry out normal outside activities of daily living, establishing cause and effect is important. Ideally, demonstrating a therapeutic response would clarify the issue.

1. Observational studies

A cross-sectional study of 3075 men from eight European centers found that those who reported chronic widespread pain (8.6%) were more likely to have low 25D levels [<15 ng/ml (37.5 nmol/liter)] (217). However, the relationship was attenuated by adjusting for age, season, activity, and other factors.
Ninety-three percent of 153 patients who were being assessed for persistent, nonspecific musculoskeletal pain in Minneapolis were vitamin D deficient [mean 25D = 12 ng/ml (30 nmol/liter)] (211). All African-American, East African, Hispanic, and American Indian patients had 25D below 20 ng/ml (<50 nmol/liter) as did 82% of Caucasian patients. None had fibromyalgia or medical conditions known to decrease production, absorption, or hydroxylation of vitamin D. This study did not include a control group of similar ethnicity.

The propensity for particular ethnic groups, including those migrating from Asia and the Indian subcontinent to Western countries, to develop myalgia and bone pain as the primary manifestation of vitamin D deficiency has been reported since the 1970s (218, 219). A study describing 33 mostly Somali female asylum seekers with musculoskeletal pain and low 25D [<8.5 ng/ml (21 nmol/liter)] found that vitamin D and calcium supplementation led to the symptom resolution in 22 (66.7%) by 3 months (220). The authors noted a 2.5-yr mean lag time between symptom onset and diagnosis. There was no control group. A study from the United Arab Emirates found that 86% of patients who were initially diagnosed with fibromyalgia or nonspecific muscle pain were vitamin D deficient. The majority reported improvement in response to supplementation (221). Another study compared cultural differences in the reporting of muscle pain among South Asians (i.e., Indian, Pakistani, and Bangladeshi) and white Europeans living in England (212). Reporting of widespread pain was significantly more common in the 1945 South Asians. However, in the 137 South Asians in whom 25D was measured, there was no association between deficiency and pain.

A report describing myalgia in six women who had migrated to the Netherlands considered vitamin D deficiency [<8 ng/ml (<20 nmol/liter) in 5 patients] to be the cause. There was a lengthy lag period (7–103 months) from the onset of symptoms to diagnosis (222). In three cases, misdiagnosis led to treatment with prednisone, estrogen, or cholecystectomy. Vitamin D and calcium supplementation was effective in reversing the myalgia in each case.

2. Case-control studies

Muscle pain was recently proposed as a marker for vitamin D deficiency among Aboriginal Australians (223). A case-control study of eight urban Aboriginal patients with muscle pain and eight matched Aboriginal controls without pain reported significantly lower vitamin D levels among those with pain [17 vs. 23 ng/ml (41 vs. 58 nmol/liter), P = 0.017].

Wide cultural and gender differences in the reporting of pain, subjective features in the diagnosis of fibromyalgia, and the presence of other features known to affect vitamin D status among patients with persistent pain syndromes confound the assessment of the role of vitamin D deficiency in muscle pain. Also, the observational nature of these studies does not equip them to address the question of causality.

3. Randomized controlled trials (RCT) for myalgia

We identified only one randomized placebo-controlled trial of supplementation where treatment of muscle pain appeared to be the primary endpoint. The study examined people with diffuse muscle pain (214). Fifty subjects with 25D below 20 ng/ml (50 nmol/liter) at baseline were randomized to placebo or vitamin D₂ 50,000 IU weekly for 3 months. There was no benefit of treatment. The authors note that 50% of the placebo group achieved normal vitamin D levels during the study; however, the improvement in pain scores was not substantial for either group. Using the PowerStat program (224), we calculate that the study had 80% power with α of 0.05 to detect a difference of ± 19 in pain score. The baseline visual analog pain score in the treatment group was high at 67 ± 23, so if there is any benefit, it is probably smaller than this.

4. Summary: myalgia

Vitamin D deficiency with osteomalacia is associated with muscle pain that in most cases resolves with treatment. The pain is more commonly located in large proximal muscle groups rather than displaying diffuse distribution (198, 222, 225, 226) and is often associated with bone pain and other features of osteomalacia and myopathy (205, 208).

Thus, for pain (proximal or diffuse) in patients without osteomalacia, other etiologies should also be considered. In patients with diffuse pain, without obvious osteomalacia, the data remain inconclusive. There are strong associations, but the only RCT found no convincing benefit of supplementation. The trial was adequately powered to detect a clinically meaningful change in pain score.

There are no RCT examining specific treatment of proximal muscle pain. Larger randomized placebo-controlled trials should be carried out, preferably with stratification by baseline vitamin D status, in people with fibromyalgia and in people with proximal myalgia. There is no evidence to support supplementation for myalgia in people with normal levels.

C. Fibromyalgia

Fibromyalgia is not purely a muscle disorder, but there are a number of studies examining the potential associa-
tion between it and vitamin D. Myalgia was examined in a cross-sectional study of 6824 white, middle-aged subjects living in the United Kingdom. A significant association between fibromyalgia, defined by the American College of Rheumatology criteria, and 25D was found among women (227). However, no such association was found in men, although they reported similar rates of pain (11.4% for men, 12.5% for women). No association with myalgia was found.

In two separate case-control studies of patients with fibromyalgia from the United States and Brazil, no statistically significant differences were found in 25D levels (214, 228).

In contrast, another case-control study that examined 40 premenopausal women with fibromyalgia and 37 controls found a significantly higher proportion of vitamin D deficiency [25D < 8 ng/ml (20 nmol/liter)] among women with fibromyalgia (229). However, this was not adjusted for physical activity, smoking, or body mass index (BMI). Fibromyalgia symptoms did not differ depending on the vitamin D status. A subset of deficient patients who received supplementation (eight of 18) reported subjective improvement that persisted at 3 months; however, 10 of 18 did not.

One placebo-controlled study examining the effect of vitamin D on fibromyalgia was identified (230). In that study of 138 patients with fibromyalgia, the subset of 100 patients with mild to moderate vitamin D deficiency and insufficiency [25D = 10–25 ng/ml (25–62.5 nmol/liter)] who were randomized to receive vitamin D₃ (50,000 IU weekly) showed significant improvement over 8 wk vs. placebo-treated controls. However, this did not persist at 1 yr, and in the same study, the subset of 38 people with severe deficiency [25D <10 ng/ml (25 nmol/liter)] who received vitamin D in an unblinded fashion did not report any improvement at either 8 wk or 1 yr (230).

Overall, there is conflicting evidence regarding the possibility of an association between low vitamin D and fibromyalgia, and clearly, having fibromyalgia may have an impact on time spent outdoors/exercising. The data from the one randomized placebo-controlled trial do not provide a clear answer, because the most deficient subjects did not benefit, but less deficient subjects did. More research is needed in this area.

D. Drug-related myopathy and vitamin D

1. Aromatase inhibitors

The effect of vitamin D supplementation on myalgia due to a drug class, namely aromatase inhibitors, has been recently reported. For this class, there is both observational and randomized controlled data, and myalgia is a very common side effect.

An observational study found significantly less musculoskeletal pain among 60 women on letrozole who had achieved median 25D levels over 66 ng/ml (165 nmol/liter) compared with women with levels below 66 ng/ml (165 nmol/liter) (19 vs. 52%) after weekly supplementation with 50,000 IU vitamin D₃ for 12 wk (231).

In a double-blind RCT, 60 patients with early-stage breast cancer with new or worsening musculoskeletal pain on aromatase inhibitor therapy were randomized to receive either high-dose vitamin D supplementation at a regimen that depended on their baseline 25D or placebo (232). Those with 25D of 10–19 ng/ml (25–47 nmol/liter) received 50,000 IU vitamin D₃ for 16 wk, whereas those with 25D of 20–29 ng/ml (50–72 nmol/liter) received 50,000 IU for 8 wk (232). There were significant improvements in pain at 2 months in the vitamin D group vs. placebo on the basis of several indices. Therapy was decreased from weekly to monthly after 2 months in most subjects. The beneficial effects did not remain at the 4- and 6-month visits. There was evidence of a dose-response effect; women who were more deficient had greater benefits, and in that subgroup, the beneficial effect was seen across the whole study period.

2. 3-Hydroxy-3-methyl-glutaryl-coenzyme A reductase inhibitors (statins)

A number of reports suggest that vitamin D deficiency may potentiate myopathy in patients on lipid-lowering statin therapy (3-hydroxy-3-methyl-glutaryl-coenzyme A reductase inhibitors) (233–242). These reports are both anecdotal and based on case series and cross-sectional studies. Not all studies have confirmed this relationship (237).

Cross-sectional and cohort studies. Among a group of 621 statin-treated patients, the 128 who reported myalgia had lower serum 25D (238). Levels were low [defined as <32 ng/ml (<80 nmol/liter) in that study] in 64% of myalgic patients vs. 43% of the others. A subset of 38 of the 82 25D-deficient myalgic patients were treated with 50,000 IU/wk of vitamin D₃ for 12 wk (unblinded) while continuing statins, and 35 had their myalgia resolve. The three nonresponders achieved similar vitamin D levels.

A prospective study of 150 statin-intolerant patients with 25D below 32 ng/ml (80 nmol/liter) reported that 83% were free of myalgia and no longer statin intolerant after approximately 8 months of vitamin D₃ treatment (50,000 IU twice weekly for 3 wk and then 50,000 IU weekly) (240). However, treatment had little effect on elevated creatine phosphokinase (CPK).

However, not all studies find an association. A retrospective study of 6808 statin users found no correlation...
between 25D levels, myalgia, or CPK levels (242). A smaller study of 129 patients on statins also found no difference in the 25D levels of those with and without myalgia (237).

Some hypotheses have been proposed regarding mechanisms by which vitamin D deficiency may potentiate statin-induced myalgia. The suggestion that statins may reduce vitamin D synthesis by inhibition of synthesis of its cholesterol precursor has not been supported by clinical studies (243, 244). The enzyme CYP34 demonstrates in vitro 25-hydroxylase activity (245), and it has been speculated that vitamin D deficiency may lead to preferential shunting or use of this enzyme for vitamin D hydroxylation, reducing its availability to metabolize (deactivate) statins (239). This hypothesis could be tested by measuring statin levels in vitamin D-deficient and vitamin D-sufficient individuals matched for the presence or absence of myopathy and/or CPK levels.

Although an association between a polymorphism of the solute carrier organic anion transporter family member 1B1 gene (SLCO1B1) and statin-induced myalgia was reported, no interaction between vitamin D deficiency and genotype was found in a group of 46 patients on statin therapy (241).

These studies of statin myalgia/myopathy are interesting; however, their unblinded nature and lack of control groups are particular limitations, especially given the well-known placebo effect in pain studies. At this time, the evidence of an association between vitamin D deficiency and statin-related myalgia is derived from observational studies and small series. Obviously, people who experience pain with muscle movement may be less likely to be participating in outdoor activities and may therefore make less vitamin D than their pain-free counterparts. Randomized placebo-controlled studies are needed. In the meantime, we suggest treating deficient patients because of the known bone and calcium benefits. There is at present no evidence to support treating people on statins who have normal vitamin D status.

3. Summary: drug-related myopathy and vitamin D

Myalgia has been observed in patients receiving statins or aromatase inhibitors. A number of these studies reveal that patients on statins who suffer from myalgia are also vitamin D deficient, and vitamin D supplementation might improve symptoms of myalgia in these patients. These findings suggest that vitamin D deficiency may potentiate statin-induced myalgia, but the molecular mechanisms underlying the interaction between vitamin D, statin treatment, and muscle function have not been elucidated. Based on the limited data, we recommend treating all deficient subjects who are receiving statins or aromatase inhibitors. The only RCT in patients taking aromatase inhibitor used high-dose therapy. If this is used, patients should be monitored for hypercalcemia and hypercalciuria.

E. Falls and vitamin D

Approximately 30% of community-dwelling people over the age of 65 fall each year, and approximately 20% of these require medical attention (246). Falls pose a substantial risk to an aging population, are a major risk factor for fracture and other injury, and impact negatively on quality of life (247). Therefore, identifying reversible factors is important. In older individuals, falls are closely related to sarcopenia (loss of muscle mass), loss of muscle tone, and a range of conditions that contribute to the complex syndrome of frailty (248). This issue has also recently been discussed in an Endocrine Society statement on extraskeletal roles of vitamin D (12).

1. Falls: observational studies

There is a seasonal variation in the incidence of falls, with more occurring in winter among older women. This raises the suggestion of a putative role for vitamin D in the occurrence of falls (249), although other factors such as decreased daylight and increased likelihood of slipping on wet or icy surfaces could also explain the differences. The possibility that vitamin D deficiency, which is highly prevalent in frail and older individuals, may contribute to falls has been examined by a number of studies. These are summarized in Table 4.

In 1619 women in low-level and high-level residential care (mean age 84 yr), a significant inverse association between serum 25D levels and the incidence of falls over approximately 5 months was reported. The authors estimated a 20% reduction in falls risk with doubling vitamin D status on analysis of log[25D] levels (250). A similarly sized study of 1231 community-dwelling individuals identified baseline 25D as a predictor for falls over a 1-yr period, particularly among those 65–75 yr of age (251). Those with 25D levels over 10 ng/ml (25 nmol/liter) displayed the highest risk of recurrent falls.

However, in a smaller study, although older individuals who fell had significantly lower 25D levels vs. the overall group of 83 subjects, this was not significant on multivariate analysis (252).

The possibility that 25D does not directly contribute to falls but is rather an associative marker of frailty remains a potential limitation in the interpretation of these observational data. As in the situation with myalgia, it is obvious that people who fall may spend less time outdoors and thereby have lower exposure to solar UV radiation. The other issue is that malnutrition is very common in elderly persons, affecting up to 40% of those living in institutions,
TABLE 4. Observational studies assessing the longitudinal effect of vitamin D levels on falls/muscle function (listed in order of number of study participants, highest to lowest)

<table>
<thead>
<tr>
<th>Study (Ref.)</th>
<th>Main results</th>
<th>Risk Adjustments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Outcomes</strong></td>
<td><strong>Main results</strong></td>
<td><strong>Risk Adjustments</strong></td>
</tr>
<tr>
<td>Faulkner et al. (367)</td>
<td>Quadriceps &amp; grip strength, walk speed, falls, others</td>
<td>IRR = 0.70, Age, clinic, season, HT, ETOH, activity, BMI, education, ethnicity, smoking, creatinine, comorb, drugs, Ca.</td>
</tr>
<tr>
<td>Faulkner et al. (367)</td>
<td>↑ 1,25D (40–80 vs. 7–26 pg/ml), ↓ falls, ↑ 25D associated with ↓ grip strength</td>
<td>HR = 0.74 (0.59–0.94), Wt, cognition, drugs, past Colles fracture, wandering behavior</td>
</tr>
<tr>
<td>Flicker et al. (250)</td>
<td>Falls: staff-reported</td>
<td>OR = 1.78 (1.06–2.99), Age, sex, ETOH, region, season, activity, education, smoking</td>
</tr>
<tr>
<td>Snijder et al. (251)</td>
<td>Falls: self-reported</td>
<td>OR = 2.23 (1.17–4.25), Activity, BMI, season, creatinine, smoking, comorb</td>
</tr>
<tr>
<td>Visser et al. (280)</td>
<td>Grip strength</td>
<td>OR = 2.57 (1.4–4.7), Activity, BMI, season, creatinine, smoking, comorb</td>
</tr>
<tr>
<td>Visser et al. (280)</td>
<td>25HD &lt;10 ng/ml ↑ risk ≥2 falls</td>
<td>OR = 2.17 (0.73–6.33), Activity, BMI, season, creatinine, smoking, comorb</td>
</tr>
<tr>
<td>Wicherts et al. (279)</td>
<td>Score: TCST, tandem stand, walking</td>
<td>OR = 2.21 (1.0–4.87), Activity, BMI, season, creatinine, smoking, comorb</td>
</tr>
<tr>
<td>Chan et al. (282)</td>
<td>ASMM, grip, walk speed, others</td>
<td>OR = 2.01 (1.06–2.99), Activity, BMI, season, creatinine, smoking, comorb</td>
</tr>
<tr>
<td>Verreau et al. (368)</td>
<td>Strength (hip, knee, grip), walking speed and TCST</td>
<td>OR = 2.23 (1.17–4.25), Activity, BMI, season, creatinine, smoking, comorb</td>
</tr>
</tbody>
</table>

ASMM, Appendicular skeletal muscle mass; Ca, calcium intake; comorb, comorbid conditions; ETOH, alcohol intake; HR, hazard ratio; Ht, height; IRR, incidence rate ratio; OR, odds ratio; TCST, timed chair stand tests; Wt, weight.

and may contribute simultaneously to vitamin D deficiency and frailty.

2. Falls: interventional studies

Interventional studies seeking to examine the effects of vitamin D supplementation or UV exposure on the risk of falls among older individuals have been performed and are summarized in Table 5.

a. Sunlight therapy. In a study of 602 residents of aged-care facilities, those who complied with a daily regimen of increased sunlight exposure had fewer falls than those randomized to the control group (253). Compliance was surprisingly poor despite the use of sunlight officers who visited the facilities (median adherence was 26%). Thus, on intention-to-treat analysis, the study was underpowered, and no effects were seen. The authors concluded that vitamin D supplementation was a more practical approach to reduce falls in residential care. It should also be noted that sunlight exposure during the early morning hours rather than at midday when UV levels are highest might not have been sufficiently effective in the synthesis of vitamin D.

b. Intervention studies: non-placebo controlled. The combination of daily 400 IU vitamin D₃ plus 1000 mg calcium reduced severe falls requiring admission in 5063 community-dwelling city residents above the age of 66 yr in Denmark (254). The number needed to treat was nine.

c. Intervention studies: placebo controlled. A reduced incidence of falls, regardless of baseline 25D, was seen in a randomized trial of vitamin D supplementation (255). Among 625 older residents of assisted-living facilities, those who received calcium (600 mg daily) and vitamin D₂ (initially 10,000 IU once weekly and then 1,000 IU daily) for 2 yr had a lower rate of falls compared with those receiving calcium alone. However, the rate of ever falling was not different, implying that the benefits were among
# TABLE 5. Interventional studies assessing the effects of vitamin D supplementation on the incidence of falls (listed in order of number of study participants, highest to lowest)

<table>
<thead>
<tr>
<th>Study (Ref.), Positive vs. Negative, Duration, Basal 25D, Other</th>
<th>n, Sex, Age</th>
<th>Vitamin D (IU), Ca (mg)</th>
<th>Outcome Proven ↑ 25D, Adjustments</th>
<th>Main Results, RR/OR/HR/IRR/ NNT where given</th>
</tr>
</thead>
<tbody>
<tr>
<td>Larsen et al. (254)</td>
<td>9605 F</td>
<td>D$_3$ 400 + Ca 1000 vs. home visit</td>
<td>Severe falls, not proven Adj: age, marital status, intervention</td>
<td>12% decrease RR = 0.88; (0.79–0.98) NNT 9</td>
</tr>
<tr>
<td>Positive 3 yr</td>
<td></td>
<td>vs. nil</td>
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<tr>
<td>NR &gt;66</td>
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<tr>
<td>Arden et al. (369)</td>
<td>6641 M, F</td>
<td>D$_2$ 300,000/yr vs. placebo</td>
<td>Falls, fracture, knee pain Adj: treatment</td>
<td>No change</td>
</tr>
<tr>
<td>Negative 3 yr</td>
<td></td>
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<td></td>
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<tr>
<td>NR &gt;75</td>
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<tr>
<td>Grant et al. (265)</td>
<td>5292 M, F</td>
<td>D$_2$ 800 vs. placebo</td>
<td>Fracture, falls ↑ Proven Adj: minimization variables</td>
<td>No change RR = 0.97</td>
</tr>
<tr>
<td>Negative 24–62 months</td>
<td></td>
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<tr>
<td>15 ng/ml (n = 16) Post fracture</td>
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<tr>
<td>Law et al. (370)</td>
<td>3717 M, F</td>
<td>D$_2$ 100,000/3 months vs. placebo</td>
<td>Fracture, falls; ↑ proven Adj: age, sex, time in the trial, cluster</td>
<td>No change RR = 1.09</td>
</tr>
<tr>
<td>Negative 10 months</td>
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<tr>
<td>19 ng/ml (47) (n = 16) Inst</td>
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<tr>
<td>Porthouse et al. (266)</td>
<td>3314 M, F</td>
<td>D$_3$ 800 + Ca 3000 + leaflet vs. leaflet</td>
<td>Fracture, falls, QOL Not proven Adj: practice</td>
<td>No change</td>
</tr>
<tr>
<td>Negative 25 months</td>
<td></td>
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<tr>
<td>NR, Inst &gt;70</td>
<td></td>
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<tr>
<td>Chapuy et al. (371)</td>
<td>3270 M, F</td>
<td>D$_3$ 800 + Ca 1200 vs. Ca alone</td>
<td>Fracture, 25OHD, PTH Yes Adj: nil Falls, fracture No change RR = 0.49 Falls risk RR 0.97</td>
<td></td>
</tr>
<tr>
<td>Positive 18 months</td>
<td></td>
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<tr>
<td>13–16 ng/ml Inst</td>
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<tr>
<td>Sanders et al. (267)</td>
<td>2256 M, F</td>
<td>D$_3$ 500,000/yr vs. placebo</td>
<td>Fracture, falls; ↑ proven Adj: nil Higher falls and fracture RR = 1.15 (1.02–1.30) RR = 1.26 (1.0–1.59)</td>
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<tr>
<td>Adverse 4–6 yr</td>
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<td>20 (49)</td>
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<tr>
<td>Glendenning et al. (290)</td>
<td>686 M, F</td>
<td>D$_3$ 150,000/3m vs. placebo</td>
<td>Falls, TUAG, grip ↑ Proven Adj: age, falls, follow-up Compliant ↓ falls risk IRR = 0.63 (0.48–0.82)</td>
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<tr>
<td>Negative 9 months</td>
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<tr>
<td>26 ng/ml (n = 40)</td>
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<tr>
<td>Flicker et al. (255)</td>
<td>625 M, F</td>
<td>D$_2$ 10,000/week + Ca 600 then D$_3$ 1,000/d + Ca vs. Ca alone</td>
<td>Fracture, falls Not proven Adj: Wt, cognition, drugs, level of care, wandering</td>
<td>99% fracture from a fall ↓ fracture Falls risk RR 0.97</td>
</tr>
<tr>
<td>Positive, post hoc subgroup 2 yr</td>
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<tr>
<td>&lt;24 (89%). Inst</td>
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<tr>
<td>Sambrook et al. (253)</td>
<td>602 M, F</td>
<td>Extra 30–40 min/day sunlight + Ca 600 vs. nil</td>
<td>Change in 25OHD and falls Not proven Adj: age, sex, falls, balance, comorb, cognition, incontinence, care level</td>
<td>Compliant ↓ falls risk IRR = 0.52 (0.31–0.88)</td>
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<tr>
<td>Positive, post hoc subgroup 12 m</td>
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<tr>
<td>13 ng/ml NH</td>
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<tr>
<td>Chapuy et al. (372)</td>
<td>583 M, F</td>
<td>D$_3$ 800 + Ca 1200 vs. D$_3$ 800 vs. placebo vs. Ca alone</td>
<td>Fracture, 25OHD, PTH ↑ Proven Adj: nil No change</td>
<td>No change</td>
</tr>
<tr>
<td>Negative 2 yr</td>
<td></td>
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<tr>
<td>&lt;20 (79%). Inst</td>
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<tr>
<td>Gallagher et al. (373)</td>
<td>489 M, F</td>
<td>1,25D 0.5 μg/d vs. placebo ± EP</td>
<td>Falls, TUAG, grip ↑ Proven Adj: age, falls, follow-up Compliant ↓ falls risk IRR = 0.63 (0.48–0.82)</td>
<td>No change</td>
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<tr>
<td>Positive 3 yr</td>
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<tr>
<td>31–33 ng/ml</td>
<td>66–77</td>
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<tr>
<td>Bischoff-Ferrari et al. (256).</td>
<td>445 M, F</td>
<td>D$_3$ 700 + Ca 500 vs. Ca alone</td>
<td>Falls (self-report) ↑ proven Adj: age, sex, BMI, Ca intake, CrCl, comorb, basal 25D and PTH, activity, ETOH, smoking Falls data not shown</td>
<td>↓ falls women only OR = 0.54 (0.30–0.97)</td>
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<tr>
<td>Positive, women only.</td>
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<tr>
<td>3 yr &gt;65</td>
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<tr>
<td>27–33 ng/ml</td>
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<tr>
<td>Dawson-Hughes et al. (257)</td>
<td>389 M, F</td>
<td>D$_3$ 700 + Ca 500 vs. Ca alone</td>
<td>Fracture, falls (self-report), BMD ↑ Proven Adj: nil Falls, falls, CrCl &lt; or &gt;60</td>
<td>Falls in women ↓ fracture (nonvertebral)</td>
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<tr>
<td>Positive fracture, adverse falls or 3 yr</td>
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<tr>
<td>&gt;65</td>
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<tr>
<td>27–33 ng/ml</td>
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<tr>
<td>Dukas et al. (374)</td>
<td>378 M, F</td>
<td>1 μg 1α−OHD$_3$ daily vs. placebo</td>
<td>Falls Ca intake &gt;512 mg/d No proven Adj: age, BMI, sex falls, activity, CCM, drugs, heart rate, Ca intake, biochemistry</td>
<td>OR = 0.46 (0.22–0.99) CrCl &lt; 65, ↓ falls OR = 0.29 (0.09–0.88)</td>
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<tr>
<td>Positive, post hoc subgroup 36 wk</td>
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<tr>
<td>&gt;70</td>
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<tr>
<td>28–30 ng/ml</td>
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(Continued)
repeat fallers. On subgroup analysis of compliant participants (defined as taking ≥50% of the doses, n = 540) the risk of ever falling was also significantly lower. The number needed to treat to prevent one fall per year was eight, similar to the above open-label study.

A 3-yr double-blinded randomized study examined combined supplementation with vitamin D₃ (700 IU) and calcium citrate malate (500 mg) vs. placebo in 445 community-dwelling older individuals (256). By intention-to-treat analysis, combined therapy was not effective. A subgroup analysis of the women found a significant reduction in the incidence of falls that was most pronounced in those who were less active. Inter-gender differences in muscle mass were postulated to lead to greater susceptibility for falls among this particular group. The trial was not originally powered to detect effect modification by sex and activity levels, and falls were also a secondary outcome. The primary outcome examined bone density, which was significantly improved (257). In another study, 242 community-dwelling older individuals were randomized to receive calcium (1000 mg daily) with or without vitamin D₃ (800 IU daily) for 1 yr and then followed 8 months without.
treatment but continuing blinding to treatment (258). On intention-to-treat analysis, subjects on dual supplementation reported 27% fewer falls at 1 yr and 39% fewer falls at 20 months. Among 122 older women in long-stay geriatric care who received calcium (1200 mg/d) with or without vitamin D$_3$ (800 IU/d), those on dual supplementation had significantly fewer falls during the 12-wk treatment period compared with the preceding 6-wk observation period. There was no difference in the proportion who fell at all, but people who fell the most appeared to obtain the greatest benefit (259).

In a particularly vulnerable group, namely older individuals with poststroke hemiplegia, a significant reduction in the number of falls per person and the total number of repeat fallers was seen among 48 women receiving vitamin D$_2$ (1000 IU daily) vs. controls who received placebo for 2 yr (85). The study included biopsies of the unaffected side and reported that the baseline proportion and diameter of type II muscle fibers were significant independent predictors for falls over the 2 yr. In demonstrating significant improvements in these parameters in a subset of individuals who received vitamin D supplementation for 2 yr, they offered a mechanistic explanation for the reduction in falls.

By contrast, recurrent fallers were the least likely to benefit from supplementation in another study (260). Community-dwelling ambulant older women were randomized to receive calcium citrate (1000 mg daily) and either vitamin D$_2$ (1000 IU) or placebo for 1 yr. After adjusting for height, which was not equal at baseline, the vitamin D group had a 19% lower risk of a single fall compared with controls. The effect was most pronounced in winter/spring rather than in summer/autumn. It was calculated that 25D levels over 22 ng/ml (54 nmol/liter) were adequate for the benefit. The results were not statistically significant without height correction.

Another study that specifically targeted vitamin D-insufficient older individuals [25D <20 ng/ml (50 nmol/liter)] reported that 800 IU vitamin D$_3$ and 1200 mg calcium daily decreased falls per subject compared with the 148 controls who received calcium alone (261). One noncompliant individual was excluded from analysis. The authors propose that the mechanism relates to a significant improvement in body sway at 8 wk (261, 262). Sagittal body sway improved with treatment, but frontal sway improved in both groups (261).

d. Negative randomized studies and falls. A number of randomized studies have also failed to demonstrate an effect of vitamin D supplementation upon falls.

Among 354 older persons in The Netherlands who were randomized to receive vitamin D$_3$ (400 IU) or placebo for 28 wk, impaired mobility was the major predictor of falls, and vitamin D treatment had no significant effect (263). This study had marked patient heterogeneity with inclusion of both institutionalized and community-dwelling persons.

A positive effect of vitamin D supplementation on falls risk was reported by Broe et al. (264) among the subgroup of 124 nursing home residents receiving high-dose vitamin D (800 IU/d) but not in the 600-, 400-, or 200-IU groups. The risk of falling was lower if those receiving 800 IU vitamin D$_2$ were compared with the combined group of smaller doses (200, 400, or 600 IU) and placebo. There was no suggestion of a normal dose-response curve with the lower doses actually having nonsignificantly higher fall rates compared with placebo (0 IU, 44%; 200 IU, 58%; 400 IU, 60%; 600 IU, 60%; 800 IU, 20%).

A large study of 5292 subjects over 70 yr of age with a recent minimal-trauma fracture did not find a beneficial effect of 800 IU of vitamin D$_3$ on falls over 26–62 months (265). Falls were a secondary endpoint with information collected only for the week before each 4-monthly questionnaire. Compliance (patients taking 80% or more of tablets) was poor (<45% at 2 yr); however, there was also no benefit if only compliant patients were examined. Baseline levels were measured in approximately 1% of patients (n = 60) and were 15.2 ng/ml (38 nmol/liter).

Another study in 3314 older women living in nursing homes did not find fewer falls among those randomized to receive a combination of vitamin D$_3$ (800 IU), calcium (1000 mg), and a falls prevention leaflet over 25 months compared with those receiving the leaflet alone (266). Falls were also a secondary outcome in this study.

In reaction to generally poor adherence to daily regimens of vitamin D supplementation as described by a number of these reports, other studies have examined the efficacy of infrequent high-dose vitamin D supplementation. In one widely reported study of 2256 community-dwelling women over 70 yr of age, an annual oral dose of 500,000 IU of vitamin D$_3$ for 3–5 yr appeared to increase the risk of falls, particularly in the first 3 months after the vitamin D dose (267). The median baseline 25D concentration was 21.2 ng/ml (53 nmol/liter), and 1 month after treatment, the median was 48 ng/ml (120 nmol/liter).

Another study using 300,000 IU, given annually by im injection, reported no effect on falls among 9440 older individuals (268). No benefit was observed in any subgroup.

3. Meta-analyses and falls

Substantial heterogeneity among these randomized trials with regard to differences in study populations, variable treatment durations, and regimens of supplemental vitamin D and whether or not calcium was coadministered...
together with inconsistencies in the identification and analysis of falls among studies (e.g. falls per subject, number of falls, and number of fallers) makes collective assessment of these data difficult. Nevertheless, several meta-analyses have been published and are the subject of debate (23, 29, 30, 269–272).

Most recently, a meta-analysis reported on 26 randomized trials that enrolled 45,782 participants, mainly elderly females (23). Studies were not excluded for lack of double randomization or strict definition of falls; hence, there were a substantially larger number of included individuals than in earlier meta-analyses. Vitamin D use was associated with statistically significant reduction in the risk of falls. However, there was no difference among those receiving higher doses (>800 IU) vs. lower doses. Vitamin D appeared to be effective in both community-dwelling and institutionalized people and in those receiving vitamin D$_2$ or D$_3$. Perhaps reassuringly, the reduction in falls was most prominent in patients who were vitamin D deficient at baseline. Studies in which calcium was co-administered with vitamin D showed a greater effect than those where vitamin D alone was given. Falls reduction in studies without calcium coadministration did not reach statistical significance. Calcium alone was the placebo in most of the combination studies.

Therefore, despite substantial heterogeneity among studies, vitamin D supplementation is probably effective in conjunction with calcium in the prevention of falls among older individuals. The positive impact of vitamin D and calcium supplementation on fall prevention appears to be best among those who are vitamin D deficient at baseline. We recommend consideration of vitamin D plus calcium therapy in people over 65 yr of age with baseline deficiency. However, appropriate supplemental dose of vitamin D and target serum levels required to prevent falls remain hotly debated issues.

F. Muscle strength and physical performance

Many studies have examined the specific effects of vitamin D on measures of muscle function and physical performance. Comparing these studies is made very difficult by the variety of outcome measures used to assess muscle function.

1. Observational studies

A number of cross-sectional studies have reported associations between 25D levels and various parameters related to muscle function including handgrip, lower limb strength, balance, 6-min walk distance, and gait speed (273–275), although not all such studies have supported the association after multivariate adjustment (276, 277). These are summarized in Table 6.

In a population-based study of 4100 ambulatory adults aged over 60 yr, an association between serum 25D and lower-extremity function assessed by 8-ft. walk and the repeated sit-to-stand test (STST) was reported (278). The improvements were modest: 3.9% worsening in STST and 5.6% in 8-ft. walk comparing the lowest vs. the highest quintiles of 25D. Interestingly, a trend toward impaired function with longer STST was seen in patients with high normal 25D [≥48 ng/ml (120 nmol/liter)] vs. lower levels [16–38 ng/ml (40–94 nmol/liter)].

Significantly poorer physical performance was seen in 1234 older individuals among those with 25D below 10 ng/ml (25 nmol/liter) and 10–20 ng/ml (25–50 nmol/liter) vs. those higher than 30 ng/ml (75 nmol/liter) (279). In a subgroup of 979 participants followed over 3 yr, those with lower 25D levels had significantly higher risk of decline in physical performance compared with those with levels above 30 ng/ml (75 nmol/liter). Those in the intermediate 25D group of 20–30 ng/ml (50–75 nmol/liter) did not display significantly greater rates of decline.

In a prospective study of 1008 older individuals in the Netherlands, baseline 25D below 10 ng/ml (25 nmol/liter) was associated with poorer grip strength and reduced muscle mass on DEXA after 3 yr compared with people with normal levels [≥20 ng/ml (50 nmol/liter)] (280). Those in the intermediate 25D group of 10–20 ng/ml (25–50 nmol/liter) demonstrated significantly greater losses of muscle mass without difference in grip strength vs. those with 25D over 20 ng/ml (50 nmol/liter).

An accelerated rate of decline in physical performance over 2.5 yr on timed up and go tests (TUAG) and timed STST were seen among 769 older women with lower baseline 25D (281). However, low 25D levels are associated with frailty (273), so a range of factors not included in adjustment models may confound data interpretation.

A recent study of 714 Chinese men (≥65 yr) found no association between baseline 25D levels and changes in performance measures or appendicular skeletal muscle mass over 4 yr (282). These men were not vitamin D deficient [mean baseline 25D = 31 ng/ml (78 nmol/liter)].

2. Nonrandomized studies

Few studies have examined the effect of vitamin D supplementation on muscle function in younger people. Glerup et al. (208) reported a case-control study in which 55 veiled Arabic women with a mean age of 32 and severe vitamin D deficiency [mean 25D = 3 ng/ml (7 nmol/liter)] were compared with 22 Danish women of similar age but higher 25D levels [19 ng/ml (47 nmol/liter)]. At baseline, all parameters of muscle function were significantly lower
in the Arabic women. Baseline 25D levels were independently associated with maximal voluntary knee extension. After vitamin D repletion, the Arabic women displayed significant improvements in parameters of muscle function at 3 and 6 months. At 6 months, a subgroup that was retested showed no difference in electrically stimulated muscle function vs. Danish controls. Subjective improvements in muscle and deep bone pain were reported by the treated Arabic women.

3. Randomized controlled studies

The randomized controlled studies are summarized in Table 7. Two studies discussed in Section VI.E and Table 6 [Bischoff et al. (259) and Pfeifer et al. (258)] reported improvements in muscle function as well as decreased falls in those randomized to receive supplemental vitamin D. Over a 12-wk treatment period, those on calcium and vitamin D demonstrated significant improvements in the summed score of knee flexor and extensor strength, grip strength, and TUAG compared with those receiving calcium alone (259). Significant improvements were reported in quadriceps strength and TUAG in another study with reduced falls after 12 months of dual supplements compared with calcium alone (258).

A two by two randomized study of patients treated with calcium 800 mg with or without resistance training, with or without vitamin D 400 IU daily for 9 months found that vitamin D improved physical performance (283). Quadriceps strength, physical performance test, and TUAG were significantly improved by vitamin D and by resistance training, with an additive benefit in the group that received both (283).

In a study of 139 ambulatory older subjects with a history of falls and vitamin D deficiency [25D <12 ng/ml (30 nmol/liter)], treatment with vitamin D2 (600,000 IU im) had a significant effect on aggregate functional performance time but no effect on either falls or quadriceps strength at 6 months follow-up vs. placebo (284).

Among 56 institutionalized persons over 60 yr of age, those randomized to receive calcium and vitamin D (two doses of 150,000 IU then 90,000 IU monthly) demonstrated significant improvements in maximal isometric strength of hip flexors and knee extensors after 6 months (285). Subgroup analysis demonstrated greater improve-
ments in muscle function among subjects with lower baseline 25D levels [<20 ng/ml (50 nmol/liter)].

Similarly, among 302 older, community-dwelling women, those in the lowest tertile of 25D levels who received daily calcium (1000 mg) and vitamin D$_2$ (1000 IU) displayed the most pronounced improvements in lower limb muscle function over 1 yr as opposed to those on calcium alone (286).

Among 69 postmenarchal adolescent females, those randomized to receive 150,000 IU vitamin D$_2$ orally every 3 months for 1 yr demonstrated significant improvements in movement efficiency, a composite of jump height and velocity measured by mechanography, compared with baseline (287). Additionally, at baseline, higher 25D levels correlated with greater jumping velocity.

In a short study of 42 postmenopausal women, 12 wk treatment with 1α-OHD$_3$ (0.5 μg) and calcium led to significant improvements in quadriceps strength compared with those receiving calcium alone (288).

4. Muscle strength and function: negative studies

In a study of 70 vitamin D-deficient women [25D = 8–20 ng/ml (20–50 nmol/liter)], 6 months of vitamin D and calcium (400 IU and 500 mg) had no effect on grip or knee strength vs. calcium alone. Baseline 25D levels showed an inverse correlation with these parameters of muscle function (289).

In a study of 686 community-dwelling women over 70 yr, treatment with oral vitamin D$_3$ (150,000 IU every 3 months) for a 9-month period had no significant effect on falls or hand grip strength compared with placebo (290). However, a randomly selected subgroup of 40 participants had a mean baseline 25D of 26 ng/ml (66 nmol/liter), suggesting that the group at large were vitamin D sufficient.

Other studies have failed to demonstrate improvements in muscle strength after vitamin D supplementation, regardless of baseline vitamin D status (291–295), as summarized in Table 7. A multicenter study of 243 frail, older patients reported no difference in parameters of physical performance between those randomized to receive a single dose of 300,000 IU of vitamin D vs. placebo (291). There was no effect in the subset with low baseline 25D levels [<12 ng/ml (30 nmol/liter)] despite significant improvements in 25D levels. Some studies have examined other forms of vitamin D, namely 1,25D and 1α-OHD$_3$, with variable effects. Among 98 older subjects with mild renal impairment, 1,25D (0.5 μg daily) resulted in no improvement over a 6-month period vs. placebo, and some subjects on 1,25D developed hypercalcemia and required dose reduction (294).

One negative study examined 179 vitamin D-deficient adolescent females in Lebanon. Those randomized to receive vitamin D$_3$ (doses of 1,400 or 14,000 IU/wk) did not demonstrate improved grip strength but did have greater increases in lean mass, bone area, and total hip bone mineral content vs. placebo after 1 yr (295).

Two studies have examined the effects of combining vitamin D supplementation with high-resistance training on muscle strength in older individuals (283, 293). In one study of 180 community-dwelling males (50–79 yr), those randomized to receive an intensive program of resistance training three times per week demonstrated improvements in strength. Those randomized to receive fortified milk alone (containing vitamin D 800 IU, calcium 1000 mg, and protein 13.2 g daily) demonstrated no additional improvements (293). Those receiving high-resistance training with vitamin D (400 IU) and calcium (800 mg) over a 9-month period showed improvements in TUAG but not in quadriceps strength compared with those who received resistance training and calcium (283).

5. Meta-analyses of muscle function

In one meta-analysis, the substantial variability in the parameters of muscle function among studies, use of measures without established validity or reliability, and lack of blinded outcome assessments were cited as reasons for inability to pool data (30).

On assessing 17 RCT involving 5072 participants, there was no significant effect of vitamin D supplementation on grip strength or proximal lower limb strength in adults with 25D levels over 10 ng/ml (25 nmol/liter) at baseline (24). However, for adults with deficiency [25D <10 ng/ml (25 nmol/liter)], a beneficial effect on hip muscle strength was found.

In another meta-analysis of 16 RCT, in which baseline 25D levels were below 20 ng/ml (50 nmol/liter) in 11 studies, the authors noted the publication of a greater number of studies that showed no effect rather than a beneficial effect of vitamin D supplementation on muscle function and that there were no obvious characteristics to differentiate studies with positive and negative findings (296).

In a more recent meta-analysis of 13 RCT involving elderly subjects who were predominantly vitamin D deficient or insufficient, vitamin D supplementation with 800–1000 IU daily was associated with improvements in lower extremity strength and balance (297). The meta-analysis included only randomized trials of older individuals in whom baseline and posttreatment parameters of muscle function were assessed. Trials in younger individuals (295) or those that included muscle training as part of the treatment were not included (293). This meta-analysis found no effect on gait.
6. Summary: vitamin D, muscle strength, and physical performance

Although some data suggest a beneficial effect of vitamin D supplementation on muscle function, particularly in vulnerable populations and those with low baseline vitamin D levels (208, 286), the evidence base is limited by highly heterogeneous studies that assess muscle function by different methods. Hence, larger studies that use standardized, reproducible assessments of muscle strength and double-blinded treatment regimens are necessary to clarify this important issue and guide recommendations. Such studies should ideally consider baseline vitamin D status and confirm adequate replacement is achieved by a rise in 25D to the normal range.

G. Muscle morphology and electromyography (EMG)

Several reports, dating back to the 1970s, have characterized the morphological appearance of skeletal muscle among vitamin D-deficient subjects and thus provided some evidence in support of a direct role for vitamin D in the morphology and development of muscle.
1. Open-label studies

In 1974, a case series of 13 patients with various degrees of proximal myopathy in the context of chronic renal failure was described (203). Ten of the 13 patients displayed significantly shorter mean action potential durations of the deltoid and quadriceps muscles on EMG. They also displayed moderate atrophy of type II (i.e. fast twitch) muscle fibers on the basis of myofibrillar ATPase staining and degenerative changes on electron microscopy with small foci of fiber necrosis, lytic vacuoles, and Z-band degeneration in four patients. Although vitamin D levels were not determined, because this was before the era of the standardized assay, substantial improvement in muscle strength after im vitamin D treatment in a proportion of the patients was reported. In another series of four uremic patients, the finding of type II muscle fiber atrophy was linked to vitamin D deficiency on the basis of significantly elevated PTH levels (298).

In 1975, gluteal muscle biopsies of 12 patients with laboratory evidence of osteomalacia displayed nonspecific muscle fiber atrophy (299). A distinction was made between patients with isolated nutritional deficiency in...
whom biopsy changes were mild compared with those with an additional condition including hyperparathyroidism, hyperthyroidism, or uremia who also demonstrated myofibrillar degeneration and infiltration with amorphous material.

One year later, Irani (201) reported a case series of 15 women with nutritional osteomalacia who demonstrated significantly shorter motor unit action potentials and a greater proportion of polyphasic potentials on EMG compared with controls. Muscle biopsies from those with osteomalacia demonstrated nonspecific muscle fiber atrophy. Complete resolution in EMG changes after a 5-wk course of high-dose vitamin D supplementation (600,000 IU of vitamin D$_2$ weekly or fortnightly) was noted in the three patients who were retested. In 1979, two patients with osteomalacia demonstrated type II muscle fiber atrophy in addition to scattered necrosis and derangement of the intermyofibrillar network on muscle biopsy (300).

Eleven patients with a condition described as bone loss of aging had muscle biopsies from the vastus lateralis before and after treatment with 1α-OHD$_3$ and calcium for 3–6 months (301). The predominant finding was an increase in the proportion and cross-sectional size of fast-twitch type IIa fibers. Measures of the oxidative capacity of muscle, succinate dehydrogenase, and total phosphorylase activity were low at baseline and increased with treatment. Lactate dehydrogenase activity, a measure of anaerobic metabolism, did not change. Interestingly, the proportion of type IIb fibers (very-fast-twitch fibers) decreased significantly with treatment. This was the first report to demonstrate changes in muscle morphology and oxidative capacity after treatment of presumably vitamin D-deficient subjects with a vitamin D analog.

Three years later, Young et al. (302) confirmed these findings by demonstrating significant increases in the proportion of type II muscle fibers in biopsies of the vastus lateralis muscle after 3 months of vitamin D supplementation among 12 patients with osteomalacia. In association with these findings, quadriceps muscle strength also improved significantly using an isodynamic dynamometer. However, this body of evidence dating back to the 1970s may be confounded by the many biochemical abnormalities associated with renal failure and osteomalacia such as hyperparathyroidism and disturbances in calcium and phosphate levels. These provide indirect mechanisms that may independently alter muscle function (Fig. 4). Nevertheless, the changes in muscle morphology and performance after vitamin D supplementation in these subjects are important preliminary observations.

Recent studies have reported an association between significantly higher skeletal muscle fat content and vitamin D deficiency. In one study of 90 postpubertal females in California, the proportion of muscle fat, assessed by comparing the attenuation signal of a 2-cm$^2$ section of the rectus femoris with the adjacent sc fat on computed tomography, was found to strongly correlate in an inverse fashion with serum 25D levels (303). This was independent of body mass or computed tomography measures of sc and visceral fat. The percentage of muscle fat was significantly lower in women with normal vs. subnormal serum 25D levels.

In another study of 366 older patients receiving magnetic resonance imaging (MRI) of one shoulder for the investigation of potential rotator cuff injury, a correlation between higher fatty infiltration of rotator cuff muscles and lower serum levels of 25D was reported (304). After multivariate linear regression analysis, this association remained statistically significant in two muscle groups (i.e. supraspinatus and infraspinatus muscles) but only among those whose MRI also demonstrated a full-thickness rotator cuff tear (228 patients).

A third study using MRI of the thigh in 20 older subjects also reported an inverse correlation between muscular fatty degeneration and 25D (305). Interestingly, selective and near-total fatty degeneration of at least one muscle was observed among 11 vitamin D-deficient patients [25D <20 ng/ml (50 nmol/liter)].

A recent cross-sectional study demonstrated a positive correlation between 1,25D levels and total skeletal muscle mass as measured on DEXA among subjects younger than 65 yr (306). This was supported by greater isometric knee extension moment in women with higher 1,25D levels. However, no association was found between 25D levels and muscle mass or strength or in those over 65 yr of age. Among 26 subjects with chronic kidney disease, thigh muscle cross-sectional area on MRI correlated significantly with a model including 1,25D levels, calcium levels, and daily physical activity (307). Functional parameters assessing gait and proximal musculature also independently correlated with 1,25D.

Although a majority of highly trained adolescent male ballet dancers had low vitamin D levels [25D <20 ng/ml (50 nmol/liter) in nine of 16 study participants], there was no correlation between 25D, body composition on DEXA, or reports of muscle injury in this study (308).

