

Declarations

The contents of this thesis are original and have not been presented for any other degree at this or any other university.

The research presented in this thesis is the work of the author, except where the contribution of others is specifically indicated.

All work on animals presented in this thesis has been carried out with the approval of The Children’s Medical Research Institute and The Children’s Hospital at Westmead combined animal care and ethics committee.

Prathibha C. Kahatapitiya ……/…../…………
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ABSTRACT

The BCNU + O\textsuperscript{6}benzylguanine (O\textsuperscript{6}BG) driven selective enrichment strategy was first established for enhanced transplantation of hematopoietic stem cells. This study describes a novel application of this BCNU + O\textsuperscript{6}BG driven selective enrichment strategy in skeletal muscle stem cell transplantation. Furthermore, this study addresses the three main limitations observed in previously reported skeletal muscle stem cell transplantation strategies. Limitation of ineffective donor cells which lack the ability for successful engraftment was overcome by using a heterogeneous population of donor cells which are present during a normal skeletal muscle regeneration response. The limitation of donor cell death upon transplantation as a result of competition from the endogenous stem cells of the host muscles was overcome by elimination of host muscle stem cells with BCNU + O\textsuperscript{6}BG treatment. Efficiency of elimination of host muscle stem cells was further demonstrated by the complete inhibition of a regeneration response up to 3 months in injured, BCNU + O\textsuperscript{6}BG treated muscles. The limitation of localised engraftment as a result of intramuscular injection of donor cells was also addressed. The transplanted donor cells demonstrated the ability to migrate via systemic circulation. This characteristic of the donor cells would allow the transplantation of cells via intraarterial or intravenous delivery which would overcome the limitation of localised engraftment. Finally, application of the BCNU + O\textsuperscript{6}BG driven selective enrichment strategy in skeletal muscle stem cell transplantation demonstrated enhanced engraftment. This is the first reported attempt of enhanced stem cell transplantation in a solid tissue achieved upon application of the BCNU + O\textsuperscript{6}BG driven selective enrichment strategy. This study provides the basis for application of the BCNU + O\textsuperscript{6}BG driven selective enrichment strategy in other tissues where stem cell transplantation is considered.
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<th>Description</th>
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<tr>
<td>AAV vector</td>
<td>Adeno-associated viral vector</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>BCNU</td>
<td>1,3-bis(2-chloroethyl)-1-nitrosourea</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic Fibroblast growth factor</td>
</tr>
<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>DAPC</td>
<td>Dystrophin associated protein complex</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DHFR</td>
<td>Dihydrofolate reductase</td>
</tr>
<tr>
<td>DMD</td>
<td>Duchenne muscular dystrophy</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EDL</td>
<td>Extensor digitorum longus</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EF1α</td>
<td>Elongation Factor 1α</td>
</tr>
<tr>
<td>EP</td>
<td>Early preplate</td>
</tr>
<tr>
<td>FACS buffer</td>
<td>Fluorescent activated cell sorting buffer</td>
</tr>
<tr>
<td>FACSorting</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence <em>in situ</em> hybridisation</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>------------</td>
<td>--------------------------------------------------------------------</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescence protein</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>i.a.</td>
<td>Intraarterial</td>
</tr>
<tr>
<td>i.m.</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
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<tr>
<td>LP</td>
<td>Late preplate</td>
</tr>
<tr>
<td>MCK</td>
<td>Muscle creating kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein 1</td>
</tr>
<tr>
<td>MDR1</td>
<td>Multi drug resistance 1</td>
</tr>
<tr>
<td>MDSCs</td>
<td>Muscle derived stem cells</td>
</tr>
<tr>
<td>mdx</td>
<td>Mouse model for Duchenne muscular dystrophy</td>
</tr>
<tr>
<td>MGMT</td>
<td>Methylguanine-methyltransferase</td>
</tr>
<tr>
<td>MGMT(P140K)</td>
<td>Methylguanine-methyltransferase, praline residue substituted with a lysine residue at position 140</td>
</tr>
<tr>
<td>MHC</td>
<td>Myosin heavy chain</td>
</tr>
<tr>
<td>MNF</td>
<td>Myocyte nuclear factor</td>
</tr>
<tr>
<td>MRF</td>
<td>Myogenic regulatory factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>N-CAM</td>
<td>Neural cell adhesion molecule (also known as CD56)</td>
</tr>
<tr>
<td>Ntx</td>
<td>Notexin</td>
</tr>
<tr>
<td>O(^6)BG</td>
<td>O(^6)benzylguanine</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>q-PCR</td>
<td>Quantitative Polymerase Chain reaction</td>
</tr>
<tr>
<td>RPM</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>Sca-1</td>
<td>Stem cell antigen 1</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immuno deficient</td>
</tr>
<tr>
<td>SDF-1</td>
<td>Stromal derived factor 1</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SP cells</td>
<td>Skeletal muscle Side Population cells</td>
</tr>
<tr>
<td>SCSC</td>
<td>Sodium Chloride – Sodium Citrate buffer</td>
</tr>
<tr>
<td>TA</td>
<td>Tibialis anterior muscle</td>
</tr>
<tr>
<td>TNF(\alpha)</td>
<td>Tumor necrosis factor (\alpha)</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
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Literature Review
1.1 Introduction

Stem cell transplantation is currently at the cutting edge of medical research due to potential application in regenerative medicine. Combined with gene therapy, stem cell transplantation can be used for the treatment of central nervous system disorders such as Parkinson’s, muscle disorders such as muscular dystrophy or cancer such as leukaemia. At present, bone marrow and haematopoietic stem cell transplantation combined with gene therapy is the most well established form of stem cell therapy in use (Bank, A., 2003, Gerull, S., et al., 2007, Persons, D.A., et al., 2003). The high success rate of bone marrow and hematopoietic stem cell transplantation is mainly due to the efficient delivery of donor cells via the circulatory system to the bone marrow. Furthermore, hematopoietic stem cells and bone marrow stem cells are highly proliferative cells, thus expansion in vivo of the transplanted cells is quite efficient resulting in substantial levels of donor cell engraftment (Bank, A., 2003). Recent developments in hematopoietic stem cell transplantation have established a strategy to selectively enrich donor cells with a survival advantage in the recipient tissues by treatment with alkylating chemotherapy (Gerull, S., et al., 2007, Reese, J.S., et al., 2008).

Skeletal muscle is not as receptive for donor cell transplantation as the haematopoietic system. However, the remarkable ability of skeletal muscle to undergo rapid regeneration following injury makes it a good candidate for successful donor cell transplantation. Although not as well established as haematopoietic stem cell transplantation, skeletal muscle stem cell transplantation has been studied since the late 1970s (reviewed by Cossu, G. and Sampaolesi, M., 2007, Partridge, T.A., 2002, Peault, B., et al., 2007). The first application of muscle stem cell transplantation in the Duchenne muscular dystrophy mouse model, mdx,
was attempted in 1989. It showed formation of genetically corrected fibers within the treated muscles (Partridge, T.A., et al., 1989). However, clinical application of muscle stem cell transplantation showed no significant functional improvements in Duchenne muscular dystrophy patients (Huard, J., et al., 1992, Karpati, G., et al., 1993, Mendell, J.R., et al., 1995, Miller, R.G., et al., 1997, Tremblay, J.P., et al., 1993). Limitations of muscle stem cell transplantation were soon discovered. Substantial cell death immediately after transplantation due to immune rejection and/or competition by endogenous cells result in inadequate engraftment (Fan, Y., et al., 1996, Karpati, G., et al., 1993). Inefficient donor cells which have limited ability for successful engraftment in host tissue has lead to an extensive search for a ‘stem-like’ cell population with the ability for contribution to myofiber formation in host muscles upon transplantation (Peault, B., et al., 2007). Most studies have performed intramuscular injection of donor cells which results in localized engraftment instead of the favorable widespread engraftment (Skuk, D., et al., 1999, Skuk, D., et al., 2002).

This study addresses the main limitations of skeletal muscle stem cell transplantation and demonstrates the application of a strategy which overcomes these limitations. The basis of the strategy used is derived from hematopoietic stem cell transplantation where donor cells which are given a selective survival advantage are enriched in the host muscles by treatment with alkylating chemotherapy. This study demonstrates the first reported successful enhancement of skeletal muscle stem cell transplantation using a selective donor cell enrichment strategy. Furthermore, this study together with hematopoietic stem cell transplantation studies can be used as a basis for application of the donor cell enrichment strategy in other organs where stem cell transplantation is considered.
1.2. **BCNU And O\(^6\)benzylguanine Driven Selective Enrichment**

*Strategy For Enhanced Stem Cell Transplantation*

1.2.1. *Methylguanine-methyltransferase (MGMT)*

MGMT protein was first purified from *E. Coli* (reviewed by Pegg, A.E., 1990). MGMT protein has the characteristic ability to repair DNA following DNA alkylation. A reactive cysteine residue on the MGMT protein allows the covalent binding of one alkyl group which leads to DNA repair (Pegg, A.E., 1990). The covalent binding of the alkyl group is irreversible and leads to inactivation of the repair protein, MGMT (Robins, P., *et al.*, 1983). This characteristic of MGMT gene expression was found to inhibit the action of alkylating chemotherapy (Nakatsu, Y., *et al.*, 1993). The human MGMT gene is approximately 170 kilobase pairs long, has 5 exons, and encodes a 950 nucleotide mRNA (Tano, K., *et al.*, 1990). The cDNA of human MGMT was cloned in 1990, and the sequence was submitted to GenBank (Dolan, M.E., *et al.*, 1990, Hayakawa, H., *et al.*, 1990). O\(^6\)benzylguanine (O\(^6\)BG) is a pseudosubstrate which also reacts with the active cysteine residue of MGMT leading to stoichiometric inactivation of the enzyme (Gerson, S.L., 2004). Therefore, O\(^6\)BG treatment leads to increased sensitivity of alkylating chemotherapy via inactivation of the DNA repair protein, MGMT (Xu-Welliver, M., *et al.*, 1998).

A mutant form of the human MGMT gene, MGMT(P140K), was first identified and isolated in 1998 (Xu-Welliver, M., *et al.*, 1998). The MGMT(P140K) mutant gene codes for a protein which substitutes a proline residue with a lysine residue at position 140. This results in steric restriction of the active site of the protein which allows covalent binding of alkyl groups but not the binding of the pseudosubstrate O\(^6\)BG (Davis, B.M., *et al.*, 2001, Gerson, S.L., 2004).
The MGMT(P140K) mutant gene therefore codes for a form of MGMT whose action cannot be inhibited by O\textsuperscript{6}BG and confers resistance against O\textsuperscript{6}BG plus alkylating chemotherapy (Gerson, S.L., 2004). Figure 1 summarises the consequences of wildtype MGMT and MGMT(P140K) expression in cells which are treated with alkylating chemotherapy plus O\textsuperscript{6}BG.

MGMT is mainly contained in the nucleus of cells, thus immunohistochemical results would show nuclear localization (Gerson, S.L., et al., 1986). Expression of MGMT was found to vary among species and between individuals of the same species (reviewed by Gerson, S.L., 2004). The study carried out by Gerson et al. (1986) found that human tissues have greater expression of MGMT than rat tissues and rat tissues have greater expression than mouse tissues. The highest level of MGMT was observed in the liver and a very low level of expression was observed in bone marrow and spleen. These results suggest that human tissues would have greater resistance to alkylating chemotherapy and mice tissues would be the most vulnerable of these three species. Expression of MGMT increases after treatment with alkylating chemotherapy, radiation and other forms of DNA damage (Bhakat, K.K. and Mitra, S., 2000, Biswas, T., et al., 1999, Gerson, S.L., 2004). The MGMT promoter has several transcription factor recognition sequences, which allows promoter activation via glucocorticoids such as dexamethasone and cAMP resulting in increased levels of MGMT expression (reviewed by Gerson, S.L., 2002).
**Figure 1: Effect of MGMT and MGMT(P140K) expression on BCNU and O\textsuperscript{6}benzylguanine treatment.**

Summarises the effect of BCNU treatment on wildtype cells and effect of BCNU plus O\textsuperscript{6}benzylguanine treatment on wildtype cells or cells transduced with the MGMT(P140K) gene. O\textsuperscript{6}BG: O\textsuperscript{6}benzylguanine.
1.2.2. Alkylating Chemotherapy

There are two categories of alkylating chemotherapeutic agents; DNA chloroethylating agents such as carmustine (BCNU) and DNA methylating agents such as temozolomide (Gerson, S.L., 2002, Tong, W.P., et al., 1982). Both types of drugs result in alkylation of DNA at the O\textsuperscript{6} position of guanine. Chloroethylating agents are much more potent cytotoxic drugs where less than 10 lesions result in cell death, whereas methylating agents require at least 6000 lesions. Chloroethylating agents result in a cross-link between the guanine and cytosine of the opposite strand of DNA thus disabling replication leading to double and single strand breaks which result in cell death (Gerson, S.L., 2002). Methylating agents cause a mismatched pairing between the modified guanine and thymidine. Mismatch repair following DNA replication results in DNA double strand breaks and cell death (Armstrong, M.J. and Galloway, S.M., 1997). Both chloroethylating and methylating chemotherapeutic agents damage DNA irrespective of the cell cycle status (Le Fevre, A., et al., 2007). However, cell death due to DNA damage occurs during mitosis.
1.2.3. In Vivo Selection Of Drug Resistant Haematopoietic Stem Cells


Achievement of enhanced donor cell survival via use of an \textit{in vivo} selective enrichment strategy with the aid of the MGMT(P140K) gene has only been studied for haematopoietic stem cell transplantation. There is a recent study which demonstrated selective enrichment of hematopoietic stem cell derived lung epithelial cells following hematopoietic stem cell transplantation (Reese, J.S., \textit{et al.}, 2008). There are no other reports of application of the BCNU plus O\textsuperscript{6}BG driven selective enrichment strategy in solid organs where stem cell transplantation is considered. Stem cell transplantation has been studied as a treatment in various tissues such as heart, liver, brain, pancreas and skeletal muscle. Enrichment of stem cell transplantation via the MGMT(P140K) and BCNU plus O\textsuperscript{6}BG driven selective enrichment strategy has not been studied in these tissues.
1.3. **Skeletal Muscle Biology**

Skeletal muscles are capable of contraction, which allow actions such as breathing and limb movement. Skeletal muscle is a complex structure where function depends on an integrated network of muscle fibers, motor neurons, blood vessels and extracellular connective tissue. Many muscle fibers, also known as myofibers, are surrounded by connective tissue and bundled together in a normal mature skeletal muscle. A myofiber is a multinucleated structure, which is formed by the fusion of individual myoblasts or muscle progenitor cells. Myofibers are the basic contractile units of skeletal muscle (Sherwood, L., 2001). Each myofiber has characteristic striations, which are brought about by the presence of thick and thin filaments. The myosin thick filaments and actin thin filaments are direct contributors to force generation. This complex organization of the structure of skeletal muscle allows for substantial amounts of force generation (Sherwood, L., 2001). A schematic representation of the structure of skeletal muscle can be seen in Figure 2. Contractions of the skeletal muscle occur through the relative movements of the thin and thick filaments of each muscle fiber (Sherwood, L., 2001). The release of acetylcholine at the neuromuscular junctions by the motor neurons causes muscle fiber membranes to become positively charged via activation of sodium/potassium channels (Figure 3). This results in generation of an action potential, which is instantaneously spread through all of the muscle fiber with the aid of T-tubules. The spread of the action potential triggers a release of Ca^{2+} that is stored in the sarcoplasmic reticulum. The release of Ca^{2+} in turn allows for the movement of the thick and thin filaments resulting in muscle contraction (Sherwood, L., 2001). The contractions of skeletal muscle is transformed to movement at the myotendinous junctions where skeletal muscle is joined to the skeleton via tendons (Sherwood, L., 2001).
Figure 2: Schematic representation of the structural organisation of skeletal muscle.

Figure 3: Schematic representation of excitation and contraction of skeletal muscle.

1.3.1 Development And Regeneration Of Skeletal Muscle

Skeletal muscle is susceptible to injury through trauma or through neuromuscular and genetic disorders. Skeletal muscle has, however, a remarkable ability to regenerate after injury. Many research studies have investigated the mechanisms behind the rapid repair and regeneration that occurs in skeletal muscle post-injury, since the 1950s (reviewed by Charge, S.B.P. and Rudnicki, M.A., 2004, Wagers, A.J. and Conboy, I.M., 2005). Regeneration of skeletal muscle bears a close resemblance to embryonic development. During embryonic development myoblasts are formed by the differentiation of mesodermal precursor cells (Charge, S.B.P. and Rudnicki, M.A., 2004). Once formed, myoblasts up regulate the expression of the Myogenic Regulatory Factor (MRF) family proteins, MyoD and Myf5 (Kablar, B., et al., 1998). A subpopulation of the myoblasts proliferate and terminally differentiate to form myocytes which express another MRF family protein, myogenin, and induce the expression of muscle specific proteins such as Myosin Heavy Chain (MHC) and muscle creatine kinase (MCK) (Charge, S.B.P. and Rudnicki, M.A., 2004). Mature muscle is formed by the fusion of these myocytes. The myoblast population that does not differentiate resides between the basal lamina and the plasma membrane as quiescent satellite cells (Charge, S.B.P. and Rudnicki, M.A., 2004).
Figure 4: Skeletal muscle repair following injury

This is a schematic representation of degeneration, regeneration and remodeling of skeletal muscle following injury.
There are three phases to muscle regeneration (Figure 4). They are degeneration, repair/regeneration and remodeling (Jarvinen, T.A.H., *et al.*, 2005). During degeneration, there is necrosis present in the myofibers and an inflammatory response is triggered. There are numerous studies which demonstrate a beneficial role of the inflammatory response towards muscle regeneration (Bondesen, B.A., *et al.*, 2004, Cantini, M. and Carraro, U., 1995, Chazaud, B., *et al.*, 2003, Lescaudron, L., *et al.*, 1999, Robertson, T.A., *et al.*, 1993). During degeneration, skeletal muscle releases chemoattractants such as monocyte chemoattractant protein 1 (MCP-1), macrophage derived chemokine, fractalkine, vascular endothelial growth factor (VEGF) and urokinase type plasminogen-activator receptor, which promotes infiltration of monocytes (immature macrophages) and macrophages (reviewed by Tidball, J.G., 2005). It has been proposed that macrophages release factors which are beneficial for muscle regeneration via promotion of myoblast proliferation (Bondesen, B.A., *et al.*, 2004, Cantini, M. and Carraro, U., 1995, Robertson, T.A., *et al.*, 1993). Macrophages also release the cytokine, tumor necrosis factor α (TNFα) (Warren, G.L., *et al.*, 2002). TNFα receptor null mice were shown to have decreased MyoD expression and slower recovery of muscle strength after freeze injury than wildtype mice (Warren, G.L., *et al.*, 2002). Injured skeletal muscle also releases stromal derived factor-1 (SDF-1) which is the ligand for CXCR4 receptor expressed on various haematopoietic cells (Pituch-Noworolska, A., *et al.*, 2003). Release of SDF-1 has been shown to recruit stem cell populations from the bone marrow which promote skeletal muscle regeneration (Aiuti, A., *et al.*, 1997).

During the repair phase of muscle regeneration, the debris is cleaned up via the phagocytosis carried out by macrophages (Jarvinen, T.A.H., *et al.*, 2005). Significance of the phagocytic
role of macrophages was further demonstrated by a study which selectively depleted monocytes/macrophages by systemic injection of liposomal clodronate (Summan, M., *et al.*, 2006). Summan et al. (2006) showed that repair of injured skeletal muscle is impaired due to absence of phagocytosis leading to prolonged clearance of necrotic fibers and increased fat accumulation. Once the debris is cleaned up via phagocytosis, the skeletal muscle undergoes regeneration. The regeneration of the muscle fiber involves proliferation, migration and fusion of satellite cells and muscle stem cells to form new myotubes (Charge, S.B.P. and Rudnicki, M.A., 2004). The myotubes are then spatially orientated, eliminating the random orientation of the fragments to form myofibers parallel to the muscle axis (Jarvinen, T.A.H., *et al.*, 2005). The remodeling phase occurs by maturation of the newly formed myofibers into contracting units and formation of new synapses by the somatic nervous system to reinnervate the regenerated muscle, thus returning the injured muscle to a functional state (Jarvinen, T.A.H., *et al.*, 2005). Reinnervation and revascularisation of skeletal muscle is believed to be supported by the basement membrane, also known as the basal lamina, surrounding the muscle fibers (Sanes, J.R., 2003).
1.3.2. Satellite Cells

Alexander Mauro discovered satellite cells in 1961, which he described as mononucleated cells located between the plasma membrane and the basal lamina in electron micrographs of frog skeletal muscle fibers (Mauro, A., 1961). He proposed that the role of the satellite cell is one of a muscle precursor cell, which would be involved in the repair and formation of myotubes during the process of muscle regeneration.

Alexander Mauro’s theory regarding the role of satellite cells was later proved by *in vitro* and *in vivo* studies of muscle fibers and muscle regeneration, where satellite cells were shown to proliferate and fuse to form multinucleated myotubes (Snow, M.H., 1978). Furthermore, M. H. Snow (1978) carried out a study where minced up mouse muscles labeled with tritiated thymidine were transplanted into a mouse muscle bed which was induced to regenerate through injury (Snow, M.H., 1978). He found that radioactive myotubes were formed in the regenerated muscle bed. Since it was known that only the mononucleated satellite cells in muscle beds undergo mitosis and that myofibres do not undergo mitosis (Moss, F.P. and Leblond, C.P., 1969), the study provided further evidence for Alexander Mauro’s theory regarding the role of satellite cells as muscle precursor cells.

Although there was evidence that satellite cells were muscle precursor cells, evidence for their stem cell-like characteristics were not established until recently. The definition of a stem cell lies in its ability to self-renew and give rise to one or many determined cell types (Wolpert, L., 1988). Various studies have shown the ability of satellite cells to differentiate into multinucleated myotubes as previously mentioned (Hawke, T.J. and Garry, D.J., 2001).
Collins et al. (2005) showed conclusive evidence that satellite cells are capable of self-renewal and are maintained as quiescent cells in a muscle bed after induced regeneration via muscle injury (Collins, C.A., et al., 2005).

Immature muscle, such as that found in neonatal mice, contains far more satellite cells than does adult muscle indicating active growth of muscle fibers (Hawke, T.J. and Garry, D.J., 2001). A quiescent satellite cell has a very high nuclear/cytoplasmic ratio (Schultz, E. and Lipton, B.H., 1982). They also have very few organelles and a higher amount of heterochromatin in the nucleus than do the nuclei of surrounding myotubes (Schultz, E. and Lipton, B.H., 1982). Activated satellite cells are morphologically different to quiescent satellite cells. Once activated, satellite cells expand which results in a lower nuclear/cytoplasmic ratio. The activated satellite cells also have more organelles and a lower level of heterochromatin owing to increased transcriptional activity in these cells (Schultz, E. and Lipton, B.H., 1982). Once they are activated, satellite cells also have more cytoplasmic processes that can be seen in electron micrographs (reviewed by Hawke, T.J. and Garry, D.J., 2001).

Satellite cells can be identified in electron micrographs as cells that are located between the basal lamina and the plasma membrane of the muscle fibers. However, during muscle regeneration, the compartments inhabited by quiescent satellite cells could also be inhabited by migrated phagocytic cells (Hawke, T.J. and Garry, D.J., 2001, Wagers, A.J. and Conboy, I.M., 2005). Therefore, alternative techniques such as the use of satellite cell markers should be used for unambiguous identification of satellite cells. There are several molecular markers
of satellite cells (Hawke, T.J. and Garry, D.J., 2001). Although some show selective expression in satellite cells, others are not specific for satellite cells. N-CAM (neural cell adhesion molecule; also known as CD56) was one of the first markers to be discovered in satellite cells (Covault, J. and Sanes, J.R., 1986). Covault, J. et al. (1986) also showed that although N-CAM is highly expressed in satellite cells, its expression is not specific to this cell type. There is also evidence of expression of VCAM-1 (vascular cell adhesion molecule-1) in satellite cells (Jesse, T.L., et al., 1998). VCAM-1 is expressed in endothelial cells and is responsible for initiation of the inflammatory response, stimulated by T-cells, monocytes and eosinophils. Jesse et al. (1998) demonstrated that VCAM-1 is responsible for the interaction between leukocytes and satellite cells during muscle regeneration following injury. MNF (myocyte nuclear factor) is another molecular marker of satellite cells (Garry, D.J., et al., 1997). MNF is highly expressed in a variety of tissues in mouse embryos, whereas in adult tissues MNF is downregulated in all cells except muscle progenitor cells. C-met and m-cadherin are two more molecules, which are not specific to, but are expressed by, satellite cells (Cornelison, D.D.W. and Wold, B.J., 1997). Mouse satellite cells also express the cell surface antigen CD34 (Beauchamp, J.R., et al., 2000, Montarras, D., et al., 2005). However, the most widely used marker to identify satellite cells is the transcription factor, Pax7. It was shown that Pax7 null mice do not have any satellite cells and also have a 10-fold increase in hematopoietic cells, suggesting Pax7 regulates the satellite cell lineage specification of early pluripotent progenitor cells (Seale, P., et al., 2000). Several markers are known that can be used to differentiate between activated and quiescent satellite cells. Activated cells were found to express MyoD and to a lesser extent myogenin and myf5 (Cornelison, D.D.W. and Wold, B.J., 1997). Cell markers of human satellite cells are different to mouse satellite cells.
The CD34 cell surface antigen is not present in human satellite cells, whereas CD56 is a reliable marker (Peault, B., et al., 2007).

A single satellite cell marker protein cannot be used independently to identify satellite cells because none of the markers are specifically expressed only in satellite cells. However, a combination of markers can be used to identify either activated, quiescent or all satellite cells. For example, CD34+/Pax-7+ positive cells isolated from skeletal muscle identify all satellite cells and Pax-7+/m-cadherin+/MyoD+/Myf5+ cells identify activated satellite cells.
1.3.3. *Skeletal Muscle Resident And Non-Resident Muscle Stem Cells*

During the last decade, other stem cell populations have been reported which are capable of entering the satellite cell niche and contributing myofiber formation (Peault, B., *et al.*, 2007). These muscle stem cell populations can be separated into two categories; (1) skeletal muscle resident (2) skeletal muscle non-resident. It is a formal possibility that some or all of the muscle stem cells described here are derived from the same haematopoietic stem cell population. A summary of various muscle stem cell populations are listed in Table 1.

