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A stem cell based approach
for vascular repair in
diabetic retinopathy

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A Stem Cell Based Approach for Vascular Repair in Diabetic Retinopathy

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A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Department of Clinical Ophthalmology and Eye Health

Save Sight Institute

The University of Sydney

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DECLARATION

I hereby declare that this thesis is my own work, except where due acknowledgement is stated below and in the thesis text. To the best of my knowledge, this thesis does not contain material previously published or written by any other person. Further, it does not contain material which has been accepted for the award of any other degree or diploma of a university or institution of higher learning.

All of the experiments in this thesis were performed at The University of Sydney at the Department of Clinical Ophthalmology and Eye Health and the Save Sight Institute (located at the Sydney and Sydney Eye Hospital campus) and the Bosch Institute (The University of Sydney), between August 2009 and August 2012.

Daniel Barthelmes

Daring ideas are like chessmen moved forward;

they may be beaten, but they may start a winning game.

Johann Wolfgang von Goethe

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ABSTRACT

Background

Diabetic Retinopathy (DR) is a sight threatening, chronic ocular disorder that develops with time in nearly all patients suffering from Diabetes Mellitus (DM). It is the commonest microvascular complication of DM and the major cause of blindness in the working-age population in the developed world. Retinal vascular endothelial cell (EC) dysfunction, an early step in the pathogenesis of DR, results in vascular leakage in some areas and capillary closure in others. Consequently, retinal ischaemia develops and causes preretinal neovascularisation, i.e. growth of new vessels from the retinal vasculature into the vitreous, which, in advanced cases, will eventually result in blindness. Although new treatment options have been developed and explored (e.g. intravitreal injection of antiproliferative substances), photocoagulation of the neuroretinal tissue using laser light is still standard of care. However, these approaches – both the laser treatment and the intravitreal injections - treat the complications rather than stopping the progression of the disease, nor do they improve vascular disease.

Endothelial progenitor cells (EPCs) renew and replace aged ECs, repair vascular damage and support the integrity of the vascular endothelium. EPCs, which are mostly derived from the bone-marrow (BM), are translocated to the peripheral blood (PB) by several stimuli associated with vascular injury. During migration from the BM to the PB, EPCs mature and fully develop their functional properties. The therapeutic potential of EPCs has been reported in conditions such as myocardial infarction or peripheral ischaemic vascular disease, where transplanted EPCs have been described to improve organ function through revascularisation. The transplantation of EPCs has also been proposed as a potential treatment for DR.

The number of EPCs in the peripheral blood is reduced and their functions are impaired in people with DM. However, experimental transplantation of EPCs from the PB to diabetic individuals has not been reported to result in the improvements in organ function that have been observed in non-diabetic individuals.

Currently it is unclear how diabetes impairs the function of EPCs and whether this impairment is already present in EPCs still located within the bone-marrow.

Aims

Since little is known about the function and potential impairments due to DM of EPCs in the BM, this thesis describes studies that aimed to I) isolate and characterise EPCs from the BM of mice with respect to tube formation *in vitro* and their ability to repair retinal vascular damage and compare the results to findings from EPCs isolated from the PB; II) evaluate an approach to increase the mobilization of EPCs from the BM of diabetic and non-diabetic mice to the PB using a drug cocktail and III) identify early diabetes-related changes in EPCs from diabetic mice which may be responsible for their impaired function and mobilisation from the BM.

Methods

For aim I, EPCs were isolated from the BM of normal mice using an immunomagnetic bead technique. The strategy to isolate EPCs specifically was to perform first a LIN^+ depletion step to remove hematopoietic stem cells and then to select for $VEGFR2^+$ cells. The isolated cells were characterised by flow cytometry, by immunohistochemistry and by *in vitro* experiments to test tube formation, colony formation and their phenotypical appearance. To investigate the ability of the EPCs isolated from the BM to participate in vascular repair in the retina, *in vivo* experiments were performed in normal C57/BL6 mice. Following laser-induced branch retinal vein occlusion, isolated EPCs were transplanted intravitreally to the eyes of mice that underwent the laser procedure and mice in which no laser procedure was performed. Eyes were enucleated and serial sections as well as flatmount preparations of the retinal tissue were prepared and examined to study the behaviour of the transplanted cells including their integration into damaged vasculature.

For aims II and III, experiments were performed using normal C57/B6 mice and two different models of DM in mice. In both models mice had been diabetic for 18 weeks when experiments were performed. The streptozotocin (STZ) induced diabetic model was used for aim II. The Akita mouse has a C57/BL6 background and a spontaneous point mutation in the insulin 2 gene on chromosome 7 - which is referred to as the Ins2Akita allele. This model of spontaneous DM was used for experiments for aim III.

For aim II, to increase the mobilization of EPCs from the BM, a cocktail of granulocyte colony-stimulating factor, erythropoietin and tetrahydrobiopterin were

tested for their ability to mobilise EPCs from the BM to the PB in 18-week-old diabetic and non-diabetic mice. A group of non-diabetic and diabetic mice that did not receive the cocktail served as controls. EPCs were collected from the BM and PB of all four groups one week after administration of the drug cocktail. Total numbers in diabetic and non-diabetic animals were evaluated as well as the changes following the cocktail administration. Cells were characterised by flow cytometry. Growth patterns of non-diabetic and diabetic EPCs were evaluated in vitro.

For aim III, to identify early diabetes-related changes in EPCs from diabetic mice, EPCs were isolated from the BM of diabetic and non-diabetic mice. RNA and protein were isolated. Changes in the expression of 36 genes crucial for EPC mobilisation and EPC function were analysed using real-time PCR. Western blot analysis was used to test whether significant changes at the mRNA level were also present at the protein level. To test whether the gene and protein changes observed were specifically occurring in EPCs as a consequence of diabetes, the results from diabetic and non-diabetic EPCs were compared to LIN^+ hematopoietic cells from the BM.

Results

A protocol was established to isolate EPCs from the BM of mice in sufficient quantity and quality for subsequent *in vitro* and *in vivo* experiments. The isolated cells exhibited typical cobblestone morphology and were positive for EPC markers such as CD34 and VEGFR2. Other phenotypical properties typical for EPCs such as uptake of Dil-acLDL and binding of Lectin were also observed. However, the cells did not spontaneously form tubes when cultured. Transplantation of the cells into eyes with retinal vessels damaged by laser resulted in migration of the cells towards the areas of the injury and integration in the damaged vessels. In uninjured eyes, cells remained in the vitreous and did not integrate into retinal vessels.

We then found that the cocktail increased mobilization of EPCs from the BM in non-diabetic but not diabetic mice. This was demonstrated by a significant decrease of EPCs in the BM and a concomitant increase of EPCs in the PB in non-diabetic animals. In diabetic animals however, this response was blunted: only a small increase in EPCs in the PB could be observed. Diabetic animals had significantly more EPCs per kg bodyweight compared to controls in the BM. This means that in diabetic animals the reduced number of EPCs in the PB is not due to reduced formation of EPCs within the BM but rather due to a mobilisation deficit. This is

reinforced by the observation that growth patterns and phenotype did not differ in the *in vitro* experiments using diabetic and non-diabetic EPCs: EPCs from diabetic and non-diabetic animals did not show any differences in growth behaviour when put into culture nor different phenotypes.

The analysis of gene-expression data of the 36 genes known to be involved in EPC function and mobilisation showed that in early diabetes (18 weeks) already in the BM significant changes in EPCs could be observed. One of the main driving factors for EPC mobilisation, SDF-1, was significantly downregulated in diabetic EPCs compared with non-diabetic EPCs. Analysis of LIN^+ hematopoietic cells from the BM showed no significant change in expression or concentration of SDF-1.

Conclusions

Several conclusions can be drawn from the experiments performed. Firstly, EPCs that are isolated from the BM share a wide range of properties that have been reported for EPCs isolated from the PB, among them phenotypical properties such as cobblestone appearance, expression of surface markers such as VEGFR2 and CD34. Furthermore, EPCs isolated from the BM are capable of participating in vascular repair. The much lower proliferative potential of EPCs from the BM and apparently lower potential to repair vascular damage than has been reported for EPCs isolated from the PB may be because EPCs from the BM are not yet fully mature. Therefore, EPCs from the BM do not seem to be a good source for transplantation to repair vascular damage as seen in DM.

It was clear that diabetic impairment of EPCs is present at the very early stages of diabetes, in these experiments after as soon as 18 weeks of diabetes. However, the results of the present studies suggest that the impairment that we found and that has been reported by others in early diabetes may be more a problem of EPC mobilisation than of genuine malfunction. The formation of EPCs does not seem to be impaired, since diabetic animals had significantly more EPCs compared to non-diabetic animals in the BM. It seems that the EPCs are "trapped" inside the BM since they could not be mobilised even by the strong drug cocktail that was employed. The activation of EPCs, i.e., the signalling to repair vascular damage, seems to be still functioning, however, as one of the main driving factors for mobilisation is downregulated (SDF-1) the EPCs may not be able to be translocated from the BM to the PB.

These findings are clinically significant as they clearly demonstrate that functional changes occur in stem cells within the BM even in very early stages of DM. The results show, in contrast to findings from studies on human EPCs from the PB, that functional impairment of EPCs may not be present in very early stages of DM. The mobilisation deficit seems to be the main result of hyperglycaemia. This is reinforced by the findings that key enzymes of EPC function such as eNOS were not altered in their expression and functional *in vivo* tests showed similar results in diabetic and non-diabetic EPCs. Further research into how high glucose levels actually reduce the expression of SDF-1 is warranted. Although it may seem feasible to transplant EPCs to repair vascular damage in DR and would for the first time provide an option which could reverse some of the critical vascular changes, it seems that EPCs from the BM are probably not an ideal source for such therapeutic applications.

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ABBREVIATIONS

ACE	Angiotensin Converting Enzyme
AGE	Advanced Glycation End products
ANG	Angiopoietin
AT	Angiotensin
AKT	Protein Kinase B
BCR	Beta-common Receptor
BDNF	Brain-derived Neurotrophic Factor
BH2	Dihydrobiopterin
BH4	Tetrahydrobiopterin
BM	Bone Marrow
BRB	Blood Retinal Barrier
CD	Cluster of Differentiation
cGMP	Cyclic Guanosine-monophosphate
COX	Cyclooxygenase
CRP	C-reactive Protein
CXCR4	C-X-C chemokine Receptor type 4
DAG	Diacyglycerol
Dil-acLDL	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate - labelled acetylated low density lipoprotein
DNA	Deoxyribonucleic Acid
DM	Diabetes Mellitus
DMO	Diabetic Macular Oedema
DR	Diabetic Retinopathy
DRS	Diabetic Retinopathy Study
EC	Endothelial Cell
ECFC	Endothelial Colony-Forming Cells
ECM	Extracellular Matrix
EGM	Endothelial Growth Medium
eNOS	endothelial Nitric Oxide Synthase (NOS3)
EPC	Endothelial Progenitor Cell

EphB4	Ephrin type-B receptor 4
EPO	Erythropoietin
EPO-R	Receptor for Erythropoietin
ERG	Electroretinography
ETDRS	Early Treatment Diabetic Retinopathy Study
FAK	Focal Adhesion Kinase (PTK2)
FDA	Food and Drug Administration of the United States of America
FGF	Fibroblast Growth Factor
FOXO1	Forkhead Box Protein O1 (FKHR1)
GAPDH	Glyceraldehyde-3-Phosphatasedehydrogenase
GCSF	Granulocyte Colony-stimulating Factor
GFP	Green Fluorescent Protein
GDNF	Glial cell line derived Neurotropic Factor
GH	Growth Hormone
GM-CSF	Granulocyte-Macrophage Colony-stimulating Factor
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
GTPCH	Guanosine 5'-Triphosphate Cyclohydrolase
HbA1C	Glycated Haemoglobin A1c
HDAC	Histone deacetylase
HDL	High Density Lipoprotein
HIF1alpha	Hypoxia Inducible Factor 1 alpha
HMG-CoA	3-Hydroxy-3-Methyl-Glutaryl-Coenzyme A
HMGB1	High-Mobility-Group-Box1
HOXA9	Homeobox protein A9
HSC	Hematopoietic Stem Cells
HSP90	Heat Shock Protein 90
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM-1	Intercellular Adhesion Molecule 1
IGF	Insulin-like Growth Factor
IGFBP	Insulin-like Growth Factor Binding Protein
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL-1	Interleukin 1

IL-6	Interleukin 6
IL-8	Interleukin 8
ILK	Integrin-linked Kinase
iNOS	inducible Nitric Oxide Synthase (NOS2)
IV	roman numeral for number 4
LDL	Low Density Lipoprotein
L-NAME	N-(G)-nitro-L- arginine methylester
MAPK	Mitogen Activated Protein Kinase
MMP2	Matrix Metalloproteinase 2
MMP9	Matrix Metalloproteinase 9
mTOR	mammalian Target of Rapamycin
NAD(H)	Nicotinamide Adenine Dinucleotide
NADP(H)	Nicotinamide Adenine Dinucleotide Phosphate
NF-κB	Nuclear Factor kappa B
NFG	Nerve Growth Factor
NMDA	N-Methyl-D-Aspartate
nNOS	neuronal Nitric Oxide Synthase (NOS1)
NO	Nitric Oxide
NOD/SCID	Non-Obese Diabetic / Severe Combined Immunodeficiency
NPDR	Non-proliferative Diabetic Retinopathy
PAD	Peripheral Arterial Disease
PAI	Plasminogen Activator Inhibitor
PB	Peripheral Blood
PDGF	Platelet Derived Growth Factor
PDR	Proliferative Diabetic Retinopathy
PEDF	Pigment Epithelium Derived Factor
PKC	Protein Kinase C
PlGF	Placental Growth Factor
PI3K	Phosphatidyl-Inositol 3 Kinase
PLC	Phospholipase C
PSGL-1	P-selectin glycoprotein ligand-1
RAC-1	RAS-related C3 botulinum toxin substrate 1
RAGE	Receptor for Advanced Glycation End products
RNA	Ribonucleic Acid

RNV	Retinal Neovascularisation
ROS	Reactive Oxygen Species
RPE	Retinal Pigment Epithelium
SCF	Stem Cell Factor
SDF-1	Stromal cell Derived Factor 1
SRB1	Scavenging Receptor B1
STAT3	Signal Transducer and Activator of Transcription 3
STZ	Streptozotocin
TGF-β	Transforming Growth Factor beta
Tie	Tyrosine kinase that contains immunoglobulin like domains and epidermal growth factor similar domains
TNF-alpha	Tumour Necrosis Factor alpha
UEA-I	Ulex Europaeus Agglutinin I
uPAR	Urokinase Receptor
VCAM-1	Vascular Cell Adhesion Molecule 1
VEGF	Vascular Endothelial Growth Factor
VEGFR1	Vascular Endothelial Growth Factor Receptor 1 (Flt-1)
VEGFR2	Vascular Endothelial Growth Factor Receptor 2 (KDR / Flk-1)
VEGFR3	Vascular Endothelial Growth Factor Receptor 3 (Flt-4)
VLA-4	Integrin alpha4beta1 (Very Late Antigen-4)
vWF	von Willebrand Factor

CHAPTER 1

INTRODUCTION

1 Chapter 1 – Introduction

1.1 Diabetes mellitus and Diabetic Retinopathy

1.1.1. Description, Prevalence and Complications of Diabetes Mellitus

Diabetes Mellitus (DM) is a metabolic disorder characterised by chronic hyperglycaemia (Alberti *et al.*, 1998). Generally two forms of DM can be differentiated: in DM type 1, progressive loss of beta-cells in the pancreas leads to lack of insulin whereas in DM type 2 decreased action of insulin ("insulin resistance") or reduced secretion is found (Alberti *et al.*, 1998). The result in both situations is hyperglycaemia: an increased level of glucose in the blood. Current estimates show that in the United States (U.S.) DM affects approximately 6.3% of the total population (Fong *et al.*, 2004) and more than 13% of adults that are 30 years or older are suffering from DM (Danaei *et al.*, 2009).

DM results in a plethora of sequelae with vascular complications taking the lead (Crofford, 1995). Macrovascular and microvascular diseases are the main causes of morbidity and mortality in patients with diabetes (Alberti *et al.*, 1998, Cade, 2008). There is a 2-4 times higher risk of death from cardiovascular problems in diabetic patients compared to non-diabetic patients (Crofford, 1995). In the year 2000, 6% of the worldwide mortality were due to diabetes-related vascular complications (Cade, 2008), and the World Health Organisation estimated that in 2004 approximately 3.4 million people died from consequences of high blood sugar (see http://www.who.int/mediacentre/factsheets/fs312/en/).

Examples of macrovascular complications include ischaemic heart disease, peripheral vascular disease and cerebral vascular disease whereas diabetic retinopathy, nephropathy and neuropathy are examples of microvascular disease (Cade, 2008). More recently it was reported that cerebral microvascular disease may develop parallel to the other complications but goes unnoticed for a long time (Tiehuis *et al.*, 2008, Uzu *et al.*, 2010, Vergote *et al.*, 2006, Wessels *et al.*, 2006). In the U.S. and Europe DM has become the most common single cause for end-stage renal disease (Molitch *et al.*, 2004) and in the U.S. DM alone accounts for 40% of all new cases of end-stage renal disease costing 15.6 billion USD in 1997 (Molitch *et al.*,

2004). Additionally, diabetic retinopathy (DR) – the most common microvascular complication of DM – accounts for up to 10,000 new cases of blindness annually in the US (Fong *et al.*, 2004) making DM the leading cause of blindness among the working population in the developed world (Ciulla *et al.*, 2003, Foster *et al.*, 2005, Klein *et al.*, 1998).

1.1.2. Diabetic Retinopathy

1.1.2.1 Incidence and Prevalence of DR

Diabetic Retinopathy is the primary microvascular complication of DM (Fong *et al.*, 2004, Foster *et al.*, 2005). This serious complication affects patients with both DM type 1 and DM type 2 (Klein et al., 1984a, Klein et al., 1984b, West et al., 1980). DR is a leading cause of blindness in working-age adults in the western world (1993, 1998a) such as in the United States, where DR is the prime cause of blindness among the working population. It is estimated that more than 60% of patients with DM Type 2 have some degree of DR (Stratton *et al.*, 2001). Approximately 4.1 Million Americans have DR and nearly one in twelve patients with DR under the age of 40 has sight-threatening retinopathy (Kempen *et al.*, 2004). As with most of the other vascular complications in DM, DR develops slowly. While only few patients show signs of DR at the time DM is diagnosed, approximately 75% of all people with diabetes have clear signs of DR within 15 years after onset of the disease (Klein *et al.*, 1984a, Sjolie *et al.*, 1997). It has been reported that long duration of DM, insufficient glycaemic control, increased glycated haemoglobin (HbA1C) and hypertension are strongly correlated with the progression of DR (2000a, 2000b, 1993, 1998a, 2000c, 1998b, Chaturvedi *et al.*, 1998).

1.1.2.2 Classification and Progression of DR

The Early Treatment of Diabetic Retinopathy Study (ETDRS) Research Group established a classification of DR which is widely accepted and used (1991e). Patients in whom DM was recently diagnosed may not show any retinal abnormalities. The earliest clinical signs of DR include regional failure of microvascular function such as blood-retinal barrier (BRB) breakdown, microaneurysms (small outpouchings from

retinal capillaries), capillary obstruction and intraretinal dot haemorrhages which eventually may lead to later complications such as macular oedema, retinal ischaemia and retinal neovascularisation (RNV) if left untreated (Frank, 2004). Once RNV is established it may form tractional membranes that lead to a detachment of the neural retina and, left untreated, result in complete loss of vision (Aiello *et al.*, 1998). As long as RNV is absent, the DR is classified as non-proliferative while the presence of RNV classifies DR as proliferative DR. Microaneurysms and intraretinal haemorrhages are visible in nearly all people with type 1 diabetes after 20 years (Klein *et al.*, 1984a) and in ~80% of people with type 2 diabetes (Klein *et al.*, 1984b). Proliferative DR may occur in 50% of type 1 (Klein *et al.*, 1984a) and 10% of type 2 patients after 15 years of disease (Klein *et al.*, 1984b). In type 2 diabetes, the risk of developing proliferative DR is higher when the patients are insulin dependent.

Macular oedema, an accumulation of fluid in the neural retina, seems to be a special entity within DR as it can occur at any stage of DR and itself causes significant visual impairment (Antcliff et al., 1999, Ciulla et al., 2003, Pelzek et al., 2002). Diabetic macular oedema is the single most common cause of severe vision loss in patients with DM (Klein et al., 1984a). Over a period of 10 years it occurred in 20.1% of people with type 1 diabetes, in 25.4% of type 2 diabetics that were dependant on insulin and in 13.9% of type 2 diabetics that were not insulin dependent (Klein *et al.*, 1995). While macular oedema is more prominent in type 2 diabetic patients, type 1 diabetic patients seem to be more affected by RNV (Klein *et al.*, 1994, Klein *et al.*, 1989a, Klein *et al.*, 1989b).

1.1.2.3 Current treatments of DR

The following section will give a brief overview of some of the treatments currently used for DR. For a comprehensive and detailed overview of treatment options, further reading is suggested, e.g. in the Ophthalmology Monographs (Scott *et al.*, 2010).

The current guidelines for treating DR recommend panretinal photocoagulation using a laser (Argon, ~530nm) once retinal neovascularisation develop (1991a, 1985, Ferris, 1996). Ischaemic peripheral retina is ablated thereby relieving local hypoxia. As a result, the concentration of vascular endothelial growth factor (VEGF) – one of

the main driving factors for establishing RNV (Aiello *et al.*, 1995a, Shweiki *et al.*, 1992) - is lowered and the RNV subsides. Unfortunately this treatment irreversibly destroys retinal tissue. Both the Diabetic Retinopathy Study (DRS) (1981, 1987a, 1978) and ETDRS (1991a, 1985, 1987b) reported a 50% reduction of severe vision loss after laser treatment.

Other treatment strategies aiming at inhibiting VEGF and thereby reducing RNV have recently been developed and are currently under investigation (http://clinicaltrials.gov/ct2/show/NCT00387582) (Adamis *et al.*, 1996). To date anti-VEGF agents are used mostly in cases where RNV is already established (Spaide *et al.*, 2006).

Randomised clinical trials have reported that macular oedema may also respond to both laser and anti-VEGF agents (Michaelides *et al.*, 2010). However, these approaches treat the complications rather than stopping the progression of the disease, nor do they improve the vascular disease, especially the ischaemia.

An important factor in controlling progression of DR is, of course, proper management of blood glucose: it has been reported that in both type 1 and type 2 diabetes the degree of hyperglycaemia correlates with the progression of DR (1995, 1993, 1998a, Reichard *et al.*, 1993). Due to improved treatment of diabetes and better glucose control the risk of people with DM to develop DR has decreased: in type 1 diabetics the 20 year cumulative incidence decreased from 100% in 1984 (Klein *et al.*, 1998, Klein *et al.*, 1984a) to 96% in 1998 (Klein *et al.*, 1998). Between 1984 and 1998 the rate of patients developing PDR in type 1 DM after more than 10 years after diagnosis decreased from 50% to 36.8 %. It was further reported that rigorous control of blood glucose delays progression of DR in type 1 (1993) and type 2 diabetic patients (1998a).

Additionally, good control of blood pressure also delays progression of DR in type 2 diabetic patients (1998b), especially angiotensin converting enzyme (ACE) inhibitors (Chaturvedi *et al.*, 1998) and beta-blockers (1998b) seem to be beneficial. Angiotensin I (AT1) inhibitors, also used for lowering blood pressure, were reported to have some beneficial effects on DR progression, yet this group of drugs delayed DR only in early and mild cases of DR (Mitchell *et al.*, 2008).

As inflammatory processes are involved in the development of DR (Carmo *et al.*, 2000a, do Carmo *et al.*, 1998a, Joussen *et al.*, 2001a), anti-inflammatory drugs such

as acetylsalicylic acid (1991c) or steroids (Gillies *et al.*, 2006, Sutter *et al.*, 2004) have been tested – especially in treating macular oedema.

Other treatments such as aldose reductase inhibitors (Caldwell *et al.*, 2003) did not show any effect on altering the development of DR although it was reported that aldose reductase polymorphisms were associated with a higher risk for DR development (Kao et al., 1999, Ko et al., 1995).

Ruboxistaurin, an inhibitor of protein kinase C β (PKC β) was also tested in patients with DR. The risk of sustained visual loss was reduced, the necessity for focal laser photocoagulation was less and the chance for improving vision was increased, however there was no delay in progression of DR from the nonproliferative to the proliferative state (Gardner *et al.*, 2006).

As mentioned above, anti-VEGF agents such as ranibizumab and the well-established laser treatment seem to be the most promising tools in managing DR, with anti VEGF agents showing promising results especially for diabetic macular oedema in early clinical trials (Elman *et al.*, 2010, Massin *et al.*, 2010, Simo *et al.*, 2008). Since laser photocoagulation is still considered the gold standard in many cases of vision threatening macular oedema, any results of trials and studies using pharmacological interventions will have to measure up to those achieved using laser photocoagulation. Although anti-VEGF treatments show very promising short-term results, the treatment is hampered by its necessity for frequent treatments potentially over a very long time, which put a heavy burden of treatment on the patient. Associated costs are high.

1.2 Pathophysiologic changes in Diabetes

As the pathophysiologic processes occurring in diabetic individuals are numerous and exceedingly intricate, it is only possible to portray some of those most relevant to the research presented. Before describing the changes occurring in diabetic retinal vessels and some of the growth factors playing pivotal parts in the development of DR in more detail, a short introduction on retinal microvascular anatomy is given.

1.2.1. Introduction to retinal microvascular anatomy

The retinal circulation is in many ways special and different from other vascular beds in the human body. As the eye is – seen embryologically - an extension of the diencephalon (Patton *et al.*, 2005), the closest resemblance to the retinal microvascular architecture is found in the brain (Patton *et al.*, 2005). It is a low-flow system (Alm *et al.*, 1973) in an area of high oxygen extraction (Tornquist *et al.*, 1986) - due to the high oxygen demands of retinal neurons. There are two layers of the retinal capillary network within the retina. The superficial layer rests within the nerve fibre layer while the deeper layer supplies the inner nuclear and outer plexiform layers (Toussaint *et al.*, 1961). The diameter of these vessels is 5-6 μm (Cogan *et al.*, 1984, Leber, 1903). As retinal arteries are end-arteries, occlusions of these vessels lead to hypoxia in the area distal to the occlusion (Yanoff *et al.*, 1989).

Retinal neurons are shielded from the blood by a structure known as the blood-retinal-barrier. This structure maintains a special neuronal extracellular milieu and protects the retina from small hydrophilic and larger molecules as well as cellular components of the blood, which are potentially harmful to the neurons (Bradbury *et al.*, 1990, Lightman *et al.*, 1987, Tornquist *et al.*, 1990). The barrier and its function are realised by metabolic as well as mechanical components. The metabolic component comprises specialised selective transport mechanisms for small molecules (Betz *et al.*, 1983, Cornford *et al.*, 1999, Cornford *et al.*, 1975) and proteins (Mann *et al.*, 2003, Tornquist *et al.*, 1986). The mechanical component is constituted by inter-endothelial tight junctions, the basement membrane, pericytes and perivascular glial cells. Endothelial cells form a single layer on the luminal side of the vessels, the endothelium, where they show no fenestrations but many tight junctions (Patton *et al.*, 2005). These tight junctions are probably the most important element of the BRB and are established by a number of proteins such as occludin (Morcos *et al.*, 2001), claudins (Morcos *et al.*, 2001), adhesion proteins (Martin-Padura *et al.*, 1998) and other associated proteins, such as *zonula occludens* proteins (Stevenson *et al.*, 1986). Transport proteins within the endothelium for molecules such as glucose (Betz *et al.*, 1983) and amino acids (Tornquist *et al.*, 1986) demonstrate the significance of the endothelium in the supply of nutrients to the neuronal tissue. An asymmetric distribution of cell membrane proteins is associated with high transendothelial electrical resistance which is a reflection of impermeability (Crone *et al.*, 1982).

The pericytes that surround the endothelial cells and share the same basement membrane are another integral part of the BRB (Martin *et al.*, 2000, Wallow *et al.*, 1980). Since pericytes possess contractile properties, due to the presence of α-smooth muscle actin, they can be considered analogous to smooth muscle cells in the peripheral retinal circulation (Herman *et al.*, 1985, Wallow *et al.*, 1980).

The endothelial basement membrane, as well as supporting the endothelium, also acts as a molecular filter, an anchorage site for cell adhesion molecules and a diffusion barrier (Perlmutter *et al.*, 1990). Thickening of the basement membrane, such as occurs in diabetes (Cai *et al.*, 2002a, Tsilibary, 2003), significantly impairs oxygen diffusion and transport of molecules across it.

Other cells surrounding the retinal vessels such as glial cells (Müller cells or astrocytes) also play an important part in vascular function and maintenance of the BRB. Astrocytes are crucial for the formation of zonulae occludens (Gardner *et al.*, 1997) and for the secretion of TGF-β, fibroblast growth factor (FGF) and glial cell line derived neurotrophic factor (GDNF). Astrocytes can release VEGF and promote the development of neovascularization under hypoxic conditions (Zhang *et al.*, 1997). Another type of retinal glial cell, retinal microglial cells which are located perivascularly, have phagocytic properties and can remove damaged retinal neurons (Schnitzer, 1989, Thanos, 1991).

1.2.2. Overview of Neuronal and Vascular changes in the diabetic retina

Alterations in retinal blood flow in retinal vessels occur at very early stages of DM before changes are visible clinically. These changes may be due to upregulation of endothelin 1 and 3 (Deng et al., 1999). Simultaneously, upregulation of VEGF is found which induces increased vascular permeability (Cukiernik et al., 2004). There seems to be a costimulatory relationship between the endothelin and VEGF upregulation (Cruz et al., 2001), which are both likely to contribute to the increased vascular permeability that is induced by high glucose (Chen et al., 2000). Upregulation of endothelin also causes downregulation of NO production (Levin, 1995, Vanhoutte, 1994) – which reduces the blood vessels' capacity to dilate.

With progressive cellular damage, early signs of DR include acellular non-perfused capillaries, pericyte ghosts and pericyte loss, foci of vessel pseudofilia and

prominent basement membrane thickening (Cai *et al.*, 2002a, Grant *et al.*, 2004, Tsilibary, 2003). The loss of pericytes is associated with an increased risk of developing retinal neovascularisation as the perfused vessels are influenced by increased levels of angiogenic factors (Hammes et al., 2004, Hughes et al., 2007, Pfister et al., 2008). Additionally, endothelial cells are lost (Kuwabara *et al.*, 1963) and microaneurysms form (Caldwell *et al.*, 2003, Frank, 2004, Otani *et al.*, 2005). Apoptosis is thought to account for the disappearance of both endothelial cells and pericytes.

Hyperglycaemia naturally is the most important factor for the changes observed in DR but also oxidative stress, mitochondrial dysfunction and apoptosis are crucial mechanisms contributing to the vasculopathy. High glucose levels themselves have been reported to be able to induce apoptosis in Müller cells, retinal endothelial cells and retinal neurons (Mohr *et al.*, 2002).

A number of inflammatory processes have been described in DR. Elevated levels of inducible nitric oxide synthase (iNOS), pro-inflammatory cytokines and nuclear factor kappa B (NF-κB) (Joussen *et al.*, 2002b) are found in conjunction with increased leukostasis and increased vascular permeability. Components of the neuroretinal tissue like Müller cells and other glia were reported to contribute to these processes by releasing inflammatory cytokines such as interleukin 1β whose production is increased via caspase-1 under hyperglycaemic conditions (Mohr *et al.*, 2002). Other reports show that retinal microglial and macroglial cells can release tumour necrosis factor α (TNF-alpha) and VEGF (Gardner *et al.*, 2002, Ishida *et al.*, 2003b, Rungger-Brandle *et al.*, 2000). TNF-alpha is responsible for leukocyte adhesion to the vessel walls (Giraudo *et al.*, 1998, Joussen *et al.*, 2002a) while VEGF upregulation in the retina can lead to upregulation of intercellular adhesion molecule 1 (ICAM-1) which facilitates leukostasis through CD18-related binding of leukocytes on the vessel wall. This leukocyte binding results in blood flow changes and eventually to occlusion of the small capillaries leading in turn to increased vascular permeability and finally to ischaemia of the adjacent tissue (Grant *et al.*, 2004).

Apart from the vascular changes occurring throughout the course of the disease, neuronal damage, particularly ganglion cell death (Barber *et al.*, 1998) is said by some to also occur in the very early stages of DR, perhaps independently of the vasculopathy. Electroretinographic (ERG) studies in humans (Frost-Larsen *et al.*, 1980, Juen *et al.*, 1990) and animals (Li *et al.*, 2002) reported abnormal retinal

function long before visible changes in the fundus appeared suggesting early neuronal and Müller cell dysfunction. Increased levels of glutamate, which accumulates in the vitreous of diabetic patients (Ambati *et al.*, 1997) and also in diabetic animals (Lieth *et al.*, 1998), may contribute to the retinal neuropathy. Glutamate activates N-Methyl-D-aspartate (NMDA) receptors, thereby inducing increased intracellular influx of calcium and the formation of reactive oxygen species (ROS), both of which promote apoptosis (Smith, 2002). The breakdown of the BRB that occurs early in DR may cause or at least exacerbate the retinal neuropathy (Antcliff *et al.*, 1999, Pelzek *et al.*, 2002).

As DR progresses, it changes from the non-proliferative to the proliferative stage. This conversion is associated with chronic inflammation as evidenced by increased inflammatory markers (Schram *et al.*, 2003), immunologic responses (Tong *et al.*, 2004) and increased endothelial leukocyte adhesion (Joussen *et al.*, 2004). Increased vascular permeability results in leakage of proteins into the retina and progressive closure of capillaries results in ischaemia which in turn triggers the development of RNV which is augmented and promoted by local angiogenic factors (Calles-Escandon *et al.*, 2001, Ciulla *et al.*, 2003, Clermont *et al.*, 1997, Fong *et al.*, 2004, Wiedemann, 1993, Yilmaz *et al.*, 2000). In later stages of DR, endothelial cells are activated and start to proliferate (Garner, 1993) with sprouting of new capillaries from existing vessels (Folkman, 1995). VEGF is the main driving force promoting growth of RNV and vascular permeability leading towards the end-stage of DR (Ciulla *et al.*, 2003, Clermont *et al.*, 1997, Fong *et al.*, 2004).

After decades of research the complex interplay of increased leukocyte adhesion which results in changes in blood flow, loss of endothelial cells and pericytes, basement membrane thickening, increased vascular permeability, localised hypoxia and subsequent release of angiogenic factors and capillary occlusion resulting in tissue hypoxia remains enigmatic. As DR progresses, an interplay of growth factors and cytokines regulating the formation of blood vessels is involved in the formation of RNV. Some of these factors will be characterised briefly in the next sections.

1.2.3. Factors involved in vascular changes in DR

1.2.3.1 Insulin-like growth factor

The insulin-like growth factor (IGF) complex comprises two IGFs (IGF-1 and IGF-2) and at least six high affinity IGF binding proteins (IGFBP) and IGFBP specific proteases (Grant *et al.*, 2004). Both IGF-1 and IGF-2 have a number of functions such as the promotion of cell division, cell migration, cell differentiation and generation of tractional forces. Normally, most of IGF-1 and IGF-2 are bound to IGFBP (synthesised in the liver), which inhibits the function of the IGFs and hence suppresses their activities. On average only about 0.1% of both IGF-1 and IGF-2 is freely circulating (Grant *et al.*, 2004).

IGF-1 was implicated in the pathogenesis of proliferative DR as studies in diabetic patients revealed that individuals with quickly developing proliferative DR had elevated serum levels of IGF-1 (Grant *et al.*, 2002). A large study of 928 patients with DM also found that higher serum levels of IGF-1 were associated with proliferative DR (Dills *et al.*, 1991). Insulin treatment can significantly impact IGF-1 levels. On one hand an acute normalisation of a long-standing hyperglycaemia can trigger early worsening of a DR possibly by increasing IGF-1 but on the other hand it was reported that maintaining normoglycaemia eventually reduces IGF-1 levels with improvement of early-worsened DR (Chantelau *et al.*, 2003). However, the precise mechanism by which IGF-1 affects DR is uncertain since other studies could not correlate serum levels of IGF-1 with the development of proliferative DR (Janssen *et al.*, 2000, Wang *et al.*, 1995).

A factor contributing to the complexity of the problem is that intraocular IGF-1 levels may be controlled locally. Higher levels of IGF-1 have been reported in the vitreous of patients with proliferative DR compared with non-proliferative DR (Grant *et al.*, 1986, Meyer-Schwickerath *et al.*, 1993) that remained high even after normalisation of blood IGF-1 levels, whereas vitreous levels of IGF-1 in eyes with non-proliferative DR have been reported to be reduced (Waldbillig *et al.*, 1994).

Experimental injection of IGF-1 into the vitreous of pigs (Danis *et al.*, 1997) and rabbits (Grant *et al.*, 1993) resulted in breakdown of the BRB, basement membrane thickening and the development of retinal neovascularisation – changes seen in

proliferative DR. Although there are some contradictory observations, it seems likely that IGF-1 is one of the key mediators in the development of proliferative DR.

The observation that in a diabetic women who suffered from spontaneous hypopituitarism due to *post partum* pituitary haemorrhage (Poulsen, 1953) her established diabetic retinopathy regressed led to the introduction of IGF-1 inhibitors (e.g. octreotide). To date, studies were however not able to show a significant effect in the overall regression of DR, only the progression from NPDR to PDR could be reduced by 36% (Grant *et al.*, 2000).

Although IGF-1 is involved in the development of DR, it seems that it may have a permissive function acting on a background of elevated VEGF levels (Grant et al., 2000). This is supported by the observation that IGF-1 is necessary for induction of maximal neovascularisation induced by VEGF, an interaction that is mediated by activation of MAPK and p44/42 (Smith et al., 1999).