2. EMG and muscle biopsies: randomized study

A study that randomized 96 elderly women with post-stroke hemiplegia and severe vitamin D deficiency [<10 ng/ml (25 nmol/liter)] to vitamin D$_2$ (1000 IU daily) or placebo for 2 yr reported significant and dramatic increases in the proportion and diameter of type II muscle fibers (85). These parameters deteriorated significantly in the placebo group.
3. EMG and muscle biopsies: summary

In summary, it appears that vitamin D deficiency results in significant and reversible changes in EMG and type II muscle fiber atrophy, the latter being an independent predictor for falls in one study (85). However, the changes are nonspecific, being similar to those seen in other conditions. Although fatty infiltration in skeletal muscle has been suggested by three recent studies, these are cross-sectional and based on imaging modalities that may not be validated for the assessment of muscle fat. Muscle becomes fatty with disuse, and thus this measure may be confounded by decreased exercise associated with both increased muscle lipid and lower vitamin D. These modalities are not equipped to identify intracellular fat, perhaps of greater pathophysiological significance.

H. Insulin sensitivity and glucose handling

A broad range of epidemiological and randomized clinical studies together with specific research on molecular pathways and animal models have drawn links between vitamin D and insulin sensitivity. This is relevant to the topic of vitamin D and muscle because under normal physiological conditions, skeletal muscle is responsible for approximately 85% of whole-body insulin-mediated glucose uptake (102).

Insulin resistance, a highly prevalent condition that contributes to the pathogenesis of type 2 diabetes, is primarily due to defective insulin-stimulated glucose uptake in skeletal muscle resulting from the production of various inflammatory mediators, adipokines, and FFA by adipocytes in predominantly overnourished and obese individuals (309). However, in recognizing the complex processes involved in insulin resistance, a number of reversible factors with potential etiological relevance to this condition are being considered. One of these factors is vitamin D deficiency. In this section, we will review human clinical studies that examine the association between vitamin D status and insulin sensitivity.

1. Cross-sectional studies: vitamin D and insulin sensitivity

Studies have examined the association between parameters of insulin resistance and vitamin D status in nondiabetic individuals. In one report, 25D was inversely correlated with the homeostasis model assessment of insulin resistance (HOMA-IR) among 214 Arab-American men, but no such association was found among the 317 women of the same ethnicity included in this study (310). Among 808 nondiabetic participants of the Framingham Offspring Study, plasma 25D concentrations were inversely associated with fasting insulin concentrations and HOMA-IR after adjustment for age, sex, and BMI (311). A similar association between 25D levels and HOMA-IR was found in a group of 712 subjects at risk of diabetes (312).

Among 1941 adolescents who participated in the National Health and Nutrition Examination Survey from 2001–2006, adjusted concentrations of insulin were significantly higher among male subjects who were vitamin D deficient [<20 ng/ml (50 nmol/liter)] compared with those with higher vitamin D levels [≥30 ng/ml (75 nmol/liter)], suggesting a potential role vitamin D status in insulin sensitivity (313).

However, a recent study that employed the gold-standard technique in the assessment of insulin sensitivity, namely the hyperinsulinemic-euglycemic clamp, found that the association between insulin sensitivity in 39 non-diabetic subjects and 25D levels become nonsignificant after adjustment for other factors including BMI (314). Similarly, among 381 nondiabetic university students in Lebanon and 510 nondiabetic subjects from a largely obese ethnic minority in Canada (i.e. Canadian Cree), the inverse association between 25D levels and HOMA-IR also became nonsignificant after adjustment for BMI in addition to other factors (315, 316). In another study of 126 healthy young adults, there was a significant association between 25D levels and insulin sensitivity on a hyperglycemic clamp study that remained after adjustment for a range of factors (317).

Therefore, it is clear that the inverse correlation between 25D and BMI, as reported in a number of studies (314, 318), may particularly confound the assessment of these observational data. In fact, a recent study suggests that it may be more accurate to consider adiposity rather than BMI per se as the particular confounding factor (319). In a study of 1882 non-diabetic individuals, it was the inclusion of a computed tomography measure of visceral adiposity rather than BMI and waist circumference in the multivariate analysis that caused the inverse association between vitamin D status and markers of insulin resistance, namely HOMA-IR and log insulin levels, to be insignificant (319).

Several mechanisms associating vitamin D deficiency with obesity have been proposed, including the great capacity of adipose tissue to store vitamin D (80, 320) and the avoidance of sunlight exposure and outdoor activity among potentially self-conscious, obese individuals (320). In confirmation of the former mechanism, a study of 116 obese women reported that fat mass measured by isotope dilution method was a strong predictor of serum 25D levels both 5 yr before and 10 yr after bilio-pancreatic diversion surgery and that vitamin D levels did not correlate with insulin sensitivity at either time on the basis of the euglycemic-hyperinsulinemic clamp studies (321).

The impact of PTH, which has an inverse relation to vitamin D status and is also associated with diabetes (322),
has been addressed in a small number of studies. A study including 15 subjects with secondary hyperparathyroidism (serum PTH > 6.4 pmol/liter) and 15 controls found that after adjustment for BMI, age, and sex, serum 25D levels were significantly associated with the insulin sensitivity index on a 3-h hyperglycemic clamp, but PTH levels were not (323). Similarly, a significant adjusted association between 25D and fasting insulin was reported in a study of 654 adult subjects from Canada, but PTH was not associated with this parameter after multivariate adjustment (324).

Apart from insulin resistance of skeletal muscle, the pathophysiology of type 2 diabetes comprises a range of other factors. Vitamin D may play a role in these other processes with cross-sectional studies and meta-analyses reporting an association between vitamin D deficiency/insufficiency and the incidence of diabetes in various populations (325–328). However, not all studies confirm this association between vitamin D and diabetes. In a study of 654 adult subjects from Canada, but PTH was not associated with this parameter after multivariate adjustment (324).

Apart from insulin resistance of skeletal muscle, the pathophysiology of type 2 diabetes comprises a range of other factors. Vitamin D may play a role in these other processes with cross-sectional studies and meta-analyses reporting an association between vitamin D deficiency/insufficiency and the incidence of diabetes in various populations (325–328). However, not all studies confirm this observation, and there is substantial heterogeneity between studies in their design and adjustment for confounders (329–331).

2. Prospective studies: vitamin D and insulin sensitivity

Observational studies have examined the relationship between 25D and the prospective risk of developing insulin resistance.

In the prospective Ely study (1990–2000), baseline 25D levels of 524 nondiabetic men and women were inversely associated with the 10-yr risk of insulin resistance, on the basis of HOMA-IR and fasting insulin after adjustment for a range of factors including age, sex, BMI, and calcium and PTH levels (332). Each 10-ng/ml (25 nmol/liter) increase in baseline 25D was associated with a significant decrease in HOMA-IR score (i.e., 0.16 U) at 10 yr.

In a recent study that assessed 5200 participants of the Australian Diabetes, Obesity, and Lifestyle (AusDiab) study, lower baseline 25D levels were associated with a higher risk of developing diabetes over the 5-yr follow-up period (333). After adjustment for a range of factors, the authors reported that each 10-ng/ml (25 nmol/liter) increment in serum 25D was associated with a 24% reduced 5-yr risk of diabetes. Regarding insulin resistance, a positive and independent association with HOMA-IR at 5 yr was also reported. In contrast to an earlier report (334), no association between dietary calcium intake and diabetes risk or the follow-up homeostasis model assessment of insulin sensitivity score was found (333).

Apart from insulin resistance, a number of studies have also reported an association between baseline 25D levels and the long-term risk of diabetes (334–336). However, not all such studies have supported this association (337–339).

3. Interventional studies: vitamin D and insulin sensitivity

Mixed results have emerged from a number of interventional studies that have sought to address the impact of vitamin D supplementation on glucose homeostasis and parameters of insulin sensitivity.

There was no difference in parameters of glucose homeostasis among 238 postmenopausal women who were randomized to receive 2 yr of treatment with vitamin D3 (2000 IU daily) or 1α-OHD3 (0.25 μg daily) or 1 yr of treatment with 1,25D (0.25–0.50 μg) daily vs. placebo (340). Similarly, no differences in insulin-mediated glucose uptake on the euglycemic clamp study were found in 18 healthy males randomized to receive either 1,25D 1.5 μg daily or placebo; however, treatment in this study was only 7 d (341).

Studies examining subjects at risk of diabetes have suggested improvements in glucose homeostasis with vitamin D supplementation. In three studies, significant improvements were reported in insulin sensitivity, insulin secretion, and/or the disposition index in subjects at risk of diabetes who were randomized to receive supplemental vitamin D3 and calcium (2000 IU and 500 mg, respectively) vs. calcium alone for 16 wk, high-dose vitamin D3 (120,000 IU) every 2 wk vs. placebo for 6 wk, and vitamin D3 and calcium (700 IU and 500 mg daily, respectively) vs. placebo for 3 yr (342–344).

A double-blind randomized trial of 81 South Asian women living in New Zealand who were found to be both insulin resistant on HOMA-IR and vitamin D deficient [25D <20 ng/ml (50 nmol/liter)] reported significant reductions in insulin resistance and fasting insulin levels among those randomized to receive vitamin D3 (4000 IU daily) vs. placebo for 6 months (345).

In a recent randomized trial including 90 diabetic subjects, those randomized to receive vitamin D-fortified yogurt twice daily (each containing 500 IU) or vitamin D- and calcium-fortified yogurt demonstrated improved glycemic control on glycated hemoglobin (HbA1c) and improved insulin resistance on HOMA-IR compared with those receiving plain yogurt for 12 wk (346). Importantly, an inverse correlation was observed between changes in serum 25D and HOMA-IR in this study.

In another study, 10 females with type 2 diabetes who were predominantly vitamin D deficient reported a significant reduction in a marker of peripheral insulin resistance after 1 month of vitamin D3 supplementation (1332 IU daily) (347). However, this study had no control group.

Conversely, a number of small studies have failed to demonstrate any benefits in association with vitamin D supplementation in patients with type 2 diabetes. In 20 diabetic subjects, a randomized trial reported no improvements in fasting or stimulated glucose, insulin, C-peptide,
or glucagon concentrations among those receiving 1,25D (1 μg daily) for 4 d vs. placebo (348). Among 28 Asian Indian patients with type 2 diabetes, those randomized to receive vitamin D supplementation for 4 wk did not demonstrate a significant difference in markers of insulin resistance (i.e. fasting insulin, post-oral glucose tolerance test serum insulin levels, and HOMA-IR) compared with those receiving placebo (349). Similarly, in 32 diabetic subjects, 6 months of supplemental vitamin D (40,000 IU/wk) had no effect on fasting insulin, C-peptide, or HbA1c levels compared with baseline or those receiving placebo (350). It is probably reasonable to conclude that these studies were underpowered to answer the question in either direction.

In one case series of three British Asians with diabetes and vitamin D deficiency [25D <6 ng/ml (15 nmol/liter)], high-dose vitamin D supplementation (300,000 IU im) was associated with subsequent deterioration in glycemic control on HbA1c and progression of insulin resistance on fasting insulin resistance index (351).

Recent post hoc analyses of eight trials including participants with normal glucose tolerance at baseline and three small trials of patients with established type 2 diabetes demonstrated no effect of vitamin D supplementation on glycemic outcomes (352). However, two trials examining patients with baseline glucose intolerance reported improvements in insulin resistance among those receiving vitamin D supplementation (349, 350).

4. Summary: vitamin D and insulin sensitivity

Substantial differences in study design, duration, and type of vitamin D supplementation and the particular populations studied in these trials make collective assessment of these results difficult. Although some large trials suggest a beneficial effect of vitamin D supplementation in the reduction of insulin resistance, others do not. Furthermore, to ascertain whether the potential glycemic benefits of supplemental vitamin D are more pronounced in those with vitamin D deficiency or poor glycemic control at baseline, larger trials of longer duration are necessary. More than 20 trials are currently under way to address this question (www.clinicaltrials.gov).

VII. Conclusions

See Table 8 for conclusions.

In his 1922 publication on the cure of rickets by sunlight, Alfred Hess (35) remarked that “although we have realized the importance of light in the growth of plant life, we have [until now] accorded it too little significance in the development of animal life.” Since that time, we have come a long way in recognizing the role of UV radiation in the photochemical synthesis of vitamin D, the role of vitamin D in calcium and mineral homeostasis, and the similarity of 1,25D to members of the steroid family with their cholesterol precursor, carbon-ringed structures, and ability to bind to specific nuclear receptors in the genomic mediation of developmental and functional effects.

Although steroid hormones are known to exert diverse effects in multiple organs and tissues, a role for vitamin D beyond its predominant effects on bone and mineral homeostasis has been hotly contested. Muscle stands at the frontier of the emerging concept of vitamin D’s extraskeletal role because it shares its ancestral origin with bone in the common mesenchymal stem cell and relies heavily on
intracellular calcium handling for contraction, insulin sensitivity, and cellular plasticity (99).

In this review, we have adopted a multilayered approach in examining evidence from human clinical studies as well as reports on animal and cell models to piece together the current knowledge of vitamin D’s effects in skeletal muscle. The broad evidence base is generally in favor of a role for vitamin D in the development and function of skeletal muscle.

The strongest evidence comes from studies that report distinct morphological changes in the muscle of vitamin D-deficient subjects, others that describe significant impairments in the muscle function of VDRKO mice, and molecular studies that have mapped out the various intracellular responses of cultured muscle cells to vitamin D (129, 173, 174). As a result, we have come closer to answering the perennial question as to whether vitamin D’s influence on muscle is direct or indirect, and the answer appears to be both.

Outstanding questions remain, including the precise role of vitamin D in muscle differentiation, the possibility of specific biological activity of 25D in muscle, and the current controversy regarding the in vivo presence of the VDR in muscle tissue.

In the clinical domain, observations of reversible myopathy in subjects with severe vitamin D deficiency have been reported for some time. Cross-sectional data reporting a high prevalence of vitamin D deficiency among subjects with falls, muscle weakness, and insulin resistance are also present (211, 274, 327). However, confounding variables are a caveat in the interpretation of this circumstantial evidence. Furthermore, the demonstration of unequivocal improvements in muscle function among subjects with mild to moderate degrees of vitamin D deficiency randomized to receive vitamin D supplementation has been elusive. Possible reasons for this include heterogeneity in study design and supplemental regimens and the general lack of large-scale trials to address this issue. These challenges and others remain to be addressed.

There is reasonable evidence from cellular, animal, and at least some human studies that muscle responds to vitamin D. Although molecular pathways by which vitamin D acts on the myocyte have been identified, there is scope for more clarification. Studies are also needed to clarify the therapeutic potential of vitamin D in the treatment of age-related sarcopenia and perhaps other myopathies. In the meantime, it would be prudent for clinicians to seek and manage vitamin D deficiency in individuals at risk of these conditions.

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Chapter 3 – Effects of vitamin D in C2C12 muscle cells

This chapter consists of an original article that was published in the February edition of *Endocrinology* (2014; 155: 347–357). The primary author and PhD candidate, Christian Girgis, carried out the majority of experiments described in this work (>90%), drafted manuscripts and responses to reviewers. This has been verified by co-authors (see Appendix). To date, this article has been cited > 10 times since publication.

The main findings of this work include:

- novel effects of 1,25(OH)₂D in C2C12 muscle cell proliferation, differentiation and myotube formation.
- elucidation of mechanisms underlying the anti-proliferative effect of 1,25(OH)₂D in C2C12 cells (i.e. modulation of cell cycle genes including myc, post-translational effects of Rb).
- the presence of functional CYP27B1 in C2C12 cells using a luciferase reporter system and demonstration of morphologic changes in C2C12 cells in response to 25OHD.
- anabolic effects of vitamin D on C2C12 myotube size and profound inhibition of myostatin, a negative regulator of muscle mass.
Vitamin D Signaling Regulates Proliferation, Differentiation, and Myotube Size in C2C12 Skeletal Muscle Cells

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Vitamin D deficiency is linked to a range of muscle disorders including myalgia, muscle weakness, and falls. Humans with severe vitamin D deficiency and mice with transgenic vitamin D receptor (VDR) ablation have muscle fiber atrophy. However, molecular mechanisms by which vitamin D influences muscle function and fiber size remain unclear. A central question is whether VDR is expressed in skeletal muscle and is able to regulate transcription at this site. To address this, we examined key molecular and morphologic changes in C2C12 cells treated with 25-hydroxyvitamin D (25OHD) and 1,25-dihydroxyvitamin D (1,25(OH)\(_2\)D). As well as stimulating VDR expression, 25(OH)D and 1,25(OH)\(_2\)D dose-dependently increased expression of the classic vitamin D target cytochrome P450, family 24, subfamily A, polypeptide 1 (CYP24A1), demonstrating the presence of an autoregulatory vitamin D-endocrine system in these cells. Luciferase reporter studies demonstrated that cytochrome P450, family 27, subfamily B, polypeptide 1 (CYP27B1) was functional in these cells. Both 25OHD and 1,25(OH)\(_2\)D altered C2C12 proliferation and differentiation. These effects were related to the increased expression of genes involved in G\(_0\)/G\(_1\) arrest (retinoblastoma protein [Rb], 1.3-fold; ATM, 1.5-fold, both \(P < .05\)), downregulation of mRNAs involved in G\(_1\)/S transition, including myc and cyclin-D1 (0.7- and 0.8-fold, both \(P < .05\)) and reduced phosphorylation of Rb protein (0.3-fold, \(P < .005\)). After serum depletion, 1,25(OH)\(_2\)D (100nM) suppressed myotube formation with decreased mRNAs for key myogenic regulatory factors (myogenin, 0.5-fold; myf5, 0.4-fold, \(P < .005\)) but led to a 1.8-fold increase in cross-sectional size of individual myotubes associated with markedly decreased myostatin expression (0.2-fold, \(P < .005\)). These data show that vitamin D signaling alters gene expression in C2C12 cells, with effects on proliferation, differentiation, and myotube size. (Endocrinology 155: 347–357, 2014)

In addition to established effects in bone and mineral homeostasis, vitamin D deficiency is linked to a range of muscle disorders (1). These include muscle weakness, myalgia, and drug-related myopathy. Type II (fast-twitch) muscle fibers atrophy in elderly individuals with vitamin D deficiency, exacerbating their tendency to fall (2).

Evidence suggests that vitamin D plays a role in muscle development (1). Children with vitamin D-deficient rickets develop profound muscle weakness and hypotonia that improves after sun exposure or vitamin D supplementation (3, 4). In animal studies, mice lacking the vitamin D receptor (VDR) have smaller muscle fibers at 3 weeks of age, with a decreased ability to maintain muscle mass in response to exercise (5). Vitamin D deficiency leads to reduced muscle strength and endurance, which is likely due to alterations in muscle fiber-type distribution, with a decrease in type II fibers and an increase in type I fibers (6). These changes are associated with decreased muscle performance and increased risk of falls in elderly individuals (7). Vitamin D supplementation has been shown to improve muscle function in elderly individuals with vitamin D deficiency (8) and to reduce the risk of falls and fractures (9). These findings suggest that vitamin D has a role in maintaining muscle health and function.
persistent expression of developmental muscle genes, and impaired motor coordination throughout adulthood (5–7).

However, the precise mechanisms by which vitamin D signaling influences muscle development and function are unclear. Importantly, the issue of whether the VDR is expressed in skeletal muscle is contentious (1, 8, 9).

The aim of this study was to assess the effects of vitamin D in an in vitro model of skeletal muscle. C2C12 muscle cells, a widely investigated model of myogenesis (10, 11), were used. To determine whether vitamin D could have direct effects on these cells, expression and functionality of key components of the vitamin D-endocrine system, specifically cytochrome P450, family 27, subfamily B, polypeptide 1 (CYP27B1), VDR, and cytochrome P450, family 24, subfamily A, polypeptide 1 (CYP24A1), was measured. We then assessed effects of 25-hydroxyvitamin D (25OHD) and 1,25-dihydroxyvitamin D (1,25(OH)$_2$D) on C2C12 muscle cell proliferation and differentiation.

Materials and Methods

Cell culture

C2C12 cells were propagated in DMEM-F12 with 10% heat-inactivated fetal calf serum at 37°C and 5% CO$_2$. On reaching 70% confluence, cells were distributed into appropriate plates for each assay and cultured in media with 10% fetal calf serum. At 24 hours after seeding (day 1), cells were treated with 100nM 1,25(OH)$_2$D, 100nM 25OHD, or vehicle (ethanol). The medium was changed daily. From day 3 onward, serum was depleted (12, 13). Serum depletion has been previously used to examine cell cycle exit and myogenic differentiation (ie, serum depletion) increased from 10% to 2% and changed to horse serum to initiate differentiation.

Cell proliferation

Cell proliferation was measured by 3 methods on day 3 before serum starvation.

1) For cell counting, cells cultured in 6-well plates were dislodged by trypsin and counted using a hemocytometer.

2) For Alamar Blue (AB) staining (Invitrogen), cells cultured in 96-well plates were stained with AB (10% vol/vol). AB is reduced in proliferating cells from resazurin to resorufin, the latter being a fluorescent compound. Measurement of AB fluorescence is therefore a marker of cell proliferation and viability (21). In our study, fluorescence excitation at 544 nm and emission at 590 to 610 nm was measured. AB fluorescence was determined at baseline and after 25OHD or 1,25(OH)$_2$D treatments as an index of cellular proliferation over this period.

3) For bromodeoxyuridine (BrdU) incorporation, using a fluorescein isothiocyanate (FITC) BrdU Flow Kit (BD Pharmingen), BrdU (100 mmol/mL) was added on day 2 for 24 hours. Staining for BrdU and 7-amino-actinomycin D (7AAD) was performed as described (22). Flow cytometric data was acquired using a FACS Canto (BD Biosciences), and fluorochromes/filters were BrdU FITC B530/30 and 7AAD R660/20. Flow Jo software (Tree Star) was then used to analyze these data.

Apoptosis and necrosis

Annexin V and propidium iodide (PI) are established markers for apoptosis and cell necrosis (23). Annexin V binds to phos-
phosphatidylserine that becomes exposed on the cell surface during apoptosis, and PI is an intercalating agent that gains entry into the cell via membrane defects during necrosis (23). On day 3, cells cultured in 6-well plates were trypsinized and then re-suspended in solution containing 10mM HEPES, 140mM NaCl, 2.5mM CaCl$_2$, and FITC-labeled annexin V antibody (1:50 dilution; BD Pharmingen). After 30 minutes incubation, cells were treated with PI (1:50 dilution). Flow cytometric data was acquired using a FACS Canto (BD Biosciences), and fluorochromes/filters were annexin V FITC B530/30 and PI B655lpor. FlowJo software (Tree Star) was then used to analyze these data.

**Vitamin D luciferase reporter studies**

Plasmids for GAL-4-VDR, UASTK-Luciferase, and pcDNA were generated by R.J.C.-B. (Kolling Institute). Plasmids were transformed in chemically competent Top-10 $\text{H}11032^+$/E. coli (Invitrogen) and extracted using the Plasmid Mini Kit (QIAGEN) according to the manufacturer’s protocols. C2C12 cells were split into 24-well culture plates and transfected at a high density before adhesion as previously described (24). Lipofectamine 2000 (Gibco) was used to transfect 800 ng of GAL4-VDR and 800 ng of UASTK-luciferase reporter into 21 wells per plate. Cells were also transfected with 800 ng $\beta$-galactosidase reporter to correct for transfection efficiency. The remaining 3 wells were transfected with pcDNA empty vector as negative control. Twenty-four hours after transfection, growth medium was replaced with serum-free medium, and cells were treated with 25OHD (1nM–100nM), 1,25(OH)$_2$D (1nM–100nM), or ethanol control (0.1% of media solution). Twenty-four hours later, luciferase activity was detected using the Steady-Glo luciferase assay system (Promega) and luminometry on a microplate scintillation counter (Packard). In this system, luciferase activity results from 1,25(OH)$_2$D binding to GAL-4-VDR and subsequent activation of the UASTK-luciferase gene via its GAL4 promoter. Detection of luciferase activity after treatment with 25OHD therefore shows conversion to 1,25(OH)$_2$D. Luciferase readings were corrected for $\beta$-galactosidase activity as a transfection control. This was detected using the Galacto-Star System (Applied Biosystems).

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Components of the vitamin D-endocrine system in C2C12 muscle cells. A, C2C12 cells express CYP27B1, VDR, CYP24A1, and DBP mRNA as seen on semiquantitative PCR. Duplicates for each are shown. B, On RT-PCR, expression of VDR and CYP24A1 mRNA is stimulated in a dose-dependent fashion by 48 hours treatment with 1,25(OH)$_2$D (data are mean ± SEM, n = 3 per group). C and D, Western blots (C) and densitometric quantitation (D) show that VDR expression (normalized for $\beta$-actin) increased 2.2-fold in response to 72 hours treatment with 1,25(OH)$_2$D (P < .005, n = 6–8 per group). E and F, On RT-PCR, expression of VDR and CYP24A1 mRNA is stimulated in a dose-dependent fashion by 24 hours treatment with 25(OH)D (data are mean ± SEM, n = 3 per group). *, P < .05; **, P < .005.

<table>
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<th>Protein Target</th>
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<th>Manufacturer, Catalog No.</th>
<th>Species and Clonality</th>
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<td>Mouse monoclonal</td>
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</table>
CYP27B1 in C2C12 cells converting 25OHD to 1,25(OH)$_2$D.

Real-time PCR

RNA was isolated using the RNasy Mini-kit (QIAGEN), and equal amounts were reverse transcribed using Superscript III first strand kit (Invitrogen) as previously described (25, 26). Real-time quantitative PCR was performed in 384-well plates. The protocol included melting for 10 minutes at 95°C and 40 cycles of 2-step PCR including melting for 15 seconds at 95°C and annealing for 1 minute at 60°C. Primers were designed using Primer 3 and BLAST (National Library of Medicine) and obtained from Invitrogen. Primer sequences are listed in Table 1. Every plate included housekeeping genes (TATA-box binding protein [TBP] and/or cyclophilin) for every sample. Semiquantitative PCR was also performed by separation of PCR products via agarose gel electrophoresis. Images were taken using a Gel Doc (Bio-Rad).

Western blot

Cell lysates (60 μg protein) were separated by SDS-PAGE as previously reported (27). A 10% gel was used, proteins were transferred to PVDF membrane, and the membrane was blocked with 5% skim milk powder in PBS plus 0.1% Tween 20. Primary antibody was applied overnight at 4°C. Washed membranes were incubated for 1 hour at room temperature with 1:1000 of horseradish peroxidase-conjugated secondary antibody in blocking buffer. After washing, immune-reactive bands were visualized using enhanced chemiluminescence (Santa Cruz Biotechnology) in a Bio-Rad chemiluminescence detection system. Bands were quantified using ImageJ (National Institutes of Health).

A list of primary antibodies, the dilutions used, and manufacturers’ details have been included in Table 2. The VDR-D6 antibody was chosen for its previously reported specificity (28) and our own validation experiments that confirm the absence of signal in VDR-null tissues. The cell cycle antibodies targeting retinoblastoma protein (Rb), phospho-Rb, and c-myc have been widely used in cancer studies and validated by Western blot in small interfering RNA knockdown cell models (29, 30). We used protein lysates of MCF-7 human breast cancer cells as positive controls when assessing cell cycle protein expression.

Statistical analysis

Statistics were calculated in Excel or SPSS version 20. Unless otherwise specified, Student’s unpaired t test with unequal variance was used to compare 2 groups. ANOVA with post hoc testing and Bonferroni correction was used where multiple comparisons were made. For all figures, data are presented as mean ± SEM. P values <.05 were considered significant.

Results

C2C12 cells express components of the vitamin D-endocrine system

There is ongoing debate about whether the effects of vitamin D on muscle are direct or indirect via effects in other tissues. To determine whether direct effects were possible, components of the vitamin D-endocrine system were measured in the absence or presence of 1,25(OH)$_2$D or 25(OH)D.

At a transcript level, C2C12 myotubes express VDR, CYP27B1, CYP24A1, and vitamin D-binding protein (DBP) (Figure 1A). Expression of VDR increased in a dose-dependent manner after 48 hours treatment with 1,25(OH)$_2$D (P < .005, Figure 1B). Translation of VDR mRNA into protein was confirmed using the VDR-D6 antibody (Santa Cruz). VDR protein increased 2-fold by 72 hours (P < .005, Figure 1, C and D), and, consistent with functional vitamin D signaling, the classic VDR target gene CYP24A1 was markedly upregulated as well (P < .005, Figure 1B).
C2C12 cells express functional CYP27B1

Apart from demonstrating the expression of CYP27B1 mRNA, we sought to determine whether this enzyme was functional in C2C12 cells. After treatment with 25OHD, there were dose-dependent increases in VDR and CYP24A1 mRNAs, strongly implying the local metabolism of 25(OH)D into 1,25(OH)_2D (Figure 1, E and F). Further evidence of functional CYP27B1 protein was sought. Luciferase reporter studies were performed in C2C12 cells that were transfected with GAL4-VDR (switch) and UASTK-luciferase reporter. After 24 hours treatment with 25OHD, there was a dose-dependent increase in luciferase activity (P < .05, Figure 2A), indicating the intracellular conversion of 25OHD to 1,25(OH)_2D by functional CYP27B1 and the subsequent activation of luciferase expression via 1,25(OH)_2D-bound GAL4-VDR.

In general, luciferase activity in response to 25OHD was comparable to that seen with the same concentrations of 1,25(OH)_2D (Figure 2A). Luciferase reporter studies have been previously used to demonstrate functional CYP27B1 in other cell types (31).

25OHD and 1,25(OH)_2D exert antiproliferative effects in C2C12 myoblasts

After 72 hours treatment with 25OHD, there were visibly fewer myoblasts in culture compared with control cultured samples (Figure 2B). This antiproliferative effect was seen at 48 hours in cells treated with 1,25(OH)_2D (Figure 3A), was dose-dependent with both 25OHD and 1,25(OH)_2D (Figures 2C and 3B), and was confirmed with AB fluorescence (Figure 3C).

Decreased cell counts can be due to decreased proliferation, increased cell death, or both. Proliferation was assessed by flow cytometric analysis of BrdU incorporation and 7AAD staining. Myoblasts treated with 100 nM 1,25(OH)_2D had a higher percentage of cells in quiescent phases of the cell cycle (G0/G1) and a lower proportion of cells in the active (S and M) phases (Figure 4A). This suggested that the antiproliferative effect of 1,25(OH)_2D was associated with cell cycle arrest.

1,25(OH)_2D does not induce apoptosis or necrosis in C2C12 myoblasts

In addition to the antiproliferative effect described above, the reduced cell number after 1,25(OH)_2D treatment could be due to increased cell death. To exclude this possibility, flow cytometry analysis was performed. Treatment with 1,25(OH)_2D had no effect on the proportion of apoptotic or necrotic cells on annexin V and PI staining (Figure 4B). In addition, there was no difference in the proportion of cells in the pre-G0/G1 phases on BrdU incorporation and 7AAD staining (P = .54, Figure 4A). As this phase contains both dying and apoptotic cells, this further confirms the lack of toxic effect of 1,25(OH)_2D in this study.

1,25(OH)_2D alters expression of cell cycle markers

To assess mechanisms by which 1,25(OH)_2D exerted its antiproliferative effect, mRNA levels of genes controlling the cell cycle were measured. Treatment of C2C12 myoblasts with 1,25(OH)_2D (range 1nM–100nM) for 48 hours dose-dependently increased ATM and Rb and reduced c-myc and cyclin D1 mRNA levels (Figure 4D). These genes control the G1-S restriction point, and the described changes are consistent with cell cycle arrest as seen in Figure 3A (32). VDR expression increased at the 48-hour time point (Figure 4D). Other cell cycle markers, including p19 and p27, were unchanged by 1,25(OH)_2D treatment.

Treatment with 1,25(OH)_2D (48 hours, 100nM) did not alter levels of total Rb protein (Figure 4C). This could be related to increased turnover of Rb or perhaps...
to delay between mRNA and protein synthesis. Interestingly, despite similar total protein, there was reduced phosphorylation of Rb protein (Figure 4C). Dephosphorylated Rb binds to critical regulatory proteins including E2F transcription factors and induces cell cycle arrest (32, 33).

Treatment with 1,25(OH)₂D reduced c-myc (Figure 4C), a transcription factor that controls cell proliferation. C-myc activity is frequently elevated in cancer (34). In epithelium (35), the c-myc pathway represents a mechanism by which 1,25(OH)₂D and VDR influence proliferation, and decreased c-myc is likely to contribute to the decrease in proliferation.

25OHD and 1,25(OH)₂D decrease myotube formation

On day 3, myogenic differentiation was induced by changing 10% fetal calf serum to 2% horse serum. The rationale for this was to examine effects of 1,25(OH)₂D on myogenesis independent of its effects on proliferation, by inducing cell cycle arrest in general. Continued treatment with 1,25(OH)₂D delayed myotube formation and reduced the number of myotubes by 38% from days 3 to 10 (P < .05, Figure 5, A and C). This coincided with down-regulation of myogenic regulatory factors (MRFs) myf5, myogenin (Figure 5B), and desmin (data not shown). These genes play central, overlapping roles in myogenesis, influencing cell cycle arrest and myotube formation.
consistent with this, 1,25(OH)\(_2\)D suppressed fusion of C2C12 myocytes to form myotubes. Fewer myotubes were also seen in response to 25OHD, indicating persistent CYP27B1 function and conversion to 1,25(OH)\(_2\)D throughout C2C12 cell differentiation (Figure 2D).

**25OHD and 1,25(OH)\(_2\)D increase C2C12 myotube diameter, and 1,25(OH)\(_2\)D downregulates myostatin**

Despite delayed proliferation and lower absolute number of myotubes, by day 10, cells treated with 1,25(OH)\(_2\)D were markedly larger (Figure 5, C and D, 1.8-fold increase in cross-sectional area, \(P < .005\)). A similar increase in myotube size was also seen in response to 25OHD (Figure 2, D and E, 2-fold increase in cross-sectional area, \(P < .005\)). Myostatin, a negative regulator of muscle mass, was downregulated 10-fold on day 7 in response to 1,25(OH)\(_2\)D (Figure 5B, \(P < .005\)), providing a potential mechanism for the notable difference in myotube size.

**Discussion**

The presence of a functional vitamin D system in C2C12 cells (Figures 1 and 2) implies that vitamin D signaling may play a direct role in muscle regulation. We found 3 distinct effects of treating C2C12 cells with 25OHD and 1,25(OH)\(_2\)D: 1) inhibition of proliferation (Figures 2 and 3), 2) inhibition of myotube formation during serum starvation (Figures 2 and 5), and 3) increased size of individual...
Figure 6. Simplified model of mammalian myogenesis and proposed effects of 25OHD and 1,25(OH)₂D. Genes regulating myoblast commitment at the mesenchymal stem cell level, myoblast proliferation and cell cycle withdrawal, myocyte fusion, myofiber size determination, and satellite cell activity are summarized. Potential effects of 25OHD and 1,25(OH)₂D on myogenesis based on data from C2C12 cells have also been shown (dashed lines). These include an antiproliferative effect (−), inhibition of myotube formation (−), and increased fiber size (+). The question that arises is whether vitamin D signaling may play a role in muscle regeneration at the level of the satellite cell. Abbreviations: BMP, bone morphogenetic protein; EYA, eyes absent transcription factor; Pax3, paired box protein 3; Pax7, paired box protein 7; PCNA, proliferating cell nuclear antigen; SHH, sonic hedgehog.

myotubes (Figures 2 and 5). VDR stimulation was effective at various time points (Figures 4 and 5), again suggesting a direct role in the transcriptional regulation of muscle development. Transcription factors that are known to regulate key steps in mammalian myogenesis have been summarized in Figure 6, including those involved in myoblast commitment, proliferation, differentiation, and the determination of muscle fiber size. Potential effects of 1,25(OH)₂D and 25OHD on myogenesis based on this work have also been depicted.

The antiproliferative effects of 1,25(OH)₂D in muscle cells were first described in 1985 (36). They are consistent with antiproliferative effects of 1,25(OH)₂D in a number of other cells and tissues including skin (35), cancer cells (37), and immune cells (38). We report a novel antiproliferative effect of the prehormone 25OHD in C2C12 cells associated with the presence of functional CYP27B1 as demonstrated by luciferase reporter studies. We also report, for the first time, underlying mechanisms for the antiproliferative effect of vitamin D signaling in C2C12 cells. By direct regulation of cell cycle gene expression (ATM, myc, Rb, and cyclin D1) and posttranslational Rb hypophosphorylation, 1,25(OH)₂D promotes cell cycle arrest and quiescence in C2C12 cells as displayed in BrdU/7AAD flow cytometry analysis (Figure 4). The antiproliferative effect of 1,25(OH)₂D was not related to an increase in cell death, making toxicity unlikely (Figure 4). Consistent with this, treatment with 1,25(OH)₂D markedly upregulated CYP24A1, which degrades 1,25(OH)₂D (Figures 1 and 5).

Another novel finding is the suppression of C2C12 myotube formation after 25OHD and 1,25(OH)₂D treatment and serum deprivation. These findings stand in contrast to the recent study by Garcia and colleagues (18). Without serum starvation, prolonged treatment of C2C12 cells with 1,25(OH)₂D resulted in a stimulatory effect on myotube formation via increased expression of MRFs in this earlier study (18). This discrepancy is interesting, because serum deprivation and prolonged confluent culture represent 2 distinct models of myogenesis. The former relies on withdrawal of mitogenic stimuli to induce cell cycle arrest and myogenin expression (12), and the latter relies on expression of endogenous IGFs (12, 39). Due to the protracted nature of C2C12 myogenesis in high-serum conditions, it is possible that Garcia et al (18) reporting a stimulatory effect of 1,25(OH)₂D on myogenesis corresponds with earlier cell cycle arrest, as seen before serum deprivation (ie, day 3) in our study.

In broader terms, the inhibitory effects of vitamin D signaling on myocyte proliferation and myotube formation may indicate the promotion of cell quiescence and protection from senescence. In a recent study, 1,25(OH)₂D (100nM) inhibited proliferation and delayed replicative senescence of human mesenchymal stem cells on the basis of β-galactosidase staining and p16 expression, without...
affecting their clonogenic capacity (40). Another study found direct links between the antiproliferative effect of VDR and proteins involved in cell survival, namely FoxO and Sirt1 (41). This may be particularly important in muscle, in which age-related dysfunction of stem cells is directly related to downregulation of their quiescent, self-renewing capacity (42).

A separate effect in this study, also seen with the high-sodium method (18), was an anabolic effect of 25OHD and 1,25(OH)\_2D on myotube size. We saw a pronounced downregulation of myostatin, a negative regulator of muscle mass, and upregulation of an upstream transcription factor, follistatin, has also been described (18). Effects of 1,25(OH)\_2D and VDR on the TGF-\beta family, of which myostatin is a member, have also been demonstrated in mesenchymal stem cells (43), skin (44), and liver (45), suggesting widespread links between these pathways. 1,25(OH)\_2D may also have effects on fiber size via insulin signaling pathways, relating specifically to insulin receptor substrate-1 and Akt phosphorylation (46).

There are limitations to this study. Although C2C12 cells express proteins necessary for muscle contraction and display the morphology of individual fiber units, there are striking differences between these cells and adult muscle, particularly in their degree of maturation (47) and mode of glucose transport (48). Therefore, effects in C2C12 cells do not always translate to adult muscle. Second, treatment of cells with pharmacological doses of 1,25(OH)\_2D in vitro may not necessarily correspond with physiologic responses in vivo. Nevertheless, this in vitro model may provide insight into the role of the VDR in skeletal muscle development and transcriptional events mediated by its activation.

In vivo studies also support a role for vitamin D signaling in the development of skeletal muscle. Mice with deletion of VDR displayed muscle fibers that were smaller and more variable in size than wild-type mice (5). This was associated with higher expression of myf5, myogenin, and E2A in quadriceps muscle of the knockouts. Myostatin was not reported in this study (5). European sea bass that expressed a lower level of VDR displayed muscle fibers that were smaller and more variable in size than wild-type mice (5). This was recently been suggested by the upregulation of VDR in a mouse model of muscle injury (54) and the modulation of key angiogenic factors vascular endothelial growth factor and fibroblast growth factor-1 in C2C12 cells treated with 1,25(OH)\_2D (55).

In summary, this work reports the presence of functional CYP27B1 in C2C12 cells on the basis of luciferase reporter studies and novel effects of 25OHD in C2C12 cell proliferation and differentiation. This work also elucidates novel effects of vitamin D signaling in C2C12 cell cycle regulation, with effects in the expression and post-translational modification of genes controlling G\_0/G\_1 arrest and G\_1/S transition. This offers mechanistic insight into the established antiproliferative effect of 1,25(OH)\_2D in these cells. Independent of this effect, 1,25(OH)\_2D inhibited myogenesis by the suppression of MRFs and had an anabolic effect on myotube formation, previously unreported findings in C2C12 cells after serum deprivation. Taken together, these findings raise the possibility of a direct effect of vitamin D on muscle. Further studies are needed to examine developmental effects of vitamin D in skeletal muscle and elucidate relevant signaling pathways at this site.

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Chapter 4 – The vitamin D receptor and skeletal muscle

This chapter consists of an original article that was published in the September edition of *Endocrinology* (2014; 155: 3227–3237). The primary author and PhD candidate, Christian Girgis, carried out the majority of experiments described in this work (>80%), drafted manuscripts and responses to reviewers. A component of this work (radiolabelled vitamin D uptake assay) was performed in collaboration with the laboratory of Prof. Rebecca Mason. To date, this article has been cited > 15 times since publication and in 2014, received the Endocrine Society Best of Basic Research Award.

The main findings of this work include:

- detection of VDR in four models of murine skeletal muscle by the use of four independent techniques (i.e. RT-PCR, western blot, immunohistochemistry, and radiolabelled vitamin D uptake assay).

- experimental conditions and technical factors necessary for detection of VDR in muscle.

- significantly higher levels of VDR in young muscle and isolated muscle cells corroborating with its purported role in muscle development and pleiotropy.

- novel effect of VDR in the ligand-mediated uptake of 25OHD in primary muscle fibres.

News/Views and Counterpoint articles published in the same edition as this article, together with our rebuttal to the Counterpoint, have also been included in this chapter.
The Vitamin D Receptor (VDR) Is Expressed in Skeletal Muscle of Male Mice and Modulates 25-Hydroxyvitamin D (25OHD) Uptake in Myofibers

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Vitamin D deficiency is associated with a range of muscle disorders, including myalgia, muscle weakness, and falls. In humans, polymorphisms of the vitamin D receptor (VDR) gene are associated with variations in muscle strength, and in mice, genetic ablation of VDR results in muscle fiber atrophy and motor deficits. However, mechanisms by which VDR regulates muscle function and morphology remain unclear. A crucial question is whether VDR is expressed in skeletal muscle and directly alters muscle physiology. Using PCR, Western blotting, and immunohistochemistry (VDR-D6 antibody), we detected VDR in murine quadriceps muscle. Detection by Western blotting was dependent on the use of hyperosmolar lysis buffer. Levels of VDR in muscle were low compared with duodenum and dropped progressively with age. Two in vitro models, C2C12 and primary myotubes, displayed dose- and time-dependent increases in expression of both VDR and its target gene CYP24A1 after 1,25(OH)₂D (1,25 dihydroxyvitamin D) treatment. Primary myotubes also expressed functional CYP27B1 as demonstrated by luciferase reporter studies, supporting an autoregulatory vitamin D-endocrine system in muscle. Myofibers isolated from mice retained tritiated 25-hydroxyvitamin D₃, and this increased after 3 hours of pretreatment with 1,25(OH)₂D (0.1nM). No such response was seen in myofibers from VDR knockout mice. In summary, VDR is expressed in skeletal muscle, and vitamin D regulates gene expression and modulates ligand-dependent uptake of 25-hydroxyvitamin D₃ in primary myofibers. (Endocrinology 155: 3227–3237, 2014)
with vitamin D deficiency independently lead to muscle disease. However, emerging evidence suggests that vitamin D may play a direct role. In vitro studies demonstrate various effects of 25OHD or 1,25(OH)₂D on calcium flux, intracellular signaling, and gene expression in muscle cells in addition to uptake of 25OHD in muscle fibers (6, 7).

The vitamin D receptor (VDR), a member of the nuclear receptor superfamily, regulates expression of numerous genes involved in calcium/phosphate homeostasis and cellular proliferation/differentiation in a predominantly ligand-dependent manner (2). The question of whether skeletal muscle expresses VDR, and may therefore be a direct target of 1,25(OH)₂D, is controversial. Several studies report the presence of VDR in muscle cell lines (6, 8–11), whereas others examining the in vivo presence of VDR have yielded contradictory results (12–16).

In this study, we address the critical issue of whether VDR is present in skeletal muscle and examine variations in its expression in young and old mice. We also elucidate a novel role of VDR in the ligand-mediated modulation of 25OHD uptake in muscle fibers, further strengthening the case in favor of its presence and function at this site.

### Materials and Methods

#### Cell culture

Primary cells were isolated from the quadriceps of 3-week-old male mice by explant culture as previously described (17). Explant cells were then trypsinized and sorted (Aria U2; Becton Dickinson-BD) using a Neural Adhesion Cell Marker/CD56 antibody (MEM-188; Thermo Scientific/Pierce) as we have recently described (18). The enriched population of primary muscle cells was then propagated in DMEM-F12 with 20% heat-inactivated fetal calf serum (FCS) and 10% Amniomax at 37°C and 5% CO₂. Serum depletion was used to induce myotube formation. These primary myotubes differ from C2C12 myotubes, because they are derived from healthy rather than dystrophic muscle (19) and are not subject to mutations arising due to immortalization (20, 21). Six days after serum depletion, myotubes were fully committed using a Gel Doc (Bio-Rad) to determine the presence or absence of mRNA transcripts.

Primary myotubes with a low passage count (ie, 5 and 6) were used in these studies. C2C12 myoblasts were propagated as previously reported (10) in DMEM-F12 with 10% heat-inactivated FCS at 37°C and with 5% CO₂. On reaching 80% confluence, cells were trypsinized and subcultured in 6-well plates (30,000 cells per well). To produce myotubes, after day 3, serum was decreased from 10% to 2%, and FCS was changed to horse serum to initiate cell cycle exit and myogenic differentiation (ie, serum depletion) (20, 21). Six days after serum depletion, myotubes were fully formed and were treated with 1,25(OH)₂D (1 nM–100 nM) vehicle (ethanol). VDR mRNA and protein expression were measured after 48 and 72 hours, respectively.
concentrations were measured. Lysates (20- to 60-μg protein) were separated by SDS-PAGE as previously reported (25). A 10% gel was used, proteins were transferred to polyvinylidene fluoride, and the membrane was blocked with 5% skim milk powder in PBS plus 0.1% Tween 20. Primary antibody was applied overnight at 4°C. Washed membranes were incubated for 1 hour at room temperature with 1:1000 of horseradish peroxidase-conjugated secondary antibody in blocking buffer. After washing, immune-reactive bands were visualized using enhanced chemiluminescence (Santa Cruz Biotechnology, Inc) in a Bio-Rad chemiluminescence detection system. Bands were quantified using ImageJ (National Institute of Health). The VDR-D6 antibody (sc13133; Santa Cruz Biotechnology, Inc) was chosen for its previously reported specificity (26). Validation experiments confirm the absence of signal in tissues from our VDRKO mice. To correct for protein loading, membranes were additionally probed with β-actin antibody (A2228, 1:20 000; Sigma-Aldrich) or total protein staining using Coomassie reagent. Protein extracted from duodenum and kidney was used a positive control for detection of VDR.