Skeletal muscle derived side population (SP) cells were identified in 1999 which were able to contribute to myofiber formation upon intramuscular transplantation (Gussoni, E., *et al.*, 1999). SP cells are isolated by their characteristic appearance when run on a flow cytometer after labeling with Hoechst dye (Asakura, A., *et al.*, 2002). Further characterisation has shown that skeletal muscle SP cells are CD34⁺ve or –ve, Sca-1⁺ve and CD45⁻ve (Montanaro, F., *et al.*, 2004, Peault, B., *et al.*, 2007). Transplantation of skeletal muscle SP cells into injured or diseased muscle resulted in expression of activated and quiescent satellite cell markers (Bachrach, E., *et al.*, 2004, McKinney-Freeman, S.L., *et al.*, 2002). Although engraftment of skeletal muscle SP cells was seen in dystrophic muscle after intravenous and intra-arterial transplantation, it was not therapeutically significant (Asakura, A., *et al.*, 2002, Bachrach, E., *et al.*, 2006). Successful methodology for isolation of human skeletal muscle SP cells has also been reported (Pavlath, G.K., *et al.*, 1994).

Another skeletal muscle resident muscle stem cell population is muscle derived stem cells (MDSCs). A study carried out by Qu-Petersen et al. (2002) has shown that there are three
subpopulations of muscle-derived cells that can be classified according to their adherence properties (Qu-Petersen, Z., et al., 2002). Purification of primary cultures of muscle precursor cells is achieved using pre-plating techniques, where the majority of the fibroblasts can easily be eliminated due to their prominent adherence characteristics (Richler, C. and Yaffe, D., 1970). Using the same pre-plating techniques Qu-Petersen et al. (2002) successfully separated Early-Preplate (EP) cells, Late-Preplate (LP) cells and long time proliferating cells, which were named muscle derived stem cells (MDSC). EP and LP cells showed the characteristics of satellite cells indicated by their myogenic potential in addition to expression of markers such as desmin, myogenin, MNF and m-cadherin. However, the MDSC population showed remarkable differences to satellite cells. Not only do the MDSCs lack several of the satellite cell markers that are present in EP and LP cells, they also demonstrated more proliferation and differentiation potential than the EP and LP cells (Qu-Petersen, Z., et al., 2002). MDSCs were reported to express CD34 and Sca-1 cell surface antigens while lacking CD45 expression (Deasy, B.M., et al., 2005). Transplantation of MDSCs into dystrophic muscle resulted in successful engraftment into the host tissue (Deasy, B.M., et al., 2005, Deasy, B.M., et al., 2007, Mueller, G.M., et al., 2002, Qu-Petersen, Z., et al., 2002). There is further evidence that MDSCs contribute to innervation and revascularisation of injured muscle upon transplantation while not triggering an immune rejection response within host muscle (Qu-Petersen, Z., et al., 2002).

Montarras et al. (2005) reported an isolation procedure for a novel subpopulation of satellite cells. They isolated a subpopulation of satellite cells using fluorescence activated cell sorting (FAC Sorting). They have used a green fluorescence protein (GFP) tagged Pax3 mouse line
<table>
<thead>
<tr>
<th>Cell population</th>
<th>Cell markers</th>
<th>Delivery method (Reference)</th>
<th>Engraftment (time) (injected cell number)</th>
</tr>
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<tr>
<td>Skeletal muscle SP (mouse)</td>
<td>CD34&lt;sup&gt;+&lt;/sup&gt;/&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Intravenous (Arakura, A., et al., 2002)</td>
<td>1.4% of donor cells/TA, 14 days (3-6x10&lt;sup&gt;4&lt;/sup&gt;)</td>
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<td></td>
<td>CD&lt;sup&gt;sca-1&lt;/sup&gt;</td>
<td>Intraternal (Bachach, E., et al., 2006)</td>
<td>5-8% fibers/quads, 4-12 wks (5x10&lt;sup&gt;4&lt;/sup&gt;)</td>
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<tr>
<td></td>
<td>CD45&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
<td></td>
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<tr>
<td>MDSCs (mouse)</td>
<td>CD34&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Intramuscular (Gu-Petersen, Z., et al., 2002)</td>
<td>2178 fibers/gastroc, 10 days (3-4x10&lt;sup&gt;5&lt;/sup&gt;)</td>
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<td></td>
<td>Sca-1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Intramuscular (Mueller, G.M., et al., 2002)</td>
<td>407 fibers/EDL, 9 wks (1x10&lt;sup&gt;5&lt;/sup&gt;)</td>
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<td></td>
<td>CD45&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>Satellite cell Progenitor cells</td>
<td>CD34&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Intramuscular (Montrellas, D., et al., 2005)</td>
<td>587 fibers/TA, 3 wks (2x10&lt;sup&gt;4&lt;/sup&gt;)</td>
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<tr>
<td>(mouse)</td>
<td>Sca-1&lt;sup&gt;+&lt;/sup&gt;</td>
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<td></td>
<td>CD45&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>Myoendotheial (human)</td>
<td>CD34&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Intramuscular (Zheng, B., et al., 2007)</td>
<td>89 fibers/gastroc, 10 days (1x10&lt;sup&gt;4&lt;/sup&gt;)</td>
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<tr>
<td></td>
<td>CD56&lt;sup&gt;+&lt;/sup&gt;</td>
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<td></td>
<td>CD144&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>Mesoangioblasts (mouse, dogs)</td>
<td>CD34&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Intraternal (Sampalessi, M., et al., 2003)</td>
<td>50% fibers/soleus, 4 months (5x10&lt;sup&gt;5&lt;/sup&gt;)</td>
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<td></td>
<td>CD45&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Intraternal (Sampalessi, M., et al., 2006)</td>
<td>0-50% fibers/multiple, &gt;4 months (5x10&lt;sup&gt;5&lt;/sup&gt;)</td>
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<td>Pericytes (human)</td>
<td>CD56&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Intraternal (Dellavalle, A., et al., 2007)</td>
<td>430 fibers/TA, 28 days (1.5x10&lt;sup&gt;5&lt;/sup&gt;)</td>
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<td></td>
<td>ALP&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>CD133 progenitors (human)</td>
<td>CD133&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Intraternal (Gavina, M., et al., 2006)</td>
<td>~4% fibers/quads, 60 days (5x10&lt;sup&gt;5&lt;/sup&gt;)</td>
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<td></td>
<td>CD34&lt;sup&gt;+&lt;/sup&gt;/&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Intramuscular (Torrente, Y., et al., 2004)</td>
<td>574 fibers/TA, 60 days (2x10&lt;sup&gt;4&lt;/sup&gt;)</td>
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<tr>
<td></td>
<td></td>
<td>Intramuscular (Torrente, Y., et al., 2004)</td>
<td>23 fibers/TA, 60 days (5x10&lt;sup&gt;5&lt;/sup&gt;)</td>
</tr>
</tbody>
</table>

**Table 1: Muscle stem cell populations used in various transplantation studies**

This table summarises various cell populations used in transplantation studies. Cell markers originally used for identification of these cell populations along with delivery methods in transplantation as well as engraftment data reported by these studies are also summarised. SP: side population, MDSCs: muscle derived stem cells, ALP: alkaline phosphatase, TA: muscles, quads: quadricep muscles, EDL: extensor digitorum longus, gastroc: gastrocnemius, multiple: sartorius, tibialis cranialis and gastrocnemius. [time]: when recipient tissues were collected for analysis post transplantation.
to isolate satellite cells from the mouse diaphragm using flow cytometry. Then they have characterized parameters for isolation of a subpopulation of satellite cells from this population using FAC sorting (Montarras, D., et al., 2005). The parameters were such that the cells can be isolated by sorting for CD34\(^+\)/Sca-1\(^-\)/CD45\(^-\), small and non-granular cells. Using these parameters, cells were isolated from the diaphragm. The CD34\(^+\)/Sca-1\(^-\)/CD45\(^-\) small and non-granular cells from FAC Sorting were also 93% Pax-7\(^+\) and myogenic, where only 8% expressed MyoD, the marker for activated satellite cells. The isolated subpopulation of satellite cells were transplanted into the skeletal muscle of \(mdx\) mice which resulted in successful engraftment in the dystrophic muscle (Montarras, D., et al., 2005).

Myoendothelial cells were identified in human skeletal muscle biopsies which express CD144 and CD34 cell surface antigens while lacking CD56 cell surface antigen expression (Zheng, B., et al., 2007). These cells were transplanted intramuscularly into gastrocnemius muscle of severe combined immune deficient (SCID) mice, which were injured with cardiotoxin injection. The study demonstrated that myoendothelial cells contribute to host muscle regeneration more than human satellite cells (CD144\(^-\)/CD34\(^-\)/CD56\(^+\)) or endothelial cells (CD144\(^+\)/CD34\(^+\)/CD56\(^-\)) (Zheng, B., et al., 2007).

Recently, there have been several reports of skeletal muscle non-resident muscle stem cell populations which are capable of myofiber formation. These cells have been studied with great interest due to their potential for transplantation via the systemic circulation, where donor cells may have the ability to ‘home’ to injured muscles. Among these cells,
mesoangioblasts have been reported as the most successful at ‘homing’ into downstream dystrophic muscle once transplanted via intraarterial delivery (Galvez, B.G., *et al.*, 2006, Sampaolesi, M., *et al.*, 2003, Sampaolesi, M., *et al.*, 2006). Mesoangioblasts were first discovered in 1999 and isolated from the wall of mouse embryonic dorsal aorta (De Angelis, L., *et al.*, 1999). In recent studies, mesoangioblasts have been isolated from the vessels of postnatal tissues of mice (Sampaolesi, M., *et al.*, 2003) and dogs (Sampaolesi, M., *et al.*, 2006). Mesoangioblasts express CD34, CD31 and Sca-1 cell surface antigens while lacking CD45 cell surface antigen expression (Sampaolesi, M., *et al.*, 2003, Sampaolesi, M., *et al.*, 2006). Transplantation of healthy mesoangioblasts isolated from golden retriever dogs into a canine model for Duchenne muscular dystrophy (DMD) showed formation of dystrophin expressing fibers in downstream skeletal muscles (Sampaolesi, M., *et al.*, 2006). Furthermore, this study shows the improvement of functional strength of dystrophic muscles in these canine models. Despite the demonstrated success of mesoangioblast transplantation into a DMD canine model, this study has been heavily criticised (Bretag, A., 2007, Davies, K.E. and Grounds, M.D., 2006, Grounds, M.D. and Davies, K.E., 2007). Immunosuppressant drugs were used on dogs which received heterologous donor cells in order to avoid immune rejection (Sampaolesi, M., *et al.*, 2006). There is substantial evidence, demonstrated by previous studies performed in muscular dystrophy models, for decreased dystrophic symptoms resulting from immunosuppressant drugs and steroid treatment (Bushby, K., *et al.*, 2004, Miller, R.G., *et al.*, 1997). Criticisms of the study carried out by Sampaolesi, M. et al. (2006) arose due to the absence of controls to account for the effect of immunosuppressant drugs on dystrophic symptoms.
Another skeletal muscle non-resident muscle stem cell population is pericytes. Pericytes are perivascular cells isolated from capillaries and microvessels from adult skeletal muscle (Dellavalle, A., et al., 2007). These cells were first isolated from human muscle biopsies and were shown to express alkaline phosphatase cell surface antigen while lacking CD56 cell surface antigen expression (Dellavalle, A., et al., 2007). Transplantation of human pericytes via the femoral artery into severely combined immune deficient-\textit{mdx} (SCID-\textit{mdx}) mice resulted in generation of dystrophin positive muscle fibers in the downstream muscles (Dellavalle, A., et al., 2007). Pericytes isolated from microvasculature also demonstrated progenitor characteristics for chondrocytes, adipocytes, osteocytes and odontoblasts (Collett, G.D.M. and Canfield, A.E., 2005, Farrington-Rock, C., et al., 2004, Peault, B., et al., 2007).

Furthermore, the ability of pericytes to differentiate down the myogenic lineage was shown to be independent of the resident tissue. Pericytes isolated from adipose and pancreatic tissues were capable of generating myotubes in culture and myofibers in SCID mouse muscles (Peault, B., et al., 2007). Pericytes are believed to be the human equivalent of mesoangioblasts (Cossu, G. and Sampaolesi, M., 2007).

CD133$^{+ve}$/CD34$^{+ve/-ve}$ human circulating cells have been isolated from umbilical cord blood (Koponen, J.K., et al., 2007), peripheral blood (Gavina, M., et al., 2006, Torrente, Y., et al., 2004) or skeletal muscle (Torrente, Y., et al., 2007). These cells, upon intramuscular injection, demonstrated an improvement in regeneration in ischemic muscle (Koponen, J.K., et al., 2007). Transplantation of CD133$^{+ve}$ cells intraarterially into exercised \textit{mdx} mice showed enhanced ‘homing’ of these cells into the exercised muscles due to an increased expression in VCAM-1 ligand expression (Gavina, M., et al., 2006). There is also evidence
of CD133\(^{+}\)ve cells entering the satellite cell niche under the basal lamina of muscle fibers, upon intraarterial transplantation, which demonstrates long term engraftment of these cells in the host SCID-mdx mouse muscles (Torrente, Y., et al., 2004). A clinical trial of CD133\(^{+}\)ve cell transplantation in Duchenne muscular dystrophy patients via intramuscular injections showed no side effects of the therapy, however, no functional improvement was reported (Torrente, Y., et al., 2007). Some CD133\(^{+}\)ve cells also express the CXCR4 receptor (Gavina, M., et al., 2006). CXCR4 is also expressed on skeletal muscle satellite cells and bone marrow derived stem cells (Pituch-Noworolska, A., et al., 2003). Stromal derived factor-1 (SDF-1) is the ligand for the CXCR4 receptor. Injured skeletal muscle releases various chemokines and among these is SDF-1 (Ratajczak, M.Z., et al., 2003). SDF-1-CXCR4 axis has been thoroughly studied and there is a substantial amount of evidence which supports the ‘homing’ of CXCR4 positive cells from bone marrow to various tissues including skeletal muscle and neural tissues (Aiuti, A., et al., 1997, Pituch-Noworolska, A., et al., 2003, Ratajczak, M.Z., et al., 2004).

Recently, embryonic stem cell transplantation has also been shown to successfully contribute to fiber formation and increased functional capacity in dystrophic mice (Darabi, R., et al., 2008). This study demonstrated the first successful use of embryonic stem cell transplantation in mdx mice with no formation of teratomas.
1.4 Duchenne Muscular Dystrophy

1.4.1 Disease Onset And Characteristics

Duchenne muscular dystrophy (DMD) is caused by a mutation in the dystrophin gene located on the X chromosome (Lovering, R.M., et al., 2005). DMD affects 1 in 3500 males who have an average life expectancy of 20 to 30 years (Nowak, K.J. and Davies, K.E., 2004). Dystrophin is the largest gene in the human genome and the DMD patients lack the dystrophin protein due to a large insertion or deletion or a point mutation in the gene (Nowak, K.J. and Davies, K.E., 2004). The DMD patients show progressive skeletal muscle weakness. First signs of weakness are observed in the limb-girdle muscles by the age of 5 years which progress towards inability to walk by the age of 8 to 12 years, leading to eventual death in most cases due to respiratory or cardiac failure by the age of 20 to 30 years (Lovering, R.M., et al., 2005, Nowak, K.J. and Davies, K.E., 2004).

Dystrophin protein in healthy skeletal muscles forms a complex with other proteins such as dystroglycans, sarcoglycans, integrins and caveolin at the sarcolemma of muscle fibers (Ervasti, J.M. and Campbell, K.P., 1991). The dystrophin-associated protein complex (DAPC) acts as a link between the contractile apparatus in the muscle fiber with the extracellular matrix and provides stability to the sarcolemma during muscle contractions (Lovering, R.M., et al., 2005). Absence of dystrophin in DMD patients causes instability in the sarcolemma and leads to muscle fiber membrane damage and eventual fiber necrosis. Necrotic fibers can not undergo normal regeneration due to a limited regenerative capacity in DMD muscles and eventually leads to fat and connective tissue accumulation in the muscles (Lovering, R.M., et al., 2005).
1.4.2. Therapies For Duchenne Muscular Dystrophy

1.4.2.1 Gene Therapy

Duchenne muscular dystrophy (DMD) is caused by the lack of dystrophin protein expression. Therefore, most preclinical strategies have used gene therapy approaches, where a normal dystrophin gene could be inserted into the abnormal muscle cells. This approach has had limited success for several reasons. The dystrophin gene is large (2.4 mega bases) and cannot be inserted into a viral vector (Li, S., et al., 2006). DMD affects all skeletal muscles of the body and cardiac muscle, therefore a successful gene therapy approach requires the correction of the mutation in all of these muscles. This poses more complications since muscle fibers are post mitotic and viral vectors are immunogenic. However, Adeno-associated viral (AAV) vectors have shown the capacity of infecting post mitotic muscle fibers and reduced immunogenicity (Gregorevic, P., et al., 2004, Wang, Z., et al., 2007).

Recent research in gene therapy strategies for DMD treatment involves correcting the mutation of the gene in patients with DMD via delivery of a small nuclear RNA targeting the mutant exons resulting in a small but functional dystrophin protein (Fletcher, S., et al., 2007, Goyenvalle, A., et al., 2004). Pre-clinical studies where this strategy was applied to mdx mice demonstrated successful rescue of the dystrophin protein (Aartsma-Rus, A., et al., 2003, Alter, J., et al., 2007, Bremmer-Bout, M., et al., 2004, Lu, Q.L., et al., 2003, Tidball, J.G. and Spencer, M.J., 2003). Phase I and II clinical trials are being carried out for this strategy using a small nuclear U7 RNA targeting exons 6-8 delivered systemically in an AAV vector (reviewed by Cossu, G. and Sampaolesi, M., 2007). Research in order to improve the efficiency of this gene therapy strategy is ongoing. One of the limitations of this strategy is
inefficient delivery of the oligonucleotides which are responsible for exon skipping in the mutated dystrophin gene (McClorey, G., et al., 2005). Systemic administration of the oligonucleotides does not result in uptake and dystrophin expression by cardiac muscle (Moriuchi, T., et al., 1993).

Another form of gene therapy is using a mini-dystrophin gene that can be successfully packaged into viral vectors and results in expression of a functional dystrophin protein in mdx mice (Decrouy, A., et al., 1997, Dunckley, M.G., et al., 1993, Li, S., et al., 2006, Lochmuller, H., et al., 1996, Vincent, N., et al., 1993, Yang, L., et al., 1998). This strategy is also being trialed in a Phase I clinical trial where a mini-dystrophin gene is delivered intramuscularly into patients in an AAV vector (reviewed by Cossu, G. and Sampaolesi, M., 2007). However, mini-dystrophin genes are yet to demonstrate the ability for successful treatment of DMD patients. At present, inefficient delivery of the mini-dystrophin gene and inefficient amelioration of dystrophic phenotype by the resulting dystrophin protein are the main limitations of this strategy (Kapsa, R., et al., 2003).
1.4.2.2. Pharmacological Therapies

Current treatment of DMD is administration of corticosteroids such as Prednisone or Deflazacort (Radley, H.G., et al., 2007). These drugs reduce inflammation and reduce muscle wasting, however the exact mechanism of action is unknown. Inflammation present within skeletal muscles of DMD patients is thought to be detrimental to the dystrophic muscles resulting in further muscle fiber damage (Tidball, J.G., 2005). Immunosuppressants have also been shown to result in increased muscle strength and reduced pathological symptoms in mdx mice (De Luca, A., et al., 2005, Sharma, K.R., et al., 1993). Other pharmacological interventions for DMD includes anti-cytokine drugs, antioxidants, anabolic agents and protease inhibitors (reviewed by Radley, H.G., et al., 2007). Further nutritional interventions such as amino acid supplements are also available for DMD patients in an attempt to promote protein synthesis in order to increase muscle mass and reduce muscle wasting (Radley, H.G., et al., 2007).

Utrophin is a homolog of dystrophin and upregulation of this gene has been shown to compensate for the lack of dystrophin in DMD mouse model mdx (Tinsley, J., et al., 1998). Several synthetic compounds that have shown to reduce inflammation, eccentric damage and fibrosis in dystrophic muscles due to an upregulation of utrophin are currently being tested in preparation for clinical trials (Cossu, G. and Sampaolesi, M., 2007).
1.4.2.3. **Skeletal Muscle Stem Cell Transplantation**

Cell therapy for DMD attempts to replace the genetically abnormal satellite and muscle stem cell population within DMD muscles with cells from a healthy donor or genetically corrected donor cells. The aim of transplantation is to achieve engraftment of healthy or genetically corrected donor cells which may consequently alleviate muscle weakness and muscle wasting of DMD skeletal muscles. Initial studies using an mdx mouse model for DMD demonstrated that formation of dystrophin positive muscle fibers within dystrophic muscle is possible upon transplantation of healthy muscle stem cells (Partridge, T.A., et al., 1989). Since this finding, various studies have utilised different strategies in order to achieve successful skeletal muscle stem cell engraftment upon transplantation (Figure 5).

There has been an extensive search for a population of muscle stem cells that may result in enhanced engraftment upon transplantation. Muscle derived stem cells (MDSCs), skeletal muscle derived side population (SP) cells, myoendothelial cells, CD133+ve cells, mesoangioblasts and pericytes are a few of the cell populations which have shown potential for enhanced engraftment (reviewed by Cossu, G. and Sampaolesi, M., 2007, Peault, B., et al., 2007). Skeletal muscle stem cell transplantation involves either intramuscular or systemic injection of donor cells. Intramuscular skeletal muscle stem cell transplantation results in localised engraftment of cells due to limited migration of donor cells within the host tissue (Skuk, D., et al., 1999, Skuk, D., et al., 2002). Duchenne muscular dystrophy affects all skeletal muscles of the body and results in death primarily due to respiratory failure caused by weakness in the diaphragm muscle (Nowak, K.J. and Davies, K.E., 2004).
Figure 5: Skeletal muscle stem cell transplantation strategies.
Intramuscular transplantation of skeletal muscle stem cells as a therapy for DMD remains inadequate since this does not allow widespread rescue of DMD skeletal muscles. Recently, many studies have reported systemic delivery of skeletal muscle stem cells via the host circulatory system (Bachrach, E., et al., 2006, Dellavalle, A., et al., 2007, Galvez, B.G., et al., 2006, Sampaolesi, M., et al., 2003, Torrente, Y., et al., 2001). All of these studies show restoration of dystrophin in muscle fibers of downstream skeletal muscles, although some studies have demonstrated loss of donor cells in the filter organs such as lungs, liver and spleen (Galvez, B.G., et al., 2006). Many studies have shown that inflammation present within the injured muscles enhances skeletal muscle stem cell ‘homing’ upon systemic delivery of donor cells (reviewed by Peault, B., et al., 2007). Intraarterial delivery is proposed to be more efficient in allowing migration of donor cells into downstream injured muscles due to the intricate skeletal muscle capillary network (Peault, B., et al., 2007). Intravenous delivery of donor cells is thought to be less efficient resulting in a greater loss of cells in filter organs (Peault, B., et al., 2007).

In order to achieve an enhanced contribution of donor cells in formation of muscle fibers after transplantation, the existing muscle fibers need to be injured or destroyed. Host skeletal muscle fiber injury creates a ‘niche’ for transplanted skeletal muscle stem cells where the degenerating muscle fibers release agents which activate the donor stem cells and promote engraftment (Skuk, D. and Tremblay, J.P., 2003). There are several strategies used in skeletal muscle stem cell transplantation at present which injure the recipient skeletal muscle and induce a regeneration response. Intramuscular injection of tiger snake venom, notexin is the most commonly used strategy in muscle stem cell transplantation studies (Huard, J., et al.,
1994, Kinoshita, I., et al., 1994). Notexin treatment causes homogenous degeneration of muscle fibers while satellite cells, basal lamina, nerves and blood vessels remain intact (Skuk, D. and Tremblay, J.P., 2003, Vignaud, A., et al., 2005). An intact basal lamina is quite important in skeletal muscle regeneration since it provides a ‘framework’ for the satellite cells to align in correct orientation and fuse to form new myofibers (Sanes, J.R., 2003). Intact nerves and blood vessels aid in faster regeneration of skeletal muscle (Vignaud, A., et al., 2004). Other muscle injury strategies such as freezing and denervation/devascularisation were all shown to be less efficient regeneration models (Vignaud, A., et al., 2005). Freezing results in basal lamina, nerve and blood vessel damage whereas denervation/devascularisation results in a slower regeneration response where the muscle damage is not homogenous (Vignaud, A., et al., 2005). Another agent which is used to injure skeletal muscle is Bupivacaine (marcaine). Bupivacaine was shown to cause a similar regeneration response to notexin (Orimo, S., et al., 2004, Wakata, N., et al., 2001).

1.5 Aim Of The Study

The aim of this study is to successfully repopulate a regenerating muscle bed with transplanted donor muscle stem cells by overcoming the limitations of skeletal muscle stem cell transplantation observed in other reported studies. In order to achieve this aim, a novel enrichment strategy is utilised to selectively enhance donor cell engraftment. The novelty of the strategy lies in the ability to selectively enrich the transplanted cells \textit{in vivo}. It is hypothesised that the enrichment strategy would allow the successful expansion of the donor cells which survive the transplantation procedure. Furthermore, the strategy is based on selective elimination of host muscle stem cells, leading to enhanced engraftment of donor cells.

The approach is derived from a successful strategy developed for haematopoietic stem cell transplantation. There is conclusive evidence reported for selective enrichment of haematopoietic stem cells \textit{in vivo}, post transplantation, using a mutant form of the drug resistance gene methylguanine methyltransferase (MGMT). The mutant MGMT(P140K) gene expression provides resistance against alkylating chemotherapy while the wildtype cells remain sensitive to the cytotoxicity. Consequently, selective enrichment of cells which express MGMT(P140K) can be achieved among a population of wildtype cells using alkylating chemotherapy.

This study has used muscle stem cells isolated from MGMT(P140K) transgenic mouse skeletal muscles. The host muscle is treated with the muscle toxin, notexin prior to transplantation, in order to induce regeneration. This promotes the receptivity of the host
muscle bed for the alkylating chemotherapeutic drugs and the transplanted cells. The main hypothesis of the study is that transplantation of muscle stem cells which express the MGMT(P140K) gene provides a survival advantage for the transplanted donor cells over the endogenous cells against alkylating chemotherapy. Consequently, there will be selective expansion of donor cells within the host muscle bed leading to enhanced engraftment.
CHAPTER 2

Methods & Materials
2.1 Cell Culture

C2C12 cells were cultured in growth medium containing low glucose DMEM (Invitrogen; product number 11885-084), 20% Fetal Bovine Serum (JRH Biosciences; product number 12003-1000M) and 0.25% chicken embryo extract (MP Biomedicals, Inc., France; product number 285015). Culture medium was replaced every two days and the cells were passaged when the culture is approximately 80% confluent. To induce differentiation cells were cultured in growth medium till approximately 80% confluent and then grown in differentiation medium with low glucose DMEM + 2% horse serum (Biosciences Pty, Ltd.; product number 15-040-0500V). The medium was replaced every two days.