While elevated level of IGF seem to contribute to the development of DR, recent research suggested a vasoprotective role for IGFBP3 (Kielczewski *et al.*, 2011, Yan *et al.*, 2010). It was reported that IGFBP3 exerts its vasoprotective role by recruiting endothelial progenitor cells (EPCs) to areas of vascular injury and by increasing the release of nitric oxide (NO). Injecting plasmids carrying the gene for IGFBP3 into the vitreous of mice in a model of oxygen induced retinopathy showed a clear neuroprotective effect (Yan *et al.*, 2010) evidenced by a reduced rate of apoptosis of retinal neurons (Kielczewski *et al.*, 2011). IGFBP3 was said to be able to activate endothelial nitric oxide synthase (eNOS) by phosphorylation at SER1177 (IGFBP3 also induced phosphorylation of AKT at SER437 and THR308). Eventually neurovascular protection was accomplished through a scavenging receptor B1 (SRB1) dependent nitric oxide release resulting in increased NO.

1.2.3.2 Vascular endothelial growth factor and its receptors

As indicated above, VEGF is one of the key players in the pathogenesis of DR. When referring to VEGF, most people mean VEGF-A. VEGF-A is a member of the VEGF family of growth factors comprising of VEGF-A (Mattei *et al.*, 1996), VEGF-B (Olofsson *et al.*, 1996), VEGF-C (Joukov *et al.*, 1996, Lee *et al.*, 1996), VEGF-D (Achen *et al.*, 1998, Orlandini *et al.*, 1996), viral homologs of VEGF that are

collectively called VEGF-E (Meyer *et al.*, 1999, Ogawa *et al.*, 1998, Wise *et al.*, 1999) and placental growth factor (PlGF) (Maglione *et al.*, 1991). The action of all members of the VEGF family is mediated by binding to receptors of the tyrosine kinase type (Petrova *et al.*, 1999), not by triggering the release of other growth factors (Ortega *et al.*, 1997).

There are three known receptors: VEGFR1 (Flt-1) (de Vries *et al.*, 1992), VEGFR2 (KDR) (Terman *et al.*, 1992) and VEGFR3 (Flt-4) (Galland *et al.*, 1992). All three are almost exclusively expressed on endothelial cells (Petrova *et al.*, 1999). A co-receptor that associates with VEGFR2 called neuropilin has also been described (Aiello *et al.*, 2000, Ferrara *et al.*, 2001).

VEGF-A was characterised much earlier than the other members of the VEGF family. It was originally named vasculotropin (VAS) or vascular permeability factor (VPF) due to its ability to promote vascular leak (Guerrin *et al.*, 1995, Shen *et al.*, 1993). For the sake of simplicity, VEGF will be used here as a synonym for VEGF-A. VEGF is a dimeric glycoprotein that is encoded by the VEGF gene. Isoforms are generated by alternate splicing of the mRNA and named according to the number of amino acids: VEGF-A_{121}, VEGF-A_{145}, VEGF-A_{165}, VEGF-A_{189}, VEGF-A_{206} (Ferrara *et al.*, 1997, Poltorak *et al.*, 1997).

VEGF is a cytokine (Lee *et al.*, 2006a) that acts as an endothelial cell mitogen (Keck *et al.*, 1989, Leung *et al.*, 1989). Under physiological conditions, VEGF also functions as a vasodilator, promoter of endothelial cell migration, endothelial cell survival factor and is antiapoptotic (Alon *et al.*, 1995, Borgstrom *et al.*, 1999, Ku *et al.*, 1993). Besides its function as a survival factor for neurons, especially in hypoxic conditions or after ischaemia (Samii et al., 1999, Sondell et al., 1999a, Sondell et al., 1999b, Sondell et al., 2000), and endothelial cells, VEGF is particularly important in developing blood vessels (Grant *et al.*, 2004). Developing vessels seem to lose their dependency on VEGF and become established as they are closely enveloped with pericytes (Grant *et al.*, 2004). VEGF can however also have proapoptotic properties (Guerrin *et al.*, 1995) and is able to increase oxidative stress associated substances (El-Remessy *et al.*, 2003).

The antiapoptotic function of VEGF is mainly due to stimulation of the phosphatidyl-inositol 3-kinase (PI3K)/AKT pathway (Gerber et al., 1998a). Blocking VEGF-mediated activation of the PI3K/AKT pathway (Rousseau et al., 1997) results in increased apoptosis due to potentiation of p38 mitogen activated protein-kinase

activation (Gratton et al., 2001). The importance of VEGF in development is underlined by the observation that knockout of the VEGF gene or blockage of VEGF action during embryogenesis is lethal (Carmeliet *et al.*, 1996, Ferrara *et al.*, 1996, Fong *et al.*, 1995).

VEGF is expressed by a number of cells within the eye, such as retinal pigment epithelium cells, pericytes, astrocytes, Müller cells and endothelial cells (Adamis *et al.*, 1993, Aiello *et al.*, 1995b, Behzadian *et al.*, 1998, Simorre-Pinatel *et al.*, 1994). Some of these cells, such as the epithelial cells of the RPE, not only produce VEGF, they also express its respective receptor (Guerrin et al., 1995). The production of VEGF varies amongst these different cell types. While neuroretinal cells produce approximately 15-20pg VEGF per milligram of protein, the choroid and RPE cells produce roughly 50pg VEGF per milligram of protein (Kim *et al.*, 1999). Interestingly, VEGF production in RPE cells is asymmetric within the cell: the basal side produces much more than the apical side, supposedly fenestrating the underlying choriocapillaris (Bernstein *et al.*, 1965, Blaauwgeers *et al.*, 1999).

VEGF is able to increase endothelial fenestration and thereby create a route for transcellular permeability (Senger *et al.*, 1983). This effect is observed within 4 to 6 hours after treating endothelial cells with VEGF *in vitro*, after increased permeability has been established (Behzadian *et al.*, 2003). This is associated with induction of the urokinase receptor (uPAR) (Mandriota *et al.*, 1995). Subsequent plasmin formation and activation of proteolytic properties disrupt the cell-to-cell attachments as evidenced by a decrease of occludin levels from tight junction proteins (Antonetti *et al.*, 1998, Barber *et al.*, 2000, Leal *et al.*, 2007). The effect of VEGF to increase vascular permeability was reported under both diabetic (Do carmo *et al.*, 1998b) and non-diabetic conditions (Senger *et al.*, 1983). Events leading to increased vascular permeability are associated with increased oxidative stress from peroxynitrite and superoxide anion formation (El-Remessy *et al.*, 2003).

Other proapoptotic processes in endothelial cells are triggered by VEGF, such as the activation of stress-activated serine/threonine protein kinase and the p38 mitogen activated protein kinase (Gerber *et al.*, 1998b). Experimental inhibition of VEGF-mediated activation of PI3K/AKT (Rousseau *et al.*, 1997) pathway resulted in increased apoptosis due to potentiation of p38 mitogen activated protein-kinase activation (Gratton *et al.*, 2001), illustrating the dual function of VEGF with respect to apoptosis.

In pathological conditions such as DR, however, VEGF is regulated differently. Several studies have reported a close involvement of VEGF in the pathogenesis of DR (Adamis *et al.*, 1994, Aiello *et al.*, 1994, Amin *et al.*, 1997, Funatsu *et al.*, 2001, Ishida *et al.*, 2000, Malecaze *et al.*, 1994, Shimada *et al.*, 2002, Tanaka *et al.*, 1997).

VEGF is one of the key factors in the development of retinal vascular complications in DR and is exerting its function especially in later stages of DR. Downregulation of VEGF is found in early DM, possibly resulting in the loss of survival signals and apoptosis of retinal cells. Later, as DR develops, retinal ischaemia leads to a compensatory VEGF expression in retinal cells; increased VEGF levels are found in the vitreous of eyes with PDR (Aiello *et al.*, 1994). VEGF is expressed in DR mainly as a response to ischaemia (Aiello *et al.*, 1995a, Shweiki *et al.*, 1992), which is a major stimulus for retinal neovascularisations (Henkind, 1978, Mizutani *et al.*, 1996) and therefore promotes revascularisation and increases reperfusion (Grant *et al.*, 2004).

Studies reported that VEGF is responsible for a breakdown of the BRB in early DR (Murata *et al.*, 1996, Xia *et al.*, 1996) and that blockage of the VEGF action reverses the diabetes-induced vascular leak (Tilton *et al.*, 1997). VEGF can experimentally be used to induce RNV (Tolentino *et al.*, 2002) and conversely intravitreal injections of anti-VEGF antibodies can prevent their development (Adamis *et al.*, 1996). Systemic administration of anti-VEGF agents however, may have clear clinical disadvantages (Duh *et al.*, 1999).

A key component for VEGF upregulation is the hypoxia inducible factor 1 α (HIF1alpha) (Aiello *et al.*, 1995b, Behzadian *et al.*, 1998, Brooks *et al.*, 1998, Forsythe *et al.*, 1996) but also inflammatory mediators are able to increase VEGF levels (Cheng *et al.*, 1998, Hata *et al.*, 2000, Nie *et al.*, 2000). HIF1alpha itself is upregulated as a result of tissue ischaemia (Arjamaa et al., 2006). VEGF mediated hyperpermeability in early DR is clearly correlated with HIF1alpha induced upregulation of VEGF (Poulaki et al., 2002), eNOS and nNOS (El-Remessy *et al.*, 2003, Joussen *et al.*, 2002c, Kroll *et al.*, 1998, Takeda *et al.*, 2001).

VEGF is not only a potent inducer of vascular permeability (Connolly et al., 1989) but also of angiogenesis (Plouet et al., 1989). Development of RNV is driven by VEGF in the later stages of DR in both humans and animal models (Aiello *et al.*, 1997, Miller *et al.*, 1997) in which VEGF inhibitors could blunt the neovascular response. In other animal models the development of RNV was correlated with VEGF

levels and it was reported that anti-VEGF treatment prevented pathological vascular growth (Aiello et al., 1995b, Donahue et al., 1996, Dorey et al., 1996, Ozaki et al., 2000, Robbins et al., 1997, Robinson et al., 1996). Strong immunohistochemical localisation of VEGF (Malecaze et al., 1994) in proliferative tissue from patients with late stage DR further confirmed its close involvement in the development of RNV in humans (Boulton *et al.*, 1998).

While increased levels of VEGF were found in the blood of patients with established DM (Mahdy *et al.*, 2010), no correlation with either the presence of DR or the severity of DR could be established (Lee *et al.*, 2006a). However patients with proliferative DR had significantly higher levels of VEGF in the serum than patients with non-proliferative DR (Mahdy *et al.*, 2010). Interestingly, while there was no correlation of systemic VEGF with the stage of DR (Chaturvedi et al., 2001), there was a good correlation between systemic VEGF levels and the presence of diabetic nephropathy in type 2 diabetic patients (Wasada *et al.*, 1998). These observations suggest there is strong local control and production of VEGF within the eye to maintain a balance between too much and too little. Other events, such as protein kinase C (PKC) activation, are of great importance in the elevation of VEGF in diabetic individuals (Aiello *et al.*, 1997, Spyridopoulos *et al.*, 2002, Xia *et al.*, 1996, Xia *et al.*, 1994).

Additional factors that contribute to the pathogenesis of DR include activation of the polyol pathway, accumulation of advanced glycation end products and, as mentioned above, oxidative stress (Obrosova *et al.*, 2001).

Both high glucose and oxidative stress in DM increase Intercellular Adhesion Molecule 1 (ICAM-1) (Leal *et al.*, 2007) which is also induced by VEGF (Ishida *et al.*, 2003a, Joussen *et al.*, 2002b, Kim *et al.*, 2001, Lu *et al.*, 1999, Miyamoto *et al.*, 2000, Proescholdt *et al.*, 1999, Radisavljevic *et al.*, 2000). Conversely, inhibition of VEGF by neutralising antibodies in diabetic rats was reported to reduce ICAM-1 levels by 83% compared with untreated diabetic animals (Joussen *et al.*, 2002b). Leukocytes express CD18, the counterpart for the receptor ICAM-1 (Barouch et al., 2000), which explains why ICAM-1 mediated leukocyte adhesion is one of the earliest events in animal models of DR (Joussen *et al.*, 2002b, Miyamoto *et al.*, 1999) and ICAM-1 mediates increased vascular permeability in diabetic individuals (Miyamoto *et al.*, 1999).

This upregulation of ICAM-1 in diabetes is specifically found in the ECs (Joussen et al., 2002a, McLeod et al., 1995, Miyamoto et al., 1999). Such increased expression of ICAM-1 leads to increased leukocyte adhesion to the endothelium, occlusion of small retinal capillaries (i.e. capillary non-perfusion) and accumulation of leukocytes in the retinal tissue – all of which eventually contribute to BRB breakdown and EC apoptosis (El-Remessy *et al.*, 2003, Joussen *et al.*, 2001b, Joussen *et al.*, 2003, Joussen *et al.*, 2002b, Joussen *et al.*, 2002c, Miyamoto *et al.*, 1998a, Miyamoto *et al.*, 1999, Miyamoto *et al.*, 1997, Nishiwaki *et al.*, 1995, Nonaka *et al.*, 2000, Sander *et al.*, 1994). These observations were made at a very early stage experimentally. In a rat model of diabetes, a 2-3-fold increase in leukocyte adhesion was observed within 1 week of induction of diabetes (Joussen *et al.*, 2002b). Inhibiting ICAM-1 led to a 47% reduction of leukocytes in retinal arterioles and 36% in the retinal capillaries (Joussen *et al.*, 2002b). Moreover, experimental inhibition of ICAM-1 resulted in an improvement in BRB function and established BRB breakdown could be partially reversed (Miyamoto *et al.*, 1999). It is of note that eNOS is an important promoter of ICAM-1 expression since upregulation of ICAM-1 requires NO (Buras *et al.*, 2000, Joussen *et al.*, 2001b, Joussen *et al.*, 2002a, Joussen *et al.*, 2002b, Niu *et al.*, 1994). Upregulation of eNOS by VEGF in the diabetic retina (Joussen *et al.*, 2002b) as well as the function of eNOS and its involvement in the pathogenesis of DR will be discussed in detail in a separate section.

Besides hypoxia, oxidative stress and inflammation are critical in the development of DR. It was reported that accumulation of inflammatory factors in the rat retina occurs in DM via activation of the cyclooxygenase pathway (Ayalasomayajula et al., 2003, Joussen et al., 2002a). VEGF can induce the formation of prostacyclin and, as mentioned above, the expression of ICAM-1, which leads to amplification of inflammatory processes (He et al., 1999, Joussen et al., 2002b). The inflammatory cytokine IL1beta also induces VEGF expression in human endothelial cells (Hallscheidt et al., 2001, Jung et al., 2001) and is able to mediate ischaemic injury in the retina and is upregulated in the retina in early diabetes (Joussen *et al.*, 2001a, Yoneda *et al.*, 2001).

Due to the autocrine action of VEGF it is able to trigger its own overexpression in endothelial cells, which is normally moderated by VEGF inhibiting proteins. High glucose and oxidative stress are able to block these VEGF inhibiting proteins

resulting in VEGF *overexpression that* is found in the vitreous of diabetic patients (Funatsu et al., 2003).

VEGF has a critical antiapoptotic role and acts as a survival factor for the endothelium. Paradoxically in DM a high level of VEGF is found associated with capillary dropout and small vessel occlusion (Mizutani et al., 1996) which leads to the hypothesis that VEGF actions in diabetes must be altered in endothelial cells. Endothelial cells cultured in high glucose behave similarly when VEGF-mediated activation of the PI3K/AKT (Rousseau *et al.*, 1997) pathway is blocked, despite exogenous VEGF supplementation (El-Remessy *et al.*, 2005, Gu *et al.*, 2003). These pro-apoptotic effects of high glucose and oxidative stress are associated with increased phosphorylation of the p38-mitogen-activated-kinase, decreased AKT-kinase activity and tyrosine-nitration of both the P85 and P110 subunit of PI3K. The P85 regulatory subunit of PI3K is a target for peroxynitrite-induced protein nitration on tyrosine which leads to inhibition of the P85 with the P110 catalytic subunit suggesting a key role for peroxynitrite in high glucose environments in impairing EC survival by impacting the PI3K/AKT pathway (El-Remessy et al., 2005).

VEGFR1, one of the three receptors that VEGF binds to, seems to be mainly involved in cell differentiation and recruitment of endothelial progenitor cells (EPCs) (Grant *et al.*, 2004, Hattori *et al.*, 2002, Heissig *et al.*, 2002) as well as EPC mobilisation from the bone marrow (Hattori et al., 2002, Luttun et al., 2002). VEGFR1 is also essential for organising the developing embryonal vasculature (Fong *et al.*, 1995) and for arteriogenesis (Waltenberger, 2001): VEGFR1$^{-/-}$ knockout mice develop an abnormal vasculature with endothelial cells filling the lumen of the vessel leading to the conclusion that VEGFR1 also acts as a kind of negative regulator responsible for normal vessel structure formation (Fong et al., 1995, Shalaby et al., 1995). In hypoxic conditions, activation of the HIF-1alpha upregulates VEGFR1 expression (Grant *et al.*, 2004). PlGF can also activate VEGFR1 (Grant *et al.*, 2004).

A cascade is triggered when VEGF binds to VEGFR1 expressed on endothelial cells, resulting in the activation of phosphatidylinositol-3-kinase (PI3K) (Takahashi *et al.*, 2003, Wang *et al.*, 2011a). Activation of PI3K will induce phosphorylation of eNOS at SER1177 which activates eNOS and leads to the production of nitric oxide (Ackah *et al.*, 2005, Dimmeler *et al.*, 2000, Fulton *et al.*, 1999). In EPCs, this NO production activates matrix metallo-proteinases 2 (MMP2) and 9 (MMP9) both of which will then be involved in the degradation of extracellular material (Egeblad *et*

al., 2002, Libby *et al.*, 2000, van Kempen *et al.*, 2002) which will lead to mobilisation of EPCs to the systemic circulation (Aicher et al., 2003, Murohara et al., 1998). This involvement of VEGFR1 EPC mobilisation was also reported in model of diabetic retinopathy (Grant et al., 2002, Luttun et al., 2002).

Upon VEGF-binding, VEGFR2 undergoes a process of dimerisation and transphosphorylation that eventually leads to multiple cell responses. Vasodilation, increased vascular permeability and increased endothelial cell migration proliferation and survival are associated with the release of mitogenic, chemotactic and survival signals for endothelial cells including phosphorylation of proteins in EPCs such as phospholipase C (PLC), PI3K, RAS, GTPase and SRC (Zachary *et al.*, 2001). Within the bone marrow the VEGF-VEGFR2 interaction mediates the survival of hematopoietic and EPCs through the VEGFR2 (Gerber et al., 2002, Larrivee et al., 2003).

VEGFR2 is thought to be the main regulator for angiogenesis and vascular permeability (Fong *et al.*, 1995, Grant *et al.*, 2004). It is essential for EPC function of circulating monocytes (Elsheikh et al., 2005). The early involvement of VEGFR2 in the development of DR was demonstrated by its upregulation that was reported in to be associated with the onset and the progression of DR (Hammes et al., 1998, Witmer et al., 2002). The activation of VEGFR2 by binding of VEGF leads to the formation of superoxide anions in endothelial cells through the NADPH oxidase, thus contributing to the oxidative stress (Caldwell *et al.*, 2003) and hence to the early breakdown of the blood-retinal barrier seen in DR (Antonetti et al., 1998). It is of note that in DM the number of VEGFR2 receptors on endothelial cells is increased (Hammes *et al.*, 1998).

Activation of VEGFR2 has many other effects. For example, it stimulates EPC growth by activating the RF-MEK-ERK pathway, in which PKC is strongly involved (Grant *et al.*, 2004). VEGFR2 activation is also responsible for the antiapoptotic effects of VEGF observed in human umbilical cord vein cells (HUVECs). Additionally, involvement of VEGFR2 in endothelial lineage determination was suggested by grossly deranged angioblast differentiation in a murine knockout model for VEGFR2 (Fong et al., 1999).

While VEGFR1 seems to be more important for tube formation, a characteristic feature of EPCs that will be discussed later, VEGFR2 is more important for retinal vessel proliferation (Grant *et al.*, 2004). Although the affinity of VEGF for VEGFR1

is higher than for VEGFR2 (de Vries *et al.*, 1992, Terman *et al.*, 1992), absence of VEGFR1 (e.g. in knockout models) has little effect whereas absence of VEGFR2 prevents VEGF function completely (Ferrara, 2001).

While all three VEGF receptors are mainly found on endothelial cells (Petrova *et al.*, 1999), VEGFR1 and VEGFR2 are mainly on vascular endothelial cells (de Vries *et al.*, 1992, Seetharam *et al.*, 1995, Terman *et al.*, 1991, Terman *et al.*, 1992) whereas VEGFR3 seems to be mainly expressed by adult lymphatic endothelial cells.

VEGFR3 is crucial during embryogenesis for the development of the cardiovascular system (Iljin *et al.*, 2001). Interestingly, recent research indicates that VEGFR3 is also involved in establishing vascular networks in tumours (Padera *et al.*, 2008). Blocking VEGFR3 in the developing murine retina resulted in less dense vascularisation with fewer endothelial sprouting points (Tammela *et al.*, 2008).

The apparent major role of VEGF signalling in the pathogenesis of DR led to the development of approaches to reduce VEGF levels within the eye. A range of options have been evaluated: soluble forms for VEGF receptors, anti-VEGF antibodies, VEGF receptor proteins, antisense oligonucleotides and inhibitors of VEGF-specific protein kinases (2002, Aiello *et al.*, 1995b, Caldwell *et al.*, 2003, Ozaki *et al.*, 2000, Sone *et al.*, 1999).

Suppressing VEGF can also be achieved by reducing the activity of HIF1alpha. Inhibition of HIF1alpha by Rapamycin reduces VEGF expression (Guba et al., 2002). Rapamycin, also known as Sirolimus, is as negative regulator of PI3K/AKT dependent mTOR (mammalian target of Rapamycin) activation which reduces the transactivation function of HIF1alpha (Hudson et al., 2002, Treins et al., 2002, Zhong et al., 2000). Bucillamin (N-[2-mercapto-2-methylpropionyl]L-cysteine), which inhibits the transcriptional activity of HIF1alpha (Koyama et al., 2002), successfully blocked the diabetic upregulation of VEGF and the associated increase in vascular permeability in a STZ induced rat model of diabetes (Hikichi et al., 2002).

While the approaches described above to inhibit VEGF are more suitable in a laboratory setting, intravitreal administration of VEGF antibodies has been developed for clinical use. Ranibizumab, a human antibody fragment that was developed for intraocular use (Lucentis®, Novartis, Basel, Switzerland; Genentech Inc., South San Francisco, CA, USA), blocks all isoforms of VEGF (Simo *et al.*, 2008, Singh *et al.*, 2007). Although originally approved by the FDA in January 2007 for the treatment of wet age-related macular degeneration after having been reported to improve visual

acuity with monthly injections in the MARINA (Rosenfeld *et al.*, 2006) and ANCHOR (Brown *et al.*, 2006) trials, ranibizumab was soon considered by clinicians for use in DR (Simo *et al.*, 2008). Ranibizumab has been reported to be effective for diabetic macular oedema, with results better than laser treatment when vision was reduced (Elman *et al.*, 2010, Massin *et al.*, 2010, Mitchell *et al.*, 2011).

1.2.3.3 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors (Statins)

The group of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitors (statins) which are used to treat high cholesterol levels were also studied for their effects on the vasculature – especially in the context of diabetic retinopathy and their impact on VEGF. They are not involved in the development of DM or DR but as they show a number of beneficial effects especially in diabetes they are discussed here briefly.

Statins have a number of other biochemical effects – besides inhibiting HMG-CoA. They inhibit the Rho-guanosine triphosphatase (Rho-GTPase), for instance, leading to upregulation of PI3K/AKT pathway and hence increased NO production (Laufs *et al.*, 1998b). Inhibition of the Rac-1 GTPase by statins inhibits NADPH oxidase activity and hence reduces oxidative stress (Delbosc *et al.*, 2002). Statins were also reported to inhibit the VEGF expression that is induced by advanced glycation end products (Okamoto *et al.*, 2002). These effects however are not unidirectional as statins act in a biphasic, dose-dependent way (Urbich *et al.*, 2002a, Weis *et al.*, 2002). In studies where clinically relevant doses of statins were administered, the same dose that could suppress pathological angiogenesis could induce angiogenesis in models of acute ischaemia (Horiuchi *et al.*, 2003, Kawata *et al.*, 2001). Statins were also reported to reduce inflammation , decrease platelet aggregation and thrombus deposition and increase endothelial NO production (Dimmeler *et al.*, 2001, Gerber *et al.*, 1998a, Llevadot *et al.*, 2001, Morales-Ruiz *et al.*, 2000).

Increased adhesion of leukocytes to the endothelium in diabetes and at inflammatory sites may also be attenuated by statins (Miyahara *et al.*, 2004, Teupser *et al.*, 2001, Yoshida *et al.*, 2001) resulting in improved BRB function. A suggested mechanism is the statin-induced suppression of adhesion molecules (Miyahara *et al.*,

2004) on ECs by activating eNOS (Kaesemeyer et al., 1999, Laufs et al., 1997, Mueck et al., 2001, Pruefer et al., 2002, Romano et al., 2000). As mentioned, eNOS is an important factor of ICAM-1 expression as it is mediated by NO (Joussen *et al.*, 2002b). Additionally, statins were also reported to promote EPC proliferation, migration and cell survival *in vitro* as well as promoting the restoration of ECs and the genesis of new blood vessels (Dimmeler et al., 1998, Dimmeler et al., 1999) – which will be discussed in a separate section.

1.2.3.4 Pigment epithelium-derived factor

Pigment epithelium-derived factor (PEDF) was originally isolated from fetal RPE cells (Chader, 2001, King *et al.*, 2000). PEDF is produced in the apical region of RPE cells (Becerra *et al.*, 2004) in contrast to VEGF which is produced more on the basal side (Blaauwgeers *et al.*, 1999). PEDF was reported to promote differentiation of primitive retinoblastoma cells into neuron-like structures and to have neurotrophic functions (Steele *et al.*, 1993). More importantly – in the context of DR - it is able to inhibit neovascularisation substantially (Dawson *et al.*, 1999) and angiogenesis (Ogata *et al.*, 2002). Thus VEGF and PEDF seem to have reciprocal effects in the eye. It has been reported that PEDF is correlated negatively with angiogenic diseases such as DR: in diabetic retinopathy, VEGF increases while PEDF decreases (Gao *et al.*, 2001). PEDF reduces vascular permeability, especially if induced by VEGF (Liu *et al.*, 2004).

1.2.3.5 Stromal cell-derived factor-1

Stromal cell-derived factor-1 (SDF-1) exerts its actions through its receptor CXCR4, an alpha-chemokine transmembrane G-protein coupled receptor (Grant *et al.*, 2004). VEGF induces the expression of CXCR4 and SDF-1 in turn triggers the expression of VEGF in cells that are of both hematopoietic and endothelial origin (Kijowski *et al.*, 2001, Neuhaus *et al.*, 2003). SDF-1 promotes endothelial cell migration and plays a major role in the pathogenesis of diabetic macular oedema and PDR. The concentrations of SDF-1 in the vitreous correlate with VEGF concentrations and the degree of retinopathy and macular oedema (Grant *et al.*, 2004).

Interestingly, both VEGF and SDF-1 have a synergistic effect in recruiting endothelial progenitor cells to ischaemic regions in the diabetic retina (Grant *et al.*, 2004). In diabetic humans with macular oedema and proliferative retinopathy, the intravitreal administration of triamcinolone (a synthetic corticosteroid) resulted in a significant decrease in both SDF-1 and VEGF together with regression of proliferative changes and macular oedema (Brooks *et al.*, 2004a, Brooks *et al.*, 2004b).

1.2.3.6 Somatostatin and Somatotropin

Somatostatin (Brazeau *et al.*, 1973) (Somatotropin release-inhibiting factor, SRIF) and its analog (Ferone *et al.*, 1999) are inhibitors of Somatotropin (or growth hormone [GH]) secretion from the pituitary gland (Roth *et al.*, 1963). It has been reported that both GH and IGF-1 are involved in the progression of DR. Studies in GH deficient dwarfs suffering from diabetes showed that these individuals did not develop any form of diabetic retinopathy even after 25 years of observation (Merimee, 1978). This provided a rational to treat diabetic patients with GH inhibitors. Administration of GH antagonists in diabetic patients on intense insulin therapy was reported to improve their HbA1C significantly to levels below <8%. Unfortunately this treatment also resulted in an increase of IGF-1 which dramatically accelerated diabetic retinopathy (early worsening) (Chantelau, 1998). This early worsening was also observed in the ETDRS and DCCT studies (1993, Davis *et al.*, 1998). Further studies proposed that intensive insulin therapy leads to transcriptional activation of VEGF via p38MAPK, PI3K and HIF1alpha pathways, thus producing the breakdown of the BRB (Dell *et al.*, 2004). Another study reported that diabetic patients with proliferative DR had significantly lower levels of Somatostatin in the vitreous but significantly higher levels in the blood plasma than normal controls. It was concluded that lower levels of Somatostatin in the vitreous was contributing to the development of RNV (Simo et al., 2002).

1.2.3.7 Platelet derived growth factor

Platelet derived growth factor (PDGF) is a potent mitogen that has been implicated in the development of proliferative DR. Immunocytochemical studies reported that PDGF and its receptors are present in epiretinal membranes isolated from patients with DR (Robbins *et al.*, 1994) and increased levels of PDGF antibodies were described in vitreous samples of patients with proliferative DR (Freyberger *et al.*, 2000). The hypoxia that may be found in DR may upregulate PDGF-B in endothelial cells (Kourembanas *et al.*, 1990).

1.2.3.8 Fibroblast growth factor

Acidic fibroblast growth factor 1 (FGF-1) and basic fibroblast growth factor (FGF-2), both members of the heparin binding growth factors family, have been implicated in the development of retinal neovascularisation. FGFs are normally localized in the ganglion cell layer and the inner nuclear layer of the retina (Gao et al., 1992, Kostyk et al., 1994). Studies in diabetic patients showed FGFs were present in retinal tissue during the formation of neovascularisation (Nyberg et al., 1990) and increased FGF levels were found in the vitreous of patients with PDR (Sivalingam et al., 1990). Moreover, FGFs were found in neovascular membranes in patients with proliferative DR (Frank et al., 1996, Hanneken et al., 1991). In patients with coronary heart disease, the administration of FGF-2 resulted in improved revascularisation of ischaemic myocardium (Laham et al., 1999).

1.2.3.9 Tumour necrosis factor alpha

Tumour necrosis factor alpha (TNF-alpha) also contributes to the pathogenesis of DR. It was reported to be expressed in human retinae with proliferative DR (Armstrong *et al.*, 1998, Limb *et al.*, 1996) as well as in animal models of retinal neovascularisation (Armstrong *et al.*, 1998, Majka *et al.*, 2002). An analysis of the expression patterns of TNF alpha in hypoxic retinae developing RNV detected it mostly in the outer nuclear layer in association with the expression of MMPs (Majka *et al.*, 2002). A recent study reported that TNF-alpha is an essential factor for

progressive breakdown of the BRB. Apoptosis of endothelial cells, pericytes and neurons in diabetic mouse retinae were significantly reduced in the absence of TNF-alpha and leukostasis was also largely reduced (Huang *et al.*, 2011). It has been reported that VEGF-related changes in vascular permeability depend on TNF-alpha (Clauss *et al.*, 2001).

1.2.3.10 Plasminogen activator inhibitor

Plasminogen activator inhibitor 1 (PAI-1) is a member of the serine proteinase inhibitor family. PAI-1 has been reported to be involved in angiogenesis (Balsara *et al.*, 2006, Lambert *et al.*, 2003, McMahon *et al.*, 2001) while PAI-2 is a fibrinolytic factor which is associated with endothelial cell proliferation and migration (Irigoyen et al., 1999). Since PAI-1 was also implicated in the early pathogenesis of DR (Grant *et al.*, 1991) it was considered as a possible inhibitor of angiogenesis (Penn *et al.*, 2003). Recent research on diabetic endothelial progenitor cells found that the cytostatic activity of TGF-beta1 requires PAI-1. It was reported by the same group that elimination of PAI-1 in a knockdown model resulted in TGF-beta1 induced inhibition of neovascularisation (Grant *et al.*, 2010). Currently strategies are being evaluated to establish whether blocking PAI-1 would protect endothelial cells in diabetes (Basu et al., 2009, Grant et al., 2010).

1.2.3.11 Oxidative Stress, Superoxide and Peroxynitrite

One of the critical factors in the development of DR is oxidative stress (Kowluru et al., 2001). Oxidative stress leads to ROS formation, glucose auto-oxidation, the activation of the polyol pathway, prostanoid production and eventually the formation of advanced glycation endproducts (Brownlee, 2001, Caldwell *et al.*, 2003). Oxidative stress is a trigger for increased VEGF expression, since the latter can be inhibited by antioxidants (Ellis et al., 1998, Ellis et al., 2000, Kuroki et al., 1996, Lu et al., 1998).

Hyperglycaemia induces superoxide (O_2^-) overproduction via NADPH or NADH (Mohazzab et al., 1994, Rajagopalan et al., 1996) in the mitochondrial respiratory chain (Caldwell *et al.*, 2003). It also induces the production of prostenoid mediators of inflammation by retinal vascular endothelial cells *via* the cyclooxygenase (COX)-

pathway (Sone et al., 1999, Sone et al., 1996). Another source of (O_2^-) is the uncoupling of eNOS which occurs in diabetes, leading to increased production of (O_2^-) instead of NO only (Bauersachs et al., 2005, Guzik et al., 2002, Hink et al., 2001). This will be discussed in more detail later. Hyperglycaemia also increases intracellular Calcium-ion (Ca^{2+}) concentration in endothelial cells, which in turn results in the activation of eNOS, thereby increasing NO and superoxide which promote the formation of peroxynitrite and consequently inactivation of NO (Graier et al., 1999, Graier et al., 1997).

Superoxide anions and peroxynitrate are described as reactive oxygen species (ROS). Peroxynitrate results from a chemical reaction of free NO with free superoxide anions ($NO + O_2^- \rightarrow ONO_2^-$). This highly reactive molecule is both a nitrating as well as an oxidising agent. It can cause protein modifications by oxidising protein-associated thiol groups or nitrating tyrosine residues (nitrotyrosine) (El-Remessy *et al.*, 2007, Halliwell, 1997). Peroxynitrate inhibits metabolic enzymes, causes lipid peroxidation, decreases proteolytic breakdown of proteasomes, reduces cellular antioxidants, induces DNA breaks and eventually apoptosis (Ellis *et al.*, 2002, Salgo *et al.*, 1995a, Şalgo *et al.*, 1995b, Zhuang *et al.*, 2000). Peroxynitrite can be generated in various cell types such as Müller cells, astrocytes, microglia, ECs and neurons (Leal *et al.*, 2007). The sites where the nitration of tyrosine occurs depends on the site of superoxide production since superoxide cannot cross cell membranes (Espey et al., 2000). ECs and EPCs seem to be very susceptible to oxidative damage as they both have high levels of NO and superoxide – especially in DM (Leal *et al.*, 2007). It has been reported that peroxynitrite can mimic VEGF action by phosphorylating the VEGFR2 thus stimulating endothelial cell growth and new blood vessel growth (El-Remessy *et al.*, 2007). Superoxide is usually neutralised by superoxide dismutase (McCord *et al.*, 1969), a highly efficient enzyme converting superoxide. However, the formation of peroxynitrite is about three times faster than this enzymatic dismutation of superoxide (Beckman et al., 1996).

The importance of ROS in the development of RNV in diabetes was reported by studies in animals models (mice and rats) of DR where inhibitors of ROS could prevent the establishment of diabetic changes (Du et al., 2002, El-Remessy et al., 2007, El-Remessy et al., 2003, Kowluru et al., 2000) and inhibit the expression of VEGF (Ando et al., 2002, Brooks et al., 2001). Other studies linked microvascular

complications of DM to peroxynitrite found in the plasma of diabetic patients and diabetic rats (Ceriello et al., 2001, El-Remessy et al., 2003, Kossenjans et al., 2000). ROS and peroxynitrite were increased in diabetic rat retinae (Carmo et al., 2000a, Du et al., 2002, Kowluru et al., 2000) in association with increased leukocyte adhesion and BRB breakdown (El-Remessy *et al.*, 2003, Joussen *et al.*, 2002b, Sugawara *et al.*, 2004). As the formation of ROS leads to reduction of NO, reduced vasodilation and bioavailability of NO for endothelial cells result in an overall deterioration of blood supply and hence oxygenation, further accelerating the cycle of hypoxia induced changes in DR (Graier et al., 1999, Graier et al., 1997).

One of the driving components for VEGF expression in endothelial cells exposed to oxidative stress and peroxynitrite formation is the signal transducer and activator of transcription 3 (STAT3), a transcription factor, whose binding sites are located within the VEGF promoter. It plays a pivotal role in heart and tumour angiogenesis by inducing VEGF expression (Platt et al., 2003). In small vessels and especially retinal microvascular endothelial cells, VEGF itself can induce STAT3 and hence stimulate its own production in an autocrine manner, whereas larger vessels such as the aorta are unaffected (Bartoli et al., 2000, Bartoli et al., 2003).

1.2.3.12 Advanced Glycation End products

Advanced glycation end products (AGE) are proteins or lipids that are nonenzymatically glycated and oxidized by aldose sugars (Schmidt et al., 1994, Singh et al., 2001). AGEs play an important role in the development of diabetic complications and were reported to accelerate diabetes related atherosclerosis (Goldin *et al.*, 2006, Jandeleit-Dahm *et al.*, 2008). Some of the pathways and pathophysiological processes they are involved in are discussed in the following sections.