**Immunohistochemistry**

Frozen 8-μm muscle sections from 3-week-old VDRKO or WT mice were cut using a Cryostat (Leica) and mounted on slides. Sections were simultaneously fixed and permeabilized by incubation in 3% paraformaldehyde and 0.1% Triton X-100 in PBS for 30 minutes. After thorough washing in PBS, sections were then blocked with 2% BSA in PBS for 30 minutes. Sections were then incubated in VDR-D6 antibody (at a dilution of 1:100) in PBS containing 2% BSA in a moist chamber at 4°C overnight. The next morning, sections were blocked again in 2% BSA for 30 minutes at room temperature. They were then incubated in PBS with secondary antibody Alexa Fluor 488-conjugated goat antimouse IgG (1:250; Molecular Probes), 4',6-Diamidino-2-phenylindole (DAPI) (1:150; Molecular Probes, Life Technologies) was incubated with the secondary antibody to stain nuclei. Muscle sections from 3-week-old VDRKO or WT mice were used. Optimal antibody dilutions and incubation times were determined by earlier pilot experiments. For control sections, the primary or secondary antibody was omitted, and absence of signal was confirmed. Images were taken using a fluorescent microscope (Leica LAS Power-Mosaic). Duodonal sections from adult WT mice were used as positive control.

**Vitamin D luciferase reporter studies**

In addition to VDR, we assessed the presence of functional CYP27B1 in primary(533,949),(893,963)(537,964),(893,979) primary myotubes to further investigate an innate muscle system in this model of skeletal model. The expression construct Gal4-VDR was made by cloning the ligand-binding domain of VDR downstream of the Gal4 DNA-binding domain in pGL3Basic (Promega). Expression vectors for the Gal4-responsive reporter gene, UASTK-luciferase, and transfection control reporter gene (β-galactosidase) were kind gifts from Professor V.K.K. Chatterjee (University of Cambridge, United Kingdom). Plasmids were transformed in “chemically competent: top 10” Escherichia coli (Invitrogen) and extracted using the Plasmid Mini kit (QIAGEN) according to the manufacturer’s protocols. Primary myocytes were split into 96-well culture plates at high density (30 000 cells per well) and transfected 1 day later (confluence, ~90%). Lipofectamine-2000 ( Gibco) was used to transfect 800 ng each of Gal4-VDR, UASTK-luciferase reporter, and β-galactosidase reporter into 21 wells per plate. The remaining 3 wells were transfected with pcDNA empty vector as negative control. Forty-eight hours after transfection, primary myocytes had fused to form contractile myotubes due to confluent culture. These myotubes were subsequently treated in serum-free media with 25OHD (1nM–100nM), 1,25(OH)₂D (1nM–100nM), or ethanol (0.1% of media solution) as indicated. Twenty-four hours later, luciferase activity was detected using the Steady-Glo Luciferase Assay system (Promega) and luminometry in a microplate scintillation counter (Packard). In this system, luciferase activity results from 1,25(OH)₂D binding to GAL-4-VDR and subsequent activation of UASTK-luciferase gene via its GAL4 promoter. Detection of luciferase activity after treatment with 25OHD, therefore, indicates conversion to 1,25(OH)₂D. Luciferase readings were corrected for β-galactosidase as a transfection control. This was detected using the Galacto-Star System (Applied Biosystems).

**Effect of 1,25(OH)₂D on tritiated 25OHD uptake in muscle models**

Apart from examining the presence of VDR in skeletal muscle, we also sought to determine its functional significance. Whole myofibers were isolated from the flexor digitorum brevis muscle of euthanized WT and VDRKO mice, as previously described (27). The isolated myofibers were cultured in 24-well plates coated with 20-μg/mL laminin at a density of approximately 10–30 myofibers per well and maintained in DMEM supplemented with 10% FCS. They were preincubated with 0.1nM 1,25(OH)₂D or control for 3 hours. Uptake studies were conducted by incubation of myofibers with 25-[26,27 ³H]OHD₃ (PerkinElmer) at a concentration of 240 nCi/mL in DMEM supplemented with 0.1M 1,25(OH)₂D or control and 20% serum replacement 1 (Sigma-Aldrich) for 4 hours. Fibers were washed and lysed as described (7). Radioactivity was measured by scintillation counting, and the results were expressed as counts per minute per primary myofibers counted in each well. These studies were also performed in C2C12 myotubes, which were preincubated for 1 hour with 50μM 4,4’-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) or dimethyl sulfoxide control. DIDS is a chloride channel blocker known to inhibit nongenomic actions of 1,25(OH)₂D-VDR (28).

**Statistical analysis**

Statistics were calculated in Excel or SPSS version 20. Unless otherwise specified, Student’s unpaired t test with unequal variance was used to compare 2 groups. ANOVA with post hoc testing and Bonferroni correction was used where multiple comparisons were made. For all figures, data are presented as mean ± SEM. P < .05 was considered significant.

**Results**

**VDR is expressed and declines during differentiation in C2C12 muscle cells**

To test whether VDR is expressed in a skeletal muscle cell line, C2C12 cells were studied. These immortalized cells are derived from dystrophic murine muscle and dif-
ferentiate into multinucleated myotubes upon serum deple- 

tion. C2C12 cells express VDR mRNA in addition to 
mRNAs of vitamin D-related enzymes CYP27B1 encoding 1-α-hydroxylase and CYP24A1 encoding 24-hydroxy-

ylase (Figure 1A). The expression of VDR mRNA was inducible, increasing in a dose-dependent manner after 48 hours of treatment with its ligand, 1,25(OH)_2D (P < .005) (Figure 1B). VDR expression dropped sequentially throughout differentiation but remained detectable throughout (P < .005) (Figure 1C). Its expression was 0.4-fold lower in fully differentiated myotubes compared with myoblasts. 

This has also been reported in G8 and H9C2 muscle cell lines (11). VDR protein was detectable in differentiated C2C12 myotubes and increased more than 2-fold after 72 hours of treatment with 1,25(OH)_2D at a dose of 100nM (P < .005) (Figure 1, D and E).

**VDR and functional CYP27B1 are expressed in primary myotubes**

Primary myotubes appear as elongated, multinucleated syncytia akin to muscle fibers (Figure 2A). They express cytoskeletal proteins necessary for contraction and may contract spontaneously in culture. At a transcript level, primary myotubes express VDR in addition to vitamin D-related enzymes CYP27B1 and CYP24A1 (Figure 2, B–D). The expression of VDR and its classic target gene, CYP24A1, were inducible, increasing in a dose-dependent manner after 48 hours treatment with 1,25(OH)_2D (P < .005) (Figure 2B). In a time-course study, expression of VDR was increased by 4 hours after treatment with 1,25(OH)_2D (100nM), and the increase was maintained at 8 and 16 hours (P < .005) (Figure 2D). CYP24A1 also increased significantly but not until 16 hours (P < .005) (Figure 2D). There was no regulation of CYP24A1 by 1,25(OH)_2D in primary myotubes from VDRKO mice (Figure 2D), confirming that this effect is mediated by VDR.

Apart from demonstrating the expression of CYP27B1 mRNA (Figure 2C), we sought to determine whether the enzyme encoded by this gene (1-α-hydroxylase) was functional in primary myotubes and could convert 25OHD to 1,25(OH)_2D. Luciferase reporter studies were performed in primary myotubes that were transfected with GAL4-VDR (switch), UASTK-luciferase reporter, and β-galactosidase reporter (transfection control). After 24 hours of treatment with 25OHD, there was a dose-depen-
dent increase in luciferase activity (P < .05) (Figure 2E), indicating the intracellular conversion of 25OHD to 1,25(OH)_2D. This demonstrates functional 1-α-hydroxylase converting 25OHD to 1,25(OH)_2D and the subsequent activation of luciferase expression via 1,25(OH)_2D-bound GAL4-VDR. Luciferase activity in response to 25OHD was comparable with that seen with 1,25(OH)_2D used as positive control (Figure 2E). Doses of 25OHD used (5nM–100nM) were substantially lower than those known to directly acti-
vate VDR independent of CYP27B1 (29). Luciferase re-
porter studies have been previously used to demonstrate functional 1-α-hydroxylase in C2C12 myoblasts (10), but this is the first report in primary contractile myotubes.

**VDR transcript is detectable in skeletal muscle**

We performed RT-PCR on whole muscle extracts from adult WT and VDRKO mice. VDR transcript was detectable in WT but not in VDRKO muscle (Figure 3A). Levels of VDR transcript in (WT) skeletal muscle were low com-
pared with a classical site of VDR expression, the duode-
um (relative transcript levels were obtained by correcting for muscle VDR mRNA in Figure 3B). However, tran-
scription factors may be functionally active in the regula-
tion of gene expression at very low expression levels (30).
Therefore, this difference in transcript levels does not preclude a functional role for VDR in muscle. To examine this, we assessed differences in the expression of cell cycle and calcium handling genes in WT and VDRKO muscle. These pathways were chosen because they are regulated by VDR in other organ systems (31). Significant increases in the expression of sarcoplasmic reticulum calcium channels and an intracellular calcium-binding protein were noted in VDRKO muscle (see figure 5 below). This was unexpected because the opposite effect is seen in intestine and kidney (32). This highlights the tissue-specific nature of VDR regulation of calcium handling, which has also been demonstrated in brain (33). Expression of myc and cyclins were significantly higher in the muscle of VDRKO mice, consistent with changes seen in skin and intestine, indicating altered cell cycle regulation (see Figure 5 below) (34).

VDR protein is detectable in skeletal muscle

We compared the effects of 2 different lysis solutions upon the ability to detect VDR on Western blotting under the same experimental conditions. Protein bands were barely detectable in lysates made using RLB (Figure 3C). However, in HLB samples, bands were detectable in WT but undetectable in muscle from VDRKO mice (Figure 3C). HLB may facilitate release of DNA-bound proteins, including VDR, and be more effective for protein unfolding and denaturation (35, 36). Similar urea-containing lysis buffer is used for the detection of heat-shock transcription factors in muscle (37). These findings denote the importance of specific conditions in the detection of nuclear proteins expressed at low levels in muscle, such as VDR. In Western blottings comparing VDR in muscle, duodenum, and kidney, VDR detection in muscle required relatively long exposure time (15 min) and greater amounts of muscle protein per sample (ie, 50 vs 10 µg for duodenum and kidney per lane) (Figure 3D). However, it was detectable.

VDR is detectable at higher levels in cell models than whole muscle

VDR expression levels differed substantially between whole muscle and in vitro models. Primary and C2C12 myotubes expressed significantly higher levels of VDR mRNA than whole muscle (ie, ~8- and ~300-fold, respectively; P < .005) (Figure 3B). The CT values for the real-time PCR were 22.8 ± 0.1 for C2C12 cells, 28.1 ± 0.3 for primary myotubes, and 31.1 ± 0.2 for primary muscle. At a protein level, there was approximately 10-fold more VDR in primary and C2C12 myotubes compared with whole muscle (P < .05) (Figure 3, E and F). This finding suggests that VDR expression is activated after muscle cell isolation and immortalization, respectively. Alternatively, its presence specifically within muscle fibers and not in other components of whole muscle may possibly explain this discrepancy.

VDR is detectable within muscle fibers

Skeletal muscle is a heterogeneous tissue composed of myofibers, fibroblasts, immune cells, and adipocytes. We therefore sought to determine whether the expression of VDR in muscle was specifically located in muscle fibers. On immunohistochemistry of muscle taken from young WT mice, VDR was detected within muscle fibers and...
partly localized to fiber nuclei as seen on DAPI counterstaining (Figure 4, A–C). No signal was detected in VDRKO muscle (Figure 4, D and E). As positive control, VDR staining in duodenum of WT mice was performed (Figure 4, F and G).

**VDR expression in muscle is greater in young mice**

VDR exerts pleiotropic effects in the development of bone, skin, and the immune system (38, 39). Recent data also suggest that upon activation, VDR regulates proliferation and differentiation in C2C12 muscle cells (9, 10). We therefore sought to determine whether the expression of VDR in muscle was influenced by developmental age. To do this, VDR transcript and protein were compared in quadriceps muscles of mice of different ages, from newborn pups to 3-month-old adults. VDR transcript levels were significantly lower in the quadriceps muscles of 3-week-old mice (ie, postweaning) and 3-month-old mice compared with newborn WT mice (0.7- and 0.14-fold, respectively; \( P < .005 \)) (Figure 5B). This indicated a sequential drop in the expression of VDR in muscle throughout development (ie, from newborn to 3 wk of age) and into maturity (ie, from 3 wk to 3 mo of age). These changes were also confirmed at a protein level. On Western blotting, VDR protein was significantly lower in muscles of 3-month-old mice compared with newborn pups (0.4-fold difference; \( P < .005 \)) (Figure 5, C and D). These findings suggest the possibility of a role for VDR in muscle development and, perhaps, a different role in fully developed, adult muscles. This also denotes the importance of age in the detection of VDR in skeletal muscle.

**VDR modulates 1,25(OH)\(_2\)D-modulated uptake of 25OHD in muscle**

We have recently elucidated a novel role for skeletal muscle in the net uptake of 25OHD (7). This occurs in a time-dependent fashion and relies on the local expression of megalin and cubilin, endocytotic receptors for the vitamin D-binding protein (DBP).

WT myofibers preincubated with 1,25(OH)\(_2\)D for 3 hours showed 40% higher net uptake of \(^3\)H-25OHD\(_3\) than WT myofibers preincubated with control solution.
There was no increase in the uptake of $^{3}$H-25OHD$_3$ in VDRKO myofibers exposed to 1,25(OH)$_2$D (Figure 6). In addition, DIDS, an inhibitor of the non-genomic effects of the VDR, reversed 1,25(OH)$_2$D-mediated increases in $^{3}$H-25OHD$_3$ uptake in C2C12 myotubes (Figure 6B). Together, these data indicate that VDR modulates ligand-mediated uptake of 25OHD in skeletal muscle. This occurs via a nongenomic VDR mechanism and confirms its presence at this site.

WT and VDRKO myofibers not treated with 1,25(OH)$_2$D showed similar levels of net $^{3}$H-25OHD$_3$ uptake. This suggests that VDR-independent processes may also affect the retention of 25OHD in skeletal muscle at baseline.

**Discussion and conclusions**

This study uses 4 models to investigate the presence of VDR in skeletal muscle: C2C12 cells, primary myotubes, mature muscle fibers, and whole muscle. This is the first report of an innate vitamin D-endocrine system in primary muscle cells that possess functional CYP27B1, VDR, and CYP24A1. We report 3 factors confounding VDR detection in muscle. These include: 1) differences in VDR signal on Western blotting depending on protein extraction methods (Figure 3C); 2) age-related differences in VDR expression in muscle (Figure 5, B–D); and 3) the augmentation of VDR in muscle cell models compared with whole muscle (Figure 3E). Consistent with an earlier report (12), VDR was not detectable on Western blotting after the use of RLB in whole muscle. However, upon using a HLB, VDR was detectable but at a substantially lower level than in duodenum and kidney (Figure 3D). As further evidence of the functionality of VDR in muscle, we demonstrated its role in modulating ligand-dependent uptake of 25OHD in muscle fibers (Figure 6).

Contradictory reports regarding the presence of a 1,25(OH)$_2$D-binding protein in muscle date back 4 decades (summarized in Table 1). Before the discovery of VDR in 1974 (40), Neville and DeLuca (41) reported the localization of $^{3}$H-25OHD$_3$ in the membrane of skeletal muscle, but Stumpf et al (42) failed to identify the nuclear localization of $^{3}$H-1,25(OH)$_2$D at this site. Studies using anti-VDR antibodies were also conflicting, some report-
ing detection of VDR in skeletal muscle, which was not confirmed by others (12–15, 43). In the notable work by Wang et al. (26), differences in the specificity of commercially available VDR antibodies were reported. Using the highly specific D6 antibody, VDR was not detected in the muscle of mature animals, but young animals were not examined (12). Other studies were limited by reliance on muscle cell models alone, use of nonvalidated VDR antibodies, and/or lack of appropriate controls (Table 1). In a recent randomized study of 21 older women with vitamin D deficiency (mean 25OHD, ∼45 nmol/L), vitamin D supplementation for 4 months resulted in 30% increase in intramyonuclear VDR staining and 10% increase in muscle fiber size (16). However, the specificity of the antibody used in this study (VDR-NR1I1) is uncertain, and it has not been validated by the absence of signal in VDRKO tissue (15).

Vitamin D deficiency is associated with muscle weakness, abnormal muscle physiology, and muscle mitochondrial defects in humans and animals (2, 44–49). VDR polymorphisms in humans are associated with muscle strength and falls (2), and VDR ablation in mice leads to impaired motor coordination, shorter stride length, and abnormal swimming (50, 51). An important question raised by these studies is whether the effects of vitamin D in muscle are direct or indirect. Our findings in primary myotubes indicate that direct effects are possible, because these cells express functional CYP27B1 and respond to 1,25(OH)₂D by activation of VDR and its classic target gene CYP24A1. We have also recently reported direct effects of 25OHD and 1,25(OH)₂D in C2C12 muscle cell proliferation and differentiation (10). The increase in VDR after muscle cell isolation in this study is interesting (Figure 3E). This is not unique to muscle cells (52, 53) and may result in amplified responses to vitamin D in vitro. The relatively low levels of VDR in adult muscle do not preclude a direct role in many of its physiological effects. In liver, under normal circumstances, VDR is also expressed at levels which are so low that it is also difficult to detect by Western blotting (43). Despite this, it has recently been clearly demonstrated that VDR plays an important role in liver fibrosis (54).

We demonstrate here that VDR is necessary for 1,25(OH)₂D-modulated uptake of 25OHD in freshly isolated muscle fibers (Figure 6A). These ex vivo experiments differ from in vitro techniques, because whole muscle fibers were used and experiments were performed directly after isolation, excluding effects of long-term altered gene expression. Whole muscle fibers responded significantly to physiological levels of 1,25(OH)₂D, validating the relevance of this model. In addition to our previous description of skeletal muscle as an extravascular site for uptake of 25OHD (7), we now demonstrate a role for VDR in this process. This finding raises the pertinent question: What

Figure 5. Effects of VDRKO in muscle gene expression and age-related differences in muscle VDR in WT mice. A, Muscle from WT vs VDRKO mice displayed significant differences in the mRNAs of calcium-handling genes, including Serca2a, Serca2b, Serca3, and Calbindin-28K (Sc2a, Sc2b, Sc3, and Cal.), and cell cycle regulatory genes, including myc and Cyclins D2, D3, and E1 (Cd2, Cd3, and Ce1). B, VDR transcript levels were significantly higher in muscles of newborn WT mice compared with 3-week- and 3-month-old mice (n = 3 per group; P < .005). Using samples prepared in HLB, Western blotting (C) and densitometric quantitation (D) demonstrated higher VDR expression in muscles of newborn WT mice compared with 3-month-old mice (n = 4 per group; P < .005). Muscle from newborn VDRKO mice was used as negative control. Pr, protein.

Figure 6. ³H-25OHD₃ uptake in WT and VDRKO myofibers. A, Preincubation of WT myofibers with 1,25(OH)₂D resulted in 40% greater ³H-25OHD₃ uptake compared with WT myofibers preincubated with control solution (P < .005). No such increase in ³H-25OHD₃ uptake in response to 1,25(OH)₂D was seen in VDRKO mice (n = 3 mice per group). B, In C2C12 myotubes, DIDS reversed the 1,25(OH)₂D-mediated increase in uptake of ³H-25OHD₃, cpm, counts per minute.
“happens” to 25OHD upon entry into the muscle fiber? Bound to DBP, it could remain attached to actin (7). Upon degradation of DBP (55), 25OHD may diffuse back into the circulation, where it becomes available for biodegradation of DBP. Upon entry into the muscle fiber?

Abbreviations: IHC, immunohistochemistry; WB, Western blot; IP, immunoprecipitation; co-IP, co-immunoprecipitation; NB, Northern blot.

Table 1. Studies Investigating VDR in Skeletal Muscle

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<td>VDR present; translocates to membrane after 1,25(OH) D; interacts with membrane scaffolding protein, caveolin-1</td>
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<td>Endo et al (56)</td>
<td>Mouse</td>
<td>Muscle extracts; C2C12 cells</td>
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<td>VDR mRNA in 3-wk- but not 8-wk-old mice; developmental differences in VDRKO vs WT mice</td>
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<td>Bischoff et al (13)</td>
<td>Human</td>
<td>Muscle extracts (20 subjects)</td>
<td>IHC (VDR-9A7 Ab)</td>
<td>VDR present, localized to nucleus; decreases with age, no correlation with 25OHD or 1,25(OH) D</td>
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<td>Ceglie et al (15)</td>
<td>Human</td>
<td>Muscle extracts (4 subjects) C2C12 cells</td>
<td>IHC (VDR-NR111 Ab; p-6 and 333C6a Ab used as control); fiber-typing</td>
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<td>Garcia et al (9)</td>
<td>Mouse</td>
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<td>PCR, IHC, WB (VDR-C20 Ab)</td>
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<tr>
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<tr>
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<td>IHC, WB (VDR-H81 Ab); transfection, BaCl2, muscle injury</td>
<td>VDR and CYP27B1 present and are increased in regenerating fibers Subjects randomized to vitD3 (4000 IU/d); increased myonuclear VDR and fiber size</td>
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<td>Muscle extracts (12 subjects)</td>
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<td>Girgis et al (10)</td>
<td>Mouse</td>
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<td>PCR, IHC, WB, luciferase reporter studies</td>
<td>VDR and CYP27B1 present; 25OHD and 1,25(OH) D antiproliferative, antmyogenic, larger myotubes</td>
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Abbreviations: IHC, immunohistochemistry; WB, Western blot; IP, immunoprecipitation; co-IP, co-immunoprecipitation; NB, Northern blot.

In rat embryos, maternal vitamin D deficiency is associated with reduced arm-muscle area in offspring (57), and in rats, smaller muscle fibers and altered expression of developmental genes was noted in pups born to vitamin D-deficient dams (58). In utero transfer of radiolabeled 25OHD across placenta and into muscle of rat embryos also supports a role for vitamin D in embryonic muscle development (59).

The main limitation of this study is the focus on murine muscle. Although there is high interspecies homology in the structure and ligand-binding properties of VDR (60), differences exist in the metabolic rate, contractile speed, and morphology of murine vs human muscle. To date, only 1 study has examined the presence of VDR in human muscle using the highly specific VDR-D6 antibody, and a
single sample was used (12). Future studies examining human muscle will be of great interest.

In summary, this study reports conditions necessary for the detection of VDR in murine muscle on the basis of 4 different models. We confirm that muscle is a direct target of vitamin D with an autoregulatory vitamin D-endocrine system in primary myotubes and the 1,25(OH)₂D-modulated uptake of 25OHD in WT myofibers, an effect which is absent in VDRKO myofibers. A novel role for VDR in the regulated uptake of 25OHD in muscle confirms its presence at this site; further studies are needed to determine the biological significance and mechanisms underlying this process.

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Vitamin D has been known for nearly a century to be essential for bone health because it can prevent and cure endemic rickets and osteomalacia (1–3). Currently, the complex implications of deficiency or excess vitamin D on bone modeling and remodeling are well documented by a wealth of preclinical and clinical studies (reviewed in 4). The vitamin D receptor (VDR) as well as the key activating (CYP27B1) or inactivating (CYP24A1) enzymes are, however, expressed in many cells. Moreover, a very large number of genes—from zebra fish up to mice and man—are under direct or indirect control of the active hormone, 1,25(OH)₂D₃. Therefore, it looks attractive to hypothesize that the vitamin D endocrine system would have many extraskeletal effects. Such hypothesis fits with the very broad spectrum of activity of most ligands of nuclear receptors (1–3). Poor vitamin D status has also been associated with most major diseases of mankind, ranging from immune diseases and infections, cancer, diabetes, and the metabolic syndrome, as well as cardiovascular risk factors and events, energy homeostasis, bile acid metabolism, to (last but not least) muscle function and falls (1, 3, 5). However, following an extensive evaluation of these data, the Institute of Medicine concluded, that “Scientific evidence indicates that calcium and vitamin D play key roles in bone health. The current evidence, however, does not support other benefits” and “There is inconsistent evidence that supplemental vitamin D reduces falls in postmenopausal women and older men” (6). This conclusion was highly debated because several meta-analyses concluded that vitamin D supplementation of elderly subjects decreased the risk of falls by approximately 20% and improved proximal muscle strength of severely vitamin D–deficient (serum 25OHD < 10 ng/ml) subjects (7, 8).

Vitamin D’s direct action on muscle became doubtful when a careful analysis of VDR expression in adult human muscle as well as mouse skeletal and cardiac muscle, performed in H. DeLuca’s laboratory (9), vitamin D receptor showed only very low gene expression (10 000-fold lower than in the intestine) without detectable protein expression even with a highly specific anti-VDR antibody, in contrast with older publications using less stringent antibodies (9). Therefore, any potential effect of vitamin D metabolites on muscle could at most be indirect given that both genomic and potential nongenomic effects require the presence of VDR.

In the current issue of Endocrinology, Girgis et al (10) demonstrate that VDR protein (using the same highly specific antibody) can be clearly demonstrated in adult mouse skeletal muscle, although only when using a hyperosmolar lysis buffer. Such buffer releases VDR from its tight binding to DNA, as it has been demonstrated previously that even unliganded VDR mainly resides in the nucleus. The gene and protein expression of VDR was higher in muscle cell precursors (in vitro) compared with adult mature muscle and was also higher in muscle from younger than in older animals (in vivo). Nevertheless, the expression was still several-fold lower in skeletal muscle than in the intestine. Moreover, the authors confirmed the expression as well as the functionality of cyp27b1 in muscle cell cultures. The activity of the vitamin D receptor was also confirmed because both 25OHDL and 1,25(OH)₂D₃ stimulated the expression of VDR and Cyp24a1, both known targets of VDR action, as well as the uptake of 25OHD in muscle. Negative controls for all these end points were obtained in VDR-null mouse tissue. All the data are in line with decreasing VDR expression, as well as decreasing
number of VDR binding sites in the cistrome and decreasing transcriptome during maturation of other cells such as osteoblasts (11) and osteoclasts. Indeed, the expression of VDR is lost in multinucleated osteoclasts whereas 1,25(OH)₂₃’s is a very potent stimulator of osteoclast progenitors (12).

These novel data will clearly reopen the discussion on muscle as a potential target for vitamin D’s action. The gene and protein expression of VDR is also in line with several other in vitro studies. Indeed, 1,25(OH)₂₃ decreased proliferation and enhanced differentiation of myocyte precursors, including the stimulation of follistatin and inhibition of myostatin (13) (Figure 1). Moreover, VDR-null mice show a clear muscle phenotype, with smaller muscle fiber size and abnormal expression of all major muscle-specific genes including myogenin, myoD, and Myf5 (14). Furthermore, cardioselective VDR deletion (15) also causes similar cardiac hypertrophy and fibrosis as in systemic VDR-null mice (Table 1). Finally, vitamin D deficiency accelerates muscle protein degradation by stimulation of the ubiquitin pathway in male rats (16).

Do Girgis’ (10) observations in mouse muscle also apply to human skeletal, cardiac, and smooth muscle? Careful clinical observations indeed linked hypotonia, myopathy, and especially proximal muscle weakness to rickets already in the early descriptions in the 17th century. More recently, muscle weakness was confirmed in subjects with inborn deficiency of CYP27B1 (hereditary pseudovitamin D deficiency rickets (18)) and in patients with severe chronic renal failure and deficiency of all vitamin D metabolites. Severe muscle weakness, up to the point of need for a wheelchair, also rapidly disappears when given 1,25(OH)₂₃ (3). Randomized controlled trials, however, could not demonstrate beneficial effects of vitamin D supplementation on either grip strength or proximal muscle strength (except in patients with serum 25OHD levels < 10 ng/ml at baseline). Elegant in vivo ³²P nuclear magnetic resonance spectroscopy data, however, showed more rapid energy (ATP) recovery after modest exercise, concomitant with correction of complaints of severe muscle weakness following vitamin D supplementation in deficient Asian United Kingdom residents (5, 19). Finally, the risk of falls could be reduced by approximately 20% after vitamin D supplementation of elderly, mostly vitamin D–deficient subjects, at least according to some (7, 8, 20) but not all meta-analyses (21). As for most ligands of nuclear receptors, too much may be equally deleterious as too little. Indeed an increased risk of falls and fractures

Figure 1. Muscle gene regulation by the vitamin D endocrine system as revealed by in vitro and in vivo rodent studies.
was observed during the first 3 months following a yearly loading dose (500 000 IU of 25OHD₃) (17).

Therefore, muscle may indeed be a real target for vitamin D action but its mechanism of action may well be a combination of indirect [eg, myocardial dysfunction of vitamin D–deficient chicks could be corrected by calcium alone (22)], and direct genomic actions (23, 24) (Figure 1) or even nongenomic actions (24, 25).

The study of Girgis et al (10) might generate more questions than answers. Protein expression of other nuclear receptors may also have to be reconsidered by using hyperosmolar conditions to better release such receptors from the nucleus, and this may be especially relevant for (un)liganded receptors residing permanently in the nucleus, or for clinical situations whereby receptor expression in cancer tissues is crucial for therapeutic decisions. A hyperosmolar lysis buffer was also needed to visualize heat shock proteins. Tissue-specific deletion of VDR or Cyp27B1, at early or late stage of muscle development or conditional postnatal deletion may help to clarify mechanism(s) of action of the vitamin D endocrine system. The Girgis study (10) also suggested that 1,25(OH)₂D₃ may stimulate the uptake of 25OHD in muscle, thereby addressing the intriguing question of the storage site of vitamin D (metabolites). Indeed, the summer accumulation of vitamin D apparently enables surviving lack of sunlight exposure during autumn and winter longer than expected from the short half-life of only two weeks of the serum pool of 25OHD.

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The diverse biological actions of the vitamin D hormone, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), that are beyond its contribution to the maintenance of mineral metabolism are now well recognized (1). These actions include significant roles in skin maturation, protection, and function, the immune system, cardiovascular activity, neuromuscular function, bile acid metabolism, xenobiotic detoxification, muscle activity, and hepatic function (2). They also include broad cellular growth control mechanisms that include blockade of proliferation, prodifferentiation, induced apoptosis, and other fundamental cellular processes that may be of therapeutic relevance in cancer (3). Many of these activities have been described over a span of several decades both in cultured cells of specific lineage origin and in vivo. In the latter case, these studies have made frequent use of genetic strains of mice that are either globally or tissue specifically deficient in the expression of the vitamin D receptor (VDR), the central mediator of vitamin D action in all tissues (2, 4, 5). Perhaps most importantly, the beneficial biological effects of vitamin D in many of these systems appear to have translational and clinical components as well, because clinical pathologies associated with these systems frequently correlate with vitamin D deficiency, and at least, a subset have been shown to respond positively to increased vitamin D intake (6). Accordingly, the actions of 1,25(OH)₂D₃ in many of these tissues in humans are generally not in dispute.

The mechanism through which vitamin D acts in tissues centers on the presence and activity of the VDR, a transcription factor that is activated by hormonal vitamin D and functions at the level of the genome in a cell-specific manner to regulate the transcription of genes (5). This receptor is highly expressed in tissues, such as intestinal epithelial cells and proximal and distal tubules of the kidney, and mesenchymal lineage cells, such as chondrocytes, early osteoblast precursors, mature osteoblasts, and osteocytes, and in many other cell types as well (7). Molecularly cloned and the protein product studied over several decades, many of the principles of the VDR’s modulatory actions to regulate the expression of specific genes are now well established, most recently at the genome-wide level (8–11). Although not fully understood, our advanced appreciation of the mechanisms through which the VDR operates at the genetic level stands in stark contrast to those that have been proposed to account for the so called rapid, nongenomic actions of the hormone, where the VDR or other “receptors” have been suggested to regulate specific membrane-associated activities in cells (12). Despite suggestions to the contrary, both the mechanisms that underlie these latter activities and their relevance in vivo remain to be determined, although considerable mechanistic precedent has been established for nongenomic activity through the study of nuclear receptors for estrogen, the androgens, and progesterone.

With these issues in mind, it is not surprising that studies aimed at understanding the actions of the vitamin D hormone in such tissues as the nervous system, the cardiovascular system, and the skeletal muscle system have focused upon detection of the VDR as an initial prerequisite for defining a direct mechanism of action of the hormone in these systems. Although this may appear to the outsider to be a relatively easy task, the VDR protein is expressed at extremely low levels and thus is not easily detected even in bone fide vitamin D-sensitive target tissues. Therefore, it is even more difficult to identify the receptor in atypical tissues where VDR abundance may be restricted to rare cell subtypes or is generally low for unknown reasons. Accordingly, these features lead to more
fundamental questions: Is the VDR expressed in a particular tissue above cellular background, can its activity(s) be identified in unequivocal terms, is its activity localized to a specific cell type and not to a contaminating or invading cell type, and is it possible that the VDR manifests biological roles in tissues that are completely independent of those that involve transcription? These questions need to be answered before excluding the possibility that in certain tissues vitamin D’s actions are indirect, perhaps through the regulation of local or systemic modulators, such as peptide or steroid-like hormones from other tissues or through the maintenance of extracellular calcium and phosphorus levels, which are known to impact muscle, bone, and nerve cell function. What also must be considered is the possibility that VDR expression can be selectively regulated in cell types that are temporally unique to tissues and organs, such as those that occur during development, growth, differentiation, and/or during physiologic states, such as pregnancy, lactation, and aging. Alternatively, VDR expression may appear in a specific cell type(s) as a consequence of disease. A final challenge is to interpret the expression of the VDR in cell lines and in primary cells in culture, where lineage relationships to the originating tissue are often times uncertain and frequently problematic. Efforts to identify the VDR in liver, cardiovascular tissues, skeletal muscle cells, and neurons of the central nervous system are particularly prone to these latter issues, because the VDR is generally found in cell lines derived from these tissues but has been difficult to detect in the primary tissues.

In this current volume, Girgis et al (13) examine whether the VDR is present in skeletal muscle both in vitro and in vivo and assess whether the receptor is capable of mediating biological responses. In view of the numerous and often conflicting studies that have attempted to address this question over the years, as summarized, in part, in table 1 of the manuscript, one would have hoped that the current study would resolve these issues experimentally once and for all. Although the observations are interesting, however, this is really not the case. In short, some of the major issues still remain major points of controversy. Experiments in this study are initiated using the mouse C2C12 cell line, and early mesenchymal lineage cell has been employed by muscle biologists for decades to study myoblast to myotube differentiation and by many previous investigators to support the concept that muscle cells contain the VDR (14–17). As in these earlier studies, the present authors show that the VDR is indeed readily expressed in this cell line (at the mRNA level and protein levels) and regulated by 1,25(OH)2D3. Because the VDR can be dynamically up-regulated in many cell lines passed in culture as well as during differentiation, however, these observations documented by Girgis et al (13) do not really resolve the question of whether the VDR is expressed in skeletal muscle targets in vivo.

Thus, is this question answered in the current study? An attempt is made. The authors show using traditional semi-quantitative RT-PCR analysis that VDR transcripts are detected, however, at concentrations 3–4 logs lower than that found in the intestine. Unfortunately, this technique does not measure the VDR protein itself and perhaps more importantly, is many logs more sensitive than those that do. Thus, it is not surprising that these investigators and many before them have struggled to identify the VDR using Western blot analysis or through immunocytochemistry (see Ref. 13 and table therein). This seems to be highlighted by the Western blot analyses in the current work, where it is unclear whether the immunoband detected in normal adult skeletal muscle is in fact the VDR, given the requirement for an unusual hyperosmolar (denaturing) extraction buffer (18), the presence of additional bands in a size range that is similar to the VDR, and the fact that virtually all of the bands that are detected are absent in the VDR-null control extract. With regard to the use of the hyperosmolar buffer, although the authors suggest that it may remove the VDR more efficiently from DNA, most of the VDR extracted here is not likely to be bound to the genome, because normal levels of gene occupancy were shown years ago to be surprisingly low (~15%) in vitamin D-sufficient animals (19). In addition, most nuclear receptors are known to be extracted efficiently in high salt buffers. In the case of the ligand-free VDR, concentrations of 0.15M NaCl are generally sufficient (20, 21), although higher levels (~0.3M) are necessary when the VDR is bound to DNA via activation by 1,25(OH)2D3. In the current case, the addition of detergents, such as Triton X-100 and NP-40 (very similar nonionic detergents), only increase VDR sensitivity to extraction. One has to wonder whether it is technically feasible using Western blot analysis to detect VDR protein in crude extracts of muscle such as these when VDR transcripts are present at 1/4284th the concentration of those observed in the duodenum.

Interestingly, a recent series of well-controlled immunocytochemical studies has been reported wherein VDR was detected in a highly robust fashion in intestinal tissue and other VDR-positive tissues (22–24). These experiments, however, failed to detect the VDR in skeletal muscle, numerous cardiovascular sites, including the heart and liver, and in other cell types as well, suggesting its absence in these tissues. Interestingly, these reports have been widely used to call into question the presence of the VDR in muscle. Ironically, however, they simply reiterate the historical inability of investigators to detect the VDR in muscle in studies using sucrose gradient analysis, hor-
mone-binding assays, scintillation autoradiography, the earliest Western blot analyses, and finally various forms of mRNA analysis, culminating in highly sensitive reverse transcriptase, real time PCR approaches that have spanned almost 4 decades. Perhaps the most important lesson to be learned from the studies of DeLuca and co-workers (22–24) is the absolute requirement for highly robust positive and negative controls when assessing VDR levels in test tissues such as the muscle. In the current study, this does not appear to be the case, because the fluorescence intensity of signals generated from VDR-positive tissue such as the intestine are not particularly remarkable and the cellular and subcellular sources of the signals are unclear. Even in the most recent studies (22–24), however, the question of whether low levels of VDR in muscle and other tissues would be detectable by immunological means should still be raised. Is it possible that mRNA levels and VDR proteins levels might be strikingly discordant, and thus the levels of receptor protein in muscle tissue are higher than would be predicted as a result of low transcript levels? The answer is of course yes; but if so, such a relationship needs to be demonstrated. What additional observations are made regarding the receptor in this study? Perhaps the most important is the finding that the concentrations of the VDR in neonatal mouse muscle is much higher than that found in adults. Although further exploration is necessary, it is possible that this interesting discovery could provide an important entre into defining the mechanism through which vitamin D exerts its actions on muscle in adults as well.

The VDR functions in a traditional sense to regulate the expression of genes by virtue of its ability to bind to sites on DNA, to recruit coregulatory complexes that modify chromatin structure and function, and to interact with other transcription factors in a DNA-independent manner (5). In the current study, the authors show that both the VDR and Cyp24a1 genes are modestly up-regulated by 1,25(OH)2D3, thereby establishing that this traditional function is apparently intact in passaged primary myotubes. Unfortunately, the regulation of these or other genes by 1,25(OH)2D3 is not followed up by in vivo studies. Rather, the authors explore the possibility that 1,25(OH)2D3 might stimulate the uptake of 25OHD3 into isolated muscle myofibers, an interaction that has been previously described in muscle, which occurs between the vitamin D-binding protein-bound vitamin D metabolite and membrane-bound endocytotic receptors megalin and cubilin, and which may mediate storage of 25OHD3 (25).

Accordingly, the authors show that there is a modest involvement of the VDR in this process in that pretreatment of both myofibers as well as C2C12 cells with low levels of 1,25(OH)2D3 are indeed stimulatory for uptake. Unfortunately, the nature of this stimulatory effect is entirely obscure, because despite the suggestion that this phenomenon may represent a rapid nongenomic activity, the requirement for a 3-hour hormone pretreatment of the myofibers (and cells) is hardly the stuff of a nongenomic action. Because neither megalin nor cubilin are up-regulated, it would appear that this activity is not dependent upon transcription either. Clearly, further studies will be necessary to facilitate an understanding of this proposed role for the VDR in muscle tissue.

The present studies highlight the difficulties of using the presence of the VDR in cells and tissues as a centerpiece for defining the mechanism that underlies the biological actions of vitamin D in nontraditional vitamin D-responsive tissues. At this stage, hard experimental evidence will be necessary to define a regulatory mechanism in muscle tissue and to conclude that 1,25(OH)2D3 plays a direct rather than an indirect biological role in this tissue, perhaps through the regulation of extracellular phosphate levels. These effects must also be distinguished from the more subtle yet indirect mechanisms that have already been shown to occur. For example, 1,25(OH)2D3 affects bile acid metabolism in the liver not via a direct effect but rather by inducing intestinal expression of fibroblast growth factor 15, an endocrine fibroblast growth factor that is rapidly transported via the portal system to the liver, where its effects on bile acid metabolism are actually observed (26). They must also be distinguished from mechanisms related to distinct VDR up-regulation during a disease process. Accordingly, a recent study suggests a direct action of 1,25(OH)2D3 in the liver centered on the hormone’s ability to counter TGFβ-induced liver fibrosis (27). However, TGFβ activates normally quiescent liver stellate cells. Thus, the possibility exists that the VDR is up-regulated during activation, and that the protein’s presence is actually representative of a disease process rather than a physiological model of normal hepatic tissue response. Finally, studies must also be undertaken to quantitate the levels of the VDR in specific cell types as well. For example, although VDR-specific peptides have recently been demonstrated in whole rat brain using mass spectrometry, the starting material was derived from 20 separate mice (28). Clearly, additional details similar to those generated recently will be necessary to interpret this interesting result (29). Studies of this nature will be necessary to understand 1,25(OH)2D3 action in muscle tissues as well. Perhaps careful genetic deletion of the VDR gene product in adult myocytes and/or their precursors will be revealing, as has been accomplished in cardiac tissue (30). These latter studies have provided supportive evidence for the direct effects of vitamin D on the heart.
There is no doubt that vitamin D is beneficial for the maintenance of normal muscle form and function based upon both animal models as well as overwhelming positive clinical experience (31). Unfortunately, the biological effects observed in mice and men do not really speak to the mechanism through which the vitamin D hormone acts in this tissue. Thus, resolution will likely be achieved using creative basic approaches both in vitro and in vivo. Fortunately, the biochemical, molecular biological, genetic, and genomic techniques are now available such that this issue should be resolved in the next few years.

Response to J.W. Pike by C.M. Girgis, N. Mokbel, K.M. Cha, P.J. Houweling, M. Abboud, D.R. Fraser, R.S. Mason, R.J. Clifton-Bligh, and J.E. Gunton

We thank Professor Pike for his comments above in response to our work (13) and Professor Bouillon for his comments in the “News and Views” (32). We agree that clear detection of VDR in skeletal muscle has been controversial due to a number of technical factors, including protein extraction methods, variability in different muscle models, past problematic antibodies, and of course, the low level of VDR that is present in mature muscle at baseline. Our work shows clear absence of VDR in VDR knockout mice by immunohistochemistry and Western blotting, and perhaps more importantly, conclusive proof of functional presence comes with the demonstration of a novel physiological function, specifically the VDR-mediated uptake of 25-hydroxyvitamin D in muscle fibers (ie, nongenomic as indicated by inhibition using the chloride-channel blocker 4,4'-disothiocyanatostilbene-2,2'-disulfonic acid). We agree that the substantially higher levels of VDR in muscle of younger mice and immature muscle cells is intriguing; this suggests the possibility for a pleiotropic role for VDR in muscle and its potential activation after muscle injury from relatively low baseline levels of expression. We hope that the findings of our study bring some closure to this controversial field and may assist in future work examining roles of VDR in muscle development, regeneration, and 25-OHD uptake, ultimately justifying the generation of a skeletal muscle-specific VDR knockout model.

Acknowledgments

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Response to Counterpoint on: “The Vitamin D Receptor (VDR) is Expressed in Murine Skeletal Muscle and Modulates 25-Hydroxyvitamin D (25OHD) Uptake in Myofibers”

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We thank Professor Bouillon for his comments in the "News and Views" and Professor Pike for his comments above. We agree that clear detection of VDR in skeletal muscle has been controversial due to a number of technical factors including protein extraction methods, variability in different muscle models, past problematic antibodies and of course, the low level of VDR that is present in mature muscle at baseline. Our work shows clear absence of VDR in VDR knockout mice by immunohistochemistry and Western blotting, and perhaps more importantly, conclusive proof of functional presence comes with the demonstration of a novel physiological function, specifically the VDR-mediated uptake of 25-hydroxyvitamin D in muscle fibers (i.e. non-genomic as indicated by inhibition using the chloride-channel blocker DIDS). We agree that the substantially higher levels of VDR in muscle of younger mice and immature muscle cells is intriguing; this suggests the possibility for a pleiotropic role for VDR in muscle and its potential activation following muscle injury from relatively low baseline levels of expression. We hope the findings of our study bring some closure to this controversial field and may assist in future work examining roles of VDR in muscle development, regeneration and 25-OHD uptake, ultimately justifying the generation of a skeletal muscle-specific VDR knockout model.
Chapter 5 – Effects of vitamin D in muscle strength and fibre size

This chapter consists of a manuscript that was submitted for publication (November 2014). The primary author and PhD candidate, Christian Girgis, carried out the majority of experiments described in this work (>90%), drafted this manuscript and constructed figures. Figures and figure legends are found at the end of the manuscript.

The main findings of this work include:

• progressive weakness, demonstrated by grip strength testing, in whole-body VDR knockout mice (VDRKO) and vitamin D deficient mice.

• smaller muscle fibres and increased nuclei in muscles from VDRKO mice with dysregulation of myogenic regulatory factors, indicating impaired muscle maturation.

• reduced expression of components of the calcium-handling apparatus in muscle of VDRKO and vitamin D deficient mice.

• activation of myostatin and the E3-ubiquitin ligase MuRF1 in VDRKO and vitamin D deficient mice, suggesting activation of atrophy pathways and increased proteolysis in muscle in these models.
Manuscript Submitted For Publication

Vitamin D Receptor Ablation and Vitamin D Deficiency Result in Reduced Grip Strength, Altered Muscle Fibers and Increased Myostatin in Mice

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ABSTRACT
Vitamin D deficiency is associated with muscle weakness, pain and atrophy. Serum vitamin D predicts muscle strength and age-related muscle changes. Here we examine the controversial question of whether vitamin D directly affects muscle function by characterizing the muscle phenotype of mice with deletion of vitamin D receptor (VDRKO) and mice with diet-induced vitamin D deficiency. VDRKO and vitamin D-deficient mice had significantly weaker grip strength than their controls. Weakness progressed with age and duration of vitamin D deficiency respectively. Histological assessment showed that VDRKO mice had muscle fibers that were significantly smaller in size and displayed hyper-nuclearity. Real time PCR (RT-PCR) also indicated muscle developmental changes in VDRKO mice with dysregulation of myogenic regulatory factors (MRFs) and increased myostatin in quadriceps muscle (>2-fold). Vitamin D-deficient mice also showed increases in myostatin and the atrophy marker E3-ubiquitin ligase MuRF1. As a potential explanation for grip strength weakness, both groups of mice had down-regulation of genes encoding calcium-handling and sarco-endoplasmic reticulum calcium transport ATPase (Serca) channels. This is the first report of reduced strength, morphological and gene expression changes in VDRKO and vitamin D deficient mice where confounding by calcium, magnesium and phosphate have been excluded by direct testing. Although suggested in earlier in vitro work, this study is the first to report an in vivo association between vitamin D, myostatin and the regulation of muscle mass. These findings support a direct role for vitamin D in muscle function and corroborate earlier work on the presence of VDR in this tissue.
INTRODUCTION

In addition to established effects in bone and mineral homeostasis, increasing evidence suggests that vitamin D exerts effects in skeletal muscle.\(^1\) Muscle weakness and pain are prominent features of vitamin D deficiency that respond to replacement.\(^1\) Serum 25(OH)-vitamin D (25OHD) levels correlate with muscle strength in healthy individuals and predict the risk of functional decline and sarcopenia in older populations.\(^2,3\) A cross-sectional observational study also reported a positive correlation between serum levels of 25OHD and myostatin, a negative regulator of muscle mass, in older men.\(^4\)

The biologically active form of vitamin D, 1,25(OH)\(_2\)D, relies on the photochemical conversion of 7-dehydrocholesterol to vitamin D in skin by ultraviolet type-B radiation and subsequent hydroxylation steps. Active vitamin D then binds to its cognate receptor: the vitamin D receptor (VDR). VDR is a member of the nuclear receptor superfamily that regulates expression of many genes including those involved in calcium/phosphate homeostasis and cellular proliferation and differentiation.\(^1\) The 1,25(OH)\(_2\)D-VDR complex also exerts rapid, non-genomic effects on intracellular signaling and calcium flux.