Human myoblasts were cultured on calf skin collagen (Calbiochem; product number 234112) coated tissue culture dishes or plates in growth medium (F10 Ham’s nutrient medium [Invitrogen; product number 11550-043] with 20% fetal bovine serum, 1% chicken embryo extract and 1% 100X PenStrep [Invitrogen; product number 15070-63]). In order to induce differentiation, human myoblasts were first cultured in starvation medium (1% 100X-PenStrep + 0.025% of 2.5x10⁻⁶M dexamethasone [Sigma; product number D4902] + 1% of 1x10⁻⁶M insulin [Sigma; product number I6634] in DMEM) overnight. The starvation medium was then replaced by differentiation medium (5% horse serum + 1% 100X PenStrep + 0.5% chicken embryo extract + 0.025% of 2.5x10⁻⁶M dexamethasone + 1% of 1x10⁻⁶M insulin). Medium was replaced every two days.
MGMT(P140K) viral vector packaging PA137 cells were cultured in low glucose DMEM or F10 Ham’s nutrient medium with 10% heat inactivated fetal bovine serum until ~90% confluent and then used for transductions.

CD34\textsuperscript{+ve} cells were cultured after isolation from skeletal muscles. 60mm culture dishes were coated with gelatin prior to culture by incubating the dishes with a sufficient volume of 1% calf skin gelatin (AJAX; Product number 1080) made up in sterile Milli Q water at 37\(^\circ\) Celsius for 30 minutes. 1.5x10\textsuperscript{6} cells were plated in the gelatin coated dishes. The cells were cultured for 24 hours in muscle stem cell culture medium (40% DMEM high glucose, glutamax [GIBCO/Invitrogen; Product number 61965], 40% Ham’s F12 glutamax nutrient mix [GIBCO/Invitrogen; Product number 31765], 20% FBS, 10ng/ml bFGF and 1X Insulin/Transferrin/Selenium (Sigma Aldrich; Product number I3146). The floating cells were then transferred into a new dish and cultured separately. Medium of both cultures were replaced every 2 days with fresh medium.
2.2 Retroviral Transduction Of Cells

Concentrated vector (25 µl) was added to C2C12 cultures or human myoblasts (100 mm dishes) in the presence of 20 µg/ml polybrene for overnight culture. To produce MFG-MGMT(P140K) vector supernatant, plasmid was transfected into PA317 packaging cells by calcium phosphate precipitation and vector producing cells enriched for by exposure to O\textsuperscript{6}BG (10 µM) and 100 µM BCNU for 2 hours. Supernatant collected from transfected PA317 cells was then used to transduce C2C12 cells and human myoblasts. C2C12 cell transduction: PA137 cell culture medium was collected and 20 µg/ml polybrene added. The composition of fetal bovine serum and chicken embryo extract was adjusted as per C2C12 growth medium. The medium was filtered though a 0.45 µM syringe filter and used to replace the medium of C2C12 cell cultures. C2C12 cells were cultured overnight and the medium replaced with normal growth medium the following day. Human myoblast transduction: PA137 cell culture medium was collected and 20 µg/ml polybrene added. The composition of fetal bovine serum, chicken embryo extract and PenStrep was adjusted as per human myoblast growth medium. Medium was filtered through a 0.45 µM syringe filter and used to replace the medium of human myoblast cultures. Human myoblasts were cultured overnight and the medium replaced with normal growth medium the following day.
2.3 \textit{Notexin Treatment Of CD34}^{+ve} \textit{Skeletal Muscle Cells}

Notexin treatment of CD34$^{+ve}$ skeletal muscle cells was carried out in 96 well plates. The cells were plated at approximately 30\% density and cultured overnight. The following day, 25ul, 50ul or 100ul of notexin (Latoxan, France; product number L8104) was added to the cultures at 40ng/ul. Negative controls were carried out where instead of notexin. An equivalent volume of saline was added to the cultures. The cells were exposed to notexin for 48 hours. A cell proliferation assay was done to determine the viable cells.
2.4 BCNU + O\textsuperscript{6}BG Treatment Of C2C12 Cells And Human Myoblasts

Transfected and non-transfected C2C12 cells and human myoblasts were cultured in 96 well plates with equivalent cell numbers in all wells. Sub-confluent, proliferating cultures were treated with BCNU (at 0 µM, 10 µM, 25 µM, 50 µM, 100 µM and 200 µM) for 2 hours or the cells were treated with a combination of O\textsuperscript{6}benzylguanine (Sigma-Aldrich; product number B2292) at 40 µM for 1 hour and BCNU (at 0 µM, 10 µM, 25 µM, 50 µM, 100 µM and 200 µM) for 2 hours. Two thirds of the volume of BCNU was then removed from the wells and replaced with growth medium. C2C12 cells were cultured for 24 hours and human myoblasts were cultured for 48 hours after treatment. A cell proliferation assay was done to determine the viable cells.
2.5 **Cell Proliferation Assay**

50μl of 5μg/ml MTT (Sigma-Aldrich; product number M5655) solution was added per well for cultures done in 96 well plates and incubated for 4 hours at 37°C. Liquid was aspirated from wells and the purple precipitate was dissolved with 200ul of DMSO (Invitrogen; product number 14040-133). Amount of dissolved precipitate was measured with a plate reader at 590 nm as an indicator of viable cells.
2.6 **Skeletal Muscle Cell Isolation**

Cell isolation protocol was adopted from a previously published study (Montarras, D., *et al.*, 2005). The methodology was slightly modified to give the maximum efficiency for our purpose. Mouse skeletal muscles (either tibialis anterior [TA], extensor digitorum longus [EDL], diaphragm or a regenerating TA 3 days post notexin treatment) were collected from mice into sterile Dulbecco’s PBS and kept on ice till ready to use. All procedures after dissections were carried out in sterile conditions. A sterile biohazard hood was used whenever possible. Muscles were minced using microscissors and transferred to a fresh tube containing F12 nutrient mix (Invitrogen; Product number 31765035). Minced muscle was then washed in the F12 medium by inverting several times. The tube was left on a holder for the muscle to spontaneously deposit at the bottom. The supernatant was aspirated. An enzyme solution containing 0.1% Collagenase D (Roche; Product number 11088866001) and 0.1% trypsin (Roche; Product number 10210234001) in F12 nutrient mix was added to the minced muscle (5ml to 4 TAs or 5 diaphragms, 2ml to 4 EDLs). Solution was incubated at a temperature ranging between 28 – 30 °C with gentle agitation for 20 minutes. At the end of the incubation period the tube was left on a holder until the undigested solid tissue deposits at the bottom. The supernatant containing cells was transferred into a fresh tube and Fetal Bovine Serum (JRH Biosciences; product number 12003-1000M) was added to a final concentration of 20%. The cell suspension was always kept on ice till used. The digestion step was repeated with fresh enzyme solution 3 more times and the supernatant was accumulated into one tube while keeping the final concentration of FBS 20%. The cell suspension was filtered through a 40um cell strainer (BD Biosciences; Product number 352340) and centrifuged at 1800 rpm for 20 minutes at RT.
2.7 Selection Of CD34\textsuperscript{+}ve Skeletal Muscle Cells

Muscle cells were isolated from 3 days post notexin treated muscles as mentioned before. The cell pellet at the end of the isolation process was resuspended in 500ul of 2% FBS in Dulbecco’s PBS. The cells were incubated with 10ul of anti-CD34 antibody (eBioscience; Product number 13-0341) for 30 minutes on ice. Cell suspension was then washed using 2% FBS in Dulbecco’s PBS, pelleted by centrifugation and resuspended in 500ul of fresh 2% FBS in Dulbecco’s PBS. 10ul of Streptavidin-RPE (DAKO; Product number R0438) was then added to the cell suspension and incubated for a further 30 minutes on ice. Washing step was repeated after the incubation period. The CD34 positive cells were then selected using a magnetic EasySep-PE Cell separation kit (Stem Cell Technologies; product number 18554). Cell counts were performed using the Z 1 Coulter Particle Counter from Beckman Coulter.
2.8 Fluorescent Membrane Labeling Of CD34⁺ve Cells

CD34⁺ve skeletal muscle cells were isolated from regenerating skeletal muscles as described previously. Cell membranes were then labeled using a PKH67 Green Fluorescent Cell linker Mini Kit (Sigma; Product number MINI67) according to the manufacturer’s instructions. Prior to labeling the cell concentration was adjusted to 1x10⁷ cells/ml. Following the labeling procedure cells were resuspended in PBS at 6x10⁴ cells/10µl to use in intramuscular cell injections.
2.9 **Differentiation Of CD34\(^{+}\)ve Cells To Achieve Angiogenesis**

CD34\(^{+}\)ve skeletal muscle cells were isolated from regenerating skeletal muscles as described in section 2.6 and 2.7. CD34\(^{+}\)ve cells were induced to differentiate towards angiogenesis using an In vitro Angiogenesis Assay Kit (Chemicon International; Product number ECM625). Cells were suspended in CS-C medium (Sigma; Product number C1431) supplemented with 1X endothelial growth factor (Sigma; Product number E9640) Cells were plated at $1 \times 10^3$ cells per well in a 96 well plate which was coated with the ECM matrix solution according to manufacturer’s instructions. Cells were then observed and images were taken using a light microscope Olympus light microscope (Model IX70) at 6 hours, 48 hours, 72 hours and 96 hours in culture.
2.10 Flow Cytometry

2.10.1 In Vitro Selective Enrichment Study

BCNU + O6BG treated C2C12 cells and human myoblasts were washed in Dulbecco’s PBS, trypsinised with Trypsin-EDTA (Invitrogen; product number 25300-954), collected and washed in Dulbecco’s PBS and fixed for 30 minutes in 1% paraformaldehyde (Pro SciTech, QLD, Australia; C007) at RT. Cells were then washed in FACS buffer (Ca$^{2+}$ and Mg$^{2+}$ free PBS with 0.1% bovine albumin [Sigma-Aldrich; product number A3059] and 0.1% sodium azide [Sigma-Aldrich; product number S2002]). Cells were permeabilised in 0.1% Tween-20 (Sigma-Aldrich; P7949) in PBS at 37°C for 1 hour. The cells were then labeled with the MGMT antibody (Neomarkers, CA, USA; 8µg/ml; Clone MT 3.1) at 1:25 dilution in FACS buffer for 2 hours at 4°C. After labeling, the cells were washed in FACS buffer and labeled with goat anti-mouse IgG conjugated with FITC (1:100; CALTAG Laboratories, CA, USA) for 1 hour at 4°C. Cells were washed thoroughly and MGMT positive cells were measured using a FACScan (Becton Dickinson Biosciences with an Argon ion laser).
2.10.2 CD34^{+}ve Cell Characterisation Study

The isolated muscle cells and bone marrow cells were fixed in 1% paraformaldehyde (ProSciTech, QLD, Australia; C007) in PBS for 30 minutes at room temperature. The cells were then washed with FACS Buffer (CMF PBS + 0.1% BSA, 0.1% Na Azide). Muscle cells that are to be labeled with an antibody against an intracellular maker were further incubated with 0.1% Tween-20 in PBS for 1 hour at 37 °C in order to permeabilise the membrane. The cells were again washed in FACS Buffer. The cell concentrations were adjusted to approximately 1x10^5 cells/ml in FACS Buffer. Cells were first incubated with the primary antibodies followed by washing with FACS buffer. Cell suspension was always kept at 400µl for each incubation. If the primary antibodies were biotinylated cells were then incubated with streptavidin-RPE (DAKO; Product number R0438) or streptavidin fluorescein (Perkin Elmer Life Sciences; Product number NEL720. If an unconjugated primary antibody was used the cells were further incubated with a secondary antibody conjugated with FITC followed by washing in FACS buffer. Each antibody labeling was controlled for non specific binding by including an isotype control. The cells labeled with the isotype control antibodies received identical treatment to their corresponding primary antibody. The list of antibodies used, dilutions and incubation conditions are; rat anti mouse CXCR4 fluorescein conjugated antibody (R&D Systems; Product number FAB21651F) at 1:5 overnight at 4°C, rat anti mouse CD133 FITC conjugated antibody (eBioscience; Product number 11-1331) at 1:50 overnight at 4°C, rat anti mouse CD144 Alexa fluor 647 conjugated antibody (eBioscience; Product number 51-1441) at 1:50 overnight at 4°C, rat anti mouse Sca-1 (Ly-6A/E) PE-Cy5.5 conjugated antibody (CALTAG Laboratories; Product number MSCA18) at 1:100 for 30 minutes on ice, rat anti mouse CD45 FITC conjugated antibody (CALTAG Laboratories;
Product number MCD4501) at 1:100 for 30 minutes on ice, mouse anti mouse Pax7 antibody (R&D Systems; Product number MAB1675) used with rat anti mouse IgG FITC conjugated antibody (eBioscience; Product number 11-4011), anti NCAM (CD56) PE conjugated (Abcam; product number ab18277) and biotinylated anti alkaline phosphatase (R&D Systems; Product number BAM1448). Anti Pax7 antibody labeling was performed using Zenon Mouse IgG Labeling Kit (Invitrogen; Product number Z25002) according to manufacturer’s instructions. Cells were labeled with the primary/secondary Zenon conjugate overnight at 4°C. FACS sorting was performed using a FACScan (Becton Dickinson Biosciences).
2.11 Animals

Effects of BCNU + O\(^{6}\)BG on regenerating muscles were studied using MGMT(P140K) transgenic mice and C57BL/6 mice at 8-10 weeks of age. Male MGMT(P140K) transgenic mice and male C57BL/6 mice of age 8-12 weeks were used as the cell donors in transplantation studies while female C57BL/6, or C57BL/10ScSn-Dmd\(^{mdx}\)/Arc of age 8-12 weeks were used as recipients. MGMT(P140K) transgenic mice were bred in the Animal Facility at the Children’s Medical Research Institute (kindly provided to us by Belinda Kramer at the Children’s Hospital at Westmead, NSW, Australia), while all other mouse strains were purchased from ARC, Perth, Australia. The MGMT(P140K) transgenic mice demonstrate variegated expression of the transgene (Kramer, B.A., et al., 2006). Expression of the MGMT(P140K) expression varies among individual MGMT(P140K) transgenic mice as well as within different tissues of the same animal (unpublished data; Honours Thesis by Renjing Liu, 2005). Figure 29 in supplementary data shows the structure of the hEF1\(\alpha\)MGMT(P140K) construct used for the generation the MGMT(P140K) transgenic mice.

Following surgery, mice were monitored for 14 days for their health unless culled for tissue collection prior to the end of the monitoring period. If treated with cytotoxic drugs such as BCNU, the mice were housed in micro isolator cages for 5 days after treatment. Monitoring of these mice was carried out in a fume hood. The cages were replaced with normal cages after the initial 5 days.
2.12 Animal Surgery

All surgical procedures were approved by the Animal Care and Ethics Committee and aseptic surgical techniques were practiced wherever possible. All surgical procedures were carried out by Josephine Joya (Muscle Development Unit, Children’s Medical Research Institute) and Antonio Lee (Oncology Research Unit, Children’s Hospital at Westmead, Westmead, Australia). The mice were anaesthetised with Ketamine (0.1mg/g BW) and Xylazine (0.01 – 0.02 mg/g BW) mixture and pedal withdraw reflex was tested prior to surgery. Hind limbs were shaved and skin was alcohol-swabbed. For the intra-muscular and intra-venous injections, 1 cm skin incision was made on either the anterior or posterior aspect of the hindlimb. Following the injection, skin incisions were closed using non-absorbable surgical suture. Animals were then injected with saline (0.01 ml/g BW) subcutaneously to aid re-hydration and let to rest on a heating pad until recovering from the anesthesia.
2.12.1 In Vivo Study To Determine The Effect Of BCNU + O⁶BG On Regeneration Of Injured Muscle

For the in vivo study to determine the effect of BCNU & O⁶BG on the regeneration of the muscle injured by Notexin, 0.2µg of Notexin (40µg/ml stock solution in PBS; Latoxan, France: L8104) and 0.1 – 0.5mg of BCNU (33.3mg/ml in sterile ethanol; Bristol-Myers-Squibb, VIC, Australia) were mixed together into an injection volume of 10µl. Notexin-BCNU mixture was injected intramuscularly into the hindlimb muscle extensor digitorum longus (EDL) of 8-12 week-old C57BL/6J wild-type or MGMT(P140K) transgenic mice using 50µl Hamilton syringe with 33G Hamilton needle (Hamilton Company, Nevada, USA). O⁶BG (Sigma: B2292 50MG) was delivered intraperitoneally at 30 mg/kg of body weight following the recovery of the animals from the anaesthesia.

For donor cell preparation, 8-12 week-old male C57BL/6J wild-type or MGMT-P140K<sup>14/14</sup> transgenic mice were injected with 0.1µg and 0.4µg of Notexin intramuscularly into the EDL and TA muscles, respectively. The muscles were allowed to regenerate for 3 days before being collected for the donor cell isolation.
2.12.2 Transplantation Of Donor CD34+ve Cells And Notexin, BCNU + O6BG Treatment Of Recipient Muscles

2.12.2.1 Pilot Study: Injured EDL Muscles Of C57BL/6 Mice As Recipient Muscles For Transplantation

Recipient female C57BL/6J wild-type mice (8-12 week-old) were anaesthetised and the skin incision was made on the anterior aspect of the hindlimb to expose the EDL muscle. Donor CD34+ve cells (60,000 cells in 10 µl of sterile PBS) were injected intra-muscularly using 50 µl Hamilton syringe with 33G Hamilton needle (Hamilton Company, Nevada, USA). BCNU (0.1mg) plus notexin (0.1µg) in 10µl of sterile PBS was also injected intramuscularly using a 50µl Hamilton syringe. Skin incision was sutured. O6BG (Sigma: B2292 50MG) was delivered intraperitoneally at 30mg/kg of body weight.
2.12.2.2 Application Of BCNU + O6BG Driven Selective Enrichment Strategy In Transplantation Of CD34+ve Cells

Recipient female C57BL/6J wild-type mice or C57BL/10ScSn-Dmd\textsuperscript{mdx}/Arc (8-12 week-old) were anaesthetised and the skin incision was made on the anterior aspect of the hindlimb to expose the EDL muscle. Donor CD34+ve cells (60,000 cells in 5μl of sterile PBS) were mixed with 5μl of Notexin (40μg/ml) prior to injection and injected intra-muscularly using 50 μl Hamilton syringe with 33G Hamilton needle (Hamilton Company, Nevada, USA). Skin incision was sutured and the second incision on the posterior aspect of the hindlimb was made to expose great saphenous vein. In order to temporarily block the blood flow, a tourniquet was placed at the base of the hindlimb and 0.2mg of BCNU (Bristol-Myers-Squibb, VIC, Australia) in 50μl (4 mg/ml) was injected intravenously and a sterile cotton bud was placed on the puncture site and pressure applied to prevent hemorrhage. The blood flow was blocked for further 2 minutes following the injection after which, the tourniquet was removed and the skin incision was sutured. O6BG (Sigma: B2292 50MG) was delivered intraperitoneally at 30mg/kg of body weight.
2.13 Tissue Collection

Tissue collection was carried out with the assistance from either Josephine Joya (Children’s Medical Research Institute, Westmead, NSW, Australia) or Antonio Lee (Children’s Hospital at Westmead, Westmead, NSW, Australia). Mice were culled via cervical dislocation and their body weights were recorded. The muscle(s) of interest was exposed by removing the skin and dissected and their weights recorded. The muscles that were required for cryosectioning were embedded in Tissue-Tek O.C.T. Microscopy Compound (Electron Microscopy Sciences; PA, USA; product number 625501) and frozen in liquid nitrogen cooled isopentane. The muscles that were required for quantitative PCR were dissected and transferred into autoclaved 1.5ml Eppendorf microcentrifuge tubes. The tubes were then snap-frozen in liquid nitrogen. All tissues were stored -80°C until used.
2.14 H&E Staining Of Frozen Muscle Sections

Cryostat sections (7µM) were cut from the mid belly of frozen muscle, collected on to poly-lysine coated microscope slides (Biolab Scientific; product number P4981) and melted at RT for 15 minutes. Sections were fixed in 2% paraformaldehyde (pH 7.4) for 30 minutes, washed three times in PBS followed by water for 5 minutes and immersed in haemotoxylin (Fronine Laboratory Supplies, Australia; product number II02) for 2.5 minutes. Sections were washed under running water, immersed in acid alcohol (0.5% HCl in 75% Ethanol) for 30 seconds and transferred to Scotts bluing solution (3.5g Sodium bicarbonate + 20g Magnesium sulphate made up to 1L of solution). Sections were washed under running water, immersed in alcoholic eosin (Fronine Laboratory Supplies; product number 11017) for 5 minutes, washed once more, immersed in 70% alcohol for 1min and 90% alcohol twice for 1 min each. Sections were immersed in histolene (Fronine Laboratory Supplies; product number 2052) twice for 2 minutes each and mounted with DePeX mounting medium (BDH Chemicals; product number 36125), cover-slipped and left to air dried.
2.15 Immunohistochemistry

2.15.1 MGMT(P140K) Expression In Muscle Tissue

Immunohistochemistry to detect MGMT(P140K) expression was carried out by Josephine Joya (Muscle Development Unit, Children’s Medical Research Institute, Westmead, Australia). Cryostat sections (7µM) were cut and slides were prepared as for H&E staining. Sections were fixed in 4% paraformaldehyde (pH 7.4) for 30 minutes on ice, washed in cold PBS, treated with cold methanol for 30 minutes and washed in cold PBS. A Mouse-on-Mouse Kit (Vector Laboratories: FMK-2201) was used according to manufacturer’s instructions with the exception of the primary antibody incubation period. Tissue sections were incubated with mouse anti-human MGMT (Neomarkers, CA, USA; Clone MT 3.1) for 1 hour in a humid chamber at room temperature. DAPI (at 1:10000 dilution) was added to the last wash after incubation with the secondary antibody. Stained slides were viewed using a Leica® epifluorescence microscope (Type 020-519.102).
2.15.2 Dystrophin Expression In Muscle Tissue

Immunohistochemistry to detect dystrophin expression was carried out by either Josephine Joya or Antonio Lee (Oncology Research Unit, Children’s Hospital at Westmead, Westmead, Australia). Cryostat sections (7-10µm) were fixed in 4% paraformaldehyde (pH 7.4) for 30 minutes at 4°C and washed in cold PBS. A Mouse-on-Mouse Kit (Vector Laboratories: FMK-2201) was used according to manufacturer’s instructions. The anti-dystrophin antibody (Novocastra, UK: NCL-DYS2) was incubated for 1 hour at RT in a humid chamber. DAPI (1:10000) was added to the final wash following the secondary antibody incubation. Stained slides were viewed using a Leica® epifluorescence microscope (Type 020-519.102).
2.15.3 α-actinin-2 Expression In Myotubes

Differentiated C2C12 (5 days in differentiation medium) and human myoblast cultures (11 days in differentiation medium) were analysed for α-actinin-2 expression in myotubes. First, culture medium was aspirated and the cells were washed in 5 ml of warm Ca\textsuperscript{2+} and Mg\textsuperscript{2+} free PBS. The myotubes were then fixed and permeabilised in PBS containing 2% saponin and 3% paraformaldehyde for 15 minutes at room temperature. This was followed by another wash in PBS and incubation in 2% bovine serum albumin for 15 minutes at room temperature. The myotubes were then incubated overnight with a polyclonal rabbit anti mouse α-actinin-2 antibody (Kindly provided by Alan Beggs) at 1:400 dilution at 4º Celsius. The dishes were then washed in PBS three times and the myotubes were labeled with goat anti rabbit IgG (H+L) – Alexa fluor 555 (Molecular Probes; Product number A21429). The dishes were then washed 3 times with PBS. The labeled cells were observed under a Leica inverted fluorescence microscope (Model DMIRB).
2.16 Western Blot Analysis

Protocol was adopted from previously published work. Cells were washed once with Dulbecco’s PBS, then scrapped using a cell scraper and collected in PBS. Cells were then pelleted by a quick centrifugation and the pellets were snap frozen in liquid nitrogen for storage at -80º Celsius till used. The cell pellets were then thawed to room temperature and resuspended in 300ul of extraction buffer (25mM Tris-HCl pH 7.8, 2mM EDTA, 10% Glycerol, 1% Triton-X100 made up in Milli Q water) and incubated on ice for 1 hour. The samples were then centrifuged in a Sigma 1-15 table top centrifuge for 10 minutes at 14000 RPM. Supernatant was transferred to a new tube. The protein concentrations in samples were determined using a BCA™ Protein Assay Kit (Pierce, Rockford IL, USA) according to the manufacturer’s instructions and protein concentrations were adjusted to be equivalent in all samples. 10ul of samples were run on a 5% stacking and 8% resolving gel (used for α-actinin-2 and sarcomeric tropomyosin detection) or 10% resolving gel (used for MyoD and myogenin detection) at 90V for 1 hour in running buffer (25mM Tris-pH 8.3, 250mM glycine, 0.1% v/v SDS). Proteins were transferred to a PVDF membrane (Amersham Biosciences; product number RPN303F) by semi-dry electrophoresis using a SD Electrophoretic Transfer cell (Bio-Rad Laboratories; product number 170-3940), run at 20V for 1 hour. The membrane was then blocked for non-specific binding overnight with 5% skim milk in 1X TBS (10X TBS: 250mM Tris and 1.92M Glycine, pH 8.5) with 0.1% Tween-20 at 4ºC. Once blocking was completed, the membrane was probed with the primary antibody overnight at 4º C. The membrane was washed for a further 1 hour in TBS + 0.1% Tween-20 and incubated for another hour with HRP-conjugated anti mouse IgG (Pierce Biotechnology; product number 1858413) or anti rabbit IgG secondary antibody (Pierce Biotechnology; product number 1858413).
Biotechnology; Product number 1858415) diluted 1:10000 in TBS. The membrane was washed for a further 1 hour before reacting with the West Pico chemiluminescent substrate (Pierce; product number: 34080) for 1 minute and exposed to film. Primary antibodies used are, anti sarcomeric tropomyosin Clone CH1 (Sigma; Product number: T9283) at 1:200 dilution; anti α-actinin-2 (Kindly provided by Alan Beggs) at 1:200 dilution; anti myogenin (Abcam; Product number: ab15232) at 1:100 dilution; anti MyoD (Santa Cruz Biotechnology Inc; Product number: sc-760) at 1:200 dilution.
2.17 Quantitative PCR

DNA was extracted from the frozen EDL muscles by adding 400µl of TE/SDS buffer (100mM Tris pH 8, 1mM EDTA, 0.5% SDS) containing 400µg freshly added Proteinase K (Roche; Product number 03115801001). The muscles were incubated at 56ºC overnight with agitation. After digestion, 75µl of NH₄OAc and 1ml of absolute ethanol were added. The tubes were mixed by inverting several times then centrifuged at maximum speed on a Sigma 1-15 table top centrifuge for 20 minutes. The supernatant was removed and the DNA pellet was then washed with 1ml of 70% ethanol in autoclaved Milli-Q water and centrifuged for a further 10 minutes at maximum speed. The supernatant was then removed and the DNA pellet was air-dried. DNA was then resuspended in 100µl of autoclaved Milli-Q water and stored at 4º C for a minimum of 48 hours.