The formation of AGEs is a multistep process involving several factors. Oxidative stress is a major contributor to the formation of AGEs (Brownlee, 2001, Caldwell *et al.*, 2003). AGEs are formed in a diabetic environment as a consequence of long-term hyperglycaemia (Csiszar et al., 2008, Schleicher et al., 2007, Unoki et al., 2008) and hence are found in diabetic patients (Ahmed *et al.*, 2005, Nogueira-Machado *et al.*, 2008). There are two steps in the formation of AGEs . The first step after glycation

and oxidation is the formation of a Schiff-base or an Amadori product. If glycation continues, AGEs are formed in a second step called the Maillard reaction, (Schmidt *et al.*, 1994). AGEs can fluoresce, they are able to produce reactive oxygen species thereby itself increasing oxidative stress (Yan et al., 1994), they can bind to molecules on the cell surface and form cross-links between other molecules (Brownlee *et al.*, 1985, Schmidt *et al.*, 1994).

AGEs can also exert effects by binding to their specific receptor (RAGE), a 35kD transmembrane receptor of the immunoglobulin super family (Neeper *et al.*, 1992, Schmidt *et al.*, 1992a). Under physiologic conditions, RAGE is expressed in very low levels and found in regular tissue and quiescent blood vessels (Schmidt et al., 2001). The accumulation of AGEs in DM upregulates RAGE in endothelial cells, smooth muscle cells and mononuclear phagocytes found in diabetic blood vessels (Schmidt et al., 2000, Schmidt et al., 2001).

It is of importance that both NFkappaB and IL6 are located within the RAGE promoter (Li et al., 1997). Activation of RAGE is linked to an inflammatory response (Li et al., 1997) via NFkappaB (Tikellis et al., 2008) and to induction of oxidative stress (Su et al., 2008). The location of NFkappaB and IL6 genes explains why activation of RAGE by AGE causes upregulation of both NFkappaB and IL6 and their respective target genes (Goldin *et al.*, 2006) such as VCAM-1, Endothelin-1, ICAM-1, E-selection, VEGF, IL1alpha, IL6, TNFalpha and RAGE itself (Basta et al., 2004, Neumann et al., 1999). Moreover, AGEs were also reported to upregulate COX2 which, in conjunction with NFkappaB, is associated with apoptosis and inflammation (Kern *et al.*, 2007, Su *et al.*, 2008). The relation of RAGE-related activation with NFkappaB was demonstrated when blocking RAGE with IgG prevented NFkappaB activation (Schmidt et al., 1999).

In summary, AGEs increase the risk of developing diabetic microvascular complications (Negrean et al., 2007) by: 1) a receptor independent intrinsic action such as crosslinking of key molecules and 2) a receptor dependent activity through AGE-RAGE interaction with intracellular signalling (Scheubel et al., 2006).

Ultimately, AGE formation is regarded as one of the main mechanism for vascular damage in patients with diabetes (Brownlee, 2000). A study in diabetic patients reported that the levels of AGEs were inversely correlated with the degree of endothelium dependent and endothelium independent vasodilation (Rojas et al., 2000). Thickening of the vascular basement membrane, a prominent feature of DR is

a result of major modifications of its constituent proteins by AGE crosslinking (Gardiner et al., 2003, Grant et al., 2004). Crosslinking of collagen I and elastin results in increased stiffness of vessels (Corman et al., 1998, Kass et al., 2001). Another factor that contributes directly to basement membrane thickening is an AGE/RAGE interaction that increases extracellular matrix deposition (Thallas-Bonke et al., 2004). Glycation itself results in increased synthesis of collagen III, alpha3collagen IV, collagen V, laminin and fibronectin via a TGFbeta intermediate (Kushiro et al., 1998, Makino et al., 1996, Striker et al., 1996, Throckmorton et al., 1995). Furthermore the glycation of laminin and collagen I / collagen IV inhibits the adhesion of endothelial cells (Haitoglou et al., 1992, Paul et al., 1999). The basement membrane modifications may result in reduced EPC attachment and incorporation in sites with vascular damage, thus impairing repair of damaged endothelium and facilitating the development of acellular capillaries (Heissig et al., 2003).

AGEs can also be formed within endothelial cells. In association with glycation of fibroblast growth factor (FGF), this can reduce the mitogenic activity of endothelial cells by 70% (Giardino et al., 1994). It was further reported that the functional impairment of mature ECs by AGEs (Xu *et al.*, 2003a) occurs mainly though the interaction with RAGE (Goldin et al., 2006).

Apart from effects on the endothelium, AGEs also affect EPCs. *In vitro* studies reported that endothelial progenitor cells are decreased in numbers by treatment with AGEs (Chen *et al.*, 2009a, Scheubel *et al.*, 2006, Sun *et al.*, 2009a). AGEs can induce EPC dysfunction by activation of p38 and ERK1/2 mitogen activated protein kinase (MAPK) (Sun *et al.*, 2009a). Furthermore, AGEs were able to increase EPC apoptosis in a time- and dose-dependent manner (Scheubel *et al.*, 2006, Shen *et al.*, 2009, Yan *et al.*, 1994) – although no direct effects on eNOS function and proliferation could be observed (Chen *et al.*, 2009a).

AGEs can also inhibit EPC maturation and integration into sprouting endothelium (Scheubel et al., 2006). These effects may also be mediated by RAGE expression and activation of p38 and MAPK pathways which eventually leads to a reduction of available NO (Sun *et al.*, 2009a). Experimental inhibition of MAPK was reported to reverse the increase in apoptosis and partially restore NO production in human EPCs isolated from the peripheral blood (Shen et al., 2009).

Effects of AGEs include increased monocyte migration across the endothelium (Edelstein et al., 1992) and if AGEs activate RAGE, endothelial permeability is

increased (Bierhaus et al., 1997). Also the bioavailability of NO is reduced by AGEs, which itself inhibits leukocyte adhesion, smooth muscle cell growth and platelet adhesion (Bucala et al., 1991).

Since AGEs are an important factor in DR development and atherosclerosis, a brief overview of possible therapeutic approaches in reducing AGEs is given. As it became clear that AGEs play a pivotal role in the pathogenesis of DR and atherosclerosis, several inhibitors of AGE formation such as aminoguanidine were studied. Aminoguanidine is a hydrazine compound that prevents the formation of AGE (Yan et al., 1994). It reacts with derivatives of early glycation products that are not yet bound on proteins like 3-deoxyglucosone (Yan et al., 1994). Aminoguanidine attenuates the effects of diabetes on large arteries, increases aortic elasticity, improves left ventricular afterload and also improves static compliance of the heart (Corman et al., 1998). Furthermore it also reduced extracellular matrix accumulation of fibronectin and laminin in a streptozotocin (Rerup, 1970) induced diabetes model in rats (Thallas-Bonke et al., 2004). Additionally, a decrease in vascular AGE accumulation and plaque severity in the STZ mice treated with aminoguanidine has been reported (Forbes et al., 2004). Unfortunately aminoguanidine is also a inhibitor of the nitric oxide synthase which may offset some of its benefits (Bucala et al., 1991, Wilkinson-Berka et al., 2002). Aminoguanidine was reported to prevent the development of diabetic retinopathy in dogs (Kern *et al.*, 2001).

A different substance, N-(2-Acetamidoethyl)hydrozinecarboximidamid-hydrochlorid (ALT-946) has also been reported to be an effective inhibitor of AGE-induced crosslinks (Forbes et al., 2001) but the NOS inhibition is less than observed using aminoguanidine (Forbes et al., 2001).

Trapping carbonyl intermediates and hence preventing the alteration of nucleophilic residues is achieved using (±)-2-isopropylidenehydrazono-4-oxothiazolidin-5-ylacetanidide (OPB-9195) (Kass, 2003). Animal studies using OPB9195 reported a decreased glycated albumin in rats and increase the excretion of NO and increase the expression of eNOS mRNA compared with control rats (Mizutani et al., 2002).

Breaking crosslinks of AGEs by breaking carbon bonds between carbonyls is achieved by 4,5-Dimethyl-3-phenacylthiozolium-chloride (ALT-711) (Kass, 2003, Kass et al., 2001). Diabetic rats treated with ALT-711 for a period of 4 months had increased collagen III solubility, reduced RAGE and AGE-R3 RNA compared to

controls (Candido et al., 2003). A clinical trial in humans reported that ALT-711 reduced the arterial pulse amplitude and improved the arterial compliance (Kass et al., 2001).

Cerivastatin, a 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitor (statin), known also as Lipobay®, prevents AGE stimulated increases in VEGF, NFkappaB and also prevents AGE-induced angiogenesis by interfering with the intracellular AGE pathway (Okamoto et al., 2002). Due to reports of fatal rhabdomyolysis, Cerivastatin was withdrawn from the market in 2001 (Furberg *et al.*, 2001).

Other drugs such as ACE inhibitors and AT2-blockers, clinically used to control blood pressure, were reported to reduce the carbonyl precursors (Miyata et al., 2002) and Ramipril (ACE inhibitor) was reported to attenuate AGE accumulation in diabetic animals (Forbes et al., 2002).

The soluble form of RAGE (sRAGE) is an extracellular ligand-binding domain of RAGE that is able to block AGE from binding to RAGE (Wautier et al., 1996). In doing so, sRAGE suppresses atherosclerotic plaque formation and decreases vascular permeability (Goova et al., 2001, Park et al., 1998, Wautier et al., 1996). In mice it was reported to decrease plaque formation in blood vessels (Schmidt et al., 2001). On the other hand, studies in diabetic humans showed that sRAGE is increased in DM in association with an increased levels of inflammatory cytokines (Nakamura *et al.*, 2007).

One of the first drugs used in the treatment of DM that also showed beneficial effects in reducing AGEs and protecting endothelial cells was rosiglitazone, a thiazolidinedione that acts as an insulin sensitiser (Liang et al., 2009). Studies reported that rosiglitazone, as an agonist for the peroxisome proliferator-activated receptor gamma (PPARgamma) increased the number, proliferative and migratory potential of cultured EPCs from the PB (Liang *et al.*, 2009, Pistrosch *et al.*, 2005), improved differentiation of EPCs from the bone-marrow (Wang *et al.*, 2004b), reduced NADPH oxidase activity (Sorrentino *et al.*, 2007) and additionally prevented the H_2O_2 induced apoptosis of EPCs in a PI3K dependent but NO independent manner (Gensch et al., 2007b). Rosiglitazone was also reported to increase AKT and eNOS phosphorylation thus leading to an increased production of NO (Liang *et al.*, 2009). This temporary improvement of EPC numbers and migratory potential was independent of glycaemic control (Pistrosch et al., 2005, Wang et al., 2006a).

Another effect of rosiglitazone on EPC function is *via* influencing C-reactive protein (CRP). CRP was reported to increase EPC apoptosis, alter the antioxidant defences and decrease the ability to secret atherogenic chemokines (Fujii et al., 2006, Suh et al., 2004, Verma et al., 2004). Rosiglitazone can attenuate these effects of CRP on EPC function (Verma et al., 2004). Though promising results, such as increased EPC numbers and migration potential in patients with atherosclerosis and diabetes type 2 (Werner et al., 2007), have been reported with rosiglitazone, another clinical study found it did not delay the progression of DR (Shen *et al.*, 2008). Rosiglitazone should be used with caution since it increases the risk of myocardial infarction (Nissen et al., 2007).

Pyridoxamine, which is converted to the biologically active form of vitamin B6, pyridoxal 5-phosphate, was reported to be able to suppress the formation of AGEs at three different levels: by preventing the degradation of protein-Amadori products to protein-AGEs (Voziyan et al., 2003), by reducing hyperlipidaemia and hence AGE formation (Alderson et al., 2004, Degenhardt et al., 2002, Stitt et al., 2002) and finally by scavenging carbonyl by-products of glucose and lipid degradation (Alderson et al., 2003).

Another vitamin-like substance, Benfotiamin – a derivative of vitamin B1 – was reported to reduce AGE formation by inhibiting parts of the pathway leading to AGE formation (Hammes et al., 2003, Pomero et al., 2001). As just outlined, a reduction or normalisation of glucose and lipids seem to be an integral part of reducing AGEs and not surprisingly, a reduction of both glucose and lipids through a special diet result in decreased AGEs (Koschinsky et al., 1997).

1.2.3.13 Sorbitol pathway

Under hyperglycaemic conditions increased influx of glucose into the neurons of the retina occurs by an insulin-independent pathway (Brownlee, 2001, Van den Enden *et al.*, 1995). Inside the neuron, aldose reductase converts glucose to sorbitol thereby oxidising NADPH to $NADP^+$. Oxidation of sorbitol to fructose by the sorbitol dehydrogenase in turn increases the cytosolic $NADH/NAD^+$ ratio. This increased cytosolic $NADH/NAD^+$ quotient leads to increased superoxide production which is called "hyperglycaemia induced pseudohypoxia" (Ellis *et al.*, 2005a, Williamson *et*

al., 1993). This inhibits the glyceraldehyde-3-phosphatasedehydrogenase (GAPDH), hence increasing triose phosphate which in turn increases methylglyoxal, an AGE precursor, and also increases diacylglycerol (DAG) which subsequently activates PKC. This is the link between the sorbitol pathway and the DAG-PKC activation (Brownlee, 2001). The sorbitol pathway hence increases oxidative stress and is responsible for AGE formation and PKC activation. Early diabetic changes seen in the ERG of rats (prolongation of peak latencies and amplitude reduction of the b-wave) can be reversed by drugs such as aldose reductase inhibitors, insulin and acetyl-l-carnitine (Hotta *et al.*, 1996a, Hotta *et al.*, 1996b, Lowitt *et al.*, 1993, Segawa *et al.*, 1988).

1.2.3.14 Focal adhesion kinase

Focal adhesion kinase (FAK), also known as protein tyrosine kinase 2 (PTK2) is a protein involved in cell adhesion and cell movements. Activation of FAK is associated with migration and improved survival of several different cell types; detachment of cells from the extracellular matrix results in dephosphorylation of FAK, then apoptosis (Schaller, 2001). Migration of cells involves cyclic attachment and detachment to the ECM. Binding of VEGF to VEGFR2 in EPCs activates FAK, (Maru et al., 2001, Rousseau et al., 2000) and the additional VEGF-induced activation of SRC leads to a physiological interaction of FAK and the integrin alpha(V)beta5 (Eliceiri et al., 2002). Thus FAK is considered to be the link between integrin and growth factor mediated signalling pathways, (Grant *et al.*, 2004). IGF-1 promotes cell migration and survival by increasing PI3K/AKT activity (Wilson et al., 2001). It can also alter the status of FAK activity by phosphorylation and dephosphorylation through an insulin-dependent mechanism (Casamassima et al., 1998, Kim et al., 1998, Manes et al., 1999).

1.2.3.15 Angiopoietin and its receptors

A group of molecules that have recently attracted more attention with respect to the development of DR are angiopoietins. These are growth factors that promote angiogenesis and are involved in vessel remodelling, i.e. they are involved in the

process where endothelial cells form lumina and communicate with their environment to form a network (Thurston, 2003). Angiopoietins bind to Tie receptors (Tie1 and Tie2; tyrosine kinases that contains immunoglobulin-like and epidermal growth factor-like domains) (Grant *et al.*, 2004, Thurston, 2003).

So far four angiopoietins have been described: Ang-1 (Davis *et al.*, 1996), Ang-2 (Maisonpierre *et al.*, 1997), Ang-3 and Ang-4 (Valenzuela *et al.*, 1999). It is thought that Ang-1 and Ang-4 are agonists, whereas Ang-2 and Ang-3 are naturally occurring antagonists for Tie2 (Thurston, 2003). Although the mechanisms of action and downstream signalling of Tie receptors is not yet fully understood, it is hypothesized that Ang-2 is a destabilizing factor that is required to initiate angiogenic remodelling: in the presence of VEGF, Ang-2 stimulated ECs to undergo angiogenic steps (Maisonpierre et al., 1997). An increase in Ang-2 in the developing retina and in neovascular tissue has been described (Das et al., 2003). Ang-2, which is found in the inner nuclear layer of the retina, was reported to follow a similar temporal expression pattern as VEGF during neovascularisation. Since Ang-2 stimulates MMP9 expression (Das et al., 2003) it may be involved in EPC mobilisation.

ECs have the Tie2 receptor that normally couples with the Ang-1 protein exposed by the supporting cells (Davis et al., 1996, Suri et al., 1996). In mice deficient for Ang-1 ECs fail to correctly associate with their supporting tissue (Grant et al., 2004). Ang-1 was reported to have anti-inflammatory and vasoprotective effects in DM (Joussen *et al.*, 2002c): the vessels of mice overexpressing Ang-1 are resistant to the development of leakage.

Since Ang-2 is an antagonist to Ang-1, mice overexpressing Ang-2 have a phenotype similar to Ang-1 deficient mice (Maisonpierre et al., 1997). They have increased retinal vascular pathology, suggesting a critical role of Ang-2 in the development of DR *via* destabilisation of pericytes (Pfister *et al.*, 2010). Other data point to a pericyte recruitment function of Ang-2 and a role as a modulator of intraretinal and preretinal vessel formation both under physiological and pathological conditions (Feng *et al.*, 2007). Experiments in diabetic rats (Hammes *et al.*, 2004) reported a 2.5 fold upregulation of Ang-1 and a 30 fold upregulation of Ang-2 in diabetic retinae which preceded the loss of pericytes. These authors further reported that knockout of Ang-2 (heterozygous animals) resulted in reduced pericyte loss and fewer acellular capillaries whereas an intravitreal injection of Ang-2 lead to a dose dependant loss of pericytes. Ang-2 was reported to be upregulated in diabetic rats

after fewer than 8 weeks of diabetes (Rangasamy *et al.*, 2011) in association with decreased VE-Cadherin function and increased vascular permeability, suggesting a central role for Ang-2 in increased vasopermeability in DR (Rangasamy *et al.*, 2011).

1.2.3.16 Erythropoietin

Erythropoietin (EPO) is a glycoprotein that is produced by the liver during fetal life and by the kidney in the adult human (Lin *et al.*, 1985). Under normal conditions, EPO is found in the blood at a concentration of 1-7 pmol/L (Lin *et al.*, 1985, McVicar *et al.*, 2010). Hypoxia is the main trigger for an upregulation of EPO, causing levels to increase up to 100 fold (McVicar et al., 2010). The action of EPO is exerted *via* its receptor (EPO-R) which is in some cases co-activated with the beta-common receptor (BCR) that requires the expression of VEGFR2 for downstream signalling (Brines *et al.*, 2004, Sautina *et al.*, 2010). The expression of EPO is regulated *via* hypoxia inducible factor 1 alpha (HIF1alpha) (Siren et al., 2001) which in turn is upregulated by hypoxia, i.e., EPO is a target gene of HIF1alpha (Bernaudin et al., 2002, Cai et al., 2003, Grimm et al., 2002, Ruscher et al., 2002).

EPO acts in a paracrine and autocrine way and promotes tissue survival in ischaemic conditions and also in toxic or traumatic tissue damage (Gassmann et al., 2003). Neuroprotective properties of EPO were reported in mice where EPO administration reduced ischaemia induced neurodegeneration by preventing apoptosis of neurons and glia (Kilic *et al.*, 2005, Li *et al.*, 2004). In the eye it was reported that exogenous EPO has neuroprotective effects on the retina by preventing apoptosis caused by a variety of retinal injuries (Ghezzi et al., 2004, Grimm et al., 2005, Grimm et al., 2002, Kilic et al., 2005, Rex et al., 2004, Tsai et al., 2005).

In DM it was reported that HIF1alpha levels (Arjamaa et al., 2006) and EPO levels are increased in the vitreous of patients with proliferative DR compared with patients with minimal or no DR (Asensio-Sanchez et al., 2008, Watanabe et al., 2005). Similarly, high EPO levels were reported in patients with diabetic macular oedema but without significant ischaemia (Hernandez et al., 2006) suggesting that in these patients EPO levels may not be solely increased by ischaemia but by additional mechanisms.

Histological studies on proliferative membranes from patients with PDR showed high expression of $CD34^{+}$, HIF1alpha and VEGF but not EPO (Abu El-Asrar et al., 2007). Based on these findings it was suggested that EPO is not directly involved in the development of neovascularisation in PDR. The EPO/EPO-receptor system may not work properly in proliferative stages of DR (Zhang et al., 2008), possibly becoming disconnected from the normal pathways (Gewirtz et al., 2006), thus its neuroprotective effects might be absent.

Hence, external supplementation with EPO could be neuroprotective in DR. In diabetic rats EPO doses of 500-2000mU/mL could successfully suppress VEGF at mRNA and protein level in the diabetic retina by a negative feedback loop via HIF1alpha (Xu et al., 2009). Other studies in diabetic rats showed that a single EPO injection at the onset of diabetes could prevent death of retinal neurons and support the integrity of the blood-retinal-barrier (Zhang et al., 2008).

These observations led to the use of EPO to treat recalcitrant diabetic macular oedema. In a clinical trial where rHuEPO, which is approved by FDA for treatment of anaemia (Flaharty et al., 1989), was used to treat patients with chronic diabetic macular oedema (Li et al., 2010), study subjects received 5IU /50µl of rHuEPO intravitreally in 3 doses each 6 weeks apart with an additional follow up time of 6 weeks. Four out of 5 eyes had improved visual acuity, hard exudates were reduced but leakage as seen in the fluorescein angiogram was mainly unchanged - the improvement observed occurred within 1 week after the first injection (Li et al., 2010). None of the patients developed new neovascularisation.

1.3 Endothelium and Endothelial Progenitor Cells

As already mentioned, vascular changes occur in the very early stages of diabetes, setting off a cascade of changes that eventually result in the clinically visible signs of DR. This together with the fact that endothelial dysfunction is one of the first steps in the pathogenesis of extraocular diabetic vascular complications, including the development of atherosclerosis (Feener *et al.*, 1997), emphasises the necessity to study the effects of diabetes on the vascular endothelium. The following sections will give an outline of these changes, focusing on endothelial progenitor cells – the main subject of this thesis.

1.3.1. Introduction to the endothelium and endothelial repair

The vascular endothelium, the inner lining of blood vessels, comprises a single layer of endothelial cells (EC) (Cines *et al.*, 1998) that form a barrier between the blood and the surrounding tissue. In an average adult human of 70kg bodyweight, it is estimated that the endothelium covers an area approximately between 1000m^2 (Jaffe, 1987) and 10000 m^2 (Augustin *et al.*, 1994). It consists of approximately 1-6 x10^{13} endothelial cells with a weight of ~1kg (Augustin *et al.*, 1994).

The lack of endothelial fenestrations and presence of many tight and adherens junctions between the endothelial cells of the retinal vasculature formed by occludin (Morcos *et al.*, 2001), claudin (Morcos *et al.*, 2001), adhesion proteins (Martin-Padura *et al.*, 1998) and other associated proteins, e.g. *zonula occludens* proteins (Stevenson *et al.*, 1986), suggests the endothelium is the anatomic site of the BRB (Patton *et al.*, 2005).

Generally, the endothelium prevents inflammatory cell infiltration, modulates vascular tone and controls smooth muscle cell proliferation (Gimbrone *et al.*, 1997, Gimbrone *et al.*, 2000, Traub *et al.*, 1998). The ECs also produce a variety of hematopoietic cytokines that play an important role in maintaining the properties of stem cells as well as in regulating stem cell proliferation and differentiation (Hu et al., 2006, Kopp et al., 2005).

ECs depend on an underlying surface in which they can anchor, the extracellular matrix (ECM), as well as growth factors for survival, growth and differentiation. Many of the adhesive contacts with the vascular basement membrane are made through integrins: heterodimeric transmembrane receptor molecules formed by ion-dependent non-covalent binding of an alpha and a beta transmembrane glycoprotein subunit (Frisch et al., 1997). The extracellular domains bind to fibronectin, collagen and vitronectin. The integrins are hence the major receptors for the binding of ECs to the ECM (Grant et al., 2004).

Damage to the endothelium can be repaired by proliferation and migration of nearby mature endothelial cells (Hristov *et al.*, 2007, Miller-Kasprzak *et al.*, 2007). However, the regenerative capacity of these nearby resident ECs is limited (Ballard *et al.*, 2007, Werner *et al.*, 2006). Endothelial progenitor cells (Asahara *et al.*, 1997)

originating from the bone marrow (BM) (Asahara *et al.*, 1997) can migrate to the peripheral blood (Asahara *et al.*, 1997, Werner *et al.*, 2005) and repair injured endothelium (Asahara *et al.*, 1999a, Rookmaaker *et al.*, 2002, Walter *et al.*, 2002, Werner *et al.*, 2002). These EPCs play an important role in regenerating the endothelium through migration, proliferation, differentiation and by secreting pro-angiogenic cytokines (Fadini *et al.*, 2006c, Mukai *et al.*, 2008).

1.3.2. Endothelial Progenitor Cells

1.3.2.1 Overview of research development on EPCs

The presence of a progenitor cell population in the bone marrow that contributed to the formation of endothelial cells was suggested in 1979 (Dexter, 1979). It was further postulated that within the developing embryo pluripotent progenitor cells must exist that are capable of blood and blood vessel formation and hence are responsible for vasculogenesis (Beck et al., 1997, Choi, 1998, Noden, 1989, Takakura et al., 2000).

Later research postulated that these embryonic stem cells contribute to vasculogenesis (formation of new blood vessels from angioblasts) and angiogenesis (formation of blood vessels from existing vessels) (Risau *et al.*, 1988). But it was in 1997 that these cells, the EPCs, were first isolated and described (Asahara *et al.*, 1997). This seminal work by Asahara including the description of methods to isolate EPCs sparked an exponential growth of research on EPCs (Figure 1).

Until circulating EPCs were discovered it was believed that angiogenesis was the result of migration and *in situ* differentiation of mature ECs (Liew *et al.*, 2008). The discovery of circulating EPCs overthrew this dogma as well as the belief that vasculogenesis only occurs *in utero*. It was reported that circulating EPCs can promote both postnatal vasculogenesis and angiogenesis (Grant *et al.*, 2002, Isner *et al.*, 2001, Luttun *et al.*, 2002, Rafii *et al.*, 2002, Ruzinova *et al.*, 2003).

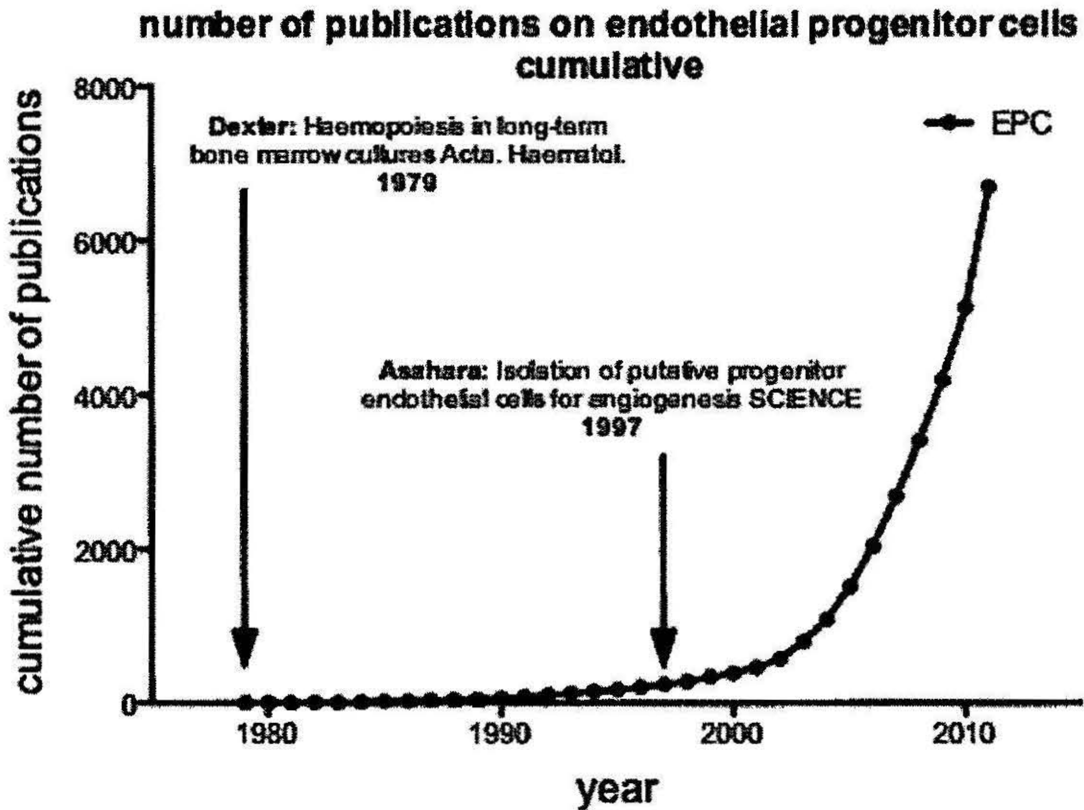

Figure 1: Number of articles published on EPCs cumulative / per year from 1979 to 2011. This graph was produced by the author of this thesis, Daniel Barthelmes. Data were acquired by searching the PubMed database.

By May 2012, more than 12000 articles could be found in PubMed using the search term "endothelial progenitor cell". In 2011 alone more than 4 scientific articles on EPCs were published per day - which illustrates the enormous interest this special field of research has attracted. This vast amount of scientific literature implies that the present review can cover only the fraction pertinent to the subject of the thesis.

1.3.2.2 Origins of EPCs

EPCs and hematopoietic stem cells (HSC) share a common putative precursor – the hemangioblast in the bone marrow (Asahara *et al.*, 1997, Asahara *et al.*, 1999b, Choi *et al.*, 1998, Flamme *et al.*, 1992, Rafii *et al.*, 1994, Risau *et al.*, 1995, Weiss *et al.*, 1996). Early EPCs and HSCs logically share a number of markers such as

VEGFR2, Tie2, cKIT, SCA1, CD133 and CD34 (Matthews *et al.*, 1991, Millauer *et al.*, 1993, Murasawa *et al.*, 2005, Yamaguchi *et al.*, 1993). To date no specific marker has been described to distinguish these very early EPCs and HSCs. However, soon after the different fate for EPCs and HSCs is established, VE-Cadherin, Tie2 and VEGFR2 disappear in HSCs and remain in EPCs (Murasawa et al., 2005). Other endothelial markers such as CD146 (Bardin *et al.*, 2001) and von Willebrand factor (vWF) appear (Fadini et al., 2008b, Urbich et al., 2004) as EPCs mature.

EPCs can be found in many tissues: bone marrow (BM) (Heeschen *et al.*, 2003, Reyes *et al.*, 2002, Shi *et al.*, 1998), peripheral blood (PB) (Asahara *et al.*, 1997, Ingram *et al.*, 2004, Rehman *et al.*, 2003, Rohde *et al.*, 2006), vessel wall between the media and adventitia layers (Alessandri *et al.*, 2001, Ingram *et al.*, 2005, Lin *et al.*, 2000, Zengin *et al.*, 2006), aortic root (Hu et al., 2004), cord-blood (Ingram *et al.*, 2004, Murohara, 2001, Murohara *et al.*, 2000) and spleen (Cherqui et al., 2006, Dimmeler et al., 2001). Interestingly, EPCs isolated from vessel walls have lower proliferative capacity compared with EPCs isolated from PB (Lin et al., 2000). Intestinal and hepatic tissue also have high levels of residing EPCs (Aicher et al., 2007) as well as adipose tissue (DiMuzio *et al.*, 2007, Miranville *et al.*, 2004, Sengenes *et al.*, 2007, Yoder *et al.*, 2007). It has been reported that monocytes in the PB can express endothelial lineage markers and may form tube-like structures under angiogenic conditions (Elsheikh et al., 2005, Schmeisser et al., 2001). $CD14^+$ monocytes may transdifferentiate into ECs (Hristov et al., 2003a).

A large proportion of circulating EPCs were reported to be of non-bone-marrow-origin (e.g. from the spleen) and to show properties similar to BM-EPCs (tube formation and surface marker expression) (Wassmann et al., 2006). Interestingly, splenectomy in animal models resulted in an increase of the circulation time of EPCs in PB suggesting that the spleen is one of the places where EPCs are removed (Wassmann et al., 2006).

1.3.2.3 Isolation of EPCs

Since EPCs can be found in various tissues, different isolation methods have been established, of which a brief overview is given here. Although the seminal paper by Asahara (Asahara *et al.*, 1997) described the isolation of EPCs from the peripheral

blood of humans using a magnetic bead isolation approach to select CD34$^+$ and VEGFR2$^+$ cells, other isolation techniques have been developed.

Generally two different methods are currently used: A) magnetic bead isolation using specific antibodies against EPC surface markers or B) gradient centrifugation combined with specialised growth media favouring the growth of EPCs. Both approaches are suitable for isolation of EPCs from the BM and the PB. As it is easier to obtain blood from a peripheral vein of a human compared to isolation of EPCs from the BM (human or animal), most studies on EPCs have been done using human PB. Table 1 gives an overview of the different techniques and materials used.