Precise mechanisms to explain vitamin D’s effects in muscle are unclear. Biochemical abnormalities associated with vitamin D deficiency, particularly altered serum phosphate and calcium levels, independently lead to muscle disease.\(^5\) Whether skeletal muscle expresses VDR and may therefore be a direct target of 1,25(OH)\(_2\)D has been controversial.\(^6-10\) We recently reported that VDR is expressed in skeletal muscle and modulates the uptake of vitamin
D in this tissue.\(^{(11)}\) Upon entry into muscle, vitamin D alters calcium flux by mechanisms involving protein kinase C and rapid inositol triphosphate (IP3)-dependent calcium shifts from the sarcoplasmic reticulum to the cytosol, thereby potentially influencing muscle contraction and relaxation.\(^{(12, 13)}\) Vitamin D treatment of cultured muscle cells leads to doubling in the size of differentiated myotubes\(^{(14)}\) and opposing effects are seen following VDR knockdown by siRNA.\(^{(15)}\) These in vitro effects rely on interaction between vitamin D signals and myostatin, a member of the TGF-β superfamily that negatively regulates muscle mass.\(^{(14, 16)}\) However, in vivo studies examining direct effects of VDR in muscle function and fiber size are lacking.

In this study, we examine the effect of vitamin D deficiency and deletion of VDR on grip strength, muscle bulk, fiber size and gene expression. In the absence of altered mineral levels following adequate dietary supplementation, these mice displayed a distinct muscle phenotype with morphological differences, reduced strength and altered gene expression. This provides further evidence in support of a functional role of vitamin D signaling in muscle, corroborating the presence of VDR in this tissue.

**MATERIALS AND METHODS**

**Animals and Ethics**

Whole-body VDR knockout (VDRKO), heterozygous and wild-type (WT) mice were maintained on a γ-irradiated “rescue chow” (SF08-002, Specialty Feeds, Glen Forest, NSW) containing 2% calcium, 1.2% phosphorus, 0.2 g/g lactose and 1 IU vitamin D/g from weaning. Rescue chow is essential to
normalize the blood mineral ion levels of VDRKO mice.\textsuperscript{(17)} These were male C57/BL6 mice, VDR knockout was generated by ablation of exon 3 as previously described \textsuperscript{(28)} and the colony was maintained by successive heterozygous mating. We also induced vitamin D deficiency in a second group of male C57/BL6 mice by housing them under incandescent lighting and administering vitamin D-free diet (SF085-003, Specialty Feeds, Glen Forest, NSW) from 3 weeks age. This diet contains increased calcium (2%), magnesium (0.2%) and phosphorus (1.2%) to prevent abnormal mineral levels in association with vitamin D deficiency. Control vitamin D-replete mice received vitamin D (cholecalciferol) 2.2 IU/g, 1% calcium, 0.2% magnesium and 0.7% phosphorus (SF085-034, Specialty Feeds, Glen Forest, NSW). Mice were fed vitamin D-free and –replete diets and serum levels of vitamin D and related minerals were tested at various time-points. To examine effects of prolonged vitamin D deficiency, muscle mass, histology and gene expression of vitamin D deficient and replete mice were examined at 6 months of age. These parameters were examined at a younger age in VDRKO mice (7-8 weeks) as they have a reduced life span. Use of animals was approved by the Animals Ethics Committee of the Garvan Institute of Medical Research (AEC 12/26). All animals were sacrificed by general anesthesia followed by cervical dislocation.

\textbf{Serum parameters}

Serum calcium, phosphate and magnesium levels were measured using Roche reagents on a Roche Modular analyser. Serum 25OHD levels were measured by Diasorin Liaison assay (Royal North Shore Hospital, Sydney, Australia).
Grip Strength Measurement

A grip strength meter (Columbus Instruments, OH, USA) was used to test mouse forearm grip strength as recorded in Newtons (N). Mice were held by the base of the tail and allowed to grip the trapeze with their front paws and then pulled with their body parallel to the floor. Each mouse was trialed 15 times in sets of 3 with a short rest in between sets. The highest and lowest readings were excluded for each mouse and the remaining readings were averaged and corrected for individual body weight. Measurement of grip strength was blinded for group with the exception of VDRKO mice as their distinct phenotype (hairlessness and stunted growth) makes this impossible.\(^{(17)}\) Heterozygous VDRKO mice were therefore included in grip strength testing blinded to genotype.

Muscle histology

Quadriceps muscles were isolated from VDRKO mice and their WT littermates (7-8 weeks of age), vitamin D deficient mice and their controls (6 months of age). Upon isolation, muscles were rapidly frozen in isopentane (2-methylbutane) cooled with liquid nitrogen. Frozen 8 µm muscle sections were cut using a Cryostat (Leica). Sections were incubated with Mayer’s hematoxylin solution for 4 mins, washed in deionized water, and then incubated with eosin solution for 4 min. Sections were then washed in deionized water, dehydrated in ethanol, and then mounted. Using ImageJ (National Institutes of Health), diameters of muscle fibers and nuclei per fiber were measured in ~150 fibers per mouse.
Real-time PCR (RT-PCR)

Total RNA was isolated from whole quadriceps muscle using TRIZOL Reagent (Sigma-Aldrich, Australia), as previously described\(^{18}\). cDNA was synthesized from 1 µg of total RNA using the Maxima First Strand cDNA synthesis kit (Thermo Scientific). RT-PCR was performed in 10 ul reactions using the SYBR Green master mix (Promega) within 384-well plates (ABI Prism 7900HT Sequence Detection System). The amplification protocol included 10 min at 95°C and 40 cycles of two-step PCR including melting for 15 sec at 95°C and annealing for 1 min at 60°C. Triplicates were used for each sample. Primers were designed using Primer 3 and BLAST (National Library of Medicine) and obtained from Invitrogen. Every plate included house-keeping genes (TATA-box binding protein (Tbp), cyclophilin) for every sample. For each experiment, a house-keeping gene that did not differ significantly between groups was used to normalize CT (cycle threshold) values. CT is the number of PCR cycles at which fluorescence above background crosses a set threshold. Relative expression levels were calculated by comparing the logarithm of the difference of total cycle number and CT for specific groups (i.e. $\Delta\Delta$CT).

Statistical analyses

Data are presented as means ± SEM. Statistical tests are Student’s t-tests conducted at a significance level of 5%. Statistical tests and graphs were performed using Excel and/or Prism (GraphPad Prism Version 6 for Windows; GraphPad Software, San Diego, CA, USA).
RESULTS

Serum Values

Serum calcium, magnesium and phosphate levels were measured in a subgroup of mice (Figures 1A, B). Due to adequate dietary supplementation, VDRKO and vitamin D-deficient mice did not display differences in mineral levels compared to their respective controls. Vitamin D deficiency was initially noted at 1 month following the commencement of the vitamin D-free diet and by 3 months, these mice had no detectable 25OHD (Figure 1C).

Grip Strength

VDRKO and heterozygote mice were significantly weaker than WT controls with 43% and 30% reduction in grip strength respectively at 7 weeks of age (Figure 2A). At 14 weeks of age, the differences were greater at 48% and 40% reduction in grip strength respectively (p<0.005, Figure 2A). The effect of VDR ablation on grip strength was supported by a dose-response effect with a decline from WT to heterozygote to VDRKO mice at both ages (Figures 2A). Age-related decline in grip strength from 7 to 14 weeks was greatest in heterozygote mice (25%, p<0.005), followed by VDRKO and WT mice (18 and 12%, both p<0.005).

There were also differences, to a lesser extent, in vitamin D-deficient versus replete mice. After 7 weeks on vitamin D-free diet, mice were 15% weaker than controls (p<0.05, Figure 2B). At 10 weeks, vitamin D-deficient mice were 25% weaker than controls (p<0.05, Figure 2B).
Muscle Mass and Histology

VDRKO mice had significantly lighter quadriceps muscles even after adjustment for total body mass (Figure 3C). They displayed smaller quadriceps muscle fibers (Figure 3 A, B, C D) and a higher nuclei to fiber ratio compared to WT littermates (Figure 4). These changes imply defects in the regulation of muscle fiber size and myocyte proliferation in the absence of VDR, respectively. Similar changes were seen in tibialis anterior muscles (data not shown), suggesting diffuse muscle defects in VDRKO mice. This staining, however, did not allow for differentiation between myonuclei and satellite cell nuclei. There were no histologic changes to indicate necrosis in muscle from VDRKO mice, nor was there an increase in the proportion of central nuclei in fibers from VDRKO mice (i.e. an indication of fiber remodeling/regeneration). By contrast, quadriceps muscle mass proportionate to total body mass did not differ in mice with vitamin D deficiency (Figure 5C). Muscle fiber size did not differ between vitamin D deficient and replete mice (Figure 5).

Gene expression

Differential expression of several groups of mRNAs was examined in the quadriceps muscles of VDRKO, vitamin D deficient mice and their controls (Figure 6). Myogenic regulatory factors MyoD and Myf5 were significantly upregulated in VDRKO mice (Figure 6A, p<0.05), consistent with impaired muscle development and maturity. Expression of Myostatin mRNA was greater than two-fold in VDRKO mice compared to WT littermates (Figure 6A, p<0.005), explaining the substantial reduction in fiber size and muscle mass
seen in these mice. To a lesser extent, vitamin D deficient mice also displayed a significant increase in *Myostatin* mRNA (Figure 6B, p<0.05). This was associated with increase in the E3-ubiquitin ligase *MuRF1* (Figure 6B, p<0.005), suggesting upregulation of proteolysis and atrophy pathways in the muscles of these mice. Both vitamin D deficient and VDRKO mice showed significant down-regulation of calcium handling genes, specifically genes encoding Calbindin and sarcoendoplasmic reticulum calcium transport ATPase (*Serca*) channels (Figure 6, p<0.05). This supports a critical role for vitamin D in intramuscular calcium handling via genomic mechanisms in skeletal muscle.

**DISCUSSION**

This is the first study to examine muscle strength, histology and gene expression in VDRKO and vitamin D deficient mice with demonstration of normal serum calcium, phosphate and magnesium levels. By assessing function, morphology and mRNAs in these mouse models, we addressed the important question: does vitamin D directly alter muscle strength and size?

Previous studies reported functional differences in VDRKO mice such as impaired swimming, motor coordination and reduced stride length compared to WT littermates.\(^{19-21}\) Other studies reported impaired muscle contraction and recovery in vitamin D-deficient rats and chicks compared to their vitamin D-replete counterparts.\(^{22-24}\) With the exception of one study\(^ {25}\), these studies did not correct for associated biochemical abnormalities and could therefore not conclude *direct effects* of vitamin D. These studies also did not associate functional differences in vitamin D deficient or VDRKO mice with specific
morphologic changes in their skeletal muscle.

On grip strength testing, vitamin D deficient and VDRKO mice were significantly weaker than their controls and this difference increased with duration of vitamin D deficiency and age. A stepwise decline in grip strength from WT to heterozygote to VDRKO mice strongly supports a role for VDR in muscle strength. Interestingly, differences in grip strength were greater in VDRKO versus WT than vitamin D deficient versus replete mice, perhaps explained by complete ablation of vitamin D signaling in the former. We hypothesized that muscle weakness in these mice may be related to defects in Ca^{2+} flux and intracellular handling during excitation-contraction coupling. Indeed, rapid, non-genomic effects of vitamin D on calcium handling in cultured muscle cells have been reported by the Boland group for over 30 years.\(^{(12, 26, 27)}\) In this study, we report that vitamin D also exerts genomic effects in the calcium-handling apparatus within skeletal muscle. Although this has been previously reported in tissues with classic vitamin D responses, namely intestine and kidney \(^{(28)}\), this is the first time such an effect has been shown in skeletal muscle. Muscle from VDRKO and vitamin D deficient mice showed reduced mRNAs for the calcium-binding gene, Calbindin-28K, and SERCAs, potentially resulting in defective Ca^{2+} flux from sarcoplasmic reticulum (SR) into cytosol following excitation, impaired muscle contraction and reduced strength. Consistent with greater impairment in strength, VDRKO mice showed more pronounced down-regulation of these genes than vitamin D deficient mice compared to respective control groups. \textit{In vivo} contractile studies may shed
further light on effects of vitamin D in muscle force, fatigue and recovery, all of which are intrinsically related to calcium signals.

Muscles from VDRKO mice were significantly lighter, even after correction for lower body weight, than WT mice. On histology, VDRKO mice displayed smaller muscle fibers and had increased muscle nuclei, arising potentially from muscle fibers and/or satellite cells. A role for VDR in the regulation of muscle fiber size is supported by anabolic effects of 1,25(OH)₂D in C2C12 myotubes and opposing growth-inhibitory effects following VDR knockdown in these cells. Similarly, muscle hyper-nuclearity in the absence of VDR concurs with anti-proliferative effects of VDR in cultured muscle cells. In the notable study by Endo and colleagues, smaller muscle fibers were seen in mice with a different model of VDR deficiency (exon 2 deletion) and this progressed in the absence of a high-calcium/phosphorus rescue diet. Our study is the first to demonstrate an in vivo role for VDR in muscle fiber size that is independent of phosphate or calcium levels. Increased expression of myogenic regulatory factors (MRFs) Myf5 and MyoD was also seen in adult VDRKO mice, supporting defects in myogenesis and post-natal muscle maturation in these mice. Most strikingly, Myostatin mRNA was greater than 2 fold higher in muscle from VDRKO mice, explaining the significant reduction in muscle mass and fiber size in these mice.

Mice with vitamin D deficiency showed increased expression in the atrophy-related gene MuRF1. This ubiquitin ligase triggers muscle protein degradation and is considered to play a role in pathological muscle wasting. However,
muscle fiber size and MAFBx, another ubiquitin ligase, were not significantly altered by dietary vitamin D deficiency. Interestingly, a modest increase in *Myostatin* mRNA was also seen, favoring the activation of atrophy-related mechanisms in vitamin D deficient mice despite the absence of morphologic changes. Possible explanations for this discrepancy are that a longer duration of vitamin D deficiency (> 6 months) may be necessary for atrophy-related mechanisms to lead to overt atrophy. Alternatively, normal mineral levels in these mice may have muted the overt development of muscle wasting in vitamin D deficiency. In support of this, the use of a high-calcium diet in Sprague-Dawley rats with vitamin D deficiency led to partial reversal in increased ubiquitin proteosomal activity in muscle.\(^{31}\) However this study showed no change in the expression of myostatin and this may be related to the shorter duration of vitamin D deficiency in this study (i.e. 4 months). It is therefore possible that vitamin D deficiency and associated calcium defects exert overlapping, interconnected effects in muscle atrophy via activation of the ubiquitin-proteosome and myostatin.

Importantly, both models showed significant increases in myostatin expression. Myostatin, a member of the TGF-β superfamily which negatively regulates muscle mass, was first discovered in 1997.\(^{32}\) A great deal of research has examined the immense potential of targeting myostatin or its receptor (ActRIIB) in the treatment of muscle wasting and sarcopenia.\(^{33,34}\) We have previously shown that treatment of cultured muscle cells with vitamin D results in pronounced inhibition of myostatin and subsequent doubling in myotube size.\(^{14}\) The STRAMBO study displayed a significant positive correlation
between serum myostatin and 25OHD levels in older human subjects. The current work advances the in vivo link between vitamin D signaling and myostatin in muscle and raises the pertinent question: may vitamin D present another pathway for myostatin inhibition and the reversal of muscle wasting?

Differences in the muscle phenotypes of VDRKO versus vitamin D deficient mice reflect diverse effects of aberrant vitamin D signaling. Reduced muscle mass and smaller muscle fibers in mice with congenital ablation of VDR highlight the developmental role of vitamin D signaling. By contrast, changes seen in 6-month old mice with vitamin D deficiency may relate to the muscle effects of aging, as supported by the activation of the atrophy-related gene MuRF1 in this model. Conversely, VDR deficiency may also play a role in muscle aging as suggested by down-regulation of VDR in muscles of older subjects. Vitamin D may also exert effects in muscle development. This is supported by the association of Vitamin D deficiency during pregnancy with reduced muscle fiber size in newborn offspring. These observations suggest that vitamin D signaling plays concerted roles in skeletal muscle at opposing ends of the age spectrum.

In conclusion, this study demonstrates, for the first time, coordinated effects of vitamin D and VDR in muscle strength, morphology and gene expression, independent of systemic mineral changes. This provides additional proof that vitamin D directly targets skeletal muscle, a point of heated contention until now. The precise delineation of these effects, namely in muscle development and aging, will be the subject of future research.
ACKNOWLEDGMENTS

CMG received salary support from postgraduate scholar awards (APA) from University of Sydney and the Joseph Thornton Tweddele Research Scholarship 2014 (Royal Australasian College of Physicians). JEG and RJC-B are supported by NHMRC. Authors’ roles: Study design: CMG, JEG, RJC-B, PJH, NM. Study conduct: CMG, KMC, ML. Data collection: CMG, KMC, ML, RR, NM. Data interpretation: CMG, JEG, RJC-B. Drafting manuscript: CMG. Revising manuscript content: JEG, RJC-B, NM, PJH. Approving final version of manuscript: CMG, PJH, ML, NM, RR, KMC, RJC-B, JEG. CMG and JEG take responsibility for the integrity of the data analysis.
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FIGURE LEGENDS

Figure 1 Serum Mineral and vitamin D levels  A) Due to rescue diet, serum calcium, magnesium and phosphate levels were not significantly different between WT and VDRKO mice at 7 weeks age. B) After being on vitamin D-free and replete diets for 3 months, serum calcium, magnesium and phosphate levels were not significantly different between these mice, owing to increased supplementation of these minerals. C) Serum 25(OH)Vitamin D levels dropped significantly after 1 month of the diet and was undetectable at 3 months (n= 4 mice per group, error-bars SEM, **p<0.005).

Figure 2 Grip Strength Analysis  A) At 7 and 14 weeks age, VDRKO and heterozygous mice displayed significantly reduced grip strength corrected for body weight. Age-related decline over this period was also more prominent in VDRKO and heterozygous mice (22% decline in grip strength) compared to WT littermates (12% decline in grip strength). B) At 7 weeks following commencement of vitamin D-free diet, vitamin D-deficient mice were significantly weaker than replete mice and this difference increased by 10 weeks (n= 6-12 mice per group, error-bars SEM, *p<0.05, **p<0.005).

Figure 3 Muscle Histology in VDRKO mice  A, B) On H&E stain, VDRKO mice showed smaller muscle fibers than WT counterparts (quadriceps, 7 week-old male mice, n= 5 per group). C) After correction for body mass, muscles remained significantly lighter in VDRKO mice. D) On ImageJ quantification, VDRKO muscles displayed ~ 30% reduction in fiber diameter (n = 5 mice per
group, 150 fibers analyzed per mouse, scale bars 200 µm, error-bars SEM, *p<0.05, **p<0.005).

**Figure 4 Muscle Histology in VDRKO mice A, B, C)** Muscle fibers from VDRKO mice displayed a significant increase in nuclear number compared to WT mice. (n = 5 mice per group, 150 fibers analyzed per mouse, scale bars 200 µm, error-bars SEM, *p<0.05, **p<0.005).

**Figure 5 Muscle Histology in Vitamin D deficient mice A, B)** On H/E stain, there was no significant difference in muscle fiber size in vitamin D deficient versus replete mice. **C)** Quadriceps muscle mass, normalized to total body mass, did not differ between vitamin D deficient versus replete mice. **D)** On Image-J analysis, muscle fiber size was not significantly different (quadriceps, 6-month old mice, n = 5 mice per group, 150 fibers analyzed per mouse, scale bars 100 µm).

**Figure 6 Gene Expression in Muscle from VDRKO and Vitamin D Deficient Mice A)** VDRKO mice showed significant upregulation of mRNAs for myogenic regulatory factors, >2-fold increase in Myostatin mRNA and significant reduction in Calbindin-28K, and genes encoding Serca2a and 2b mRNAs **B)** Vitamin D deficient mice showed upregulation of the atrophy marker MuRF1, modest increase in Myostatin mRNA and down-regulation of Calbindin-28K, Serca2a, 2b and 3 mRNAs. (n=6 mice per group, error-bars SEM, *p<0.05, **p<0.005).
Figure 1

A

![Graph A]

B

![Graph B]

C

![Graph C]
Figure 2

A

Grip Strength mN/gm

<table>
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B

Grip Strength mN/gm

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<td>VitD deficient</td>
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Figure 3

A. WT
B. VDRKO

C. % Quad mass/ total mass

D. Fiber Diameter (µm)

WT vs VDRKO
Figure 4

A  WT  

B  VDRKO

C  

Myonuclei per fiber

![Graph showing the comparison of myonuclei per fiber between WT and KO groups. The bars indicate that the KO group has significantly more myonuclei per fiber compared to the WT group.](image)
Figure 5

A  Vit D replete

B  Vit D deficient

C  % Quad mass/total mass

D  Fiber diameter (µm)

VitD replete  VitD deficient

0.2 0.4 0.6 0.8

Fiber diameter (µm)

VitD replete  VitD deficient
Figure 6

A

Myf5  MyoD  Myogenin  TGFβ1  Myostatin  MuRF1  MAFbx  Calbindin  Serca 1  Serca 2a  Serca 2b  Serca 3

mRNA fold-change

WT  KO

B

Myf5  MyoD  Myogenin  TGFβ1  Myostatin  MuRF1  MAFbx  Calbindin  Serca 1  Serca 2a  Serca 2b  Serca 3

mRNA fold-change

VitD replete  KO

VitD deficient  KO

*  **
Chapter 6 – Direct effects of vitamin D on bone mass

This chapter consists of a research letter published online in *Endocrinology* in August 2013. The primary author and PhD candidate, Christian Girgis, drafted this letter in response to an original article by Yamamoto *et al.* on the bone phenotype of osteoblast-specific VDR knockout and whole-body VDR heterozygote mice.

The main findings of this research letter include:

- no difference in bone mineral density or bone mineral content of VDR heterozygote (VDR+/−) male mice and their littermates.
- discrepancy with the findings of Yamamoto in which VDR heterozygote mice reportedly displayed increased bone density.
- potential contribution of the different genetic models of VDR ablation (i.e. exon 3 versus exon 2) in altering bone phenotype.

This research letter discusses the complexity of VDR’s effects in bone and raises the possibility that an integrated, inter-system approach in examining *musculoskeletal* roles of VDR may yield further information.
Direct effects of VDR on bone mass

Christian M Girgis, Sue Lynn Lau, Roderick J Clifton-Bligh, Jenny E Gunton

Published online on Endocrinology website, August 2013

Letter in response to: Yamamoto Y, Yoshizawa T, Fukuda T et al. Vitamin D receptor in osteoblasts is a negative regulator of bone mass control. Endocrinology 2013; 154:1008-20

We read with interest the study by Yamamoto and colleagues on the effects of osteoblast-specific VDR ablation on bone mass (1).

Contrary to their finding of increased bone mass in systemic VDR heterozygotes (VDR$^{+/}$), our experiments have revealed no such difference. The offspring of chow-fed heterozygote pairs from the Boston VDRKO line formed the experimental litters. All offspring received high calcium, high phosphorus rescue diet from weaning. At DXA scanning at 27 weeks, there was no difference in weight, bone mineral density or content between 7 VDR heterozygote (VDR$^{+/}$) males and 14 WT littermates. (BMD: 0.058 vs 0.059 g/cm$^2$, p=0.7 and BMC: 0.49 vs 0.50 g, p=0.6) A similar lack of difference was found between 11 female 27 week old VDR heterozygotes and 7 WT littermates, (BMD: 0.060 vs 0.060 g/cm$^2$, p=0.9 and BMC: 0.45 vs 0.48 g, p=0.5)

We hypothesize that these contrasting findings may relate to differences in the genetic models. In the Boston strain of mice, exon 3 of VDR was deleted. It encodes the second zinc finger of the DNA-binding domain (2). The mice used
in (1) (Tokyo strain) were generated by ablating exon 2 (the first zinc finger). The Tokyo strain expresses a truncated form of VDR which can bind 1,25(OH)₂D and thereby potentially exert biologic activity (3), even if traditional signaling through vitamin D response elements in gene promoters is prevented. Without ‘rescue’ diet, differences in survival time between homozygous Tokyo (15 weeks), Boston (6 months) and Leuven (12 months) VDRKO strains are noted, though direct comparisons between strains have not been performed. Genetic differences may therefore contribute to phenotypic variability between strains, for example, in the age of onset and extent of hypocalcaemia and bone abnormalities in relation to weaning.

The question of how vitamin D directly affects bone is complex. *In vitro* studies demonstrate changes in osteoblast proliferation, differentiation and mineralization in VDR knockout mice (1). However, osteoclasts and chondrocytes also express VDR, CYP27B1 and CYP24A1 and display profound responses to VDR ablation or 1,25(OH)₂D treatment (4). Effects of VDR on bone mass are therefore likely to result from intricate multi-cellular interactions rather than simply involve osteoblasts.

Also, the stage of osteoblast differentiation directly influences effects of 1,25(OH)₂D on osteoblast activity. This has been nicely demonstrated in a study where osteoblasts isolated from neonatal mouse calvariae and juvenile mouse long bones showed different properties and responses to 1,25(OH)₂D (5). VDR knockout at later stages of osteoblast differentiation *(eg using Dmp (dentin
matrix protein) 1-promoter Cre) may therefore result in a different phenotype from that described in this study.

To add further complexity, VDR over-expression in osteoblasts also increases bone mass (6). This raises questions about VDR activity in the physiologic setting where its expression is tightly regulated. There may be a ‘window’ of activity for optimal bone homeostasis.

The Yamamoto study adds very useful information to our understanding of the direct effects of VDR in bone mass but interesting and unresolved issues remain. A double osteoblast, osteoclast-specific VDR knockout mouse model may further enlighten the field.
References


Chapter 7 – Novel technique in satellite cell isolation

This chapter consists of an original article published in *Journal of Cellular Physiology* in November 2014. The PhD candidate, Christian Girgis, was a co-author on this publication and contributed to a specific component of this work (i.e. cell cycle analysis of Grb10 knockout primary muscle cells).

The main findings of this original article include:

- a novel technique for isolating pure populations of primary muscle/satellite cells using anti-CD56 (NCAM) cell staining. This technique was first described in this article and also used to isolate muscle cells from VDRKO and WT mice (discussed in Chapter 4).

- differences in primary muscle cell proliferation, differentiation and GLUT4 translocation in the absence of growth-factor receptor-bound protein 10 (Grb10).
Skeletal muscle is a highly dynamic tissue that makes up 25%–40% of our body weight (Janssen et al., 2000). It is capable of increasing in size and improving in function after various physiological stimuli (e.g., exercise, weight bearing), and injuries. Muscle is also a major tissue contributing to energy balance, and can be responsible for up to 80% of insulin-stimulated glucose uptake and storage after a meal (DeFronzo et al., 1985; Shulman et al., 1990). Change in muscle mass is one of the factors shown to significantly affect glucose homeostasis and metabolic health. An increase in muscle mass after exercise training is associated with overall improved glucose utilization and reduced insulin resistance (Srikanthan and Karlamangla, 2011). Conversely, reduced muscle bulk during aging and severe myopathic diseases is associated with insulin resistance, a risk factor for type 2 diabetes (Evans, 1997; Cruz Guzman Odel et al., 2012). Thus, identifying factors or mechanisms that regulate muscle growth, regeneration, and metabolism could provide therapeutic targets to improve metabolic health, and has implications for treatment of muscle diseases, and maintaining general muscle function during aging.

The regenerative capacity of muscle is attributed to the activation of a specialized population of cells termed “satellite cells” that reside between the basal lamina and sarcolemma of muscle fibers. Muscle satellite cells share the same origin as embryonic muscle precursor cells (Gros et al., 2005) and act as adult stem cells in skeletal muscle. Therefore, they are considered the major contributors to skeletal muscle growth and regeneration in adults. Under normal conditions, satellite cells are in a quiescent state (G0 phase), and in response to trauma, injury, or stress, they become activated and enter mitosis to proliferate then exit the cell cycle to differentiate and form new fibers (hyperplasia) or fuse to existing myofibers (hypertrophy) (Hawke and Garry, 2001). The main growth factors that have been shown to regulate satellite cell proliferation and differentiation include IGF-1 & IGF-2 (Duclos et al., 1991; McFarland et al., 1993), the fibroblast growth factor (FGF) family (Bendall et al., 2007), hepatocyte growth factor (HGF) (Maina et al., 1996; Tatsumi et al., 1998), and transforming growth factor (TGF)-beta (Bischoff, 1990; Odel et al., 2012). Thus, identifying factors or mechanisms that regulate muscle growth, regeneration, and metabolism could provide therapeutic targets to improve metabolic health, and has implications for treatment of muscle diseases, and maintaining general muscle function during aging.
Yablonka-Reuveni and Rivera, 1997). These growth factors trigger a cascade of downstream signaling that phosphorylate myogenic regulatory factors (MRF) leading to the regulation of proliferation and/or differentiation of satellite cells (Yablonka-Reuveni and Rivera, 1994; Sabourin and Rudnicki, 2000; Le Grand and Rudnicki, 2007).

The Grb10 gene encodes for a growth factor receptor-binding protein that is abundantly expressed in major insulin-sensitive tissues such as muscle and adipose tissue, as well as pancreas (Smith et al., 2007; Wang et al., 2007). Grb10 binds, and negatively regulates signal output from the insulin receptor (IR) (Liu and Roth, 1995; O’Neill et al., 1996; Laviola et al., 1997; Ramos et al., 2006), and IGF-1 receptor (IGF-1R) (Liu and Roth, 1995; Morrione et al., 1996; Laviola et al., 1997). Grb10 also interacts in vitro with other growth factor receptors such as those for epidermal growth factor (EGF) (Ooi et al., 1995), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and HGF receptors (Wang et al., 1999; Holt and Siddle, 2005; Liu et al., 2012), but the physiological relevance of Grb10 binding in this context is unclear. Studies from our laboratory and others have demonstrated that deletion of Grb10 in mice results in improved glucose metabolism mediated by enhanced insulin signaling and increased phosphorylation of insulin receptor substrate-1 (IRS1) in muscle (Smith et al., 2007; Wang et al., 2007; Holt et al., 2009). More recently, we have identified a novel role for Grb10 in regulating muscle mass (Holt et al., 2012). Mice lacking Grb10 have significantly elevated muscle mass compared to wild-type (WT). This is mainly due to hyperplasia, with no change in fiber size or proportion. This model of hyper-musclearity is unlike other models such as IGF-1 transgenic mice (Coleman et al., 1995; Musaro et al., 2001) and myostatin knock-out mice (McPherron et al., 1997), which exhibit both hyperplasia and hypertrophy.

The knowledge of the role of Grb10 in muscle outside of insulin signaling pathway regulation is limited. The aim of the current study was to understand the mechanisms underlying the increase in muscle mass and glucose homeostasis in the Grb10–/– mice. Thus, we investigated the impact of Grb10 deletion on muscle cell proliferation, differentiation, and glucose metabolism in an in vitro model of primary muscle cells isolated from WT and Grb10–/– mice. The results supported a role for Grb10 in playing a regulatory link between muscle growth and metabolism.

Materials and Methods
Animal maintenance

Mice with homozygous deletion of Grb10 (Grb10–/–) have been described previously in (Charalambous et al., 2003) and were maintained on a mixed C57BL/6 x CBA background at the Australian Bioresources (ABR) as described in Holt et al., 2012. All animal studies were performed with the approval of the Garvan Institute/ST Vincent’s Hospital Animal Ethics Committee.

In vitro isolation and maintenance of mouse-derived primary muscle cells

Mouse primary myoblast cell lines were established from WT and Grb10–/– mice using explant culture (Decary et al., 1997) with some modifications as outlined below. Quadriceps muscles from four-week-old mice were dissected out and rinsed in PBS containing 40 μg/ml gentamicin. Muscle was cleaned from surrounding tissue, fat, and vasculature, and then transferred into ~0.5–1.0 ml of plating media (DMEM/HAMS-F12 media 1:1, 40 μg/ml gentamicin, 10% v/v heat-inactivated FBS (Thermo Fisher Scientific), 10% v/v amniomax, 40 μg/ml gentamicin). The muscle was then cut into 1 mm³ pieces and placed as explants in culture dishes (Becton Dickinson) coated with collagen–matrigel (DMEM/HAMS-F12 1:1. 0.17 mg/ml collagen type I (Becton Dickinson), 1/50 dilution of matrigel (Becton Dickinson), 40 μg/ml gentamicin). Dishes were kept in a humidified chamber at 37 °C, 5% CO₂ and after 48 h, when the explants had sufficiently adhered, 2 ml of myoblast media (DMEM/HAMS-F12 1:1, 10% v/v amniomax, 20% v/v FBS, 40 μg/ml gentamicin) was added. Once outgrowth from the explants was visible (day 2 or 3), cells were gently washed twice with 1xPBS and detached using TrypLE. The cells were then transferred and expanded in a T75 flask. At 60%–70% confluency, expanded cells were detached with TrypLE and centrifuged at 300 g for 15 min. Media was then aspirated and the pellet frozen down in myoblast media + 10% DMSO. For differentiation of primary myoblasts into myotubes, myoblast media was replaced with a differentiation media (DM) (DMEM/HAMS-F12 1:1 media (12.5 mM glucose), antibiotics, 40 μg/ml gentamicin, 3% heat-inactivated horse serum). Cells were allowed to differentiate into mature myotubes over 6 days. For all assays, a total of six clones from WT and Grb10–/– mice was used, and clones were maintained in the same culture media conditions with similar passage number.

Purification of satellite cells by FACS

Enrichment of primary muscle cells was achieved by FACS using a Neural Adhesion Cell Marker/CD56 (NCAM/CD56) antibody (MEM-188, Thermo Scientific/Pierce). Isolated primary cells were left in culture for 48 h before sorting in order to enhance the expression of cell surface markers. Cells were centrifuged at 300 g for 1 min, then the pellet washed with 1 ml of FACS buffer (1 x sterile PBS + 0.1% BSA). The cell suspension was centrifuged at 300 g for 1 min, and the pellet suspended in 100 μl FACS buffer containing NCAM/CD56 antibody, 1:10. Cells were incubated on ice for 20 min and were washed twice in FACS buffer in preparation for sorting. Cell sorting was performed using a FACS Aria U2 (Becton Dickinson) at 4 °C with a 100 μm nozzle. To eliminate false positive cells, the preparation was sorted against auto fluorescence parameters with a 530 nm filter. For all WT and Grb10–/– clones included in this study, the top 20th percentile of the NCAM/CD56 positive cells was collected containing the most intensely staining NCAM/CD56 positive cells. All satellite cells were used at low passage (1–9).

RNA isolation, cDNA synthesis and real time qPCR

Total RNA isolation from mouse tissues, primary cultured myoblasts and myotubes was performed using the TRIzol Reagent® method (Sigma-Aldrich, Castle Hill, Australia) as per the manufacturer’s instructions, followed by ethanol precipitation. cDNA was synthesized from 1 μg of total RNA using the Omniscript RT Kit (200) (Qiagen, VIC, Australia) and Random Primer 9 (New England Biolabs, MA). The intron-spanning primer set used were as follows: for mouse Grb10 (UPL probe#63), forward 5'–CGGTGCTCCTAGCTCTTT–3', reverse 5'–CTGAAGCTCCAAGGAAAT–3', for mouse p21 (UPL probe#108), forward 5'–ATCTGTCTGAAATACCCTGT–3', reverse 5'–CTGGAGGAATTGGAAGAA–3', for mouse p21 (UPL probe#108), forward 5'–ATCTGTCTGAAATACCCTGT–3', reverse 5'–AGGGCCCTACGCCTACTA–3'. Data analysis was performed using the comparative 2-delta Ct method and normalized to cyclophilin transcript.

Immunohistochemical analysis

Cells grown on collagen/Matrigel coated plastic Thermox coverslips (Nunc, Rochester, NY) were fixed and permeabilized in 3% PFA/0.1% Triton-X 100/PBS. Immunocytochemistry was performed as previously described (Ilkovski et al., 2004). Imaging was undertaken using a Zeiss AxioPlan1 upright microscope and a Nikon Super Resolution Microscope N-SIM. Primary antibodies used for immunohistochemical analysis were incubated overnight.
at 4 °C and included: PAX7 (1:50, Hybridoma Bank, UIOWA) and MyoD (1:100, Santa Cruz). Antibodies incubated at room temperature for 3 h included: sarcomeric actin (SCS 1:500, Sigma-Aldrich), α-actinin 2 (4B3, 1:500,000, kindly provided by Prof. Kathryn North, Institute of Neuroscience and Muscle Research, Westmead, Australia) and total myosin heavy chain (MF 30,1:500, Hybridoma Bank, UIOWA).

**Immunoblotting**

Protein lysates were collected from primary myoblasts at day 2 (D2), myotubes at day 6 of differentiation (Diff 6) and muscle tissue isolated from WT and Grb10−/− mice. Samples from cells and tissue were solubilized in a lysis buffer containing (4% SDS, 62.5 mM Tris pH 6.8, 10% glycerol, 1:500 protease inhibitors cocktail). Lysates collected from cells were left on ice for ~15 min then sonicated to shear chromosomal DNA. Muscle samples collected from WT and Grb10−/− mice were processed as described in (Hoy et al., 2007). Primary antibodies used for immunoblotting included: Grb10 antibody (K20, 1:1000, Santa Cruz), total retinoblastoma (t-Rb) (1:1000, BD Pharmingen), phospho-retinoblastoma S807 (p-Rb) (1:1000, CST), total Akt (t-Akt) and phospho-Akt S473 (p-Akt) (1:1000, CST), total ERK (t-ERK) and phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (p-ERK) (1:1000, CST), skeletal actin 5C5 (1:2000, Sigma-Aldrich), vimentin (1:1000, Sigma-Aldrich), myogenin (F5D, 1:1000, Hybridoma Bank, UIOWA), β-tubulin (E7, 1:1000, Hybridoma Bank, UIOWA).

**In vitro proliferation assay**

Cell proliferation rate was measured by a BrdU ELISA (Roche, USA). Briefly, satellite cells were seeded at ~6000 cells/cm² on collagen–matrigel coated 96 well plates and pulsed with 10 μM of BrdU 2 h after seeding (T0) and incubated for 4 h. BrdU antibody was used at 1:100 and fluorescence was read at 570 nm using a fluorescent microtiter plate reader (Fluostar Omega-BMG Labtech, Ortenberg, Germany). For the manual cell count, cells were seeded at low density at ~200 cells/cm² and counted using a haemocytometer at 24 h intervals.

**Growth factor studies**

Primary myoblasts and myotubes were serum starved for 4 h and 6 h respectively (baseline) followed by growth factor stimulation with insulin (6 min), IGF-1 (6 min) and HGF (20 min), and protein lysates collected. Growth factors were used at concentrations: insulin (100 nM, Actrapid Penfill, 100 IU units/ml), IGF-1 (10 ng/ml, EAG-c03, Gropep, Adelaide, Australia), HGF (50 ng/ml, 130-093-872, Miltenyi Biotech, Macquarie Park, Australia). These concentrations were determined as optimal concentrations for activation of Akt and ERK.

**Glucose uptake assay**

To assess the rate of glucose uptake in cells, differentiated primary myotubes-Diff 6 were serum starved for 6 h in a media containing DMEM/HAMS-F12 (6 mM glucose). The assay was performed as described in (Tan et al., 2010) with minor modifications outlined below. Briefly, cells were stimulated with insulin (100 nM) for 15 min in Krebs buffer and for the final 5 min of stimulation 0.2 mM of 2-deoxyglucose (D3179-1G, Sigma-Aldrich) + 10 μCi/ml 1,2-3H radioactively labelled 2-deoxyglucose (2DG, Perkin Elmer, Massachusetts) was added to assess glucose uptake. After 5 min 2DG stock was removed and lysis buffer (2% SDS, 125 mM Tris-HCl pH 6.8) was added. Specific activity (dpm 3H/pmole) of glucose was read using a Beta counter (Beckman LS 6500) and results were normalized to total protein content.

**GLUT4 translocation assay**

GLUT4 translocation assay was performed as described in (Govers et al., 2004), (Shewan et al., 2003) with minor modifications outlined below. Satellite cells were transduced with HA-GLUT4 retrovirus and measurement of cell surface
HA-GLUT4 as a percentage of total cellular HA-GLUT4 was performed. Differentiated myotubes were serum-starved for 4 h in 1:1 DMEM/HAMS-F12, and maintained in this medium during subsequent treatments. Myotubes were stimulated with 100 nM insulin for the final 20 min of serum starvation. All values were expressed as a percentage of total GLUT4 that was determined from anti-HA antibody labeling of permeabilized cells.

Statistical analysis
All data presented are expressed as means ± SEM where appropriate. Statistical analysis was performed by two-way analysis of variance (ANOVA) where appropriate using GraphPad Prism Version 6. Unpaired t-tests, two tailed distribution, and unequal variance were applied. Statistical significance was set a priori at P ≤ 0.05.

Results
Isolation and characterization of a highly myogenic NCAM/CD56+ subpopulation of cells from mouse skeletal muscle

Primary cells isolated from muscle of four-week-old WT and Grb10−/− mice represent a highly enriched, but mixed, myogenic cell population. Using PAX7, as a marker of satellite cells, we demonstrated that ~20% and 40% respectively of WT and Grb10−/− cells isolated are satellite cells (Fig. 1). To further enrich for primary muscle cells, cells from these cultures were sorted using FACS based on the expression of the neural cell adhesion marker NCAM/CD56 previously described to be expressed on muscle progenitor cells derived from porcine (Wilschut et al., 2008) and human muscle (Meng et al., 2011). To our knowledge, this is the first paper describing the purification of murine muscle cells with the NCAM-CD56
NCAM/CD56 is a marker. FACS analysis demonstrates that NCAM/CD56 is expressed in mouse primary muscle cells (Fig. 2A, red peak). The isolated NCAM/CD56 positive population consists of spindle-shaped cells with a reduced cytoplasmic volume typical of satellite cells (Fig. 2B.i), and express the myogenic transcription factor MyoD (Fig. 2B.ii), indicating that these cells are actively proliferating. Differentiation of WT and Grb10-/- NCAM/CD56 positive cells in low serum conditions (3% horse serum) results in mature multinucleated primary myotubes as revealed by the striation pattern for α-actinin2 (z-line marker) (Fig. 2C, inset). To verify the purity of sorted cells, immunofluorescence against PAX7 and MyoD was performed (Fig. 2D.i&ii) and the percentage of PAX7/MyoD positive cells determined (Fig. 2D.iv), confirming that our method results in >95% purity (Fig. 2E). The fact that these cells co-express MyoD indicates that they have been activated in culture. Therefore, they will be referred to as activated satellite cells or primary myoblasts henceforth.

**Fig. 3.** Grb10 is present in primary myoblasts and differentiated myotubes and is developmentally regulated in vivo. A) (i) Quantitative PCR (qPCR) analysis showing expression of Grb10 mRNA in WT day 2 (D2) myoblasts and day 6 differentiated myotubes (Diff6) relative to cyclophilin (n = 3 WT, three independent experiments, *P < 0.05, t-test). (ii) Grb10 was also detected at the protein level in D2 myoblasts and Diff6 myotubes. (iii) Expression profile of IGF-1R & IR in WT myoblasts and myotubes. B) (i) qPCR and (ii) Western blot analysis show that Grb10 is highly expressed in new born pup muscle (P0) with levels decreasing as the muscle matures (3 wo, 3 mo), (n = 3 WT from each time point, two independent experiments, ***P < 0.001, t-test), (iii) Expression profile of IGF-1R and IR in muscle from mice at stage P0, 3 wo and 3 mo.
Grb10 is expressed in activated satellite cells and expression levels in skeletal muscle decrease with muscle maturity

To ascertain the expression of Grb10 in primary myoblasts and differentiated myotubes in vitro, and to determine the expression levels of Grb10 in maturing muscle in vivo, qPCR and Western blot analysis were performed. Both methods demonstrated that Grb10 is expressed in primary myoblasts (D2) and differentiated myotubes (Diff6) in vitro (Fig. 3A.i&ii). In vivo, Grb10 appeared to be developmentally regulated with highest Grb10 expression levels detected by qPCR and immunoblot in muscle of newborn pups and lower levels in 3 week old mice and 3 month old mice (Fig. 3B.i&ii). Two known binding partners of Grb10, the IR and the IGF-1R, are also expressed in myoblasts and myotubes (Fig. 3A.iii). In muscle tissue, the expression pattern of these receptors parallels that of Grb10 (Fig. 3B.iii).

Grb10 deletion enhances proliferation of primary muscle cells and alters the expression of key cell cycle markers

To investigate the proliferative capacity of Grb10<sup>−/−</sup> primary myoblasts, we examined the mean population doubling time using both a manual cell count and BrdU incorporation assay. After 48 h in culture, more Grb10<sup>−/−</sup> primary myoblasts were detected compared to WT (Fig. 4A.i&ii). Manual cell count demonstrated a twofold increase in Grb10<sup>−/−</sup> cell number

![Fig. 4. Absence of Grb10 enhances muscle cell proliferation and alters the expression of key cell cycle markers. A) Brightfield images showing a larger number of (ii) Grb10<sup>−/−</sup> cells compared to (i) WT after 48 h in culture, scale bar 50 m. B) Manual cell count of WT and Grb10<sup>−/−</sup> cells at 48 and 96 h. C) BrdU incorporation at 8, 24, and 48 h (n = 3 WT & Grb10<sup>−/−</sup> clones, three independent experiments, *<i>P</i> < 0.05, **<i>P</i> < 0.01, ***<i>P</i> < 0.001, t-test). D) qPCR analysis of p21 mRNA (n = 3 WT & 3 Grb10<sup>−/−</sup> clones, three independent experiments, ***<i>P</i> < 0.001, t-test). E) Representative Western blots from WT & Grb10<sup>−/−</sup> protein lysates at 48 h blotted for p-Rb and total Rb. F) densitometry analysis of p-Rb in Grb10<sup>−/−</sup> myoblasts compared to WT when normalised to total levels of Rb (t-Rb) (n = 3 WT & 3 Grb10<sup>−/−</sup> clones, two independent experiments, *<i>P</i> < 0.05, t-test).
Grb10 deletion enhances Akt and ERK signaling in primary myoblasts

To investigate a role for Grb10 in primary myoblast signaling, we examined phosphorylation of the mitogenic signaling pathway targets Akt and ERK. Following insulin stimulation, there was no significant difference in the response between WT and Grb10<sup>-/-</sup> myoblasts for either p-Akt or p-ERK (Fig. 5A). However, a modest difference in the phosphorylation of these signaling proteins was observed between the two genotypes in the basal state (growth factor-free media). To test the response to well-established myogenic growth factors, stimulation with IGF-1 and HGF was also performed. WT myoblasts were responsive, with increased phosphorylation of Akt and ERK in the presence of growth factors compared to the basal state (1.8- to 7-fold increase of p-ERK for IGF-1 or HGF, respectively, and 2.9- to 2.6-fold increase of p-Akt for IGF-1 or HGF respectively) (Fig. 5B). No significant difference was detected in the levels of p-ERK and p-Akt between WT and Grb10<sup>-/-</sup> myoblasts following stimulation by either IGF-1 or HGF (Fig. 5B). However, markedly higher levels of phosphorylation of ERK (1/2) and Akt were again seen in the basal state in Grb10<sup>-/-</sup> myoblasts compared to WT (3- and 2.5-fold increase, respectively) (Fig. 5B).
points of myoblast differentiation were determined. To induce differentiation, cells were switched to differentiation media (DM) and mRNA collected from WT cells after 1 (Diff1), 2 (Diff2) and 6 days (Diff6) of differentiation. qPCR showed an increase in the level of Grb10 transcripts in differentiating myoblasts, with levels peaking significantly after 2 days of differentiation (Fig. 6A, \( P < 0.05 \)). Grb10 levels were lower in mature myotubes (Diff6), but similar to previous results, remained higher compared to undifferentiated myoblasts (D2). Morphological assessment of Diff6 Grb10\(^{-/-}\) myotubes showed no apparent differences to WT, suggesting that Grb10\(^{-/-}\) cells are able to differentiate fully (Fig. 6B,i&ii). To assess the rate of differentiation in WT and Grb10\(^{-/-}\) myoblasts, subconfluent WT and Grb10\(^{-/-}\) myoblasts were switched to DM and pulsed with BrdU at 12, 24 (Diff1), and 48 h (Diff2) of differentiation. The percentage difference in BrdU incorporation relative to cells growing in normal growth media (cells still cycling) was then measured. After 12 h of switching to differentiation media, a 52% decrease in BrdU incorporation was observed in Grb10\(^{-/-}\) myoblasts compared to 27% in WT (Fig. 7A,i). A significant decrease in BrdU incorporation was observed at 24 h (Grb10\(^{-/-}\), 65%, WT 43%) (Fig. 7A.ii). However, no difference was observed between WT and Grb10\(^{-/-}\) cells at 48 h (Diff2) of differentiation (Grb10\(^{-/-}\), 64%, WT 66%, Fig. 7A.iii). These results indicate that when induced to differentiate, Grb10\(^{-/-}\) myoblasts can exit the cell cycle at a faster rate than WT cells, but only for a short time interval. We then examined the expression levels of myogenin, a marker of early differentiation. Lysates from WT and Grb10\(^{-/-}\) differentiating myoblasts were collected 24 h (Diff1) after switching to DM (Fig. 7B), and Western blot analysis of myogenin demonstrated a 2-fold increase in expression levels in Grb10\(^{-/-}\) myoblasts compared to WT (Fig. 7C&D).