Quantitative PCR (q-PCR) was performed using a protocol adopted from a previous study (Fan, Y., et al., 1996). primers with the following sequence; Y chromosome-forward: TGG AGA GCC ACA AGC TAA CCA and Y chromosome-reverse: TCC CAG CAT GAG AAA GAT TCT TC. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers were used to quantify the total amount of DNA present within the samples with the following sequence; GAPDH-forward: GAA GGT GGT GAA GCA GGC AT and GAPDH-reverse: GCA TCG AAG GTG GAA GAG TG. Primer concentrations were adjusted to 2µg/µl in autoclaved Milli Q water immediately prior to setting up of the q-PCR reaction. The DNA samples to be analysed were quantified using a spectrophotometer and the DNA concentrations were adjusted to 50ng/µl in autoclaved Milli Q water. Four Y chromosome standards for q-PCR were prepared using male DNA and concentrations were adjusted to 5ng/µl, 0.5ng/µl,
0.05ng/µl and 0.005ng/µl. Four GAPDH standards were also prepared using the same male DNA with concentration adjustments made to 50ng/µl (undiluted), 25ng/µl, 12.5ng/µl and 6.25ng/µl. Each reaction was made up to 25µl consisting: 9.5µl of autoclaved Milli Q water, 12.5µl of SYBR™ Green® master mix (Qiagen; Product number 204145), 0.5µl of forward primer, 0.5µl of reverse primer and 2µl of DNA sample. A negative control using female DNA and a no template control using autoclaved Milli Q water were also included in the assay. q-PCR reaction was carried out using the Rotorgene® (Corbett Research; Rotorgene 2000). The reaction settings are as follows: Denature at 95°C for 15 minutes; Cycling 40 repeats – step 1 at 95°C for 20 seconds, step 2 at 56°C for 20 seconds and step 3 at 72°C for 30 seconds acquiring SYBR™ green signal; Hold at 60°C for 1 minute; Melt from 60-95°C – hold 5 seconds on 1st step, hold 5 seconds on next steps then acquiring the SYBR™ green signal. The acquired Y-chromosome concentrations of each sample were normalised against 50ng/µl GAPDH.
2.18 Fluorescence In Situ Hybridisation (FISH) To Detect Y Chromosome Positive Nuclei

Fluorescence in situ hybridisation (FISH) was done in order to detect Y chromosome positive donor nuclei in recipient muscle sections. These experiments were carried out by Antonio Lee (Oncology Research Unit, Children’s Hospital at Westmead) Cryostat sections (7-10µm) were fixed in 4% paraformaldehyde (pH 7.4) for 10 minutes at RT then permeabilised in 0.3% Triton X-100 for 10 minutes. The slides were put through three 10 minute washes in PBS. The slides were incubated in 1 µg/ml of Proteinase K in Buffer (0.1M Tris-HCl, 50mM EDTA, pH 8.0) at 37ºC for 30 minutes followed by a 2 minute PBS wash. Dehydration of the sections in 70%-70%-90%-90%-100% ethanol series for 2 minutes each was performed. The slides were air-dried overnight then incubated at 65ºC for 1 hour. Equal volumes (5µl) of mouse Y chromosome paint probe (ID Labs Inc.) and hybridisation buffer were mixed, denatured at 65ºC for 10 minutes and held at 37ºC for up to 2 hours for annealing. The sections were then denatured in 70% formamide in 2 x SCSC (Sodium Chloride - Sodium Citrate buffer containing 3M NaCl and 0.3M Sodium Citrate at 20 x concentration) at 86ºC for 2 minutes followed by immediate quenching in ice-cold 70% ethanol for 5 minutes. The slides were then dehydrated in ice-cold 70%-70%-90%-90%-90% ethanol series for 2 minutes each then in 100% ethanol at RT for 5 minutes. The slides were air-dried. Followed by the application of the prepared probe, the slides were cover-slipped and sealed using rubber cement. The sections were hybridised at 37ºC overnight. Then cover-slips were removed and sections washed twice in 2 x SCSC for 2 minutes each at RT followed by 3 washes in 50% formamide (in 2 x SCSC) at 45ºC for 5 minutes each and 3 washes in 1 x SCSC at 45ºC for 5
minutes each. The sections were then incubated in Avidin-FITC (Vector Laboratory) for 30 minutes followed by 2 washes in 4 x SCSC + 0.1% Tween-20 at RT for 5 minutes each and 2 washes in 4 x SCSC at RT for 5 minutes each. DAPI was added in the final wash and the slides were mounted in Vectashield (Vector Laboratories) and cover-slipped.
CHAPTER 3

Effect Of Alkylating Chemotherapy Treatment

On Skeletal Muscles
3.1 Introduction

3.1.1 Overview Of The Chapter

The BCNU + O\textsuperscript{6}benzylguanine (O\textsuperscript{6}BG) driven selective enrichment strategy has been studied for haematopoietic stem cell transplantation for the last decade and is currently in the process of moving to clinical application. The sensitivity of proliferating haematopoietic stem cells to BCNU + O\textsuperscript{6}BG together with the resistance provided by the expression of the MGMT(P140K) gene has been well characterised (Gerson, S.L., 2005, Kramer, B.A., et al., 2006). In order to test the applicability of this selective enrichment strategy in skeletal muscle stem cell transplantation, the first step was to study the response of muscle cells to treatment with BCNU + O\textsuperscript{6}BG. This chapter reports the studies that were done to establish the sensitivity of muscle cells to the alkylating chemotherapeutic drugs and explores the \textit{in vitro} and \textit{in vivo} enrichment of MGMT(P140K) expressing muscle cells that can be achieved using the BCNU + O\textsuperscript{6}BG driven selective enrichment strategy. The experimental procedures mentioned in section 3.2.5 were carried out by Josephine Joya (Muscle Development Unit, Children’s Medical Research Institute, Westmead, Australia).
3.1.2 Hypotheses And Aims Of The Chapter

Hypothesis 1: Normal skeletal muscle cells are sensitive to the cytotoxicity of alkylating chemotherapy and MGMT(P140K) expression will provide protection against the cytotoxicity.

Aim 1: To determine the effects of alkylating chemotherapy treatment on normal skeletal muscle cells and MGMT(P140K) expressing skeletal muscle cells.

Hypothesis 2: Retroviral transduction of myoblasts with an MGMT(P140K) gene and treatment with alkylating chemotherapy do not alter the growth characteristics and differentiation capacity of myoblasts.

Aim 2: To determine the effect of retroviral transduction of an MGMT(P140K) vector and alkylating chemotherapy treatment on growth characteristics and differentiation capacity of myoblasts.

Hypothesis 3: Upon treatment with BCNU + O6BG, there will be selective enrichment of MGMT(P140K) expressing skeletal muscle cells within a population of mitotically active cells.

Aim 3: To determine the ability of skeletal muscle cells to undergo selective enrichment for MGMT(P140K) expressing cells upon treatment with BCNU + O6BG.
3.2 **Results**

3.2.1 **Sensitivity Of Muscle Cells To Alkylating Chemotherapy**

Haematopoietic stem cells have shown cytotoxicity towards alkylating chemotherapy. This characteristic allows the application of the BCNU + O\textsuperscript{6}BG driven selective enrichment strategy in haematopoietic stem cell transplantation in order to enhance engraftment. The first step in application of this selective enrichment strategy in skeletal muscle stem cell transplantation is to determine whether normal skeletal myoblasts are sensitive to the cytotoxic effects of alkylating chemotherapy. In order to achieve this, an *in vitro* study was carried out using C2C12 cells (Blau, H.M., *et al.*, 1983) and human myoblasts. Cells were plated at a density of 10\textsuperscript{5} cells/ml in a 96 well plate. 24 hours after plating, the cells were treated with 40µM O\textsuperscript{6}benzylguanine (O\textsuperscript{6}BG) plus various doses of BCNU. A cell proliferation assay was carried out in order to measure the proportion of viable cells in both C2C12 (24 hours after treatment) and human myoblast (48 hours after treatment) cultures. Cell proliferation assay showed that skeletal muscle cells, both mouse C2C12 cells and human myoblasts are sensitive to the cytotoxic effects of alkylating chemotherapy (BCNU + O\textsuperscript{6}BG) in a dose dependent manner. Increasing doses of BCNU resulted in an increased cell death (Figure 6).
Figure 6: BCNU treatment is cytotoxic to normal skeletal muscle cells.

C2C12 cells and human myoblasts were exposed to various doses of BCNU. All cell cultures were treated with 40μM O6-benzylguanine. A cell proliferation assay was done to determine the viable cells and percentage of cell survival was calculated as a proportion of cell viability in untreated cells. Error bars are standard deviations of 3 separate experiments where each experiment consisted n=8.
3.2.2 Effect Of Alkylating Chemotherapy On Cells Which Express The MGMT(P140K) Gene

MGMT(P140K) expression on haematopoietic stem cells have been shown to confer resistance against alkylating chemotherapy (Kramer, B.A., et al., 2006). In order to determine whether the same is possible in muscle cells, C2C12 cells and human myoblasts were transduced with the MGMT(P140K) gene using a retroviral vector. Both untransduced wildtype cells and transduced cells were treated with various doses of BCNU (0µM, 50µM, 100µM and 200µM) and 40µM O\(^6\)BG (O\(^6\)benzylguanine). A cell proliferation assay was performed to determine the cell viability in each culture after 24 hours for C2C12 cells and 48 hours for human myoblasts. Results are graphically illustrated in Figure 7. Both C2C12 cells and human myoblasts which expressed the MGMT(P140K) gene showed resistance against BCNU + O\(^6\)BG treatment. MGMT(P140K) expressing C2C12 cells are approximately 3 times more resistant to the cytotoxic effects of alkylating chemotherapy than the wildtype C2C12 cells. Human myoblasts also show a similar resistance where the MGMT(P140K) expressing cells are approximately 4 times more resistant to drug treatment. These results demonstrate that MGMT(P140K) expression in myoblasts confers resistance against alkylating chemotherapy.
Figure 7: MGMT(P140K) expressing C2C12 cells and human myoblasts are resistant against alkylating chemotherapy.

(A) C2C12 cells. (B) Human myoblasts. Both C2C12 cells and human myoblasts were transduced with a MGMT(P140K) gene using a retroviral vector and compared with wildtype cells for the effect of BCNU treatment. A cell proliferation assay was done to determine the viable cells (24 hours after treatment for C2C12 cells and 48 hours after treatment for human myoblasts). Percentage cell survival was calculated as a proportion of cell viability in untreated cells. Error bars are standard deviations of 3 separate experiments where each experiment consisted of n=8.
3.2.3 Application Of The BCNU + O\textsuperscript{6}BG Driven Selective Enrichment Strategy To Muscle Cells

The BCNU + O\textsuperscript{6}BG driven selective enrichment strategy is well established in the haematopoietic stem cell system (Gerson, S.L., 2005, Kramer, B.A., et al., 2006). In order to determine if this strategy can also be applied to skeletal muscle cells, a mixture of MGMT(P140K) transduced and untransduced myoblasts were treated with alkylating chemotherapy. Both C2C12 and human myoblast cultures were studied. C2C12 cultures had either 4% or 13% MGMT(P140K) expressing cells on the day of BCNU (0µM, 50µM, 100µM and 200µM) + O\textsuperscript{6}BG (40µM) treatment. Samples were taken to perform FACScan analysis to determine the percentage of MGMT(P140K) positive cells on the day of treatment, 3, 5 and 7 days post treatment. FACScan analysis revealed that post BCNU + O\textsuperscript{6}BG treatment there is a steady increase in MGMT(P140K) expressing C2C12 cells in culture (Figure 8). Selective enrichment is BCNU dose dependent where lower concentrations resulted in only a small increase and higher concentrations resulted in a larger increase in the percentage of MGMT(P140K) positive cells in the cultures. C2C12 culture that had 13% MGMT(P140K) cells on the day of treatment showed close to 90% MGMT(P140K) cells at 7 days post treatment with 200µM BCNU and 40µM O\textsuperscript{6}BG. Human myoblasts were also treated with various doses of BCNU, however 200µM proved to be too toxic. These cells were therefore treated with 0µM, 25µM, 50µM or 100µM BCNU plus 40µM O\textsuperscript{6}BG. The treated human myoblast cultures had 8% MGMT(P140K) expressing cells on the day of treatment. These cell cultures also showed enrichment of MGMT(P140K) positive cells which reached a maximum percentage of just above 70% (Figure 9). These
results demonstrate that it is possible to successfully apply the BCNU + O$^6$BG driven selective enrichment strategy for skeletal muscle cells.
Figure 8: MGMT(P140K) expression allows the *in vitro* selective enrichment of C2C12 myoblasts upon treatment with BCNU + O6-benzylguanine.

Cell cultures were treated with BCNU (either 0μM, 50μM, 100μM or 200μM) at day 1. All cell cultures were treated with 40μM of O6-benzylguanine at day 1.

C_Day 1 - C_Day 7: Control C2C12 cells which are not transduced with the MGMT(P140K) gene, cultured for either 1, 3, 5 or 7 days post treatment.

4%_Day 1 - 4%_Day 7: C2C12 cells containing 4% MGMT(P140K) transduced cells mixed in untransduced cells at the beginning of experiment, cultured for 1, 3, 5 or 7 days post treatment.

13%_Day 1 - 13%_Day 7: C2C12 cells containing 13% MGMT(P140K) transduced cells mixed in untransduced cells at the beginning of experiment, cultured for either 1, 3, 5 or 7 days post treatment.
Figure 9: MGMT(P140K) expression allows the in vitro selective enrichment of human myoblasts upon treatment with BCNU + O⁶-benzylguanine.

Cell cultures were treated with BCNU (either 0μM, 50μM, 100μM or 200μM) at day 1. All cell cultures were treated with 40μM of O6benzylguanine at day 1. C_Day 1- C_Day 13: Control human myoblasts which are not transduced with the MGMT(P140K) gene, cultured for either 1, 5, 9 or 13 days post treatment. 5%_Day 1 - 5%_Day 7: human myoblasts containing 5% MGMT(P140K) transduced cells mixed in untransduced cells at the beginning of experiment, cultured for either 1, 5, 9 or 13 days post treatment.
3.2.4 Effect Of Alkylating Chemotherapy On Injured Wildtype And MGMT(P140K) Transgenic Skeletal Muscles

BCNU shows cytotoxicity on proliferating but not quiescent cells (Gerson, S.L., 2004, Le Fevre, A., et al., 2007). In order to create an environment sensitive to BCNU, wildtype muscles have to be induced to regenerate prior to BCNU treatment which results in proliferation of muscle progenitor cells. The first stage of the in vivo studies required the establishment of efficient experimental conditions. Prior to drug testing, the histology of a wildtype regenerated EDL muscle was studied. Regeneration was induced by intramuscular injection of notexin. At 10 days post treatment there is good fiber formation and muscle regeneration (Figure 10C & D). Normal muscle regeneration was identified by the presence of centralized nuclei in the fibers as opposed to peripheral nuclei seen in untouched normal muscles.

In order to determine the effects of BCNU treatment on MGMT(P140K) transgenic muscles compared to wildtype muscles, muscles of wildtype and MGMT(P140K) transgenic mice were treated with notexin + O6BG + BCNU. Treatment with notexin was necessary in order to injure the muscle bed and to promote progenitor cell proliferation thus promoting the muscle sensitivity to BCNU. H&E staining was done in muscles which were collected 10 days after treatment (Figure 10E & H). H&E staining of muscle sections showed that a regeneration response was present in the transgenic muscle (Figure 10G & H) whereas the wildtype muscle showed very little new fiber formation (Figure 10E & F). There were many mononucleated cells present which are most likely infiltrated inflammatory cells (Figure 10E). It was also observed that the BCNU + O6BG treated transgenic muscle was bigger in
Figure 10: Effect of BCNU + O\textsuperscript{6}benzyldguaamine (O\textsuperscript{6}BG) treatment on wildtype and MGMT(P140K) transgenic muscles at 10 days post treatment.

(A) – (F) EDL muscles of wildtype C57BL/6 mice. (G) & (H) EDL muscles of MGMT(P140K) transgenic mice. (A) & (B) untreated. (C) & (D) notexin (intramuscular injection). (E) – (H) BCNU (intramuscular injection) + O\textsuperscript{6}BG (intraperitoneal injection) + notexin (intramuscular injection). (A), (C), (E) and (G) 40X magnification. (B), (D), (F) and (H) 10X magnification. ‘n’ value for each experiment group was greater than 3.
size than the wildtype BCNU + O\textsuperscript{6}BG treated muscle (Figure 10F vs H). Although not as efficient as the normal regeneration response shown in figure 10C & D, there were many newly formed fibers with centralised nuclei present in the BCNU + O\textsuperscript{6}BG treated transgenic muscle. These results show that expression of the MGMT(P140K) gene confers resistance against alkylating chemotherapy \textit{in vivo}. Immunohistochemistry was performed on MGMT(P140K) transgenic muscle sections of untreated, notexin treated and notexin + BCNU + O\textsuperscript{6}BG treated muscles, 20 days post treatment. The sections were labelled with an anti-dystrophin antibody (labels muscle fiber membrane in green), anti-human MGMT (labels MGMT(P140K) positive nuclei in red) and DAPI (stains all nuclei). In a merged image of anti-dystrophin, anti-human MGMT and dapi staining, the MGMT(P140K) positive nuclei appear pink. The untreated muscles shown in Figure 11A & B show that there is some non-specific labelling, however, there is no genuine expression of MGMT(P140K) shown by the lack of pink nuclei in Figure 11B. The results showed that there was a slight increase in the number of MGMT(P140K) positive nuclei in notexin treated compared to the untreated muscles (Figure 11A vs C). The notexin + BCNU + O\textsuperscript{6}BG treated muscles showed a substantial increase positive nuclei compared to both the notexin treated and untreated muscles (Figure 11E). These observations indicate that BCNU plus O\textsuperscript{6}BG treatment leads to selective enrichment of MGMT(P140K) expressing cells \textit{in vivo}. Figure 11D & E also show that the regenerated muscle fibers in notexin + BCNU + O\textsuperscript{6}BG treated muscles are smaller in diameter than in notexin only treated muscles. This is possibly due to elimination of muscle progenitor cells which do not express MGMT(P140K) after BCNU + O\textsuperscript{6}BG treatment due to variegated expression of the transgene. This in turn results in a smaller proportion of progenitor cells to contribute to the formation of new fibers.
Figure 11: *In vivo enrichment of MGMT(P140K) positive nuclei with BCNU + O\(^6\)benzylguanine treatment (20 days post treatment).*

(A) – (F) EDL muscles of MGMT(P140K) transgenic mice. (A), (C) & (E) MGMT(P140K) antibody labeling. (B), (D) & (F) MGMT(P140K) positive nuclei (pink nuclei), nuclear staining (blue nuclei) and dystrophin labeling of muscle fiber membrane (green). (A) & (B) untreated. (C) & (D) notexin (intramuscular injection). (E) – (F) BCNU (intramuscular injection) + O\(^6\)benzylguanine (intraperitoneal injection) + notexin (intramuscular injection).
3.2.5 Effect Of Alkylating Chemotherapy On Differentiation Of Myoblasts Which Express MGMT(P140K)

MGMT(P140K) transduced myoblasts which were treated with alkylating chemotherapy were studied in order to determine if the differentiation capacity and/or growth characteristics of these cells have been altered. C2C12 cells and human myoblasts that were transduced with MGMT(P140K) were first treated with alkylating chemotherapy. Following drug treatment, the cells were cultured in differentiation medium. The transduced/drug treated myoblast cultures were compared with wildtype/untreated myoblasts. The morphology of transduced/treated C2C12 cells and human myoblasts showed no difference in comparison to untransduced/untreated control cultures at any time point during differentiation (Figure 12a & 12b). The transduced/treated C2C12 cells and human myoblasts formed multinucleated, contracting myotubes by the end of the differentiation period. The differentiated cells were studied for expression of myogenic proteins using western blot analysis. Expression of α-actinin-2 and sarcomeric tropomyosin in C2C12 cells and expression of α-actinin-2 in human myoblasts were analysed at different time points after induction of differentiation (Figures 13 & 14). Expression of α-actinin-2 and sarcomeric tropomyosin were first observed in C2C12 cells at 3 days post differentiation and there was a gradual increase in the amount of protein present thereafter (Figure 13A & 13B). The expression patterns of α-actinin-2 and sarcomeric tropomyosin were similar in both untransduced/untreated and transduced/treated samples. Human myoblasts showed a similar pattern of expression with onset of α-actinin-2 expression at 5 days after induction of differentiation (Figure 14A). Immunohistochemistry for α-actinin-2 expression was done on
Figure 12a: BCNU treatment does not alter differentiation of skeletal muscle cells which express MGMT(P140K).

(A) - (E) untransduced/untreated C2C12 cells. (F) - (J) MGMT(P140K) transduced, BCNU + O6-benzylguanine treated C2C12 cells. (A) & (F) 0 days, (B) & (G) 2 days, (C) & (H) 3 days, (D) & (I) 4 days, (E) & (J) 5 days culture in C2C12 myoblast differentiation medium.
Figure 12b: BCNU treatment does not alter differentiation of skeletal muscle cells which express MGMT(P140K).

(A) – (E) untransduced/untreated human myoblasts. (F) – (J) MGMT(P140K) transduced, BCNU + O6-benzylguanine treated human myoblasts. (A) & (F) 1 day, (B) & (G) 3 days, (C) & (H) 5 days, (D) & (I) 8 days, (E) & (J) 11 days culture in differentiation medium.
mature myotubes in order to determine whether there are any alterations in formation of sarcomeres caused by the retroviral transduction and drug treatment. C2C12 myotubes formed after 5 days post induction of differentiation and myotubes formed from human myoblasts after 11 days post induction of differentiation were labeled with an anti \( \alpha \)-actinin-2 antibody. Transduced/treated myotubes formed by both C2C12 (Figure 13C & 13D) and human myoblasts (Figure 14B) showed normal sarcomere formation with comparable z-lines to the untransduced/untreated control myotubes. Cumulatively these results show that there is no alteration in growth characteristics and in differentiation capacity due to MGMT(P140K) transduction of myoblasts and alkylating chemotherapy.
Figure 13: Expression of muscle proteins do not get altered due to alkylation chemotherapy during differentiation of MGMT(P140K) transduced C2C12 myoblasts.

(A) & (B) Western blot analysis: α-actinin-2 and sarcomeric tropomyosin expression levels during differentiation of C2C12 cells. (A) wildtype untreated C2C12 myoblasts. (B) MGMT(P140K) transduced, BCNU + O6-benzylguanine (O6BG) treated C2C12 myoblasts. (C) & (D) Immunohistochemistry of C2C12 myotubes: α-actinin-2 labeling which shows z-line staining of sarcomeres. (C) wildtype untreated C2C12 myotubes. (D) MGMT(P140K) transduced, BCNU + O6BG treated C2C12 myotubes.
Figure 14: Expression of muscle proteins do not get altered due to alkylation chemotherapy during differentiation of MGMT(P140K) transduced human myoblasts.

(A) Western blot analysis: α-actinin-2 and sarcomeric tropomyosin expression levels during differentiation of human myoblasts. (B) Immunohistochemistry of myotubes formed from human myoblasts: α-actinin-2 labeling which shows z-line staining of sarcomeres. Wildtype: untreated – untransduced/untreated cells. Transduced: BCNU + O6BG – MGMT(P140K) transduced, BCNU and O6-benzylguanine (O6BG) treated cells.
3.3 Discussion

BCNU + O$^6$BG driven selective enrichment strategy has been well established in the haematopoietic stem cell system, evident by various in vitro and in vivo studies that have been published (Gerson, S.L., 2005, Neff, T., et al., 2006, Neff, T., et al., 2005, Neff, T., et al., 2003, Persons, D.A., et al., 2003). The results shown in this chapter demonstrate that the same selective enrichment strategy could successfully be applied to the skeletal muscle system.

Initial findings showed that skeletal muscle myoblasts such as C2C12 cells and human myoblasts are sensitive to the cytotoxic effects of alkylating chemotherapy (BCNU + O$^6$BG), however the extent of sensitivity is not as great as that reported for haematopoietic stem cells. Percentage cell survival of C2C12 cells plus human myoblasts treated with 50µM BCNU (plus 40µM O$^6$BG) was approximately 50% whereas the survival of haematopoietic stem cells was close to 0.1% (Kramer, B.A., et al., 2006). Yet, the protection shown by MGMT(P140K) expressing cells was still quite significant where MGMT(P140K) transduced C2C12 cells showed 3 times more resistance and human myoblasts showed 4 times more resistance to alkylating chemotherapy than normal untransduced cells. Further investigation revealed that the sensitivity of untransduced normal myoblasts and the protection shown by MGMT(P140K) transduced myoblasts were sufficient for successful enrichment of MGMT(P140K) transduced cell upon BCNU + O$^6$BG treatment. With one treatment of BCNU + O$^6$BG, a starting culture containing 13% MGMT(P140K) expressing C2C12 cells showed approximately 77% selective enrichment of MGMT(P140K) cells after 7 days of BCNU + O$^6$BG treatment. Selective enrichment was also apparent, although to a
lesser extent, in human myoblast cultures. This is most likely because human myoblasts have a much slower population doubling time than C2C12 cells (unpublished observations).

Protection provided by the expression of MGMT(P140K) is further demonstrated by the *in vivo* studies using the transgenic mice. A regeneration response was present in the MGMT(P140K) transgenic muscles at 10 days post treatment with notexin and BCNU plus O6BG, whereas it was completely inhibited in similarly treated wildtype muscles. The overall size of the transgenic muscle after notexin and BCNU plus O6BG treatment was smaller than a normally regenerated muscle at 10 days after treatment with notexin alone. This is likely due to death of muscle stem and satellite cells residing within the transgenic muscle which are not resistant to alkylating chemotherapy due to the lack of MGMT(P140K) expression. Flow cytometry performed on muscle stem cells which were directly isolated from the MGMT(P140K) transgenic muscles showed that only 25.1 ± 7.3% cells express the transgene. Therefore, only this population of muscle stem and satellite cells will survive and consequently contribute to muscle fiber formation following the BCNU + O6BG treatment. This will result in a smaller muscle than a normally regenerated muscle.