Table 1: Overview of different isolation techniques, materials and surface markers used to isolate EPCs from various species.

peripheral blood from humans		
Isolation method	*Surface Markers to characterise EPCs*	*Reference*
Magnetic beads (CD34 or KDR)	CD45, CD34, CD31, VEGFR2, Tie2 E-selectin, UEA-I, acLDL	(Asahara *et al.*, 1997)
density gradient centrifugation	acLDL-DiI, UEA1	(Bahlmann *et al.*, 2004)
density gradient centrifugation, then selection via medium and fibronectin-coating of culture dishes	acLDL-DiI, UEA-I, CD34, CD133, VE-Cadherin	(Balestrieri *et al.*, 2008c)
density gradient centrifugation, then selection via medium and fibronectin-coating of culture dishes	L-electin, CD14, CD34, CD133, CD62e, CD62P, Tie2, VEGFR2, VEGFR3, vWF	(Biancone *et al.*, 2004)
density gradient centrifugation, then selection via medium and fibronectin-coating of culture dishes	acLDL-DiI, UEA-I, VEGFR2, vWF, VE-Cadherin	(Chavakis *et al.*, 2005)
density gradient centrifugation, then selection via medium and fibronectin-coating of culture dishes	acLDL-DiI, UEA-I, VEGFR2, vWF, VE-Cadherin	(Chavakis *et al.*, 2007)
density gradient centrifugation, then selection via medium and fibronectin-coating of culture dishes	acLDL-DiI, UEA-I, VEGFR2, VE-Cadherin, CD34, CD133	(Chen *et al.*, 2004b)
density gradient centrifugation, then selection via medium and fibronectin-coating of culture dishes	acLDL-DiI, UEA-I, VEGFR2, VE-Cadherin, CD34, CD133	(Chen *et al.*, 2004a)
density gradient centrifugation, then selection via medium and fibronectin-coating of culture dishes	acLDL-DiI, UEA-I, VEGFR2, VE-Cadherin, CD34, CD133, PECAM1, CD31, vWF	(Chen *et al.*, 2007)
density gradient centrifugation, then selection via medium and fibronectin-coating of culture	UEA-I	(Choi *et al.*, 2004)

dishes		
density gradient centrifugation, then selection via medium and fibronectin-coating of culture dishes	acLDL-DiI, VEGFR2, eNOS	(Dernbach *et al.*, 2004)
density gradient centrifugation, then selection via medium and fibronectin-coating of culture dishes / apheresis	CD34-beads VE-Cadherin, VEGFR2, CD31, vWF,	(Dimmeler *et al.*, 2001)
density gradient centrifugation, magnetic bead isolation	CD34, CD133, acDiLDL, UEA-I, VEGFR2, CD14, CXCR4, CD31	(Friedrich *et al.*, 2006)
density gradient centrifugation, then selection via medium and fibronectin-coating of culture dishes	VEGFR2, Tie2, VE-Cadherin, CD14, CD31	(Gulati *et al.*, 2003)
density gradient centrifugation, then selection via medium and fibronectin-coating of culture dishes	vWF, VEGFR2, CD31	(He *et al.*, 2004a)
density gradient centrifugation, then selection via medium	acDiLDL, UEA-I, CD34, CD133, VEGFR2	(Heeschen *et al.*, 2003)
density gradient centrifugation, then selection via medium	acDiLDL, UEA-I, CD31 VEGFR2	(Imanishi *et al.*, 2003)
density gradient centrifugation, then selection via medium and fibronectin-coating of culture dishes	acDiLDL, UEA-I, CD31 VEGFR2	(Imanishi *et al.*, 2005)
density gradient centrifugation, then selection via medium and fibronectin-coating of culture dishes	CD31, VEGFR2, Tie2	(Ito *et al.*, 1999)
density gradient centrifugation, then selection via medium and fibronectin-coating of culture dishes	acDiLDL, UEA-I	(Iwaguro *et al.*, 2002)
density gradient centrifugation, then selection via medium and fibronectin-coating of culture dishes	acDiLDL, UEA-I, CD31, CD34, CD14, VE-Cadherin, VEGFR2, CD62E, CD51/61	(Kalka *et al.*, 2000a)
density gradient centrifugation, then selection via medium and fibronectin-coating of culture dishes	acDiLDL, UEA-I, VE-Cadherin, VEGFR2, CD83, CD3, CD31, CD34, CD51/61, CD19	(Kalka *et al.*, 2000b)
density gradient centrifugation, then selection via medium and fibronectin-coating of culture dishes	acDiLDL, UEA-I, VE-Cadherin, VEGFR2, CD62E, CD34, CD51/61, CD31	(Kalka *et al.*, 2000c)
density gradient centrifugation, then selection via medium	acDiLDL, CD31	(Kawamoto *et al.*, 2001)
density gradient centrifugation, then selection via medium and fibronectin-coating of culture dishes	acDiLDL, UEA-I	(Laufs *et al.*, 2004)
density gradient centrifugation, then selection via medium and collagenI-coating of culture dishes	CD14, CD31, CD34, CD45, CD133, VE-Cadherin, VEGFR2	(Lavoie *et al.*, 2010)
density gradient centrifugation, then magnetic beads CD133, then	VEGFR2, CD31, CD133	(Leshem-Lev *et al.*, 2010)

selection via medium and fibronectin coating of the culture dishes		
density gradient centrifugation, selection via medium and fibronectin coating of the culture dishes	acDiLDL, UEA-I, VEGFR2, CD34, CD133	(Liang *et al.*, 2009)
density gradient centrifugation, selection via medium and fibronectin coating of the culture dishes	VE-Cadherin, VEGFR2, anti-integrin, CD62E, CD3, CD68, CD31, CD34, CD19	(Llevadot *et al.*, 2001)
density gradient centrifugation, selection via medium and fibronectin coating of the culture dishes	UEA-I, acDiLDL, VEGFR2, CD31	(Marchetti *et al.*, 2006)
density gradient centrifugation, selection via medium and fibronectin coating of the culture dishes	UEA-I, acDiLDL	(Min *et al.*, 2004)
density gradient centrifugation, selection via medium and fibronectin coating of the culture dishes	CD31, VEGFR2, vWF, CD45,	(Mukai *et al.*, 2008)
density gradient centrifugation, selection via medium	acDiLDL	(Murasawa *et al.*, 2002)
density gradient centrifugation, selection via medium and fibronectin coating of the culture dishes	acDiLDL, UEA-I, VE-Cadherin, E-Selectin, CD11b, CD11c, CD14, CD45, CD133, cKIT, CD31, , CD34,	(Rehman *et al.*, 2003)
magnetic bead isolation then selection via medium and fibronectin coating of the culture dishes	CD34, CD133, CD31, CD146,	(Rosso *et al.*, 2006)
gradient centrifugation, selection via medium and fibronectin coating of the culture dishes	vWF, VEGFR2, acDiLDL,	(Schuh *et al.*, 2008)
gradient centrifugation, selection via medium and fibronectin coating of the culture dishes	CD34, CD133, VEGFR2, acDiLDL, UEA-I	(Shen *et al.*, 2009)
gradient centrifugation, selection via medium and fibronectin coating of the culture dishes	CD14, CD31, VEGFR2, vWF	(Sorrentino *et al.*, 2007)
gradient centrifugation, selection via medium and fibronectin coating of the culture dishes	acDiLDL, UEA-I	(Strehlow *et al.*, 2003)
gradient centrifugation, selection via medium and gelatine coating of the culture dishes	acDiLDL, UEA-I, vWF, VE-Cadherin, VEGFR2, CD34, CD133,	(Suh *et al.*, 2004)
gradient centrifugation, selection via medium and fibronectin coating of the culture dishes	acDiLDL, UEA-I, VEGFR2, CD34, CD133,	(Sun *et al.*, 2009a)
gradient centrifugation, selection via medium and gelatine coating of the culture dishes	vWF, VEGFR2, CD34, CD31, acDiLDL	(Tang *et al.*, 2008)
gradient centrifugation, selection via medium and fibronectin coating of the culture dishes	VE-Cadherin, CD34, CD31, VEGFR2, P1H12, acDiLDL, UEA-I	(Tepper *et al.*, 2002)
gradient centrifugation, selection via medium and fibronectin coating	acDiLDL, UEA-I, vWF	(Thum *et al.*, 2007)

of the culture dishes		
gradient centrifugation, selection via medium and collagen I coating of the culture dishes	CD45, CD14, CD31, Tie2, vWF, VEGFR2,	(Togliatto *et al.*, 2010)
gradient centrifugation, selection via medium and fibronectin coating of the culture dishes	acDiLDL, UEA-I, VEGFR2, VE-Cadherin, vWF, eNOS	(Urbich *et al.*, 2005a)
gradient centrifugation, selection via medium and fibronectin coating of the culture dishes	acDiLDL, UEA-I, VEGFR2, vWF, CD105, CD45, CD14	(Urbich *et al.*, 2003)
gradient centrifugation, selection via medium and fibronectin coating of the culture dishes	CD49E, CD29, CD51/61, CD106, CD68, CD31, P1H12, CD133	(Walter *et al.*, 2002)
gradient centrifugation, selection via medium and fibronectin coating of the culture dishes	cKIT, CD31, SCA1, VEGFR2, acDiLDL, UEA-I	(Wang *et al.*, 2006b)
gradient centrifugation, selection via medium and fibronectin coating of the culture dishes	acDiLDL, UEA-I, CD34, VEGFR2	(Werner *et al.*, 2007)
gradient centrifugation, selection via medium and fibronectin coating of the culture dishes	acDiLDL, UEA-I, CD34, VEGFR2, VE-Cadherin, CD133, CD45	(Xia *et al.*, 2008)
gradient centrifugation, selection via medium	acDiLDL, UEA-I, CD14, CD31, CD45, CD105, CD144, CD146, VEGFR2, CD115, vWF	(Yoder *et al.*, 2007)
gradient centrifugation, selection via medium and fibronectin coating of the culture dishes	CD34, CD14, VEGFR2, VE-Cadherin, E-Selectin, vWF	(Zhang *et al.*, 2006)
gradient centrifugation, selection via medium and fibronectin coating of the culture dishes	acDiLDL, UEA-I, VEGFR2, CD34, CD133	(Zhu *et al.*, 2004)
	bone marrow from humans	
density gradient centrifugation, then selection via medium	acDiLDL, UEA-I, CD34, CD133, VEGFR2	(Heeschen *et al.*, 2003)
	peripheral blood from mice	
staining of whole blood	SCA1 / VEGFR2	(Balestrieri *et al.*, 2008a)
density gradient centrifugation, selection via medium and fibronectin coating of the culture dishes	UEA-I, acDiLDL	(Llevadot *et al.*, 2001)
gradient centrifugation	VEGFR2, VE-Cadherin, CD34, acDiLDL, UEA-I	(Asahara *et al.*, 1999b)
gradient centrifugation	acDiLDL, UEA-I, SCA1, VEGFR2,	(Iwakura *et al.*, 2003)
	bone marrow from mice	
magnetic bead	$SCA1^{+}$ (Ly-6A/E^{+})	(Awad *et al.*, 2005)
magnetic beads	SCA1, LIN cocktail, VEGFR2	(Chavakis *et al.*, 2005)
selection via medium and fibronectin coating of the culture dishes	acDiLDL, UEA-I	(Wang *et al.*, 2008)
gradient centrifugation, selection via medium	acDiLDL, UEA-I, VEGFR2	(Yoon *et al.*, 2006)
gradient centrifugation and magnetic bead separation	Lin^{-} SCA1	(Otani *et al.*, 2002)
	spleen from mice	
mechanical mincing of the spleen, density gradient centrifugation, then selection via medium and fibronectin-coating of culture	CD117, SCA1, UEA-I, acDiLDL	(Dimmeler *et al.*, 2001)

dishes		
mechanical mincing of the spleen, density gradient centrifugation, then selection via medium and fibronectin-coating of culture dishes	UEA-I, acDiLDL, SCA1, VEGFR2	(Gensch *et al.*, 2007a)
gradient centrifugation and magnetic bead separation	CD11, CD45, SCA1, CD31, VEGFR2	(Wassmann *et al.*, 2006)
	peripheral blood from rabbit	
density gradient centrifugation, then selection via medium	UEA-I, acDiLDL	(Fan *et al.*, 2005)
magnetic beads, culturing on fibronectin	negative selection using CD5, IgM, CD11, acDiLDL, UEA-I	(Takahashi *et al.*, 1999)
	bone marrow from rabbit	
gradient centrifugation, selection via medium and fibronectin coating of the culture dishes	acDiLDL, CD34	(Shintani *et al.*, 2001b)
	peripheral blood from rat	
gradient centrifugation, selection via medium and vitronectin+gelatine coating of the culture dishes	acDiLDL, UEA-I,	(Walter *et al.*, 2002)
	bone marrow from rat	
gradient centrifugation, selection via medium and fibronectin coating of the culture dishes	acDiLDL, CD31, PECAM-1, CD146, CD133, CD106	(Kahler *et al.*, 2007)

As can be seen from Table 1, most studies were performed in humans using PB. Most groups seem to favour a centrifugation and cell medium-based approach, although it is not entirely clear why. One reason may be that EPCs can not be characterised by a single surface marker and that the range of cell surface markers that are used, changes during the development of EPCs (Critser *et al.*, 2011). The centrifugation/cell media methods allow the rare population of EPCs in the PB (Fadini *et al.*, 2008b, Peichev *et al.*, 2000) and BM (Gehling et al., 2000) to be captured in its entirety irrespective of their stage of development.

1.3.2.4 Characterisation of EPCs

Table 1 makes it clear that there is no uniform set of cell surface markers that characterises EPCs. To complicate matters, studies on cultured EPCs and studies on freshly isolated EPCs do not report the same surface markers, probably due to growth factors in cell culture medium and the change of environment. Also, EPCs isolated from BM and PB do not show identical cell surface markers. This may be related to different stages of maturation. ECs and EPCs have specialised scavenger receptors

that can be used to identify them, still today this is one of the cardinal properties required for characterisation.

Microvascular cells contributing to new vessels need different growth factors and surfaces compared with established ECs (Folkman et al., 1979). An assay was developed using 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate - labeled acetylated low density lipoprotein (Dil-acLDL) (Voyta et al., 1984) to identify these cells. Dil-acLDL uptake and binding of endothelium specific Lectin have long been used to identify EPCs (Asahara *et al.*, 1997, Bellik *et al.*, 2005, Ingram *et al.*, 2004, Kaushal *et al.*, 2001, Laufs *et al.*, 2004, Liew *et al.*, 2006, Liew *et al.*, 2008, Loomans *et al.*, 2004, Murohara *et al.*, 2000, Walenta *et al.*, 2005, Werner *et al.*, 2003, Werner *et al.*, 2002). When the Dil-acLDL is taken up into the cell via the scavenger pathway, the molecule is processed by lysosomal enzymes and the Dil accumulates in the lysosomal membranes. Higher metabolic rate will lead to higher accumulation of Dil-acLDL. Dil can be visualized with a Rhodamine fluorescence filter. Dil-acLDL does not inhibit endothelial cell growth (Voyta et al., 1984). Scavenger receptors, such as the macrophage scavenger receptors type 1 and 2 (Kodama et al., 1990, Rohrer et al., 1990) are responsible for the endocytosis of chemically altered lipoproteins such as oxidised LDLs or Dil-acLDL. Endothelial cells express a specialised scavenger receptor specific for acetylated LDL (acLDL) (Adachi et al., 1997). Binding of acLDL leads to the release of endothelin (Boulanger et al., 1992) and stimulation of the receptor induces the expression of ICAM-1, VCAM1 and E-selectin (Palkama et al., 1993).

Another important differentiating feature of EPCs is their *in vitro* characteristics. These include tube formation: when placed on Matrigel®, EPCs spontaneously form tube-like structures – a unique feature not observed in cells other than EPCs (Asahara *et al.*, 1997, Capla *et al.*, 2007, Chen *et al.*, 2009a, Chen *et al.*, 2007, Churdchomjan *et al.*, 2010, Kaushal *et al.*, 2001, Murohara *et al.*, 2000, Tepper *et al.*, 2002, Thum *et al.*, 2006).

Besides Dil-acLDL uptake, binding of Lectin and tube formation assay, a number of cell surface markers have been used to identify and characterise EPCs. The authors first describing the isolation of EPCs from human PB (Asahara *et al.*, 1997) used CD34 and VEGFR2 as markers to isolate and identify EPCs. VEGFR2 is found on almost any type of EPCs, being a functional marker of angiogenic endothelial cells (Ortega *et al.*, 1997) and one of the key molecules on EPCs. It was reported to be a

marker for early hematopoietic stem cells and angioblasts (Matthews et al., 1991, Millauer et al., 1993, Yamaguchi et al., 1993) and is one of the few factors that is present at almost any maturation stage of EPCs. It was further described that immature EPCs mainly express CD34 and CD133 (Brunner et al., 2009), less immature EPCs express CD34, CD133 and VEGFR2 (Fadini *et al.*, 2006a, Gehling *et al.*, 2000, Hristov *et al.*, 2003b, Peichev *et al.*, 2000, Salven *et al.*, 2003, Stevens *et al.*, 2001) and more mature EPCs CD34, CD133, VEGFR2 and CD31 (Brunner et al., 2009).

The time period at which CD34 is expressed is still a matter of debate. CD34 does not seem to be present on very immature EPCs (Friedrich *et al.*, 2006) and CD133 seems only to be present from a relatively immature state to a more mature state – CD133 is then lost as EPCs become mature (Friedrich *et al.*, 2006, Gehling *et al.*, 2000, Handgretinger *et al.*, 2003, Peichev *et al.*, 2000, Stevens *et al.*, 2001). Based on the foregoing, a consensus could be suggested such as follows:

$CD34^-$ / $CD133^+$ / $VEGFR2^+$	= very immature EPCs
$CD34^+$ / $CD133^+$ / $VEGFR2^+$	= immature EPCs
$CD34^+$ / $CD133^+$ / $VEGFR2^+$	= less immature EPCs
$CD34^+$ / $CD133^-$ / $VEGFR2^+$ / $CD31^+$	= more mature EPCs
$CD34^+$ / $CD31^+$ / $VEGFR2^+$ / $CD309^+$	= mature EPCs

Other studies have described other markers to identify EPCs. cKIT and SCA1 have been used to identify EPCs isolated from the BM (Awad *et al.*, 2005, Bailey *et al.*, 2004, Briddell *et al.*, 1992, Jackson *et al.*, 2001, Wassmann *et al.*, 2006). A combination of a lineage negative selection (Lin^-, which includes markers such as CD3e, CD11, CD45R/B220, Ly-76, Ly-6G and Ly-6C) and $SCA1^+$ positive selection was used to isolate EPCs as SCA1 is a typical marker for a common precursor for both endothelial and hematopoietical cells (Grant *et al.*, 2002, Wang *et al.*, 2004c). Similarly, Otani et al. (Otani *et al.*, 2002) isolated BM EPCs based on gradient centrifugation followed by Lin^- $SCA1^+$ magnetic bead isolation. Their study also showed that the isolated Lin^- $SCA1^+$ BM cells contained other myeloid progenitor cells so they were therefore not a pure population of EPCs.

Recent research has cast doubt on the utility of cKIT and SCA1 to identify EPCs. It is now widely accepted that cKIT is highly expressed on the earliest progenitors in

the thymus and on hematopoietic stem cells, and common myeloid progenitors (Leong et al., 2008) as well as in cardiac stem cells (Orlic et al., 2001, Peichev et al., 2000). Similarly, since SCA1 was identified on progenitor cells for cardiomyocytes (Matsuura *et al.*, 2004) and smooth muscle cells (Xiao *et al.*, 2007), it may not be an ideal marker for EPCs. Today the use of cKIT and SCA1 for EPC isolation is very limited, because they are also found on HSCs.

With increasing knowledge and a growing number of studies on EPCs, other markers have been identified to be expressed at various maturation stages of EPCs. Conflicting results have, however, been reported (Yoder, 2009) with different approaches to identify EPCs *via* flow cytometry showing poor agreement (Van Craenenbroeck *et al.*, 2008). It has been reported that VEGFR2$^+$ / CD34$^+$ / CD133$^+$ cells may be of hematopoietic origin and not true EPCs (Case *et al.*, 2007). Other studies (Friedrich *et al.*, 2006, Kuci *et al.*, 2003) reported that a CD34$^-$ and CD34$^+$ population existed within the CD133$^+$ population. CD34$^-$ cells were reported to be able to differentiate into CD34$^+$ cells (Friedrich *et al.*, 2006). Furthermore the CD34$^-$ population was more responsive *to SDF-1, showed more rapid migration to sites of* ischaemia and vascular lesions and showed better re-endothelialisation compared with CD34$^+$ populations. CD34$^-$ cells were reported to differentiate into endothelial cells (Nakamura et al., 1999) and CD34$^-$/CD133$^+$ cells showed a more sustained effect and higher long term engraftment than CD34$^+$ cells when implanted into irradiated Non-Obese Diabetic / Severe Combined Immunodeficiency (NOD/SCID) mice (Kuci et al., 2003).

Despite these observations, the use of CD34, CD133 (Asahara *et al.*, 1997, Del Papa *et al.*, 2004, Milkiewicz *et al.*, 2006, Rafii *et al.*, 2003) and VEGFR2 (Gehling *et al.*, 2000, Peichev *et al.*, 2000, Zampetaki *et al.*, 2008) seems to be the most generally accepted approach to characterising EPCs (Hristov *et al.*, 2003b) and CD34 is the most frequently used single marker (Sekiguchi et al., 2009). It is now agreed that the loss of CD133 and the expression of VE-Cadherin, CD31 and von Willebrand factor (vWF) marks the step towards more mature EPCs, i.e. EPCs found in the PB (Hristov *et al.*, 2003b, Yin *et al.*, 1997).

Other markers that have been detected on EPCs in the PB besides CD34 and VEGFR2 include CD146, eNOS and E-selectin (Asahara *et al.*, 1997, Hristov *et al.*, 2003b, Kaushal *et al.*, 2001, Quirici *et al.*, 2001).

As EPCs isolated from the BM lack expression of CD31, CD146, vWF, VE-Cadherin and CD31 (PECAM-1) but show CD34, CD133 and VEGFR2 (Quirici et al., 2001) the use of the terms "circulating EPCs" and "bone-marrow derived EPCs" was suggested (Hristov *et al.*, 2003b). Absence of CD31 in BM EPCs is expected as this is a marker for more mature EPCs (Medina et al., 2010a) and ECs (Fong et al., 1995).

Further characterisation and differentiation of subtypes of EPCs isolated from PB has been achieved by studying their growth behaviour and their ability to form colonies *in vitro*. Based on this approach, seeded EPCs were categorised into early and late EPCs (Chen *et al.*, 2009a, Gehling *et al.*, 2000, Gulati *et al.*, 2003, Hur *et al.*, 2004, Ingram *et al.*, 2004, Liew *et al.*, 2008, Lin *et al.*, 2000, Medina *et al.*, 2010d, Mukai *et al.*, 2008, Reinisch *et al.*, 2009, Timmermans *et al.*, 2007, Yoder *et al.*, 2007). Early EPCs are spindle shaped and show a peak in growth 2 weeks after seeding and often die after 4weeks (Chen *et al.*, 2009a, Hur *et al.*, 2004, Liew *et al.*, 2006). Another interesting feature is that early EPCs from human PB retain their monocytic function in culture (Zhang et al., 2006) and secrete more angiogenic factors compared with late EPCs (Hur et al., 2004). Other studies confirmed these findings and reported that early EPCs have low proliferative potential and readily adopt-characteristics of ECs such as eNOS expression but fail to form tube like structures (Zampetaki *et al.*, 2008).

Late EPCs, on the other hand, are morphologically distinct. They have cobblestone shaped morphology with a smooth cytoplasmatic outline. They grow in clusters and appear after approximately 3 weeks of culture with a growth peak at 4-8 weeks (Chen *et al.*, 2009a, Gulati *et al.*, 2003, Hur *et al.*, 2004, Liew *et al.*, 2006, Murohara, 2001, Quirici *et al.*, 2001). Late EPCs were reported to express more VEGFR1, VEGFR2, eNOS, VE-Cadherin and vWF (Hur et al., 2004) and were also capable of forming tube-like structures and of incorporating into mature vessels much better than early EPCs (Hur et al., 2004). They have higher proliferation potential, can be maintained in culture for extended periods of time (Hur et al., 2004) and contribute to neoangiogenesis (Yoder *et al.*, 2007). In comparison to the early EPCs, they produce lower amounts of growth factors (Urbich *et al.*, 2005a). This low production of angiogenic cytokines was proposed as a reason why these late outgrowth cells can contribute to neoangiogenesis but only to a small amount (Urbich *et al.*, 2005a) when

used singularly, i.e. not in conjunction with early EPCs that supply higher amounts of growth factors.

The observation that at least two types of EPCs (early and late) (Duan et al., 2006, Hur et al., 2004) exist and that these both types interact closely has been reported experimentally. It has been reported that only combined transplantation of early and late EPCs results in a therapeutic effect which was not achieved when transplanting either of them alone in New Zealand White Rabbits or ischaemic hind limbs of nude mice (Fan et al., 2005, Yoon et al., 2005). Based on these findings, a hypothesis was established that early EPCs support late EPCs *via* the release of cytokines and only late EPCs eventually contribute to the endothelial graft and interact with resident ECs (Medina *et al.*, 2010a, Medina *et al.*, 2010d, Mukai *et al.*, 2008, Sieveking *et al.*, 2008, Zampetaki *et al.*, 2008).

Early and late EPCs were further studied to reveal differences at the molecular level that may help to explain the differences observed in cell culture. Gene array analyses (Medina *et al.*, 2010d) revealed that early EPCs have more monocytic features and express a number of genes that are related to immunological processes, especially the complement system (Medina *et al.*, 2010d). At the protein level early EPCs show a close relationship to monocytes. Late EPCs express more endothelial markers and also show at the protein level a close relationship to ECs (Medina *et al.*, 2010d). Only late EPCs form tight junctions and zonulae adherentes with ECs (Medina et al., 2010a).

Based on the studies performed so far (Medina *et al.*, 2010a), a short tabular summary of early and late EPCs isolated from human PB is presented in Table 2.

Table 2: Overview of different markers and properties of early and late EPCs (Medina *et al.*, 2010a).

early EPCs	**late EPCs**
appear with 1-2 weeks	appear after 3-4 weeks
die after ~4 weeks	can be kept in culture for long periods
spindle shaped	cobble-stone shaped
low proliferative potential	high proliferative potential
low clonogenic potential	high clonogenic potential
Lectin binding	Lectin binding
DiL-acLDL uptake	DiL-acLDL uptake

CD31	CD31
no vWF expression, low VEGFR2 expression	vWF expression, high VEGFR2 expression
CD45, CD14 (haem marker)	CD105, CD146 (endo marker)
CD133, Stro-1	no CD133, Stro-1
low CD34, low CD117	CD34, CD117 > more immature
features of monocytic cells	features of endothelial cells

In summary, EPCs express a range of cell surface markers. CD34, CD133 CD146, vWF and VEGFR2 are the most frequently used markers to identify them (Fadini *et al.*, 2008b, Fadini *et al.*, 2006a, Urbich *et al.*, 2004). As no single or unique marker for EPCs has yet been identified as being specific (Barber *et al.*, 2006a, Timmermans *et al.*, 2009), researchers use a range of markers and phenotypic properties to define EPCs (Bellik *et al.*, 2005, Critser *et al.*, 2011, Friedrich *et al.*, 2006, Ingram *et al.*, 2004, Ishikawa *et al.*, 2004, Medina *et al.*, 2010c, Murohara *et al.*, 2000, Walenta *et al.*, 2005). Regardless of this lack of accuracy, the term "EPC" is used and it is acknowledged that "EPC" refers to a heterogeneous group of cells rather than a single population (Barber *et al.*, 2006b, Hirschi *et al.*, 2008, Medina *et al.*, 2010c). Nevertheless, the differing results from clinical trials and inconsistent outcomes (Beeres et al., 2008) emphasizes the necessity for a clear definition of what EPCs actually are (Yoder, 2009).

1.3.2.5 Function of EPCs

EPCs, closely related to hematopoietic stem cells (Orlic *et al.*, 1993, Peichev *et al.*, 2000), have been reported to have a number of unique functions. Among them are postnatal vasculogenesis (formation of new blood vessels, neovasculogenesis) and angiogenesis (formation of blood vessels from existing vessels by migration and proliferation of mature ECs) (Carmeliet, 2000b, Grant *et al.*, 2002, Hristov *et al.*, 2003b, Isner *et al.*, 2001, Luttun *et al.*, 2002, Rafii *et al.*, 2002, Ruzinova *et al.*, 2003, Schmidt-Lucke *et al.*, 2005, Shirota *et al.*, 2003). It has also been reported that a pool of circulating EPCs is continuously repairing damaged, dysfunctional or denuded endothelium (Kong *et al.*, 2004a, Laufs *et al.*, 2004, Rosenzweig, 2003, Strehlow *et al.*, 2003, Walter *et al.*, 2002, Werner *et al.*, 2003, Werner *et al.*, 2002).

The participation of EPCs in neovasculogenesis has been studied in various conditions such as tumour neovascularisation (Peters *et al.*, 2005), retinal neovascularisation in DR (Lee *et al.*, 2006a) and choroidal neovascularisation in exudative age-related macular degeneration (Machalinska *et al.*, 2011, Sengupta *et al.*, 2005, Sheridan *et al.*, 2006, Yodoi *et al.*, 2007). Most importantly, EPCs contribute to angiogenesis in ischaemic tissues – including the retina (Butler et al., 2005, Grant et al., 2002, Lee et al., 2006a, Otani et al., 2002, Takahashi et al., 1999, Tamarat et al., 2004). In DR, circulating EPCs can differentiate into mature ECs at the site of the retinal neovascularisation (Grant et al., 2002, Lee et al., 2006a, Otani et al., 2002, Takahashi et al., 1999).

Angiogenesis is a multistep process which involves ECM degradation, proliferation of ECs as well as EC and EPC migration (Carmeliet, 2000b). Cooperation of EPCs and mature ECs in angiogenesis was proposed as EPCs alone could not always form vessels de novo (Crosby et al., 2000).

The potential of EPCs to contribute to neovascularisation is of great importance since many endogenous repair processes rely on it. EPCs from mononuclear cells from the peripheral blood which were expanded *ex-vivo* have been reported to be beneficial for myocardial ischaemia and during infarct-remodelling (Britten *et al.*, 2003, Kamihata *et al.*, 2001, Kawamoto *et al.*, 2001, Kocher *et al.*, 2001, Schachinger *et al.*, 2004), repair vasculature after myocardial infarction (Rafii *et al.*, 2003, Sekiguchi *et al.*, 2009, Tateishi-Yuyama *et al.*, 2002, Urbich *et al.*, 2004), peripheral ischaemic conditions (limb ischaemia) (Rafii et al., 2003, Tateishi-Yuyama et al., 2002), in cerebral neovascularisation after stroke (Zhang et al., 2002) and ischaemic cerebrovascular disease (Jung *et al.*, 2008). Although a contribution to new vessel development by the secretion of angiogenic factors (Hristov *et al.*, 2003b) and activation of resident ECs (Rehman et al., 2003) seems to be the predominant function of EPCs, there is also evidence that EPCs may inhibit angiogenesis as reported experimentally *in vitro* using EPCs from diabetes type 1 patients (Loomans *et al.*, 2004). The postulated contribution of EPCs to neovascularisation in ischaemic tissues (Urbich et al., 2004) has been reported in animal models. In rabbits and mice, EPCs have been reported to promote new vessel formation in areas of injury, enhance perfusion and recover ischaemic tissue (Kalka et al., 2000b, Takahashi et al., 1999) or augment neovascularisation (Shintani *et al.*, 2001b). EPCs have been reported to integrate into damaged blood vessels in the ischaemic hind limb model (Couffinhal *et*

al., 1999, Kalka *et al.*, 2000b, Sasaki *et al.*, 2006), areas of myocardial infarction (Schuh et al., 2008), ischaemic retinopathies (Caballero *et al.*, 2007) and tumour vessels (Nolan et al., 2007).

The contribution and involvement of EPCs in tumour neovascularisation is well studied: a correlation between numbers of circulating EPC and multiple myeloma (Zhang *et al.*, 2005a) and increased numbers of $CD133^{+}$ EPCs in patients with lung cancer (Hilbe et al., 2004) has been reported. Blocking the recruitment of EPCs inhibits tumour growth (Lyden et al., 2001).

While the contributory role of EPCs to angiogenesis is clear, the role of EPCs in the development of atherosclerosis is less obvious. The role of EPCs in atherosclerosis seems to be of a dual nature: they contribute both to re-endothelialisation and plaque formation (Zampetaki *et al.*, 2008). The protective role of EPCs was reported in APO-E knockout mice treated with EPCs from normal mice in which atherosclerosis formation could be prevented despite significant hypercholesterinaemia (Rauscher et al., 2003). It has been suggested that EPCs may contribute to reducing smooth muscle cell activation and neointima formation (Kong et al., 2004a). Furthermore, an increased NO bioavailability associated with EPC transplantation improved vasculoprotective properties and inhibited neo-intimal hyperplasia (Kong et al., 2004b). Despite these positive effects just described, a negative effect of EPCs in atherosclerosis was also observed. Decreased blood flow through adventitial *vasa vasorum* can trigger atherosclerotic intimal thickening (Hu et al., 2003, Khurana et al., 2005). The increased contact time of circulating EPCs with proinflammatory properties can lead to a reduction in plaque stability (George et al., 2005). A possible release of mitogens for smooth muscle cells and a transdifferentiation of EPCs into smooth muscle cells further accelerating atherosclerosis could not be excluded (Yeh et al., 2003). However, it seems that the isolation protocols to characterise EPCs and their properties with respect to a possible atherogenesis are important (Seeger et al., 2007) as differences in isolation protocols can cause alterations the properties of EPCs and in some cases have negative effects on functional properties or cause selection of proinflammatory EPCs.

In an animal model of arteriosclerosis it has been reported that circulating EPCs repaired endothelium (Hu et al., 2003). The same could be reported in animals undergoing vein graft surgery (Xu *et al.*, 2003b). Further experiments reported that EPCs promote *in vivo* re-endothelialisation of damaged vessels (Gulati et al., 2004)

and can incorporate into newly formed vessels in the hindlimb ischaemia model in animals (Asahara et al., 1999a, Schatteman et al., 2000).

EPCs are also involved in maintaining the integrity of the vasculature (Takahashi et al., 1999, Takamiya et al., 2006). This task includes regular endothelial renewal (Asahara *et al.*, 1999a, Asahara *et al.*, 1997) and repair of damaged endothelium (Hristov et al., 2003a) due to trauma, shear stress or other causes such as atherosclerosis (Rookmaaker et al., 2002, Walter et al., 2002, Werner et al., 2002) (Wassmann et al., 2006). EPCs can directly integrate into newly formed vessels (Otani et al., 2002) but may also contribute indirectly by augmenting neovascularisation (Shintani et al., 2001b) without being physically incorporated (De Palma et al., 2003).

It should be noted, however, that EPCs do not always integrate into the vasculature (Purhonen *et al.*, 2008, Ziegelhoeffer *et al.*, 2004) and may even transdifferentiate into smooth muscle cells (Frid et al., 2002). This may be related to the fact that only few markers to identify EPCs are used in some instances: if only CD34 is used as a cell surface marker to characterise BM- derived progenitor cells, they may not only form endothelial cells but also hematopoietic cells, neurons, fibroblasts and even muscle (Asahara *et al.*, 1997, Hashimoto *et al.*, 2004, Hattori *et al.*, 2001, Kawamoto *et al.*, 2001, LaBarge *et al.*, 2002, Otani *et al.*, 2002, Ott *et al.*, 2005, Phillips *et al.*, 2004, Priller *et al.*, 2001, Rafii *et al.*, 2003, Religa *et al.*, 2002, Taguchi *et al.*, 2004, Torrente *et al.*, 2004). When CD34 is combined with CD133, the resulting population is less heterogeneous and the cells contribute only to neovascularisation and differentiation into ECs (Asahara *et al.*, 1997, Hattori *et al.*, 2001, Kawamoto *et al.*, 2001, Otani *et al.*, 2002, Ott *et al.*, 2005, Rafii *et al.*, 2003).

1.3.2.6 Mobilisation and Migration of EPCs

Stem cells in the BM can remain in their niche or differentiate upon stimulation by a wide range of factors such as hormones or growth factors (Calvi *et al.*, 2003, Li *et al.*, 2005, Yin *et al.*, 2006). The mobilisation and differentiation of EPCs from the BM is a most complex process that involves many growth factors such as VEGF, IFG1, PDGF, FGF, Ang-1 and IL-8 (Grant et al., 2004) as well as the local environment in the BM including fibroblasts and ECs (Kiger et al., 2000, Lapidot et

al., 2002, Morrison et al., 1997). Since only partial knowledge exists about the underlying mechanisms of mobilisation and differentiation of BM EPCs, only a few important aspects will be discussed in this section.

Generally, the turnover of ECs (endothelial renewal) is thought to be low in healthy subjects (Dignat-George et al., 2000), however any kind of stress / shear stress, endothelial damage, ischaemia, burn injury or surgery increases the number of circulating EPCs (Gill et al., 2001, Shintani et al., 2001a, Takahashi et al., 1999) in an immediate response (Hristov et al., 2003a). Once the EPCs are mobilised from the BM, circulating EPCs home to sites of ischaemia and contribute to the formation of new blood vessels (Asahara et al., 1997, Shi et al., 1998, Takahashi et al., 1999). It has been reported that VEGF mediates proliferation, differentiation and chemotaxis of mature ECs (Asahara et al., 1999b, Hanahan et al., 1996). VEGF is also essential for EPC mobilisation and function.

The actual mobilisation of EPCs is supposed to start with activation of MMP9 (Hattori *et al.*, 2002, Heissig *et al.*, 2002, Hristov *et al.*, 2003a), which itself is activated by NO (Aicher et al., 2003, Murohara et al., 1998), or by the direct action of VEGF (Gu et al., 2002, Hiratsuka et al., 2002). NO-mediated pathways are critical for EPC mobilisation (Aicher et al., 2003, Heissig et al., 2002). MMP9 will then be involved the degradation of extracellular material (Egeblad *et al.*, 2002, Libby *et al.*, 2000, van Kempen *et al.*, 2002) and in the transfer of EPCs from the osteoblastic to the vascular niche (Heissig et al., 2002) which will lead to a mobilisation of EPCs from the BM to the systemic circulation (Aicher et al., 2003, Murohara et al., 1998). The effect of VEGF, to act as a chemoattractant for EPCs (Asahara et al., 1999a, Kalka et al., 2000a) and to trigger EPC release from the bone marrow, has been well studied (Asahara *et al.*, 1999b, Hattori *et al.*, 2001, Iwaguro *et al.*, 2002, Kalka *et al.*, 2000c, Peichev *et al.*, 2000, Takahashi *et al.*, 1999). The critical role of VEGF for vasculogenesis and angiogenesis (Carmeliet et al., 1996, Ferrara et al., 1996, Shalaby et al., 1995) was reported by the ability of VEGF to mobilize EPCs in humans with limb ischaemia (Kalka et al., 2000a) and myocardial ischaemia (Kalka et al., 2000b).

Another effect of MMP-9 activation is the release of sKiT-ligand which in turn improves survival, mobilisation and proliferations of stem cells (Heissig et al., 2002). As outlined in the section "Vascular endothelial growth factor and its receptors", MMP9 activation occurs a result of VEGF binding to VEGFR1, the subsequent activation of PI3K, phosphorylation of eNOS and production of NO (Ackah *et al.*,

2005, Dimmeler *et al.*, 2000, Fulton *et al.*, 1999). Increased phosphorylation of eNOS results in increased production of NO and better migratory capacity of EPCs (Sasaki et al., 2006). It seems that the increase in NO and MMP9 activity mediate the angiogenic response (Zampetaki *et al.*, 2008).

As mentioned, VEGF induces the expression of SDF-1 (Kijowski *et al.*, 2001, Neuhaus *et al.*, 2003). Both VEGF and SDF-1 were reported to contribute to EPC mobilisation (Asahara *et al.*, 1999b, Friedrich *et al.*, 2006, Heissig *et al.*, 2002, Moore *et al.*, 2001, Peichev *et al.*, 2000) and to upregulate endothelial markers in EPCs (Rossig et al., 2005, Wang et al., 2005, Yamamoto et al., 2003, Zeng et al., 2006). Apart from the mobilisation of EPCs by SDF-1 (De Falco et al., 2004), VEGF, PlGF and Ang-1 and SDF-1 induce expansion of EPCs and HSCs (Rabbany et al., 2003). The colonisation of the foetal bone marrow by EPCs is SDF-1 dependent (Ara *et al.*, 2003, Kopp *et al.*, 2005).

SDF-1 plays an important role in the mobilization and integration of EPCs. It has been reported that SDF-1 is expressed by a variety of cells as a response to stress, especially in the central nervous system (Hatch et al., 2002, Lazarini et al., 2003). It facilitates migration of EPCs towards sites of endothelial damage or ischaemic tissue (homing) (Butler *et al.*, 2005, Segal *et al.*, 2006). Once they have arrived at the site of damage, SDF-1 is crucial for the integration of EPCs into the compromised vasculature in diseases such as in DR (Butler et al., 2005). The ability of EPCs to produce SDF-1 was reported for EPCs isolated from both the BM (Yun *et al.*, 2003) and PB (Urbich *et al.*, 2005a). As EPCs express both the SDF-1 and its receptor (CXCR4) (Yamaguchi *et al.*, 2003, Zheng *et al.*, 2007), an autocrine/paracrine regulation loop for SDF-1 (Yin *et al.*, 2007) within EPCs was proposed. The function of the SDF-1/CXCR4 interaction in EPCs within the bone marrow is still being investigated, however it seems that this ligand/receptor interaction within the EPCs plays an important role in the mobilisation of EPCs to the PB and also in EPC maturation (De Falco *et al.*, 2009, Yin *et al.*, 2007). *In vitro* interruption of the SDF-1/CXCR4 regulation loop was reported to impair EPC function (Yin *et al.*, 2007).

Outside the BM SDF-1 and its receptor CXCR4 play a major role in the recruitment of stem cells to ischaemic areas (Askari et al., 2003, Kollet et al., 2003, Lapidot et al., 2005). SDF-1 is upregulated by inflammation, mechanical force and hypoxia (Ceradini et al., 2004) but also in DR (Butler et al., 2005). Platelets, which also strongly express SDF-1, are responsible for chemotaxis of EPCs to areas of

vessel injury: upon vessel damage, platelets bind to the damaged area and attract *via* SDF-1 the EPCs, this mechanism is also VEGF dependent (Madlambayan *et al.*, 2009). It was reported that interaction of SDF-1 with CXCR4 led to mobilisation of hibernating cells that require activation of proteases to be able to migrate into the PB (Petit et al., 2007).