**Grb10\(^{-/-}\) primary myotubes display an increase in glucose metabolism and GLUT4 content at the plasma membrane**

We evaluated the potential mechanisms by which Grb10 ablation impacts glucose uptake in muscle. H\(_2\)O\(_2\)-deoxyglucose uptake in Diff6 differentiated myotubes was measured under basal conditions, and following insulin stimulation. Grb10\(^{-/-}\) myotubes showed a 2-fold increase in glucose uptake under both conditions (Fig. 8A) compared to WT. GLUT4 translocation assays were performed to investigate the mechanisms underlying the increase in glucose uptake. Cell surface HA-GLUT4 constituted ~25% of total HA-GLUT4 content in WT myotubes, while in Grb10\(^{-/-}\) myotubes, it represented 55% (Fig. 8B). No significant difference in surface HA-GLUT4 level was seen between WT and Grb10\(^{-/-}\) myotubes following insulin stimulation. Signaling pathways were also examined. In the basal state, there was a tendency for higher levels of p-ERK and p-Akt in Grb10\(^{-/-}\) myotubes, and the same was found following insulin stimulation (Fig. 8C,D).

**Discussion**

Factors that regulate muscle mass have the potential to offer therapeutical benefits in treating muscle diseases and improving metabolic health. Our laboratory has recently identified Grb10 as one such protein (Holt et al., 2012). In the present study, we developed an in vitro model of primary murine muscle cells to investigate the mode of action of Grb10 and provide insight into mechanisms that contribute to the growth of muscle mass and the phenotype of improved insulin action observed for Grb10 KO mice in vivo.

The satellite cell population in skeletal muscle is also referred to as adult muscle stem cells. As well as having a common origin with embryonic muscle precursor cells, they share many cellular and molecular features, making them an appropriate model for the study of muscle development in vitro. However, such investigations require isolation of a highly pure population of satellite cells to be able to specifically evaluate muscle cell proliferation, differentiation, and intracellular signaling cascades. Here, we provide a method combining explant isolation with FACS to specifically enrich for muscle satellite cells. To date, only two cell surface markers (CD34 and alpha 7 integrin) have been used to purify mouse muscle cells using FACS (Cooper et al., 1999; Beauchamp et al., 2000; Ieronimakis et al., 2010). Based on studies using human muscle (Meng et al., 2011), we investigated the use of NCAM/CD56 as a marker for the isolation of muscle progenitor cells from mouse tissue. NCAM/CD56 was initially considered as a marker of Natural Killer (NK) cells (Hercend et al., 1985; Jacobs et al., 1992) and later identified to be expressed in a variety of tissues including brain, nerve, and muscle (McCain and Edelman, 1982; Cunningham et al., 1987). We have shown that NCAM/CD56 is expressed on primary muscle cells from mouse tissue. Sorting with this marker yielded over 95% Pax7/MyoD positive cells that can actively engage in the terminal differentiation program (Fig. 2C). To our knowledge, this is the first paper describing the isolation of a pure and highly myogenic population of primary myoblasts from mouse tissue using this marker. Furthermore, we have demonstrated the utility of this method for application to a genetically modified mouse model, to facilitate comparative studies of satellite cell function. We suggest that NCAM/CD56 cell surface marker can be considered by itself or in combination with other established markers when isolating primary muscle cells from mice.

While the role of Grb10 has been previously studied in the context of adult muscle function, its role in myogenesis has been unclear. We demonstrated that Grb10 protein is more
highly expressed in activated satellite cells than in differentiated myotubes. mRNA level did not correlate with protein, but this could be attributable to post-transcriptional and/or post-translational regulation of Grb10 (Hsu et al., 2011; Yu et al., 2011). In addition, we showed in vivo that in skeletal muscle, the highest expression for Grb10 mRNA and protein is at birth (P0), and these levels significantly decrease during muscle maturation to adulthood (3 months). These results are consistent with previous work showing that Grb10 transcripts, as well as other imprinted genes, are downregulated in adult muscle compared to postnatal day five (P5) skeletal muscle (Berg et al., 2011). The decline in Grb10 expression levels from the postnatal to adult period may also be associated with a drop in the proportion of satellite cells during postnatal muscle growth. At birth, satellite cells represent ~30% of myocyte nuclei, while they only constitute ~2%-7% of nuclei within adult skeletal muscle (Hawke and Garry, 2001; Halevy et al., 2004). Taken together, these results suggest that the expression of this imprinted gene is retained in satellite cells and support a role for Grb10 in early muscle development and satellite cell function.

The hypermuscular phenotype of Grb10−/− mice is related to an increase in muscle fiber number with no apparent changes in fiber size or fiber type proportion (Holt et al., 2012). Fiber number is thought to be established by birth (Ontell and Kozeka, 1984) and is dependent on the number of available

Fig. 7. Deletion of Grb10 produces faster transition to a myogenin-positive state. A) Assessment of the difference in BrdU incorporation after (i) 12 h, (ii) 24 h and (iii) 48 h of switching from growth media (GM) to differentiation media (DM) (n = 3 WT & three Grb10−/−, two independent experiments, **P < 0.01, ***P < 0.001, t-test). B) (i) Representative bright field images of Diff WT and Grb10−/− differentiating myotubes, scale bar 50 μm. (ii) Western blotting of myogenin on protein lysates extracted from Diff WT and Grb10−/− myotubes. (iii) Densitometry analysis of myogenin in WT and Grb10−/− Diff1 myotubes (P = 0.055, n = 3 WT & three Grb10−/−, two independent experiments).
myogenic progenitor cells. Therefore, we hypothesized that the increase in fiber number is likely due to an increase in the proliferation of muscle progenitor cells during early stages of muscle development. Studies have shown both positive (O’Neill et al., 1996; Wang et al., 1999) and negative (Liu and Roth, 1995; Morrione et al., 1997) roles for Grb10 in regulating cellular proliferation. However, it is possible that these differences result from the different cellular model examined or the differences in experimental procedure. Our results demonstrate that Grb10 acts as a negative regulator of muscle cell proliferation. We show that Grb10−/− primary muscle cells proliferate faster than WT cells by transitioning rapidly from the G0 to the S (DNA synthesis) phase of the cell cycle. Molecularly, the increased proliferative capacity of Grb10−/− cells was associated with downregulation of p21 transcript levels and hyperphosphorylation of Rb. Changes in p21 levels and Rb activity are known to affect cell cycle dynamics, with upregulation of p21 being associated with permanent cell cycle arrest of muscle cells (Nevins, 1992; Sherr, 1996; Walsh et al., 1996), whilst phosphorylation of Rb releases E2F from the complex to enhance transition through the cell cycle (Gottifredi et al., 2004). Our findings provide compelling evidence that ablation of Grb10 in muscle cells stimulates the proliferative potential. They are also consistent with those described for myostatin ablated mice (myostatin−/−) where the increase in muscle mass was associated with an enhanced proliferative capacity of satellite cells and changes in cell cycle markers (McPherron et al., 1997; Joulia et al., 2003; McCroskery et al., 2003).

In addition to the changes we observed in cell cycle markers, we also demonstrated that absence of Grb10 affects upstream proteins in proliferation signaling pathways including Akt and ERK. Phosphorylation of both Akt and ERK is markedly enhanced in Grb10−/− myoblasts in the basal state. Stimulation with growth factors IGF-1 or HGF in Grb10−/− myoblasts did not further increase phosphorylation of these targets indicating that Grb10−/− cells are near maximal phosphorylation even under basal conditions. It is not known which signaling molecules upstream of Akt and ERK are activated to maintain this phosphorylation in the basal state, nor which growth factor receptor is likely to be involved in triggering these effects. Grb10 has an established role in binding the IR and IGFR (Liu and Roth, 1995; Morrione et al., 1996; O’Neill et al., 1996; Laviola et al., 1997; Ramos et al., 2006). Moreover there is some evidence from in vitro studies showing that Grb10 binds to FGFR and HGF (Wang et al., 1999). Therefore it is likely that any one or all of these growth factor receptors are residually activated and could be involved in the enhanced basal activation of Akt and ERK when the Grb10 interaction is abolished.

In addition to the increase in cell proliferation, our data demonstrate that Grb10 does not affect the ability of myoblasts...
to differentiate, but rather impacts on when terminal differentiation is initiated. Grb10 transcript levels peak during early time points of differentiation suggesting that Grb10 may be required to initiate differentiation. However, Grb10-/- myoblasts do differentiate and form functional myotubes similar in morphology and size to WT myotubes. It is known that terminal differentiation requires permanent withdrawal from cell cycle and is regulated by the expression of the early differentiation marker myogenin (Kitzmann and Fernandez, 2001). Our studies from the BrU labeling experiments show that Grb10-/- myoblasts withdraw faster from the cell cycle compared to WT. In addition, the molecular basis for the increased withdrawal is due to an early increase in the expression levels of myogenin. Taken together, these results provide evidence that absence of Grb10 does not directly modify the differentiation program; but rather provides an environment that facilitates a faster differentiation of myoblasts to myotubes. Based on the proliferation and differentiation data presented here, we propose Grb10 as a regulator of distinct intracellular signaling pathways involved in both proliferation and differentiation of primary myoblasts.

Previous studies have demonstrated that Grb10-ablated mice have an improved insulin action and improved whole body glucose homeostasis compared to WT mice (Smith et al., 2007; Wang et al., 2007). To investigate whether Grb10 ablation has a direct effect on muscle cell metabolism, glucose uptake was investigated in differentiated myotubes from WT and Grb10-/- cells. Our results demonstrated that Grb10-/- myotubes have a twofold higher glucose uptake in the basal state compared to WT. This improved glucose uptake in Grb10-/- myotubes was accompanied by an increase in GLUT4 transporters at the plasma membrane and a tendency toward an enhanced Akt and ERK signaling. Reduced GLUT4 translocation to the plasma membrane is thought to be a major contributor to the defect in glucose transport in skeletal muscles of insulin resistant and type 2 diabetic patients (Ryder et al., 2000). Therefore, these results provide further evidence that lack of Grb10 enhances insulin signaling and glucose transport in myotubes in vitro and supports in vivo data suggesting that improved insulin action in Grb10-/- mice may be due to differences in muscle metabolism.

Myoblast proliferation and differentiation are early critical processes that regulate muscle growth and also regeneration. A defect in satellite cell proliferation or differentiation is associated with different metabolic diseases including type II diabetes and obesity, as well as with muscular diseases and with reduced muscle mass during aging (Allbrook et al., 1971; Purchas et al., 1985; Mozdziak et al., 1994; Aguiari et al., 2008). In the current study, we developed and utilized an in vitro model of highly purified primary muscle cells to study mechanisms of action of Grb10 in muscle cells. We provide evidence supporting a role for Grb10 as a factor that regulates the balance between muscle cell proliferation and differentiation via modulating signaling pathways and expression of key cell cycle and differentiation markers (Fig. 9). In addition, we demonstrated that absence of Grb10 increases glucose uptake via enhanced translocation of GLUT4 transporters to the plasma membrane. These data identify Grb10 as a potential therapeutic target to improve glucose metabolism and alleviate the reduction in muscle mass associated with muscle disorders and aging.

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Fig. 9. A model for the role of Grb10 in muscle cell proliferation and glucose metabolism. Absence of Grb10 results in an enhanced growth factor signaling leading to increase in Akt and ERK activity. The downstream effect of ERK is an increase in cell proliferation through regulation of key cell cycle markers (p21 and Rb). In addition, enhanced Akt activity leads to an improved glucose metabolism and insulin action.

hybridoma antibodies: PAX7, Myogenin, β-tubulin, and myosin chain were respectively developed by investigators Atsushi Kawakami, Klymkowsky, M., Wright, W.E., Fischman, D.A. and were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained at The University of Iowa. This work was supported by funding from the National Health and Medical Research Council (NHMRC) of Australia (481335). C.M.G. received salary support from a postgraduate scholar award (APA) from the University of Sydney. N.T. is supported by an Australian Research Council Future Fellowship. G.J.C and R.J.D are supported by research fellowships, from the NHMRC of Australia. The authors declare no conflicts of interest.

Literature Cited
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Chapter 8 – Clinical and therapeutic aspects of vitamin D, muscle and bone

This chapter consists of 3 solicited review articles that were published in 2014. As primary author on these articles, the PhD candidate, Christian Girgis, conducted the literature reviews, analysed the evidence-base for the respective articles, prepared manuscripts/figures and reviewer responses.

The article published in *Clinical Endocrinology* (Feb 2014: 80; 169–181) covers clinical studies, including observational and randomised controlled trials, on effects of vitamin D on a range of muscle outcomes, specifically falls, strength, athletic performance and metabolic function (i.e. insulin sensitivity). This is followed by description of mechanisms and translational data on vitamin D and muscle.

The article published in *Current Osteoporosis Reports* (June 2014: 12; 142–153) discusses novel therapies that target both muscle and bone, including activin signalling inhibitors, GH secretagogues and Vitamin D Receptor Agonists. The emerging notion that bone and muscle form a finely integrated unit is discussed here.

The article published in *Current Opinion in Clinical Nutrition and Metabolic Care* (November 2014: 17; 546-550) discusses recent evidence on effects of vitamin D in muscle function in elderly subjects. The debate on optimal serum levels of vitamin D in the elderly is reviewed.
REQUESTED REVIEW

Effects of vitamin D in skeletal muscle: falls, strength, athletic performance and insulin sensitivity

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Summary

Accompanying the high rates of vitamin D deficiency observed in many countries, there is increasing interest in the physiological functions of vitamin D. Vitamin D is recognized to exert extraskeletal actions in addition to its classic roles in bone and mineral homeostasis. Here, we review the evidence for vitamin D’s actions in muscle on the basis of observational studies, clinical trials and basic research. Numerous observational studies link vitamin D deficiency with muscle weakness and sarcopenia. Randomized trials predominantly support an effect of vitamin D supplementation and the prevention of falls in older or institutionalized patients. Studies have also examined the effect of vitamin D in athletic performance, both inferentially by UV radiation and directly by vitamin D supplementation. Effects of vitamin D in muscle metabolic function, specifically insulin sensitivity, are also addressed in this review. At a mechanistic level, animal studies have evaluated the roles of vitamin D and associated minerals, calcium and phosphate, in muscle function. In vitro studies have identified molecular pathways by which vitamin D regulates muscle cell signalling and gene expression. This review evaluates evidence for the various roles of vitamin D in skeletal muscle and discusses controversies that have made this a dynamic field of research.

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Introduction

Long before the discovery of vitamin D, the sun was revered as a source of physical strength and vitality. In ancient Greece, heliotherapy (sun exposure) was prescribed as a cure for ‘weak and flabby muscles’. Sun exposure was considered a performance enhancer; ancient Olympians were instructed to train in sunlight to increase their muscle size.

It was in 1922 that the American physician, Alfred Hess, realized that children with rickets had profound muscle weakness and that direct sunlight exposure improved their ‘general vigour and nutrition’. Adolf Windhaus was awarded the 1928 Nobel prize for his work on sterols, including the discovery of vitamin D. The name ‘vitamin D’ is a historical misnomer. The active form, calcitriol, is a hormone; it is synthesized in humans, undergoes auto-crime regulation and it interacts with a nuclear receptor. Following the photochemical conversion of 7-dehydrocholesterol to vitamin D under the influence of UV radiation, this molecule circulates bound to vitamin D-binding protein (DBP) and undergoes two hydroxylation steps, firstly in the liver to form 25(OH) Vitamin D (25OHD) and subsequently in the kidney to form 1,25(OH)2Vitamin D (1,25(OH)2D) by the 1-alpha-hydroxylase enzyme (encoded by CYP27B1). 1,25(OH)2D is the biologically active form that binds to the vitamin D receptor (VDR) to regulate gene expression, with major effects on calcium and mineral homeostasis. 1,25(OH)2D regulates its own catabolism via upregulation of the expression of CYP24A1 gene (which encodes 24-hydroxylase). Although VDR is present in cell types involved in mineral homeostasis, it is present in other cell types, suggesting that there may be nontraditional roles of vitamin D.

Vitamin D deficiency is now recognized to be common. In Australia, 31% of participants in a study of 11 247 adults were deficient (25OHD < 50 nmol/L), and an incidence of 41% was recently reported in the United States. Other parts of the world, such as Asia, India and the Middle East, have also reported high rates of vitamin D deficiency. With increasing recognition of this pandemic, a plethora of observational studies have associated deficiency with several conditions including autoimmune diseases, diabetes, cancer and muscle weakness.

A direct link between vitamin D deficiency and muscle weakness was first described in the 1960s. In adults with
osteomalacia, muscle strength improved following supplementation.\textsuperscript{5} Whether this improvement represented a direct effect on muscle or an indirect result of vitamin D-mediated changes in calcium homeostasis was unclear. In the years since this paper, clinical studies examining the effects of vitamin D on muscle performance, mass and the risk of falls in various populations have emerged.\textsuperscript{9,10} There are also a variety of studies that have examined mechanisms responsible for vitamin D’s effects in muscle. However, controversy remains regarding the significance of this research and whether the VDR is expressed in adult muscle.\textsuperscript{11–13} In this review, we will cover the clinical evidence for effects of vitamin D in skeletal muscle function and potential mechanisms underlying a role for vitamin D at this site.

Vitamin D and muscle function in older populations

Low muscle mass (sarcopaenia) is characteristic of the ageing process and is associated with a greater risk of disability, falls and mortality in older people.\textsuperscript{14} Vitamin D deficiency is common in the elderly and institutionalized and may contribute to sarcopaenia.\textsuperscript{15} Reduced capacity for UV-mediated vitamin D synthesis in older skin may be partly responsible. Additionally, VDR expression in human muscle declines with age.\textsuperscript{11} This may render the muscles of elderly individuals more vulnerable to low vitamin D levels. Higher serum levels may be required to achieve comparable physiological effects. In this section, we discuss observational studies linking vitamin D deficiency and muscle dysfunction in elderly subjects and interventional studies examining potential effects of vitamin D supplementation in the risk of falls and muscle weakness.

Observational studies

Several authors report an association between baseline 25OHD and subsequent falls risk in elderly people. In a prospective, longitudinal study of 1600 elderly women living in assisted care, Flicker and colleagues found a 20% reduction in falls risk over \(\geq 150\) days with a doubling of 25OHD levels.\textsuperscript{16} Similarly, amongst 1231 people over age 65, those with 25OHD levels \(< 25 \text{ nM} \) were more likely to have recurrent falls in the subsequent year (OR \(1.78 \text{ for } \geq 2 \text{ falls}) \textsuperscript{17}.

Prospective studies have also reported an association between baseline 25OHD levels and declining muscle function. In a Dutch study of 1000 subjects over age 65, baseline 25OHD level \(< 50 \text{ nM} \) correlated with a greater 3-year decline in physical performance (sum score of walking test, chair stands and tandem stand i.e. ability to stand with feet in tandem position with eyes closed for 10 s).\textsuperscript{18} The 3-year risk of sarcopaenia in older subjects was two times greater with baseline 25OHD levels \(< 25 \text{ nM} \).\textsuperscript{15}

However, not all studies confirm these associations\textsuperscript{19} and despite adjustments, potential confounding factors exist. Low baseline 25OHD levels may be a marker of pre-existing frailty, as the frail elderly are less likely to spend time outdoors, exposed to UV radiation. Malnutrition is common in the elderly; 40% of those living in institutions are at risk.\textsuperscript{20} Dietary deficiencies may contribute simultaneously to low vitamin D and to frailty. Factors not considered in adjustment models may confound data interpretation, making interventional studies important.

Intervention studies

Many studies have examined the effect of exposure to UV radiation or vitamin D supplementation in the prevention of falls and muscle strength in older individuals.

Amongst 602 residents of aged-care facilities living in Sydney, Australia, those who increased sunlight exposure had fewer falls than those randomized to the control group over 12 months (incidence rate ratio 0.52, \(P = 0.01\)).\textsuperscript{21} However, compliance with sunlight exposure was poor (26%), and no effect was observed on intention-to-treat analysis.

Several studies reported beneficial effects of vitamin D on falls. Amongst 625 older residents of assisted-living facilities, those who received calcium (600 mg daily) and ergocalciferol (initially 10 000 IU weekly, then 1000 IU daily) for 2 years were less likely to ever fall than those receiving calcium alone.\textsuperscript{22} Similarly, in 122 older women in geriatric care, calcium (1200 mg/day) and cholecalciferol (800 IU/day) caused a 49% reduction in falls during the 12-week treatment vs the preceding 6-weeks, vs calcium alone.\textsuperscript{23} Those who fell most had the greatest benefit. Other studies have also suggested benefits in at-risk individuals such as less active older women\textsuperscript{24} and those suffering stroke.\textsuperscript{25}

Not all studies, however, are concordant.\textsuperscript{26,27} A randomized study of 5292 subjects \(\geq 70\) years showed no benefit with cholecalciferol (800 IU daily) in the reduction of falls over 26–62 months.\textsuperscript{27} However, in both negative studies,\textsuperscript{26,27} patients had recently sustained minimal-trauma fracture or were at risk of fracture, suggesting impaired mobility and falls were not the primary study end-point.

One study suggested that ‘mega-doses’ increased falls risk. An annual oral dose of 500 000 IU of cholecalciferol increased falls in 2256 community-dwelling older women, particularly in the 3 months following the dose (incidence rate ratio 1.31 compared to placebo).\textsuperscript{28} The mechanism was unclear as serum levels were not toxic. As the vitamin D system is autoregulated by induction of 24-hydroxylase which inactivates 25OHD and 1,25OHD, we speculate that large single doses of cholecalciferol may result in excess 24-hydroxylase induction, rapidly degrading the administered dose and potentially contributing to subsequent deficiency.

Regarding muscle performance, studies show improved lower limb strength following vitamin D supplementation in institutionalized\textsuperscript{29} and community-dwelling older individuals.\textsuperscript{30} Those with baseline 25-OHD levels \(< 50 \text{ nM} \) had the greatest benefits. However, a study of single mega-dose therapy (300 000 IU) in 243 frail, older patients did not improve performance even amongst those with baseline levels \(< 30 \text{ nM} \).\textsuperscript{31} For a detailed list of studies examining vitamin D supplementation and falls, we refer readers to Tables 4 and 5 in a recent review.\textsuperscript{4}
**Meta-analyses**

In the widely cited meta-analysis by Bischoff-Ferrari, a dosedependent effect of vitamin D supplementation in the reduction in falls was found when examining eight trials and 2426 elderly individuals. Compared with placebo, higher doses of vitamin D (700–1000 IU) reduced falls risk by 19%, but lower doses had no effect. Likewise, people who achieved serum 25OHD concentrations ≥60 nm had reduced falls by 23%, but those who achieved concentrations ≤60 nm had no effect.

The recent Institute of Medicine report criticized this meta-analysis for inconsistencies in study inclusion, perceived errors in statistical analysis and reliance on two studies to produce the positive effect, one of which was reportedly not well-powered. On reanalysis with two statistical alterations, the positive effect was not found.

The most recent and largest meta-analysis of 45 782 people, mainly elderly females, found a significant reduction in falls risk amongst those randomized to vitamin D supplementation (OR 0.86 for ≥1 fall). There was no difference between higher (>800 IU) vs lower doses. Vitamin D was effective in both community-dwelling and institutionalized people with cholecalciferol or ergocalciferol. Reduction in falls was most prominent in patients who were deficient at baseline and with calcium co-administration. In studies included in this meta-analysis, the calcium doses ranged from 500 to 1200 mg daily and elemental calcium and calcium carbonate were used.

Meta-analyses dealing with vitamin D and muscle strength are limited by substantial study heterogeneity in design, including in the parameters of muscle function. A recent meta-analysis that assessed 17 RCTs involving 5072 participants reported no effect on grip strength or proximal lower limb strength in adults with 25OHD levels >25 nm. However, on pooling data from two studies on vitamin D-deficient adults (i.e. 25OHD < 25 nm), a large effect on hip muscle strength was found although the studies used different measures.

**Summary**

Although observational studies link falls, muscle weakness and sarcopaenia with vitamin D deficiency, randomized intervention-al trials have yielded conflicting results. This may be explained by the heterogeneity in study design, variability in treatment dose, duration and the different analyses of falls and muscle function. Nevertheless, the data generally support a benefit of vitamin D supplementation in reducing falls amongst at-risk populations, specifically those with vitamin D deficiency and a history of falls. On this basis, we suggest the use of vitamin D in combination with calcium at a daily dose of at least 500 mg for falls prevention in older people with baseline vitamin D deficiency. The dose of vitamin D supplementation and target 25OHD levels are controversial. Whilst the Institute of Medicine recommends 25OHD target levels of 50 nm and daily vitamin D doses of 800 IU in older adults (>70 years), the US Endocrine society advocates higher serum target level of 75 nm and daily doses of at least 1500–2000 IU in this age group. Those targets are based on skeletal and biochemical outcomes, and the evidence for muscle outcomes does not particularly favour one target over the other. It appears that single-dose mega-therapy is not beneficial for muscle function, and we recommend daily therapy in favour of intermittent mega-dosing.

**Vitamin D and muscle function in the young**

Children with rickets, a condition characterized by chronic vitamin D deficiency, display profound muscle weakness and hypotonia. Similarly, those affected with the rare condition type II vitamin D dependent rickets display muscle weakness in association with mutations in the VDR. These observations suggest that together with a critical role in bone mineralization and metaphyseal fusion, vitamin D is important in muscle development.

Beyond the developmental stage, adults with osteomalacia display proximal myopathy characterized by proximal weakness, pain, a ‘waddling gait’ and difficulty rising from a seated or squat position.

Here, we review evidence for a role of vitamin D in muscle function and mass in those not yet affected by age-related muscle loss.

**Physical performance**

There is extensive literature dating back to the 1930s reporting significant improvements in physical performance following exposure to UV radiation. Although these studies do not directly refer to vitamin D, UV-mediated changes in vitamin D status may have played a role in muscle function amongst study participants. In 1938, a Russian group reported significant improvements in 100-m sprint times amongst students receiving UV radiation (7.4% vs 1.7% improvement in controls). In 1944, a German group found that medical students receiving UV radiation over 6 weeks had a 13% improvement in performance on a bicycle ergometer. In a study from USA, 11 male students experienced a 19% increase in cardiovascular fitness following a course of UV radiation.

Contemporary studies have reported surprisingly high rates of vitamin D deficiency amongst athletes, including Middle-Eastern sportsmen (58% deficient), professional British athletes (57%), and Australian gymnasts (33%). However, studies examining vitamin D supplementation in athletes are sparse. In a study of thirty UK athletes randomized to cholecalciferol 20000, 40000 IU or placebo once per week for 12 weeks, despite significant increases in 25OHD levels at 6 and 12 weeks in the active arms, there was no significant improvement in muscle performance. In a larger study including 61 male athletes and 30 healthy male nonathletes in the UK, cholecalciferol (5000 IU/ day) led to significant improvements in 10-m sprint times and vertical jump over the 8-week period, with no improvement in the placebo group. Baseline 25OHD levels were lower in this study than in the previous one (mean approximately 40 vs 50 nm), and higher 25OHD levels were achieved (mean 103 vs
approximately 85–91 nm), possibly explaining the discrepancy. With the potential involvement of vitamin D in metabolic pathways, athletes may require a higher intake to ensure adequate availability for muscle metabolism and physical performance. Once again, there are suggestions that high-dose supplemental vitamin D does not improve muscle function compared with daily lower doses.42,44

A study of 14 recreationally active subjects found an association between baseline vitamin D levels and muscle recovery, assessed by leg isometric force following a bout of intense exercise.46 Pre-exercise 25OHD levels predicted immediate and persistent muscle weakness (i.e. 48 and 72 h postexercise), but there was no correlation at 24 h. Interestingly, 25OHD levels initially increased (by approximately 5 nm) and then decreased following exercise, and the authors hypothesized this was due to exercise-related shifts in cytokine and protein levels.

In nonathletes, supplementation studies have yielded mixed results. Amongst 69 adolescent females, those randomized to receive 150 000 IU ergocalciferol orally every 3 months for 1 year demonstrated significant improvements in movement efficiency, a composite of jump height and velocity measured by mechanography, compared with baseline.47 Additionally, higher baseline 25OHD correlated with greater jumping velocity.

In another study of 179 vitamin D-deficient adolescent females in Lebanon, those randomized to receive cholecalciferol (doses 1400 or 14 000 IU weekly) did not improve grip strength.48 Adequate 25OHD levels were achieved in the high-dose group but not in the low-dose group (≈ 95, 40 nm, respectively, placebo 40 nm), implying that increases in lean mass and bone mineral content seen in both groups vs placebo at 1 year were not directly due to serum 25OHD levels.

In a case–control study, 55 veiled Arabic women with severe vitamin D deficiency (mean 25OHD 7 nm) were weaker on all tested parameters of muscle function than 22 Danish women with higher levels (47 nm).49 Following vitamin D repletion (IM ergocalciferol: 100 000 IU per week for 1 month then monthly for 5 months and 400–600 IU orally daily), the Arabic women had significant improvements in muscle function and pain at 3 and 6 months. This may have been due to improvement in related biochemical defects and does not prove a direct role for vitamin D. In any case, mega-dose supplementation was helpful in this group.

Therefore, vitamin D deficiency is common in young populations, and vitamin D supplementation may improve physical performance in athletes depending on the dose.42,44 Such an effect is less clear is nonathletic populations.47,48

**Myalgia**

Studies examining a putative association between vitamin D deficiency and myalgia (i.e. muscle pain) have generally been small, observational and potentially confounded by factors such as physical activity, smoking and body mass index (BMI).

A single randomized trial of ergocalciferol supplementation (50 000 IU weekly for 3 months) in 50 subjects with 25OHD < 50 nm failed to demonstrate improvement in pain scores compared with baseline or placebo.52 As the study was adequately powered to detect a significant change in pain, results would suggest that high-dose supplementation has no benefit in the management of generalized pain in the absence of osteomalacia. Therefore, despite an association between vitamin D deficiency and myalgia, the single RCT addressing this question shows no effect.

**Vitamin D receptor (VDR) polymorphisms**

Vitamin D receptor is the nuclear receptor to which 1,25(OH)2D binds to regulate gene expression. Several VDR polymorphisms exist that are associated with a range of biological effects, including bone mineral density and muscle function.53 The FokI polymorphism, found in approximately 35% of Caucasians,52 is a T/C transition in exon 2 that results in a shorter protein with enhanced transactivation capacity. Although this greater activity might suggest improved muscle strength, the FokI polymorphism is in fact associated with reduced muscle strength. In 107 patients with chronic obstructive pulmonary disease (COPD), homozygosity was associated with reduced quadriceps strength compared with heterozygosity.54 In another study, the Fok I genotype associated with reduced muscle mass and sarcopenia in 302 Caucasian men aged 58–93 years.55

The BsmI polymorphism affects the 3’ region, which is important in the regulation of gene expression. It is reported in approximately 40% of Caucasians.53 In studies of older individuals, the bb genotype was associated with greater strength and reduced risk of falls. Amongst 121 healthy women >70 years, the bb genotype was linked to higher quadriceps and wrist strength compared with the BB genotype.56 In two population-based studies from Italy and the UK, the bb genotype of the BsmI polymorphism appeared to protect against falls.57

However, in younger people, studies link the bb genotype to reduced strength. Amongst 175 women (20–39 years), bb genotype associated with lower hamstring strength and lower fat-free mass compared with the BB genotype.58 In a Chinese study of 109 female university students, bb genotype was associated with significantly lower peak torque in concentric knee flexors.59

Whilst these polymorphisms are interesting, the associations are not consistent and lack mechanistic explanation. Larger studies to assess the associations are needed. Importantly, polymorphisms of other components of the vitamin D system may also be relevant: DBP polymorphisms have been associated with different responses to vitamin D3 supplementation60, and CYP27B1 polymorphisms have been linked to a greater risk of osteoporotic fracture.61

**Mechanisms linking vitamin D and muscle function**

A variety of mechanisms by which vitamin D impacts on muscle cells and fibres have been elucidated. This research includes the
description of muscle morphology in vitamin D-deficient subjects, signalling pathways by which vitamin D effects muscle cells in vitro, and in vivo studies that have sought to delineate effects and mechanisms of aberrant vitamin D signalling on muscle function. These have been summarized in Fig. 1.

**Changes in muscle morphology**

Several reports dating back to the 1970s described striking morphological changes in the skeletal muscle of subjects with osteomalacia, likely related to severe vitamin D deficiency. The main change described is atrophy of the type 2 (i.e. fast twitch) muscle fibres with some reports of scattered necrosis and derangement of the intermyofibrillar network. These changes appear to be reversible. Eleven patients with a condition described as ‘bone loss of ageing’ displayed significant increases in the proportion and cross-sectional size of fast-twitch type IIa fibres of the vastus lateralis following treatment with the vitamin D analogue, 1α-OHD₃ and calcium for 3–6 months. Interestingly, vitamin D supplementation resulted in changes to the oxidative capacity of muscle – succinate dehydrogenase activity and total phosphorylase activity were low at baseline and increased with treatment whilst lactate dehydrogenase activity, a measure of anaerobic metabolism, did not change.

At a functional level, Sato and colleagues suggested that the reduction in falls in a group of 48 women with poststroke hemiplegia who received vitamin D₂ (1000 IU daily) supplementation could be explained by the increase in the proportion and diameter of type II muscle fibres at 1 year. A recent study also implicates improved mitochondrial function as a possible mechanism in the prevention of falls by vitamin D. In twelve individuals with severe vitamin D deficiency (<15 nm), 10–12 weeks of treatment with cholecalciferol 20 000 IU on alternate days resulted in improved muscle mitochondrial function, as assessed by NMR spectroscopy. There is an association between fatty infiltration of skeletal muscle and vitamin D deficiency. In 90 postpubertal females in California, the proportion of quadriceps muscle fat, assessed by computed tomography (CT), was strongly inversely correlated with serum 25OHD levels, independent of body mass or subcutaneous and visceral fat. Similar findings were reported in 20 older subjects who received MRI (magnetic resonance imaging) of the thigh. Interestingly, selective and near total fatty degeneration of at least one muscle was observed amongst 55% of patients with 25OHD <50 nm. These studies raise the interesting possibility that vitamin D deficiency may increase the deposition of intramyocellular lipids, either directly or potentially as a consequence of the reduced physical activity or via reduced muscle mitochondrial capacity. As defects in mitochondrial fuel metabolism and increases in intramuscular lipids are thought to play a role in the pathogenesis of skeletal muscle insulin resistance, these findings may potentially explain the association between vitamin D deficiency and insulin resistance.

**VDR and muscle**

The presence of the VDR in avian, murine and human muscle cells is described by immunohistochemistry, Western blot and detection of VDR mRNA by RT-PCR. However, a recent paper suggested that the VDR is not expressed in skeletal, cardiac or smooth muscle. Differences in experimental conditions and the possibility of tight protein binding of VDR to DNA may have accounted for this finding. Another possible explanation is that VDR is expressed at low levels in resting muscle but is activated by particular conditions. In support of this, a recent paper reported that VDR expression increases following muscle injury in mice. Interestingly, this paper also reported the presence of 1-alpha hydroxylase (CYP27B1) in muscle and that it too is activated during muscle injury. These findings support the intriguing possibility that VDR and CYP27B1 play roles in muscle regeneration and that muscle possesses the capacity for local production of 1,25(OH)₂D.

More recently, a pilot study linked vitamin D supplementation (4000 IU/day for 4 months) with an increase in muscle VDR expression and fibre size in older, mobility-limited women. Although this was a small study and the specificity of the particular antibody used is unclear (VDR-NR1I1), this study provides tantalizing evidence that vitamin D may influence muscle fibre size in humans by upregulating local VDR expression.

Vitamin D receptor defects also influence muscle function. Mice with congenital absence of VDR display a number of deficits in muscle function. On forced swim tests, they display more sinking episodes and fatigue compared with wild-type mice. These changes are probably largely related to impaired muscle development with generalized atrophy of muscle fibres in this mouse model. One important caveat is that these mouse models of VDR defects also display secondary biochemical abnormalities in calcium, phosphate and parathyroid hormone that could affect muscle function.

One study examined the precise aetiology of vitamin D deficiency myopathy by rendering rats vitamin D₃, phosphorus and calcium deficient by dietary methods. Phosphorus levels correlated independently with the reduction in soleus muscle force in these rats, and phosphorus repletion in the presence of persistent vitamin D deficiency resulted in complete restoration in slow-twitch muscle force. Another study reported that calcium
was predominantly important in the type 2 muscle fibre atrophy and the muscle protein degradation seen in vitamin D-deficient rats.

Although calcium repletion mostly corrected these abnormalities, persistent vitamin D deficiency was itself associated with a degree of muscle fibre atrophy.

It therefore appears that muscle defects are due, in part, to vitamin D deficiency but predominantly to the associated hypocalcaemia and hypophosphataemia. Repletion with vitamin D remains the most effective treatment as it corrects all of those defects.

**Vitamin D and intracellular pathways in muscle**

A variety of molecular pathways by which vitamin D may affect muscle cells have been elucidated. These effects may be rapid, occurring within seconds to minutes of vitamin D treatment and include the release of calcium from intracellular stores and its subsequent entry via voltage-gated membrane channels. This suggests a role for vitamin D in the calcium-mediated functions of muscle, namely contraction, plasticity, mitochondrial function, insulin signalling and fuel handling. Whilst evidence for a link between vitamin D and mitochondrial function is only recent, potential alterations in muscle substrate metabolism may help explain the occurrence of insulin resistance, intramuscular fatty deposition and muscle weakness in vitamin D-deficient subjects. Serum 25OHD levels correlate with recovery rates of phospho-creatine muscle stores after exercise, suggesting a broader link with ATP production and oxidative function.

Vitamin D could lead to delayed effects on muscle via gene expression. This involves binding of the 1,25OHD-VDR-RXR (retinoid X-receptor) complex to vitamin D response elements of DNA and includes effects in the expression of contractile proteins and myogenic transcription factors which influence muscle development.

More recently, it has emerged that skeletal muscle serves a storage site for 25OHD. Skeletal muscle was found to express megalin and cubulin, proteins necessary for the endocytic internalization of DBP-bound 25OHD and muscle fibres were noted to retain substantially higher proportions of tritium-labelled 25OHD than bone cells.

Therefore, evolving research points to both rapid and genomic effects of vitamin D in skeletal muscle and its storage at this site that may have a range of effects that remain unknown at this time.

**Vitamin D and insulin sensitivity**

Apart from the generation of force, skeletal muscle is a highly metabolic tissue that responds to a range of hormones including growth hormone, IGF-1, corticosteroids and insulin. Under normal physiological conditions, skeletal muscle is responsible for approximately 85% of whole-body insulin-mediated glucose uptake and is therefore of primary importance to insulin resistance. In this section, we discuss the evidence linking vitamin D deficiency with insulin resistance and the broader implications for the pathogenesis of type 2 diabetes.

**Observational studies**

Amongst 808 nondiabetic participants of the Framingham Offspring Study, plasma 25OHD was inversely associated with fasting insulin concentrations and homeostasis model assessment of insulin resistance (i.e. HOMA-IR) after adjustment for age, sex and BMI (summarized in Table 1). A similar association between 25OHD and HOMA-IR was found in 712 subjects at risk of diabetes. HOMA is a mathematical model, based on data from physiological studies, which is used to estimate insulin resistance (HOMA-IR) and insulin sensitivity (HOMA-S). In a group of 126 healthy young adults, there was a significant association between 25OHD and insulin sensitivity assessed by hyperglycaemic clamps, after adjustment for a range of factors including BMI.

Prospective studies show an association between baseline 25OHD levels and the long-term risk of developing insulin resistance (Table 1). In a study of 5200 participants, a 25 nm increment in baseline serum 25OHD levels was associated with a 24% reduced 5-year risk of diabetes. A positive and independent association with HOMA-S at 5 years was also reported ($r = 0.16, P < 0.001$).

These observational studies suggest, but do not prove, a causal or directional relationship between vitamin D status and insulin resistance. Multiple possible confounders exist. In particular, adiposity has an independent inverse association with 25OHD, in many (though not all) studies. This may relate to storage of fat-soluble compounds, the avoidance of sunlight exposure and outdoor activity amongst potentially self-conscious obese individuals. Adiposity is a key factor in insulin resistance. Other potential confounders include physical activity, parathyroid hormone levels, serum calcium and dietary intake, all of which may influence or reflect 25OHD levels and also independently influence insulin sensitivity.

**Interventional studies**

In people without diabetes or underlying insulin resistance, there is no clear evidence that vitamin D supplementation results in improved insulin sensitivity (summarized in Table 2). However, limited evidence suggests that subjects at risk of diabetes may benefit. At-risk subjects randomized to receive various regimens of cholecalciferol and calcium over 6 weeks–3 years, displayed significant improvements in insulin sensitivity, secretion and/or the disposition index (an integrated measure of insulin secretion and action).

The evidence is mixed for subjects with established diabetes. In a recent trial of 90 diabetic subjects, those randomized to receive daily vitamin D (1000 IU) in fortified yoghurt demonstrated improved glycaemic control and insulin resistance (HOMA-IR) vs those receiving plain yoghurt. Importantly, an inverse correlation was observed between changes in serum 25OHD and HOMA-IR in this study.

As summarized in Table 2, the large number of small interventional studies in different populations with baseline vitamin D status, using different modes of supplementation, creates
### Table 1. Cross-sectional studies assessing the correlation between serum vitamin D levels and parameters of insulin sensitivity and glucose homeostasis

<table>
<thead>
<tr>
<th>Study (ref)</th>
<th>Positive or negative</th>
<th>No.</th>
<th>Age, sex</th>
<th>Vitamin D nm (ng/ml)</th>
<th>Tests</th>
<th>Findings</th>
<th>Risk or correlation</th>
<th>Adjustments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kositsawat91</td>
<td>Positive, subgroup</td>
<td>9773</td>
<td>&gt;18, M, F</td>
<td>NHANES</td>
<td>HbA1c, HbA1c</td>
<td>Inverse association 35–74 years</td>
<td>PE = −0.0035</td>
<td>Age, race, sex, DM, activity, supplements, PTH</td>
</tr>
<tr>
<td>Scragg92</td>
<td>Negative</td>
<td>6228</td>
<td>&gt;20 M, F</td>
<td>All</td>
<td>Log (HOMA-IR)</td>
<td>No association</td>
<td>PE = −0.0014</td>
<td>Age, sex, BMI, activity, season</td>
</tr>
<tr>
<td>Positive, subgroup</td>
<td></td>
<td>2766</td>
<td>&gt;20 M, F</td>
<td>Caucasian</td>
<td>Log (HOMA-IR)</td>
<td>Positive association</td>
<td>β = −0.009</td>
<td>Age, sex, BMI, activity, season</td>
</tr>
<tr>
<td>Negative subgroup</td>
<td></td>
<td>1736</td>
<td>&gt;20 M, F</td>
<td>African Americans</td>
<td>Log (HOMA-IR)</td>
<td>No association</td>
<td>β = 0.001</td>
<td>Age, sex, BMI, activity, season</td>
</tr>
<tr>
<td>Positive, subgroup</td>
<td></td>
<td>1726</td>
<td>&gt;20 M, F</td>
<td>Mexican Americans</td>
<td>Log (HOMA-IR)</td>
<td>Positive association</td>
<td>β = −0.016</td>
<td>Age, sex, BMI, activity, season</td>
</tr>
<tr>
<td>Cheng93</td>
<td>Negative</td>
<td>3890</td>
<td>40, M, F</td>
<td>Caucasian</td>
<td>Insulins, HOMA-IR</td>
<td>No association</td>
<td></td>
<td>Adipose tissue, season, WC, activity, D intake</td>
</tr>
<tr>
<td>Ford94</td>
<td>Positive, subgroup</td>
<td>1941</td>
<td>12–17 M, F</td>
<td>&gt;75 (30) vs &lt;50 (20)</td>
<td>Fasting insulin, Hba1c</td>
<td>Association with insulin in males</td>
<td>24% lower insulin</td>
<td>Age, sex, BMI, HDL, ethnicity, activity, time, supplements, cholesterol</td>
</tr>
<tr>
<td>Kayanil92</td>
<td>Positive</td>
<td>960</td>
<td>50, M, F</td>
<td>Caucasian</td>
<td>56</td>
<td>HOMA-IR, ISI on OGTT</td>
<td>Both markers associated</td>
<td>r = −0.29</td>
</tr>
<tr>
<td>Liu95</td>
<td>Positive, subgroup</td>
<td>808</td>
<td>60 M, F</td>
<td>&gt;54 (22) vs &lt;39 (16)</td>
<td>HOMA-IR, ISI, Insulins at OGTT</td>
<td>Improved fasting insulin and HOMA-IR</td>
<td>(10% vs 13%)</td>
<td>Age, sex, BMI, waist circumference, smoking</td>
</tr>
<tr>
<td>Pinelli96</td>
<td>Positive, subgroup</td>
<td>542</td>
<td>38 M, F</td>
<td>46% Ins Res, 42% IGT</td>
<td>M 45 (18), F 35 (14)</td>
<td>HOMA-IR, FPG, HbA1c</td>
<td>Inverse association, men only</td>
<td>M r = −0.19</td>
</tr>
<tr>
<td>Del Gobbo97</td>
<td>Negative</td>
<td>510</td>
<td>&gt;18 M, F</td>
<td>Canadian Cree</td>
<td>52 (21)</td>
<td>HOMA-IR</td>
<td>No association</td>
<td>r = 0.03</td>
</tr>
<tr>
<td>Garnage Yared98</td>
<td>Positive, subgroup</td>
<td>381</td>
<td>24 M, F</td>
<td>Lebanon</td>
<td>77 (31)</td>
<td>HOMA-IR, FPG</td>
<td>Association with FPG</td>
<td>r = −0.15</td>
</tr>
<tr>
<td>Baynes99</td>
<td>Positive</td>
<td>142</td>
<td>70–88 M</td>
<td></td>
<td>42 (17)</td>
<td>Insulin at OGTT</td>
<td>Inverse association</td>
<td>r = −0.18 to −0.23</td>
</tr>
<tr>
<td>Manco100</td>
<td>Negative</td>
<td>116</td>
<td>F</td>
<td>Italian</td>
<td>39 (16)</td>
<td>EH clamp</td>
<td>No association</td>
<td>r = 0.25</td>
</tr>
<tr>
<td>Chiu101</td>
<td>Positive</td>
<td>126</td>
<td>Approximately 25 M, F</td>
<td></td>
<td>47–70 (19–28)</td>
<td>ISI on H clamp</td>
<td>Significant association</td>
<td></td>
</tr>
</tbody>
</table>

BMI, body mass index; BP, blood pressure; EH clamp, euglycaemic–hyperinsulinaemic clamp; ETOH, alcohol consumption; FPG, fasting plasma glucose; H clamp, hyperglycaemic clamp; ISI, insulin sensitivity index; IR, insulin resistance; MetS, metabolic syndrome; IGT, impaired glucose tolerance; OGTT, oral glucose tolerance test; HbA1C, glycated haemoglobin; HOMA-IR, homeostasis model assessment of insulin resistance; NR, not reported; PE, parameter estimates; SES, socio-economic status; WC, waist circumference; WHR, waist-to-hip ratio.
<table>
<thead>
<tr>
<th>Study (ref)</th>
<th>Positive or negative</th>
<th>No.</th>
<th>Age, sex</th>
<th>Other</th>
<th>Basal 25OHD nm (ng/ml)</th>
<th>Intervention D in IU/day, Ca in mg</th>
<th>↑ VitD?</th>
<th>Follow-Up</th>
<th>Outcomes</th>
<th>Findings</th>
<th>Adjustments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nondiabetic at baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>De Boer 99</td>
<td>Negative</td>
<td>33951</td>
<td>50–79 F</td>
<td></td>
<td>&lt;80 (32) in 89%</td>
<td>D3 400 + Ca 1000 vs placebo</td>
<td>No</td>
<td>7 years</td>
<td>T2D onset</td>
<td>No change</td>
<td>Nil</td>
</tr>
<tr>
<td>Nilas et al 100</td>
<td>Negative</td>
<td>238</td>
<td>45 months 54 F</td>
<td>NR</td>
<td></td>
<td>D3 2000, 1α-OHD 0·25 µg, 1,25OHD 0·25–0·5 µg or placebo</td>
<td>NR</td>
<td>2, 1 years</td>
<td>BGL, weight</td>
<td>No change</td>
<td>Nil</td>
</tr>
<tr>
<td>At risk of diabetes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pittas 86</td>
<td>Positive, subgroup</td>
<td>314</td>
<td>Approximately 71 M, F</td>
<td>92 IFG, 222 NGT</td>
<td>&gt;65 (26) in most</td>
<td>D3 700 + Ca 500 or placebo</td>
<td>Yes</td>
<td>3 years</td>
<td>FPG, HOMA-IR</td>
<td>IFG arm ↓ rises in FPG and HOMA-IR</td>
<td>Age, sex, BMI, activity, smoking</td>
</tr>
<tr>
<td>Mitri 84</td>
<td>Positive</td>
<td>92</td>
<td>57 M, F</td>
<td>BMI &gt;25, IGT &amp;/or IFG</td>
<td>61 (24·5)</td>
<td>D3 2000 or Ca 800 mg</td>
<td>Yes</td>
<td>16 weeks</td>
<td>Clamp</td>
<td>↑ insulin release and DI</td>
<td>Age, sex, BMI, baseline values, race, time</td>
</tr>
<tr>
<td>Nagpal 85</td>
<td>Positive</td>
<td>71</td>
<td>Approximately 45 M</td>
<td>Central obesity, Indian</td>
<td>&lt;50 (20)</td>
<td>D3 120 000 2 weekly or placebo</td>
<td>Yes</td>
<td>6 weeks</td>
<td>OGIS</td>
<td>Improvement</td>
<td>Age, WHR, baseline 25OHD, betel-nut use</td>
</tr>
<tr>
<td>Ljunghall 101</td>
<td>Negative</td>
<td>65</td>
<td>61–65 M</td>
<td>IGT</td>
<td>‘Normal’ 1αOHD, 0·75 µg or placebo</td>
<td>D3 400 or placebo</td>
<td>Yes</td>
<td>3 months</td>
<td>FPG, IVGTT, HbA1c HOMA-IR, Insulin</td>
<td>No change</td>
<td>Nil</td>
</tr>
<tr>
<td>von Hurst 102</td>
<td>Positive</td>
<td>81</td>
<td>23–68 F</td>
<td>South Asian</td>
<td>&lt;50 (20)</td>
<td>D3 400 or placebo</td>
<td>Yes</td>
<td>6 months</td>
<td>Improved. More effect if 25OHD &gt;32 ng/ml</td>
<td>Baseline values</td>
<td></td>
</tr>
<tr>
<td>Subjects with diabetes</td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Nikooeyeh 87</td>
<td>Positive</td>
<td>90</td>
<td>51 M, F</td>
<td>Iranian</td>
<td>Approximately 44 (18)</td>
<td>Yoghurt: plain vs 1000 D3 vs D3 1000 + Ca 300</td>
<td>Yes</td>
<td>12 weeks</td>
<td>HbA1c, HOMA-IR</td>
<td>↓ HbA1c and HOMA-IR</td>
<td>Nil</td>
</tr>
<tr>
<td>Sabherwal 103</td>
<td>Positive</td>
<td>52</td>
<td>33–73 M, F</td>
<td>South Asian Retro</td>
<td>15 (6)</td>
<td>D3 400 and Ca 1200 No placebo group</td>
<td>Yes</td>
<td>3 months</td>
<td>HbA1c</td>
<td>ΔHbA1c and Δ25OHD correlated. ( r = -0.305 )</td>
<td>Nil</td>
</tr>
</tbody>
</table>

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(continued)
difficulty in drawing definite conclusions. Methods of assessing insulin sensitivity range from measuring fasting insulin to euglycaemic–hyperinsulinaemic clamps. Whilst insulin resistance is a factor in the genesis of type 2 diabetes, there is a complex interaction between glucose and insulin levels, with glycaemic outcomes reflecting the relationship between both insulin resistance and insulin secretory capacity.