Variegated expression of transgenes in transgenic animals of the same line has previously been reported. It has been proposed that the variation of transgene expression is dependent on the transgene integration locus (Dobie, K.W., *et al.*, 1996) or the transgene sequence (Ramirez, A., *et al.*, 2001). This phenomenon has also been observed in the MGMT(P140K) transgenic mice. The MGMT(P140K) transgenic mice have been characterised and it has been found that within bone marrow not all cells will be expressing MGMT(P140K)
Furthermore, MGMT(P140K) transgenic mice were shown to have variation in transgene expression levels within different tissues of the same animal and between individuals (unpublished data; Honours Thesis by Renjing Liu, 2005).

Treatment with BCNU plus O⁶BG results in death of cells which do not express the MGMT(P140K). Therefore, once regenerated, a transgenic muscle treated with notexin and BCNU plus O⁶BG should exhibit an enrichment of MGMT(P140K) positive cells. This is demonstrated by the immunohistochemical analysis of the MGMT(P140K) expression within sections of untreated, notexin treated and notexin + BCNU + O⁶BG treated transgenic muscles. The untreated transgenic muscle shows little MGMT(P140K) expression, however upon treatment with notexin there is a slight increase in the MGMT(P140K) expressing nuclei. This increase is most likely due to the activation of the EF1α promoter driving the MGMT(P140K) gene within the transgenic mouse DNA. The EF1α promoter activation occurs when a cell is mitotically active (Jefferies, H.B.J., et al., 1994). Therefore, in an untreated transgenic muscle where all muscle fibers are post mitotic and muscle stem and satellite cells are quiescent, there will be little MGMT(P140K) expression. However once the muscle is injured by notexin treatment, the muscle stem and satellite cells become mitotically active and expression of the MGMT(P140K) gene is expected to increase. MGMT(P140K) expressing nuclei were further increased in muscles which were treated with notexin + BCNU + O⁶BG. This increase is most likely due to the selective enrichment of cells which express MGMT(P140K) upon treatment with BCNU + O⁶BG. BCNU + O⁶BG treatment leads to selective survival of the MGMT(P140K) expressing cells which would
undergo proliferation thus resulting in an increased fraction of the MGMT(P140K) expressing cells.

Finally, expression of the mutated gene MGMT(P140K) plus the stress of undergoing BCNU + O\textsuperscript{6}BG treatment did not have any adverse effects on the skeletal muscle cells. Myoblasts which were transduced with an MGMT(P140K) gene followed by treatment with BCNU + O\textsuperscript{6}BG showed no impact on differentiation of these cells into myotubes. The morphology of both transduced/treated C2C12 and human myoblasts were comparable to the untransduced/untreated controls at all time points during differentiation. Further analysis on protein expression of $\alpha$-actinin-2 and sarcomeric tropomyosin showed there are no differences in muscle specific protein expression in transduced/treated and untransduced/untreated myoblast cultures during differentiation. Furthermore, transduced/treated myoblast cultures produced spontaneously contracting myotubes that have similar expression pattern of $\alpha$-actinin-2 to an untransduced/untreated control. These results demonstrate that MGMT(P140K) expression and BCNU + O\textsuperscript{6}BG treatment does not cause any gross structural or morphological alterations to the cells and that despite the manipulations these myoblasts are capable of assembling a functional contractile apparatus.

In conclusion, the BCNU + O\textsuperscript{6}BG driven selective enrichment strategy can successfully be used in the skeletal muscle system in order to selectively allow the survival of cells which express MGMT(P140K). In addition, MGMT(P140K) expression and BCNU + O\textsuperscript{6}BG treatment of skeletal muscle cells do not grossly effect myoblast differentiation \textit{in vitro} and muscle fiber formation \textit{in vivo}.
CHAPTER 4

CD34^{+}ve Skeletal Muscle Cells As Prospective Donor Cells For Transplantation
4.1 Introduction

4.1.1 Overview Of The Chapter

Muscle stem cells are widely studied due to their potential use in treatment of diseases such as Duchene’s Muscular Dystrophy (Cossu, G. and Sampaolesi, M., 2007, Peault, B., et al., 2007). Since the identification of satellite cells various transplantation studies have utilised these cells to achieve donor contribution to host skeletal muscle regeneration (Karpati, G., et al., 1993, Partridge, T.A., et al., 1978, Snow, M.H., 1978). The lack of engraftment using satellite cells as donor cells lead to the characterisation of other stem cell populations that demonstrated the ability for myogenic differentiation. Various recent studies have reported the isolation of stem cells from sources such as skeletal muscle, peripheral blood, umbilical chord blood, bone marrow and blood vessels (reviewed by Cossu, G. and Sampaolesi, M., 2007, Peault, B., et al., 2007). These stem cells have demonstrated a better capacity for engraftment into host skeletal muscles upon transplantation than initial studies with satellite cells.

Many of these recently identified stem cell populations show expression of the CD34 cell surface antigen (Peault, B., et al., 2007). This chapter reports the characterisation of a heterogeneous population of CD34$^{+\text{ve}}$ cells that are isolated from a regenerating skeletal muscle bed. Subpopulations of stem cells and other cell populations were also described that are found within the CD34$^{+\text{ve}}$ cells isolated from a regenerating skeletal muscle. The significance of the heterogeneity observed in this population of CD34$^{+\text{ve}}$ cells and its implications for the role of donor cells in transplantation are also discussed.
Hypotheses And Aims Of The Chapter

Hypothesis 1: CD34^{+}ve cells isolated from a regenerating mouse skeletal muscle would have the capacity to differentiate and form functional myotubes in culture.

Aim 1: To determine the potential of CD34^{+}ve cells isolated from a regenerating skeletal muscle to undergo terminal myogenic differentiation.

Hypothesis 2: CD34^{+}ve cells isolated from a regenerating mouse skeletal muscle would be more heterogeneous and would consist of various stem cell populations compared to CD34^{+}ve cells isolated from a normal skeletal muscle.

Aim 2: To compare the composition of CD34^{+}ve cells isolated from normal and regenerating skeletal muscle and identify the subpopulations of stem cells present.

Hypothesis 3: CD34^{+}ve cells isolated from a regenerating mouse skeletal muscle would contain cells that are capable of angiogenesis.

Aim 3: To determine the potential of cells within the CD34^{+}ve cells isolated from a regenerating skeletal muscle to undergo vascular formation.
4.2 Results

4.2.1 Behavioral Characteristics Of Skeletal Muscle Derived CD34$^{+}$ve Cells In Culture And Their Capacity For Differentiation

CD34$^{+}$ve cells isolated from normal muscle and regenerating muscle (3 days post notexin treatment) were compared to determine whether the cell populations are the same from both sources. First the behavioral characteristics in culture were determined by isolating the cells and culturing them on collagen coated dishes. Differences between the two populations were evident 24 hours post culture in muscle stem cell medium. CD34$^{+}$ve cells isolated from the regenerating muscle showed two distinct subpopulations of cells (Figure 15). The first population adhered to the collagen coating of the dish and had a fibroblast like morphology. The second population cells remained in suspension and had a spherical morphology. Furthermore these floating cells were highly refractive and appeared bright under a light microscope. CD34$^{+}$ve cells isolated from a normal skeletal muscle showed dissimilarity to both the adherent and floating cell populations derived from the regenerating skeletal muscle. 24 hours post culture, they remained in suspension and lacked the adherent cells that were isolated from the regenerating muscle. They also appeared significantly smaller in size than the floating cells from the regenerating muscle (Figure 15).

The next step was to determine the potential of adherent and floating CD34$^{+}$ve cells isolated from regenerating muscle to undergo terminal myogenic differentiation. 24 Hours post culture, the floating cells from the regenerating muscle were transferred into a new collagen coated dish and were separately grown in muscle stem cell culture medium. The adherent cells from the regenerating muscle spontaneously differentiated and formed myotubes after 3
Figure 15: CD34<sup>+</sup> cells isolated from a regenerating muscle differs to cells isolated from a normal muscle

(A) CD34<sup>+</sup> cells isolated from a normal tibialis anterior (TA) muscle, 24 hours in culture. (B) CD34<sup>+</sup> cells isolated from a regenerating TA muscle (3 days post notexin treated), 24 hours in culture. Black arrows: floating cells; White arrow: adherent cells.
Figure 16: Both adherent and floating CD34$^{+ve}$ cells are capable of differentiating and formation of contracting myotubes.

(A) Adherent CD34$^{+ve}$ cells. (B) Contracting myotubes formed by adherent cells after 3 days in culture. (C) Floating CD34$^{+ve}$ cells. (D) Contracting myotubes formed by floating cells after 3 days in culture.
days in culture. The floating cells were much slower to form myotubes, these cells took approximately 2 days to adhere to the gelatin coated dish and a further 3 days to differentiate into myotubes (Figure 16). Myotubes formed by both adherent and floating CD34\(^{+ve}\) cells were capable of spontaneous contraction in culture medium.

A high number of CD34\(^{+ve}\) cells are required for muscle stem cell transplantation. CD34\(^{+ve}\) cells were isolated from various muscles and cell counts were performed to determine the best source for high cell numbers. Various muscles such as regenerating tibialis anterior (TA) muscles (3 days post notexin treated), normal TA muscles, normal extensor digitorum longus (EDL) muscles, normal soleus muscles and normal diaphragms were used. The highest number of CD34\(^{+ve}\) cells isolated was \(1.09 \times 10^6 \pm 9.9 \times 10^3\) per regenerating TA muscle (Figure 17). A normal TA had approximately 12 times less cells, an EDL and soleus both had approximately 30 times less cells whereas a diaphragm had 5 times less cells than a regenerating TA. The proportion of CD34\(^{+ve}\) cells among the mononucleated cells isolated is also highest in the regenerating TA at just above 20\% where as both a normal TA and a normal diaphragm have just above 5\% CD34\(^{+ve}\) cells (Figure 18).
Figure 17: Regenerating tibialis anterior (TA) muscle is the highest source for skeletal muscle CD34<sup>+</sup> cells

CD34<sup>+</sup> cells were isolated from various muscles of C57BL/6 male mice aged approximately 8 weeks. Normal = untreated muscles; Regenerating = 3 days post notexin treated (0.4mg in 40μl of saline, injected intramuscularly). TA = tibialis anterior muscle; EDL = extensor digitorum longus muscle. Error bars are based on standard deviations of more than 3 different samples. Number of CD34 positive cells was calculated after sorting for CD34 antibody labeled skeletal muscle mononucleated cells using magnetic bead separation.
Figure 18: A regenerating skeletal muscle has a higher proportion of CD34<sup>+</sup> cells compared to normal skeletal muscles

Percentages of CD34<sup>+</sup> cells were determined using flow cytometry. TA muscles and diaphragm muscles of C57BL/6 male mice, aged approximately 8 weeks, were used. Normal = untreated muscles; Regenerating = 3 days post notexin treated (0.4mg in 40μl of saline; injected intramuscularly). TA = tibialis anterior muscle. Error bars are based on standard deviations of more than 3 different samples.
4.2.2 Level Of Myogenic Commitment Of The Adherent And Floating CD34$^{+ve}$ Skeletal Muscle Cells

Adherent CD34$^{+ve}$ cells formed myotubes much faster than the floating cells in culture. We determined whether this difference in the progress of differentiation is due to varying levels of myogenic commitment. In order to study myogenic commitment, expression of myogenin and MyoD in adherent compared to floating cells was investigated through western blot analysis. CD34$^{+ve}$ cells were isolated from tibialis anterior muscles which had been injected with notexin 3 days prior to tissue collection. Cells were then cultured for 24 hours. Floating cells and adherent cells were collected separately and the cell pellets were processed for western blot analysis. Protein expression was determined using anti myogenin and anti MyoD antibodies (Figure 19). Both floating and adherent CD34$^{+ve}$ cells expressed MyoD, although the amount of MyoD protein present in adherent cells appeared to be higher than that in the floating cells. Floating CD34$^{+ve}$ cells did not express myogenin, whereas the adherent CD34$^{+ve}$ cells appeared to have a small amount of myogenin expression. This indicates that the adherent cells are further committed towards terminal myogenic differentiation than floating cells.
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Figure 19: Adherent CD34⁺ve cells are further committed towards myogenic terminal differentiation than floating CD34⁺ve cells

CD34⁺ve cells were isolated from 3 days post notexin treated tibialis anterior muscles. Cells were cultured in muscle stem cell medium on collagen coated dishes overnight. Floating cells and adherent cells were collected separately and processed for western blot analysis to detect myogenin and MyoD expression. Coomassie staining shows the loading of each sample. Sample #1, #2 and #3 are samples taken from 3 different mice.
4.2.3 Heterogeneity Of CD34^{+}ve Cells Isolated From A Regenerating Compared To A Normal Skeletal Muscle

Many muscle stem cell populations have been characterised and are currently being used in skeletal muscle stem cell transplantation studies. Flow cytometry was carried out in order to determine whether there are cells present within CD34^{+}ve cells isolated from skeletal muscle, which express markers of known muscle stem cell populations. As reported in the section 4.2.1, a regenerating skeletal muscle has 12 times more CD34^{+}ve cells than a normal muscle. In order to determine whether the proportion and the number of various stem cell populations present are different, flow cytometry was carried out on cells isolated from both regenerating and normal skeletal muscles. Flow cytometry results are summarised in Table 2.

Muscle Derived Stem Cells (MDSCs) have been shown to contribute to skeletal muscle regeneration upon transplantation (Qu-Petersen, Z., et al., 2002). Mesoangioblasts are another population of stem cells that have shown remarkable success in contribution of skeletal muscle regeneration upon transplantation (Sampaolesi, M., et al., 2003, Sampaolesi, M., et al., 2006). Skeletal muscle side population (SP) cells have also been identified to successfully engraft upon transplantation into regenerating muscle (Gussoni, E., et al., 1999, Jackson, K.A., et al., 1999). MDSCs, mesoangioblasts and skeletal muscle SP cells were characterised to express CD34 and Stem Cell Antigen 1 (Sca-1) cell surface antigens while lacking the expression of the panleukocyte marker CD45 (reviewed in Peault, B., et al., 2007). In order to determine the proportion of cells which express MDSC, mesoangioblast and skeletal muscle SP cell markers, CD34^{+}ve cells isolated from normal and regenerating (3
<table>
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<th>Cell population</th>
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<td>Satellite cell-Progenitor cells</td>
<td>Sca-1&lt;sup&gt;−/−&lt;/sup&gt; CD45&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>31.8 ± 2.2% (2.83x10&lt;sup&gt;6&lt;/sup&gt; ± 1.1x10&lt;sup&gt;6&lt;/sup&gt;)</td>
<td>13.4 ± 1.1%&lt;sup&gt;**&lt;/sup&gt; (1.47x10&lt;sup&gt;6&lt;/sup&gt; ± 1.7x10&lt;sup&gt;6&lt;/sup&gt;)</td>
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<td>MDSCs</td>
<td>Sca-1&lt;sup&gt;−/−&lt;/sup&gt; CD45&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>59 ± 2.4% (5.35x10&lt;sup&gt;6&lt;/sup&gt; ± 2.1x10&lt;sup&gt;6&lt;/sup&gt;)</td>
<td>56 ± 6.6% (6.13x10&lt;sup&gt;6&lt;/sup&gt; ± 9.3x10&lt;sup&gt;6&lt;/sup&gt;)</td>
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<td>Mesoangioblasts</td>
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<td>SP cells</td>
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<td>Pericytes</td>
<td>CD56&lt;sup&gt;−/−&lt;/sup&gt; ALP&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>2.2 ± 0.1% (1.56x10&lt;sup&gt;6&lt;/sup&gt; ± 7.9x10&lt;sup&gt;5&lt;/sup&gt;)</td>
<td>4.4 ± 1.3% (6.78x10&lt;sup&gt;6&lt;/sup&gt; ± 1.5x10&lt;sup&gt;6&lt;/sup&gt;)</td>
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<td>Myo-endothelial</td>
<td>CD144&lt;sup&gt;−/−&lt;/sup&gt; CD56&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>10.9 ± 0.3% (9.49x10&lt;sup&gt;6&lt;/sup&gt; ± 3.9x10&lt;sup&gt;6&lt;/sup&gt;)</td>
<td>0 *** (0)</td>
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<td>Endothelial</td>
<td>CD144&lt;sup&gt;−/−&lt;/sup&gt; CD56&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>0 (0)</td>
<td>0.3 ± 0.1% * (3.89x10&lt;sup&gt;5&lt;/sup&gt; ± 8.2x10&lt;sup&gt;5&lt;/sup&gt;)</td>
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<td>CD133&lt;sup&gt;−/−&lt;/sup&gt; progenitor cells</td>
<td>CD133&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>2.7 ± 0.5% (2.37x10&lt;sup&gt;6&lt;/sup&gt; ± 1.1x10&lt;sup&gt;6&lt;/sup&gt;)</td>
<td>30 ± 4.5%*** (3.28x10&lt;sup&gt;6&lt;/sup&gt; ± 5.7x10&lt;sup&gt;6&lt;/sup&gt;)</td>
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<td>Infiltrated cells in response to SDF-1</td>
<td>CXCR4&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>4.6 ± 1.3% (4.12x10&lt;sup&gt;6&lt;/sup&gt; ± 2.6x10&lt;sup&gt;6&lt;/sup&gt;)</td>
<td>23.8 ± 2.5%** (2.63x10&lt;sup&gt;6&lt;/sup&gt; ± 3.6x10&lt;sup&gt;6&lt;/sup&gt;)</td>
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<td>Monocytes</td>
<td>CD11b&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>2.0 ± 0.5% (0.46x10&lt;sup&gt;6&lt;/sup&gt; ± 1.2x10&lt;sup&gt;6&lt;/sup&gt;)</td>
<td>65.5 ± 1.1%*** (7.77x10&lt;sup&gt;6&lt;/sup&gt; ± 6.6x10&lt;sup&gt;6&lt;/sup&gt;)</td>
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<td>Myogenic cells</td>
<td>Pax 7&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>12.4 ± 1.1% (3.10x10&lt;sup&gt;6&lt;/sup&gt; ± 4.5x10&lt;sup&gt;6&lt;/sup&gt;)</td>
<td>25 ± 1.9% * (2.74x10&lt;sup&gt;6&lt;/sup&gt; ± 3.2x10&lt;sup&gt;6&lt;/sup&gt;)</td>
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Table 2: Stem cell subpopulations present within the CD34<sup>−/−</sup> skeletal muscle cells

CD34<sup>−/−</sup> cells were isolated from either normal or 3 day post notexin treated (regenerating) TA muscles. Percentages of subpopulations were determined by flow cytometry. Since certain cell markers can be expressed by more than one lineage of cells, cells from multiple lineages could be present within one cell subpopulation. Values in brackets are the total number of cells per muscle within the isolated CD34<sup>−/−</sup> cell population. *<i>p</i> < 0.05, **<i>p</i> < 0.005, ***<i>p</i> < 0.0005. MDSCs: Muscle derived stem cells; SP cells: skeletal muscle side population cells.
days post notexin treated) tibialis anterior (TA) muscles were labeled with anti CD34, anti Sca-1 and anti CD45 antibodies followed by flow cytometry. Flow cytometry revealed that 56.0 ± 13.5% cells within the CD34^+ve cells isolated from regenerating TAs were Sca-1^-ve and CD45^-ve. This proportion was not significantly different to what was found in normal TAs. However, since the total cell number of CD34^+ve cells is much higher in a regenerating skeletal muscle than a normal muscle, the absolute number of cells which express markers of MDSCs, mesoangioblasts and skeletal muscle SP cells present in a regenerating TA was approximately 10 times higher than in a normal TA.

Another muscle stem cell population that has been characterised by Montarras, et al. (2005) is the CD34^+ve/Sca-1^-ve/CD45^-ve satellite cell progenitor cells isolated from mouse skeletal muscles (Montarras, D., et al., 2005). The proportion of cells which lack Sca-1 and CD45 within the CD34^+ve cells isolated from regenerating TAs was 13.4 ± 1% which was a significantly smaller proportion than the 31.8 ± 2.2% found in normal TAs. However, the absolute number of cells which expressed satellite cell progenitor cell markers was 5 times higher in a regenerating TA compared to a normal TA.

Recently, pericytes have been identified for their ‘stem’ like characteristics and their ability to engraft into host skeletal muscles upon systemic transplantation. These cells express the cell surface marker alkaline phosphatase (ALP) and lack the expression of CD56. CD34^+ve cells from normal muscles and regenerating muscle were labeled with an anti ALP antibody and anti CD56 antibody. Flow cytometry showed that 2.2 ± 0.1% of CD34^+ve cells from normal TAs and 4.4 ± 1.3% of CD34^+ve cells from regenerating TAs express pericyte
markers. The difference of cell proportions which express pericyte markers within normal and regenerating TAs were not statistically significant, however the absolute number of cells was approximately double in a regenerating TA compared to a normal TA.

There are other stem cell populations which have shown engraftment in host skeletal muscle upon transplantation via the systemic circulation. Among these are the CD133+ve cells which some studies have shown to co-express CD34 (Peault, B., et al., 2007). In order to determine the proportion of CD34+ve cells that express CD133, cells were isolated from normal and regenerating TAs, followed by labeling with an anti CD34 and anti CD133 antibody. Flow cytometry revealed that 2.7 ± 0.5% of CD34+ve cells isolated from normal TAs expressed CD133, whereas regenerating TAs consisted of a significantly higher proportion of 30 ± 4.5% CD133 positive cells (p = 0.006). The absolute number of CD133+ve/CD34+ve cells from a regenerating TA was approximately 100 times more than that found in a normal TA.

Another population of stem cells that can be transplanted via the systemic circulation co-express CD34 and CD144 cell surface antigens (Zheng, B., et al., 2007). They can be further categorised into two subpopulations depending on the expression of CD56 cell surface antigen. CD34+ve/CD144+ve and CD56+ve cells are called myoendothelial cells whereas CD56–ve cells are called endothelial cells. In order to identify the proportion of CD34+ve cells which co-express myoendothelial cell markers in regenerating TAs compared to a normal muscle, cells were isolated and labeled with anti CD144, anti CD34 and anti CD56 antibodies. Flow cytometry performed on the labeled cells showed that there are undetectable levels of myoendothelial cells present within the CD34+ve cells isolated from regenerating muscles.
However, CD34\textsuperscript{+ve} cells isolated from normal muscles consisted of a significantly higher proportion of cells which express myoendothelial markers. Cells expressing endothelial cell markers were absent in CD34\textsuperscript{+ve} cells isolated from normal muscles, however, there were approximately 3000 cells which expressed endothelial cell markers within the CD34\textsuperscript{+ve} cells isolated from a regenerating TA.

When a skeletal muscle is injured it releases stromal derived factor -1 (SDF-1) (Ratajczak, M.Z., et al., 2003). CXCR4 is the receptor for the cytokine SDF-1. Therefore release of SDF-1 results in infiltration of CXCR4 positive cells from the systemic circulation (Aiuti, A., et al., 1997, Pituch-Noworolska, A., et al., 2003). Cells isolated from a normal and a regenerating TA were labeled with an anti CD34 and anti CXCR4 antibody in order to determine the proportions of CD34\textsuperscript{+ve} cells which co-express CXCR4. The proportion of CD34\textsuperscript{+ve} cells which co-express CXCR4 from normal TAs was 4.6 ± 1.3% whereas regenerating TAs consisted of 23.8 ± 2.5%. The increase in CD34\textsuperscript{+ve} cells which co-express CXCR4 in regenerating TAs compared to normal TAs was highly significant (p = 0.004) and the absolute number of CXCR4\textsuperscript{+ve} cells was approximately 60 times higher than in a normal TA.

Skeletal muscle regeneration is a complex procedure, which initially requires the removal of cell debris followed by formation of new muscle fibers. Monocytes and macrophages have been shown to aid in muscle regeneration via phagocytosis as well as promotion of muscle stem cell infiltration and formation of new muscle fibers (Tidball, J.G., 2005). Monocytes which are immature macrophages, express the cell surface markers CD34 and CD11b.
(Arnold, L., et al., 2007). In order to determine the proportion of cell which express this monocyte marker within the CD34\(^{+ve}\) cells isolated from a normal and regenerating TA, the cells were labeled with anti CD34 antibody and anti CD11b antibody prior to running through flow cytometry. CD34\(^{+ve}\) cells from a normal TA consisted of 2.8 ± 0.8% and CD34\(^{+ve}\) cells from a regenerating TA consisted of a significantly higher proportion of 65.5 ± 1.1% cells which co-express CD11b cell surface antigen (p = 0.004). The absolute number of cells which express the monocyte marker CD11b within a regenerating TA was approximately 300 times more than that was in a normal TA.

Pax 7 expression in adult skeletal muscles identifies satellite/myogenic cells (Hawke, T.J. and Garry, D.J., 2001). In order to determine the proportion of myogenic cells within the CD34\(^{+ve}\) cells in normal and regenerating muscles, cells isolated from normal and regenerating TAs were labeled with anti CD34 and anti Pax 7 antibodies. Flow cytometry revealed that 12.4 ± 1.1% of CD34\(^{+ve}\) cells from normal TAs and 25 ± 1.9% CD34\(^{+ve}\) cells from regenerating TAs were Pax 7\(^{+ve}\). The absolute number of myogenic cells within the CD34\(^{+ve}\) cells isolated from a regenerating TA has increased to approximately 25 times more than that was in a normal TA.