Interestingly, this SDF-1 / CXCR4-related mobilisation and proliferation of EPCs can be facilitated by estrogens (Iwakura et al., 2003). In mouse models of proliferative DR (Madlambayan *et al.*, 2009) and laser induced retinal damage (Grant et al., 2002), it was reported that BM derived progenitors contributed largely to retinal neovascularisation. It was reported that the local expression of SDF-1 was critical chemoattractant stimulus for angiogenesis by the BM derived progenitors; inhibition of SDF-1 repealed the neovascular response (Madlambayan *et al.*, 2009). It was also reported that not all neovascularisation could be suppressed, hence the authors suggested that redundant mechanisms must exist for postnatal neovascularisation (Madlambayan *et al.*, 2009).

Although SDF-1 seems to play a pivotal role in the migration process for EPCs, other factors such as VEGF, EPO, FGF, PDGF are also potent stimuli for EPC mobilisation and homing to ischaemic tissues (Aicher *et al.*, 2005, Butler *et al.*, 2005, Freyberger *et al.*, 2000, Jin *et al.*, 2006a, Kopp *et al.*, 2005, Rafii *et al.*, 2003, Takahashi *et al.*, 1999, Watanabe *et al.*, 2005). Granulocyte colony-stimulating factor (GCSF) is another cytokine capable of stimulating EPC mobilisation from the BM to the PB (Gehling et al., 2000, Murasawa et al., 2005, Tepper et al., 2002) as well as granulocyte-macrophage colony-stimulating factor (GM-CSF) (Takahashi et al., 1999).

Statins have a number of positive effects on ECs. These include upregulating eNOS, increasing NO levels and improving of eNOS function (Laufs et al., 1997, Laufs et al., 1998a). Simvastatin was also reported to attenuate EC apoptosis and to augment angiogenesis in the ischaemic hindlimb model in the rabbit (Kureishi et al., 2000). Statins rapidly activate the AKT signalling in ECs, which in turn stimulates their bioactivity and enhances their angiogenic capacity *in vivo* (Kureishi et al., 2000). This activation of AKT by statins is also important for EPC mobilisation from the BM: it was reported that EPCs from the BM can be mobilized and contribute to postnatal neovascularisation through stimulation of the AKT pathway via statins

(Dimmeler *et al.*, 2001, Landmesser *et al.*, 2004, Llevadot *et al.*, 2001, Vasa *et al.*, 2001a, Walter *et al.*, 2002, Werner *et al.*, 2002).

In a rat model of myocardial infarction (Li *et al.*, 2009), the numbers of $CD34^+$ / $CD133^+$ EPCs could be significantly increased by administering simvastatin. Although myocardial infarction itself lead to an increase of EPCs in the PB, this increase was significantly higher in animals treated with simvastatin. VEGF and AKT were significantly increased as well by simvastatin (Li *et al.*, 2009). The effects of simvastatin in this model could be confirmed when the isolated EPCs from the rats were studied *in vivo*: phosphorylation of eNOS and forkhead transcription (FKHR) were significantly increased.

It is important to know that AKT regulates the transcription of FOXO1 (forkheadtranscription factor). FOXO1 (=FKHR) is a transcription factor that regulates downstream genes that are involved in the cell cycle, apoptosis, aging and metabolism. Normally AKT phosphorylates FOXO1 which decreases its transcriptional activity (Urbich *et al.*, 2002a). AKT increase from simvastatin treatment leads to increased phosphorylation of FKHRL1 (FKHR ligand 1), therefore decreasing its transcriptional activity and potentially preventing apoptosis signalling.

The ability of statins to mobilise EPCs from the BM was reported by a number of other authors (Dimmeler *et al.*, 2001, Landmesser *et al.*, 2004, Llevadot *et al.*, 2001, Vasa *et al.*, 2001a). Activation of the PI3K / AKT was confirmed as the underlying mechanism to mobilise EPCs in various conditions in humans such as endothelial damage in myocardial infarction (Landmesser et al., 2004, Llevadot et al., 2001) or after balloon-dilation trauma to the coronary arteries (Walter *et al.*, 2002, Werner *et al.*, 2002). In these situations, increasing EPCs in PB was reported to improve the re-endothelialisation. So, both suppression of apoptosis signalling in EPCs and their increased mobilisation from the BM are augmented by simvastatin (Li et al., 2009).

ACE inhibitors and Angiotensin 2 (AT2) receptor blockers were reported to improve EPC numbers and function independent of their blood-pressure lowering properties in patients without DM (Bahlmann *et al.*, 2005, Min *et al.*, 2004). Enalapril, an ACE blocker, increased ischaemia-induced EPC mobilisation by affecting the CD26 system (Wang *et al.*, 2006b). AT2 accelerated the ageing of EPCs, which could be reversed by AT2 receptor blockers such as valsartan (Imanishi *et al.*, 2005). AT2 also induced smooth muscle cell proliferation as well as upregulation of VEGF expression by retinal pericytes (Daemen *et al.*, 1991, Otani *et al.*, 2000), its

actions are mediated via the AT2 type 1 receptor. AT2 and VEGF are found in high levels inpatients with proliferative DR (Funatsu *et al.*, 2002) – given this, and for other reasons, inhibition of AT2 is desirable, especially in people with DR. However, AT2 receptor blockers have demonstrated antiproliferative effects, especially in coronary endothelial cells possibly increasing the risk of insufficient endothelial repair (Stoll *et al.*, 1995), which may limit the desirability of using AT2 blockers in people with DR.

A molecule involved in EPC recruitment to ischaemic areas is ICAM-1. It was reported that ICAM-1 was upregulated in diabetic retinas (Leal *et al.*, 2007) and that ICAM-1 upregulation enhanced EPC recruitment to ischaemic areas (Yoon *et al.*, 2006). Additionally, integrin-linked-kinase (ILK) overexpression induces ICAM-1 expression as well as SDF-1 expression in EPCs thus facilitating EPC recruitment to ischaemic tissues (Lee *et al.*, 2006b).

Another type of adhesion molecules, integrin $\alpha_4\beta_1$ (VLA-4) also promotes homing of circulating EPCs to the $\alpha_4\beta_1$ ligand VCAM and cellular fibronectin which are expressed in remodelling neovasculature (Jin *et al.*, 2006b). Moreover, $\alpha_4\beta_1$ improves blood flow recovery and tissue preservation (Qin et al., 2006) through stimulation of EPC migration. It was reported that EPCs expressing $\alpha_4\beta_1$ homed to sites of active tumour neovascularisation (Jin *et al.*, 2006b). Furthermore, integrin $\alpha_4\beta_1$ (also known as lymphocyte integrin) is necessary for intercellular adhesion and survival of EPCs during blood vessel formation (Jin *et al.*, 2006b): the BM derived EPCs were detected within vessel walls near tumour margins, indicating a special homing response of EPCs to growing vessels. EPCs were detected in the tumour margins as soon as 10 minutes after injection of EPCs through a tail vein of mice and they remained in place stably up to 3 days later. EPCs express $\alpha_4\beta_1$ specifically (other cell types have different combinations of the α and the β subunit) and integrin $\alpha_4\beta_1$ mediated the adhesion of EPCs ($CD34^+$) to adult ECs. Connecting segment-1 (CS-1) fibronectin and cellular fibronectin are ligands for $\alpha_4\beta_1$ (Jin *et al.*, 2006b).

Beta-2 integrins, which are expressed on the surface of EPCs, are also involved in the firm adhesion and transmigration of circulating EPCs to areas of damaged endothelium thereby contributing to the neovascularisation capacities of EPCs (Chavakis et al., 2005). The interaction between beta-2 integrin on EPCs and the damaged ECs is facilitated by the high-mobility-group-box1 protein (HMGB1) which is released by necrotic, but not by apoptotic, cells into the extracellular space where it

activates beta1 and beta2 integrins and hence induces homing and adhesion of EPCs (Chavakis et al., 2007).

Other adhesion receptors such as L-selectin, usually regulating lymphocyte homing and leukocyte migration (Torrente *et al.*, 2003, Tu *et al.*, 1999), were reported to be present in EPCs and involved in EPC homing (Biancone *et al.*, 2004). P-selectin and E-selectin were supposed to mediate parts of the initial steps of EPC homing (Zampetaki *et al.*, 2008). Increased adhesion of EPCs to P- and E-selectins is the result of an activation of ephrin type-B receptor 4 (EphB4) (Flanagan *et al.*, 1998) in EPCs which triggers high expression of P-selectin glycoprotein ligand-1 (PSGL-1) (Foubert et al., 2007). E-selectin then mediates a EPC-EC interaction that improves angiogenic capacity – as reported in the ischaemic hindlimb model (Oh et al., 2007). The expression of E-selectin was described to be a sign of activation of EPCs, i.e. to actively participate in angiogenesis (Avci-Adali *et al.*, 2009, Dignat-George *et al.*, 2011, Nguyen *et al.*, 2009), which is consistent with the observation that unstimulated EPCs isolated from BM do not express E-selectin (Reyes *et al.*, 2002).

The marker cKIT (KIT/CD117) (Yarden *et al.*, 1987), which was discussed and described as marker for the isolation of EPCs (Awad *et al.*, 2005, Jackson *et al.*, 2001), was also studied with respect to its function in EPC recruitment in inflammatory processes involving the endothelium (Dentelli *et al.*, 2007). cKIT (KIT) is the receptor for the naturally occurring ligand KITL (or stem cell factor, SCF) (Fintl *et al.*, 2010). This receptor has great similarities to other tyrosine kinase receptors such as the receptor for VEGF or PDGF (Reilly, 2002). The ligand for KIT (KITL) is expressed in 2 splice variants which are localised at the cell surface (mKIT) – the larger of the two containing a cleavage site allowing for a release of KITL from the cell surface (Flanagan *et al.*, 1991). When MMP9 is activated, it processes the mKIT to a resulting soluble form of KITL (sKIT) which triggers proliferation and mobilisation of EPCs in the BM (Heissig *et al.*, 2002). Binding of sKIT to the receptor cKIT leads to receptor dimerisation and activation of the intrinsic receptor kinase which in turn can activate a range of downstream signalling cascades that are responsible for e.g. proliferation, migration and cell survival, AKT activation being one of them (Broudy, 1997, Dentelli *et al.*, 2007). In cells where KITL is cell membrane bound (mKIT), cells remain in a quiescent state (Flanagan *et al.*, 1991). Normal ECs do not express mKIT constitutively, but when exposed to inflammatory

agents or oxidative stress, mKIT will be expressed (Koenig et al., 1994), then as a consequence of mKIT processing sKIT will be able to bind to cKIT.

Other enzymes involved in the migration process of EPCs are selectins (Biancone et al., 2004, Vajkoczy et al., 2003) and integrins (Chavakis et al., 2005, Vajkoczy et al., 2003) and adhesion molecules such as VCAM which is upregulated in ischaemic areas and promotes EPC homing (Jin *et al.*, 2006b). In *in vitro* inflammatory models (TNF-alpha, IL-1beta) used, ECs were activated with upregulation of mKIT, E-selectin and ICAM-1. Blocking cKIT by imatinibmesylate prevented the adhesion of EPCs to the damaged and activated ECs; EPCs with blocked cKIT could not be recruited to neovascular tissue (Dentelli *et al.*, 2007). Of the cKIT mediated actions, VCAM1, ICAM-1 and E-selectin seemed to cooperate with cKIT to stabilise adhesion of EPCs in areas of damaged or activated endothelium.

The invasiveness of EPCs also depends on the expression of cathepsin-L (Shimada *et al.*, 2010, Urbich *et al.*, 2005b) and MMP2 (Cheng et al., 2007). Both are strongly expressed in EPCs and are essential for matrix degradation and invasion. Knockout of cathepsin-L prevents EPCs from homing to ischaemic sites and augments neovascularisation (Urbich *et al.*, 2005b), an effect very similar to what was reported for the ischaemic hindlimb model in the MMP2-knockout mouse (Cheng et al., 2007).

Histone deacetylase 3 (HDAC3) plays a central role in the transformation of EPCs to fully functional ECs (Zampetaki *et al.*, 2008). HDAC3 upregulates p53 and p21. Inhibition of HDAC by homeobox protein A9 (HOXA9) downregulation consequently diminishes the effect of HDAC (transformation of EPCs to ECs, expression of EC markers on EPCs) whereas a forced expression of HOXA9 re-establishes the HDAC effect on EPCs, emphasising the important regulatory role of HDAC3 in the VEGF induced differentiation of EPCs to ECs (Xiao et al., 2006, Zeng et al., 2006).

A range of other growth factors and drugs (Leone *et al.*, 2009, Liew *et al.*, 2008) are capable of increasing or decreasing the number of EPCs in PB, i.e. they are able to mobilise EPCs or to suppress mobilisation. Table 3 gives an overview.

Table 3: Growth factors and drugs affecting the numbers of EPCs in the PB.

increase in EPCs in PB	
Growth factor / medication	*Reference*
Angiopoietin-1	(Lemstrom et al., 2004)
basic FGF	(Conklin et al., 2004)
Constitutive telomerase reverse transcriptase (hTERT)	(Murasawa et al., 2002)
GM-CSF	(Takahashi et al., 1999)
Hepatocyte growth factor (HGF)	(Ishizawa et al., 2004)
PDGF-B	(Keswani et al., 2004)
SDF-1	(De Falco et al., 2004)
TGF-beta1	(Henrich et al., 2004)
VEGF	(Kalka et al., 2000a)
angiotensin converting enzyme (ACE) inhibitors	(Min *et al.*, 2004)
ACE receptor blockers	(Bahlmann et al., 2005, Imanishi et al., 2005)
erythropoietin (EPO)	(Bahlmann *et al.*, 2004, Bahlmann *et al.*, 2003, Calvillo *et al.*, 2003, d'Uscio *et al.*, 2007, Landmesser *et al.*, 2004, Urao *et al.*, 2006)
estrogen	(Fadini *et al.*, 2008c, Sugawara *et al.*, 2005)
Ginko bilboa	(Chen et al., 2004a)
phosphodiestrase (PDE) 5 inhibitor	(Foresta et al., 2005)
peroxisome proliferator-activated receptor (PPAR) gamma agonists	(Sorrentino *et al.*, 2007, Wang *et al.*, 2004b, Werner *et al.*, 2007)
Puerarin	(Zhu et al., 2004)
Statins	(Dimmeler *et al.*, 2001, Landmesser *et al.*, 2004, Li *et al.*, 2009, Llevadot *et al.*, 2001, Walter *et al.*, 2002)
physical exercise	(Laufs *et al.*, 2004)
Benfotamine	(Gadau et al., 2006, Marchetti et al., 2006)
decrease in EPCs in PB	
Growth factor / medication	*Reference*
Angiostatin	(Ito et al., 1999)
131-iodine therapy	(Palumbo et al., 2003)
Rapamycin	(Butzal et al., 2004)

1.3.2.7 Cytokines released by the neuroretina involved in EPC mobilisation

Several studies have reported that neurons within the retina and also glial cells are able to interact with the vasculature and contribute to the development of pathological neovascularisation by creating a special cytokine environment (Duda et al., 2005, West et al., 2005). These observations led to a greater interest in uncovering the processes underlying neuronal-driven angiogenesis (Carmeliet et al., 2005, Eichmann et al., 2005, Suchting et al., 2006, Vogel, 2005).

One of the major triggers for this process is hypoxia. Under low oxygen conditions neurons secrete angiogenic factors such as PEDF or VEGF which are involved in the angiogenic sprouting (Eichler et al., 2000, Eichler et al., 2001, Gerhardt et al., 2003, Ijichi et al., 1995, Mukouyama et al., 2002, West et al., 2005).

Factors that are less well studied than VEGF, such as neurotropins which are normally responsible for the mediative role in axonal guidance, were found to play a critical role in the regulation of angiogenesis by neuronal tissue (Calza et al., 2001, Carmeliet et al., 2005, Donovan et al., 2000, Torres-Vazquez et al., 2004) and in reparative neovascularisation (Emanueli et al., 2002a, Emanueli et al., 2002b). Examples of neurotropins include brain-derived-neurotrophic-factor (BDNF), glial-line-derived-neurotropic-factor (GDNF) and nerve-growth-factor (NGF), all of which were found to exert pro-angiogenic effects, not only by directly affecting ECs but also by recruiting EPCs (Jadhao *et al.*, 2012, Liu *et al.*, 2010). BNDF in particular has been reported to have proangiogenic effects by recruiting BM-derived precursors (Emanueli et al., 2002a, Kermani et al., 2005). Both BDNF and NGF were identified as strong angiogenic molecules that target ECs in the whole vascular system (Cantarella et al., 2002, Emanueli et al., 2002b, Turrini et al., 2002).

Neurotropins play a role in vasculogenesis and blood vessel maintenance – and they are also involved in the development of DR. It was reported that in diabetic patients systemic levels of NFG and BDNF were increased (Liu et al., 2010) which was associated with increased EPCs in the PB. Human isolated retinal cells when cultured under hypoxic conditions released significantly more NGF, BDNF and GDNF (Liu et al., 2010). Neurotrophic factors, either alone or in combination with other angiogenic molecules, were reported to increase the angiogenic activity of ECs (Calza et al., 2001, Donovan et al., 2000) and an administration of BDNF and NGF significantly enhanced angiogenesis both *in vivo* and *in vitro* (Cantarella *et al.*, 2002,

Emanueli *et al.*, 2002b, Jadhao *et al.*, 2012, Nakamura *et al.*, 2006, Turrini *et al.*, 2002, Wilson *et al.*, 2006).

1.3.3. Nitric Oxide and endothelial nitric oxide synthase

Nitric oxide and endothelial nitric oxide synthase are crucial for normal endothelial and EPC function. Loss of endothelial NO bioactivity and increased production of reactive oxygen species are features of diabetic and other vascular disorders (Harrison, 1997). This section will briefly elaborate on the function of eNOS and NO and their importance in the context of pathophysiological changes in DM and DR. The discovery of the importance of NO, its actions and the description of the signalling involved led to the award of The Nobel Prize in Physiology or Medicine in 1998 to Robert F Furchgott, Louis J Ignarro and Ferid Murad (see http://www.nobelprize.org/nobel_prizes/medicine/laureates/1998/illpres/).

1.3.3.1 Functions of Nitric Oxide

The most important function of NO is the relaxation of smooth muscle cells surrounding the blood vessels, thus leading to an increase of vessel diameter and hence increased perfusion – NO is the strongest vaso-dilating agent known (Albrecht et al., 2003). Impairment of vessel relaxation in DM is present before structural changes occur and mirror a decreased bioavailability of NO (Kawashima *et al.*, 2004). NO produced in endothelial cells can freely diffuse across cell membranes. NO can bind to the haem centre of the soluble guanylyl cyclase, e.g. in smooth muscle cells. If guanylyl cyclase is activated by this mechanism, it will produce cyclic guanosine monophosphate (cGMP) which then as second messenger can activate other pathways eventually relaxing smooth muscle cells (Albrecht *et al.*, 2003). NO can also directly activate Ca^{2+} dependent K^{+} channels (Bolotina et al., 1994) which causes an endothelium-dependent hyperpolarisation of vascular smooth muscle cells which results in vasodilation. Hence, NO is one of the most important regulators of vascular tone (Palmer et al., 1987).

NO has a number of other effects that may be deranged in vascular disease. It regulates leukocyte adhesion to the endothelium (Kubes et al., 1991), maintains

normal basal membrane thickness (Ellis *et al.*, 2005a) and inhibits vascular smooth muscle cell proliferation (De Caterina *et al.*, 1995, Garg *et al.*, 1989, Gross *et al.*, 1995, Lloyd-Jones *et al.*, 1996, Rikitake *et al.*, 1998, Sarkar *et al.*, 1996, Scott-Burden *et al.*, 1992). NO inhibits platelet aggregation (Radomski et al., 1987) and regulates bone mass and bone turnover (Armour et al., 2001). It also regulates renal oxygen consumption (Laycock et al., 1998). Importantly, NO is involved in the regulation of angiogenesis (Lee et al., 1999, Ziche et al., 2000).

NO also serves as an important signalling molecule in host defence and neuronal communication (Gross et al., 1995). The NO produced by eNOS was reported to be closely associated with the mobilisation of EPCs from the BM to the PB (Aicher et al., 2003). As can be seen, NO is involved in a number of quite diverse activities such as regulation of vessel tone, angiogenesis, wound healing, inflammation, ischaemic cardiovascular disease and malignancies (Duda *et al.*, 2004)

NO is not only produced by ECs, it can also be generated in low levels by bone marrow cells (Aicher *et al.*, 2003). NO was reported to influence growth, differentiation and colony forming ability of $CD34^+$ BM stem cells (Shami et al., 1996) - underlining its importance and early involvement in EPC biology.

1.3.3.2 Synthesis of Nitric Oxide and Nitric Oxide Synthase isoforms

NO is produced by Nitric Oxide Synthases (NOS), a group of enzymes that catalyse the production of L-Citrullin and NO from L-Arginin using Tetrahydrobiopterin (BH4) as a cofactor (Knowles *et al.*, 1994). In this reaction, NO is split from the terminal guanidino nitrogen of L-arginine leaving L-citrullin (Hemmens et al., 1998, Palmer et al., 1988): NO synthesis starts with hydroxylation of L-arginine to NG-hydroxy-L-arginine followed by oxidation to L-citrullin and NO through the oxidase domain of NOS (Ghosh et al., 1995, Klatt et al., 1993, Palmer et al., 1988, Stuehr et al., 1991). This reaction, in which NO is generated, is dependent on a number of cofactors: NADH, Ca^{2+}/Calmodulin, flavin adenin dinucleotid (FAD, a redox agent), riboflavin-5'-phosphate (FMN, the prostetic group of oxidoreductases) and tetrahydrobiopterin (BH4) (Moncada et al., 1993, Moncada et al., 1991, Nathan et al., 1994, Stuehr, 1999). The catalytic mechanisms of NOS involve a flavin-mediated electron-transport from the C-terminal-bound NADPH to the N-terminal haem centre

of NOS – where the oxygen is reduced and incorporated into the guanidine group of L-Arginine, thus producing NO and L-Citrullin (Kawashima *et al.*, 2004). It appears that BH4 facilitates electron transfer from the eNOS reductase unit and maintains the heme prosthetic unit in its active redox form (Stuehr et al., 2001, Stuehr, 1999, Vasquez-Vivar et al., 1998).

To date, three isoforms of NOS have been described (Duda *et al.*, 2004, Knowles *et al.*, 1994):

1. neuronal NOS (nNOS), type I NOS, mediates transmission of neuronal signals
2. inducible NOS (iNOS), type II NOS, is transcriptionally regulated by inflammatory cytokines; is Ca^{2+} independent and produces high levels of NO which results in cytostatic or toxic effects
3. endothelial NOS (eNOS), type III NOS, constitutively expressed in vascular ECs, Ca^{2+} dependent and produces low levels of NO

There are a number of important similarities and differences between the 3 NOS isoforms. They all require BH4 as an essential cofactor (Cosentino *et al.*, 1999). The two constitutively expressed Ca^{2+}/Calmodulin dependent enzymes, eNOS and nNOS, produce NO in short bursts at very low concentrations (nM). The inducible enzyme iNOS produces NO in high concentrations (μM) as long as the enzyme is activated. The activity of iNOS is independent of Ca^{2+} and normally inflammatory cells express iNOS after stimulation by cytokines and other inflammatory mediators – however in lung epithelium and the distal tubulus of the kidney iNOS is expressed constitutively (Andrew et al., 1999, Hecker et al., 1999).

The expression of iNOS and the resulting excess production of NO have been implicated in the vascular complications of diabetes (Goldstein et al., 1996, Tilton et al., 1993). Increased iNOS levels have been associated with blood retinal barrier breakdown (Carmo *et al.*, 2000b, Leal *et al.*, 2007). In early DR, iNOS is the dominant isoform of NOS and is responsible for increased leukocyte adhesion by a mechanism involving ICAM-1 upregulation and tight junction downregulation, PKC activation and reactive oxygen species generation (Leal *et al.*, 2007). In proliferative DR, iNOS was reported to mediate retinal cell apoptosis (Sennlaub et al., 2002) and to modulate EPCs and resident EC function (Ellis *et al.*, 2005b). While high levels of NO may be harmful (iNOS) (Hernandez-Pando et al., 2001), low levels of NO (from eNOS and nNOS) may be protective (Heeringa et al., 2002).

1.3.3.3 eNOS function under normal and pathological conditions

Although eNOS is mainly expressed in endothelial cells (Palmer *et al.*, 1988, Palmer *et al.*, 1987) it is also found in platelets (Radomski *et al.*, 1990), smooth muscle and cardiac myocytes (Balligand *et al.*, 1995), bone cells (Armour *et al.*, 2001, Fox *et al.*, 1998) and neurons (Bredt *et al.*, 1990, Dinerman *et al.*, 1994). eNOS is an important factor involved in neovascularisation, vessel wall tension regulation, platelet aggregation, vascular permeability and leukocyte-endothelial interaction (Fukumura et al., 1998, Moncada, 1992) and EPC mobilisation (Gallagher *et al.*, 2007b). The expression and phosphorylation of eNOS are essential for the survival, migration and angiogenesis facilitated by EPCs and ECs (Dimmeler *et al.*, 2000, Urbich *et al.*, 2002b).

The importance of eNOS for EPC function was demonstrated experimentally *in vitro* in human EPCs engineered to overexpress eNOS. These cells had increased migratory potential, a higher ability to differentiate into endothelial spindle like structures and an increased ability to incorporate into tube-like like structures (Kaur et al., 2009). The expression of eNOS in EPCs is not uniform over time. eNOS expression is absent in very early EPCs and is increased as EPCs get more mature so that mature ECs have the highest levels of eNOS expression (Dernbach *et al.*, 2004, He *et al.*, 2004a, Thum *et al.*, 2006).

The enzyme eNOS is a dimer consisting of two identical 134kD monomers but only a dimeric form is fully functional (Albrecht *et al.*, 2003). The human gene is located on chromosome 7q35-36 and contains 26 exons at a total size of ~21kb (Albrecht *et al.*, 2003). Two monomers bind a haem (Klatt et al., 1993) and form a dimer to which tetrahydrobiopterin (BH4) can bind, leading to stabilisation of the dimer (List et al., 1997, Venema et al., 1997). BH4 itself promotes formation of active NOS homodimers (Tzeng et al., 1995). Furthermore, zinc ions are required to stabilize the BH4 binding site (Raman et al., 1998). Each eNOS monomer can bind one BH4, the complete enzyme consequently has two BH4 (Albrecht *et al.*, 2003). The production of NO depends on the amount of BH4 bound: if no BH4 is bound, only superoxide (O_2^-) is produced, when one BH4 is present NO and (O_2^-) are produced and only when two BH4 are bound is NO produced exclusively (Albrecht *et*

al., 2003), hence BH4 if available reduces superoxide production by "uncoupled" eNOS (Vasquez-Vivar et al., 1998, Wever et al., 1997).

Although it is still not clear where exactly eNOS is located within the cell, it seems likely that it is found in caveolae (Feron, 1999). When caveolin binds to eNOS, eNOS is inactivated by caveolin interfering with the Calmodulin binding and the haem electron transfer (Andrew et al., 1999). If intracellular Ca^{2+} is increased, increased formation of Ca^{2+}/Calmodulin results in increased binding to eNOS and eventual dissociation of caveolin from eNOS, thus activating eNOS (Albrecht *et al.*, 2003). If, conversely, intracellular Ca^{2+} is decreased, then Ca^{2+}/Calmodulin binding to eNOS becomes weaker and caveolin can bind again to inactivate eNOS. The activating or inactivating capabilities of caveolin are of importance when a change in lipid composition in the caveolae occurs, such as in hypercholesterinaemia, which can result in altered activation of eNOS (Feron et al., 1999).

Activated eNOS can produce NO if oxygen, NADPH/NADH and L-Arginine are present (Albrecht *et al.*, 2003). The level of eNOS expression and the activity of the enzyme are influenced by numerous factors such as shear stress (Malek *et al.*, 1999, Uematsu *et al.*, 1995), mechanical forces (Ziegler *et al.*, 1998), hypoxia (Hoffmann *et al.*, 2001, Le Crás *et al.*, 1996), estrogens (Ruehlmann *et al.*, 2000), LDL (Ramasamy et al., 1998), NO (via a positive feedback loop) (Joussen *et al.*, 2002b) and diabetes (De Vriese et al., 2001). Upregulation of eNOS in diabetic retinas due to increased levels of VEGF is well documented (do Carmo *et al.*, 1998a, Joussen *et al.*, 2001b, Joussen *et al.*, 2002a, Joussen *et al.*, 2002b, Radisavljevic *et al.*, 2000, Takeda *et al.*, 2001).

It has been reported that oxidised low density lipoprotein (oxLDL) and lipophosphatidylcholine impair eNOS activity (Hirata et al., 1991, Inoue et al., 1992, Miwa et al., 1997). As eNOS function is essential for survival and function of EPCs, increased levels of oxLDL reduce EPCs numbers and function in a time- and dose-dependent manner (Wang *et al.*, 2004e). Moreover, oxLDL inhibits VEGF-induced EPC differentiation *via* the AKT phosphorylation pathway (Imanishi et al., 2003) and accelerates EPC ageing (Imanishi et al., 2004). These deleterious effects of oxLDL could be abolished by pre-treatment with the statin atorvastatin (Imanishi et al., 2003, Imanishi et al., 2004).

In conditions such as hypercholesterinaemia and high LDL, upregulation of caveolin has been described – leading to an increase of the caveolin-eNOS complex

thus reducing the NO production in ECs (Feron et al., 2001, Feron et al., 1999). Endogenous eNOS inhibitors such as asymmetric dimethylarginine (ADMA) or N-monomethylarginine (NMA) also tend to increase vascular tone by reducing NO levels (Cooke, 2000, Miyazaki et al., 1999).

Reduced eNOS expression has been linked to hypertension (Panza, 1997), hypercholesterinaemia and atherosclerosis (Blair et al., 1999, Kuhlencordt et al., 2001), diabetes (Oyadomari et al., 2001), heart failure (Watanabe et al., 2000) and poor wound healing (Lee et al., 1999). Conversely, experimentally induced overexpression of eNOS in ECs increased the vasculoprotective effects of reconstituted endothelium after vascular injury (Kong *et al.*, 2004b).

As mentioned, the process of eNOS activation is generally mediated *via* increasing intracellular Ca^{2+} levels, but other mechanisms such as activator protein 1 (AP1, a transcription factor) activation (Hoffmann *et al.*, 2001) have been described.

1.3.3.4 eNOS function in angiogenesis and vasculogenesis

eNOS is involved both in angiogenesis and vasculogenesis (Duda *et al.*, 2004, Fukumura *et al.*, 2001). Angiogenesis under pathological conditions such as ischaemia or inflammation (Carmeliet et al., 2000) involves many angiogenic factors, with NO taking a lead role (Duda et al., 2004). It was reported that NO alone is able to induce endothelial migration (Duda et al., 2004). The critical role of VEGF with respect to vasculogenesis and angiogenesis (Ferrara et al., 2003) is better appreciated when it is known that VEGF activates eNOS by stimulating intracellular Ca^{2+} influx, recruitment of heat shock protein 90 (HSP90) and eventually phosphorylation of eNOS at serine (position 1,177) *via* the PI3K/AKT pathway (Fulton et al., 1999, Govers et al., 2001). In hypoxia eNOS is activated (phosphorylated) through the PI3K/AKT pathway after binding of increased levels of VEGF to VEGFR2 (Dimmeler et al., 1999, Fulton et al., 1999).

This activation of PI3/AKT in turn may prevent death of EPCs (Kim et al., 2000) by increased NO production through an activation of eNOS (Fulton et al., 1999). This underlines the pivotal role of the PI3K pathway in EPC regulation (Chen et al., 2007, Xia et al., 2008). EPCs with a defect in PI3K ($PI3K^{-/-}$) show defects in proliferation,

survival, integration in to endothelial networks and migration towards SDF-1 (Madeddu et al., 2008).

While VEGF triggers eNOS gene transcription and hence results in increased NO production, NO itself can downregulate VEGF (Bouloumie et al., 1999, Ghiso et al., 1999), indicating the likely presence of a negative feedback mechanism. This feedback mechanism was reported to be involved in the regulation of postnatal EPC mobilisation from the BM and neovascularisation formation (Asahara *et al.*, 1999b) through eNOS dependent effects (Aicher et al., 2003).

The importance of eNOS in the processes of angiogenesis and vasculogenesis has raised interest in the therapeutic potential of manipulating it (Jain, 2003). Selective block of eNOS function with cavtratin inhibited angiogenesis and subsequently tumour progression in a mouse models of Lewis Lung Carcinoma and Human Hepatocarcinoma (Gratton et al., 2003). Not surprisingly, reduced activation of eNOS by cavtratin also resulted in a reduced mobilisation of EPCs from the BM (Gallagher *et al.*, 2007b).

The critical involvement of eNOS in EPC mobilisation and angiogenesis was reported experimentally in a mouse model of eNOS deficiency ($NOS3^{-/-}$). These mice showed markedly decreased MMP9 activity and membrane KIT-ligand cleavage leading subsequently to reduction of sKIT-ligand (Aicher et al., 2003). The basal numbers of EPCs in control mice and eNOS deficient mice were similar, however only control mice could mobilise EPCs into the PB after stimulation with VEGF, which did not facilitate mobilisation in eNOS deficient mice (Aicher et al., 2003). Transplanted eNOS deficient EPCs could not restore function in an ischaemic hind limb model (Aicher et al., 2003) whereas EPCs from control mice could restore circulation, improve capillary density and incorporate into newly formed vessels. The cultured eNOS deficient EPCs formed fewer colonies and proliferated more slowly (Aicher et al., 2003). Interestingly, typical stem cell regulatory factors beta1-integrin CD29 (Prosper et al., 1998), adhesion molecules such as CD44 (Vermeulen et al., 1998) and the chemokine receptor CXCR4 (Peled et al., 1999) were not altered in eNOS deficient mice.

It was furthermore reported that, apart from mobilising EPCs from the bone marrow, eNOS activation is crucial for basal membrane degradation (Aicher et al., 2003, Murohara et al., 1998). This observation lead to the suggestion that eNOS is

essential for a full EPC function, for both angiogenesis and vasculogenesis (Aicher *et al.*, 2003, Lyden *et al.*, 2001).

Another study in NOS3$^{-/-}$ mice found poor wound healing and a poor response to growth factor stimulated angiogenesis (Lee et al., 1999). Further effects of an absence of eNOS activity on the vasculature were reported in double knockout mouse models (apoE/eNOS) where mice showed hypertension, increased and accelerated formation of atherosclerotic lesions, enhanced smooth muscle proliferation and increased platelet aggregation (Knowles et al., 2000, Kuhlencordt et al., 2001).

Similar to the knockout mouse model, pharmacological inhibition of eNOS using N-(G)-nitro-L- arginine methylester (L-NAME), which blocks the arginase, showed similar results of reduced eNOS function (Pfeiffer et al., 1996). Loss of eNOS function also resulted in increased microvascular permeability and protein leakage (Kubes et al., 1992). Given this, it is clear that eNOS has vasoprotective functions which become relevant in conditions damaging vessels such as inflammation (Heeringa et al., 2000), arterial hypertension (Huang et al., 1995), glucose and lipid disorders (Duplain et al., 2001).

1.3.3.5 eNOS polymorphisms and associations with vascular dysfunction

A number of eNOS polymorphisms have been described in humans (Albrecht *et al.*, 2003, Miyamoto *et al.*, 1998b, Nakayama *et al.*, 1999). In most cases a single nucleotide substitution was observed which did not affect the normal function of the enzyme. More significant polymorphisms, however, may affect the function of eNOS (Albrecht et al., 2003). Studies in humans reported that a change in intron 13 is associated with a higher risk of coronary artery disease (Stangl et al., 2000), and patients with diabetes type 1 who have a change in intron 4 have a higher risk of developing diabetic nephropathy (Zanchi et al., 2000) and a higher progression rate of IgA nephropathy (Morita et al., 1999). Single nucleotide changes in the exon 7 (Wattanapitayakul et al., 2001) could lead to a changed eNOS protein but whether this increases the risk of developing coronary artery disease or hypertension remains controversial. No alteration in eNOS function or disease correlations were reported for changes occurring in the promoter region.

In Japanese type 2 diabetic patients three polymorphisms were studied with respect to their association with DR and macular oedema (Awata *et al.*, 2004). The different polymorphisms of eNOS were not associated with the presence of DM, presence of DR or the severity (non proliferative / proliferative) of DR.

1.3.4. Tetrahydrobiopterin (BH4) and eNOS function

The importance of BH4 was already outlined in the previous section, where the function of BH4 as a cofactor for eNOS and its effects on the production of superoxide (O_2^-) were discussed (Albrecht *et al.*, 2003). Lack of BH4 results in uncoupling of eNOS which results in a production of superoxide anions instead of NO (Cosentino et al., 1997), thus making BH4 an essential cofactor for eNOS to produce NO.

1.3.4.1 BH4 and its function under physiological and pathological conditions such as DM or oxidative stress

The following section will give a further introduction to BH4 and its impact on endothelial function and production of NO under normal and pathological conditions. The importance of BH4 availability with respect to oxidative stress was briefly discussed in the section about eNOS function under normal and pathological conditions. BH4 is endogenously produced de novo by the enzyme Guanosine 5'-Triphosphate Cyclohydrolase (GTPCH) (Shinozaki et al., 2000) and the activity of GTPCH is the limiting factor in the biosynthesis process (Thony *et al.*, 2000). The activity of GTPCH is increased by inflammatory cytokines such as TNFalpha and IL1beta, a reduction of activity is caused by oxidised low density lipoproteins (oxLDL) (Kawashima *et al.*, 2004) and by high glucose – as reported in an animal model of insulin resistance (Shinozaki et al., 2000).