Thus, the question of whether vitamin D supplementation is effective in the amelioration of insulin resistance, and with particular respect to a role in muscle, remains unanswered. A number of large clinical trials are currently underway, which may definitively answer this question (e.g. NCT00736632, NCT01354964, NCT01315366).

Potential mechanisms

Mouse models of type 2 diabetes show improvements in insulin sensitivity following treatment with 1αOH₃. In vitro studies help to explain this link. 1,25D leads to increased expression of insulin receptors and amelioration of insulin resistance via effects on Akt and insulin receptor phosphorylation. Nongenomic effects of vitamin D may also be important. In addition to intracellular calcium regulation, vitamin D leads to the release of arachidonic acid, a polyunsaturated fatty acid, from the cell membrane and into the cytoplasm of muscle cells. This further links vitamin D with insulin sensitivity as does the possibility that vitamin D may influence caveolin-I, a scaffolding protein within the membrane that plays roles in metabolism.

Conclusions

Our understanding of the role of vitamin D in skeletal muscle results from the combined efforts of basic scientists and clinical researchers (summarized in Table 3). Reports of profound muscle weakness in children with rickets more than 80 years ago and more recent descriptions of muscle weakness and myalgia in adults with severe vitamin D deficiency have provided prima facie evidence of a role for vitamin D in muscle. Although historical literature reports substantial improvements in physical performance amongst athletes exposed to UV irradiation, a direct association with vitamin D status was not established in these early studies and remains to be substantiated by contemporary research.

Amongst older individuals, studies examining the effect of vitamin D on strength and falls have had mixed results. The general consensus is that correction of vitamin D deficiency is beneficial in preventing falls, in combination with calcium supplementation and with the recommended aim of achieving serum 25OHD levels of >50 nm according to the IOM or >75 nm according to the US Endocrine society.

Animal studies have demonstrated that congenital lack of the VDR and models of vitamin D deficiency are associated with defects in muscle function. From these studies, it has been difficult to dissect the individual contribution of vitamin D signalling to muscle function, distinct from its role in mineral homeostasis. At a molecular level, muscle cells show both rapid
and delayed responses to the active form of vitamin D, 1,25OH\(_2\)D, that provides mechanistic support for these clinical and animal studies. Recent research showing the activation of VDR in regenerating muscle and the storage of 25OHD in skeletal muscle raise intriguing questions regarding its unknown roles at this site. In conclusion, vitamin D supplementation prevents falls in susceptible populations, vitamin D pathways regulate at this site. In conclusion, vitamin D supplementation prevents falls in susceptible populations, vitamin D pathways regulate VDR in regenerating muscle and the storage of 25OHD in skeletal muscle. The elucidation of vitamin D's effects in muscle promises to remain a vibrant and evolving field.

VDR, vitamin D receptor.

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Therapies for Musculoskeletal Disease: Can we Treat Two Birds with One Stone?

Christian M. Girgis · Nancy Mokbel · Douglas J. DiGirolamo

Abstract Musculoskeletal diseases are highly prevalent with staggering annual health care costs across the globe. The combined wasting of muscle (sarcopenia) and bone (osteoporosis)—both in normal aging and pathologic states—can lead to vastly compounded risk for fracture in patients. Until now, our therapeutic approach to the prevention of such fractures has focused solely on bone, but our increasing understanding of the interconnected biology of muscle and bone has begun to shift our treatment paradigm for musculoskeletal disease. Targeting pathways that centrally regulate both bone and muscle (eg, GH/IGF-1, sex steroids, etc.) and newly emerging pathways that might facilitate communication between these 2 tissues (eg, activin/myostatin) might allow a greater therapeutic benefit and/or previously unanticipated means by which to treat these frail patients and prevent fracture. In this review, we will discuss a number of therapies currently under development that aim to treat musculoskeletal disease in precisely such a holistic fashion.

Keywords Muscle · Bone · Anabolic agents · Osteoporosis · Sarcopenia

Introduction

Musculoskeletal diseases are highly prevalent, affecting up to 1 in every 2 individuals in western countries [1, 2]. Moreover, the annual cost of these diseases is staggering, estimated at nearly 8 % GDP in USA ($850 billion) and an even greater proportion of GDP in other countries (eg, 10 % GDP or $4.5 billion in Australia). As the world’s population ages, the sequelae of musculoskeletal wasting, falls, and fractures are a highly concerning health problem; not only for their financial impact, but as well for the significant increases in patient morbidity, the need for assisted care, and mortality [3]. Falls/fractures represent the common end-point in the age-related involution of bone (osteoporosis) and muscle (sarcopenia) [4••]. In this regard, the combined wasting of muscle and bone—both in normal aging and pathologic states—can lead to vastly compounded risk for fracture in patients. Reduced muscle mass can lead to poor balance and falls, and these falls are then more likely to result in fractures due to the osteoporotic bone’s inability to withstand load.

Until now, our therapeutic approach to the prevention of low-energy fracture has focused solely on bone. While osteoporosis has been clearly defined, sarcopenia and its end-points remain open to debate [5]. Sarcopenia has been provisionally defined on the basis of anthropomorphic parameters (appendicular lean mass relative to height or corrected for body weight/fat mass) [6], performance-based parameters (lower limb strength, timed up and go test, walking speed) or a combination of both (lower limb strength/leg lean mass on DXA) [7]. The unclear relationship between muscle mass and function and sex-specific differences highlight difficulties in reaching a consensus definition that corresponds to clear outcomes.

A paradigm shift may be underway with increasing recognition of the interaction of 2 adjacent tissues, bone and muscle. As we are becoming increasingly aware, these interactions are not merely at their anatomic interface, bone and muscle. Rather, bone-muscle interaction encapsulates an intimate relationship, in which bone and muscle communicate via complex paracrine
and endocrine signals to coordinate their growth and development from their earliest embryologic stages to involution, as well as to adapt in response to loading and injury [8••]. Research in bone-muscle interactions opens an immense field of potential therapeutic targets and the possibility of addressing osteoporosis and sarcopenia as a single disorder, rather than parallel pathologies, and may present the possibility of a way to ‘treat 2 birds with 1 stone.’ In this review, we will discuss factors involved in bone-muscle interactions and their therapeutic implications.

Muscle and Bone Development

The musculoskeletal system grows, functions, and ages as a finely coordinated unit. Muscle and bone are derived from a common mesenchymal progenitor during embryogenesis, and their development is closely coordinated by the action of myriad overlapping genes and growth factors [9, 10•]. In addition to these biochemical cues—and likely intertwined with them—mechanical force from developing muscle drives periosteal bone growth, bone density, and bone geometry; even during embryogenesis. Evidence of this integral association of bone and muscle during development can be observed in various mouse models, in which mice with paralyzed or nonfunctional muscle display severe impairments in bone growth and remodeling. In support of this notion, individuals exposed to a gravity-free environment, such as astronauts, experience dramatic bone loss due to lack of muscle loading [19]. However, this “mechanostat theory,” as it is commonly known, presents an incomplete picture of bone-muscle interactions. Importantly, appendicular muscle mass correlates with bone cortical thickness even at remote sites and not just adjacent, mechanically loaded bone [20••], suggesting additional paracrine or endocrine cross talk, by which bone and muscle coordinate their mass.

Further support for bone-muscle cross talk can be observed in fracture repair, where it has been repeatedly demonstrated that the presence of healthy muscle tissue is a positive factor for fracture healing. For example, the use of muscle flaps in the treatment of open fractures results in faster rates of bone healing in both mice and humans [21, 22]. In addition, the rate of nonunion is markedly higher in fractures associated with acute compartment syndrome, where muscle viability is compromised [23]. In this regard, skeletal muscle may represent a kind of “second periosteam”, providing trophic factors, morphogens, and even cells to aid bone repair. Several myokines with potential effects on bone have been proposed, including myostatin, interleukin 6 (IL6), fibroblast growth factor 2 (FGF2), and matrix metalloproteinase 2 (MMP2), amongst others [24–26]. Communication between bone and muscle is likely bi-directional, and bone may also ‘talk back’ to muscle via a range of osteokines, such as FGF21 produced by osteocytes and other factors [27••]. Additionally, common pathways such as GH/IGF-1, sex steroids and Wnt signaling can centrally coordinate the bone-muscle unit during development and adaptation to mechanical stimuli [20••, 28].

Thus, a complex interplay of mechanical, endocrine, and paracrine signals exists between muscle and bone that serves to coordinate their mass and function throughout life. In the following sections, we will discuss some of these common pathways that have been, or are currently being investigated, as possible targets to treat musculoskeletal diseases. Unraveling the individual effects of these pathways and stimuli poses significant experimental challenges. However, achieving a more thorough understanding of the biochemical links that intertwine bone and muscle physiology is critical for the discovery of therapeutic targets that may lead to a more holistic approach to musculoskeletal disease.

Growth Hormone (GH) and GH Secretagogues

GH plays a fundamental role in bone and muscle growth during childhood and puberty. It also exerts important effects throughout life in glucose and lipid metabolism [29], body composition and bone mineralization [30]. GH is secreted in a pulsatile manner by the pituitary gland and acts by specific growth hormone receptors (GHR) in peripheral tissues, or indirectly through induction of insulin-like growth factor-1 (IGF-1) [31••]. Circulating IGF-1 is produced mainly in the liver, but it is also produced locally in numerous peripheral tissues, including muscle during exercise [32] and regeneration [33]. GH/IGF-1 signaling is complex and tissue-specific, involving JAK/STAT, PI3K, and ERK pathways [34, 35]. Effects of GH in muscle cell proliferation, fiber size and fiber type depend on IGF-1, whilst effects on insulin sensitivity are
IGF-1-independent [31••]. In bone, GH/IGF-1 promotes osteoblast proliferation and differentiation, inhibits osteoclast activity, and modulates renal 1α-hydroxylase, (which activates 25-OH-Vitamin D) and phosphate reabsorption [36–39].

Patients with GH deficiency or congenital mutations of GH signaling display short stature, impaired muscle development, and failure of epiphyseal fusion, which respond to GH or IGF-1 replacement, respectively [40]. Even in healthy, GH-replete patients, serum GH and IGF-1 levels decline during aging and are correlated with losses in muscle, bone, and an increased risk of osteoporotic fracture [41]. Furthermore, muscle levels of growth hormone receptor (GHR) drop in proportion to reduced muscle fiber size in older adults [42], and bone responsiveness to IGF-1 also decreases with age [43]. Given these correlates, its central role in postnatal growth, and examples of effective treatment in pathologic states, GH would seem a logical therapeutic for musculoskeletal disease.

However, treatment of older adults with recombinant human growth hormone (rhGH) to reverse age-related changes in muscle, bone, and fat is controversial. In the landmark study by Rudman and colleagues, 12 older men treated with rhGH for 6 months showed increases in lean mass (8.8 %) and lumbar bone density (1.6 %), reduced fat mass (14.4 %), and no change in femoral neck bone density [44]. These results were consistent with effects of GH treatment in adults with hypopituitarism [45] and sparked intense interest in GH as an ‘anti-aging’ therapy. However, subsequent studies and a meta-analysis of 18 randomized controlled trials reported more modest changes in lean mass, inconsistent effects in bone density and physical function, and a number of side effects of rhGH treatment in older patients, including arthralgias, edema, carpal tunnel syndrome, and diabetes [46–48]. It should be noted that these studies were generally small, the treatment duration was short (~6 months) and follow-up times and rhGH dosing were variable.

Additional concerns surround the possibility that GH therapy might increase mortality. Reduced GH/IGF-1 signaling has been demonstrated to increase lifespan in worms, flies, and rodents [49]. A similar observation can be made in humans, where GH deficiency and resistance are associated with advanced longevity [40], and short individuals are more likely to live longer than tall individuals from the same population [50]. Conversely, acromegaly (GH-secretory pituitary adenoma) leads to increased mortality due to cardiovascular disease and cancer. The question of whether GH therapy increases mortality has yet to be adequately addressed.

Despite this uncertainty, equivocal effects in body composition, and reported side effects (eg, edema, diabetes), a multi-billion dollar industry based on the off-label use of rhGH as anti-aging therapy has emerged in the US. The case of an 86 year-old male with Crohn’s disease who developed metastatic colon cancer 7 years after commencing rhGH for anti-aging is concerning [51]. The tumor showed greater expression of IGF-1 receptor, suggesting a direct link with rhGH. Larger and longer-term studies are needed to determine the risk: benefit ratio of rhGH in elderly patients, its functional effects in osteoporosis and sarcopenia, and address long-term safety concerns. Proteins involved in tissue-specific GH/IGF-1 signaling in muscle and bone, such as Grb10 [52], SOCS proteins, and local isoforms of IGF and IGF binding proteins (IGFBP) [53] may provide future therapeutic targets that could circumvent undesirable side effects of GH therapy.

Another alternative to rhGH therapy is the use of GH secretagogues. In principle, these agents are “more physiological” than administration of rhGH, as they result in pulsatile—rather than prolonged—elevation of GH and preserve negative feedback by IGF-1. Small studies of GH secretagogues (including GHRH-1,44-amide and ghrelin mimetic MK-677) confirmed increases in GH and IGF-1 levels, showed improvements in lean mass, no change in bone density, and inconsistent effects in physical function [54, 55]. In the largest clinical trial of a GH secretagogue, 395 older individuals were randomized to capromorelin or placebo for a planned 2-year period [56]. The trial was ceased prematurely as significant increases in weight gain (1.4 kg at 6 months) offset improvements in lean body mass. This probably resulted from an appetite-stimulating effect of this drug, a ghrelin mimic. Interestingly, 2 of 6 functional parameters improved significantly by 12 months, namely tandem walking and stair climbing [56], but older patients in this trial were healthy with mild functional decline. It remains to be seen whether GH secretagogues demonstrate similar functional effects, or improvements in bone parameters, in a more frail population.

Androgens

Sex steroids are another critical player in regulating growth that might serve as a potential bone-muscle therapeutic, in particular, androgens. Apart from their established effects in the reproductive system, androgens exert anabolic effects in muscle and bone—the former being quite easy to appreciate in professional bodybuilders. The mechanisms by which androgens exert their anabolic actions in muscle and bone are complex and extend beyond simply androgen receptor (AR) activation in these tissues. In bone, testosterone must first be converted to estrogen (aromatization) to exert effects on osteoclast activity via estrogen receptors [57]. In muscle, testosterone stimulates protein synthesis, leads to muscle fiber hypertrophy, and increases myonuclei and satellite cell number, suggesting effects on pluripotent precursors [58].

Clinically, men with classic hypogonadism develop muscle wasting and osteoporosis that are reversible with testosterone therapy [59••]. HIV-positive men and glucocorticoid-treated men also display increases in lean mass and muscle strength following testosterone supplementation [60]. Elderly males
with reduced testosterone levels are more likely to have muscle/bone loss and a higher fracture risk [61], but testosterone replacement is controversial in this group. Studies demonstrate significant increases in lumbar BMD in older men receiving testosterone [62, 63]. This effect was more pronounced in those receiving intramuscular rather than transdermal formulations, and in general, there was no improvement in femoral neck BMD. Despite increases in lean mass, effects of testosterone on muscle strength are heterogeneous with a tendency to improved leg/knee extension and handgrip strength [63]. In 1 randomized trial of frail, older men, transdermal testosterone led to improved physical function and increased fat-free mass after 6 months [64]. However, no clinical trials have evaluated the effects of testosterone on hard outcomes such as falls or fractures.

There are also safety concerns about long-term use of testosterone in vulnerable, older patients. In particular, data on cardiovascular events and prostate cancer are limited; trials are also not sufficiently powered to assess such effects [65•].

The risk of obstructive sleep apnea and polycythemia in individuals using testosterone is also higher. In 2003, the Institute of Medicine (IOM) reported that the existing evidence-base was so equivocal that it could not even recommend large-scale clinical trials without better short-term evidence [66]. However, the US Endocrine Society advocates an individualized approach in the consideration of testosterone therapy in older men [59••]. Despite the uncertainty, prescription sales of testosterone in the US have grown by about 25% annually between 1993 and 2002, suggesting that increasing proportions of older males are using these medications [66].

Selective Androgen Receptor Modulators (SARMs)

The ‘holy grail’ of decades of preclinical research has been a highly tissue selective and safe agent that does not inhibit gonadotropins [67•]. Selective Androgen Receptor Modulators (SARMs) have been developed to produce anabolic effects in muscle and bone without the dose-limiting androgenic effects associated with testosterone (eg, prostate growth, acne, oily skin). These compounds achieve tissue selectivity by differences in gene regulation, tissue distribution, and local interactions with aromatase and 5-alpha-reductase [60]. In general, nonsteroidal SARMs (eg, aryl propionamides, quinolines) have greater AR specificity, oral bioavailability, and tissue selectivity than their steroidal counterparts (eg, 17-alpha-methyl-testosterone, 19-nortestosterone) and have, therefore, progressed further. Andarine (also known as 8 or S-4) has been described as the ideal SARM due to single daily dosing, complete oral bioavailability and a wealth of preclinical data reporting anabolic muscle and bone effects [68]. Early clinical data were also encouraging, and a related compound, Ostarine (GTX-024, enobosarm), showed increases in lean mass and physical function in elderly men, postmenopausal women, and cancer patients in randomized controlled trials [69, 70••]. There was no improvement in BMD, but this may have been due to the relatively short study period of 3 months [69]. A phase III trial is currently underway for Ostarine, focusing on cancer cachexia in particular. Another agent, LGD-4033, increased lean mass and strength in healthy males after 3 weeks [71], and according to the company, increased bone mass in preclinical studies (www.ligand.com/). A phase II trial for this agent is currently in development for disorders associated with muscle wasting (eg, cancer, fracture). Other nonsteroidal SARMs such as BMS-564929 and LGD-2941 are currently in phase I trials for age-related functional decline.

The first steroidal SARM to enter clinical trials, MK-0773, showed increases in lean mass but no change in physical function or bone mineral content over 6 months in women aged >65 years [72•]. It has now entered a phase II trial for sarcopenia. Clinical data on the efficacy and safety of SARMs continues to emerge, and they hold great promise as anabolic and function-promoting agents in a range of musculoskeletal conditions. However, functional outcomes and long-term side effects of these agents remain to be seen.

Vitamin D

In addition to sex steroids, a number of other hormone pathways impinge on bone and muscle development and may present viable therapeutic targets to treat musculoskeletal diseases. Vitamin D is one such hormone, and while its importance in bone physiology is quite well established, our understanding of its involvement in muscle physiology and function is only emerging. The biologically active form of vitamin D, 1,25(OH)2D, is a bona fide hormone that binds to a nuclear receptor (VDR), regulates gene expression, and exerts effects on mineral homeostasis, tissue development, and cell cycle [73•]. Effects of vitamin D in bone and muscle are mainly indirect, resulting from effects on calcium and phosphate homeostasis [74, 75]. In bone, direct effects of vitamin D are also possible, as both osteoblasts and osteocytes express VDR [76•]. Osteoblast VDR inhibits bone mineralization to preserve normal serum calcium levels [77] and consistent with this, osteoblast-specific VDR knockout mice display increased bone density [78]. Conversely, VDR overexpression in osteoblasts and osteocytes protects against the bone effects of vitamin D deficiency [79]. By contrast, whether the VDR is expressed in muscle remains controversial, but studies in cultured muscle cells and VDR knockout mice suggest that vitamin D signaling does play a role in muscle differentiation and fiber size regulation [80, 81].

In humans, severe vitamin D deficiency leads to osteomalacia and muscle weakness due to type II muscle fiber atrophy...
Vitamin D deficiency is common in the elderly, owing to both nutritional deficits and lack of sun exposure, and has been associated with falls, sarcopenia, and osteoporosis. One study even observed a reduction in the levels of VDR in muscle with age, suggesting an even greater vulnerability of older individuals to low vitamin D levels. Randomized trials have demonstrated that vitamin D supplementation reduces the risk of falls and fractures in older, institutionalized individuals. However, the effects of vitamin D supplementation are less clear amongst those living in the community. Although vitamin D supplementation may increase femoral neck and hip BMD in such individuals, this effect is small and not associated with reduction in fracture risk. Interestingly, vitamin D supplementation may increase muscle fiber size in frail, older patients, confirming effects demonstrated at a cellular level. Whether these effects on fiber size translate into any functional benefit (e.g., muscle strength or improved physical performance measures) is not clear without standardized end points for muscle function in these trials.

While generally well tolerated, a greater incidence of kidney stones and increased falls and fractures have been report ed in individuals receiving mega-doses of vitamin D. Such reports have raised questions and vigorous debate about what precisely constitutes vitamin D sufficiency, and safe doses to achieve positive benefit from vitamin D. Indeed, discord persists regarding recommendations for vitamin D. For example, the IOM recommends 25 OHD target levels of 50 nmol/L and daily vitamin D doses of 800 IU in older adults (>70 years). The US Endocrine society advocates higher serum target level of 75 nmol/L and daily doses of at least 1500–2000 IU in this age-group. Perhaps most attractive for treating musculoskeletal disease because of its availability and ease of use, the ongoing uncertainty regarding risks and benefits of vitamin D supplementation, together with continued controversy regarding optimal serum levels, point to a need for further study; especially in the context of its potential effects on skeletal muscle.

Exercise and Nutrition

Perhaps the simplest of all possible therapies to treat—or in this case, even more importantly, prevent—musculoskeletal disease is also one of the most difficult to implement. For many years, health professionals have been advising patients with osteoporosis to engage in weight-bearing exercise. The benefits of exercise in elderly patients are quite clear: improved muscle tone and balance to prevent falls and attenuation of bone loss, particularly at the femoral neck. Sufferers of chronic diseases, such as breast cancer, may also prevent muscle and bone loss by regular strength training and exercise. Unfortunately, the positive effects of exercise on bone and muscle can only be maintained through continued engagement in the activity; a fact with which many of us who sit at desks and write papers about musculoskeletal therapies are all too familiar. For example, a study of premenopausal women demonstrated that 6 months after ceasing regular exercise, positive effects in muscle strength and BMD were lost. An additional confounder in recommending exercise, there is no clear consensus on the type, intensity, or duration of exercise that is most effective. However, regular walking has shown positive effects on muscle and bone in elderly individuals. Even low magnitude mechanical signals have been demonstrated to have positive effects on bone and muscle, providing an encouraging prospect for those who have restricted mobility due to prior injury or concomitant disease. Electrical muscle stimulation may also prevent muscle and bone loss, as demonstrated in patients with spinal cord injury.

Nutrition provides substrates necessary for bone matrix and mineral (protein, calcium, magnesium, phosphate) and muscle accretion (protein). Nutrition is of particular concern in the elderly, where malnutrition affects up to 40% of those living in institutions. Moreover, 20% of older individuals in the USA consume inadequate protein, as defined by <0.66 g/kg/actual body weight per day. Although an association between dietary protein intake and lean mass exists, the use of protein supplementation to reduce sarcopenia is controversial. Small trials suggest that 25–30 g of high-quality protein is necessary to maximize skeletal muscle protein synthesis. However, a meta-analysis of 62 trials found no improvement in physical function in elderly patients on high-energy protein supplements.

Another concern has been the co-occurrence of muscle wasting and visceral adiposity, known as “sarcopenic obesity”. This is associated with functional disability and osteoporosis, possibly related to adipocyte infiltration in bone and muscle and subsequent pro-inflammatory state. The addition of exercise training to energy restriction preserves muscle mass during periods of weight loss in older adults. Activin signaling inhibitors, discussed below, show promising results in the reduction of fat mass whilst increasing lean mass.

The use of calcium supplements and their benefit in bone health is similarly controversial. Calcium supplements may lead to small benefits in bone mineral density, but they do not clearly reduce fracture risk and their effects do not persist beyond their duration of use. A potentially increased risk of myocardial infarcts with calcium supplements has also called the benefits of calcium supplementation into question. Taken together, exercise, and dietary interventions would seem to produce equivocal results, at best, in elderly patients with existing osteoporosis and sarcopenia. However, their value as both preventative and concurrent approaches to help maintain bone and muscle mass should not be
overlooked, especially given the additional health benefits of exercise and proper nutrition in other organ systems (eg, cardiovascular).

**Activin Signaling Inhibitors**

In addition to the more ‘classical’ pathways involved in muscle and bone development discussed already, recent studies have suggested that the activin signaling pathway—well known for the suppressive effects of myostatin on muscle mass—may represent another shared pathway between muscle and bone. Myostatin is a member of the TGF-β superfamily and a muscle-derived hormone that was first discovered in 1997 [108]. Myostatin deficiency results in increased muscle mass in several species, including humans [108, 109]. Conversely, increases in myostatin may partially explain the muscle wasting observed in patients with chronic diseases such as renal failure [110], HIV [111], and chronic obstructive pulmonary disease [112]. Myostatin exerts these effects on muscle by binding to a transmembrane receptor, activin receptor IIB (ActRIIB), ultimately activating Smad family proteins and downstream signals that lead to muscle protein breakdown via the ubiquitin-proteasome system. There is also a closely related ActRIIA that binds additional activin ligands (but can weakly bind myostatin) and shares some functional overlap with ActRIIB in muscle [113, 114]. More recently, the activin signaling pathway has also been shown to affect bone development and remodeling. Polymorphisms in the myostatin gene are associated with peak bone mineral density [115]. Myostatin knockout mice display increased BMD and bone mineral content (BMC) [24, 116] and greater callus size following osteotomy [117]. These anabolic effects on bone were predominantly believed to be related to increased mechanical loading, secondary to increased skeletal muscle mass. However, direct effects of activin/myostatin on bone are also possible, as bone marrow stromal cells and osteoblasts express activin receptors, and modulating the pathway in vitro appears to affect bone cell differentiation [118–119].

The activin signaling pathway is an attractive therapeutic target for musculoskeletal disease, given the evidence to suggest that it might function to negatively regulate both bone and muscle mass. Indeed, several inhibitors of this pathway have already been developed, including myostatin-neutralizing antibodies/propeptide, recombinant follistatin (an endogenous inhibitor that binds and sequesters ligands), follistatin derivatives, and soluble activin receptors [114]. Mice treated with such agents demonstrated substantial increases in muscle mass and strength [26, 118]. Positive effects on muscle mass were also reported in mouse models of androgen deficiency [120], muscular dystrophy [121], and cancer cachexia [122]. In addition to the expected effects on skeletal muscle, ActRIIB-Fc also increased bone formation rates and bone mineral density in mice and demonstrated direct effects on osteoblast activity [118, 123]. Similar bone anabolic responses were also seen in primates administered soluble ActRIIA [124]. Interestingly, a myostatin propeptide had no effect on bone parameters in mice, despite increasing muscle mass [125]. This difference in tissue response highlights the possibility that specific components in the pathway could be exploited therapeutically to achieve different benefits (eg, bone and muscle anabolic, only muscle anabolic, etc.), depending on the disease context.

In addition to these preclinical studies, inhibitors of the activin signaling pathway have also been tested in human phase 1 and 2 trials. A recombinant human myostatin antibody (MYO-029, Staulumab) was found to be generally safe in healthy individuals (NCT00563810) and adults with muscular dystrophy [126]. Although MYO-029 resulted in improved contraction in single muscle fibers [127], increases in muscle mass on DXA were not statistically significant. Moreover, no improvement in muscle strength was observed in 116 patients with muscular dystrophy [126], although this study was not adequately powered to detect changes in muscle function. In a double-blind, placebo-controlled study of 48 postmenopausal women, a single dose of ACE-031 (soluble ActRIIB decoy receptor) resulted in significant increases in lean mass (3.3 %) and thigh muscle volume (5.1 %) on DEXA and MRI after 1 month [128]. Although grip strength was measured at baseline, changes following treatment were not reported. ACE-031 also resulted in a significant increase in bone-specific ALP and decrease in C-telopeptide, indicating increased bone remodeling. The company reported a significant 3.4 % increase in bone mineral density (BMD) at 113 days in a phase 1b trial of 60 postmenopausal women on ACE-031 (www.acceleron.pharma.com/), although this trial has not been published or subject to peer review.

Targeting this pathway is not without its issues however. First noted in trials of children with DMD (NCT01099761, clinicaltrials.gov) and postmenopausal women receiving higher doses of ACE-031 [128], side effects include nosebleeds and skin telangiectasia. Although not serious in itself, this phenomenon does raise concerns about unrecognized, systemic effects of ActRIIB inhibition. Other off-target effects include significant reduction in serum FSH levels (43 %) after a single dose of ACE-031 (3 mg/kg), most likely related to suppression of activin/GnRH signaling [128], and alteration of fat mass and metabolism [105]. In this regard, antibodies directed against activin receptors could potentially offer a means to avoid off target effects seen with soluble receptor administration by allowing more specific targeting of IIA vs IIB receptor and varied blockade kinetics. A recent study showed that a novel anti-ActRIIA antibody (BYM338) was twice as effective as a myostatin-specific inhibitor (D76A) in increasing muscle mass in mice [129]. The effect of BYM338 was partly myostatin-independent as confirmed by its effects...
myokines include myostatin, LIF, IL-6, IL-7, BDNF, IGF-1, in influence other organ systems, including bone. These pathways have been elucidated that connect bone metabolism well beyond just one another. In recent years, endocrine organs, as the interconnectedness of bone and muscle extends and muscle secondarily, through actions on other tissues and skeletal disease.

**Myokines and Future Directions**

In addition to myostatin, a number of recent studies have demonstrated other muscle-secreted factors—termed myokines—that can serve as paracrine/endocrine factors to influence other organ systems, including bone. These myokines include myostatin, LIF, IL-6, IL-7, BDNF, IGF-1, FGF-2, FSTL-1, and irisin [25]. Given that we have already discussed therapeutics targeted at 2 of these myokines (myostatin and IGF-1, albeit secondary to GH), it is likely that further study of muscle-bone interactions will reveal other myokines as candidate therapeutic targets to treat musculoskeletal disease.

Importantly, many of these myokines could impact bone and muscle secondarily, through actions on other tissues and organs, as the interconnectedness of bone and muscle extends well beyond just one another. In recent years, endocrine pathways have been elucidated that connect bone metabolism to the pancreas, fat, and brain; all organs also interconnected with muscle. It is not unreasonable to suspect that impinging upon a “middle man” could exert profound effects on muscle and/or bone. One such example can be envisaged for the myokine IL-6, which has been demonstrated to increase the secretion of insulin from the pancreas [130]. Insulin could then feed into the bone–pancreas endocrine loop to exert secondary effects upon bone [27••]. Such systems biology-based approaches to understanding the interaction of muscle and bone—with each other and other organs—could even result in treatments for musculoskeletal disease that target entirely different organ systems to exert their effect on muscle and bone (eg, CNS or fat). This represents a truly exciting future direction for study.

**Conclusions**

Osteoporosis and sarcopenia are closely related conditions characterized by age-related involution of the bone-muscle unit. Functionally, this progressive muscle and bone loss leads to falls, fractures, deconditioning, and further muscle wasting, all of which can be exacerbated by additional disease pathology. While previous efforts to reduce fracture were heavily geared toward treating bone as a separate organ, our increasing understanding of bone-muscle interactions has highlighted that targeting the bone-muscle unit as a whole may break this ‘vicious cycle’ of musculoskeletal atrophy even more effectively. There has been significant progress in the development of novel anabolic agents for bone and muscle, most notably SARMs and activin pathway inhibitors, and exciting new opportunities for targeting myokines may be on the horizon.

Developing therapeutic treatments to holistically treat musculoskeletal disease is not without significant challenge however, and one of the largest hurdles is related neither to the targets nor their biology. Rather, it lies within the definition of the condition itself; sarcopenia and its endpoints are poorly defined. Moreover, functional outcomes and markers are needed to clarify a positive outcome in the ‘musculoskeletal unit’ and guide efficacy trials. These topics are under vigorous debate and are of critical importance for advancing musculoskeletal therapeutics. As with any therapeutic development, safety has also been a concern. Telangiectasia, bleeding, and gonadotropin suppression in patients on activin pathway inhibitors highlight our incomplete understanding of systemic activin/myostatin signaling [128•]. Similar issues have been overcome for other pathways, however. To avoid undesirable systemic effects of androgens, SARMs selectively target muscle and bone. Concerns of nonphysiological GH levels, and possible related side effects, with GH administration have been addressed with GH secretagogues, which preserve IGF-1-mediated negative feedback of GH [60]. Finally, systems biology-based research may prompt us to consider other tissues that participate in bone-muscle interactions, such as fat and nerves, as future potential therapeutic targets [8••]. Collaborative efforts by basic scientists, clinicians, and industry are needed to address these complex issues and energize the clinical development of novel bone-muscle therapies.

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**Compliance with Ethics Guidelines**

**Conflict of Interest** CM Girgis and N Mokbel declares that they have no conflicts of interests. DJ DiGirolamo has received a speaker’s honorarium from Eli Lilly and Company.

**Human and Animal Rights and Informed Consent** All studies by the authors involving animal subjects were performed after approval by the appropriate institutional review boards.
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- Of importance
- Of major importance


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118.• Digirolamo D SV, Clemens T, Lee S-J. Systemic administration of soluble 483 activin receptors produces differential anabolic effects in muscle and bone in mice. J Bone Miner Res. 2011;1167 (Suppl). This abstract presented at ASBMR meeting 2011 reported direct effects of activin receptor signaling in osteoblasts. Mice treated with ActRII-Fc fusion proteins showed both increases in muscle (15%–40%) and trabecular (~13%) bone over 4 weeks.


Vitamin D and muscle function in the elderly: the elixir of youth?

Christian M. Girgis

INTRODUCTION

Vitamin D deficiency is highly prevalent in elderly subjects, affecting up to 50% of those living in institutions [1] and in the community [2] [that is deficiency defined as 25 hydroxy vitamin D (25OHD) levels <50 nmol/l, 20 ng/ml]. Elderly individuals are at risk of vitamin D deficiency owing to intrinsic age-related changes in vitamin D synthesis [3], reduced exposure to sunlight and malnutrition, which is rife amongst those living in institutions [2,4]. The active form of vitamin D [1,25(OH)2D] binds to a nuclear receptor, vitamin D receptor (VDR), to mediate effects in calcium homeostasis in addition to wide-ranging extracalcaemic effects (e.g. in tissue development, cell cycle regulation). The expression of VDR declines with age in skeletal muscle and vitamin D deficiency is associated with age-related involution of muscle in elderly subjects [3]. However, the precise role of vitamin D in the ageing process is unclear. In this review, clinical studies seeking to clarify the relation between vitamin D and muscle function in the elderly and potential mechanisms will be discussed. Muscle function is a broad entity and individual components including muscle mass, strength, functional parameters and falls will be reviewed. Controversies in this field will be discussed together with conflicting guidelines for vitamin D supplementation in the elderly.

OBSERVATIONAL STUDIES

Observational studies report an association between vitamin D, muscle function and physical performance in the elderly. In two recent studies from the...
Vitamin D deficiency is associated with muscle weakness, age-related muscle loss (that is sarcopenia) and functional decline in the elderly. Vitamin D supplementation may reduce falls and improve muscle function in frail elderly people, particularly those living in institutions.

Randomized controlled trials and meta-analyses do not demonstrate a convincing effect of vitamin D supplementation in muscle function in the elderly. This may be because of heterogeneity in study design, variability in vitamin D supplementation regimens and different vitamin D assays amongst the studies. Future studies should use standardized measures of proximal muscle function, rather than grip strength, to assess effects of vitamin D.

Vitamin D is a marker of general health. Establishing direct effects of vitamin D in muscle function in the elderly may be confounded by its potential effects in general health and frailty.

Until muscle outcomes of vitamin D and its therapeutic window are clarified, a conservative approach aiming for vitamin D levels that maintain normal calcium homeostasis is the safest option (that is serum vitamin D >50 nmol/l, 20 ng/ml).

Netherlands, serum 25OHD levels less than 75 nmol/l (20 ng/ml) correlated with impairments in gait and the ability to conduct activities of daily living in older individuals [5*]. In individuals who had recently experienced a fall, vitamin D levels correlated with classical parameters of physical function (e.g. timed-up-and-go test) and vitamin D deficiency was very common (i.e. 44% of study participants) [6].

Associations between specific tests of muscle function – such as grip strength testing – and vitamin D levels in the elderly are not consistently found [6,7*]. This may relate to the predominant involvement of proximal muscles in vitamin D deficiency and the possibility that vitamin D has a more generalized effect in coordinating muscle function, balance and well-being.

Low vitamin D levels are associated with reduced muscle mass, a condition referred to as sarcopenia [8,9]. This association is specific to appendicular lean mass rather than whole lean mass, supporting vitamin D’s predominant effect in proximal limb muscles [8]. However, no association was found between vitamin D intake and muscle mass in two studies [8,10], raising doubts as to whether vitamin D supplementation may increase muscle mass.

Vitamin D levels associated with optimal muscle function in the elderly are not clearly defined. One study suggested that 25OHD level of 75 nmol/l (30 ng/ml) in elderly men was associated with better physical performance than lower levels [5*]. A larger study of ~1500 community-dwelling older men found that a vitamin D range of 50–75 nmol/l (20–30 ng/ml) correlated with significantly better physical performance and reduced falls than lower levels [7*]. No additional benefit was seen in those with levels higher than 75 nmol/l (30 ng/ml). In fact, further analysis of this study found a higher incidence of fractures in those with levels higher than 72 nmol/l (29 ng/ml) compared with those in the intermediate range of 60–72 nmol/l (24–29 ng/ml), suggesting a U-shaped association between vitamin D and fracture incidence [11]. This increased fracture risk was not related to a greater incidence of falls or changes in bone mineral density, raising questions about potential mechanisms for this finding.

Although these studies support an association between vitamin D and muscle strength, they are not equipped to establish a cause-and-effect relationship. Their cross-sectional nature and the possible presence of confounding factors, not included in adjustment models, are limitations. Vitamin D deficiency is also associated with frailty, a syndrome characterized by the combination of reduced muscle strength, impaired gait and exhaustion in older individuals [7*]. It may therefore be difficult to disentangle direct effects of vitamin D in muscle function, independent of its association with frailty. Interventional studies may clarify these issues and more clearly examine the relation between vitamin D and muscle function.

INTERVENTIONAL STUDIES

Several studies have examined effects of sunlight exposure and vitamin D supplementation in the prevention of falls and muscle strength in older individuals [12,13]. These studies generally support effects of vitamin D supplementation in vulnerable elderly populations. For example, in institutionalized elderly subjects, daily sunlight exposure reduced falls [14] and the daily consumption of fortified bread (containing vitamin D 5000 IU, calcium 320 mg) improved locomotion and reduced general pain compared with baseline [15]. However, these studies were limited by poor compliance [14], lack of a control group [15] and increases in serum vitamin D levels were not demonstrated [15].

Apart from general improvements in physical performance, muscle strength has also been shown to respond to vitamin D supplementation. In older, institutionalized subjects, 6 months of vitamin D supplementation led to increases in hip flexor and knee extensor strength (~20–25%) [16]. Similar
increases in proximal muscle strength were demonstrated in community-dwelling subjects, predominantly in those who were weakest at baseline [17]. Impairments in balance may also respond to vitamin D supplementation, thereby reducing the incidence of falls [18]. This effect in falls is seen predominantly in the institutionalized elderly [12,13], whereas healthy, postmenopausal women showed no change in grip strength or falls risk in response to vitamin D supplementation (400 and 1000 IU/day) [19]. Therefore, an individualized approach to vitamin D supplementation, depending on a subject’s baseline muscle function, may be necessary.

From a mechanistic viewpoint, vitamin D supplementation (4000 IU/day) was found to increase muscle fibre size and expression of vitamin D receptor within muscle nuclei of frail older women with baseline vitamin D deficiency [20**]. This did not translate to tangible improvements in muscle function, possibly because of the short study duration of 4 months. In another study, vitamin D supplementation improved muscle mitochondrial function in deficient subjects, explaining improvements in muscle fatigue reported in this study [21].

Megadoses of vitamin D are not recommended. In a study of 243 frail, older patients, megadose vitamin D (300 000 IU) did not improve performance even amongst those with baseline levels less than 30 nmol/l (12 ng/ml) [22]. An annual oral dose of 500 000 IU increased falls in 2256 community dwelling older women, particularly in the 3 months following the dose (incidence rate ratio 1.31 compared with placebo) [23]. One potential explanation is that sudden improvement in muscle function and increased physical activity following supplementation led to an increased risk of falling. Alternatively, supraphysiological levels of vitamin D may be deleterious to muscle function by inducing muscle protein breakdown via calpain proteolytic pathways [24].

Vitamin D’s positive effects in skeletal muscle may be limited by its dose-dependent effect in increasing serum calcium levels. To support this, a vitamin D analogue with lower calcaemic potency, eldecalcitol, increased bone mineral density, reduced falls/fractures and improved lower limb muscle power in older individuals [25*,26]. The use of vitamin D analogues may present a viable option for optimizing muscle function in the elderly, but this needs further evaluation.

In summary, recent studies suggest that moderate-dose daily vitamin D supplementation reduces falls and improves muscle function in vulnerable elderly subjects such as the institutionalized and those with baseline weakness.

### META-ANALYSES

In a widely cited meta-analysis, vitamin D supplementation was found to reduce falls amongst 2426 elderly individuals from eight trials [27]. Higher doses (700–1000 IU) reduced falls risk by 19%, but lower doses had no effect. Likewise, people who achieved serum 25OHD concentrations 60 nmol/l (24 ng/ml) or higher reduced falls by 23%, but those who achieved concentrations 60 nmol/l (24 ng/ml) or lower had no effect. This meta-analysis was criticized by the recent Institute of Medicine report for inconsistencies in study inclusion, perceived errors in statistical analysis and reliance on two studies to produce the positive effect, one of which was reportedly not well powered [28]. On reanalysis with two statistical alterations, the positive effect was not found.

Another meta-analysis including 45 782 people, mainly elderly females, found a significant reduction in falls amongst those randomized to vitamin D supplementation (odds ratio 0.86 for ≥1 fall) [29]. The effect was most prominent in those who were deficient at baseline and with calcium co-administration. However, the effect was not related to the dose or form of vitamin D supplementation (that is D2 versus D3) or the target population (community dwelling versus institutionalized subjects).

Meta-analyses dealing with vitamin D and muscle strength are limited by substantial heterogeneity in study design, including the parameters of muscle function. A recent meta-analysis that assessed 17 randomized controlled trials (RCTs) involving 5072 participants reported no effect on grip strength or proximal lower limb strength in adults with 25OHD levels higher than 25 nmol/l (10 ng/ml) [30]. However, on pooling data from two studies on vitamin D-deficient adults (that is 25OHD <25 nmol/l, 10 ng/ml), an effect on hip muscle strength was found, although the studies used different measures.

More recently, an umbrella review collectively assessed more than 250 reviews and meta-analyses on vitamin D, exploring its effects in 137 outcomes [31**]. From these studies, evidence that vitamin D supplementation reduced nonvertebral fractures and improved muscle strength was suggestive but not convincing (P values 0.04 and 0.02, respectively). In general, no effect was seen in falls or fractures, raising doubts about vitamin D’s widely accepted effects.