Characterisation of CD34\(^{+ve}\) cells isolated from a regenerating muscle compared to a normal muscle showed that a regenerating muscle has higher proportions of cells infiltrated from blood such as CD133\(^{+ve}\) cells, CXCR4\(^{+ve}\) cells and monocytes. Cell populations which express MDSC markers, satellite cell progenitor cell markers, pericyte markers and endothelial cell markers have also increased in numbers within a regenerating skeletal muscle.
compared to a normal muscle. Expression of cell markers on certain cell populations is not mutually exclusive, therefore, proportions of various populations of cells analysed is likely to include cells from multiple cell lineages. Nevertheless, flow cytometry performed in order to detect various cell markers revealed that the CD34$^{\text{ve}}$ skeletal muscle cell population is highly heterogeneous, consisting of various stem cells, myogenic cells and cells which express the monocyte marker CD11b.
4.2.4 Potential Of CD34$^{+ve}$ Cells Isolated From A Regenerating Skeletal Muscle To Undergo Angiogenic Differentiation

The presence of endothelial (CD144$^{+ve}$) and endothelial progenitor cells (CD133$^{+ve}$, CXCR4$^{+ve}$, Sca-1$^{+ve}$) within the CD34$^{+ve}$ cell population has been established in the previous section. In order to determine the capacity of these cells to form blood vessels, CD34$^{+ve}$ cells isolated from 3 day post notexin injected tibialis anterior muscles were induced to differentiate using an \textit{in vitro} angiogenesis kit. Cell cultures were observed under a light microscope at 6 hours, 48 hours, 72 hours and 96 hours in culture (Figure 20). No change in morphology was observed in cells at 6 hours in culture. However, by 48 hours, the majority of the cells appeared to be dying and the remaining cells were attached to the gel matrix. 72 hours in culture showed evidence of rapid cell proliferation of the remaining cells where distinct colonies were present. The first appearance of angiogenesis was observed at this time point, where some cells were aligning to form vessels. Vessel formation was observed at 96 hours in culture. Sprouting of new capillaries from mature vessels was also observed at this time point. This demonstrates the ability of a subpopulation of CD34$^{+ve}$ cells isolated from a regenerating skeletal muscle to undergo angiogenesis and provides further confirmation of the presence of endothelial cells and endothelial progenitor cells within the CD34$^{+ve}$ cell population.
Figure 20: *In vitro* angiogenesis from CD34<sup>+</sup>ve skeletal muscle cells

CD34<sup>+</sup>ve cells were isolated from 3 day post notexin treated (regenerating) tibialis anterior muscles. Using the Chemikon *In vitro* Angiogenesis kit, the cells were induced to differentiate towards vessel formation in endothelial cell culture medium. (A) 6 hours in culture. (B) 48 hours in culture. White arrows: dead cells, Black arrow: live cells. (C) 72 hours in culture. Black arrow: aligning of cells. (D) 96 hours in culture. Black arrow: vessel, asterisk: branching or sprouting of a new vessel.
4.3 Discussion

The CD34 cell surface antigen has been associated with mouse satellite cells and many other mouse muscle stem cell populations (reviewed by Cossu, G. and Sampaolesi, M., 2007, Peault, B., et al., 2007). Many published studies demonstrate the capacity of individual CD34+ve stem cell populations to differentiate towards muscle fiber formation and some studies have further demonstrated multilineage potential (Peault, B., et al., 2007). The results shown in this chapter demonstrates the heterogeneity of CD34+ve cells isolated from a regenerating mouse skeletal muscle. These cells showed multilineage potential where appropriate stimulation resulted in terminal myogenic differentiation and angiogenesis.

CD34+ve cells, isolated from regenerating skeletal muscles, cultured overnight in muscle stem cell culture medium revealed the presence of two distinct subpopulations of cells. Approximately two thirds of the cells adhered to the collagen coating overnight and had a flat fibroblast like morphology whereas the rest of the cells remained in suspension and appeared small in size and round in morphology. Although both populations demonstrated the ability to spontaneously differentiate into contracting myotubes, formation of myotubes was much faster in the adherent CD34+ve cell cultures compared to the floating cells. There are many studies which have reported that muscle stem cell populations such as MDSCs (Qu-Petersen, Z., et al., 2002), pericytes (Dellavalle, A., et al., 2007) and CD133 progenitor cells (Nunes, V.A., et al., 2007) have a small, round morphology and demonstrate a lack of adherence in culture. Characterisation of MDSCs published by Qu-Peterson, et al. (2002) reported early preplate (EP) and late preplate (LP) cells which are more adherent in culture than the MDSCs. EP cells and LP cells were shown to be more committed to myogenic differentiation than the
MDSCs (Qu-Petersen, Z., et al., 2002). Another study has shown that round, floating cells isolated from mouse skeletal muscle converted into thick, adherent cells prior to myogenic terminal differentiation (Hashimoto, N., et al., 2004). A human muscle stem cell study also reported round, floating skeletal muscle cells which have muscle stem cell characteristics and thick, adherent cells which have more satellite cell like characteristics (Alessandri, G., et al., 2004). Analysis of myogenin and MyoD expression in the floating versus adherent CD34^{+}ve cells revealed that adherent cells, which expressed both myogenin and MyoD, are in fact further committed towards terminal myogenic differentiation than the floating cells, which expressed a small amount of MyoD and no myogenin. It is notable that CD34^{+}ve cells isolated from a normal muscle lack the adherent cell population. This can be explained since a normal muscle does not require mononucleated cells that are committed towards myogenic terminal differentiation. Furthermore, the floating CD34^{+}ve cells isolated from normal skeletal muscles appeared much smaller in size than the floating CD34^{+}ve cells from regenerating skeletal muscles. It is known that upon activation of satellite cells, the nuclear/cytoplasmic ratio reduces, thus resulting in bigger cells with smaller nuclei (Hawke, T.J. and Garry, D.J., 2001). Therefore, the phenomenon of smaller CD34^{+}ve cells which were isolated from normal muscles compared to the CD34^{+}ve cells isolated from regenerating muscles is quite possibly due to the quiescent state of cells in normal muscles as opposed to the activated cells in regenerating muscle.

At present, there are numerous populations of stem cells being used in skeletal muscle stem cell transplantation studies. Among these there are skeletal muscle resident stem cells such as MDSCs (Qu-Petersen, Z., et al., 2002), skeletal muscle SP cells (Gussoni, E., et al., 1999,
Jackson, K.A., et al., 1999), myoendothelial cells (Zheng, B., et al., 2007) and satellite cell progenitor cells (Montarras, D., et al., 2005). Furthermore, there are skeletal muscle non-resident stem cells such as pericytes (Dellavalle, A., et al., 2007), mesoangioblasts (Sampaolesi, M., et al., 2003, Sampaolesi, M., et al., 2006), and CD133^+ve progenitor cells (Gavina, M., et al., 2006, Koponen, J.K., et al., 2007, Torrente, Y., et al., 2007). Characterisation of CD34^+ve cells using flow cytometry demonstrated the presence of cell populations which expressed cell markers of MDSCs, mesoangioblasts, skeletal muscle SP cells, satellite cell progenitor cells, pericytes and CD133^+ve progenitor cells. Although expression of these cell markers may indicate the presence of a specific muscle stem cell subpopulation, this is not conclusive since the isolation procedure and source of cells are different to the originally characterised MDSCs, mesoangioblasts, skeletal muscle SP cells, satellite cell progenitor cells, pericytes and CD133^+ve progenitor cells.

Skeletal muscle regeneration has been studied extensively using various models such as injection of the myotoxin, notexin (Huard, J., et al., 1994, Kinoshita, I., et al., 1994) or marcaine (Wakata, N., et al., 2001), cryodamage and deinervation/devascularisation (Vignaud, A., et al., 2004). Various muscle stem cell populations have also been studied which have demonstrated successful engraftment into injured host muscles upon transplantation (Cossu, G. and Sampaolesi, M., 2007, Peault, B., et al., 2007). However, there is a lack of knowledge in regards to the involvement of various muscle stem cell populations during skeletal muscle regeneration. The results in this chapter have compared various cell populations present within a regenerating skeletal muscle (3 days post notexin injection) to a normal skeletal muscle. Cells which express markers of satellite cell
progenitor cells, MDSCs, mesoangioblasts, skeletal muscle SP cells, pericytes, endothelial cells and myogenic cells (Pax 7+ve) appear to have undergone extensive proliferation compared to a normal muscle. Extensive proliferation of muscle stem cells and myogenic cells are expected since it is well known that upon injury muscle stem and satellite cells undergo extensive proliferation (reviewed by Wagers, A.J. and Conboy, I.M., 2005). However, cells which express myoendothelial markers are absent within a regenerating skeletal muscle where as a normal tibialis anterior muscle has approximately 9000 such cells. There are two possible hypotheses that may explain this phenomenon. The first hypothesis is that at 3 days post notexin injection these cells have already differentiated down the myogenic pathway and no longer expresses the myoendothelial cell markers. The second hypothesis is that the cells which express myoendothelial cell markers have not proliferated in response to the injury caused by notexin, thus due to expansion of other cells, they may not be detectable through flow cytometry.

Another finding was that approximately 65% of the CD34+ve cells from a regenerating skeletal muscle expressed the monocyte marker CD11b, compared to the small proportion of 3% that was observed in the normal muscles. This increase in the proportion of cells which express CD11b within CD34+ve cells is also reflected in the absolute number of these cells where regenerating muscles occupied approximately 300 times more CD11b+ve cells than normal muscles. There is a substantial amount of evidence which supports the fact that an injured muscle secretes certain chemoattractants causing infiltration of monocytes and macrophages (Chazaud, B., et al., 2003, Lescaudron, L., et al., 1999). Furthermore, it has also been suggested that the monocytes and macrophages release certain factors which
promote myoblast proliferation (Bondesen, B.A., et al., 2004, Cantini, M. and Carraro, U., 1995, Robertson, T.A., et al., 1993). Another study reported that absence of macrophages and monocytes results in impairment of skeletal muscle regeneration (Summan, M., et al., 2006). Although it is possible that not all CD11b positive cells are monocytes, the fact that a regenerating skeletal muscle has infiltrated 300 times more cells which express the monocyte marker than a normal muscle reiterates the significance of the role of monocytes and macrophages in skeletal muscle regeneration.

Considering the evidence which supports the role of monocytes and macrophages in satellite cell activation, myoblast differentiation, and myoblast proliferation (Tidball, J.G., 2005), it is not unreasonable to theorise that the factors released by these cells may also have a role in stem cell infiltration from the systemic circulation. Although the role of monocyte and macrophage released factors in recruitment of stem cells from the systemic circulation is yet to be investigated, the release of the chemoattractant, stromal derived factor-1 (SDF-1) from injured muscle has been well documented (Ratajczak, M.Z., et al., 2003). CXCR4 is the receptor for SDF-1. Release of SDF-1 results in recruitment of CXCR4+ve cells from the systemic circulation thereby ‘homing’ these stem cells into injured tissue (Aiuti, A., et al., 1997, Pituch-Noworolska, A., et al., 2003, Ratajczak, M.Z., et al., 2004). This phenomenon is compatible with the results reported in this chapter which demonstrates an approximately 60 fold increase in CXCR4+ve cells in a regenerating muscle compared to a normal muscle. It has been reported that some CD133+ve stem cells isolated from the peripheral blood, which are capable of myogenic differentiation, co-express CXCR4 (Gavina, M., et al., 2006). Results reported in this chapter demonstrated approximately a 130 times increase in
CD133^{+ve} cells within a regenerating skeletal muscle compared to a normal muscle. It is likely that some of these cells may have been recruited in response to SDF-1 secretion from the injured skeletal muscle.

The results in this chapter also demonstrate a slight increase in endothelial cells in a regenerating skeletal muscle as opposed to a normal muscle, however these cells are only comprised of approximately 0.3% of the CD34^{+ve} cells. Furthermore, endothelial progenitor cells have been shown to express cell surface antigens such as Sca-1, CD133 and CXCR4 (Dome, B., et al., 2007). Endothelial and endothelial progenitor cells contribute to angiogenesis (Leri, A. and Kajstura, J., 2005), which is an important role in regeneration and repair of any damaged tissue. Characterisation of CD34^{+ve} cells isolated from a regenerating skeletal muscle demonstrated significant increases in the number of Sca-1^{+ve}, CD133^{+ve} and CXCR4^{+ve} cells compared to a normal muscle. Angiogenesis achieved via differentiation of CD34^{+ve} cells isolated from regenerating skeletal muscles demonstrated that the small proportion of endothelial cells and endothelial progenitor cells present within this population are capable of differentiation down the vascular pathway. It is well known that muscle injury caused by notexin injection does not damage the microvasculature of the skeletal muscle bed (Harris, J.B., 2003, Vignaud, A., et al., 2004). However, the increased number of endothelial cells and endothelial progenitor cells in a regenerating skeletal muscle bed which are capable of vascular formation indicates that angiogenesis may have a crucial role in skeletal muscle regeneration following notexin induced injury.
Muscle stem cell transplantation studies using various stem cell populations have shown limited engraftment. However, cell populations such as pericytes, mesoangioblasts, MDSCs and CD133\textsuperscript{+} progenitor cells have recently shown potential for improved engraftment (Cossu, G. and Sampaolesi, M., 2007, Peault, B., et al., 2007). The findings reported in this chapter reiterate the significant role of a heterogeneous population of stem cells, monocytes and macrophages in successful regeneration of an injured skeletal muscle bed. Therefore, it can be hypothesised that use of this heterogeneous population of cells as donors in muscle stem cell transplantation would promote regeneration and enhance engraftment of donor cells within the host muscle.
CHAPTER 5

Selective Enrichment Of Transplanted CD34^{+}ve Donor Cells In Recipient Skeletal Muscles
5.1 Introduction

5.1.1 Overview Of The Chapter

Muscle stem cell transplantation has been studied with limited success since the 1980’s as a treatment for skeletal muscle diseases such as Duchenne muscular dystrophy (Cossu, G. and Sampaolesi, M., 2007, Peault, B., et al., 2007). Clinical application has shown very limited success. The three main limitations which result in unsuccessful transplantation are: (1) inefficient donor cells that demonstrate a limited ability for host incorporation after transplantation (Peault, B., et al., 2007), (2) competition from endogenous cells which causes donor cell death or poor engraftment (Fan, Y., et al., 1996, Karpati, G., et al., 1993), (3) limited migration of donor cells within the host system leading to localised engraftment (Skuk, D., et al., 1999), all leading to insufficient or short term recovery of dystrophic host muscles.

The BCNU + O\textsubscript{6}BG driven selective enrichment strategy has demonstrated enhanced engraftment of donor hematopoietic stem cells within the host bone marrow upon transplantation (Gerson, S.L., 2005, Gerull, S., et al., 2007, Reese, J.S., et al., 2008). Furthermore, the initial studies described in this thesis reported successful enrichment of skeletal muscle cells using \textit{in vitro} and \textit{in vivo} models using the BCNU + O\textsubscript{6}BG driven selective enrichment strategy. This chapter reports the application of this strategy in skeletal muscle stem cell transplantation in order to achieve enhanced engraftment of donor cells within host tissue. This chapter further addresses the three main limitations in muscle stem cell transplantation mentioned above and shows a significant improvement in donor cell engraftment as a direct result of the strategy used.
5.1.2 Hypotheses And Aims Of The Chapter

Hypothesis 1: The application of BCNU + O\textsuperscript{6}BG driven selective enrichment strategy would enhance the engraftment of CD34\textsuperscript{ve} donor cells within injured host skeletal muscle upon transplantation.

Aim 1: To determine the effects of BCNU + O\textsuperscript{6}BG treatment of injured host skeletal muscle on engraftment of CD34\textsuperscript{ve} donor cells upon transplantation.

Hypothesis 2: Transplanted CD34\textsuperscript{ve} donor cells isolated from a regenerating skeletal muscle would demonstrate the ability for migration via the systemic circulation to injured skeletal muscles.

Aim 2: To determine whether transplanted CD34\textsuperscript{ve} donor cells isolated from a regenerating skeletal muscle are capable of migration via the systemic circulation to injured skeletal muscles.
5.2 Results

5.2.1 Pilot Study: Injured EDL Muscles Of C57BL/6 Mice As Recipient Muscles For Transplantation

Female C57BL/6 EDL muscles were treated with either notexin or notexin plus BCNU with either MGMT(P140K) transgenic or wildtype CD34\(^{+}\) male donor cells. Cells and drugs were injected intramuscularly. In order to determine the level of donor cell engraftment, DNA were analysed for the presence of Y chromosome DNA using quantitative PCR on recipient muscles collected 1 hour, 3 days, 7 days or 14 days post transplantation. The data is shown in Figure 21a and 21b. The muscles which received notexin, BCNU plus MGMT(P140K) transgenic donor cells showed 2 ± 0.9\% donor DNA within recipient muscle DNA 1 hour after transplantation. The percentages of donor DNA levels within individual recipient muscles varied considerably where 4 muscles showed close to no donor DNA. This indicates that donor cells undergo death or leakage following injection into the recipient muscles. This is reflected by DNA samples from recipient muscles collected at all time points. There was a considerable variation in the percentages of donor DNA levels where the majority of the samples consisted of less than 0.5\% donor DNA within recipient muscle DNA. However, differences between treatment groups could be observed when averages were studied with the corresponding standard errors. The recipient muscles which received notexin plus cells showed a maximum amount of donor DNA at 3 days post transplantation. MGMT(P140K) transgenic donor cell recipient muscles had 1.1 ± 0.4\% donor DNA within recipient muscle DNA and wildtype donor cell recipient muscles showed approximately 0.4 ± 0.1\% donor
Figure 21a: Variable engraftment 3 days post transplantation resulting from loss of cells immediately after injection.

Quantitative PCR analysis was done to determine the amount of donor DNA (Y chromosome positive) present within the female recipient muscles. Concentrations of recipient muscle DNA samples were adjusted to 50ng/μl. Notexin: treated with notexin (0.1μg), BCNU: treated with BCNU (0.1mg), Mgmt cells: transplanted with $6\times10^4$ male MGMT(P140K) transgenic donor cells, WT cells: transplanted with $6\times10^4$ male wildtype donor cells.
Figure 21b: Variable engraftment 7 & 14 days post transplantation resulting from loss of cells immediately after injection.

Quantitative PCR analysis was done to determine the amount of donor DNA (Y chromosome positive) present within the female recipient muscles. Concentrations of recipient muscle DNA samples were adjusted to 50ng/μl. Ntx: treated with notexin (0.1μg), BCNU: treated with BCNU (0.1mg), m. cells: transplanted with 6x10⁴ male MGMT(P140K) transgenic donor cells, wt cells: transplanted with 6x10⁴ male wildtype donor cells.
DNA within recipient muscle DNA. The percentage of donor DNA in these muscles showed a reduction with time reaching approximately 0.3% within recipient muscle DNA at 14 days post transplantation. The recipient muscles which received notexin, BCNU plus MGMT(P140K) transgenic donor cells at 3 days post transplantation had less donor DNA than the muscles which received notexin plus cells only. However at 7 and 14 days post transplantation the muscles which received notexin, BCNU plus MGMT(P140K) transgenic donor cells had a substantially higher percentage of donor DNA than the muscles which received notexin and cells only. The recipient muscles which received notexin, BCNU plus wildtype cells had a very low percentage of donor DNA which never reached higher than 0.5% within recipient muscle DNA at any of the time points. In summary, the data reported in this section indicates that there is a substantial loss of donor cells immediately after transplantation. Nevertheless, there is also evidence of enhanced engraftment in the recipients which received notexin, BCNU plus MGMT(P140K) transgenic cells compared to muscles which received notexin plus cells only or notexin, BCNU plus wildtype cells.

Further studies were done to determine the cause of donor cell loss immediately after transplantation. The transplantation protocol is such that notexin plus BCNU solution is injected intramuscularly in a total volume of 10µl followed by cells in another 10µl intramuscular injection. Observation of the second injection under a dissecting microscope showed slight leakage of solution. In order to determine whether the loss of cells is caused by the leakage of the cell injection volume, the cells were labeled with a membrane dye. The labeled cells were injected intramuscularly into an EDL via two 10µl injections. The injection sites were observed under a fluorescent dissecting microscope and images were
taken of the superficial site of the injection (Figure 22). There was a slight leakage of the first injection shown in Figure 22A and 22B, however there was a substantial amount of cell leakage with the second intramuscular injection (Figure 22C & D). This indicates that a total intramuscular injection volume of 20µl is excessive for an EDL muscle and leads to leakage of the second injection through the site of first needle entry.

In summary, application of the BCNU + O6BG driven selective enrichment strategy in CD34<sup>+</sup>ve skeletal muscle cell transplantation demonstrated an enhancement of donor cell engraftment in some animals. However, the improvement in engraftment was not statistically significant due to high variability of engraftment within individual recipient muscles. The high variability of engraftment was found to be caused by loss of cells due to leakage of the intramuscular cell injection during transplantation. It was found that an injection volume of 20µl exceeds the capacity of an EDL. Furthermore, it was found that two injections results in leakage of the constituents of the second injection through the needle entry site of the first injection.
Figure 22: Two 10μl injections of solution into an EDL muscle causes leakage of the constituents of second injection.

(A) EDL muscle injected with cells labeled with a fluorescent tag (6x10^4 cells in 10μl of PBS). (B) Higher magnification of the boxed region in (A). (C) EDL muscle injected twice with cells labeled with a fluorescent tag (2 times intramuscular injections; each injection with 6x10^4 cells in 10μl of PBS) (D) Higher magnification of the boxed region in (C). Boxed regions are the needle entry sites of intramuscular injections.
5.2.2 Application Of The BCNU + O⁶BG Driven Selective Enrichment Strategy In Cd34⁺Ve Skeletal Muscle Cell Transplantation

In order to overcome the loss of donor cells, the number of intramuscular injections per muscle should be limited to one and the total injection volume should remain 10µl. In order to achieve this, an alternative strategy was used where BCNU was delivered via a restricted intravenous injection followed by a combination of notexin plus cells in a single intramuscular injection of 10µl. Cytotoxic effects of notexin on CD34⁺ve skeletal muscle cells are unknown. In order to study the viability of notexin exposed cells, an in vitro study was performed where CD34⁺ve cells were overexposed to excessive doses of notexin (0.5µg or 1µg per 2x10⁵ cells). Notexin overexposed cells were then analysed using a cell-viability assay to determine the cell survival 2 days after treatment. There was a significant reduction in cell viability after exposure to notexin. However, there was also a reduction in cell viability in cell cultures treated with an equivalent volume of saline, which indicates an adverse effect of dilution of the culture medium (Figure 23). Nevertheless, once the effect of diluted culture medium was accounted for, the cytotoxicity of notexin on cells was approximately 15% for 0.5µg and 22% for 1µg of notexin treated cultures. Since this small reduction in cell viability was after overexposure to excessive doses of notexin for 48 hours, the toxicity shown was considered acceptable.

Once the transplantation protocol was established the next step was to determine the level of engraftment of donor cells after application of the BCNU + O⁶BG driven selective enrichment strategy. In order to study the potential of the selective enrichment strategy to enhance engraftment, 6x10⁴ CD34⁺ve cells (cells from either MGMT(P140K) transgenic male
Figure 23: Overexposure of CD34^{+ve} skeletal muscle cells to notexin results in a slight decrease in cell viability.

CD34^{+ve} cells isolated from regenerating C57BL/6 tibialis anterior muscles were cultured in the presence of notexin or saline for 48 hours. An MTT assay was carried out to determine the cell viability. Untreated: cells cultured in normal culture medium, 50μl ntx: treated with 0.5μg of notexin in 50μl of saline, 100μl ntx: treated with 1μg of notexin in 100μl of saline, 50μl saline: treated with 50μl of saline, 100μl saline: treated with 100μl saline.
mice or wildtype C57BL/6 male mice) plus notexin in a final injection volume of 10µl were injected intramuscularly into EDL muscles of female C57BL/6 mice. A number of these mice also received BCNU (0.1mg) which was injected via restricted, localised, intravenous delivery. All mice received O6benzylguanine (30mg/kg of body weight). Percentage of donor DNA was determined using quantitative PCR on DNA extracted from recipient muscles that were collected at 7 and 14 days post transplantation (Figure 24). The recipient muscles which received notexin plus cells had only a very low proportion of 0.39 ± 0.05% donor DNA present within the recipient muscles at 7 days post transplantation. The recipient muscles which received wildtype cells, notexin plus BCNU showed a higher proportion of 2.1 ± 0.6% donor DNA within the recipient muscles at 7 days post transplantation. The recipient muscles which received the MGMT(P140K) transgenic donor cells, notexin plus BCNU had the highest proportion of donor DNA at 7 days post transplantation, which was 6.4 ± 1.7% within recipient muscle DNA. This indicates that application of BCNU + O6BG driven selective enrichment strategy in CD34+ve skeletal muscle cell transplantation results in enhanced engraftment.

Similarly, at 14 days post transplantation the lowest proportion of 0.2 ± 0.1% donor DNA was seen in recipient muscles which received notexin plus cells only. The recipient muscles which received notexin, BCNU plus wildtype cells had a slightly higher proportion of 0.9 ± 0.3% donor DNA, although this difference was not statistically significant. The highest proportion of 1.8 ± 0.5% donor DNA was seen in recipient muscles which received notexin, BCNU plus MGMT(P140K) transgenic donor cells. This is significantly higher than the proportion of donor DNA seen in recipient muscles which received notexin plus cells only.
Figure 24: Enhanced engraftment of transplanted CD34	extsuperscript{+ve} donor cells following application of BCNU + O\textsuperscript{6}BG driven selective enrichment strategy.

Quantitative PCR was used to determine the amount of Y chromosome positive DNA present within the female host recipient muscles. (A) 7 days post transplantation. (B) 14 days post transplantation. WT cells: treated with 6x10\textsuperscript{6} wildtype male CD34	extsuperscript{+ve} donor cells, MGMTP140K cells: treated with 6x10\textsuperscript{6} MGMT(P140K) transgenic male CD34	extsuperscript{+ve} donor cells. # p = 0.004; ## p = 0.001; ### p = 0.002; * p = 0.028. Error bars represent standard deviations of more than 6 different samples.
At 14 days post transplantation, there is an overall reduction in the proportion of donor DNA in recipient muscles of all treatment groups compared to what was seen at 7 days post transplantation. The proportion of donor DNA at 14 days post transplantation in the recipient muscles which received notexin, BCNU plus MGMT(P140K) transgenic cells was 28% of that seen at 7 days post transplantation.

Recipient muscle weights were also noted since this can be used as an indication of muscle recovery after treatment (Figure 25). Recipient muscles which received only notexin plus cells demonstrated an increase in muscle weight at 7 and 14 days post transplantation. In contrast, recipient muscles which received alkylating chemotherapy in combination with notexin showed a substantial decrease ($p < 0.005$) in muscle weight at 7 and 14 days post transplantation. Wildtype cell recipient muscles showed a large decrease in muscle weight at 14 days post transplantation compared to muscles which received MGMT(P140K) transgenic donor cells. The average weight of recipient muscles which received notexin, BCNU plus wildtype donor cells was 38% of the weight of MGMT(P140K) transgenic donor cell recipients (Figure 25).

At 14 days post transplantation, the recipient muscles which received notexin, BCNU plus MGMT(P140K) transgenic cells appeared healthy and had normal gross morphology, however, the recipient muscles which received notexin, BCNU plus wildtype cells showed a dramatic decrease in muscle size (Figure 26). Appearance of muscles which received notexin, BCNU plus wildtype cells were quite similar in size to an EDL which received notexin,
Figure 25: MGMT(P140K) transgenic donor cell transplantation allows better recovery of recipient muscle weight following notexin, BCNU plus O6BG treatment.