BH4 availability is critical as this can easily be changed, e.g. in DM. In normal vessels the endothelium contains approximately 60% of the total BH4 (Katusic, 2001, Tiefenbacher et al., 2000) whereas endothelial cells from diabetic rats show a marked reduction of BH4 (Meininger et al., 2000). Reduced levels of BH4 were also found in aortas from an insulin-resistance model (high fructose diet) in rats (Shinozaki et al.,

1999) and levels of oxidized forms of BH4 (7,8dihydrobiopteridin and biopterin) were significantly higher in diabetic versus nondiabetic animals (Kawashima *et al.*, 2004).

When BH4 levels are reduced, NOS may become "uncoupled", producing superoxide rather than NO (Stroes et al., 1998, Vasquez-Vivar et al., 1998, Wever et al., 1997, Xia et al., 1998). With "uncoupled" NOS the electrons that would normally be transferred from the reductase domain to the haem domain are diverted from the L-Arginine to the Oxygen thus generating superoxide – this has been reported for both nNOS and eNOS (Pou *et al.*, 1992, Schmidt *et al.*, 1992b).

This tight relationship between BH4 levels and NO production was reported in endothelial cells. It seems that optimal concentrations of BH4 levels are required for normal NO production. It was suggested that BH4 donates electrons from the reductase domain to the ferrous-dioxygen complex in the oxygenase domain – although the precise mechanism it not known (Bec et al., 1998, Vasquez-Vivar et al., 2002b). The relationship of BH4 levels and NO / superoxide production could be tested experimentally in endothelial cells: it was reported that exogenous supplementation of BH4 increased NO levels and decreased superoxide production, thereby shifting the "balance" towards NO and away from superoxide (Wever et al., 1998).

To understand the impact of BH4 on oxidative stress in pathological conditions, a brief overview on superoxide production in ECs has to be given. Superoxide anions can be generated by a number of enzymes. NADPH, which is a cofactor for eNOS in the generation of NO, is present in endothelial cells where, besides other enzymes such as xanthine oxidase and cyclooxygenase, it is an important generator of superoxide (Griendling *et al.*, 2000, Inoue *et al.*, 1998, Zalba *et al.*, 2005). The superoxide produced by NADPH seems itself to be involved in the uncoupling of eNOS (Kawashima *et al.*, 2004).

A different way for superoxide production is via eNOS, which is regulated by the availability of BH4. This eNOS-dependent production of superoxide resulting from decreased availability of BH4 (Cosentino *et al.*, 1999) was reported in situations such as hyperglycaemia (Cosentino *et al.*, 1997), hypertension, hypercholesterolemia, smoking, and ischemia-reperfusion (Katusic, 2001, Tiefenbacher, 2001) and atherosclerosis (Wever *et al.*, 1998). Superoxide production via the sorbitol pathway also contributes to oxidative stress in DR (Ellis *et al.*, 2005a, Williamson *et al.*, 1993).

Although NAPDH is responsible for a great amount of the superoxide generated in ECs and EPCs, the potential production of superoxide by eNOS uncoupling becomes very relevant in disease conditions such as diabetes or atherosclerosis (Cosentino et al., 1999, Katusic, 2001, Mugge et al., 1991, Ohara et al., 1993, Ohara et al., 1995, Rikitake et al., 2001, Sorrentino et al., 2007, Tiefenbacher, 2001, Wever et al., 1998).

Endothelial dysfunction is induced by oxidative stress and the resulting reduced NO availability (Cai et al., 2000). In ApoE-knockout mice, for instance, where a marked cholesterol level led to the rapid formation of atherosclerotic plaques, it was reported that superoxide was produced by eNOS resulting in impaired endothelial derived relaxation of the vessels (Kawashima *et al.*, 2004). Also in diabetic EPCs it was reported that uncoupling and increased production of superoxide led to a decrease in function and ability to migrate (Thum *et al.*, 2007). It can hence be concluded that the required functionality of eNOS is only available under normoglycaemic conditions. In a rabbit model of hypercholesterinaemia, administration of a precursor of BH4 – sepiapterin – led to a reduction of superoxide and vessel relaxation was improved. However, this effect seemed to be of only short duration and dose dependant and may even have contradictory effects (Vasquez-Vivar et al., 2002a): high levels of sepiapterin are pro-oxidative and can change BH4 into BH2 – the increased BH2 levels then competed with BH4 for eNOS-binding and hence exacerbated eNOS uncoupling (Bec et al., 1998).

As well as the absolute availability of BH4 it was proposed that the ratio of BH4/BH2 may affect the production of NO versus superoxide from uncoupled eNOS (Shinozaki et al., 2001, Shinozaki et al., 1999). Oxidative stress facilitates oxidation of BH4 and reduces levels of BH4. Oxidized analogues of BH4 accelerate superoxide production, thus oxidative stress causes uncoupling of eNOS by decreasing BH4 and by changing the BH4/BH2 ratio (Laursen et al., 2001). Apart from the shift of the BH4/BH2/ratio, the increased superoxide production in humans and animals with atherosclerosis (Mugge et al., 1991, Ohara et al., 1993, Ohara et al., 1995, Rikitake et al., 2001) is perhaps also influenced by decreased transcription of eNOS caused by instability of the eNOS mRNA due to pre-existing oxidative agents (Oemar et al., 1998).

1.3.4.2 Effects of BH4 supplementation and modulation of enzymes involved in BH4 function

Mature ECs are much less resistant to oxidative stress than EPCs (He *et al.*, 2004a). Studies of external BH4 supplementation reported improvement of endothelial dysfunction in vessel rings of diabetic animals (Hink et al., 2001, Shinozaki et al., 1999), and of animals with atherosclerosis (Laursen et al., 2001) as well as in mammary artery rings from patients with diabetes (Guzik et al., 2002). Acute BH4 administration seemed to improve NO-mediated effects on blood flow in the forearm of patients with diabetes (Heitzer et al., 2000) or hypercholesterinaemia (Stroes et al., 1997).

The true relationship between exogenously administered BH4 and endothelial BH4 levels and eNOS regulation *in vivo* remains unclear. High BH4 levels themselves may have nonspecific antioxidative effects that increase NO bioactivity indirectly by ROS-scavenging rather than eNOS modulation. The impact of total BH4 or the balance BH4/BH2 needs to also be considered (Vasquez-Vivar *et al.*, 2002b).

The provision of antioxidants such as vitamin C can enhance NOS activity by increasing its reaction rate without effects on L-Arginine. Based on this some authors suggested that saturated vitamin C levels in ECs are necessary to protect BH4 from oxidation and to allow for regular NO production (Carr et al., 2000, Heller et al., 2001, Huang et al., 2000, Kuzkaya et al., 2003). This hypothesis was subsequently verified experimentally (Drexler, 1997, Ito et al., 1998). Especially in diabetic patients, but also in smokers and patients with anaemia, vitamin C is effective in restoring the relaxation function of vessels (Levine et al., 1996, Ting et al., 1996, Ting et al., 1997). Other options to optimise eNOS function by supplementing enough substrate for NO production such as in chronic treatment with L-Arginine, results in reduction of atherosclerotic lesions (Aji et al., 1997, Candipan et al., 1996) whereas inhibiting NOS (with L-NAME) increases progression of atherosclerosis (Holm et al., 1997, Kauser et al., 2000).

In an animal model overexpressing GTPCH solely in the endothelium it was reported that BH4 levels were augmented in the endothelium of STZ diabetic mice (Alp et al., 2003). Improved vascular function and decreased superoxide production were also observed. This study also reported that diabetes led to a conversion of almost all BH4 to BH2 and a great increase in superoxide, changes which were not

found in diabetic animals overexpressing GTPCH. Overexpression of GTPCHI in cultured ECs was reported to be sufficient to augment BH4 levels (Cai *et al.*, 2002b). It seems that for mature ECs, supplementation with BH4 or antioxidants may improve eNOS function and reduce oxidative stress.

Statins play a role in EPC mobilisation. They were also reported to increase GTPCHI in ECs resulting in increased BH4 (Hattori et al., 2003). Statins have also been reported to increase eNOS expression (Laufs *et al.*, 1998a) resulting in increased NO production and improved eNOS function in EPCs (Laufs *et al.*, 1997, Laufs *et al.*, 1998a). Simvastatin administration was not only reported to increase eNOS expression and to decrease P-selectin expression (Lefer, 2002) – signifying a strong anti-inflammatory effect – but also to increase the half-life of eNOS mRNA from 13 to 38 hours (Laufs *et al.*, 1998a). As mentioned before, statins are also able to reduce negative effects of oxLDL on EPCs (Imanishi et al., 2003, Imanishi et al., 2004). Therefore, statins can improve EPC ageing and promote proliferation (Assmus et al., 2003)

Another effect of statins in improving NO availability is their action on RHO - an endogenous inhibitor of endothelial NO generation. Inhibition of RHO by preventing the RHO geranylgeranylation using e.g. simvastatin increases eNOS expression and NO production (Laufs et al., 1998b). These effects occur additionally to the direct activation of the AKT kinase resulting in enhanced eNOS phosphorylation and NO generation via eNOS (Kureishi et al., 2000). This was experimentally verified by blocking the PI3K/AKT pathway with LY294002 (Li et al., 2009) – when the PI3K/AKT pathway was blocked, eNOS phosphorylation was blocked as well (Li et al., 2009)

1.3.5. Erythropoietin in the regulation of eNOS and EPC function

EPO has been reported to have neuroprotective properties (Ghezzi *et al.*, 2004, Grimm *et al.*, 2005, Grimm *et al.*, 2002, Kilic *et al.*, 2005, Li *et al.*, 2004, Rex *et al.*, 2004, Tsai *et al.*, 2005). EPO is also involved in the regulation of EPCs and impacts eNOS function. The following section will give an overview on EPO and its involvement in EPC physiology.

The effects of EPO are numerous: within the BM, EPO stimulates erythrocyte production and acts as an oxygen-regulated pleiotropic growth factor for EPCs as well (Sasaki, 2003). EPO has pro-angiogenic properties and promotes revascularisation (Marti, 2004, Wang *et al.*, 2004d) and microvascular remodelling (Haroon et al., 2003). Steps in EPO-regulated angiogenesis include phosphorylation of eNOS (Heeschen et al., 2003, Santhanam et al., 2005) and mobilisation of EPCs (Calvillo et al., 2003, d'Uscio et al., 2007, Hirata et al., 2006, Landmesser et al., 2004, Urao et al., 2006).

To date several recombinant EPOs are available, among them Epoetin Alfa and Darbepoetin Alfa (Steensma *et al.*, 2006), Epoetin Delta (Smith *et al.*, 2007) and Epoetin Beta (Storring *et al.*, 1998). A number of experiments with these recombinant variants of EPO were performed to test their ability to improve EPC function and vascular remodelling. In a mouse model of ischaemic retinopathy (McVicar *et al.*, 2010), all forms of EPO could increase the number of SCA1$^+$ cells significantly, though only Epoetin Delta could normalise the damaged retinal vasculature whereas Epoetin Beta increased pathological vascularisation (McVicar *et al.*, 2010).

Levels of EPO need to be fine-tuned because abnormally high levels can result in pathological neovascularisation. It was reported that exogenous EPO administration induces such pathological neovascularisation and may produce severe systemic side effects (Lappin et al., 2007). In patients with PDR, high intravitreal levels of EPO were detected (Watanabe *et al.*, 2005). In other patient groups high systemic levels of EPO were associated with cardiovascular disease and tumour growth (Bohlius et al., 2005, Bohlius et al., 2006, Singh et al., 2006, Wright et al., 2007).

This emphasises the link between EPO and neovascularisation and the role of EPO in EPC mobilisation – which was studied in animals. In a mouse model of hypoxia induced retinopathy (Chen et al., 2008) it was reported that EPO increases CD34$^+$ cells in the retina by 80% and that EPO activates NFkappaB pathways. Other studies showed similar results as well as the ability of EPO to mobilise EPCs (Bahlmann *et al.*, 2004, Bahlmann *et al.*, 2003, Chong *et al.*, 2002, Heeschen *et al.*, 2003, Hirata *et al.*, 2006, Urao *et al.*, 2006, Westenbrink *et al.*, 2007). This mobilisation of EPCs by EPO may explain the involvement of EPO in the wound healing process (Heeschen et al., 2003).

Although patients with DR were reported to have higher systemic levels of EPO (Lee *et al.*, 2006a), intravitreal administration of EPO had beneficial effects.

Reduction of exudates (Berman et al., 1994) and protection of both vasculature and neurons in DR, possibly by an anti-VEGF effect (Xu *et al.*, 2009, Zhang *et al.*, 2010, Zhang *et al.*, 2008), have been reported. Astonishingly, a possible worsening of DR due to growth of RNV in these patients did not occur nor did systemic side effects.

1.3.6. Diabetes related changes in EPCs

1.3.6.1 Introduction to EPC changes related to various conditions

Before the changes within EPCs in DM are discussed it has to be kept in mind that a range of factors and conditions influence the number and function of EPCs – which may occur in association with or independently of DM.

As well as hyperglycaemia (Ingram et al., 2008), several other conditions such as smoking, physical exercise, age and gender can alter EPCs both in number and function. For example, it was reported that EPCs from middle-aged postmenopausal woman have a higher colony forming and migratory activity than in men of the same age-group (Hoetzer et al., 2007a, Hoetzer et al., 2007b). In sedentary adult men, age inversely correlated with EPC clonogenic and migratory capacity (Hoetzer *et al.*, 2007b, Vasa *et al.*, 2001b). With increasing age a decrease in EPC function was observed in otherwise healthy patients despite normal EPC numbers in PB (Heiss et al., 2005). Physical exercise for about 30 min increases EPC numbers and function also in healthy individuals (Laufs *et al.*, 2005) and in patients with coronary artery disease (Laufs *et al.*, 2004).

Cigarette smokers were reported to have fewer EPCs (Kondo *et al.*, 2004) in PB. It was reported that smoking impairs EPC function (Michaud *et al.*, 2006). EPC number and the number of cigarettes smoked correlate inversely (Kim *et al.*, 2006b). Cessation of smoking rapidly increased EPC numbers: 4 weeks after stopping, levels of EPCs in PB were comparable to normal subjects. Light smokers were reported to show a greater recovery in EPC numbers as compared to heavy smokers which may be related to the differing nicotine levels found in the blood of light and heavy smokers (Kondo *et al.*, 2004). Interestingly, nicotine can increase EPC numbers at levels of 10^{-12} to 10^{-8}mol/l *in vitro* but is cytotoxic at 10^{-6} mol/l (Liew et al., 2008).

Hypercholesterinaemia as an influence on EPCs was briefly mentioned. Patients with hypercholesterinaemia have a reduced number of EPCs compared to healthy

subjects and an inverse correlation of EPC numbers to the total level of LDL cholesterol could be demonstrated (Chen *et al.*, 2004b). EPCs isolated from subjects with hypercholesterinaemia show impaired function even when cultured in normal media (Chen *et al.*, 2004b). Also, high density lipoproteins (HDL) affect EPC numbers and their function: *in vitro* HDL increases eNOS expression and inhibits EPC apoptosis (Pellegatta et al., 2006). Patients with low HDL were reported to have low EPC counts (Pellegatta et al., 2006); administration of HDL in mice augmented EPC recruitment for endothelial repair (Tso et al., 2006).

These examples show that in many conditions, and in normal and healthy individuals, number and functions of EPCs differ.

1.3.6.2 DM related changes in EPCs

Diabetic patients have fewer EPCs than non-diabetic patients. Diabetic EPCs are significantly reduced in their capacity for tube formation *in vitro*. Despite culturing them in normoglycaemic conditions, impairment in EPCs from people with diabetes remains.

Until recently, it was generally accepted that EPCs are reduced in numbers and are dysfunctional (reduced ability to form tubes, proliferate, incorporate into damaged endothelium) in people with diabetes type 1 (Loomans et al., 2004) and type 2 (Capla *et al.*, 2007, Chen *et al.*, 2007, Fadini *et al.*, 2005, Fadini *et al.*, 2006a, Fadini *et al.*, 2006c, Gallagher *et al.*, 2007a, Hill *et al.*, 2003, Loomans *et al.*, 2004, Tepper *et al.*, 2002). It is believed that this dysfunction is related to decreased eNOS activity and NO availability (Aicher *et al.*, 2003, Ding *et al.*, 2000, Guo *et al.*, 2000, Lloyd *et al.*, 2001). This decreased EPC function was associated with increased apoptosis in high glucose environments (Balestrieri *et al.*, 2008b, Chen *et al.*, 2009a). Later it will be discussed that these assumptions may not be true in patients with DR.

It was reported that hyperglycaemia alters the redox-state intracellularly which is associated with EC dysfunction and vascular complications (Srinivasan et al., 2004). As well as increased aldose reductase activity and activation of PKC, oxidative stress contributes to endothelial dysfunction in diabetic individuals (Chakrabarti et al., 2000, Kowluru, 2001, Stitt et al., 1997, Xia et al., 1994). The increased oxidative stress found in diabetic patients e.g. due to ROS further contributes to the vascular

complications (Baynes, 1991, Jennings *et al.*, 1991, Kowluru *et al.*, 2001), changes of the vessel structure (Cai *et al.*, 2000, Suh *et al.*, 1999) and to the diabetes related dysfunction of EPCs and their reduction in PB (Sorrentino *et al.*, 2007, Thum *et al.*, 2007). The effects of ROS on EPCs (in contrast to ECs) seem however not as prominent as the effects of eNOS impairment or NO reduction – the experimental supplementation of antioxidants did not significantly reverse the effects of high glucose on EPCs (Chen et al., 2007). The lower impact of ROS compared with reduced eNOS/NO availability on EPCs may be explained by an intrinsic antioxidant system within human EPCs (Dernbach et al., 2004) which increases their ability to resist better direct oxidative stress (He *et al.*, 2004a).

The direct effect of high glucose on EPCs is sill controversial. *In vivo* studies using EPCs from healthy volunteers showed a reduction in number and function when put in high glucose medium (Chen *et al.*, 2007, Ii *et al.*, 2006, Seeger *et al.*, 2005). The exposure to high glucose in the culture medium resulted in a 33% reduction of EPCs and an increased rate of apoptosis based on free histones and caspase activity (Krankel et al., 2005). It was also reported that high glucose decreased NO production, eNOS expression, and AKT phosphorylation in both early and late outgrowth EPCs (Chen et al., 2007). These effects of high glucose could be partially reversed by the administration of a NO donor (sodium nitroprusside) or by adding a p38 MAPK inhibitor (Chen et al., 2007). Others studies suggested that high glucose did not increase apoptosis (based on Annexin V measurements) and that reduced EPC proliferation was due to activation of p38 MAPK (Seeger et al., 2005). Following these hypotheses it was reported that p38 MAPK activation accelerated ageing of EPCs (Kuki et al., 2006) and that these aged EPCs showed impaired function, decreased NO production and decreased MMP9 activity when put into high glucose culture (Krankel et al., 2005). Similar results were obtained when EPCs from diabetic rats (Tamarat et al., 2004) or mice (Ii et al., 2006) were studied. However, short-term exposure to high glucose did not seem to affect the homing of EPCs. Inhibition of the p38 MAPK pathway (Kuki et al., 2006, Seeger et al., 2005) and AKT/FOXO1 signalling (Marchetti et al., 2006) led to a restoration of hyperglycaemia-induced impairment of early EPC function. A different pathway that was reported to be involved in the accelerated aging of EPCs is the AKT - p53 - p21 pathway (Rosso et al., 2006).

As mentioned above, most studies on normal EPCs have been done on human material, i.e. EPCs were isolated from PB. The same applies to studies on diabetes-induced changes in EPCs. As is the case for research into surface markers of EPCs, in which conflicting results have sometimes been presented (Critser *et al.*, 2011, Yoder, 2009), the field of diabetes induced changes in EPCs is replete with paradoxical or even contradictory results (Brunner *et al.*, 2011, Brunner *et al.*, 2009, Fadini *et al.*, 2005, Fadini *et al.*, 2006b, Hill *et al.*, 2003, Jialal *et al.*, 2010, Liu *et al.*, 2010). One factor contributing to the confusion is the fact that the condition "diabetes" when used in experiments or studies is ill-defined. It comprises patients with quite different stages of the disease and different complications, hence it is not surprising that studies in patients with diabetes and peripheral arterial occlusive disease and arteriosclerosis (Hill *et al.*, 2003) show different results compared with those of patients with diabetic retinopathy (Brunner *et al.*, 2011, Brunner *et al.*, 2009). Another critical point naturally is the definition of EPCs, which also varies from study to study. Despite a certain degree of confusion, the mechanisms of diabetes related changes become more clear as more and more studies examining EPCs in diabetes from different angles become available.

1.3.6.3 Diabetes related changes in EPCs in patients with or without atherosclerosis and peripheral arterial disease including stroke and coronary heart disease but without DR

Endothelial cells are prone to be affected by hyperglycaemia-induced changes as they are in direct contact with glucose circulating in the blood. Glucose enters the retina by facilitated diffusion which cannot be regulated by the endothelium (Brownlee, 2005). The development of EPC dysfunction and the reduction of numbers in PB in people with diabetes seems to be a gradual process. Newly diagnosed patients with type 2 DM, a HbA1C of < 7.5% and no medical therapy have similar numbers of EPCs in the PB to non-diabetic controls (Pistrosch et al., 2005). In diabetic patients on insulin treatment, in whom diabetes had been diagnosed at least 1 year previously but who had no clinical evidence of complications such as DR, reduction of EPCs and their ability to adhere and differentiate was found (Loomans *et al.*, 2004).

Studies in patients with diabetes suffering from peripheral macrovascular disease (PAD), including coronary heart disease, showed that EPCs from diabetic individuals with PAD had more pronounced functional impairment with respect to proliferation, tube formation and integration into vascular networks compared with EPCs isolated from healthy controls (Schatteman *et al.*, 2000, Tepper *et al.*, 2002). Patients with diabetes and macrovascular complications such as PAD and coronary heart disease had lower numbers (~40% less) of EPCs in PB compared to healthy patients and the number of EPCs correlated with the degree of carotid stenosis and claudication (Fadini *et al.*, 2005, Fadini *et al.*, 2006a).

The observed reduction of number and function of EPCs in DM observed *in vitro* (Krankel et al., 2005, Seeger et al., 2005) was also reported *in vivo* (Fadini et al., 2005, Loomans et al., 2004, Tepper et al., 2002). For patients with both type 1 and type 2 DM it was found that the less well DM was controlled, the lower the numbers of EPCs that were detected in the PB (Fadini et al., 2005, Tepper et al., 2002) and the number of EPCs was inversely correlated with the fasting glucose levels (Fadini *et al.*, 2005) and HbA1C (Churdchomjan *et al.*, 2010, Loomans *et al.*, 2004, Tepper *et al.*, 2002). An even stronger reduction of EPCs is seen in patients with peripheral vascular complications compared with patients without such complications and levels of EPCs are inversely correlated with the severity of the disease (Fadini *et al.*, 2006a): the presence of PAD accounted for a reduction of 47% of EPCs in PB compared with controls (Fadini et al., 2005). Culturing isolated EPCs from the PB revealed a significant decrease in the clonogenic potential and adherence capacity in EPCs from diabetic patients with PAD but not in non-diabetic patients with PAD (Fadini et al., 2006a).

Changes in EPCs in the PB of patients with DM were also studied *in vitro*. EPCs from a diabetic environment showed an impaired potential for migration, proliferation and tube formation. Although phenotypical appearance of cultured EPCs from diabetic and nondiabetic individuals did not show clear differences, EPCs from diabetic patients took much longer to establish colonies compared with EPCs from non-diabetics. Despite good glycaemic control, the potential to migrate, proliferate and form tubes was lower in EPCs from people with diabetes compared with EPCs from nondiabetic individuals (Thum *et al.*, 2007). EPCs from diabetic patients were found to be more susceptible to high glucose concentrations with higher rates of apoptosis and lower viability (Churdchomjan et al., 2010). It seemed that

hyperosmolality due to high glucose did not significantly affect EPCs (Krankel et al., 2005). Hyperglycaemia could, however, induce dysregulated activation of transcription factors such as ETS (E-twenty six) which is important for proliferation, survival, and differentiation (Sharrocks, 2001), thus inducing a functional block of EPCs (Seeger et al., 2009). As mentioned, activation of PI3/AKT *via* VEGF prevented cell death in EPCs (Kim et al., 2000) by increasing eNOS activation, which facilitated EPC survival by increasing NO production (Fulton et al., 1999). Recently it was reported that activation of the PI3/AKT pathway also contributes to prevention of high glucose induced cell injuries (Ho et al., 2006). It was mentioned that diabetic EPCs have a higher superoxide content compared to non-diabetic EPCs. High glucose levels lead to a reduction of BH4 availability in the EPCs. The resulting uncoupling of eNOS due to high glucose and the resulting increase of superoxide was improved by supplementation of BH4 (Thum *et al.*, 2007).

In cardiovascular research the number of EPCs in the PB has been established as a predictor for cardiovascular complications (Hill *et al.*, 2003, Vasa *et al.*, 2001b). Reduction in EPC numbers was reported in non-diabetic patients who had suffered a stroke (Ghani et al., 2005). Conversely, higher EPC numbers are associated with lower risk of major cardiovascular events and hospitalisation (Werner et al., 2005). In DM, a low number of circulating EPCs confers higher risk for developing peripheral complications of DM (Fadini et al., 2006a). Based on these results, the number of circulating EPCs has been established as a good predictor and surrogate marker for cardiovascular events (including death), being even more robust than the Framingham cardiovascular risk score (Hill *et al.*, 2003, Schmidt-Lucke *et al.*, 2005, Werner *et al.*, 2005).

Other studies confirmed these results and found that EPC numbers and their function are already affected very early in the course of diabetes, cardiovascular disease (Fadini et al., 2007, Fadini et al., 2008a) and ischaemic cardiomyopathy (Kissel *et al.*, 2007, Vasa *et al.*, 2001a). Reports from non-diabetic patients with coronary artery disease and chronic myocardial ischaemia did not show any differences in EPC numbers compared with controls (Heeschen *et al.*, 2003) but EPCs had lower angiogenic potential than those of controls. This is of importance as it was reported that patients with severe hypertension and acute coronary syndrome have more apoptotic endothelial cells (Bernal-Mizrachi et al., 2003, Preston et al., 2003). The impairment of EPC function and the decrease in numbers of EPCs in PB was

reported both for diabetes type 1 (Loomans et al., 2004) and diabetes type 2 (Tepper et al., 2002) patients. It seems therefore that DM specifically affects EPC function.

As the number of EPCs was reported to be linked to cardiovascular events and the pathogenesis of diabetic vascular complications (Ding et al., 2005, Loomans et al., 2004, Tepper et al., 2002), administration of EPCs and ways to increase them pharmacologically in PB were proposed as treatments for diabetic vascular complications (Schmidt-Lucke et al., 2005, Werner et al., 2005). Therapies were proposed to enrich the EPC pool in ischaemic diseases and to block EPCs in pathological angiogenesis (Butler et al., 2005, Schachinger et al., 2004).

One of the first approaches to improve EPC function and numbers in people with diabetes was normalisation of blood glucose levels or good glycaemic control. This was reported to efficiently recover EPC function (Churdchomjan et al., 2010) and EPC numbers (Kusuyama et al., 2006), however even under optimal treatment conditions, pathological vascular changes could not be prevented in diabetic patients (Snyder, 2007). It was hence suggested that recovery of EPC function after improved glycaemic control was only partial, since there were more EPCs in the PB of patients with good glycaemic control than in those with poor glycaemic control, but the numbers in well controlled diabetic patients were still below the numbers observed in non-diabetic individuals (Churdchomjan et al., 2010). These findings are in accordance with experiments where EPCs from diabetic patients and did not show improved angiogenic capacity when they were cultured under normoglycaemic conditions (Loomans et al., 2004).

These observations indicate that in diabetes both function and mobilisation of EPCs is abnormal. Several factors such as SDF-1, VEGF or IGF-1 are able to trigger migration of EPCs – this response is impaired in diabetic patients (Caballero *et al.*, 2007, Segal *et al.*, 2006). The reduced number of EPCs in the PB of diabetic patients (Awad et al., 2005, Loomans et al., 2004) may be a result of the impaired response towards factors such as SDF-1 or VEGF leading to reduced mobilisation of EPCs from the BM (Awad *et al.*, 2005, Waltenberger *et al.*, 2000).

1.3.6.4 Diabetes related changes in EPCs in patients with DR

Reduction of number and function of EPCs (Fadini *et al.*, 2007, Fadini *et al.*, 2008a, Loomans *et al.*, 2004), including impairments in tube formation, adhesion and proliferation (Asnaghi *et al.*, 2006, Tepper *et al.*, 2002), have been reported in diabetic patients. It should be noted that most of the studies discussed above describing decreased number and function of EPCs were done in patients suffering from DM but who do not exhibit DR. In the area of EPC function in DR, results from the various studies are again often paradoxical or contradictory.

In diabetic patients with DR the situation regarding EPC function and number in the PB seems to be reversed compared with patients with DM but without DR. Numbers of EPCs were increased in the peripheral blood in the presence of proliferative DR (Asnaghi *et al.*, 2006, Brunner *et al.*, 2011, Brunner *et al.*, 2009, Fadini *et al.*, 2006b, Tan *et al.*, 2010). It was reported that type 2 diabetic patients who were categorised into groups regarding the presence of PAD and PDR showed decreased EPC numbers when PAD was present and increased numbers when PDR was present (Fadini et al., 2006b). These EPCs from patients with PDR were described to contribute to the development of retinal neovascularisation (Lee et al., 2006a). This increase was related to higher plasma levels of VEGF and substance P (Lee *et al.*, 2006a). EPCs from patients with DM and DR showed enhanced differentiation and increased clonogenic potential compared with those from patients with DM but without DR (Fadini et al., 2006b). This coexistence of ischaemia (e.g. coronary heart disease, PAD) and increased angiogenesis (e.g. proliferative DR) in diabetes (Abaci et al., 1999, Ciulla et al., 2003) is called the "diabetic paradox" (Fadini et al., 2006b).

The negative correlation of EPCs and HbA1C levels, which is observed in diabetics, is not found any more once DR is established (Asnaghi *et al.*, 2006). It was hypothesised that the reduced number of EPCs put patients at risk to develop DR, but once the damage is established in the retina a number of cytokines are produced that trigger a BM response which leads to increased EPCs in the PB – however these EPCs may possibly not be fully competent with respect to their ability to participate in neovascularisation. Hence, high numbers of EPCs in PB of patients with DM and DR were proposed as a risk factor for developing retinal neovascularisation (Murasawa et al., 2005).

More recent research (Brunner *et al.*, 2011, Brunner *et al.*, 2009) evaluated the relationship of EPC numbers in PB of diabetic patients with different stages of DR

according to the ETDRS classification (1991b, 1991d, 1991e, Davis *et al.*, 1998). It was reported that EPCs were in fact reduced in patients with mild nonproliferative DR and moderate-severe nonproliferative DR. EPC numbers were comparable to healthy controls in patients with mild to moderate proliferative DR and significantly increased in high risk proliferative DR (Brunner et al., 2009), i.e. with increasing severity of DR the EPC numbers in PB increased. These results further differentiated previous findings in type 2 DM patients with PDR who had increased peripheral EPCs (Fadini *et al.*, 2006b, Lee *et al.*, 2006a) compared to those without PDR (Lee *et al.*, 2006a) and specified that increased numbers of EPCs are found when active neovascularisation is present as in high risk PDR.

As well as the numbers, the function of EPCs has also been studied in the presence of DR. For EPCs isolated from both type 1 (Asnaghi et al., 2006) and type 2 (Fadini et al., 2006b) diabetic patients, increased clonogenic potential was reported. Although the increased clonogenic potential was demonstrated, the ability of EPCs from patients with proliferative DR to migrate and integrate into vascular networks was reported to be reduced (Tan et al., 2010). It was hypothesised that damage, or structural impairment, of EPCs – despite their increased clonogenic potential - is responsible for the late stage complications in DR rather than just the high number of EPCs as previously suggested (Caballero et al., 2007, Tepper et al., 2002). It is still not known whether the increased number of EPCs is a result or the cause of advanced DR. Increased levels of SDF-1 in the plasma of patients with proliferative DR may point towards the latter (Tan et al., 2010).

Based on the different findings in patients with DM and with or without DR or peripheral vascular disease, it has been proposed that EPCs may have different levels of differentiation and function depending on the type of vascular problem (Liu et al., 2010). Based on this underlying vascular problem, specific growth factors or mediators were suggested to be involved in activating EPCs and in directing the eventual development of pathological neovascularisations such as in DR (Awad *et al.*, 2006, Schatteman *et al.*, 2007, Wang *et al.*, 2004a).

1.3.6.5 Diabetes related studies on EPC mobilisation in animals

The knowledge that EPCs contribute to neovascularisation and repair endothelial damage led to a significant number of studies in animal models in which important questions regarding mobilisation of EPCs from the BM and tissue revascularisation potency of EPCs were evaluated.

A frequently used animal model is the ischaemic hindlimb model (Awad *et al.*, 2005, Couffinhal *et al.*, 1999, De Falco *et al.*, 2009, Duplain *et al.*, 2001, Emanueli *et al.*, 2002b, Fadini *et al.*, 2006c, Kureishi *et al.*, 2000, Lee *et al.*, 2006b, Liu *et al.*, 2010, Murasawa *et al.*, 2005, Yan *et al.*, 2009). In this model, the femoral artery and vein of a hind limb are excised or ligated resulting in severe ischaemia (Murohara *et al.*, 2000). If no neovascularisation or revascularisation occurs, the limb becomes necrotic. This model is often combined with a model of streptozotocin (STZ) induced diabetes (Rerup, 1970) to mimic DM related changes. Experiments in rats with hindlimb ischaemia showed that in non-diabetic animals a compensatory increase of EPCs in the PB occurred and consequently the capillary density in the muscles of the ischaemic hindlimb improved (Fadini *et al.*, 2006c). This could not be found in diabetic animals: no mobilisation of EPCs and no increase in capillary density occurred (Fadini *et al.*, 2006c). Administration of insulin in diabetic animals, treatment with granulocyte colony-stimulating factor (GCSF) and stem cell factor (SCF) resulted only a partial recovery of EPC mobilisation in the diabetic animals using this model (Fadini *et al.*, 2006c). It was hypothesised that in diabetes a mobilisation deficit is the main reason for decreased numbers of EPCs in PB. An experiment using the ischaemic hindlimb model in combination with a diabetes type 1 (STZ) and diabetes type 2 model ($Lepr^{-/-}$) in mice reported that the perfusion recovery after establishing the ischaemia was lower in both models compared to non-diabetic mice, however the type 2 diabetic mice had the lowest perfusion (Yan et al., 2009).

Both in type 1 and type 2 diabetic mice, EPCs were studied for their eNOS expression and EPC mobilisation. In the ischaemic hindlimb model, eNOS expression in diabetic animals did not increase while a significant increase of eNOS expression was observed in non-diabetic mice. Both type 1 and type 2 diabetic animals had reduced mobilisation of EPCs. Interestingly,diabetic mice with type 2 diabetes had less efficient integration of EPCs in newly formed vessels (Yan et al., 2009) compared with type 1 diabetic mice. An examination of the capillary density revealed that type 1 diabetic mice could increase their capillary density similarly to non-diabetic animals whereas no change in capillary density was seen in the type 2

diabetic animals. Overall it seems that the response to ischaemia is less effective in type 2 diabetic animals compared to type 1 diabetic animals (Yan et al., 2009). It is of note that in this study type 2 diabetic animals had a greater number of EPCs in the BM compared to non-diabetic mice and type 1 diabetic mice (Yan et al., 2009).

1.3.6.6 Transplantation of EPCs in experimental and clinical settings

Since the discovery of the therapeutic potential of EPCs to repair vascular damage, a number of studies were performed to evaluate feasibility, practicability and the effects of EPC transplantations.

As mentioned before, the ischaemic hindlimb model in animals is frequently used to study effects such as revascularisation and EPC mobilisation and integration into damaged vasculature. It is also suited to study the behaviour of transplanted EPCs and their ability to participate in neovascularisation and vessel reformation. As it is difficult to extract sufficient numbers of EPCs for transplantation from the PB of mice or rats, human EPCs from the peripheral blood have been isolated and then transplanted into athymic nude mice (Murasawa *et al.*, 2005).

EPCs were reported to be closely involved in neovascularisation in a mouse model of hindlimb ischaemia. Cultured human EPCs were reported to incorporate at sites of neovascularisation maximally at day 3-7 after transplantation and capillary density and the index of neovascularisation was markedly increased by the transplanted cells (Couffinhal et al., 1999). Other studies confirmed those findings and furthermore reported that blood flow was restored more quickly upon EPC transplantation and limb loss could be avoided (Kalka et al., 2000b). As reported in the seminal paper by Asahara (Asahara *et al.*, 1997), transplanted EPCs incorporated into capillary vessel walls of newly formed vessels specifically in the injured areas while none of the transplanted EPCs were found in the uninjured hindlimb.

Experiments using other models such as experimental myocardial infarction demonstrated similar beneficial effects of EPCs (Kawamoto et al., 2001): transplantation of human EPCs (isolated from the PB) three hours after experimental induction of myocardial infarction in rats resulted in accumulation of EPCs in the areas of ischaemia. The EPCs found there reduced scarring of the myocardium, increased capillary density in the affected muscle and improved left ventricular

function compared with animals without EPC transplantation (Kawamoto et al., 2001). This effect was not only observed with pre-cultured EPCs from humans but also freshly isolated EPCs from the PB (Kocher et al., 2001). It was estimated that approximately 50% of the transplanted EPCs contributed to the neovascularisation observed in the rat model of myocardial infarction (Kawamoto et al., 2001).