An important consideration is that vitamin D is a marker of general health [32]. Therefore, effects of vitamin D in muscle function may be indirectly related to improvements in general well-being [15,32]. Moreover, these studies lack uniformity in parameters of muscle function, reporting of falls,
Vitamin D and muscle in the elderly Girgis

**Vitamin D and muscle in the elderly**

**GUIDELINES**

In the absence of clearly established muscle outcomes, guidelines for vitamin D supplementation in the elderly are conflicting. In a recent report, the US Institute of Medicine recommended 25OH D target levels of 50 nmol/l and daily vitamin D doses of 800 IU/day in older adults (>70 years) [28]. However, the US Endocrine Society advocated a higher serum target level of 75 nmol/l and daily doses of at least 1500–2000 IU in this age group [34]. Similar targets were recommended by the American Geriatrics Society to prevent falls and fractures in frail, elderly subjects [35]. The society also recommended a total daily intake of 4000 IU of vitamin D from all sources [35]. However, evidence to support this higher target is lacking and studies hint that adverse effects may occur with excessive doses of vitamin D [11,23]. Until these issues are clarified, guidelines for vitamin D supplementation regimens and study populations. Commercially available vitamin D assays also differ substantially in their estimation of serum levels [33]. This heterogeneity complicates the collective assessment of these studies, leading to the lack of a clear and convincing effect of vitamin D in muscle function in the elderly.

**CONCLUSION**

Although observational studies link falls, muscle weakness and sarcopenia with vitamin D deficiency [5*], interventional trials and meta-analyses have yielded conflicting results [29,30,31*]. A number of confounding factors such as heterogeneity in study design, variability in treatment dose and analyses of muscle function may explain the lack of a convincing effect. Some studies suggest that vitamin D supplementation may reduce the risk of falls and improve muscle function in more vulnerable elderly people, such as the institutionalized or those who are prone to falls [12,13,16]. Other studies suggest that excessive doses of vitamin D may have adverse effects and precipitate falls and fractures in frail elderly people [11,23]. On the basis of these conflicting data, vitamin D cannot be considered the ‘elixir of youth’ with regards to skeletal muscle and stringent guidelines for vitamin D supplementation are premature. At this time, conservative vitamin D targets aiming to preserve calcium homeostasis are the safest option (>50 nmol/l, 20 ng/ml).

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**Conflicts of interest**

There are no conflicts of interest.

**REFERENCES AND RECOMMENDED READING**

Papers of particular interest, published within the annual period of review, have been highlighted as:

* of special interest
** of outstanding interest


This large observational study of community-dwelling older men in Australia reported that vitamin D levels between 50–70 nmol/l were associated with optimal physical performance measures and reduced falls. No additional benefit was seen in those with levels >75 nmol/l.


This provides mechanistic insight into positive effects of vitamin D in muscle. Expression within muscle following vitamin D supplementation in frail, older women. This pilot study reported an increase in muscle fibre size and vitamin D receptor concentration in older women. The Journal of clinical endocrinology and metabolism 2013; 98:E509–513.


This randomized trial showed a beneficial effect of the vitamin D analogue eldecalcitol in muscle function, specifically chair-rising time, in postmenopausal women with osteoporosis. This provides hope that noncaemic vitamin D analogues may improve muscle function in the elderly.


This recent large review assessed a range of studies on vitamin D and critically assessed evidence in favour of its skeletal and extraskeletal effects.


This article discusses evidence for vitamin D target levels from recent clinical studies.
Chapter 9 – Vitamin D, bone-muscle interactions and metabolic effects

This chapter consists of 3 published review articles. The PhD candidate, Christian Girgis, was primary author on one of these manuscripts and second-author on the other two.

The manuscript published in *Molecular and Cellular Endocrinology* discusses biomolecular effects of vitamin D in muscle-bone interactions and evidence for specific roles throughout the life of the musculoskeletal systems: development, injury and ageing. The therapeutic potential of the vitamin D endocrine system in subjects with sarcopaenia and osteoporosis is discussed.

The manuscript published in *Bone* discusses mechanisms underlying bone and muscle interactions, humoral and endocrine factors responsible for their cross-talk and purported effects of vitamin D in integrating the musculoskeletal system.

The article published in *Nutrition and Dietary Supplements* discusses clinical evidence on effects of vitamin D and the anti-oxidant, vitamin C, in the prevention and treatment of type 2 diabetes mellitus. This is then followed by a discussion of potential mechanisms by which vitamins C and D may alter skeletal muscle insulin sensitivity.

Manuscript layout and referencing style differ between these articles, depending on specific journal requirements.
Vitamin D, muscle and bone: Integrating effects in development, aging and injury

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ABSTRACT

Beyond the established effects of muscle loading on bone, a complex network of hormones and growth factors integrates these adjacent tissues. One such hormone, vitamin D, exerts broad-ranging effects in muscle and bone calcium handling, differentiation and development. Vitamin D also modulates muscle and bone-derived hormones, potentially facilitating cross-talk between these tissues. In the clinical setting, vitamin D deficiency or mutations of the vitamin D receptor result in generalized atrophy of muscle and bone, suggesting coordinated effects of vitamin D at these sites. In this review, we discuss emerging evidence that vitamin D exerts specific effects throughout the life of the musculoskeletal system – in development, aging and injury. From this holistic viewpoint, we offer new insights into an old debate: whether vitamin D's effects in the musculoskeletal system are direct via local VDR signals or indirect via its systemic effects in calcium and phosphate homeostasis.

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Introduction

The link between vitamin D and musculoskeletal function is long-standing. In the first description of rickets four centuries ago, Whistler reported the combination of “flexible, waxy” bones and “flabby, toneless” muscles in young children (Whistler, 1645). Adults with vitamin D deficiency also display concurrent defects in bone and muscle, characterized by osteomalacia (i.e. reduced bone mineral) and type 2 muscle fiber atrophy (Girgis et al., 2013). These conditions are also seen in subjects with mutations of the vitamin D receptor (VDR), suggesting local genomic effects of vitamin D in the muscle–bone unit.

Studies have elucidated a range of effects of vitamin D in bone and muscle cells and transgenic mouse models have shed light on potential interconnected vitamin D signals between these tissues (Garcia et al., 2011; Girgis et al., 2014b; Lam et al., 2014; van Driel et al., 2006). Concurrently, we are becoming increasingly aware that muscle and bone interact to form a finely coordinated unit (DiGirolamo et al., 2013). This notion is supported not only by effects of muscle loading on bone function but also the emergence of multiple paracrine/endocrine factors that facilitate cross-talk between these tissues. Bone and muscle arise together from embryonic mesoderm, grow together throughout puberty and atrophy during aging.
The search for biomechanical and endocrine cues responsible for this life-long synchrony of muscle and bone is underway. Vitamin D is one endocrine system that may integrate bone and muscle function. Clinically, vitamin D levels predict the rate of functional decline, fracture risk and bone mass in young and old individuals (Sohl et al., 2013; Ward et al., 2010). At a molecular level, muscle and bone both express the 1-alpha-hydroxylase enzyme, encoded by Cyp27b1, facilitating the local synthesis of the bioactive hormone, 1,25(OH)2D (Anderson et al., 2005; Girgis et al., 2014b; Srikuea et al., 2012). Regulated by local and systemic vitamin D signals, bone is the major depot for calcium, an essential mineral in muscle contraction, morphology and plasticity. Conversely, skeletal muscle is emerging as a major storage site for vitamin D from where it may diffuse back into the circulation or possibly, into adjacent bone, following specific signals (Abboud et al., 2013; Girgis et al., 2014d). Thus, vitamin D may have complementary effects in bone and muscle, further supporting the integration of these two tissues.

In this review, we will discuss key events in the life of the musculoskeletal system – development, aging and injury – and evidence for integrated effects of vitamin D at these times. We will cover areas of controversy, particularly whether vitamin D’s effects in the musculoskeletal system are mainly indirect via systemic calcium and phosphate levels or direct via local VDR, the presence of which is debated in muscle. Finally, potential therapeutic implications of this field and outstanding questions will be raised.

**Vitamin D and musculoskeletal development**

The VDR makes its first appearance in the fetal rat at day 13 gestation (Johnson et al., 1996). At this stage, it resides within the condensing mesenchyme of the vertebral column and by day 17, within osteoblasts and proliferating, hypertrophic chondrocytes. Mesenchymal stem cells (MSCs) express VDR (Artaza and Norris, 2009). In various studies, 1,25(OH)2D results in nuclear translocation of VDR in MSCs and induction of myogenic and osteogenic pathways by effects in TGF-beta and bone matrix proteins ALP, bone sialoprotein (BSP) and osteopontin (Artaza and Norris, 2009; Honda et al., 2013; Mostafa et al., 2012). MSCs also express 1-alpha-hydroxylase (Cyp27b1). Inhibition of this enzyme reverses the effects of 250HD in human MSCs including its anti-proliferative, pro-differentiation and anti-apoptotic activity (Geng et al., 2011b). Therefore, effects of vitamin D in MSCs and the presence of vitamin D signaling components in these primordial cells provide prima facie evidence for vitamin D’s role in musculoskeletal development.

Vitamin D exerts a range of in vitro effects in committed myoblasts and osteoblasts. These effects vary widely depending on the particular cell model used (van Driel et al., 2006; Yamaguchi and Weitzmann, 2012), the stage of cellular differentiation (Tanaka et al., 2014), origin of primary cells (Yang et al., 2013) and species (Thomas et al., 2000; van Driel and van Leeuwen, 2014). In general, 1,25(OH)2D stimulates bone formation and mineralization in human osteoblasts by effects in Wnt and B–catenin pathways (Fretz et al., 2007) and the subsequent expression of RUNX2, osteocalcin, osteopontin and matrix Gla protein (Prince et al., 2001; van Driel et al., 2006; Zhang et al., 1997). By contrast, 1,25(OH)2D inhibits bone formation and mineralization in cultures of murine osteoblasts due to suppression of the same osteogenic markers (Shi et al., 2007; Yamaguchi and Weitzmann, 2012) and leads to increases in the mineralization inhibitor pyrophosphate (Drissi et al., 2002; Lieben et al., 2012). This discrepancy between responses of murine and human osteoblasts to vitamin D may result from different extracellular conditions including phosphate or cytokine levels or species-specific VDR cistromic interactions (Pike et al., 2014).

C2C12 muscle cells also respond to vitamin D by increased expression and nuclear translocation of VDR (Garcia et al., 2011; Girgis et al., 2014b). In addition to anti-proliferative effects due to altered cell cycle activity, vitamin D intriguingly leads to a doubling in the size of C2C12 myotubes by profound inhibition of myostatin (Garcia et al., 2011; Girgis et al., 2014b). Conversely, knockdown of VDR and CYP27B1 results in increased proliferation, impaired differentiation and myotube maturation in C212 and G8 cells (Srikuea et al., 2012; Tanaka et al., 2014). Together, these studies support a direct role for vitamin D signaling in bone and muscle cells but present a mixed picture of its effects. Under certain conditions, vitamin D has an anabolic effect in bone and muscle cells, promoting mineralization and myotube growth. However, the physiologic relevance of these findings is unclear and a limitation inherent to these studies is the isolated assessment of bone and muscle cells without accounting for interactions. Cross-culture studies would be helpful in assessing integrated effects of vitamin D in bone and muscle cell differentiation.

Despite the early presence of VDR in the developing embryo, the clinical features of rickets develop after weaning (Li et al., 1997; Yoshizawa et al., 1997). These features including growth plate defects, increased osteoid (i.e. unmineralized bone), and generalized atrophy of type I and II muscle fibers are seen in VDRKO mice at day 35 (Endo et al., 2003; Li et al., 1997). However, subtle changes are seen prior to weaning including expansion of the growth plate (Li et al., 1997), smaller muscle fibers and altered expression of myogenic regulatory factors at day 15 (Endo et al., 2003). Interestingly, another VDRKO model – generated by ablation of exon 2 – showed no musculoskeletal defects prior to weaning (Yoshizawa et al., 1997), possibly due to the presence of a truncated form of VDR with potential biologic activity in these mice (Bula et al., 2005). This suggests non-classical signaling may be responsible for pre-weaning musculoskeletal effects of VDR.

In humans, subtle differences in fetal bone development are observed in association with maternal vitamin D deficiency (Joannou et al., 2012; Mahon et al., 2010). Maternal vitamin D status is indirectly associated with fetal bone mineral content via methylation at 4 sites of the RRXRA in umbilical cord tissue (Harvey et al., 2014; Jaenisch and Bird, 2003). This indicates a novel epigenetic role for vitamin D in embryonic skeletal development. VDR may also prepare bone during embryogenesis for post-natal mineralization by enhancing production of mature matrix vesicles rather than directly increasing mineral deposition (Woecckel et al., 2010). Vitamin D may also regulate muscle fiber size during development. In humans, maternal vitamin D deficiency is associated with reduced arm–muscle area in newborn offspring (Krishnaveni et al., 2011). In rats born to vitamin D deficient dams, smaller muscle fibers were seen in association with altered expression of genes involved in protein catabolism, differentiation and the cytoskeleton (Max et al., 2013). These effects are not restricted to mammals as European sea bass treated with dietary 250HD after hatching also demonstrate dose-dependent increases in muscle fiber size and myogenic gene expression (Alami-Durante et al., 2011). Growth-promoting effects of vitamin D in muscle may potentially enhance loading on bone and stimulate bone mass. In addition, skeletal muscle may represent a reservoir for vitamin D from where it may diffuse to adjacent bone during embryogenesis. In support of this, a study from 1986 reported the in utero transfer of radiolabeled 250HD across the placenta and into the skeletal muscle of rat embryos (Clements and Fraser, 1988). More recently, we reported a direct effect of VDR in the uptake of 250HD into muscle fibers (Girgis et al., 2014d). Human studies also support the notion that vitamin D may be taken up and utilized by the musculoskeletal system during growth. Reductions in serum vitamin D levels concurrent with increases in lean muscle mass and bone mineral content during puberty have been reported (Breen et al., 2011; Willis et al., 2007).
Vitamin D directly alters growth plate fusion. Cyp27b1-KO mice display persistent abnormalities in long bone growth even after correcting their mineral levels (Dardenne et al., 2003). Chondrocyte-specific VDR knockout mice displayed an intriguing phenotype with transient impairment in osteoclastogenesis, reduced vascular invasion of the growth plate and increased serum phosphate (Masuyama et al., 2006). This elegantly demonstrates the role of VDR in regulating interactions between chondrocytes, osteoblasts and endothelial cells in growth plate fusion and systemic mineral levels. Mechanical loading is also important for growth plate fusion (Mirtz et al., 2011) and effects of vitamin D in muscle fiber size and mass may thereby play an indirect role in growth plate physiology.

In summary, evidence suggests that vitamin D plays a subtle role in prenatal musculoskeletal development that has perhaps been obscured by overwhelming evidence in support of a predominantly post-natal effect. VDR appears at an early stage in the developing embryo and is expressed by MSCs, osteoblasts and myoblasts, all of which are responsive to vitamin D (Artaza and Norris, 2009). In vitro studies report growth-promoting effects of vitamin D in myotubes and maturation of matrix vesicles in osteoblasts (Girgis et al., 2014b; Woeckel et al., 2010). In vivo studies support subtle musculoskeletal changes in pre-weaned VDRKO mice (Li et al., 1997). However, pre-weaning effects of VDRKO are not consistently found (Yoshizawa et al., 1997) and VDR activity in muscle/bone precursor cells is confounded by its increased expression following cell isolation (Girgis et al., 2014d). Therefore further research is required to clarify these questions. If confirmed, a role for VDR in musculoskeletal development would be consistent with its known pleiotropic effects in other tissues (Lin et al., 2002). For further discussion on development effects of vitamin D on bone, we refer readers to the following review (van Driel and van Leeuwen, 2014).

**Vitamin D and musculoskeletal involution**

Vitamin D deficiency is common in elderly and institutionalized subjects and may contribute to age-related involution of bone (osteoporosis) and muscle (sarcopenia) (Sohl et al., 2013; Visser et al., 2003). Vitamin D levels predict the risk of functional decline, loss of muscle mass and osteoporotic fracture in elderly subjects, all of which have devastating effects by increasing morbidity, the need for assisted-care and mortality (Sohl et al., 2013). Elderly individuals are also at risk of vitamin D deficiency due to intrinsic age-related changes in vitamin D synthesis, reduced exposure to sunlight and malnutrition, which is rife among those living in institutions (Girgis, 2014).

VDR expression in muscle and bone declines with age (Bischoff et al., 2001; Montero-Odasso and Duque, 2005), rendering the musculoskeletal system more vulnerable to low vitamin D levels in the elderly. In addition, Cyp27b1 levels drop in aged bone cells and MSCs, blunting their responses to 25OHD (Anderson et al., 2005; Geng et al., 2011a). Clinically, 25OHD levels correlate more tightly with bone mass than with serum 1,25(OH)2D, also supporting an effect of Cyp27b1 in skeletal aging (Anderson et al., 2013). By contrast, muscle mass correlates tighter with 1,25(OH)2D (Marantes et al., 2011), suggesting a lesser role for Cyp27b1 in this tissue.

Vitamin D also affects age-related musculoskeletal atrophy, a complex process that involves involution and adipose infiltration of the muscle–bone unit (Schelling et al., 2001; Visser et al., 2005). Vitamin D deficiency activates pathways responsible for muscle atrophy by increasing protein turnover via activation of the ubiquitininproteasome system, ubiquitin ligases (MAFbx and MuRF1), FoxO signaling and TGF-beta signaling (Bhat et al., 2013; Bonaldo and Sandri, 2013). These features are only partly reversible following correction of serum calcium, suggesting a direct VDR effect. Conversely, treatment of muscle cells with 1,25(OH)2D increases myotube size by effects on TGF-beta signaling (Garcia et al., 2011; Girgis et al., 2014b) and prevents free fatty acid (FFA)-induced atrophy by effects on Akt/C-Jun N-terminal kinase (cJNK) (Zhou et al., 2008). However, at supra-physiological doses, vitamin D may induce muscle protein breakdown by activating calpain proteolytic pathways, a mechanism that has been studied for its potential to tenderize beef from aged cattle (Montgomery et al., 2004).

Age-related decline in bone mineral density and mass involves increased osteoclastogenesis, mismatch in RANKL:OPG activity and proteasomal degradation of bone morphogenetic signaling proteins (Cao et al., 2005; Guo et al., 2008). 1,25(OH)2D exerts anti-aging effects in bone marrow pluripotent stem cells by inducing osteogenesis (Duque et al., 2004), delaying replicative senescence (Klotz et al., 2012) and modulating cell survival proteins FoxO, Sirt1 and sestrins (Amol et al., 2010; Eelen et al., 2013). In osteoblasts, 1,25(OH)2D upregulates cystathionine beta-synthase (CBS) (Kriebitzsch et al., 2011), a key enzyme that catalyzes homocysteine and thereby exerts positive effects on bone strength and collagen cross-linking in aging (van Meurs et al., 2004). This effect is supported by an inverse relationship between serum vitamin D and homocysteine levels in human subjects (Amer and Qayyum, 2014) and importantly, the discovery of a VDRE in the CBS gene in pre-osteoblastic MC3T3-E1 cells (Kriebitzsch et al., 2011).

Adipose infiltration is another pertinent factor in muscle and bone aging. Clinical studies report an inverse correlation between serum vitamin D levels, muscle adiposity and poor functional outcomes (Gilsanz et al., 2010; Redzic et al., 2014; Tagliafico et al., 2010). In muscle cells, low-doses of 1,25(OH)2D (10−11 and 10−13 M) mimicked deficiency result in upregulation of PPAR2 and formation of lipid droplets while higher doses (10−7 and 10−9 M) inhibited trans-differentiation (Ryan et al., 2013). Similarly in bone, 1,25(OH)2D inhibits marrow adipogenesis by reducing expression of PPAR2 and has concomitant pro-osteogenic effects on bone marrow stem cells (Duque et al., 2004; Kelly and Gimble, 1998). Therefore vitamin D and PPAR2 signaling are related in muscle and bone and this link requires further evaluation for its tissue-modulatory effects.

Clinical studies show that vitamin D supplementation reduces (Bischoff-Ferrari et al., 2005; Broe et al., 2007), increases (Sanders et al., 2010) or has no effect on the risk of falls and fractures (Girgis et al., 2014c), functional endpoints of musculoskeletal aging. The musculoskeletal system may respond dose-dependently to vitamin D with a U-shaped curve. Individuals with high and low serum vitamin D levels were found to have the highest risk of fracture and frailty compared to those with intermediate levels (60–70 nmol/l) (Bleicher et al., 2014; Ensrud et al., 2010). Likewise, single mega-doses of vitamin D (500,000 IU) increased the risk of falls and fractures (Sanders et al., 2010) while daily supplementation at lower doses had favorable effects in bone mineral density and muscle fiber size in older individuals (Ceglia et al., 2013; Reid et al., 2013). However, these effects did not translate to improvements in muscle function or fracture incidence possibly due to the dose-limiting calcemic effect of vitamin D. By contrast, a 1,25(OH)2D analog with lower calcemic potency, eldecalcitol increased bone mineral density, reduced falls/fractures and improved lower limb muscle power in older individuals (Iwamoto and Sato, 2014; Matsumoto et al., 2011). These findings may result from tissue-specific activation of VDR in bone and muscle, an effect that has been recently observed following vitamin D supplementation (Ceglia et al., 2013).

In summary, serum vitamin D and musculoskeletal VDR decline with age concurrent with the physiological involution of muscle and bone. Effects of vitamin D deficiency overlap with those of aging, including adipose-tissue infiltration, activation of proteolytic pathways in muscle, osteoclastogenesis and increased mineral turnover in bone. Evidence also suggests that musculoskeletal aging responds to vitamin D at a cellular level and in human clinical studies. These findings raise an important question for future consideration: can therapies targeting VDR reverse musculoskeletal aging?
Effects of vitamin D on musculoskeletal injury: direct or indirect?

We are becoming increasingly aware of the interconnected responses of muscle and bone to injury. Fractures associated with muscle injury are more likely to undergo non-union (Reverte et al., 2011) and conversely, the use of muscle flaps to treat open fractures leads to improved bone healing (Harry et al., 2008). In this regard, skeletal muscle may represent a kind of “second periosteen”, providing trophic factors, morphogens and cells to aid bone repair.

Vitamin D may also integrate the combined response to injury. Serum 25OHD levels drop in the curative phase of a fracture and increased 25,25OHD levels correlate with fracture healing (Ettehad et al., 2014; Seo and Norman, 1997). This suggests that vitamin D is being utilized and metabolized by healing bone. To support this, fracture callus displays increased Cyp24a1 activity and 24,25(OH)2D notably improves fracture repair in mice and chickens (Seo and Norman, 1997). This may relate to known effects of 24,25(OH)2D in the differentiation and maturation of growth plate chondrocytes and freeze-crush injury (Choi et al., 2013; Stratos et al., 2013). Importantly, increased VDR is localized to regenerating muscle fibers and not dependent on other cell types such as inflammatory or satellite cells seen in injury (Srikuea et al., 2012). Vitamin D supplementation also reduces muscle injury due to high-intensity exercise or crush injury in rats (Choi et al., 2013; Stratos et al., 2013). Mechanisms include reduced activation of stress-related proteins (p38 MAPK, ERK1/2, IKK, IkappaB), reduced expression of inflammatory cytokines (TNF-alpha, IL-6) and increased muscle fiber turnover (Choi et al., 2013). As such, vitamin D leads to faster recovery of contractile force in the injured muscle. At a cellular level, increases in VEGF and FGF-1 – classic factors involved in tissue regeneration and neovascularization – were seen in C2C12 myoblasts treated with 1,25(OH)2D, suggesting additional pathways by which vitamin D modulates muscle regeneration (Garcia et al., 2013). An important question is whether vitamin D also affects muscle fibrosis following injury. This is suggested by recently described effects of VDR in hepatic stellate cells and liver injury via pathways relevant to muscle (TGF-beta/SMA) (Ding et al., 2013), but is yet to be addressed in this tissue.

These intriguing effects of vitamin D in bone and muscle injury highlight the need to examine models that integrate these components. In particular, potential effects of vitamin D on paracrine factors and morphogens crossing between muscle and bone during injury and the common activation of regeneration pathways at both sites need to be addressed.

Effects on muscle and bone: direct or indirect?

The extent to which vitamin D directly affects the musculoskeletal system versus its indirect actions via calcium and phosphate homeostasis is a matter of ongoing debate. This is further complicated by controversy regarding the presence of VDR in muscle. Overwhelming evidence supports a predominantly indirect effect. This includes the observation that musculoskeletal defects in vitamin D deficiency or VDR ablation coincide with altered serum mineral levels and are reversed by correcting these levels (Bhat et al., 2013; Li et al., 1998; Schubert and DeLuca, 2010). In addition, solo expression of VDR in the intestine reverses the skeletal phenotype of VDRKO mice, highlighting its central role in the enteric absorption of calcium and phosphate (Xue and Fleet, 2009). However, there is evidence to suggest direct effects of vitamin D in muscle and bone, potentially working in parallel or complementary to its systemic effects.

The first, perhaps weakest, line of evidence is that vitamin D exerts a range of effects in cultured bone and muscle cells. Genomic effects of 1,25(OH)2D-VDR alter muscle and bone cell proliferation, differentiation, remodeling and regeneration (Girgis et al., 2014b; Tanaka et al., 2014; van Driel et al., 2006; Yang et al., 2013). Rapid effects in calcium handling and cellular proliferation occur via diverse signaling pathways including c-src, MAPK, protein kinase C, phospholipases and voltage-gated ion channels (Buitrago et al., 2003; Morelli et al., 2001; Wali et al., 2003). Receptors modulating these effects include membrane-bound VDR and non-classical proteins such as protein-disulfide isomerase-associated 3 protein (Pdi3) and membrane associated rapid response-binding protein (MARRS) (Nemere et al., 1998). However the physiological relevance of these in vitro studies is questionable and VDR levels increase during cell isolation, possibly augmenting its activity in culture (Bhalla et al., 1987; Girgis et al., 2014d).

Mice with aberrant vitamin D signaling continue to display subtle musculoskeletal defects even after correction of mineral defects. Smaller muscle fibers and changes in osteoblast number, mineral apposition rate and bone volume persist in VDRKO mice on rescue diets (Endo et al., 2003; Panda et al., 2004). However, upon transplanting femora from VDRKO bone mice into WT mice, they paradoxically form more bone (Tanaka and Seino, 2004). This raises the possibility that extra-skeletal signals, potentially originating from muscle, may modulate bone effects of VDR. Interestingly, lack of VDR in bone protects VDRKO mice from the more severe skeletal phenotype seen in intestine-specific VDRKO mice (Lieben et al., 2012). However, intestine-specific VDRKO mice showed normal calcium and phosphate levels, presumably at the expense of bone. These observations support complex interactions in local and systemic VDR signaling in modulating bone and calcium levels.

Transgenic mouse models have shed further light on local effects of VDR in bone. These effects vary depending on the differentiation stage of osteoblasts, systemic calcium levels and the particular promoter-based Cre model used (e.g. osteocalcin, collagen 1-promoter) (Eismen and Bouillon, 2014). VDR overexpression in mature osteoblasts (OSVDR model, osteocalcin promoter) enhances bone formation, reduces bone resorption and protects against bone loss due to vitamin D deficiency (Baldock et al., 2006; Gardiner et al., 2000; Lam et al., 2014). These mice respond to mechanical loading by increasing bone formation and mineralization (Anderson et al., 2013), suggesting an intriguing interaction between muscle and bone through osteoblast VDR. This effect may occur by activation of Wnt co-receptor LRPS, a transcription factor with a central role in bone mechano-transduction (Bowneald, 2007; Shi et al., 2007), supported by the presence of a putative VDRE in the LRPS gene (Fretz et al., 2007). Alternatively, 1,25(OH)2D may alter mechano-transduction via non-genomic mechanisms, suggested by its VDR-independent effect on nitric oxide (NO) production by osteoblasts (Willems et al., 2012).

In immature osteoblasts, VDR signaling may have the opposite effect in stimulating bone resorption and reducing bone mass, as suggested by a knockout model using the collagen 1-promoter
Vitamin D and muscle-bone signals

Under various stimuli, muscle and bone produce a range of hormones that facilitate cross-talk between these tissues. Vitamin D may regulate such factors, thereby indirectly affecting muscle–bone interactions (Fig. 1).

One such factor, osteocalcin is produced by osteoblasts, regulated by vitamin D and its gene contains a well-established VDRE (Morrison et al., 1989; Terpening et al., 1991). Apart from being a primary marker of bone formation, osteocalcin in its undecarboxylated form participates in glucose homeostasis and forms the basis of a putative bone–pancreas endocrine loop (Clemens and Karsenty, 2011). Osteocalcin also has potential effects in skeletal muscle by altering insulin sensitivity (Clemens and Karsenty, 2011), muscle mitochondrial function (Clemens and Karsenty, 2011) and lower limb strength in older women (Levinger et al., 2014).

Sclerostin is another potential link. This factor, secreted by osteocytes, regulates the osteogenic response to muscle loading and, interestingly, responds to vitamin D supplementation (Dawson-Hughes et al., 2014; Tu et al., 2012). Similarly, FGF23 is a vitamin D-responsive phosphaturic hormone produced by bone cells that has effects on cardiac and smooth muscle, suggesting potential effects in skeletal muscle (Six et al., 2014; Touchberry et al., 2013).

Vitamin D supplementation has been shown to alter IL-6 (Schleithoff et al., 2006), an inflammatory cytokine and hormone produced by skeletal muscle following contraction and exercise (Pedersen and Febbraio, 2008). In addition to its role in glucose homeostasis via fat and liver, IL6 stimulates bone resorption and IL6-related cytokines, Oncostatin M and ciliary neurotrophic factor (CNTF), alters osteoblast differentiation and bone strength (Johnson et al., 2013, 2014). Alterations in IL6 may also explain age-related inflammation and skeletal muscle defects in vitamin D-deficient older individuals (Sanders et al., 2014).

Myostatin, a member of the TGF-beta superfamily and a muscle-derived hormone, has been linked to vitamin D in cultured muscle cells and in a human study (Girgis et al., 2014b; Szulc et al., 2012). Myostatin negatively regulates muscle mass by effects on Smad family proteins and the ubiquitin–proteasome system and affects bone mass directly (via activin receptors) and indirectly (by muscle loading) (Bower et al., 2013; Elkasrawy and Hamrick, 2010). Vitamin D has a profound inhibitory effect on myostatin expression in cultured muscle cells, an intriguing connection which explains the doubling in myotube size in response to 1,25(OH)2D (Girgis et al., 2014b). Another member of the TGF-beta family, follistatin enhances 1,25(OH)2D-simulated mineralization in osteoblasts (Woeckel et al., 2013) and is also regulated by vitamin D in muscle cells (Garcia et al., 2011).

Therefore, vitamin D is associated with several bone and muscle-derived hormones, suggesting indirect links in muscle–bone cross-talk. While these links are tenuous, they require further evaluation and present interesting lines of enquiry. These links have been summarized in Fig. 1.

Conclusions

In his seminal publication on the cure of rickets by sunlight, the American physician Alfred Hess remarked that “although we have realized the importance of sunlight in the growth of plant life, we have [until now] accorded it too little significance in the development of animal life” (Hess, 1922). Since this time, we have made major advances in understanding the biologic activity of the “sunshine hormone”, 1,25(OH)2D, and its system-wide effects in mineral homeostasis, organ development, fibrosis and cell cycle regulation. We are also becoming increasingly aware of the interconnected biology of muscle and bone and the search for unifying factors to explain this connection has clear implications to future therapies (Girgis et al., 2014a).

Vitamin D is one such factor that potentially integrates bone and muscle. To support this contention, in vitro studies demonstrate a range of rapid and genomic effects of vitamin D in differentiation, bone mineralization and muscle fiber size (Girgis et al., 2014b; Tanaka et al., 2014; van Driel et al., 2006; Yang et al., 2013). Following injury, the muscle–bone unit displays heightened sensitivity to vitamin D with local up-regulation of Cyp27b1, VDR and associated reductions in serum 25OHD levels (Ettehad et al., 2014; Seo and Norman, 1997; Srikuea et al., 2012). In contrast, the aging muscle–bone unit displays reduction in local VDR in association with activated proteolytic pathways in muscle, increased bone resorption and adipose-tissue infiltration by common effects on PPARγ2 (Bhat et al., 2013; Duque et al., 2004; Ryan et al., 2013).

A central question that has perplexed this field for many years is whether effects of vitamin D in bone and muscle are entirely indirect – via calcium and phosphate homeostasis – or partly direct by local VDR. This is especially controversial in muscle where the presence of VDR is debated and in bone, where transgenic mouse models have not presented a clear picture of VDR’s local effects (Eisman and Bouillon, 2014). The latter highlights the complexity...
of local and inter-system VDR signals in modulating tissue responses. Another question is whether vitamin D’s effects in bone and muscle are truly integrated or rather independent. In support of the former, osteoblast VDR modulates the effect of muscle loading on bone (Anderson et al., 2013) and vitamin D, sequestered within muscle fibers, may diffuse to adjacent bone under the influence of local stimuli (Abbud et al., 2013). Furthermore, vitamin D regulates paracrine factors considered to facilitate bone–muscle cross-talk – osteocalcin and, possibly, IL6 and myostatin (Morrison et al., 1989; Schleithoff et al., 2006; Szulc et al., 2012). Perhaps the most compelling evidence of an inter-connected response is seen in elderly patients receiving the vitamin D analog alendocalcit. They demonstrated parallel improvements in muscle and bone parameters with associated functional outcomes (Iwamoto and Sato, 2014; Matsumoto et al., 2011).

Taken together, these studies corroborate the intimate relationship between bone and muscle and add to vitamin D’s burgeoning repertoire of extra-skeletal effects. Future studies are needed to unravel mechanisms in tissue-modulatory, pleiotropic effects of vitamin D and to explore the therapeutic potential of targeting this pathway in musculoskeletal disorders.

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Bone muscle interactions and vitamin D

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Abstract

Beyond the established roles of vitamin D in bone and mineral homeostasis, we are becoming increasingly aware of its diverse effects in skeletal muscle. Subjects with severe vitamin D deficiency or mutations of the vitamin D receptor develop generalized atrophy of muscle and bone, suggesting coordinated effects of vitamin D in musculoskeletal physiology. At a mechanistic level, vitamin D exerts wide-ranging effects in muscle and bone calcium handling, differentiation and development. Vitamin D also modulates muscle and bone-derived hormones, facilitating cross-talk between these tissues. In this review, we discuss emerging evidence that vitamin D regulates bone and muscle in a direct, integrated fashion, positioning the vitamin D pathway as a potential therapeutic target for musculoskeletal diseases.

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Introduction

Bone and muscle serve obvious and critical functions in our structure, strength and motion. In addition, bone serves as the major reservoir for calcium which is vital for the regulation of blood calcium levels. This tight regulation of serum calcium is an absolute requirement for normal neurological function. Muscles are not usually thought of as a reservoir, but they contain up to 80% of stored carbohydrates in healthy individuals and may contain a surprising amount of lipid in trained athletes, and in the obese. Emerging evidence also suggests that skeletal muscle may be a storage site of 25(OH)vitamin D (25D), a process that relies on innate vitamin D signaling components and transport carriers within muscle cells [1,2].

Diseases of the musculoskeletal system are becoming more common with increasing longevity and adiposity of the population in many...
countries around the world. Loss of muscle mass (sarcopenia) and decreased bone density (osteopenia or osteoporosis) with subsequent fractures cause much of the frailty and disability of aging, with enormous societal and economic costs. This has been estimated at $850 billion dollars a year in the USA [3].

During fetal life, post-natally and in adulthood, muscle mass and bone mass are linked. This link is mediated in part via direct mechanical interactions, and also via tissue cross-talk between muscle and bone. Bones weaken with decreased muscle use in people and in animals. Examples occur in humans with congenital myotonic dystrophy and with other hereditary muscle disorders. These groups of people are born with thin long-bones, relating to severely impaired fetal muscle movements [4,5]. Bone mass and strength are rapidly lost in people with denervating neurological injuries, e.g. spinal cord lesions, and in astronauts who spend prolonged periods of time in a gravity-free environment.

In the opposite direction, bones may also regulate muscle function via a range of osteokines, such as fibroblast growth factor (FGF)-21 which is produced by osteocytes, and other factors [6]. Osteocytes are osteoblasts which have become completely surrounded by bone matrix. Additionally, common pathways such as growth hormone/insulin-like growth factor (IGF)-1, sex steroids and Wnt-signaling may centrally coordinate the bone–muscle unit during development and adaptation to mechanical stimuli [7].

Vitamin D represents another pathway by which muscle and bone may interact. Individuals with vitamin D deficiency and rickets display concurrent defects in muscle and bone. Mutations in vitamin D signaling are also associated with generalized underdevelopment of the musculoskeletal system. These observations suggest an integrated role of vitamin D in muscle/bone health. Potential mechanisms may relate to effects of vitamin D in the expression of myokines and osteokines (i.e. muscle and bone-derived hormones) or regulation of mechano-stimulatory processes.

This review presents evidence for a holistic role of vitamin D in the musculoskeletal system beyond its established roles in skeletal health alone. While evidence for such an integrated role is slowly emerging, there is hope that the vitamin D pathway may present therapeutic targets for the treatment of musculoskeletal disease [8].

Development and regulation of bone and muscle

Bone and skeletal muscle both develop during early fetal life from the somatic mesoderm in close physical association with each other. This has been the subject of excellent reviews, including [6,9].

Bone forms sequentially, initially by the condensation of mesenchymal precursors at future skeletal sites and then by differentiation into chondrocytes to form a cartilage anlage (i.e. endochondral bone) or directly into osteoblasts (i.e. intramembranous bone). This early process relies on positional and mechanical cues [6]. Once formed, bone undergoes continual remodelling throughout life in response to mechanical loads and in order to repair microdamage and adapt to changing mechanical requirements. This relies on the coordinated action of bone cells: bone resorption by osteoclasts, then subsequent bone matrix formation and mineralization by osteoblasts. Osteocytes are sensitive to mechanical forces and may initiate adaptation through regulation of both bone resorption and formation.

During embryogenesis, myogenic differentiation occurs adjacent and concurrent to skeletal development. Myogenesis is specified by the master regulator Pax3 in fetal and neonatal life and by Pax7 in later life. In the trunk and limbs, mesodermal precursor cells under the control of Pax3 become committed to myogenic lineage and differentiate and fuse to form multinucleated myotubes under the control of external signals such as MyoD and Myf5 [10]. These myotubes develop into mature, multinucleated muscle fibers. Not all myoblasts fuse. A subset of myogenic precursors instead form a reservoir of Myf5/MyoD-expressing cells that remain on the periphery of myofibers as a source of new muscle cells during postnatal growth and regeneration [11]. These are known as satellite cells [12–15].

The linked developmental origins of muscle and bone suggest the possibility that common signaling pathways or networks may regulate their mass. To support this, mice with genetic defects in muscle development (MyoD knockout) display profound impairments in bone development and mineralization [16]. Signals responsible for coordinated muscle/bone development include FGFs, transforming growth factors (TGFs), IGF-1 and other morphogens [17].

Muscle and bone mass are both increased by anabolic exercise and are both decreased by disuse. The changes in mass with exercise or with disuse are mediated by some of the same factors. As well as the direct mechanical interactions between muscle and bone, there is further evidence that muscles may indirectly regulate bone repair following fracture. Covering fractures with muscle flaps, even with immobilization is well recognized to improve healing of traumatic fractures [18,19]. Conversely, injury to overlying muscle impairs fracture healing [20].

The vitamin D receptor (VDR) makes an early appearance during musculoskeletal development. In the fetal rat, it is found at day 13 of gestation [21] within the condensing mesenchyme of the vertebral column. By day 17, the VDR resides within osteoblasts and proliferating chondrocytes. Despite the early embryologic presence of the VDR, defects in vitamin D signaling do not lead to clinical features of rickets until after birth [22]. The skeletal phenotype is most manifest after weaning, coinciding with development of hypocalcemia and hyperparathyroidism in VDR knockout (VDRKO) mice. Abnormal growth plate development, impaired bone mineralization and muscle fiber atrophy are seen at day 35 [22,23]. However, subtle changes are noted in pre-weaned VDRKO mice. These include growth plate enlargement [22] and smaller muscle fibers at day 15 [23]. These effects occur prior to the onset of abnormal calcium and phosphate levels, suggesting a direct albeit subtle role for VDR in musculoskeletal growth.

Vitamin D

Vitamin D synthesis and signaling are the subject of many reviews [24]. Briefly, vitamin D may be synthesized in the body by conversion of 7-dehydrocholesterol in the skin upon ultraviolet (UV) light exposure, or obtained from the diet, where it is mainly in fatty fish or in supplemented foods. Because humans can synthesize vitamin D, it is actually not a classic vitamin, but has retained the name for historical reasons.

Vitamin D is then hydroxylated in the liver into 25-hydroxyvitaminD (25D). This is the main form of circulating vitamin D and it is measured to assess adequacy of overall vitamin D status. However, the major active hormone is 1,25-dihydroxyvitaminD (1,25D). This binds to the vitamin D receptor (VDR), a member of the nuclear steroid hormone receptor superfamily. VDR usually heterodimerizes with the retinoid X receptor (RXR) although it can homodimerize with a second VDR. There are hundreds of well-characterized targets for activated VDR in the genome. Recent use of RNA-sequencing combined with chromatin immunoprecipitation (ChIP) has given new power to examine genes regulated by vitamin D in non-classic target tissues such as liver [26] and bone cells [27] although this technology has not yet been applied to muscle.

Vitamin D in bone

Vitamin D has long-recognized actions in both bone and muscle [28–30]. The classical consequence of vitamin D deficiency is a defect of bone mineralization, causing rickets in children and osteomalacia in adults. However, vitamin D has been linked more to active calcium
Vitamin D in muscle and potential effects on bone mass

The link between the effects of vitamin D signaling in muscle/bone has long been appreciated. There are many potential factors which may play a role in mediating this interaction, some of which are shown in Fig. 1.

Indeed, one of the major components of vitamin-D deficiency-related increases in fracture rates is due to muscle weakness and falls. The greatest effects are evident in those with lowest vitamin D levels [40,41]. In addition, vitamin D status is associated with physical performance in epidemiological studies. Vitamin D deficiency can predict a decline in physical performance after 3 years [42,43]. According to the study of Need et al. [44], the calcium absorption starts to decrease and the alkaline phosphatase as a sign of deficient mineralization starts to rise when serum 25D is below 20 nmol/l. Physical performance decreases when serum 25D falls below 50 nmol/l, but the decrease accelerates when 25D is below 30 nmol/l [42].

A meta-analysis of randomized clinical trials has confirmed that vitamin D supplementation can decrease the incidence of falls [45], although this remains controversial. However, the appreciation of muscle-bone cross-talk continues to increase, and evidence is emerging of a role for vitamin D in this axis [46]. Co-regulation of bone and muscle by vitamin D is evident in co-existence of osteoporosis and sarcopenia in the elderly, vitamin D deficient population [40,47–49].

Interestingly, the notion of direct humoral interactions between bone and muscle has also been supported by studies such as those of myostatin, a powerful inhibitor of muscle growth (reviewed in [50]), which also has powerful effects upon bone mass and fracture healing, independent of the changes in muscle strength [51,52]. Vitamin D treatment decreases myostatin in cultured muscle cells, suggesting a potential relationship between these pathways in vivo.

Vitamin D-related signals have also been implicated in muscle bone interactions [53]. Osteoglycin, produced by muscle cells under the control of vitamin D, regulates osteoblastic activity [53].

Vitamin D in muscle

We reviewed the roles of vitamin D in muscle in 2013 and 2014 [40,41] and others have published excellent reviews on this area [28,54]. Whether vitamin D receptor is expressed in muscle has been a controversial question, made more difficult by the low level of expression of VDR in myocytes after neonatal life. However, by RNA, Western immunoblot and by immunohistochemistry, VDR is present at low levels in cultured C2C12 myotubes [55] and normal murine muscle [56]. VDR expression is typically much higher in cell lines and isolated myocytes than in primary muscle. VDR expression is also significantly higher in neonatal muscle and declines with age at this site. This further supports developmental roles of VDR in muscle.

It has been long-recognized that vitamin D deficiency is associated with muscle weakness, particularly proximal muscle weakness, and that this resolves with correction of deficiency. There are non-specific electromyographic (EMG) features which resolve with correction of deficiency (reviewed in [40]). In biopsy studies, most of which are non-randomized, people with deficiency have preferential loss of type 2 muscle fibers which improves with supplementation [57,58]. In children with rickets and adults with osteomalacia, muscle weakness can...
be extreme [40]. In the people with vitamin D pathway mutations, weakness is also a clinical feature prior to treatment. However, in these cases, and in severe vitamin D deficiency, the situation is frequently confounded by other factors such as hypocalcemia or hypophosphatemia [59].

In addition, in people, it is challenging to control for sunlight and diet exposure; ‘healthier’ individuals may have better diets and undertake more outdoor activities, and have correspondingly better muscle and bone function. For this reason, it is useful to turn to animal models to examine the question of bone–muscle interactions and vitamin D.

Mice with mutations in vitamin D receptor are weak and can have low bone mass and less longitudinal growth. However, these findings are confounded by the altered calcium and phosphate status of the mice. Studies of VDR-null mice where phosphate and calcium are aggressively replaced with a ‘rescue diet’ may result in near-normal bone phenotype [60,61]. However, there can be more subtle residual effects, suggesting persistent and local effects of VDR ablation in bone, including in heterozygous VDR-null mice [61]. Epidemiological studies show associations between vitamin D deficiency and poor physical performance, assessed by a walking test, 5 chair stands, and a balance test [42].

Potential muscle to bone vitamin D cross-talk

Even with use of rescue diet, there is decrease in the size of muscle fibers in mice lacking VDR [23]. This suggests that the effect on muscle is more likely to be a VDR effect rather than a calcium or phosphate effect. On ‘rescue diet’ the bone mass changes in VDR null mice are smaller than those changes seen in muscle mass and strength measures. This suggests that there may be a muscle-related or hormonal factor that is stimulating bone mass in VDR-null muscle.

Myostatin is a hormone secreted from muscle. Its role is to inhibit increased muscle mass, hence its name. It is a member of the TGFβ superfamily which acts on activin receptors and SMADs. Vitamin D inhibits myostatin production from muscle cells, for example C2C12 cells [62], and vitamin D treatment doubles myotube size in these cells [55]. Decreased myostatin is associated with greater bone mass [63] (Fig. 1).

Vitamin D may regulate responses to muscle injury and regeneration. Serum 25D levels drop directly following muscle injury, which may partly be due to the fall in vitamin D binding protein after trauma [64]. Baseline levels correlate directly with muscle recovery [65]. Studies in rodents demonstrate substantial increases in the expression of Vdr and Cyp27B1 following injury [66–68]. This has been found both in models of chemical injury [66,68], and a freeze–crush model of physical injury [67]. Increased VDR is directly localized to regenerating muscle fibers [66]. Vitamin D supplementation also reduces muscle injury due to high-intensity exercise in rats and humans [69,70]. At a cellular level, it increases VEGF and FGF1; both factors involved in tissue regeneration and neovascularization. This was seen in C2C12 myoblasts treated with 1,25D, and VEGF and FGF1 are potential pathways by which vitamin D may modulate muscle regeneration [71]. An important question is whether vitamin D also affects muscle fibrosis following injury. This is suggested by recently described effects of VDR in hepatic stellate cells and liver injury via pathways relevant to muscle (TGF-B/SMAD) [72], but is yet to be addressed in this tissue.

Vitamin D is known to stimulate local vascular endothelial growth factor (VEGF) and IGF-1 (Fig. 1) production in muscle. Both factors are well known to have potential beneficial effects in bone. How large a contribution muscle vitamin D action makes to circulating levels of either hormone is not clear.

Another potential muscle factor which is regulated by vitamin D is IL-6 (interleukin-6). It is produced following exercise or contraction. It stimulates bone resorption, and may alter bone strength. It is decreased by vitamin D [73].