All recipient mice received O6-benzylguanine (O6BG) at 30mg/kg of body weight (i.p.). Notexin (0.1μg) was given in combination with 6x10^4 donor cells in 10μl injection volume (i.m.). BCNU (0.1mg) was delivered via a restricted, localised intravenous injection. WT: wildtype, mgmt: MGMT(P140K) transgenic. Error bars are standard deviations of greater than 3 separate samples.

* p < 0.05 compared to weights of notexin + cell recipient muscles.
BCNU plus no cells (refer to chapter 3: figure 10). We next determined whether the donor cells residing within the recipient muscles were contributing to the formation of new muscle fibers during regeneration. Recipient muscles which received notexin, BCNU plus wildtype or MGMT(P140K) transgenic donor cells were collected 14 days post transplantation and histology was performed on longitudinal frozen sections of these muscles. H&E staining was done to determine the overall histology of recipient muscles (Figure 26). H&E staining of recipient muscles revealed that muscles which received notexin, BCNU plus MGMT(P140K) transgenic cells had elicited a normal regeneration response (Figure 26A-C). These muscles had abundant numbers of new fibers formed and regeneration was evident by the presence of centralised nuclei. However, the muscles which received wildtype cells showed an absence of a normal regeneration response. There were no fibers formed and the tissue appeared fibrotic with many mononucleated cells (Figure 26).

In order to determine whether the fibers incorporated the MGMT(P140K) transgenic donor cells, fluorescent in situ hybridisation (FISH) was done using a Y chromosome probe (Figure 27). There were many Y chromosome positive nuclei within the recipient muscle, however the majority of the nuclei were Y chromosome negative. This demonstrates that the MGMT(P140K) transgenic donor cells also have the ability to promote host-cell-driven skeletal muscle regeneration. Y chromosome positive nuclei were not spread evenly within the muscle bed. There were dense areas of Y chromosome positive nuclei towards the distal regions of the EDL, whereas the medial and posterior regions had a few scattered Y chromosome positive nuclei. FISH analysis also revealed that Y chromosome positive nuclei
Figure 26: Regeneration of injured skeletal muscle with transplanted MGMT(P140K) transgenic donor cells following BCNU + O\textsuperscript{6}BG treatment.

Recipient muscles received BCNU (0.1mg) and notexin (0.1μg). All mice received O\textsuperscript{6}benzylguanine (30mg/kg of body weight: i.p.). (A) – (C) Received 6×10\textsuperscript{4} MGMT(P140K) transgenic CD34\textsuperscript{+ve} donor cells. (D) – (F) Received 6×10\textsuperscript{4} wildtype CD34\textsuperscript{+ve} donor cells. (A) & (D) Gross morphology of recipient muscles. (B) & (E) H&E staining, 10X magnification. (C) & (F) H&E staining, 40X magnification.
Figure 27: Transplanted MGMT(P140K) transgenic CD34<sup>ve</sup> donor cells have fused to form myofibers in recipient EDL muscles following application of BCNU + O<sup>6</sup>BG driven selective enrichment strategy.

(A) & (B) Fluorescence <i>in situ</i> hybridisation labeling Y chromosome positive donor nuclei within female recipient muscles, 100X magnification. (C) Donor nuclei aligning to form a myofiber within the female recipient muscles, 40X magnification.
have in some instances aligned for fiber formation. This finding demonstrates the ability of CD34^{+ve} donor cells to contribute to myofiber formation upon transplantation.

In summary, application of the BCNU + O^{6}BG driven selective enrichment strategy in CD34^{+ve} skeletal muscle cell transplantation demonstrated a statistically significant enhancement of engraftment compared to the conventional transplantation strategy where donor cells are injected into injured skeletal muscles. However, the proportion of donor DNA within recipient muscles showed a reduction at 14 days post transplantation compared to 7 days post transplantation. Gross morphology and weights of recipient muscles at 14 days transplantation demonstrated that recipient muscles which received notexin, BCNU plus MGMT(P140K) transgenic cells have elicited a regeneration response whereas wildtype cell recipient muscles have not. Histology of the recipient muscles confirmed this observation where the MGMT(P140K) transgenic cell recipient muscles consisted of many newly formed myofibers with centralised nuclei whereas muscles which received wildtype cell received muscles which showed no myofiber formation. Fluorescent in situ hybridisation performed in order to detect the Y chromosome positive donor cells demonstrated that MGMT(P140K) transgenic CD34^{+ve} donor cells are capable of contributing and fusing to form new myofibers within injured recipient muscles. Furthermore, MGMT(P140K) transgenic CD34^{+ve} donor cells also demonstrated the ability to promote host-cell-driven skeletal muscle regeneration.
5.2.3 Migration Of Transplanted CD34\(^{+}\)ve Donor Cells Via Systemic Circulation To Injured Skeletal Muscles

Characterisation of CD34\(^{+}\)ve cells isolated from a regenerating skeletal muscle demonstrated that within this population there are cells expressing markers of various stem cells which are known for their ability to migrate via the systemic circulation. In order to determine whether some of the transplanted CD34\(^{+}\)ve cells travel via the systemic circulation in response to injury, donor cells were only transplanted into right EDLs while both right and left EDLs were injured by notexin treatment. Both right and left EDLs received BCNU and all animals received O\(^{6}\)benzylguanine (O\(^{6}\)BG). Recipient muscles were collected at 14 days post transplantation. The left EDLs of mice which received MGMT(P140K) transgenic donor cells in their right EDLs showed a similar muscle size and gross morphology as the right EDLs. However, the left EDLs of mice which received wildtype donor cells showed a similar inhibition of regeneration resulting in a smaller size as seen in their cell treated right EDLs (Figure 28). Quantitative PCR for Y chromosome was performed to determine the level of donor DNA within the recipient muscles, including the left EDLs which were only injected with notexin plus BCNU. Left EDLs of mice which received MGMT(P140K) transgenic donor cells in their right EDLs showed presence of some donor DNA. The level of donor DNA present within these contra-lateral EDLs was only 3% of what was seen within the right EDLs. However, the left EDLs of mice which received wildtype donor cells in their right EDLs showed no Y chromosome DNA and mirrored the sample of female DNA which was used as a negative control. These results indicate that CD34\(^{+}\)ve donor cells isolated from a regenerating skeletal muscle are capable of migration via the systemic circulation after
transplantation. Furthermore, the injected donor cells exhibited the ability to promote host-cell-driven skeletal muscle regeneration.
Figure 28: CD34\textsuperscript{+}ve skeletal muscle cells isolated from a regenerating skeletal muscle demonstrated the ability for migration via systemic circulation.

(A) – (D) EDL muscles which received notexin (0.1µg) plus BCNU (0.1mg). All mice received G\textsuperscript{6}BG (30mg/kg of body weight, i.p.). (A) Right EDL of mouse #202, received 6x10\textsuperscript{4} MGMT(P140K) transgenic CD34\textsuperscript{+}ve cells. (B) Left EDL of mouse #202, no cells received. (C) Right EDL of mouse #215, received 6x10\textsuperscript{4} wildtype CD34\textsuperscript{+}ve cells. (D) Left EDL of mouse #215, no cells received. (E) No cell received contra-lateral EDLs of mice which received MGMT(P140K) transgenic cells on right EDLs showed the presence of Y chromosome positive donor DNA.
5.3 Discussion

Skeletal muscle stem cell transplantation has been studied using various animal models and clinical trials since the 1980s with limited success (reviewed by Cossu, G. and Sampaolesi, M., 2007, Peault, B., et al., 2007). There are three main limitations of skeletal muscle stem cell transplantation. The first limitation is the use of inefficient donor cells in transplantation, which lack the ability to successfully incorporate into host muscles (Peault, B., et al., 2007). The second limitation is reduced engraftment resulting from the competition caused by host endogenous stem cells (Karpati, G., et al., 1993). The third limitation is the lack of migration of donor cells from the injection site upon transplantation, which results in localised engraftment (Skuk, D., et al., 1999, Skuk, D., et al., 2002). This chapter reported successful enhancement of donor cell engraftment achieved by adopting a donor cell enrichment strategy that has been developed for hematopoietic stem cell transplantation. This enrichment strategy was used in conjunction with a heterogeneous population of donor cells which were capable of successful incorporation into host muscle and promoting skeletal muscle regeneration.
5.3.1 Alkylating Chemotherapy Promotes Engraftment Of Resistant CD34^{+ve} Skeletal Muscle Donor Cells Upon Transplantation

This chapter reported the establishment of a skeletal muscle donor cell transplantation protocol, which involved intramuscular injection of \(6 \times 10^4\) MGMT(P140K) transgenic CD34^{+ve} skeletal muscle donor cells plus nontoxin. The restricted, localised, intravenous delivery of BCNU and intraperitoneal injection of O^{6}BG resulted in selective enrichment of MGMT(P140K) transgenic CD34^{+ve} donor cell engraftment at 7 and 14 days post transplantation. At 7 days post transplantation, CD34^{+ve} donor cells transplanted into injured recipient muscles resulted in a low engraftment level which consisted of only 0.38% donor DNA within the recipient muscle DNA. The low level of engraftment was expected because a similar lack of donor cell incorporation due to donor cell death immediately after transplantation has been previously reported (Fan, Y., et al., 1996).

Although the main reason for the immediate cell death is thought to be immune rejection, competition from the endogenous satellite and stem cells are also know to contribute to this phenomenon (Karpati, G., et al., 1993). It has been proposed that since intact satellite cells and stem cells are positioned in the correct ‘niche’, they are more efficient in contributing to skeletal muscle regeneration than injected donor cells (Karpati, G., et al., 1993). The most common methods of elimination of host muscle stem and satellite cells in order to reduce competition with donor cells are cryodamage (Irintchev, A., et al., 1997, Wernig, A., et al., 1995a, Wernig, A., et al., 1995b) and irradiation (Huard, J., et al., 1994, Kinoshita, I., et al., 1994, Wernig, A., et al., 2000) of the recipient muscle bed prior to transplantation. However, these methods have been demonstrated to result in inefficient regeneration of recipient...
This chapter demonstrated the use of alkylating chemotherapy treatment in order to eliminate the host muscle stem and satellite cells, which resulted in enhanced engraftment of donor cells within the recipient muscles. Injured recipient muscles which were not treated with alkylating chemotherapy demonstrated an average donor cell engraftment of 0.38% within the recipient muscles. However, injured muscles which received alkylating chemotherapy demonstrated approximately 2% (wildtype donor cells) and 6% (MGMT(P140K) transgenic donor cells) average donor cell engraftment within the recipient muscles. Wildtype cells show cytotoxicity towards alkylating chemotherapy whereas MGMT(P140K) transgenic cells are resistant. Therefore, the selective survival of MGMT(P140K) transgenic donor cells in combination with elimination of host muscle resident stem and satellite cells resulted in a higher level of engraftment. The efficiency of host muscle resident stem and satellite cells were further emphasised by the complete inhibition of skeletal muscle regeneration observed at 14 days post transplantation in injured recipient muscles which received wildtype cells plus alkylating chemotherapy. Thus, this strategy employs both elimination of host competition and selective survival to produce a greater than 10 fold increase in donor cell engraftment than seen using conventional transplantation of muscle cells.

There are various transplantation studies that have been reported in the literature which have used disease models such as mdx mice or α-sarcoglycan null mice as recipients (reviewed by Cossu, G. and Sampaolesi, M., 2007, Peault, B., et al., 2007). These studies have analysed
the engraftment efficiency of donor cells by observing the level of dystrophin (in \textit{mdx} recipient mice) or \(\alpha\)-sarcoglycan (in \(\alpha\)-sarcoglycan null recipient mice) expression in recipient muscle fibers. Multinucleation of muscle fibers allows the rescue of dystrophic muscle fibers by incorporation of a few healthy donor cells per fiber. However, the cell numbers required per muscle fiber for complete rescue is not yet known. Therefore, in order to directly compare the transplantation efficiency using the approach described in this chapter requires the application of this strategy in a disease model such as \textit{mdx} mice or \(\alpha\)-sarcoglycan null mice.
5.3.2 Engraftment Of CD34+ve Skeletal Muscle Cells Upon Transplantation Into Skeletal Muscles

MGMT(P140K) transgenic CD34+ve donor cells have shown successful engraftment within recipient muscles where donor cells have contributed to formation of muscle fibers. The CD34+ve donor cells were isolated from regenerating skeletal muscles and Chapter 4 has described the presence of cell subpopulations within these donor cells which express markers of various muscle stem cell populations. Among these were cells which expressed markers of mesoangioblasts, pericytes, skeletal muscle side population cells, CD133+ve cells, satellite cell progenitor cells and muscle derived stem cells. All these different populations of muscle stem cells have been demonstrated to have the capacity to contribute to recipient muscle regeneration upon transplantation (Dellavalle, A., et al., 2007 Sampaolesi, M., et al., 2006, Sampaolesi, M., et al., 2003, Torrente, Y., et al., 2004, Asakura, A., et al., 2002, Bachrach, E., et al., 2006, Montarras, D., et al., 2005, Mueller, G.M., et al., 2002, Qu-Petersen, Z., et al., 2002). Therefore, the contribution of CD34+ve donor cells to recipient muscle fiber formation is expected.

MGMT(P140K) transgenic CD34+ve donor cells also displayed the ability to migrate via the systemic circulation to injured muscles upon transplantation. Both EDLs of recipient mice were treated with alkylating chemotherapy and notexin and only the right EDLs were treated with donor cells. In mice which received MGMT(P140K) transgenic donor cells, left EDLs showed approximately 3% of donor DNA that was present within the right EDLs. CD34+ve cell characterisation reported in chapter 4 indicates the presence of various cells which
express markers of pericytes, mesoangioblasts, skeletal muscle side population cells and CD133^{+ve} progenitor cells which are all known for their ability to migrate via the systemic circulation upon transplantation (Asakura, A., et al., 2002, Bachrach, E., et al., 2006, Dellavalle, A., et al., 2007, Gavina, M., et al., 2006, Sampaolesi, M., et al., 2003, Sampaolesi, M., et al., 2006, Torrente, Y., et al., 2004). Furthermore, cell characterisation indicated approximately 65% of CD34^{+ve} cells isolated from regenerating TAs could be monocytes which also have the capacity for migration via the systemic circulation. Recruitment of muscle stem cells, monocytes and macrophages into injured muscles is a well known phenomenon (Chazaud, B., et al., 2003, Lescaudron, L., et al., 1999, Pituch-Noworolska, A., et al., 2003). Therefore, it is possible for a small population of CD34^{+ve} donor cells to migrate via the systemic circulation in response to injury and/or inflammation. Thus, CD34^{+ve} donor cell transplantation via intravenous or intraarterial delivery may also result in donor cell engraftment within multiple downstream muscles.

The level of donor DNA present within the left EDLs indicate that only a small proportion of donor cells have been recruited to these muscles. Despite the low level of donor cells that have been recruited to the left EDLs, these muscles have recovered to the same extent as the cell treated right EDLs by host cell-driven-regeneration. The ability of these muscles to undergo host-cell-driven regeneration following elimination of muscle resident stem and satellite cells indicates a significant role of the donor cells in the recruitment of muscle stem cells from the systemic circulation, bone marrow or adjacent skeletal muscle beds.
Recipient muscle weights at 7 days and 14 days post transplantation indicate that upon alkylating chemotherapy plus notexin treatment there is a dramatic loss of weight compared to normal untreated EDL muscles. This is expected since notexin treatment would damage muscle fibers and alkylating chemotherapy would eliminate the proliferating endogenous muscle stem and satellite cells and thus regeneration of the treated muscle will be compromised. However, MGMT(P140K) transgenic donor cell treated recipient muscles have maintained muscle weight from 7 days to 14 days compared to the dramatic decrease observed in muscles which received wildtype donor cells. This indicates that despite the depletion of MGMT(P140K) transgenic donor cell engraftment observed from 7 days to 14 days post transplantation, the incorporated cells were responsible for recovery of the recipient muscles. Histology of recipient muscles confirmed that in fact the MGMT(P140K) transgenic donor cell treated muscles have undergone regeneration and formed new muscle fibers whereas wildtype donor cell recipient muscles demonstrated no signs of regeneration and new fiber formation.

Detection of Y chromosome positive donor cells via fluorescence in situ hybridisation demonstrates that although donor nuclei are present within newly formed muscle fibers, the majority of the nuclei are of host origin. This poses a question regarding the regeneration of MGMT(P140K) transgenic donor cell treated muscles. Inhibition of regeneration in injured skeletal muscle following BCNU + O\textsuperscript{6}BG treatment has been established in chapter 3. Furthermore, BCNU + O\textsuperscript{6}BG treated injured skeletal muscle failed to regenerate upon wildtype donor cell transplantation. Yet regeneration was present within MGMT(P140K) transgenic donor cell recipient muscles where the majority of fibers consisted of host
contributed nuclei. This indicates that MGMT(P140K) transgenic CD34^{+ve} donor cells trigger infiltration of muscle stem cells from a source other than the treated muscle while allowing donor contributed muscle fiber regeneration. Chapter 4 demonstrated that approximately 65% of the CD34^{+ve} skeletal muscle donor cells express the monocyte marker CD11b. Monocytes and macrophages are known to have a crucial role in normal skeletal muscle regeneration where these cells are responsible for promotion of muscle stem and satellite cell proliferation in addition to their conventional role of phagocytosis and clearing of necrotic tissue (Jarvinen, T.A.H., et al., 2005, Summan, M., et al., 2006, Tidball, J.G., 2005). Monocytes and macrophages carry out all their roles in promotion of skeletal muscle regeneration, with the exception of phagocytosis, through the release of various chemokines and cytokines (Tidball, J.G., 2005). The role of monocytes in promotion of skeletal muscle regeneration together with the high numbers of cells found within CD34^{+ve} donor cells which express the monocyte marker CD11b suggests that monocytes are the most likely candidate responsible for promotion of host-driven-regeneration observed within recipient muscles which have been treated with alkylating chemotherapy.
5.3.3 Long-term Engraftment Of CD34<sup>+</sup>ve Donor Cells Within BCNU + O<sub>6</sub>BG Treated Recipient Muscles

This chapter demonstrated the ability of BCNU + O<sub>6</sub>BG driven selection enrichment strategy to enhance CD34<sup>+</sup>ve donor cell engraftment within recipient skeletal muscles. However, the percentage of MGMT(P140K) transgenic donor cell engraftment in BCNU plus notexin treated muscles reduced from 6.4% donor DNA within recipient DNA at 7 days post transplantation to 1.8% at 14 days post transplantation. This suggests a depletion of donor cell engraftment with time despite the recovery seen in the MGMT(P140K) transgenic donor cell recipient muscles. The lack of long-term donor cell engraftment due to donor cell death is a common limitation in skeletal muscle stem cell transplantation (Fan, Y., et al., 1996, Karpati, G., et al., 1993). Various studies have demonstrated that the lack of long-term engraftment is due to competition from host endogenous stem cells (Karpati, G., et al., 1993) or immune rejection (Hodgetts, S.L., et al., 2000).

Chapter 3 demonstrated to ability of BCNU + O<sub>6</sub>BG treatment on injured skeletal muscles to completely eliminate the muscle resident stem and satellite cells leading to a complete inhibition of regeneration. However, this chapter demonstrated that upon transplantation of MGMT(P140K) transgenic CD34<sup>+</sup>ve donor cells, host-driven-regeneration is promoted, possibly via infiltration of muscle stem cells from neighboring muscles, blood circulation or bone marrow. Therefore, it is likely that despite the depletion of host muscle resident stem and satellite cells, competition from the infiltrated stem cells also causes donor cell death.
thus leading to reduced long-term engraftment. In order to minimise donor cell loss due to competition from the infiltrated host stem cells, multiple rounds of BCNU + O\textsuperscript{6}BG + notexin injection may be required. This would further enhance donor cell engraftment. This hypothesis is supported by the \textit{in vivo} enrichment of MGMT(P140K) expressing nuclei within injured transgenic muscles achieved via BCNU + O\textsuperscript{6}BG treatment which was reported in chapter 3.

Transplantation studies reported in this chapter were performed using host compatible male donor cells and female recipients. Although the donor cells were host compatible, it is likely there is some immune rejection of donor cells by the host system. Immunosuppression is known to enhance long-term donor cell engraftment in skeletal muscle stem cell transplantation (Kinoshita, I., \textit{et al.}, 1994, Ming-Hui, W., \textit{et al.}, 2003, Pavlath, G.K., \textit{et al.}, 1994). Therefore, it can be hypothesised that use of immunosuppression in combination with multiple rounds of BCNU + O\textsuperscript{6}BG + notexin would most likely enhance long-term engraftment of CD34\textsuperscript{+}ve skeletal muscle donor cells.
5.3.4 Conclusion

In summary, this chapter reports the successful application of a novel skeletal muscle stem cell transplantation strategy which has the ability to selectively enhance engraftment of donor cells within injured recipient muscles. Given the \textit{in vivo} enrichment of MGMT(P140K) positive nuclei, it can be hypothesised that further enhancement of MGMT(P140K) transgenic donor cell engraftment can be achieved via multiple treatments of notexin, BCNU plus O\textsuperscript{6}benzylguanine. It is hypothesised that a combination of multiple rounds of notexin, BCNU plus O\textsuperscript{6}BG and immunosuppression would overcome the limitation of reduced long-term donor cell engraftment. Further development of the transplantation strategy is required in order to study the enhancement of donor cell engraftment in a disease model such as \textit{mdx} mice or \textit{\alpha}-sarcoglycan null mice. Furthermore, a small population of CD34\textsuperscript{+} donor cells used in transplantation demonstrated the ability for migration via systemic circulation. This finding indicates the possibility of donor cell transplantation via intravenous or intraarterial delivery which may result in widespread donor cell engraftment in multiple downstream muscles. The endogenous cell contributed regeneration observed within BCNU + O\textsuperscript{6}BG treated recipient muscles indicate a role of CD34\textsuperscript{+} donor cells in recruitment of muscle stem cells from a source other than the injured skeletal muscle. It is hypothesised that monocytes are the most likely candidate responsible for activating the observed host-driven-regeneration.
CHAPTER 6

Discussion And Future Directions
6.1 Discussion

The impact of regenerative medicine in therapies for various diseases has lead to a better understanding of stem cells during the last few decades. Stem cell-based therapies have been used for treatment of diseases involving various organs such as heart, brain, lung, muscle, pancreas, bone marrow, liver and kidney (reviewed by Mimeault, M., et al., 2007). However, most stem cell transplantation strategies have demonstrated limited success in clinical applications. A major limitation in any type of stem cell transplantation is the competition caused by endogenous stem cells which result in donor cell death and reduced engraftment. During the last decade, the BCNU + \textit{O}^6\textit{BG} driven selective enrichment strategy has been developed for \textit{in vivo} enrichment of donor hematopoietic stem cells (Gerull, S., et al., 2007, Horn, P.A., \textit{et al.}, 2004, Neff, T., \textit{et al.}, 2006, Neff, T., \textit{et al.}, 2005) and most recently \textit{in vivo} enrichment of donor hematopoietic stem cell derived lung epithelial cells (Reese, J.S., \textit{et al.}, 2008). Application of this strategy in a clinical trial for hematopoietic stem cell transplantation is scheduled to begin in year 2008 at Children’s Hospital at Westmead (Westmead, NSW, Australia). This study has demonstrated a novel application of the BCNU + \textit{O}^6\textit{BG} driven selective enrichment strategy in enhanced transplantation of cells in skeletal muscles. This finding is the first reported attempt of an application of the BCNU + \textit{O}^6\textit{BG} driven selective enrichment strategy to enhance transplantation of cells in a solid tissue. The outcome of this study could be used as a basis for the application of this strategy in numerous other solid tissues such as brain, heart, liver, pancreas and kidney where stem cell transplantation is considered.
Skeletal muscle stem cell transplantation has been studied extensively in the laboratory and in clinical trials since the 1980’s as a potential therapy for Duchenne muscular dystrophy (Cossu, G. and Sampaolesi, M., 2007, Miller, R.G., et al., 1997, Partridge, T.A., et al., 1989). The clinical application of various skeletal muscle stem cell transplantation strategies has proven unsuccessful due to three main limitations. The first limitation is the used of inefficient donor cells that demonstrate limited ability for incorporation into host muscles (Peault, B., et al., 2007). The second limitation is the competition caused by endogenous stem cells resulting in donor cell death and limited engraftment (Karpati, G., et al., 1993). The third limitation is the limited migration of donor cells from the injection site resulting in localised engraftment (Skuk, D., et al., 1999, Skuk, D., et al., 2002). This study addressed all three of the limitations, allowing enhanced engraftment of donor skeletal muscle cells in injured host muscles.

In order to overcome the limitation of donor cell engraftment caused by competition from host stem cells, most commonly used strategies that have been reported are cryodamage of the recipient muscles (Irinchev, A., et al., 1997, Wernig, A. and Irinchev, A., 1995, Wernig, A., et al., 1995) and irradiation of injured recipient muscles (Huard, J., et al., 1994, Kinoshita, I., et al., 1994, Wernig, A., et al., 2000). Cryodamage disrupts the nerves, blood vessels and most importantly the basal lamina, which results in slower, inefficient donor cell contribution to regeneration (Vignaud, A., et al., 2005). Irradiation, when used in non-lethal doses on injured or developing skeletal muscles, is reported to result in incomplete elimination of host muscle stem cells (Gross, J.G. and Morgan, J.E., 1999, Olive, M., et al., 1995). This study demonstrated that BCNU + O6BG treatment of injured wildtype mouse skeletal muscle
results in complete elimination of the ability of the muscle to regenerate for up to 3 months (unpublished data) and the treatment showed no long lasting adverse side effects on the mice. This study further demonstrated the resistance of cells which express the MGMT(P140K) gene to BCNU + O\(^6\)BG treatment using \textit{in vitro} and \textit{in vivo} models. The survival advantage exhibited by the cells which express MGMT(P140K) gene allowed selective enrichment of these cells among wildtype cells which died in response to BCNU + O\(^6\)BG treatment. It can be hypothesised that the engraftment of donor cells which express MGMT(P140K) would be further enhanced by multiple rounds of notexin, BCNU + O\(^6\)BG. This hypothesis is supported by the initial \textit{in vivo} studies performed on the MGMT(P140K) transgenic mice which demonstrated enrichment of MGMT(P140K) positive cells upon notexin, BCNU + O\(^6\)BG treatment. Further enhancement of donor cells with multiple treatments of notexin, BCNU + O\(^6\)BG would also demonstrate the ability of donor cells to enter the muscle stem and satellite cell ‘niche’.