The positive effect of transplanting healthy EPCs on endothelium-dependent vasodilation and endothelial repair was further demonstrated in $ApoE^{-/-}$ mice, a model for atherosclerosis (Wassmann *et al.*, 2006). The effects of improved vascular function after the EPC transplantation could be observed for up to 45 days after the transplantation.

The promising results from studies in the ischaemic hindlimb model and the myocardial infarction model resulted in further studies evaluating the potential of EPCs in repairing retinal vascular damage. In mice with genetically determined retinal degeneration (rd/rd mice) and subsequent vascular degeneration, the transplantation of human EPCs from the PB prevented the naturally occurring degeneration of the retinal vasculature (Otani *et al.*, 2002). In mice with developing vasculature it was reported that approximately four days after intravitreal transplantation of human EPCs (from the PB), they were found adhering to the retina, in contact with astrocytes guiding the retinal vascular patterned development (Zhang et al., 1997) and were integrating into retinal vessels (Otani *et al.*, 2002). The integration into retinal vessels occurred either in cooperation with mature ECs or by establishing completely new vessels (Otani *et al.*, 2002). Transplantation into eyes of normal mice with developed vasculature did not result in any interaction with astrocytes or integration into retinal vessels.

The fate of intravitreally transplanted EPCs does not seem to be uniform. In a laser induced retinal injury model in mice, intravitreally transplanted EPCs (isolated from the BM of mice) migrated towards areas of vascular damage and integrated into the damaged tissue (Ritter *et al.*, 2006, Wang *et al.*, 2009). The EPCs could be observed as long as 4 weeks after transplantation (Wang *et al.*, 2009). Apart from their ability to repair the vascular damage, the transplanted cells from the BM could also differentiate into retinal pigment epithelium cells, microglia, pericytes and even photoreceptors – suggesting that EPCs from the BM have reparative capacity not only for the vasculature (Chan-Ling *et al.*, 2006, Ritter *et al.*, 2006, Wang *et al.*, 2009). This ability of cells isolated from the BM to differentiate into photoreceptors was

confirmed by other studies (Kicic et al., 2003, Minamino et al., 2005, Tomita et al., 2002). It seems that the isolation method to obtain EPCs is crucial, as authors using other isolation methods reported that transplantation of cells isolated from the BM can delay degeneration of retinal tissue without actually transforming into photoreceptors (Arnhold et al., 2007, Inoue et al., 2007).

Further studies evaluated whether EPCs from non-diabetic and diabetic individuals show different behaviour with respect to their ability to repair vascular damage. In diabetes, impaired vessel growth in the periphery and impaired vascular repair maybe found alongside neovascularisation e.g. in the eye (Fadini et al., 2006b). The ability of circulating EPCs in healthy subjects to differentiate to ECs, to contribute to vascular repair and to form vascular structures (Asahara et al., 1999a, Asahara et al., 1997, Grant et al., 2002, Peichev et al., 2000, Shi et al., 1998) is impaired in diabetic individuals (Caballero *et al.*, 2004, Ellis *et al.*, 2005b, Grant *et al.*, 2002, Segal *et al.*, 2006, Sengupta *et al.*, 2003). Furthermore, the reduced capabilities for proliferation, adhesion and deformation of EPCs from diabetic subjects (Segal et al., 2006, Tepper et al., 2002) as well as the inability to reverse the diabetes-induced defects by culturing isolated EPCs in a normoglycaemic environment (Loomans et al., 2004) were reported. The severely curtailed repair function and recruitment of diabetic EPCs in vascular injury models (ischaemic hindlimb, arterial injury, microvascular injury) was reported for both diabetes type I and type II models (Awad *et al.*, 2006, Ii *et al.*, 2006, Jiang *et al.*, 2004, Schatteman, 2004, Stepanovic *et al.*, 2003, Tepper *et al.*, 2002) – these experiments did however not include an evaluation of repair capabilities in retinal vascular damage like in DR.

Early studies in laser-induced retinal vein occlusion models in mice – where as a result of the retinal vein occlusion retinal neovascularisations occur - reported that specific transplantation of hematopoietic stem cells with EPC properties results in participation of these cells in vascular repair (Grant *et al.*, 2002). Further evaluations of the potential of EPCs to repair vascular damage of retinal vessels, which were made in animal models of oxygen induced retinopathy, diabetic retinopathy and ischaemia/reperfusion injuries, clearly demonstrated that EPCs isolated from the PB of nondiabetic humans were able to repair the vascular damage while EPCs from diabetic humans did not (Caballero *et al.*, 2007). The observation that non-diabetic EPCs were able to integrate and repair damage in diabetic animals was suggested to be due to an interaction with still functioning resident ECs which guide the EPC

supported repair. The formation of neovascularisation - a sign of aberrant regulation of vessel repair - may include the participation of transplanted EPCs and monocytes with EPC function (Kamihata et al., 2001, Wollert et al., 2004).

Apart from transplantation studies in animals, several clinical trials were performed to investigate the effects of increasing EPCs in the circulation or transplanting EPCs in patients with various vascular complications – however with conflicting results. As macrovascular complications are associated with fewer EPCs in the PB, increasing the number of circulating EPCs, e.g. by using GM-CSF, seemed indicated. However, results of the effects of GM-CSF administration on EPC mobilisation and the possible benefits, i.e. vascular repair, are mixed for patients with coronary heart disease (Hill et al., 2005, Zbinden et al., 2005). While EPCs could be mobilised in nondiabetic patients, the response in diabetic patients with chronic angina was very limited (Chih *et al.*, 2012, Kovacic *et al.*, 2008).

Further experiments such as autologous infusion of EPCs (from the PB) after limb ischaemia (Tateishi-Yuyama et al., 2002) and after myocardial infarction (Assmus et al., 2002, Strauer et al., 2002) were performed, results were however ambiguous. Other studies using BM derived EPCs in patients with myocardial infarction revealed either slight improvement of left ventricular function (Schachinger et al., 2006) or no effects on left ventricular function (Lunde et al., 2006). An overview on clinical trials using EPCs in patients is given by Liew (Liew *et al.*, 2008).

1.4 Animal models for DM

Two different mouse models of diabetes were used for experiments described in this thesis, a model in which diabetes develops spontaneously (Akita mouse) and a model where diabetes is induced pharmacologically (STZ model). As both models mimic effects of DM type 1 and do not show late stage complications such as RNV, a combination with a laser-induced retinal vein occlusion was employed to imitate changes occurring in late stage DR.

1.4.1. Streptozotocin induced DM

The streptozotocin (STZ) model is a model for type 1 diabetes (Rerup, 1970). Repeated administration of STZ results in insulinitis, i.e. inflammation of the insulin producing cells of the pancreas, resulting in a loss of function of beta cells and development of hyperglycaemia due to lack of insulin (Rossini *et al.*, 1977b).

After an overnight fast, animals receive STZ, freshly prepared at a concentration of 27.5 mg/ml in 0.1 M citrate buffer (pH 4.5), by intraperitoneal injection (i.p., 55 mg/kg bodyweight) for 5 consecutive days. Diabetes is established with this approach 6 days after the first injection of STZ. In some mouse strains, resistance to STZ develops, i.e. despite injecting STZ, no diabetes develops (Rossini *et al.*, 1977a). The response to the drug is also different with respect to gender: in male mice progression of the loss of beta cells is faster and more uniform than in female mice (Yoshioka et al., 1997).

1.4.2. Akita Model

Another model to study changes in DM is the Akita model (Barber *et al.*, 2005), which is also a model for type 1 DM. The Akita mouse carries a dominant point mutation in the Insulin 2 gene ($Ins2^{Akita}$) on chromosome 7 resulting in the development of diabetes at approximately 4 weeks after birth (Yoshioka *et al.*, 1997) with almost 100% penetrance (Wang *et al.*, 1999). The diabetes is the result of a progressive loss of beta-cell function and decreased pancreatic beta-cell density.

The model is based on the C57BL/6 background, has normal fertility and does not develop obesity (such as the LEPR mouse). These properties make the Akita mouse ideal for studying diabetes-related changes (Barber *et al.*, 2005). As female mice develop diabetes more slowly and less stably compared with males (Yoshioka *et al.*, 1997) only male mice heterozygous for the $Ins2^{Akita}$ allele are used in studies. Presence of the $Ins2^{Akita}$ allele or the wildtype Ins2 gene is normally confirmed by Restriction Fragment Length Polymorphism (RFLP) analysis (Wang *et al.*, 1999). Within the eye, increased vascular permeability can be observed as well as acellular capillaries after about 36 weeks of diabetes (Barber *et al.*, 2005). Furthermore, increased apoptosis of inner retinal neurons, reduction of the inner plexiform layer and consequently reduction of cells in the ganglion cell layer can be found (Barber *et al.*, 2005). Akita mice also show an increased number of leukocytes in retinal vessels.

Overall the ocular features of STZ diabetic and Akita mice are very similar with respect to the increase in vascular permeability (Antonetti *et al.*, 1998, Barber *et al.*, 2005) as well as with respect to the occludin changes (Antonetti et al., 1998, Barber et al., 2003). STZ induced diabetic rats have more acellular capillaries than Akita mice (Kern et al., 1994, Kern et al., 2000, Kowluru et al., 2001). The degeneration of the inner retinal neurons seems to occur much earlier in Akita mice (22 weeks), whereas in STZ rats only at 7.5 months of diabetes a reduction can be observed (Barber *et al.*, 2005, Barber *et al.*, 1998).

1.4.3. Laser induced retinal vein occlusion

The use of laser light to occlude retinal veins and to study the effects and sequelae of retinal vein occlusions has been described for many years (Danis *et al.*, 1987, Danis *et al.*, 1993). As argon laser light (~530nm) directed on a retinal vein often only temporarily interrupts blood flow, rose bengal as a light sensitiser is employed to boost the effects of the laser light (Danis *et al.*, 1993). Using this approach a high rate of retinal vein occlusion can be achieved. RNV, usually located at the disc, starts to form 3-6 weeks after the laser-induced vein occlusion (Danis *et al.*, 1993).

Directly after the induction of the vein occlusion, dilation of the distal vascular bed occurs. Microvascular changes are obvious within 1 day of the occlusion, including capillary dropout, with the intermediate retinal vascular bed being most affected (Genevois et al., 2004). Collateral veins are formed after dilation of deeper retinal veins occurs. From day 3 after the induced vein occlusion, regression in microvascular changes can be detected, including recanalisation of occluded veins. In areas where no revascularisation occurs, capillary dropout, vascular remodelling and neovascularisation are observed (Danis *et al.*, 1987, Pournaras *et al.*, 1990).

CHAPTER 2

ISOLATION AND CHARACTERIZATION OF MOUSE BONE MARROW-DERIVED LIN$^-$/VEGFR2$^+$ PROGENITOR CELLS

2 Isolation and characterization of mouse bone marrow-derived Lin$^-$/VEGFR2$^+$ progenitor cells

2.1 Introduction

Until circulating endothelial progenitor cells were described in a seminal publication in 1997 (Asahara *et al.*, 1997), it was believed that angiogenesis, the formation of blood vessels from existing vessels, was the result of migration and *in situ* differentiation of mature endothelial cells (ECs) (Liew *et al.*, 2008). The discovery of circulating EPCs repudiated this dogma as well as the belief that vasculogenesis, the process of forming new blood vessels from angioblasts or endothelial progenitors, only occurs *in utero*. It has been shown that circulating EPCs can promote both postnatal vasculogenesis and angiogenesis (Grant *et al.*, 2002, Isner *et al.*, 2001, Luttun *et al.*, 2002, Rafii *et al.*, 2002, Ruzinova *et al.*, 2003). Many studies have since linked low numbers of circulating EPCs to vascular dysfunction and increased risk of cardiovascular disease (Hill *et al.*, 2003). The level of EPCs in the peripheral blood (PB) was found to be an independent risk factor for cardiovascular events or even death (Schmidt-Lucke *et al.*, 2005, Werner *et al.*, 2005). The therapeutic potential (Sekiguchi *et al.*, 2009) of circulating EPCs became obvious when studies showed that they are capable of repairing vascular damage in conditions such as myocardial infarction (Sekiguchi *et al.*, 2009), ischemia (Rafii *et al.*, 2003, Tateishi-Yuyama *et al.*, 2002) and cerebrovascular disease (Jung *et al.*, 2008). In animal models of hindlimb ischemia (Couffinhal *et al.*, 1999) or ischemic retinopathies (Caballero *et al.*, 2007), transplantation of EPCs was reported to improve circulation and partially repair damaged vasculature.

To date there are still no markers to uniquely identify EPCs, hence a combination of phenotypic properties and surface markers are used. One common phenotypic definition of EPCs includes their ability to take up 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate labeled acetylated-low density lipoprotein (Dil-acLDL), to bind to fibronectin and lectin (Bellik *et al.*, 2005, Ingram *et al.*, 2004, Walenta *et al.*, 2005) and to form tube-like structures on Matrigel® (Ishikawa *et al.*, 2004, Murohara *et al.*, 2000). In humans, a combination of cell surface markers such as CD133, VEGFR2 and CD34 have also been used to define EPCs, although it was

reported that very early EPCs do not express CD34 (Friedrich *et al.*, 2006, Liew *et al.*, 2008, Medina *et al.*, 2010a) and it was shown that none of these single markers is highly specific (Critser *et al.*, 2011, Otani *et al.*, 2002). Despite this, the term "EPC" is still used to describe a group of cells rather than a single population (Barber *et al.*, 2006b, Hirschi *et al.*, 2008, Medina *et al.*, 2010a). Recent reports indicate that the actual vasculogenic activity of "EPCs" can be attributed only to a subpopulation of circulating cells that produce endothelial colony-forming cells (ECFCs) (Critser *et al.*, 2011, Medina *et al.*, 2010a). Most previous studies used human EPCs isolated from PB for phenotypic characterization and functional studies, while only few have focused on murine BM derived EPCs (Aicher *et al.*, 2003, Butler *et al.*, 2005, Otani *et al.*, 2002). With limited studies on murine BM EPCs, isolations of BM EPCs have been based on selection of $CD45^+$ monocytes (Aicher *et al.*, 2003), depletion of lineage cells with a lineage depletion cocktail (Otani *et al.*, 2004, Otani *et al.*, 2002) or selection of cells using a growth medium that favours vascular endothelial cells (Wang *et al.*, 2008).

VEGFR2 (also known as Flk-1) is one of the few surface markers that are constantly expressed on both early and late EPCs (Friedrich *et al.*, 2006) and on ECFCs (Friedrich *et al.*, 2006, Medina *et al.*, 2010a). There is, however, little information on BM EPCs isolated using VEGFR2. In the current study, we isolated a novel early progenitor cell type, $Lin^-/VEGFR2^+$ cells, from the mouse BM and evaluated their phenotypic and functional properties as endothelial progenitor cells. We then studied their distribution and integration into damaged vasculature in a mouse model of laser-induced retinal vascular injury.

2.2 Methods

2.2.1. Animals

All animal studies were performed according to the principles of laboratory animal care and adhering to the Association for Research in Vision and Ophthalmology (ARVO) Statement as well as the NSW Animals Act (1985) and were approved by The University of Sydney Animal Ethics Committee. C57 BL/6J mice were obtained from Animal Research Centre (Canning Vale, WA, Australia) and the breeders for the transgenic mice carrying the green fluorescent protein (GFP, C57BL/6-Tg [UBC-

GFP] 30Scha/J) were kindly provided by Dr. Alison Rice (Mater Medical Research Institute, Australia) and Dr. David Curtis (Monash University, Australia).

2.2.2. Bone marrow collection

Eighteen week old male C57 BL/6 mice were euthanized using CO_2. Femora and tibiae were immediately excised and flushed with 8mL of IMag™ buffer (BD, Cat no 552362) using a 25-gauge needle. Cells were placed on ice. After centrifugation (400rcf, 5min), red blood cell lysis was performed for 5min using 2mL red cell lysing buffer (Sigma, Cat no R7757). After a second centrifugation step (400rcf, 5min) cells were resuspended in 10mL of IMag™ buffer and washed twice. After the second washing step, the isolated cells were passed through a 40μm nylon cell strainer (BD, Cat no 352340), centrifuged and resuspended in 2mL of IMag™ buffer. Cell numbers were determined using an automated cell counter (TC10, BioRad) and cell viability was assessed using a trypan blue exclusion assay (Shapiro, 1988).

2.2.3. Immunomagnetic bead separation

The cells isolated from BM were incubated on ice with NA/LE rat anti-mouse CD16/CD32 (Fc-block, 1μg/10^6 cells, BD, Cat no 553140) for 15min, then labeled with both APC mouse lineage antibody cocktail (BD, Cat no 558074; includes CD3e, CD11, CD45R/B220, Ly-76, Ly-6G and Ly-6C) and FITC rat anti-mouse Flk-1 (BD, Cat no 560680) for 20min on ice in the dark. Adding the antibodies in recommended concentrations to the isolated cells was done according to the manufacturer's instructions specified in the datasheets for both the cocktails and Flk-1. Cells were centrifuged at 4°C and washed with cold IMag™ buffer. The labeled cells were incubated with APC magnetic particles-DM (BD, Cat no 557932) for 30min at 4°C. This was followed by a magnetic Lin^+ depletion step using a Dynal MPC-S magnetic separator (Invitrogen, Cat no 12020D). The Lin^- fraction was then incubated with anti-FITC beads (Miltenyi Biotec, Cat no 130-048-701) for 30min at 4°C to select Lin^- and $VEGFR2^+$ ($Lin^-/VEGFR2^+$) cells. Cells were stored in buffer at 4°C in dark until further processing.

2.2.4. Preparation of pooled platelet lysate

In order to enhance the seeding efficiency and growth of Lin$^-$/VEGFR2$^+$ cells *in vitro*, platelet lysate was used instead of FCS. A human platelet unit (Red Cross, Australia) was thawed at 37°C, without allowing the material to warm up and then re-frozen at -20°C. To increase the rate of platelet fragmentation and the amount of released growth factors a further freeze/thaw step was performed. Then the platelet fragments were removed by centrifugation at 4000rcf for 15min at 4°C. The supernatant plasma was aliquoted and stored at -20°C. The final EGM-2 medium was supplemented with 10% platelet lysate instead of fetal calf serum (FCS).

2.2.5. Culture of Lin$^-$/VEGFR2$^+$ cells

Lin$^-$/VEGFR2$^+$ cells were washed and resuspended in modified-EGM-2 (EBM-2 basal medium plus EGM-2 SingleQuot kit, Lonza, Cat no CC-3162) containing 20% FCS (GIBCO, Cat no 16000) (Thum *et al.*, 2007), 100U/mL penicillin, 100µg/mL streptomycin (GIBCO, Cat no 15140) and 2mmol/l L-alanyl-L-glutamine (Dipeptide Glutamine, Mediatech, Cat no 25-015-CI). A total of 10^6 cells ($4x10^4$ cells/cm^2) were seeded in 25cm^2 cell culture flasks (Corning® CellBIND®, Cat no 3289) pre-coated with 2mL of 100µg/mL collagen-I solution (Sigma, Cat no C3867) for 30min at 37°C and observed for the formation of ECFCs. Lin$^-$/VEGFR2$^+$ cells were cultured in modified-EGM-2 medium containing recombinant mouse CXCL12/SDF-1α (R&D Systems, Cat no 460SD) at a concentration of 10ng/mL. The colony formation and expansion was then monitored at baseline, 1 and 3 weeks time-points. Cell medium was changed every 4th day by gently aspirating the cell medium from the cell culture flask and refilling the cell culture flask with fresh medium (7mL/25cm^2).

2.2.6. Immunocytochemistry

Living cells were incubated for 30min at 37°C with Dil-acLDL (Invitrogen, Cat no L3484, 10µg/mL in PBS), then washed twice and fixed with 4% paraformaldehyde

(Sigma, Cat no P6148). Incubation with UEA-I (Vector Laboratories, Cat no B-1065, 1:100 in PBS) for one hour was followed by an incubation with FITC-streptavidin binding to the UEA-I (Zymed, Cat no 43-4311) for 30min. Photographs were taken after washing twice with PBS.

Staining with cell surface markers was performed as follows: after fixation with 4% paraformaldehyde, cells were incubated with the primary antibodies against CD34 (Santa Cruz, Cat no SC7054, 1:200) and VEGFR2 (Santa Cruz, Cat no SC504, 1:200). The bound antibodies were detected with Alexa Fluor® 488- and 594-conjugated secondary antibodies (Invitrogen, Cat no A-11006 and A-21207, 1:1000) and cell nuclei were counterstained with Hoechst 33258 (Sigma, Cat no 861405, 5μg/mL). The stained cells were examined under an inverted fluorescence microscope (Olympus, IX71).

2.2.7. Flow cytometry

Flow cytometric acquisition was performed on a FACSCanto-II equipped with 405, 488 and 633 nm lasers using FACSDiva software (both from BD) or a MACSQuant Analyzer-10 (Miltenyi Biotec) equipped with 405, 488 and 635 nm lasers. At least 10^6 events were acquired for each sample. On FACSCanto-II data was acquired uncompensated while it was acquired compensated on the MACSQuant. Analyses were performed using the MACSQuantify Software (Miltenyi Biotec) or FlowJo (TreeStar). Cells were Fc-blocked (BD, Cat no 553140) with 1μg/10^6 cells for 15min on ice and then stained with the designated monoclonal antibody. APC mouse lineage antibody cocktail (BD, Cat no 558074), FITC rat anti-mouse Flk-1 (BD, Cat no 560680) , FITC cKIT (BD, Cat no 553354), FITC SCA1 (BD, Cat no 553335), PE SCA1 (BD, Cat no 553336), PerCpCy5.5 VEGFR2 (BD, Cat no 560681), PE CD90.1 (BD, Cat no 554898), PE CD105 (Miltenyi Biotech, Cat no 130-092-929), PE cKIT (BD, Cat no 553355), PE CD133 (Miltenyi Biotech, Cat no 130-092-334), PerCpCy5.5 CD34 (Biolegend, Cat no 128608), PE CD14 (BD, Cat no 560639) and PE CXCR4 (BD, Cat no 551966). Cells were first gated on forward scatter (FSC) versus side scatter (SSC) to collect only small mononucleated cells followed by a discrimination blot (FSC-A versus FSC-H) for singlet population. Compensation tubes were prepared using anti-mouse BD CompBeads (BD, Cat no 552843).

Biexponential transformation (FlowJo) or hyper-log (hlog, MACSQuantify) was used on all logarithmic graphs to visualize compensated data squished against the axes. Negative control tubes as well as the appropriate isotype controls were used. Fluorescence-minus-one tubes (FMOs) were prepared identically and were used to define "positive" populations. Flow cytometry absolute count beads standard (Bangs Laboratories, Cat no 580) were used as an internal counting standard for EPC enumeration on the FACSCanto-II. Beads were mixed with samples at a concentration of 10^5 beads per sample. The beads were then gated on the FSC-A vs. SSC-A, then FSC-A *vs.* FSC-H and then using channels 530 *vs.* FSC-H.

2.2.8. Tube formation assay

$Lin^-/VEGFR2^+$ cells ($5x10^4$ cells/cm^2) were seeded on Matrigel® coated plates (BD, Cat no 356234; 150µl/cm^2). Each well of the 24-well plate was supplemented with modified-EGM-2 medium. Lin^+ cells isolated from the BM and human umbilical vein endothelial cells (HUVECs) were used as negative and positive controls, respectively. Cells were incubated at 37°C, 5% CO_2 and 20% O_2. Each cell-type was seeded in triplicates. Cells were analysed for tube formation 2, 4, 6, 8, 12, 24 and 48 hours as well as one week after seeding using inverted phase-contrast microscopy (Olympus, IX71).

2.2.9. Retinal vascular injury model

Mice were anaesthetized by an intraperitoneal (i.p.) injection of a cocktail of Ketamine (50mg/kg bodyweight, Parnell Laboratories, New Zealand) and Medetomidine (1mg/kg bodyweight, Pfizer Animal Health, USA). Branch retinal vein occlusion (BRVO) was produced with an argon laser (Oculas, Alcon, ~530 nm) at a spot size of 50µm, a duration of 2 seconds and an energy level of 50mW. Five adjacent spots were placed on a retinal vein, approximately 1 disc diameter from the rim of the optic disc. This led to a temporary retinal vein occlusion and consecutive damage to the retinal vasculature distally to the occluded area. Three to four veins were treated in each mouse. After applying the laser, mice received i.p. atipamezole (2.5mg/kg bodyweight, Pfizer Animal Health, USA) to reverse the aesthetic.

2.2.10. Intravitreal cell transplantation

$Lin^-/VEGFR2^+$ cells and Lin^- cells were isolated from the BM of transgenic mice carrying the green fluorescent protein (GFP) using the magnetic bead separation technique described earlier. For each mouse, one eye received $Lin^-/VEGFR2^+$ cells while the contralateral eye received Lin^- cells. After general anaesthesia, 10^5 cells in 2μl of BSS (intraocular irrigation solution, Alcon Surgical) were injected intravitreally one day after the induction of BRVO injury. In brief, one drop of chloramphenicol (10mg/mL, Sigma Pharmaceuticals) was instilled into the eye prior to the surgical procedure. Using a sterile 30-gauge needle, a small incision in the conjunctiva and the sclera posterior to the limbus was created. Cells were injected into the vitreous cavity using a 31-gauge needle (Hamilton, Cat no 22031-01) attached to a Hamilton syringe (Cat no 7632-01). After injection slight pressure was applied to the wound to facilitate wound closure and prevent reflux and one drop of chloramphenicol was instilled to prevent infection. Mice received i.p. atipamezole for reversal of anaesthesia as described earlier and eyes were enucleated 3, 7, 14 and 28 days after cell transplantation for examinations.

2.2.11. Tissue fixation and immunohistochemistry

After euthanasia, eyes were enucleated and immediately placed in phosphate buffer saline (PBS, Amresco, Cat no E404) containing 4% paraformaldehyde (Sigma, Cat no P6148). After 30min of incubation at room temperature, eyes were dissected to remove anterior segments. The resultant eye cups were further fixed in 4% paraformaldehyde for another 30min, and washed twice in PBS and processed for flatmount or cryosection immunohistochemistry (IHC).

For flatmount IHC, retinae were dissected from the eyecup and placed for 45min at room temperature in PBS containing 5% goat serum (Sigma, Cat no G9023). Retinae were washed twice with PBS, permeabilised in PBS containing 1% TritonX-100 (Merck, Cat no 30632.4N) and 1% FCS (Sigma, Cat no F2442) for a minimum of 4 hours and then incubated with fluorescence-labeled isolectin GS-IB_4 (10μg/mL, Invitrogen, Cat no I21413) in the dark for 3 days at 4°C. After washing in PBS

containing 0.1% TritonX-100 for 3 times, retinae were flatmounted and examined using a confocal laser scanning microscope (Zeiss Axioskop / LSM-5 Pascal, Zeiss).

For IHC on frozen sections, the fixed eye cups were transferred to PBS containing 20% sucrose, incubated overnight at 4°C then embedded in optimum cutting temperature (OCT, Tissue-Tek, Cat no 4583) medium and snap frozen in liquid nitrogen. Cryosections of 10-12µm thickness were produced for IHC. In brief, cryosections were washed 3 times in PBS followed by incubation with 10% goat serum in PBS at room temperature for 1 hour. After washing sections three times in PBS, sections were incubated with a rabbit-anti-mouse collagen-IV antibody (AbD Serotec, Cat no 2150-1470, 1:250) for 2 hours at room temperature. The primary antibody was diluted in PBS containing 1% FCS and 1% TritonX-100. The bound antibody was detected with an Alexa Fluor® 594-conjugated donkey-anti-rabbit secondary antibody (Invitrogen, Cat no A-21207, 1:1000) and sections were mounted for confocal laser scanning microscopy.

2.2.12. Statistics

Data are presented as mean ± standard deviation (SD) as they were normally distributed. Data distribution was assessed using the Shapiro-Wilk test and the D'Agostino and Pearson Omnibus normality test.

2.3 Results

2.3.1. Cell numbers and viability

The average body weight of mice was 34.4 ± 2.2g (n=8). A total number of $4.18 \pm 0.95\times10^7$ cells were isolated from the BM of each mouse, which corresponds to $1.22 \pm 0.31\times10^9$ cells/kg body weight per mouse. The total number of Lin$^-$/VEGFR2$^+$ isolated from each mouse was $1.57 \pm 0.35\times10^6$, which corresponds to $4.60 \pm 1.15\times10^7$ cells/kg body weight per mouse. The viability of Lin$^-$/VEGFR2$^+$ cells was 85 ± 4%.

2.3.2. Cell culture and colony forming

After seeding in collagen-I coated flasks, $Lin^-/VEGFR2^+$ cells started to attach from day 1. Small colonies were observed after 3-4 weeks of culture but the number of cells in each colony increased very slowly thereafter (Fig 2A-D). Immunocytochemistry revealed that all attached cells were positive for CD34 and VEGFR2 (Fig 2E-H). These phenotypic properties remained unchanged even after 437 days of culture (Fig 2I-L). In addition, the isolated $Lin^-/VEGFR2^+$ cells took up Dil-acLDL and were stained positively for UEA-I (Fig 2M and N). Even after 437 days of culture, $Lin^-/VEGFR2^+$ cells did not achieve complete confluence. Any attempt to passage the cells resulted in their death. Thus, only $Lin^-/VEGFR2^+$ cells in primary culture were used in this study.

$Lin^-/VEGFR2^+$ cells cultured in modified-EGM-2 supplemented with 10% platelet lysate instead of FCS or in modified-EGM-2 supplemented with 100ng/mL of recombinant mouse CXCL12/SDF-1α did not induce different growth patterns. Nor did these manoeuvres increase the number of cell colonies compared to cells cultured in modified-EGM-2 supplemented with 20% FCS.

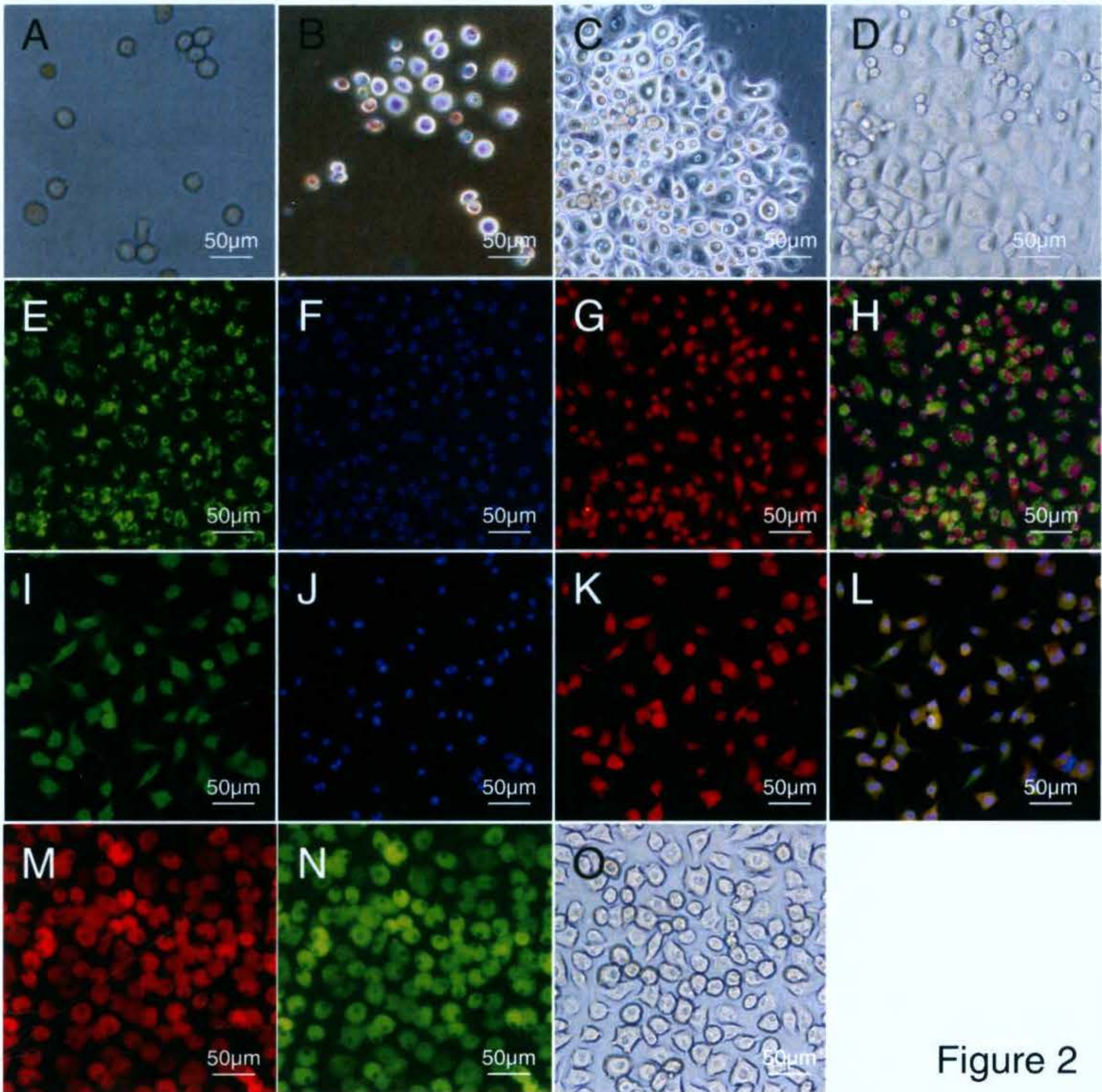

Figure 2: Culture and immunocytochemistry of BM Lin$^-$/VEGFR2$^+$ cells. Panels A to D show cultured EPCs at day 0 (A), day 3 (B), day 47 (C) and day 98 (D) after seeding. IHC at day 7 is shown in panels E to H: CD34 conjugated with Alexa Fluor® 488 secondary antibody (E), Hoechst nucleus stain (F), VEGFR2 conjugated with Alexa Fluor® 594 secondary antibody (G) and composite (H). IHC at day 437 is shown in panels I to L: CD34 (I), Hoechst nucleus stain (J), VEGFR2 (K) and composite (L). Dil-acLDL (M) and UEA-I staining (N) is shown for cells at day 47 (unstained cells in O).

2.3.3. Flowcytometry

After lineage depletion ($CD3e^-$ $CD11b^-$ $CD45R^-$ $Ly\text{-}76^-$ $Ly\text{-}6G^-$ and $Ly\text{-}6C^-$) and VEGFR2 enrichment, freshly isolated $Lin^-/VEGFR2^+$ cells were tested for expression of a number of surface markers. Of the population of $Lin^-/VEGFR2^+$ cells, 8% expressed SCA1, 4.5% expressed cKIT, 2.2% expressed CD133, 15% expressed CXCR4, 4% expressed CD105, 0.6% expressed CD90.1, 5% CD14, and 70.5% expressed EpoR. 100% of $Lin^-/VEGFR2^+$ cells expressed CD34 but none expressed CD31. As only $Lin^-/VEGFR2^+$ cells were analysed, no information on the distribution of the surface markers in Lin^+ and Lin^- fractions is available and no estimate on a possible Lin^+ contamination can be made.

2.3.4. Tube formation assay

Neither the freshly isolated Lin^+ (data not shown) nor the freshly isolated $Lin^-/VEGFR2^+$ cells formed tube-like structures when cells were seeded on Matrigel® alone or on Matrigel® supplemented with 100ng/mL SDF-1 and observed for up to 1 week, whereas the HUVECs (positive control) formed tube-like structures after 6 hours (Fig 3).

Figure 3

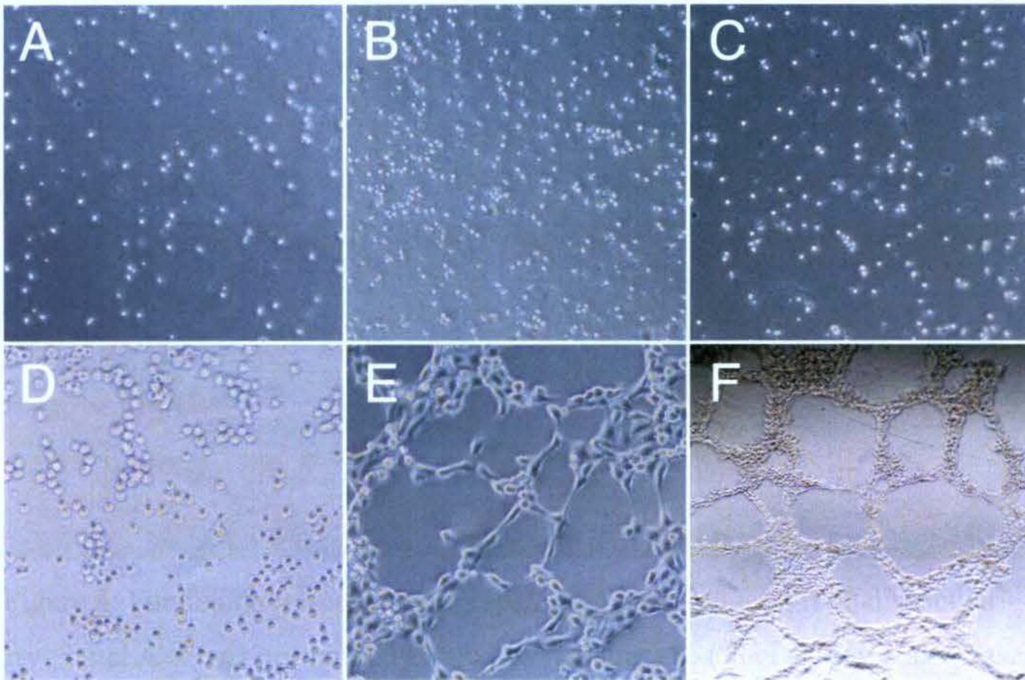

Figure 3: Tube formation assay. Panels A to C show $Lin^-/VEGFR2^+$ cells seeded on Matrigel® at 0, 6 and 20 h after seeding. Corresponding images from HUVECs are shown in panels D to E as positive controls. 10x magnification.