Potential bone to muscle vitamin D cross-talk

The osteocyte cell line MLO-Y4 expresses muscle anabolic factors IGF-1, MGF and VEGF after mechanical loading [74]. Osteocalcin which is produced by osteoblasts has recently come to a new light for its role in regulating beta-cell function [75]. Its classic use is as a marker of bone formation. It is regulated by vitamin D and its gene contains a vitamin D response element (VDRE) indicating direct regulation by vitamin D [2]. It has potential effects in muscle, in which it alters mitochondrial function, insulin sensitivity [75], and possibly strength in women.

Sclerostin is secreted by mature osteocytes during completion of osteon formation. It inhibits bone formation. Mutations in the SOST gene, coding for sclerostin, cause osteoporosis with undetectable or low sclerostin levels, increased bone formation, very high bone mass and neurological impairments due to entrapment of nerves [37]. Sclerostin antibodies have a potential for exciting use in the treatment of osteoporosis. Sclerostin secretion by osteocytes increases in response to bedrest and decreases with muscle loading [76]. Serum levels increase in people treated with vitamin D [77]. FGF23 is another vitamin D responsive hormone produced by bone which may have positive effects on cardiac and smooth muscle [78]. The effects on skeletal muscle are the subject of current investigation.

Conclusions

In addition to established roles in calcium and phosphate homeostasis, vitamin D plays a vital role in musculoskeletal health. People with severe vitamin D deficiency display the combination of reduced bone mineral density and muscle wasting. Mechanistic studies demonstrate a range of rapid and genomic effects of vitamin D on cell differentiation, bone mineralization and muscle fiber size [53,55,79,80]. Vitamin D may also exert effects in bone and muscle injury. Following injury, these tissues display heightened sensitivity to vitamin D with local up-regulation of Cyp27B1, VDR and associated reductions in serum 25D levels [68,81,82]. We are also becoming increasingly aware of the interconnected biology of muscle and bone. The search for unifying factors to explain this connection has clear implications for future therapies [8]. Vitamin D appears to be one of these factors integrating bone and muscle. To support this, vitamin D exerts effects in a range of bone and muscle-derived hormones, including osteocalcin, sclerostin, IL-6 and myostatin, and may thereby modulate bone–muscle cross-talk. Taken together, these studies confirm the intimate relation between bone and muscle and add to vitamin D’s increasing repertoire of extraskeletal effects. Future studies are needed to unravel mechanisms involved in vitamin D’s tissue-modulatory, pleiotropic effects and explore the therapeutic potential of targeting this pathway in musculoskeletal disease.

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Effects of vitamins C and D in type 2 diabetes mellitus

Abstract: Scurvy and rickets are largely considered historical diseases in developed countries. However, deficiencies in vitamins C and D are re-emerging due to increased consumption of processed foods and reduced fresh foods in the Western diet, as well as to an indoor sedentary lifestyle away from sun exposure. These dietary and lifestyle factors also predispose one to diabetes and metabolic syndrome. Our understanding of the potential roles of vitamin C (an antioxidant) and vitamin D (a biologically active hormone) in disease is increasing. In this review, we present observational, interventional, and mechanistic studies that examine the potential links between vitamins C and D in reversing defects in glucose homeostasis and the prevention of type 2 diabetes. Studies suggest an association between vitamin C deficiency and diabetes. An association between vitamin D and insulin resistance has been well described; however, the role of vitamin C and D supplementation in diabetes and its prevention requires further controlled trials.

Keywords: glucose homeostasis, diabetes, insulin resistance, vitamin C, vitamin D

Introduction

Nutrients play essential roles in health and the prevention of disease. Nutrients, including vitamins, are vital to cardiovascular health (ie, vitamin B1), nerve function (ie, vitamins B6 and B12), the production of red blood cells (ie, folate and vitamin B12), and coagulation (ie, vitamin K), among many other functions. Scurvy and rickets were largely thought to be conditions of historical interest in developed countries; however, deficiency in vitamins C and D are re-emerging due to increasingly inadequate carbohydrate- and fat-based Western diets and sedentary, indoor living. These same phenomena are contributing to the surging prevalence of obesity, metabolic syndrome, and diabetes. Early evidence suggests that vitamin D deficiency may contribute to diabetes, and that vitamin D repletion may ameliorate abnormal glucose homeostasis. Low vitamin C is associated with an increased risk of the future development of diabetes. It is likely that low vitamin C is a marker of a less healthy lifestyle, a well-known risk factor for diabetes.

Vitamin C is an antioxidant, and the structural similarity between vitamin C and glucose makes it of interest in diabetes. Oxidative stress can lead to disturbed glucose metabolism and hyperglycemia. Therefore, a benefit of antioxidants to prevent diabetes or to achieve positive outcomes in type 2 diabetes mellitus (T2DM) is biologically plausible.

Vitamin D plays a vital role in whole-body calcium homeostasis by exerting classic effects on the duodenum, bone, and kidney. Vitamin D may also alter intracellular...
calcium signals and thus plays a role in pancreatic insulin secretion and insulin sensitivity, both of which relate to calcium levels. It therefore has potential in the prevention of T2DM. The role of vitamin D in insulin resistance has been studied extensively, and vitamin C is another vitamin for which studies have demonstrated significant associations with diabetes. This review explores the observational, interventional, and mechanistic studies that address the effects of vitamins C and D in T2DM.

**Effects of vitamin C in diabetes**  
**Physiology and vitamin C deficiency**

Vitamin C, also known as ascorbic acid, is a cofactor in multiple enzymatic reactions including collagen synthesis. Humans are unable to produce vitamin C due to the absence of the enzyme, L-gulonolactone oxidase, which catalyzes the final step in the synthesis of ascorbic acid; therefore, it is an essential nutrient in humans. Vitamin C acts as a reducing agent in free radical-mediated oxidation processes; therefore, it can act as an antioxidant. Deficiency of vitamin C results in the defective formation of collagen and connective tissues in the skin, cartilage, dentine, bone, and blood vessels.

In its most severe form, vitamin C deficiency results in scurvy, which is uncommon in developed nations due to the inclusion of fresh fruits and vegetables in the diet. Nevertheless, lesser degrees of vitamin C deficiency were found to be common among healthy adults in the United States (National Health and Nutrition Examination Survey [NHANES] 2003–2004: 7.1%). Smokers and people in lower-income groups were at increased risk of deficiency. Other classic risk factors include alcoholism and renal failure.

Though diabetes is not traditionally considered a risk factor for vitamin C deficiency, patients with diabetes should all receive dietary advice about healthy eating and vitamin C dietary sources, including fresh fruits and vegetables. The recommended dietary intake of vitamin C is 45 mg per day for adults. There are some data suggesting that people with diabetes may have increased cellular uptake and turnover of vitamin C that would necessitate increased intake, and they also have an increased risk of deficiency.

**Observational studies**

The effects of vitamin C in diabetes have been an area of interest for over 50 years. A review of 23 observational studies looking at the vitamin C status of people with diabetes published between 1935 and 1996 found that people with diabetes have at least 30% lower vitamin C concentrations than do people without diabetes. However, there was heterogeneity among the studies in terms of the methods used to measure vitamin C status, and subjects were unmatched on important covariates such as dietary intake of vitamin C, sex, smoking status, and acute illness.

Observational data from NHANES 1988–1994 identified that people with newly diagnosed diabetes had significantly lower serum vitamin C concentrations than did people without diabetes; however, no difference was seen after adjusting for the dietary intake of vitamin C. On the other hand, among those with a similar dietary intake of vitamin C, those with diabetes of 2 months to 25 years’ duration did have lower levels of vitamin C when compared to controls. In the Finnish Mobile Clinic Health Examination Survey conducted during 1966–1972, the association between low vitamin C levels and diabetes was also not demonstrated to be due to low dietary vitamin C. This prospective study estimated total habitual food consumption in the previous year, and it examined the incidence of diabetes during a 23-year follow-up period. About 380 cases of diabetes were identified, but vitamin C intake was not associated with the risk of T2DM.

In a cross-sectional study where dietary vitamin C intake was the same, lower vitamin C levels in people with diabetes were seen as a consequence of diabetes itself and not due to the inadequate dietary intake of vitamin C. In a nested case-control study in Korea, there was no difference in dietary vitamin C intake between those with diabetes and controls matched for age, sex, drinking status, and smoking status. Interestingly, however, nonsmoking individuals with a new diagnosis of T2DM had lower serum vitamin C levels (22.3±16.8 μmol/L) than did controls (26.3±17.0 μmol/L) (P<0.01). Among smokers, there was no difference between serum vitamin C levels in those with diabetes and controls. Therefore, smoking – a known trigger of vitamin C deficiency – appears to modify the association between vitamin C and diabetes incidence.

An English population-based prospective cohort study of 25,639 volunteer participants demonstrated a strong inverse association between plasma vitamin C levels and incident diabetes after 8–12 years with a dose–response effect. A 29% reduction was found in diabetes risk per 19.87 μmol/L (0.35 mg/dL) change in vitamin C level, which was adjusted for important covariates including the use of vitamin supplements. However, vitamin C may actually be a marker of other protective factors found in fruits and vegetables that may decrease the risk of T2DM.

An association between vitamin C levels and glycemic control was assessed in a cross-sectional study of people...
with diabetes, which identified a weak, negative correlation between hemoglobin A1c (HbA1c) and vitamin C levels.10 The relationship between vitamin C and glucose levels was also demonstrated in a large sample of US adults without a history of diabetes from NHANES 2003–2006, in which serum vitamin C concentrations were inversely associated with HbA1c levels.20 An observational study on the use of vitamin C supplements demonstrated that a significantly lower risk of diabetes was associated with the use of daily vitamin C supplements when compared to nonusage.21 The potential benefit of a vitamin C supplement was limited to those who did not take a multivitamin or those who had a lower dietary intake of vitamin C. However, the observational nature could not demonstrate a cause-and-effect relation, nor could confounding be excluded, such as health-conscious users of supplements being less likely to develop disease. To rectify this, a post hoc analysis from the SU.VI.MAX study,22 a randomized trial to assess the effects of a combination of vitamins and minerals including vitamin C, was performed. Plasma concentrations of vitamin C were inversely (P=0.046) associated with fasting plasma glucose levels; however, over the follow-up period of 7.5 years, supplementation had no effect on age-adjusted fasting plasma glucose levels.

Observational studies have suggested that people with T2DM have lower vitamin C levels, and this was not explained by differences in dietary vitamin C intake when compared to people without diabetes. There may be an association between lower vitamin C levels and the subsequent development of diabetes, as well as higher glucose levels.

Interventional studies

In three intervention studies of vitamin C supplementation in T2DM, an improvement was seen in fasting glucose and HbA1c levels, but only with a higher dose supplementation of 2,000 mg of vitamin C per day for 90 days, with no improvement observed after 2 months of 500 mg daily vitamin C supplementation, nor with short-term supplementation over 2 weeks.23–25 This finding may be due to an inadequate increase in vitamin C levels in diabetes with standard supplementation doses or of brief duration. The difficulty with interpreting the vitamin C intervention studies is the use of study populations with adequate vitamin C levels, which significantly decreases the likelihood of observing any effects of the intervention.26,27 Another randomized controlled trial28 demonstrated that supplementation with 800 mg per day for 4 weeks in people with T2DM and low plasma vitamin C levels (<40 μmol/L) at baseline was insufficient for replenishing serum levels to what is seen in healthy subjects (>80 μmol/L). The lower vitamin C levels achieved could have explained the lack of improvement in measures of insulin resistance in the supplemented group.

The addition of vitamin C supplementation to standard therapy was assessed in 70 patients treated with metformin for T2DM who were randomized to 500 mg twice daily of vitamin C or placebo for 12 weeks.29 Those given vitamin C were identified to have lower HbA1c, fasting, and postmeal blood glucose levels when compared to the placebo group, despite all being treated with metformin.

Apart from the impact on glycemic control, antioxidant supplementation has been studied for the prevention of complications of diabetes. An improvement in neuropathic symptoms (but not in objective signs of neuropathy) and decreased odds of retinopathy were seen with the use of a combination of vitamin supplements including vitamin C.30,31 The impact of high dietary vitamin C and supplementation has been demonstrated to have variable effects on cardiovascular complications in diabetes.32,33

Interventional studies with vitamin C supplements have not consistently demonstrated improvements in glycemic control in T2DM. Vitamin C as an antioxidant could reduce the risk of complications of T2DM; however, excess supplementation may increase the risk of cardiovascular disease.32

Potential mechanisms

The significance of vitamin C in T2DM has been suggested by the hypothesis that hyperglycemia inhibits the cellular uptake of dehydroascorbic acid (DHA), which is the oxidized transportable form of vitamin C.34,35 In the red blood cell, glucose strongly inhibits the uptake of DHA; therefore, hyperglycemia in diabetes would be expected to cause vitamin C deficiency within the cell.35 DHA uptake into the cells is accomplished through glucose transporters, GLUT1 and GLUT3, which transport DHA in competition with glucose.36,37 and this effect may be overcome by a large intake of vitamin C.38

The mechanisms for reduced vitamin C levels in diabetes are suggested by animal studies. A study of rats with streptozotocin-induced diabetes39 demonstrated a decrease in serum levels of vitamin C and the increased urinary excretion of vitamin C when compared to the levels obtained prior to the induction of diabetes in the same rats, suggesting that the renal reabsorption of vitamin C is reduced in diabetes. Another study of diabetic rats40 demonstrated an increased turnover of vitamin C, and this finding was most likely due to the increased oxidation of ascorbate to DHA in tissue.
mitochondria. Furthermore, a human study of vitamin C turnover, measured by the rate of reduction of DHA to ascorbic acid, indicated that this turnover was higher in a group of people with diabetes than in volunteers without diabetes.\textsuperscript{12} This higher turnover of vitamin C in diabetes may underlie the need for higher dietary vitamin C requirements in diabetes.

Significant benefits of vitamin C supplementation have been demonstrated in an animal model of T2DM. Vitamin C supplementation in ob/ob mice caused significant reductions in food intake, plasma glucose, HbA\textsubscript{1c} levels, plasma glucose levels, and insulin concentrations when compared with untreated control ob/ob mice.\textsuperscript{39} The total insulin content and the extent of insulin glycation in the pancreas of ob/ob mice also decreased after vitamin C supplementation. These parameters did not change with vitamin C supplementation in lean mice.

The biological mechanisms underlying lower vitamin C levels in people with diabetes includes decreased cellular uptake, increased urinary losses, and increased metabolic turnover of vitamin C in diabetes. These mechanisms suggest higher dietary vitamin C requirements in diabetes, along with the benefits of supplementation, as seen in a mouse model of T2DM.

**Effects of vitamin D in diabetes**

**Vitamin D physiology**

The name “vitamin D” is a historical misnomer because a vitamin, by definition, is something that we cannot synthesize ourselves. The active form of vitamin D, calcitriol, is synthesized in humans, and is a hormone that undergoes autocrine regulation. Vitamin D is synthesized from cholesterol precursors. Moreover, 7-dehydrocholesterol is converted to vitamin D in the skin following exposure to ultraviolet radiation. This molecule binds to vitamin D-binding protein (DBP) and undergoes hydroxylation in two stages: firstly, in the liver to form 25-OH vitamin D (25OHD); and secondly, in the kidney to form 1,25(OH)\textsubscript{2} vitamin D (1,25(OH)\textsubscript{2}D). In its active form, vitamin D (1,25(OH)\textsubscript{2}D) has predominant effects in calcium and mineral homeostasis by interacting with the vitamin D receptor (VDR).\textsuperscript{3} The VDR is present in tissues involved in calcium/phosphate homeostasis (ie, the intestine, bone, kidney), and it is present at lower levels in other tissues, suggesting the nonclassical roles of vitamin D (eg, in glucose homeostasis).

**Observational studies**

The potential effects of vitamin D on insulin sensitivity and glucose tolerance have been the subject of a recent review.\textsuperscript{5} In 808 subjects without diabetes, plasma 25OHD correlated inversely with fasting insulin levels and Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) scores after adjusting for sex, age, and body mass index (BMI).\textsuperscript{41} A similar link between HOMA-IR and 25OHD was found in 712 prediabetic subjects.\textsuperscript{42} HOMA used data from physiological studies to derive equations to assess insulin resistance (HOMA-IR) and insulin sensitivity (HOMA-S) from matched fasting insulin and glucose samples.\textsuperscript{43} In an elegant study of healthy adults,\textsuperscript{44} there was a significant association between 25OHD and insulin sensitivity assessed by hyperglycemic clamps, after adjusting for BMI and a range of other factors.

Prospective studies have demonstrated that 25OHD levels correlate with the long-term risk of insulin resistance. In a study of 5,200 participants, a 25 nmol/L increment in baseline serum 25OHD levels correlated with a 24% reduced risk of developing diabetes over 5 years, although this was a nonlinear relation.\textsuperscript{45} Furthermore, a meta-analysis of 21 prospective studies identified a linear trend for each 10 nmol/L increment in 25OHD levels to be associated with a 4% lower risk of T2DM (95% confidence interval [CI]: 3–6; \(P\) for a linear trend, <0.0001).\textsuperscript{46} An independent association with HOMA-S at 5 years was also found (\(r=0.16; \ P<0.001\)). Furthermore, vitamin D status and T2DM were inversely associated in multiple observational studies.\textsuperscript{46–52}

In the Women’s Health Study,\textsuperscript{53} dietary vitamin D was inversely associated with metabolic syndrome, but this was related to total calcium intake. The Nurses’ Health Study\textsuperscript{48} showed that there was a decrease in the relative risk of incident diabetes of 0.87 (95% CI: 0.75–1.00; \(P\) for trend =0.04) among those on high-dose vitamin D supplements (>800 IU daily), as compared to those on low-dose vitamin D supplements (<400 IU daily); this was also seen with calcium intake.

Studies have demonstrated an association between vitamin D and insulin resistance, which is significant for the risk of developing T2DM. Although suggestive, these observational studies do not prove a causal relationship between vitamin D and insulin resistance. A number of factors may confound this link, such as adiposity.\textsuperscript{54} Obese individuals may avoid sun exposure, and vitamin D (a lipophilic compound) may be trapped in adipose tissue, resulting in serum deficiency. Obesity is also a major determinant of insulin resistance. Other confounders include parathyroid hormone levels,\textsuperscript{55} calcium levels, physical activity, and diet. These may influence vitamin D and independently alter insulin sensitivity.
Interventional studies
In the absence of insulin resistance, vitamin D supplementation does not alter insulin sensitivity. However, some evidence indicates that subjects with prediabetes may benefit. At-risk subjects receiving various regimens of vitamin D and calcium over 6 weeks to 3 years showed significant improvements in insulin secretion, sensitivity, and/or the disposition index (a marker of insulin secretion/activity). The evidence that vitamin D improves glycemic control in subjects with established diabetes is mixed. In a recent trial, 90 subjects with diabetes were randomized to daily vitamin D (1,000 IU), taken in fortified yogurt; these subjects showed better glycemic control and reduced insulin resistance (HOMA-IR) when compared to subjects on plain yogurt. Importantly, serum vitamin D levels and HOMA-IR scores correlated inversely in this study. Another three studies of vitamin D supplementation of 3 weeks to 3 months’ duration have demonstrated positive effects on HbA1c levels, insulin resistance, and insulin secretion compared to placebo, while other studies have shown no benefit from brief supplementation with low doses of vitamin D, nor with high doses of over 6 months’ duration.

Small interventional studies examining different populations with variable vitamin D levels, using different regimens of supplementation, make it difficult to draw definite conclusions. Parameters of insulin sensitivity are variable, ranging from measuring fasting insulin to hyperinsulinemic–euglycemic clamps. While insulin resistance leads to diabetes, a complex interaction between glucose and insulin levels exists. Glycemic outcomes reflect the interplay between insulin resistance and insulin secretion by pancreatic beta-cells.

Therefore, whether vitamin D supplementation results in clear improvement in diabetes remains unclear. A randomized controlled trial of two doses of vitamin D supplementation (5,000 IU versus 400 IU daily) did not demonstrate improved glucose levels in pregnancy, despite another study showing a link between vitamin D levels and the subsequent risk of gestational diabetes. Large trials are currently underway in other populations, which may definitively resolve this question (eg, NCT01354964, NCT01315366, NCT00736632, and Pittas et al).

Potential mechanisms
Mouse models of diabetes demonstrate improvements in insulin sensitivity following the administration of 1α-OHD3. This vitamin D analog is automatically 25-hydroxylated in the liver, forming calcitriol, and it bypasses normal endocrine regulation of the activation process. An in vitro study has shown that 1,25(OH)2D increases insulin receptor expression and insulin signaling via Akt and insulin receptor phosphorylation. Nongenomic, rapid effects of vitamin D may also play a role. Apart from calcium regulation, vitamin D leads to the release of arachidonic acid, a polyunsaturated fatty acid, from the cell membrane and into the muscle cell cytoplasm. This links vitamin D with insulin sensitivity, as does the possibility that vitamin D may modulate caveolin-1, a scaffolding protein within the membrane that exerts its effects in metabolism.

Pancreatic beta-cells, responsible for insulin secretion, express components of the vitamin D pathway, including 1α-hydroxylase enzyme, a vitamin D-dependent calcium-binding protein (calbindin), and the VDR. Vitamin D treatment increases insulin secretion in vitamin D-deficient rodents and cultured pancreatic cells. This effect occurs via intracellular calcium signals and altered gene expression, but its physiological significance is unclear. Animal models and in vitro studies provide potential mechanisms for the associations between vitamin D and insulin resistance.

Summary points
- Observational studies indicate a link between deficiencies in vitamins C and D and the prevalence of type 2 diabetes.
- There is biological plausibility for the effects of vitamins C and D in glucose homeostasis. Vitamin C prevents oxidative stress and is known to interact with glucose transporters, potential mechanisms by which it may also alter glucose homeostasis. Vitamin D may affect calcium handling in pancreatic beta-cells and skeletal muscle, thereby affecting insulin secretion and sensitivity, respectively.
- Randomized controlled trials have not clearly demonstrated the effects of vitamins C and D in the prevention or treatment of diabetes. This is due to a range of factors, including heterogeneity in study design and endpoints, and the lack of standardized supplementation regimens. Further research will address the impact of these nutrients in glucose homeostasis.

Conclusion
The relationship between diabetes and vitamins C and D have been demonstrated by human and animal studies. The limitation of observational studies is that confounders cannot be excluded to explain the relationship between vitamin...
deficiency and diabetes outcomes. Interventional studies that are randomized provide the best evidence for a benefit of supplementation, with serum vitamin levels indicating variations in the dietary intake of vitamin C and the levels of sun exposure for vitamin D. Animal models can provide an indication of the mechanisms that underlie these associations; however, metabolic processes in animals are not directly translated in humans.

Vitamin C levels are lower among people with T2DM and not completely explained by a difference in dietary vitamin C intake. A number of mechanisms underlying the decrease in vitamin C levels and increased requirements in T2DM have been proposed. Consequently, the dietary requirements or the need for supplementation of vitamin C may be greater in people with diabetes. However, studies of vitamin C supplements alone or in combination have not demonstrated sufficient benefit to support a recommendation for routine supplementation, nor higher target serum vitamin C levels, in people with T2DM. Further studies are needed to determine if vitamin C supplementation may play a role in minimizing the risk of complications of diabetes.

While strong links between vitamin D and diabetes have been demonstrated, it remains to be determined whether this is by cause or association. Increasing endogenous levels of vitamins C and D can be achieved by dietary modifications, and in the case of vitamin D, also by judicious sun exposure. The availability of supplements enables a convenient method of reaching a prespecified serum vitamin C and D level. However, as with many other nutrients, the maximum health benefit is probably obtained by achieving the recommended daily intake of vitamin C as part of a balanced diet, and maintaining vitamin D status by regular sun exposure. Further studies – in particular, randomized controlled trials – are needed to determine if there is a benefit of routine supplementation with these vitamins in diabetes or for its prevention.

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Chapter 10 – Discussion, conclusions and future directions

This thesis demonstrated that skeletal muscle responds directly to vitamin D via its receptor (VDR) in this tissue. To reach this conclusion, different models of murine muscle and experimental techniques were employed. The presence of VDR was demonstrated in immortalised and primary muscle cells, muscle fibres and whole muscle tissue (16, 17). Effects of VDR on muscle cell differentiation, the regulation of muscle fibre size and strength, and the uptake of 25OHD within muscle fibres were demonstrated (17). These novel effects, in themselves, provide compelling evidence of functional vitamin D signalling in skeletal muscle and serve to clarify the controversy on VDR’s presence in muscle.

Vitamin D and cultured muscle cells

The C2C12 line is an established *in vitro* model of skeletal muscle, originally derived from satellite cells of dystrophic murine muscle. These cells were shown to express VDR mRNA and protein. VDR expression, together with the classic target gene CYP24A1, were activated in dose- and time-dependent manners by 1,25(OH)₂D (i.e. 1- 100 nM, 8-24 hrs respectively). Luciferase reporter studies also demonstrated the presence of functional CYP27B1 in these cells, explaining demonstrated increases in VDR and CYP24A1 in response to 25OHD. Similar effects were also demonstrated in primary muscle cells, corroborating these findings from C2C12 cells. To obtain a pure population of primary cells, they were isolated from healthy quadriceps muscle of 4 week-old mice and sorted for the specific myogenic cell-surface marker, CD56. The development of this novel technique occurred as part of these doctoral studies, and the original article has therefore been added (24).
Three distinct effects of treating C2C12 cells with 1,25(OH)₂D were demonstrated: (1) inhibition of proliferation, (2) inhibition of myotube formation during serum starvation, and (3) increased size of individual myotubes. VDR stimulation was demonstrated at various time-points during this study, indicating its role in the transcriptional regulation of muscle development. Although anti-proliferative effects of vitamin D have been reported, including in muscle cells, for over thirty years, precise mechanisms are unclear (27-29). This thesis demonstrated a significant increase in the proportion of quiescent muscle cells in response to 1,25(OH)₂D, associated changes in cell cycle genes (ATM, myc, Rb, cyclin D1) and post-translational phosphorylation of Rb (16). This effect was not associated with an increase in cell death.

To suppress C2C12 myotube formation, 1,25(OH)₂D directly altered myogenic regulatory factors myogenin, myf5 and desmin which were down-regulated following serum starvation (16). However, Garcia and colleagues reported an opposite effect of 1,25(OH)₂D in stimulating myotube formation when cells were not exposed to serum starvation (30). This discrepancy is interesting and suggests interconnected effects of 1,25(OH)₂D with myogenic regulatory factors and endogenous Insulin-like Growth Factors (IGFs), the latter being essential in myogenesis due to prolonged confluent culture (31, 32).

Together, the anti-proliferative and myotube-inhibitory effects of 1,25(OH)₂D may indicate the promotion of cell quiescence and protection from senescence. Such an effect of vitamin D has been demonstrated in human mesenchymal stem cells (33) and is relevant to muscle stem cells as their ability to maintain
quiescence determines their self-renewing capacity (34). 1,25(OH)$_2$D had no effect on necrotic or apoptotic C2C12 cells in this study (16). However, previous studies have reported modulatory effects of VDR on cell survival via regulation of FoxO and Sestrins (35, 36).

Both 25OHD and 1,25(OH)$_2$D exerted anabolic effects on C2C12 cells with doubling of myotube size (37). Mechanistically, 1,25(OH)$_2$D led to pronounced down-regulation of myostatin, a negative regulator of muscle mass and a member of the TGF-β superfamily. This novel finding occurs within the broader context of VDR’s emerging links with TGF-β, particularly in mesenchymal stem cells (38), skin (39) and in liver (40). In hepatic stellate cells, fascinating interactions between VDR and TGF-β at a cistromic level mediate the response to fibrosis (40). To assess whether similar effects may be responsible for VDR’s anabolic effect in muscle cells, chromatin immunoprecipitation (ChIP) studies are necessary to examine potential vitamin D response elements (VDREs) in the myostatin gene or the presence of co-regulated genes. Separate pathways may also be responsible for vitamin D’s effect on myotube size. In the study by Zhou et al, vitamin D had a protective effect against free fatty acid (FFA)-induced C2C12 myotube atrophy by interacting with insulin signalling components IRS-1 and Akt phosphorylation (41).

Cultured muscle cells provide prima facie evidence for the presence of VDR in skeletal muscle. These studies purport novel effects of vitamin D signalling on muscle development via biologically plausible pathways including TGF-β, cell cycle genes and myogenic regulatory factors by genomic and post-translational
mechanisms (16). Whilst these in vitro findings are intriguing, they are not conclusive and therefore prompted examination of VDR in muscle using in vivo models.

**Vitamin D receptor and skeletal muscle**

Skeletal muscle expresses a range of nuclear receptors that play vital roles in metabolism (42), mitochondrial function and exercise (43). However the presence of VDR in muscle has been controversial for many years. This is due to a number of confounding factors including the use of variable muscle models (i.e. cultured muscle cells vs. whole muscle), differences in the specificity of VDR antibodies and protein extraction methods. Previous studies assessing the presence of VDR in muscle have been summarised in table 2 of the literature review included with this thesis (Chapter 2) and recently published (2).

To circumvent these technical issues and comprehensively address this controversy, this thesis employed three different techniques (i.e. RT-PCR, western blot, immunohistochemistry) to examine VDR in four muscle models (i.e. C2C12, primary muscle cells, muscle fibres and whole muscle) (17). The highly specific VDR-D6 antibody was used in addition to positive and negative controls (i.e. intestine/kidney and muscle from VDRKO mice, respectively).

VDR transcript was detected in whole muscle but at substantially lower levels than the classic site of VDR activity, the duodenum (~ 4000-fold lower) (17). Following standard tissue lysis, VDR protein was not detected in muscle from mature mice consistent with previous reports using the same antibody (44).
However, following hyperosmolar lysis, VDR was detectable in mature muscle but at substantially lower levels than in duodenum and kidney. Hyperosmolar lysis may be necessary for the release of DNA-bound proteins including VDR and is more effective in protein unfolding and denaturation (45, 46). In addition, detection of VDR in whole muscle required longer exposure time on western blot (15 mins) and greater protein per sample (50 µg vs. 10 µg for duodenum). By describing specific conditions for the detection of VDR in murine muscle, this thesis both confirms the technical basis for this long-standing controversy and seeks to resolve it.

Further efforts to examine VDR’s presence in muscle focused on its potential functions in this tissue. Primary muscle fibres responded to physiological doses of 1,25(OH)₂D by significantly increasing their uptake of ³H-25OHD₃ (17). This effect was absent in muscle fibres from VDRKO mice and reversed by DIDS, a chloride channel blocker and classic inhibitor of VDR non-genomic effects. Together, these data indicate a non-genomic role for VDR in the ligand-mediated uptake of 25OHD into muscle.

Neville and De Luca first raised the possibility that muscle stores vitamin D in 1966 (47). These authors reported that a significant proportion of ³H-25OHD₃ localised within muscle following its intravenous administration in deficient rats (47). More recently, Abboud and colleagues demonstrated that vitamin D uptake in muscle is time-dependent and relies on the local activity of megalin and cubilin, endocytic receptors of the vitamin D binding protein (DBP) (48). This thesis advances these findings by demonstrating a modulatory role for VDR in
1,25(OH)_{2}D-mediated uptake of 25OHD in muscle (17). These data also suggest that VDR rapidly regulates megalin and cubulin in muscle to produce these effects. A related nuclear receptor, RXR, modulates these endocytic receptors at other sites (49), supporting similar mechanisms in muscle.

The significance of vitamin D uptake in muscle is unclear. Bound to DBP, 25OHD could be stored in muscle attached to actin and thereby escape hepatic catabolism (48). Upon degradation of DBP, 25OHD may then diffuse back into the circulation (50), or be converted locally to 1,25(OH)_{2}D as suggested the presence of functional CYP27B1 in primary myotubes (17). This cycling of vitamin D between muscle and the circulation, dependent upon the local activities of DBP and VDR, may determine the half-life and biologic activity of 25OHD. This effect may also respond to exercise, with increases in 25OHD uptake in muscle and its subsequent recirculation potentially explaining the higher serum levels of vitamin D found following activity (48, 51). Conversely, increases in serum vitamin D may lead to its enhanced uptake by muscle, increases in local mitochondrial function and improved physical performance (52). Vitamin D uptake may also play a role in muscle development. In support of this, radiolabelled 25OHD has been shown to cross the placenta in late gestation and thereafter, reside preferentially in the muscle of rat embryos (53).

Expression of VDR in muscle was significantly greater in young mice and there was a sequential drop in levels of muscle VDR from birth (17). In 3 week-old mice, VDR was localised clearly within nuclei and cytoplasm of muscle fibres. Similarly, C2C12 myoblasts showed greater levels of VDR than fully
differentiated myotubes. These findings support a developmental role for VDR in muscle, corroborating earlier work presented in this thesis on effects of 1,25(OH)2D on C2C12 muscle cell differentiation (16). Together these data prompted further evaluation of direct in vivo effects of VDR on muscle morphology and strength.

**Vitamin D, muscle strength and morphology**

In a manuscript submitted for publication and included in this thesis, the muscle phenotypes of VDRKO and vitamin D deficient mice were characterised. These different models allowed systematic assessment of effects of the vitamin D pathway whilst correcting associated biochemical abnormalities by dietary supplementation. The former model (VDRKO) relies on congenital whole-body ablation of VDR (exon 3) to examine global effects of vitamin D signalling (54). Vitamin D deficiency was induced in a second group of mice by housing them under incandescent, UV-free lighting and administering vitamin D-free diet (SF085-003, Specialty Feeds, Glen Forest, NSW) from 3 weeks age.

On grip strength testing, vitamin D deficient and VDRKO mice were significantly weaker than their controls and this difference progressed with duration of vitamin D deficiency and age. A stepwise decline in grip strength from WT to heterozygote to VDRKO mice strongly supported a role for VDR in muscle strength. Interestingly, differences in grip strength were greater in VDRKO vs. WT than vitamin D deficient vs. replete mice, possibly explained by complete ablation of vitamin D signalling in the KO model. A potential mechanism for weakness in these mice includes altered calcium handling within
muscle fibres. In support of this, mRNA expression of calcium-handling genes, specifically Calbindin-28K and sarcoendoplasmic reticulum calcium transport ATPase (SERCA) channels, were significantly reduced in VDRKO and vitamin D deficient mice. SERCA channels are essential for the release of Ca\(^{2+}\) into the cytosol and the subsequent process by which muscle contraction occurs via the interaction of troponin-tropomyosin complex and actin filaments (2). The Boland group has previously shown that 1,25(OH)\(_2\)D exerts rapid, non-genomic effects on calcium handling by muscle cells (55, 56). However, this thesis reports, for the first time, novel genomic effects of vitamin D on altering the calcium-handling apparatus in muscle. With reduced levels of these calcium-handling components in muscle, reduced Ca\(^{2+}\) entry into the cytosol during excitation-contraction coupling may lead to defects in muscle contraction and reduced strength. Other explanations for the reduced strength seen in these mice may include effects of vitamin D on the expression of muscle contractile proteins (57, 58) and extracellular matrix proteins (59). Therefore the relationship between vitamin D and muscle strength is potentially multifactorial and requires further examination. In vivo contractile studies may shed light on effects of vitamin D on muscle force, fatigue and recovery.

In addition to functional effects, this thesis demonstrates that vitamin D also exerts critical effects on muscle mass. VDRKO mice had significantly lighter muscles, even after correction for their lower body weight, and ~ 30% reduction in muscle fibre size. This was associated with other developmental changes including muscle fibre hyper-nuclearity and dysregulation of myogenic regulatory factors (MRFs). Although Endo et al reported similar findings in
VDRKO mice before weaning (60), this thesis describes a significant muscle phenotype in adult VDRKO mice, generated by ablation of exon 3 rather than 2, on rescue diet and with unaltered mineral levels. These in vivo findings concur with earlier studies in which vitamin D treatment exerted opposing effects by inhibiting proliferation and increasing myotube size in C2C12 cells (16). By contrast, vitamin D deficient mice did not display significant changes in muscle mass or fibre size. Differences in these models – congenital ablation of VDR versus prolonged vitamin D deficiency in the post-natal setting – may account for this discrepancy. In the absence of altered calcium and phosphorus defects, vitamin D’s predominant effect in muscle mass and morphology may relate to development and pre-natal myogenesis. Observational data support this conclusion. Children born to vitamin D-deficient mothers displayed significantly smaller arm-muscle area (61). Administration of vitamin D to European sea bass, directly after hatching, resulted in dose-dependent increases in white muscle fibre size and altered expression of muscle developmental genes (62). To further support this developmental role, the first appearance of VDR during embryogenesis is within mesoderm, the structure from which muscle and bone arise (63). VDR is also expressed within mesenchymal stem cells and immature myoblasts, both of which respond in vitro to vitamin D (38).

Evidence that vitamin D plays a clear role in fetal musculoskeletal development may have clinical implications in the recommended daily intake of vitamin D in pregnant women. Recent evidence also links maternal vitamin D levels with methylation at 4 sites of the RXR-A (retinoid X-receptor alpha) in umbilical cord
tissue, suggesting an intriguing epigenetic role for vitamin D in development via alterations in the heterodimeric partner of VDR (64, 65). If confirmed, a role for VDR in musculoskeletal development would be consistent with its known pleiotropic effects in other tissues (66).

Observational data also suggest that vitamin D plays a role in musculoskeletal ageing. Serum levels of vitamin D predict the risk of functional decline, loss of muscle mass and osteoporotic fracture in elderly subjects (67). VDR levels in muscle and bone decline with age (68, 69), rendering the musculoskeletal system more vulnerable to low vitamin D levels in the elderly. In this thesis, mice placed on a vitamin D deficient diet for 6 months showed upregulation of the atrophy marker, MuRF1 and a modest increase in the TGF-β myostatin. However, another ubiquitin ligase, MAFbx, was not altered and muscle mass and fibre size were not significantly different between vitamin D deficient and replete mice. A longer duration of vitamin D deficiency (> 6 months) may be necessary for atrophy-related mechanisms to lead to overt atrophy. It is also possible that normal mineral levels in the vitamin D deficient mice may have prevented the development of muscle wasting. A recent study supports the presence of complementary effects of vitamin D and calcium deficiency on muscle fibre atrophy (15). Sprague-Dawley rats with vitamin D deficiency showed partial reversal in parameters of muscle atrophy and reduced activation of the ubiquitin proteosome in muscle in response to a high-calcium diet (15).

Musculoskeletal ageing may also result, in part, from interactions between Vitamin D, FGF23 and its co-factor klotho. Premature ageing seen in FGF23
knockout mice, including osteopaenia, sarcopaenia and atherosclerosis, was completely reversed by ablation of 1α-hydroxylase (70). This suggests that, at a tissue level, 1,25(OH)₂D mediates age-related responses to FGF23. Klotho deficient mice also display premature ageing (71) and klotho levels are reduced in FGF23 KO mice (70), leading the authors to posit vitamin D as the common humoral pathway mediating these age-related effects (70). Interestingly, klotho deficient subjects display similar functional defects to VDRKO and vitamin D deficient mice with reduced grip strength and impaired running endurance (72, 73). Alterations in TGF-β and Wnt signalling leading to muscle wasting may be responsible for these effects in klotho deficient mice (71, 74). On the basis of these similarities, it is intriguing to speculate whether vitamin D and klotho may play interdependent and connected effects on skeletal muscle morphology and function during ageing.

By examining the muscle phenotype of VDRKO and vitamin D deficient mice, this thesis reports novel effects of vitamin D signalling on muscle development and ageing. Effects on grip strength, calcium handling, muscle fibre size and developmental gene expression are described here, further confirming the local expression and diverse functional effects of VDR in skeletal muscle.

**Future directions**

This work has raised several questions beyond the original scope of this thesis. A fascinating link between vitamin D and myostatin has been reported (2, 16, 17). Myostatin is a highly conserved member of the TGF-β superfamily which negatively regulates muscle mass (75). Genetic inactivation of myostatin leads to
marked increase in muscle mass in mammalian species including humans (76) and conversely, increases in myostatin may explain muscle wasting seen in chronic diseases such as renal failure, HIV, and emphysema (26). Muscle cells treated with 1,25(OH)₂D showed pronounced 10-fold down-regulation of myostatin mRNA with a doubling in myotube size (16). Conversely, VDRKO mice showed two-fold increase in myostatin expression, associated with 30 % reduction in muscle fibre size. Together, these findings raise the pertinent question of whether targeting VDR may be a viable therapeutic mechanism to inhibit myostatin and reverse disorders of muscle wasting. Already, intense efforts are underway in the search for effective myostatin inhibitors and decoy molecules for ActRIIB (i.e. the myostatin receptor) and these are at phase I-II clinical trials (26). In this thesis, increases in myostatin in vitamin D deficient mice were not clinically significant and in vitro doses of 1,25(OH)₂D leading to myostatin inhibition were supra-physiological. Therefore the use of VDR analogues, such as calcipotriol, may be more effective in addressing this important question. Such agents would allow greater stimulation of VDR for its potential tissue-modulatory effects whilst avoiding hypercalcaemia.

Molecular effects of vitamin D on muscle ageing are also an open question. Striking similarities in the phenotype of VDRKO and klotho deficient mice, such as reduced survival, osteopaenia/sarcopaenia, muscle weakness and reduced body mass raise the possibility that these pathways intersect in ageing (54, 71), warranting further research. Another feature of muscle ageing is a decline in the number and function of muscle stem cells responsible for the reduced regenerative capacity of old muscle (77). Two observations in this thesis suggest
that vitamin D signalling may play critical roles in muscle stem cell function (known as satellite cells). Firstly, significantly higher VDR levels in muscles of newborn and 3-week old mice correspond with a higher proportion of satellite cells at these ages (i.e. 30% of muscle cells compared to 3% in adult mice) (17, 77). Secondly, using a novel technique to isolate muscle precursor cells (24), this thesis shows that VDR expression was significantly higher in these cells than in whole muscle and that these cells express functional CYP27B1 (17). However precise in vivo effects of vitamin D on satellite cell function, in particular relating to quiescence, differentiation and survival, remain unknown and will be addressed in future studies.

Another component of muscle ageing is the progressive infiltration of muscle with adipose tissue. Interestingly, clinical studies report an association between vitamin D deficiency, muscle adiposity and poor functional outcomes (78-80). In muscle cells, 1,25(OH)$_{2}$D exerts dose-dependent inverse effects on PPAR$\gamma$2 expression and adipose transdifferentiation (81). Therefore, adipose tissue may represent an important piece of the puzzle in determining vitamin D’s role in muscle ageing (82).

Although the VDRKO mouse model has greatly advanced our knowledge of diverse biological effects of the vitamin D-endocrine system (83), there are inherent limitations to this model. Global effects of VDR ablation such as growth restriction, hairlessness, and hyperparathyroidism may confound the examination of tissue-specific effects. Conditional, muscle-specific deletion of VDR is therefore necessary to confirm the local effects of vitamin D that have been
reported in thesis. A similar approach has been taken by generating mice with cardiomyocyte-specific ablation of VDR. These mice display severe cardiac hypertrophy in response to an adrenergic stimulus, similar to the phenotype seen in whole-body VDRKO mice (84). Similar studies are currently underway, employing Cre-lox recombinase technology for VDR deletion in skeletal muscle.

**Concluding remarks**

This thesis reports the unequivocal presence of VDR in skeletal muscle and provides closure to this long-standing controversy. This thesis elucidates direct effects of vitamin D on muscle function relating to development, fibre morphology, strength and local uptake of 25OHD. These findings provide hope that the vitamin D endocrine system may be a potential therapeutic target for diseases of muscle wasting, sarcopaenia and acquired myopathies.
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References listed below are for Chapters 1 and 10 (Introduction and Discussion). References for other chapters are found therein.


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Appendix

To fulfill criteria for thesis by publication as set out by the University of Sydney (Chapter 4, clause 4.22 of the University of Sydney Higher Degree by Research Rule 2011), this appendix contains signed statements by co-authors of three major published works included in this thesis.

These statements affirm the major contribution that the PhD candidate, Christian Girgis, made in the experimental design and conduct, literature review, manuscript drafting and reviewer rebuttals for these publications.
As a co-author on the following publication:

**The roles of vitamin D in skeletal muscle: form, function, and metabolism.** Girgis CM, Clifton-Bligh RJ, Hamrick MW, Holick MF, Gunton JE. *Endocrine Reviews* 2013; 34(1):33-83

I attest that Christian Girgis contributed to this work in a satisfactory manner, fulfilling his role as primary author by conducting an extensive literature review, drawing conclusions from current evidence-base and preparing this review for publication.

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Signature
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I attest that Christian Girgis contributed to this work in a satisfactory manner, fulfilling his role as primary author by conducting an extensive literature review, drawing conclusions from current evidence-base and preparing this review for publication.

Mark W. Hamrick

October 10, 2014

Name

Date

Signature
As a co-author on the following publication:


I attest that Christian Girgis contributed to this work in a satisfactory manner, fulfilling his role as primary author by conducting an extensive literature review, drawing conclusions from current evidence-base and preparing this review for publication.

Michael F Holick 10/9/14
Name Date

Signature
As a co-author on the following publication:

**The roles of vitamin D in skeletal muscle: form, function, and metabolism.** Girgis CM, Clifton-Bligh RJ, Hamrick MW, Holick MF, Gunton JE. *Endocrine Reviews* 2013; 34(1):33-83

I attest that Christian Girgis contributed to this work in a satisfactory manner, fulfilling his role as primary author by conducting an extensive literature review, drawing conclusions from current evidence-base and preparing this review for publication.

Name: Jerry Gunton  
Date: 10/11/14

Signature:
As a co-author on the following publication:

**Vitamin D signaling regulates proliferation, differentiation, and myotube size in C2C12 skeletal muscle cells.** Girgis CM, Clifton-Bligh RJ, Mokbel N, Cheng K, Gunton JE. *Endocrinology* 2014 Feb;155(2):347-57

I attest that Christian Girgis contributed to this work in a satisfactory manner, fulfilling his role as primary author by designing/carrying out experiments, analysing data and preparing this work for publication.

Name

Date

Signature

17/10/2014
As a co-author on the following publication:

**Vitamin D signaling regulates proliferation, differentiation, and myotube size in C2C12 skeletal muscle cells.** Girgis CM, Clifton-Bligh RJ, Mokbel N, Cheng K, Gunton JE. *Endocrinology* 2014 Feb;155(2):347-57

I attest that Christian Girgis contributed to this work in a satisfactory manner, fulfilling his role as primary author by designing/carrying out experiments, analysing data and preparing this work for publication.

Nancy Mokbel

Name

10/10/2014

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Kim Cheng 16/10/14

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I attest that Christian Girgis contributed to this work in a satisfactory manner, fulfilling his role as primary author by designing/carrying out experiments, analysing data and preparing this work for publication.

[Signature]

Name

Date 10/11/14
As a co-author on the following publication:


I attest that Christian Girgis contributed to this work in a satisfactory manner, fulfilling his role as primary author by designing/carrying out experiments, analysing data and preparing this work for publication. A specific component of this work (radiolabelled Vitamin D uptake assay) was performed in collaboration with Professor Rebecca Mason.

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I attest that Christian Girgis contributed to this work in a satisfactory manner, fulfilling his role as primary author by designing/carrying out experiments, analysing data and preparing this work for publication. A specific component of this work (radiolabelled Vitamin D uptake assay) was performed in collaboration with Professor Rebecca Mason.

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**MYRIAM ABBOU**
As a co-author on the following publication:


I attest that Christian Girgis contributed to this work in a satisfactory manner, fulfilling his role as primary author by designing/carrying out experiments, analysing data and preparing this work for publication. A specific component of this work (radiolabelled Vitamin D uptake assay) was performed in collaboration with Professor Rebecca Mason.

David Fraser 13 October 2014
Name Date

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As a co-author on the following publication:


I attest that Christian Girgis contributed to this work in a satisfactory manner, fulfilling his role as primary author by designing/carrying out experiments, analysing data and preparing this work for publication. A specific component of this work (radionlabelled Vitamin D uptake assay) was performed in collaboration with Professor Rebecca Mason.

Rebecca S Mason 10 Oct 2014

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