Satellite cells were first discovered in 1961 (Mauro, A., 1961) and this finding was followed by the use of satellite cells in transplantation (Partridge, T.A., \textit{et al.}, 1978, Snow, M.H., 1978). However, the clinical application of satellite cell transplantation showed no improvement in the muscle weakness of DMD patients, indicating that these cells are inefficient for engraftment (Karpati, G., \textit{et al.}, 1993). In order to overcome the limitation of inefficient donor cells, there has been an intense search during the last decade for a population of ‘stem-like’ cells which are capable of successful donor contribution to myofiber formation within host skeletal muscles. This lead to the finding of numerous cell populations which can be categorised into two groups, the first group consist of cells which
can be delivered via systemic blood vessels, the second group consist of cells that has to be delivered intramuscularly. Pericytes (Dellavalle, A., et al., 2007), mesoangioblasts (Sampaolesi, M., et al., 2003, Sampaolesi, M., et al., 2006), CD133\(^{+}\)ve progenitor cells (Torrente, Y., et al., 2004) and skeletal muscle side population (SP) cells (Asakura, A., et al., 2002, Bachrach, E., et al., 2006) are some of the cells that can be delivered to downstream muscles via blood vessels. Satellite cell progenitor cells (Montarras, D., et al., 2005), MDSCs (Mueller, G.M., et al., 2002, Qu-Petersen, Z., et al., 2002) and myoendothelial cells (Zheng, B., et al., 2007) have shown successful donor contribution to myofiber formation upon intramuscular transplantation. The CD34\(^{+}\)ve donor cells used in transplantations of this study were isolated from 3 days post notexin treated skeletal muscles in order to maximise cell numbers. Upon characterisation of the CD34\(^{+}\)ve donor cells, it was discovered to be quite heterogeneous consisting of cells which express markers of various muscle stem cell populations mentioned above. Furthermore, there were cells which expressed CXCR4, which indicates that some of the CD34\(^{+}\)ve donor cells may have been infiltrated from the systemic blood circulation or the bone marrow. CD34\(^{+}\)ve donor cells indicated further heterogeneity by their ability for angiogenesis in culture, a characteristic which would be ideal for regeneration of injured host muscles. The heterogeneity seen within the CD34\(^{+}\)ve donor cells indicates their potential efficiency in achievement of donor contribution to host skeletal muscle regeneration.

Notexin treatment of skeletal muscle results in complete muscle fiber necrosis (Vignaud, A., et al., 2005). In this study, it was hypothesised that better migration of donor cells within host skeletal muscles can be achieved upon notexin, BCNU + O\(^6\)BG treatment due to extensive
damage of myofibers and elimination of host muscle resident stem and satellite cells. However, the donor cell engraftment that was observed using fluorescent in situ hybridisation proved that this strategy has not successfully overcome the limitation of localised engraftment upon intramuscular injection of donor cells. Although donor nuclei were present throughout the host muscle, the numbers were observed to be higher in close proximity to the injection site. In order to overcome the limitation of localised engraftment, other reported transplantation studies have utilised techniques such as donor cell delivery via blood vessels to downstream host muscles (reviewed by Cossu, G. and Sampaolesi, M., 2007, Peault, B., et al., 2007). This technique results in widespread engraftment of cells within multiple downstream muscles. Intramuscular transplantation of CD34+ve donor cells in this study demonstrated that a small population of cells was capable of migration to injured contra lateral muscles, possibly in response to inflammation. Although the proportion of migrated donor cells were quite low, the ability of these cells to enter the blood circulation and migrate to contra lateral muscles indicate that successful intraarterial or intravenous delivery of CD34+ve donor cells would be possible.

Characterisation of CD34+ve donor cells which were isolated from 3 days post notexin treated skeletal muscles provided further insight into cells which are involved in regeneration. Notexin induced regeneration has been well characterised in regards to functional and structural recovery of the muscles (Vignaud, A., et al., 2005). However, there is a lack of knowledge in regards to which cell populations have the most significant roles in regeneration of injured skeletal muscle. This study compared and characterised the CD34+ve cells isolated from normal versus 3 days post notexin treated skeletal muscles. There was an
increase in numbers of the majority of the cell subpopulations present within the CD34$^{+ve}$ skeletal muscle cells upon notexin treatment. It is well known that upon injury muscle stem and satellite cells undergo extensive proliferation (reviewed by Wagers, A.J. and Conboy, I.M., 2005). Furthermore, it is known that injury and inflammation triggers infiltration of stem cells from bone marrow and blood circulation that are capable of contribution to muscle regeneration (Aiuti, A., et al., 1997, Pituch-Noworolska, A., et al., 2003, Ratajczak, M.Z., et al., 2004). Therefore an increase in cell numbers in a regenerating skeletal muscle is expected. The increase in numbers of various populations of muscle stem cells in regenerating muscle indicates that normal skeletal muscle regeneration is achieved with multiple muscle stem cell populations rather than one specific muscle stem cell population.

The ability of muscle stem and satellite cells to migrate into adjacent muscles has previously been reported (Jockusch, H., Voigt, S., 2003). Recruitment of muscle stem cells into injured tissue in response to chemokines and cytokines has also been well established (Aiuti, A., et al., 1997, Pituch-Noworolska, A., et al., 2003, Ratajczak, M.Z., et al., 2004). However, given the absence of a regeneration response, evidence of recruitment of muscle stem cells from adjacent muscles, blood circulation or bone marrow was not observed in wildtype muscles treated with BCNU + O$^6$BG. The evidence of recruitment of muscle stem cells indicated by the host contribution to myofiber formation was only observed in MGMT(P140K) transgenic donor cell transplanted muscles. This indicates that the muscle stem cell recruitment was a direct response to the presence of a surviving subpopulation of donor cells within the recipient muscles. Monocytes and macrophages are recruited to injured muscles where they promote satellite cell proliferation in addition to clearing up the necrotic muscle tissue.
The crucial role that monocytes play in skeletal muscle regeneration is further emphasised by the findings of this study where a regenerating skeletal muscle bed was demonstrated to consist of approximately 300 times more CD34^{+ve} cells which express the monocyte marker CD11b than normal muscles. Furthermore, it is also known that monocytes and macrophages release various cytokines (Tidball, J.G., 2005) and it has been emphasised that depletion of the monocytes and macrophages results in inhibition of skeletal muscle regeneration (Summan, M., et al., 2006). It is therefore a likely possibility that chemokines and cytokines released by monocytes are also contributing to recruitment of muscle stem cells from adjacent muscles, blood circulation and bone marrow. However, this characteristic of monocytes in injured muscles is yet to be investigated. Furthermore, approximately 65% of CD34^{+ve} donor cells used in transplantation consisted of cells which express the monocyte marker CD11b. Considering all the evidence provided, it can be hypothesised that monocytes are the most likely candidate within the CD34^{+ve} donor cells which are responsible for promotion of the host contribution to myofiber formation in the stem and satellite cell depleted, BCNU + O6BG treated muscles.

In summary, this study demonstrated successful enhanced engraftment of transplanted skeletal muscle cells upon application of a BCNU + O6BG driven selective enrichment strategy. The three main limitations of skeletal muscle transplantation were also addressed. The limitation of inefficient donor cells used in transplantation was addressed by utilising a heterogeneous population of skeletal muscle donor cells which demonstrated successful engraftment in host skeletal muscle. The limitation of donor cell death due to competition
from host muscle stem and satellite cells was successfully overcome by the elimination of muscle resident stem and satellite cells using BCNU + O\textsuperscript{6}BG treatment. Restricted, localised delivery of a low BCNU dose was demonstrated to be safe and efficient in mice. Limitation of localised engraftment due to intramuscular injection of donor cells were addressed utilising notexin induced myofiber injury and elimination of muscle stem and satellite cells. The ability of CD34\textsuperscript{+} donor cells to migrate via the systemic circulation indicates the potential of intraarterial or intravenous delivery of donor cells which would result in widespread engraftment in multiple downstream muscles. Furthermore, it is proposed that monocytes may have a novel role in skeletal muscle regeneration where these cells display the ability to recruit muscle stem and satellite cells from adjacent muscles and the systemic circulation.

Finally, the successful application of the BCNU + O\textsuperscript{6}BG driven selective enrichment strategy in skeletal muscle stem cell transplantation indicates that it could be applied in other tissues where stem cell transplantation is considered.
6.2 Future Directions

6.2.1 Further Enhancement Of Donor Cell Engraftment In MGMT(P140K) Transgenic Donor Cell Engrafted Recipient Muscles

The BCNU + O6BG driven selective enrichment strategy demonstrated successful enhancement of donor cells engraftment in injured recipient muscles. However, the level of donor cell engraftment reduced from 7 to 14 days post transplantation which was paralleled by an increase in host contribution to regeneration. In order to overcome the depletion of donor cells within host tissue, host contributed regeneration should be eliminated. One strategy that may achieve this goal would be multiple treatments of notexin, BCNU plus O6BG. Initial studies carried out in MGMT(P140K) transgenic mice where enrichment of MGMT(P140K) positive nuclei was achieved using notexin, BCNU + O6BG treatment supports this hypothesis. This strategy has the potential to successfully eliminate the host contribution to regeneration while allowing the donor cells to survive and proliferate leading to enhanced engraftment. Another strategy that can be used is negative selection to eliminate cells which express the monocyte marker CD11b from the CD34+ donor cell population. This study demonstrated that the CD34+ donor cells have a role in promotion of the host contribution to recipient muscle regeneration and that this phenomenon is most likely due to monocytes within the donor cells. Therefore, negative selection of monocytes prior to transplantation may reduce the host contribution to recipient muscle regeneration thus allowing enhanced donor cell engraftment. However, a combination of both strategies where transplantation of CD34+ donor cells which lack monocytes followed by multiple treatments of notexin, BCNU + O6BG may prove to be the most potent strategy for further enhancement of donor cell engraftment.
6.2.2 Developments Of The BCNU + O^6BG Driven Selective Enrichment Strategy In Order To Use In Clinical Application

In order to apply this novel skeletal muscle stem cell transplantation strategy in clinical trials further developments are required. The first requirement is the application of the BCNU + O6BG driven selective enrichment strategy in a disease model such as mdx mice or α-sarcoglycan null mice. The second requirement is that the strategy should utilise drugs that can be safely administered in humans. The third requirement is that healthy host compatible donor cells are required to be transduced with an MGMT(P140K) gene in order to give them a selective survival advantage. The fourth requirement is that isolation of donor cells from a healthy compatible donor should be relatively easy and safe for the donor.

There are various studies which investigate the therapeutic potential of skeletal muscle stem cell transplantation in muscular dystrophy (Cossu, G. and Sampaolesi, M., 2007, Peault, B., et al., 2007). In order to study the efficiency of skeletal muscle donor stem cell engraftment and consequent rescue of dystrophic muscles, various groups have utilised animal models such as mdx mice and α-sarcoglycan null mice. Application of the BCNU + O^6BG driven selective donor cell enrichment strategy using these animal models would reveal the true therapeutic potential of this novel strategy in muscular dystrophy.

Restricted, localised treatment of BCNU + O^6BG treatment was demonstrated in this study to be safe where no loss in white blood cells leading to myelosuppression was observed. BCNU + O^6BG treatment is already used in chemotherapeutic treatments for various cancers in humans. The other drug used in the transplantation strategy is notexin. Notexin is used to
injure the host muscles in order to make them more receptive to donor cells. Although dystrophic muscles have chronic damage, notexin could play a role in inflammation which maybe beneficial for ‘homing’ donor cells which are delivered via blood vessels. However, notexin, which is a snake toxin, has not yet been used in humans and the safety has not been investigated. A local anesthetic agent used in clinical applications called Bupivacaine, which is also known as marcaine, has similar effects as notexin in skeletal muscles (Wakata, N., et al., 2001). Use of Bupivacaine in this novel transplantation strategy would be more favourable in clinical trials. The efficiency of injury resulting from Bupivacaine for application of the BCNU + O6BG driven selective enrichment strategy in skeletal muscles needs to be established.

The BCNU + O6BG driven selective enrichment strategy used for enrichment of skeletal muscle stem cell transplantation in this study used donor cells from an MGMT(P140K) transgenic mouse in order to achieve selective resistance to alkylating chemotherapy. In order to apply this strategy in clinical trials, donor cells need to be transduced with an MGMT(P140K) gene. This could be achieved most effectively with a lentiviral vector encoding MGMT(P140K). Since lentiviral transduction does not require cell division, the cells can be transduced with a minimal period of time ex vivo. The number of resistant cells and therefore the transplantation efficiency would be expected to be much higher with transduced cells than what was demonstrated in this study, since on average only 25% of donor cells isolated from the transgenic mice expressed the MGMT(P140K) gene.
The CD34^{+ve} donor cells used in this study were isolated from 3 days post notexin treated skeletal muscles in order to maximise cell numbers and their engraftment efficiency due to the heterogeneity of cells present. However, this strategy has two limitations when considered for application in clinical trials. The first limitation is that the CD34 cell surface antigen expression in human cells varies from mouse cells. For example, human satellite cells do not express the CD34 antigen (Peault, B., et al., 2007). The second limitation is that the invasive notexin injection of a compatible donor in order to isolate donor cells would not be suitable for clinical application. Due to these reasons, a different strategy is required for isolation of cells which would have similar characteristics to the CD34^{+ve} donor cells used in this study. Characterisation of CD34^{+ve} donor cells demonstrated the presence of cells which express markers of MDSCs, mesoangioblasts, skeletal muscle SP cells, pericytes, endothelial cells, CD133^{+ve} progenitor cells, CXCR4^{+ve} cells, monocytes and myogenic cells. There have been studies which have reported isolation procedures of the majority of these cell populations from human and mouse tissue (reviewed by Berthold, F., 1981, Cooper, R.N., et al., 2006, Peault, B., et al., 2007, Ratajczak, M.Z., et al., 2003). Therefore, it may be possible to transplant a mixture of these cells isolated from various sources such as muscle biopsies and blood samples. In order to determine the efficiency of transplantation of a mixture of donor muscle stem cells isolated from various tissues compared to CD34^{+ve} donor cells isolated from a regenerating muscle, this strategy should first be trialed in a mouse model.
Supplementary Figures
Figure 29: The hEF1-α-MGMT(P140K) construct used in the generation of MGMT(P140K) transgenic mice (adopted from Kramer, B., et al., 2006).

'The 4193 bp ASP718-Bst1107I fragment shown was electroporated into C57BL/6 ES cells. The 624 bp Nco I – Not I fragment indicated was used to probe Southern blots of genomic DNA extracted from F2 generation MGMT(P140K)-transgenic mice.' By Kramer, B., et al., 2006.
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Abstracts Of Participated Conferences
ENRICHMENT OF MUSCLE STEM CELL TRANSPLANTATION USING CHEMOTHERAPEUTIC DRUG SELECTION: A PARADIGM FOR ENHANCED STEM CELL TRANSPLANTATION IN TISSUES

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We describe a drug-based selection strategy for enhancing the repopulation of a muscle bed with transplanted skeletal muscle stem cells. The strategy that was used combines the use of an alkylating chemotherapeutic agent, BCNU, with the gene for methylguanine methyltransferase (MGMT) that detoxifies the action of this agent. This strategy has proven successful for the repopulation of haematopoetic cells in bone marrow. We propose that it may be useful in a variety of tissues where transplantation is contemplated. The use of muscle stem cell engraftment to address genetic muscle diseases in particular would benefit from providing both a selective advantage to transplanted stem cells and elimination of endogenous stem cells. In order to determine if skeletal muscle is amenable to this selection scheme, first we established that cultured muscle cells, C2C12 and human primary myoblasts are sensitive to BCNU in a dose dependent manner. To determine if donor myoblasts can be rendered BCNU resistant by overexpressing MGMT, we virally transduced mouse and human cultured myoblasts with an MGMT vector under the control of the viral promoter. Mixtures of MGMT expressing and nonexpressing cells were exposed to BCNU. In all cultures, increasing doses of BCNU led to increased enrichment of the MGMT expressing cells. To establish proof of principle that MGMT-protected muscle stem and satellite cells can be enriched in the muscle bed, we utilized a transgenic mouse that expresses MGMT under the control of an EF1α promoter. Approximately, 5% of muscle precursor cells express detectable level of MGMT in these mice, which was increased by five-fold following induction of muscle regeneration and drug selection. We conclude that chemotherapeutic drugs applied to various activated stem cells can provide a means to select cells expressing MGMT and that viral transduction of cells with the MGMT gene may provide an effective approach to muscle stem cell transplantation.
ENRICHMENT OF MUSCLE STEM CELL TRANSPLANTATION USING CHEMOTHERAPEUTIC DRUG SELECTION: A PARADIGM FOR ENHANCED STEM CELL TRANSPLANTATION IN TISSUES

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We describe a novel application of a selective enrichment strategy, initially established for hematopoietic cells, in a skeletal muscle system. Cells expressing the mutant drug resistance gene, methylguanine methyltransferase (MGMT-P140K) are resistant against carmustine (BCNU) plus O⁶-bezylguanine (O⁶-BG) treatment, but wildtype cells show cytotoxicity. In vitro studies were done to determine the effectiveness of this strategy in a muscle cell system using C2C12 cells and human myoblasts. They showed selective enrichment of the MGMT-P140K expressing cells. An MGMT-P140K gene knock-in mouse model was used to determine whether the MGMT-P140K expressing muscle beds are resistant against BCNU plus O⁶-BG treatment. Treatment of a regenerating muscle bed showed increased MGMT-P140K expression and resulted in successful regeneration while the wildtype control lacked a regeneration response. These data suggest MGMT-P140K expression in satellite cells lead to selective cell survival in response to BCNU plus O⁶-BG treatment. In previous attempts myoblast transplantation has been unsuccessful, mainly due to reduced cell viability after transplantation. We propose that the strategy reported would allow repopulation of a regenerating muscle bed by giving the transplanted cells a survival advantage.
Enrichment of muscle satellite and stem cells with a chemotherapeutic drug advantage: A paradigm for enhanced stem cell transplantation in tissues

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Introduction: We describe a novel application of a selective enrichment strategy, initially established for hematopoietic cell transplantation, in a skeletal muscle system. Cells expressing the mutant version of the drug resistance gene methylguanine methyltransferase, MGMT-P140K, are resistant to the chemotherapeutic drugs, carmustine (BCNU) plus O6-bezylguanine (O6-BG).

Method: In vitro studies were done to determine the effectiveness of this strategy in a muscle cell system using C2C12 cells and human myoblasts. An MGMT-P140K transgenic mouse model was used to determine whether MGMT-P140K expressing satellite and stem cells in the muscle beds are resistant to BCNU plus O6-BG treatment. Regeneration was induced in a skeletal muscle to mitotically activate the satellite and stem cells.

Results: Drug selection of C2C12 cells and human myoblasts resulted in an enrichment of the population of transduced muscle cells expressing MGMT-P140K. Drug treatment also resulted in an increase in MGMT-P140K expressing cells and successful muscle regeneration in the MGMT-P140K transgenic mice while the wildtype control showed poor regeneration.

Conclusions: These data indicate that MGMT-P140K expression in satellite cells lead to selective cell survival in response to BCNU plus O6-BG treatment. Transplantation of genetically corrected muscle stem and satellite cells is being considered as a therapy for a number of human skeletal muscle diseases. In attempts so far, myoblast transplantation has been unsuccessful, mainly due to reduced cell viability after transplantation. We propose that the strategy that we report would allow enhanced repopulation of a regenerating muscle bed by giving the transplanted cells a survival advantage under drug selection.
REPOPULATION OF A REGENERATATING MUSCLE BED WITH MUSCLE STEM CELL TRANSPLANTATION WHERE THE CELLS HAVE BEEN GIVEN A SELECTIVE SURVIVAL ADVANTAGE

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We describe a novel application of a selective enrichment strategy, initially established for hematopoietic cells, in a skeletal muscle system. Cells expressing the mutant drug resistance gene methylguanine methyltransferase (MGMT-P140K) are resistant against carmustine (BCNU) plus O6-bezylguanine (O6-BG) treatment, but wildtype cells show cytotoxicity. In vitro studies were done to determine the effectiveness of this strategy in a muscle cell system using C2C12 cells and human myoblasts. They showed selective enrichment of the MGMT-P140K expressing cells. An MGMT-P140K gene knock-in mouse model was used to determine whether the MGMT-P140K expressing muscle beds are resistant against BCNU plus O6-BG treatment. Treatment of a regenerating muscle bed showed increased MGMT-P140K expression and resulted in successful regeneration while the wildtype control lacked a regeneration response. These data suggest MGMT-P140K expression in satellite cells lead to selective cell survival in response to BCNU plus O6-BG treatment, which would explain the presence of a regeneration response in transgenic mice and the absence of it in wildtype mice. In previous attempts myoblast transplantation has been unsuccessful, mainly due to reduced cell viability after transplantation. We propose that the strategy reported would allow repopulation of a regenerating muscle bed by giving the transplanted cells a survival advantage.
Enrichment of muscle stem cell transplantation using chemotherapeutic drug selection

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We describe a novel application of a selective cell enrichment strategy, initially established for hematopoietic cells, in a skeletal muscle system with a view to muscle stem cell transplantation. Cells expressing a mutant form of the drug resistance gene methylguanine methyltransferase (MGMT-P140K) are resistant to the cytotoxic effects of carmustine (BCNU) plus O6bezylguanine (O6BG) treatment; whereas, wildtype cells exhibit cytotoxicity. In vitro studies were performed to determine the effectiveness of this strategy in a muscle cell culture system using C2C12 cells and human myoblasts. Results showed selective enrichment of the MGMT-P140K expressing cells in response to the drugs. A transgenic mouse overexpressing MGMT-P140K in muscles was used to determine whether MGMT-P140K-expressing muscle stem and satellite cells when stimulated to proliferate in situ, are resistant to BCNU plus O6BG. Drug treatment of a regenerating muscle bed showed increased MGMT-P140K expression and resulted in successful regeneration while the wildtype control had a suppressed regenerative response. These data indicate that MGMT-P140K expression in satellite cells results in selective cell survival in response to BCNU/O6BG treatment. Preliminary transplantation trials were performed using CD34+ve donor cells (Montarras D. et. al. Science 2005 23;309(5743):2064-7) isolated from regenerating skeletal muscles of male MGMT-P140K transgenics. Donor cells were co-injected with notexin (myotoxic agent to induce regeneration) and BCNU intra-muscularly into syngeneic wild-type female hosts. O6BG was injected IP. Donor cell survival was significantly enhanced in host muscle beds treated with BCNU/O6BG compared with transplanted muscles that did not receive the drugs. We propose that the selective survival advantage of the donor cells demonstrated in the current strategy can be used to enhance the efficacy of muscle-derived stem cell therapy.
ENRICHMENT OF MUSCLE STEM CELL TRANSPLANTATION USING CHEMOTHERAPEUTIC DRUG SELECTION


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We describe a novel application of a selective cell enrichment strategy, initially established for haematopoietic cells, in a skeletal muscle system with a view to muscle-derived stem cell transplantation. Cells expressing a mutant form of the drug resistance gene methylguanine methyltransferase (MGMT-P140K) are resistant to the cytotoxic effects of carmustine (BCNU) plus O⁶benzylguanine (O⁶BG) treatment; whereas, wild-type cells exhibit cytotoxicity. In vitro studies were performed to determine the effectiveness of this strategy in a muscle cell culture system using C2C12 cells and human myoblasts. Results showed selective enrichment of the MGMT-P140K-expressing cells in response to the drugs. A transgenic mouse over-expressing MGMT-P140K in muscles was used to determine whether MGMT-P140K-expressing muscle stem and satellite cells, when stimulated to proliferate in situ, are resistant to BCNU plus O⁶BG. Drug treatment of a regenerating muscle bed showed increased MGMT-P140K expression and resulted in successful regeneration, while the wild-type control had a suppressed regenerative response. These data indicate that MGMT-P140K expression in satellite cells results in selective cell survival in response to BCNU/O⁶BG treatment. Transplantation trials were performed using CD34(+ve) donor cells (Montarras D. et al., Science 2005 23;309(5743):2064-7) isolated from regenerating skeletal muscles of male MGMT-P140K transgenics. Donor cells were co-injected with notexin (myotoxin) intramuscularly and BCNU intravenously into syngeneic wild-type female hosts. O⁶BG was injected IP. Quantitative RT-PCR analyses were performed on recipient muscles 7 days post-transplantation to quantify the amount of male DNA present. The muscles injected with MGMT-P140K-expressing donor cells showed significantly higher male DNA content than the muscles injected with wild-type donor cells and this difference is dependent on BCNU treatment. We conclude that it is possible to apply this selection strategy to enhance the efficacy of muscle-derived stem cell therapy.
ENRICHMENT OF MUSCLE STEM CELL TRANSPLANTATION USING CHEMOTHERAPEUTIC DRUG SELECTION

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We describe a novel application of a selective cell enrichment strategy, initially established for haematopoietic cells, in a skeletal muscle system with a view to muscle-derived stem cell transplantation. Cells expressing a mutant form of the drug resistance gene methylguanine methyltransferase (MGMT-P140K) are resistant to the cytotoxic effects of carmustine (BCNU) plus O\textsuperscript{6}benzylguanine (O\textsuperscript{6}BG) treatment; whereas, wild-type cells exhibit cytotoxicity. In vitro studies were performed to determine the effectiveness of this strategy in a muscle cell culture system using C2C12 cells and human myoblasts. Results showed selective enrichment of the MGMT-P140K-expressing cells in response to the drugs. A transgenic mouse over-expressing MGMT-P140K in muscles was used to determine whether MGMT-P140K-expressing muscle stem and satellite cells, when stimulated to proliferate in situ, are resistant to BCNU plus O\textsuperscript{6}BG. Drug treatment of a regenerating muscle bed showed increased MGMT-P140K expression and resulted in successful regeneration, while the wild-type control had a suppressed regenerative response. These data indicate that MGMT-P140K expression in satellite cells results in selective cell survival in response to BCNU/O\textsuperscript{6}BG treatment. Transplantation trials were performed using CD34\textsuperscript{(+ve)} donor cells (Montarras D. et al., Science 2005 23;309(5743):2064-7) isolated from regenerating skeletal muscles of male MGMT-P140K transgenics. Donor cells were co-injected with notexin (myotoxin) intramuscularly and BCNU intravenously into syngeneic wild-type female hosts. O\textsuperscript{6}BG was injected IP. Quantitative RT-PCR analyses were performed on recipient muscles 7 days post-transplantation to quantify the amount of male DNA present. The muscles injected with MGMT-P140K-expressing donor cells showed significantly higher male DNA content than the muscles injected with wild-type donor cells and this difference is dependent on BCNU treatment. Fluorescent in situ hybridisation (FISH) was performed using a Y-chromosome specific probe on frozen recipient muscle sections collected at 14 days post transplantation. The muscles injected with MGMT-P140K donor cells contained a significant number of muscle fibers formed by the donor cells and showed extensive regeneration. In contrast, wild-type donor cells failed to achieve a similar extent of regeneration in recipient muscles. We conclude that it is possible to apply this selection strategy to enhance the efficacy of muscle-derived stem cell therapy.