2.3.5. Retinal injury model and transplantation experiments

Applying laser to retinal veins induced retinal vein occlusion which was characterized by venous engorgement, severe tortuosity and congestion (Fig 4). Intraretinal capillary haemorrhages and retinal oedema were commonly observed in the occluded areas on the day after laser treatment, which gradually resolved within one week. Changes in the retinal capillary bed of affected areas was disclosed by fluorescein angiography showing fluorescein leakage, dilatation of the capillary bed, and several areas of capillary non-perfusion (Fig 4). Fundus fluorescein angiography (FFA) was performed using a modified clinical fundus camera (Topcon TRC-50VT) as previously described with slight modifications (Shen *et al.*, 2010a). In brief, a 40D lens was mounted in front of the fundus camera for FFA in mice. Mice were anesthetized by i.p. injection of a cocktail consisting of ketamine (50 mg/kg, Parnell Laboratories, New Zealand) and medetomidine (1 mg/kg, Pfizer Animal Health,

USA) and their pupils dilated with 1% tropicamide and 2.5% phenylephrine. FFA was conducted after i.p. injection of 0.05 ml of 10% sodium fluorescein.

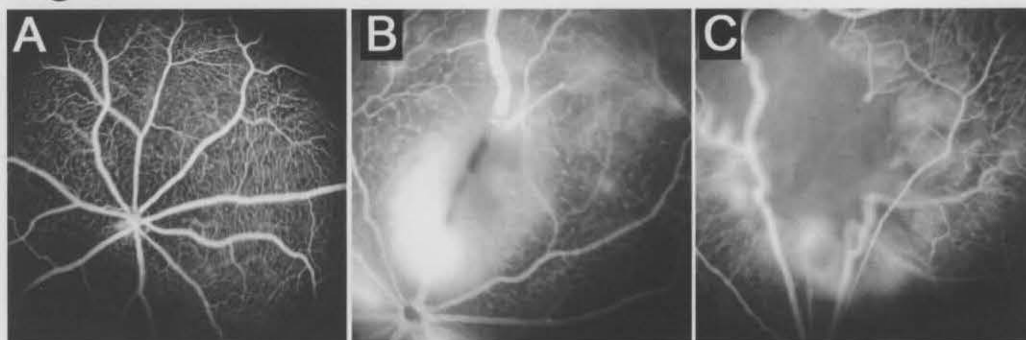

Figure 4: Fundus fluorescein angiography after laser induced-retinal vascular injury. Panel A shows normal retina (no laser treatment). Panel B (day 1 after laser treatment) shows leakage of fluorescein around the area where the laser was applied, the vein distally is engorged and the adjacent area shows diffuse leakage. Panel C (day 7 after laser-induced retinal vascular injury) shows nonperfusion and leakage of fluorescein at the borders of the nonperfused area.

In eyes without retinal vein occlusion, the majority of transplanted cells remained in the vitreous cavity though some cells were found on the retinal surface but they failed to integrate into the normal retinal vasculature (Fig 5A). In eyes undergoing laser-induced retinal vascular injury and receiving Intravitreal (IV)-transplantation of $Lin^-/VEGFR2^+$ cells, a number of phenomena were observed regarding cell movement, integration and phenotypical appearance. Firstly, most transplanted cells were located around the area where laser burns were delivered (Fig 5B). Secondly, the IV transplanted cells were able to integrate into the injured retinal vessels, both into small capillaries (Fig 5C) as well as larger retinal vessels (Fig 5D). Thirdly, a number of transplanted cells showed microglia-like appearance and wrapped around the damaged retinal vessels (Fig 5E). The microglia-like appearance seemed to be more pronounced in eyes receiving Lin^- cells compared with those injected with $Lin^-/VEGFR2^+$ cells (Fig 5F). In contrast, cell integration into the injured retinal vessels seemed more obvious in eyes receiving $Lin^-/VEGFR2^+$ cells (Fig 5C&D) compared with those injected with Lin^- cells (Fig 5G). In addition, the transplanted cells were

also found in blood vessels in the optic nerve head and episclera (Fig 5H). Cell integration into retinal vessels was commonly observed from day 7 onwards, occurring mostly in regions undergoing laser-induced retinal vascular injury. The transplanted cells seemed to more preferentially incorporate in larger vessels than in capillaries. Considering the number of cells we injected into each eye (10^5), the efficacy of cell integration seemed very low. Due to technical limitations, no further characterisation of cells integrating into bloodvessels could be made.

Figure 5

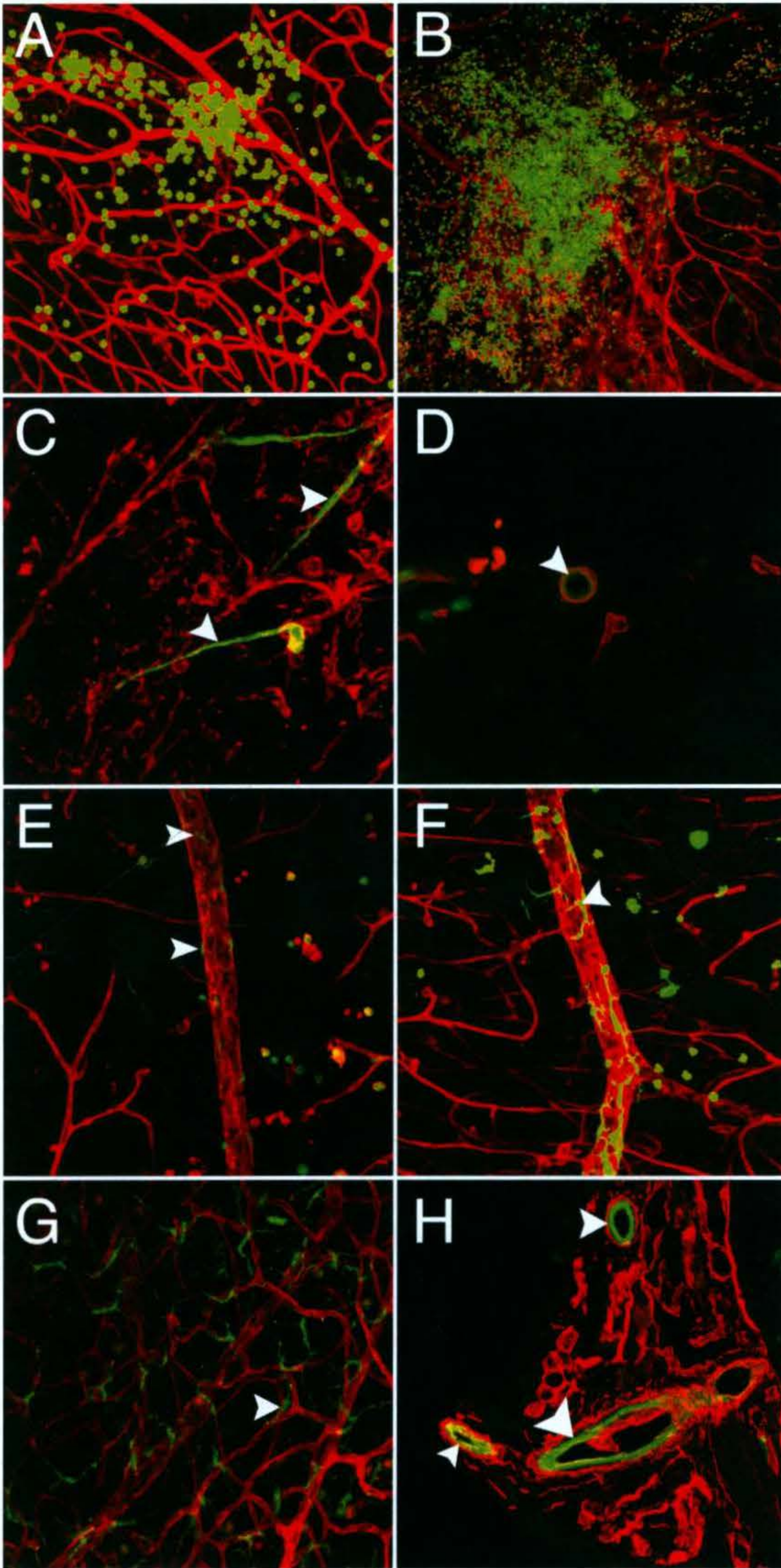

Figure 5: Distribution and incorporation of Lin⁻/VEGFR2⁺ cells, isolated from the BM of GFP transgenic mice, 14 days after intravitreal transplantation. Panel A depicts

Lin$^-$/VEGFR2$^+$/GFP$^+$ cells sitting on the retinal surface in an eye without retinal vascular injury (vasculature shown in red: Alexa Fluor® 594-conjugated secondary antibody against anti-collagen-IV primary antibody). No integration into blood vessels is observed. Panel B shows the aggregation of transplanted cells around the area of laser-induced retinal vascular injury. Integration of Lin$^-$/VEGFR2$^+$/GFP$^+$ cells into small capillaries and a larger retinal vessel is shown in Panel C and D, respectively. Some Lin$^-$/VEGFR2$^+$/GFP$^+$ cells show Glia-like appearance and wrap around large veins (E). With Lin$^-$ cells, it seems that more cells form Glia-like appearance wrapping large vessels (F) and retinal capillaries (G). Integration of Lin$^-$/VEGFR2$^+$/GFP$^+$ cells into large vessels in the optic nerve head and episclera is shown in (H).

2.4 Discussion

In this study we isolated Lin$^-$/VEGFR2$^+$ cells from the BM of mice and characterized their properties as endothelial progenitor cells *in vitro*. Furthermore, we performed IV-transplantation to study their distribution and incorporation into injured retinal vessels in a mouse model of laser-induced retinal vein occlusion. While sharing a number of widely reported features of human EPCs from PB such as Dil-acLDL uptake, positive staining for CD34 and cobble-stone morphology *in vitro* (Asahara *et al.*, 1997, Bellik *et al.*, 2005, Hristov *et al.*, 2003a, Ingram *et al.*, 2008, Ingram *et al.*, 2004, Ishikawa *et al.*, 2004, Kalka *et al.*, 2000b, Lin *et al.*, 2000, Llevadot *et al.*, 2001, Peichev *et al.*, 2000, Walenta *et al.*, 2005), murine BM-derived Lin$^-$/VEGFR2$^+$ cells neither rapidly formed colonies of ECFCs nor developed tube-like structures on Matrigel®. When transplanted into the laser-induced retinal vascular injury model, only limited numbers of Lin$^-$/VEGFR2$^+$ cells were able to integrate into damaged retinal blood vessels. Therefore it appears that murine BM Lin$^-$/VEGFR2$^+$ cells share some similarities to human EPCs isolated from the PB (Medina *et al.*, 2010a) but most of them seem to be less active both *in vitro* and *in vivo* in this study as compared to studies using human EPCs.

It has been shown that the vasculogenic activity of circulating EPCs can be attributed only to the subpopulation that has the ability to form ECFC (Critser *et al.*, 2011, Medina *et al.*, 2010a). Little information is available on the vasculogenic

activity of EPCs isolated from the BM (Reyes *et al.*, 2002), especially in mice (Otani *et al.*, 2002, Wang *et al.*, 2008). This may be because access to EPCs from humans is easier and more often described in a *de-facto* (Asahara *et al.*, 1997, Bahlmann *et al.*, 2004, Balestrieri *et al.*, 2008b, Balestrieri *et al.*, 2008c, Biancone *et al.*, 2004, Chavakis *et al.*, 2005, Chavakis *et al.*, 2007, Chen *et al.*, 2004a, Chen *et al.*, 2004b, Chen *et al.*, 2007, Choi *et al.*, 2004, Dernbach *et al.*, 2004, Dimmeler *et al.*, 2001, Friedrich *et al.*, 2006, Heeschen *et al.*, 2003, Kalka *et al.*, 2000a, Kalka *et al.*, 2000b, Kalka *et al.*, 2000c, Leshem-Lev *et al.*, 2010, Llevadot *et al.*, 2001, Tepper *et al.*, 2002, Thum *et al.*, 2007, Yodoi *et al.*, 2007) standardised way compared with the various approaches for isolation of progenitor cells from the BM of mice (Chavakis *et al.*, 2005, Gensch *et al.*, 2007b, Iwakura *et al.*, 2003, Otani *et al.*, 2002, Ritter *et al.*, 2006, Yoon *et al.*, 2006). Another reason may be the relative scarcity of ideal markers for murine EPCs.

We found that that $Lin^-/VEGFR2^+$ cells incorporated into different ocular vascular beds after IV-transplantation in the laser-induced retinal vascular injury model. While circulating EPCs may represent a relatively homogenous group that is easy to identify, the BM may contain a much wider range of progenitor cells expressing different surface markers at stages varying from very early progenitor cells to relatively mature cells about to be mobilized into the circulation. For example, CD34, a marker commonly used for EPC isolation from the PB, is rarely found on very early EPCs (Friedrich *et al.*, 2006, Liew *et al.*, 2008, Medina *et al.*, 2010a) while CD133, a marker of early EPCs, is lost after EPCs differentiate into mature vascular endothelial cells (Friedrich *et al.*, 2006, Yin *et al.*, 1997). A recent study showed that human EPCs from the PB with high proliferative potential did not express CD133 (Medina *et al.*, 2010a). The approach used here may also include some BM stromal cells since Aicher *et al.* have reported that BM stromal cells may also express VEGFR2 (Aicher *et al.*, 2003). However, the growth of stromal cells and other non-EPCs may have been inhibited in our approach using specific growth factor medium that favours EPCs growth only. The combination of lineage depletion with VEGFR2 positive selection followed by growth factor medium would make the process more specific compared with that only based on linage depletion (Ritter *et al.*, 2006) or growth factor medium to suppress the growth of or eliminate stromal cells (Wang *et al.*, 2008, Wang *et al.*, 2011b).

Many previous studies relied on SCA1 and cKIT as markers to isolate EPCs, which has been proved to be an unsuitable approach to identify a homogeneous population of EPCs. Dimmeler *et al.* (Dimmeler *et al.*, 2001) isolated EPCs from minced spleen using special cell media and adherence properties of the cells. It is well known that cKIT is highly expressed on earliest thymocyte progenitors in the thymus and on hematopoietic stem cells, multipotent progenitor cells, and common myeloid progenitors (Leong *et al.*, 2008). SCA1 is found on progenitor cells for cardiomyocytes (Matsuura *et al.*, 2004) and smooth muscle cells (Xiao *et al.*, 2007) and hence is also not an ideal marker for EPCs. Awad *et al.* (Awad *et al.*, 2005) used only SCA1$^+$ cells isolated from BM using magnetic beads and called them monocytic endothelial cell progenitors. Similarly, Otani *et al.* (Otani *et al.*, 2002) isolated BM-EPCs based on gradient centrifugation followed by Lin$^-$ /SCA1$^+$ magnetic bead isolation. Their study also showed that the isolated Lin$^-$ /SCA1$^+$ BM cells differentiated into glia-like cells which were predominately localized to the site of retinal vascular injury after IV-transplantation. Taken together, these considerations suggest that current research on BM EPCs is hindered by the lack of methods for isolation of a pure population of functional EPCs.

In this study, only small percentage of BM Lin$^-$/VEGFR2$^+$ cells expressed CD14 (5%) and CD105 (4%), which are usually expressed on macrophages and neutrophils or activated macrophages, endothelial cells and fibroblasts, respectively. The use of growth medium that favours endothelial progenitor cell growth only may eliminate the CD14$^+$ and CD105$^+$ growth. Furthermore, the lack of CD90.1 expression on Lin$^-$ /VEGFR2$^+$ cells means that they are not mesenchymal, hematopoietic stem cells, NK cells or fibroblasts. We found that murine BM Lin$^-$/VEGFR2$^+$ cells shared widely described features of EPCs. These include the formation of colonies with cobble-stone morphology (Thum *et al.*, 2006, Yoder *et al.*, 2007), Dil-acLDL uptake (Liew *et al.*, 2006, Thum *et al.*, 2006) and positive staining for CD34 (Gehling *et al.*, 2000, Gulati *et al.*, 2003, Hristov *et al.*, 2003a, Medina *et al.*, 2010a, Peichev *et al.*, 2000). Most importantly they were able to integrate into damaged retinal vasculature after IV-transplantation. The level of cell integration into injured retinal vessels seemed to be low when compared with EPCs isolated from human PB as reported by others (Caballero *et al.*, 2007, Medina *et al.*, 2010a, Otani *et al.*, 2004). The Lin$^-$/VEGFR2$^+$ cells that we isolated seemed to transform into microglia-like cells to a lesser extent than described for Lin$^-$ cells from BM (Ritter *et al.*, 2006). This may be because the

additional selection using VEGFR2 eliminates immature cells that potentially differentiate into glial cells (Yeh *et al.*, 2003). Consistent with previous reports, we found that transplantation of cells in healthy eyes did not result in integration in the normal retinal vasculature (Otani *et al.*, 2002).

In contrast to EPCs isolated from human PB (Ishikawa *et al.*, 2004, Murohara *et al.*, 2000), Lin$^-$/VEGFR2$^+$ isolated from the BM of mice did not form tube-like structure in Matrigel® assay. Wang *et al.* (Wang *et al.*, 2008, Wang *et al.*, 1998) once isolated BM derived EPCs based on their plastic adhesive property and further selected cells using a conditioned medium from vascular endothelial cells. They reported that tube formation by BM derived murine EPCs was only observed after 3 passages of culture (Wang *et al.*, 2008, Wang *et al.*, 1998). It is possible that the Lin$^-$/VEGFR2$^+$ cells described in this study are more immature and hence may not be directly able to form tube-like structures on Matrigel® as this may require specific gene activation and maturation. The process of EPC maturation and eventual translocation of EPCs from the stem cell niche to the PB is complex and involves several key enzymes found in EPCs (Aicher *et al.*, 2003, Hattori *et al.*, 2002, Heissig *et al.*, 2002, Luttun *et al.*, 2002, Murohara *et al.*, 1998) The ability to form tubes may be established during this maturation process. The differences in cell activity demonstrated in tube formation assay on Matrigel® and after IV-transplantation may reflect the requirement of certain cytokines/molecules for BM EPC activation and differentiation. The limited number of cells integrating into the damaged retinal vasculature may also be explained by this fact as well. After IV-transplantation, only few BM EPCs might be mature enough to react to the stimuli released from the laser-induced retinal vascular injury. Administration of SDF-1 *in vitro* did not induce any changes in growth patterns (colony formation, expansion) of the murine BM EPCs that we isolated, although it has been described that SDF-1 is one of the key factors for EPC mobilization and maturation (Asahara *et al.*, 1999b, Butler *et al.*, 2005, Heissig *et al.*, 2002, Moore *et al.*, 2001, Rabbany *et al.*, 2003). It seems SDF-1 alone is insufficient to promote early EPC maturation.

In summary, our results indicate that, using current methods for EPC isolation, the cell population isolated from BM may be inferior to that isolated from PB as a cell source to repair retinal vascular damage. In contrast to EPCs derived from PB, we found that murine BM EPCs did not expand significantly in primary culture. They did not form tubes on Matrigel® and limited number of cells was able to incorporate into

damaged retinal vasculature after IV-transplantation. Thus, it appears that the process of BM EPC mobilization from the BM to the PB may render the mobilized EPCs more suitable for therapeutic application. Further research is warranted to investigate how murine BM $Lin^-/VEGFR2^+$ cells could be activated *in vitro* before therapeutic application *in vivo*.

CHAPTER 3

DIABETES IMPAIRS MOBILIZATION OF MOUSE BONE MARROW-DERIVED LIN$^-$/VEGFR2$^+$ PROGENITOR CELLS

3 Diabetes impairs mobilization of mouse bone marrow-derived $Lin^-/VEGFR2^+$ progenitor cells

3.1 Introduction

Diabetes mellitus, a metabolic condition characterized by chronic hyperglycaemia (Alberti *et al.*, 1998), is estimated to affect over 13% of adults older than 30 years in the US (Danaei *et al.*, 2009) and 5.7% of the total population of Australia (2007-2008, http://www.aihw.gov.au/how-common-is-diabetes/#all). Diabetes confers a 2-4 times higher risk of death from cardiovascular disease (Crofford, 1995) and diabetic retinopathy, the commonest microvascular complication of diabetes, caused around 10,000 new cases of blindness in the US in 2004 (Fong *et al.*, 2004). Impaired angiogenic responses to injury or ischemia in diabetic subjects have been described (Waltenberger, 2001).

Endothelial progenitor cells participate in renewing vascular endothelium and contribute to neovascularization (Asahara *et al.*, 1999a, Asahara *et al.*, 1997). Their discovery has shed new light on the pathogenesis of diabetic vascular complications (Tepper *et al.*, 2002). The therapeutic potential of EPCs naturally attracts attention since they may be able to repair vascular damage in conditions such as myocardial infarction (Sekiguchi *et al.*, 2009), limb ischemia (Tateishi-Yuyama *et al.*, 2002) and cerebrovascular disease (Jung *et al.*, 2008). It is believed that EPCs are mainly recruited from the bone marrow (BM) (Shi *et al.*, 1998) and from the walls of the blood vessels (Alessandri *et al.*, 2001, Ingram *et al.*, 2005).

No single unique marker for EPCs has yet been identified. Most investigators use a combination of cell surface markers and phenotypic properties to define them (Critser *et al.*, 2011, Friedrich *et al.*, 2006, Medina *et al.*, 2010b, Walenta *et al.*, 2005). The term "EPC" is still used but it is now accepted that it may comprise a group of cells rather than a single population (Barber *et al.*, 2006b, Hirschi *et al.*, 2008, Medina *et al.*, 2010b). Previously, it was shown that human $CD34^+/CD133^+/VEGFR2^+$ cells are not true EPCs but distinct, primitive hematopoietic progenitors. The phenotypic analysis showed that endothelial outgrowth cells generating $CD34^+/CD45^-$ cells express VEGFR2 but not CD133, whereas $CD34^+/CD45^+$ hematopoietic precursors express CD133 as expected, but not VEGFR2. It is clear that the endothelial

outgrowth cells are not derived from $CD133^+$ cells or $CD45^+$ hematopoietic precursors. That coexists with the fact that limited efficacy in improving long-term clinical outcomes is observed in conditions such as cardiovascular diseases (Case *et al.*, 2007, Timmermans *et al.*, 2007). Cells that do not express hematopoietic lineage markers but possess vascular endothelial growth factor receptor 2 ($Lin^-/VEGFR2^+$) share cardinal properties of EPCs such as Dil-acLDL uptake, cobble-stone shaped colony formation *in vitro*, lectin binding and CD34 expression (Fig 6 and Fig 7). VEGFR2 is one of the few surface markers that are constantly expressed on both early and late EPCs (Friedrich *et al.*, 2006) and on endothelial colony forming cells (Friedrich *et al.*, 2006, Medina *et al.*, 2010b). Thus, depletion of cells expressing hematopoietic lineage markers followed by positive selection of cells expressing VEGFR2 then culturing the cells in EPC selective medium may be a better way to identify "true" putative EPCs.

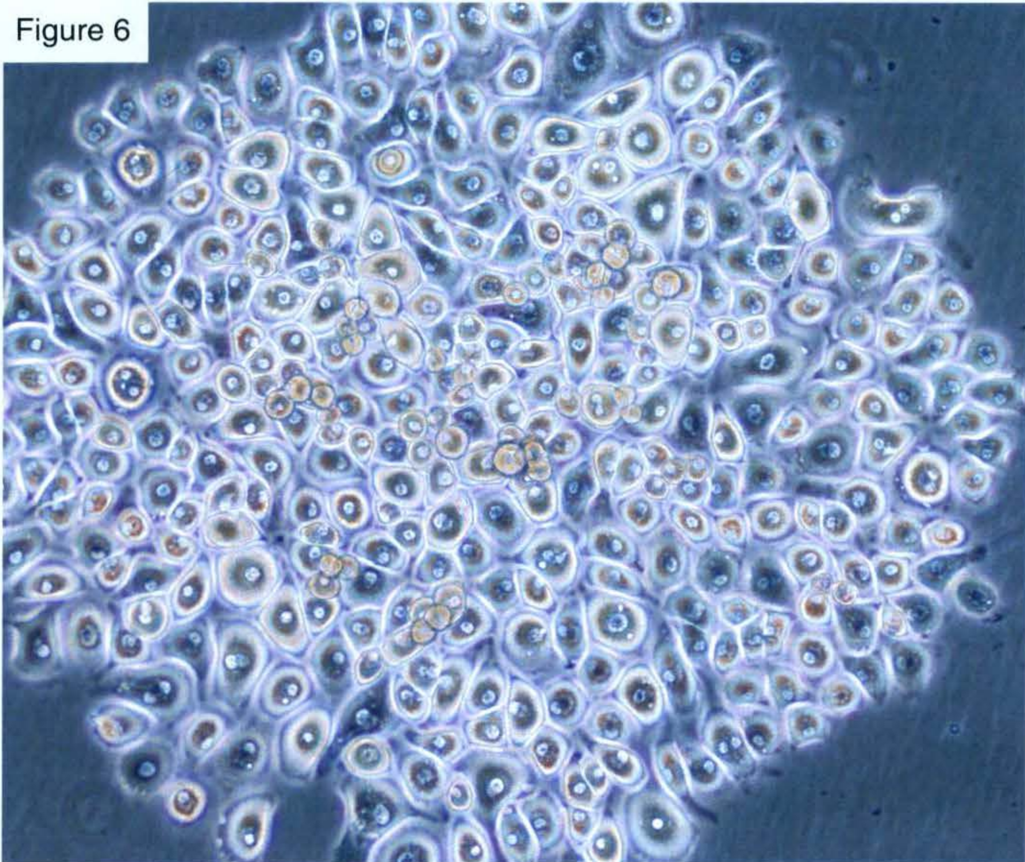

Figure 6: Colony that emerged from mouse bone marrow isolated $Lin^-/VEGFR2^+$ cells culture in modified EGM-2 after 47 days.

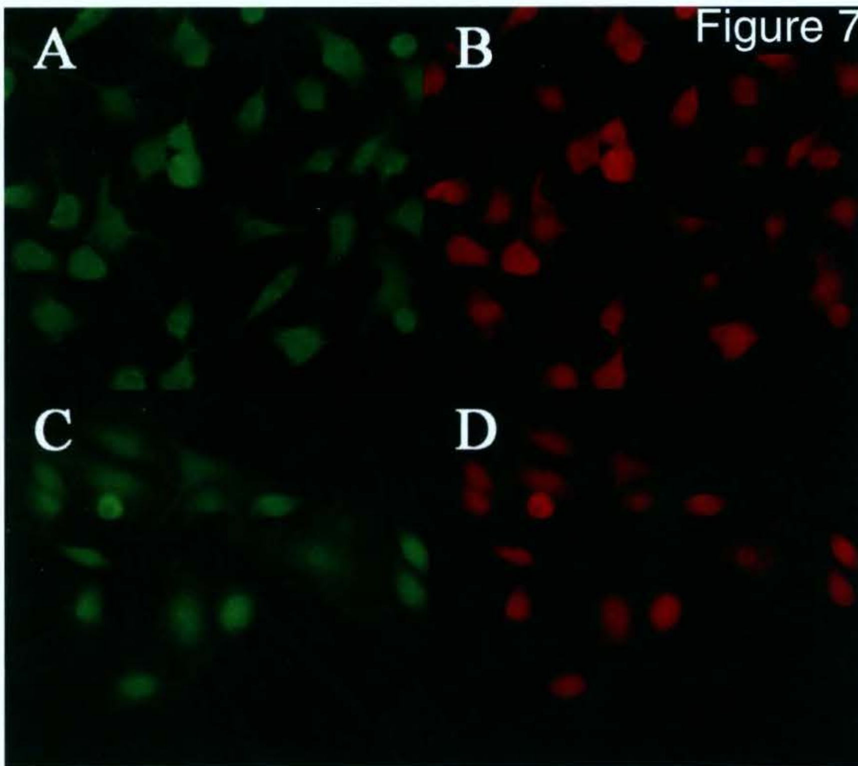

Figure 7: Immunocytochemistry of non-diabetic untreated mouse BM Lin$^-$ /VEGFR2$^+$ cell culture in modified EGM-2. Panel A shows stained cells with VEGFR2 conjugated with Alexa Fluor® 488 secondary antibody and Panel B shows the same cells stained with CD34 conjugated with Alexa Fluor® 594 secondary antibody. Panel C shows cells stained with UEA-I and Panel D shows the same cells stained with Dil-acLDL.

As it has been shown that the number of EPCs circulating in the PB predicts the risk of cardiovascular events or even death in humans (Schmidt-Lucke *et al.*, 2005, Werner *et al.*, 2005), efforts have been made to increase EPCs number in the PB for the treatment of ischemic conditions (Kondo *et al.*, 2004). A number of factors and cytokines have been employed to achieve this goal. Erythropoietin (EPO) (Heeschen *et al.*, 2003, Urao *et al.*, 2006), granulocyte colony-stimulating factor (G-CSF) (Fadini *et al.*, 2006c) and tetrahydrobiopterin (BH4) (Stroes *et al.*, 1997, Thum *et al.*, 2007) have all been implicated in EPC mobilization from BM and in improving the EPCs migratory capacity and, eventually, endothelial function in mice (Heeschen *et al.*, 2003, Urao *et al.*, 2006), rats (Fadini *et al.*, 2006c, Thum *et al.*, 2007) and humans (Stroes *et al.*, 1997, Thum *et al.*, 2007). Although a positive effect on EPCs and their mobilization has been demonstrated in non-diabetic individuals after administrations

of BH4 for SCA1$^+$/VEGFR2$^+$ EPCs (Xie *et al.*, 2010), EPO for CD45^{dim}/VEGFR2$^+$ EPCs (Urao *et al.*, 2006) and CD34$^+$ MNCs (Hirata *et al.*, 2006) or G-CSF for CD34$^+$/VEGFR2$^+$ EPCs (Yoshioka *et al.*, 2006), only very limited effects were observed in diabetic subjects on SCA1$^+$/VEGFR2$^+$/vWF$^+$ EPCs after treatment with G-CSF (Fadini *et al.*, 2006c). As the mechanisms of action of EPO, G-CSF and BH4 are different, the aim of this study was to evaluate whether a cocktail consisting of EPO, G-CSF and BH4 could successfully mobilize Lin$^-$/VEGFR2$^+$ from the BM to PB in diabetic mice.

3.2 Methods

3.2.1. Animals

Principles of laboratory animal care were followed. Animal studies were performed in accordance with the Association for Research in Vision and Ophthalmology Statement as well as the New South Wales Animals Act (1985) and were approved by The University of Sydney Animal Ethics Committee. C57 BL/6J mice were provided by the Animal Research Centre (Canning Vale, WA Australia).

3.2.2. Induction of diabetes

After an overnight fast, 40 4-weeks old male C57BL/6J mice were separated into two groups: 20 animals that received streptozotocin (STZ) to induce diabetes (Rerup, 1970) and 20 mice receiving citrate buffer as non-diabetic controls. Freshly prepared STZ, (Sigma-Aldrich, Cat no 85882) at a concentration of 27.5 mg/ml in 0.1 M citrate buffer (pH 4.5) was injected intraperitoneally (i.p., 55 mg/kg bodyweight) for 5 consecutive days. Body weight and blood glucose (Accu-Chek Performa, Roche, Germany) were monitored weekly. To promote survival and wellbeing of diabetic mice, weekly i.p. injection of 0.5 IU insulin glargine (Lantus, Sanofi-Aventis, Germany) was administered if the blood glucose was >17.3 mmol/l. Only mice maintaining blood glucose levels >13.3 mmol/l were included. Animals were monitored for 16 weeks after developing diabetes.

3.2.3. Fundus fluorescein angiography

Fundus fluorescein angiography (FFA) was performed using a modified clinical fundus camera (Topcon TRC-50VT) as previously described with slight modifications (Shen *et al.*, 2010b). In brief, a 40D lens was mounted in front of the fundus camera for FFA in mice. Mice were anesthetized by i.p. injection of a cocktail consisting of ketamine (50 mg/kg, Parnell Laboratories, New Zealand) and medetomidine (1 mg/kg, Pfizer Animal Health, USA) and their pupils dilated with 1% tropicamide and 2.5% phenylephrine. FFA was conducted after i.p. injection of 0.05 ml of 10% sodium fluorescein.

3.2.4. Mobilization of BM EPCs using a cocktail of G-CSF, EPO and BH4

After 17 weeks of diabetes (21 weeks of age), half the animals in the control and diabetic group, *i.e.* 10 mice in each group, received a daily i.p. injection of a cocktail consisting of 2500 IU/kg EPO (Eprex®, Janssen Cilag, Australia), 10 μg/kg G-CSF (Neupogen®, Amgen, Australia) and 10 mg/kg BH4 (Cayman Chemical, Cat no 81880) for 6 consecutive days. The remaining animals received injection of vehicle. Animals were euthanized two days after the last cocktail administration. There were, therefore, four groups of animals for comparison: control mice without drug cocktail treatment (CNTX), control mice treated with the drug cocktail (CTX), diabetic mice without drug cocktail treatment (DNTX) and diabetic mice treated with the drug cocktail (DTX).

3.2.5. PB and BM collections

At 22 weeks of age, mice were anaesthetized (i.p.) by a cocktail of Ketamine and Medetomidine as described earlier. Approximately 0.8 ml of blood was collected from the inferior vena cava of each mouse after intravenous injection of heparin sodium (50 IU/kg bodyweight, Pfizer, Australia) and stored on ice for further processing. To collect BM cells, femora and tibiae were immediately excised and flushed with 8 ml of cold IMag™ buffer (BD, Cat no 552362) using a 25-gauge needle. Then, red blood cells were lysed using 2 ml red cell lysing buffer (Sigma, Cat

no R7757). Cells were passed through a 40 μm nylon cell strainer (BD, Cat no 352340), centrifuged and finally resuspended in 2 ml of IMag™ buffer. Cells were counted using an automated cell counter (TC10, BioRad); viability was assessed using a trypan blue exclusion assay. Cells were stored on ice for further processing.

3.2.6. Magnetic separation of BM $Lin^-/VEGFR2^+$ progenitor cells

The cells that were isolated from the BM were put on ice and Fc-blocked (1 $\mu g/10^6$ cells, BD, Cat no 553140). The labelling step (in the dark) with both the APC mouse lineage antibody cocktail (BD, Cat no 558074) and the FITC rat anti-mouse Flk-1/VEGFR2 (BD, Cat no 560680) followed. After 20 min, the labeled cells were centrifuged and washed twice with ice cold IMag™ buffer. The first magnetic bead incubation step (30 min at 4°C) followed using APC magnetic particles-DM (BD, Cat no 557932). The Lin^+ depletion was performed using a Dynal MPC-S magnetic separator (Invitrogen, Cat no 12020D). The remaining Lin^- fraction was then incubated (30 min at 4°C) with anti-FITC beads (Miltenyi Biotec, Cat no 130-048-701). After a positive selection step using the same magnetic separator, the resulting $Lin^-/VEGFR2^+$ cells were stored at 4°C in the dark until further processing.

3.2.7. Culture of $Lin^-/VEGFR2^+$ BM-derived EPCs

To compare the growth potentials of cells isolated from diabetic and control animals, $Lin^-/VEGFR2^+$ cells from CNTX and DNTX mice were resuspended in modified-EGM-2: EBM-2 basal medium plus EGM-2 SingleQuot kit, (Lonza, Cat no CC-3162) supplemented with 20% FCS (GIBCO, Cat no 16000) (Thum *et al.*, 2007), 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO, Cat no 15140) and 2 mmol/l L-alanyl-L-glutamine (Dipeptide Glutamine, Mediatech, Cat no 25-015-CI). Cell culture flasks (25 cm^2, Corning® CellBIND®, Cat no 3289) were pre-coated using 2 ml of 100 μg/ml collagen-I solution (Sigma, Cat no C3867) for 30 min at 37°C. After pre-coating, a total of 10^6 cells (4×10^5 cells/cm^2) were seeded in the culture flasks. Flasks were then monitored for the formation of colonies.

3.2.8. Immunocytochemistry

Isolated $Lin^-/VEGFR2^+$ cells were incubated *in vitro* with Dil-acLDL (Invitrogen, Cat no L3484, 1:100 in PBS) for 30 min at 37°C. They were then washed twice and fixed with 4% paraformaldehyde (Sigma, Cat no P6148) then incubated with biotinylated Ulex Europaeus Agglutinin I (UEA-I; Vector Laboratories, Cat no B-1065, 1:100 in PBS) for 1 hour. Cells were washed then incubated for 30 min with FITC-streptavidin (Zymed, Cat no 43-4311) to allow binding to the UEA-I. After washing twice with PBS, photographs were taken using an inverted fluorescence microscope (Olympus, IX71). Staining with cell surface markers VEGFR2 and CD34 was performed as follows: after fixation with 4% paraformaldehyde, cells were incubated with the primary antibodies against CD34 (Santa Cruz, Cat no SC7054, 1:200) and VEGFR2 (Santa Cruz, Cat no SC504, 1:200). Alexa Fluor® 488- and 594-conjugated secondary antibodies (Invitrogen, Cat no A-11006 and A-21207, 1:1000) were used to bind to the primary antibodies; cell nuclei were counterstained with Hoechst 33258 (Sigma, Cat no 861405, 5 μg/ml). Then cells were photographed using an inverted fluorescence microscope.

3.2.9. PB analysis and flow cytometry

Full blood count was performed with an automated haematology analyser (KX-21N, Sysmex, Japan). Flow cytometry was done on a FACSCanto-II flow cytometer analyser using FACSDiva software (both from BD). Data analysed using FlowJo (v.7.6.4, Tree Star, USA). Cells were labeled after Fc-blocking with different antibodies including APC Lin, FITC Flk-1/VEGFR2, PE CD133, PerCpCy5.5 CD34 and PE CXCR4 (Fig 8).

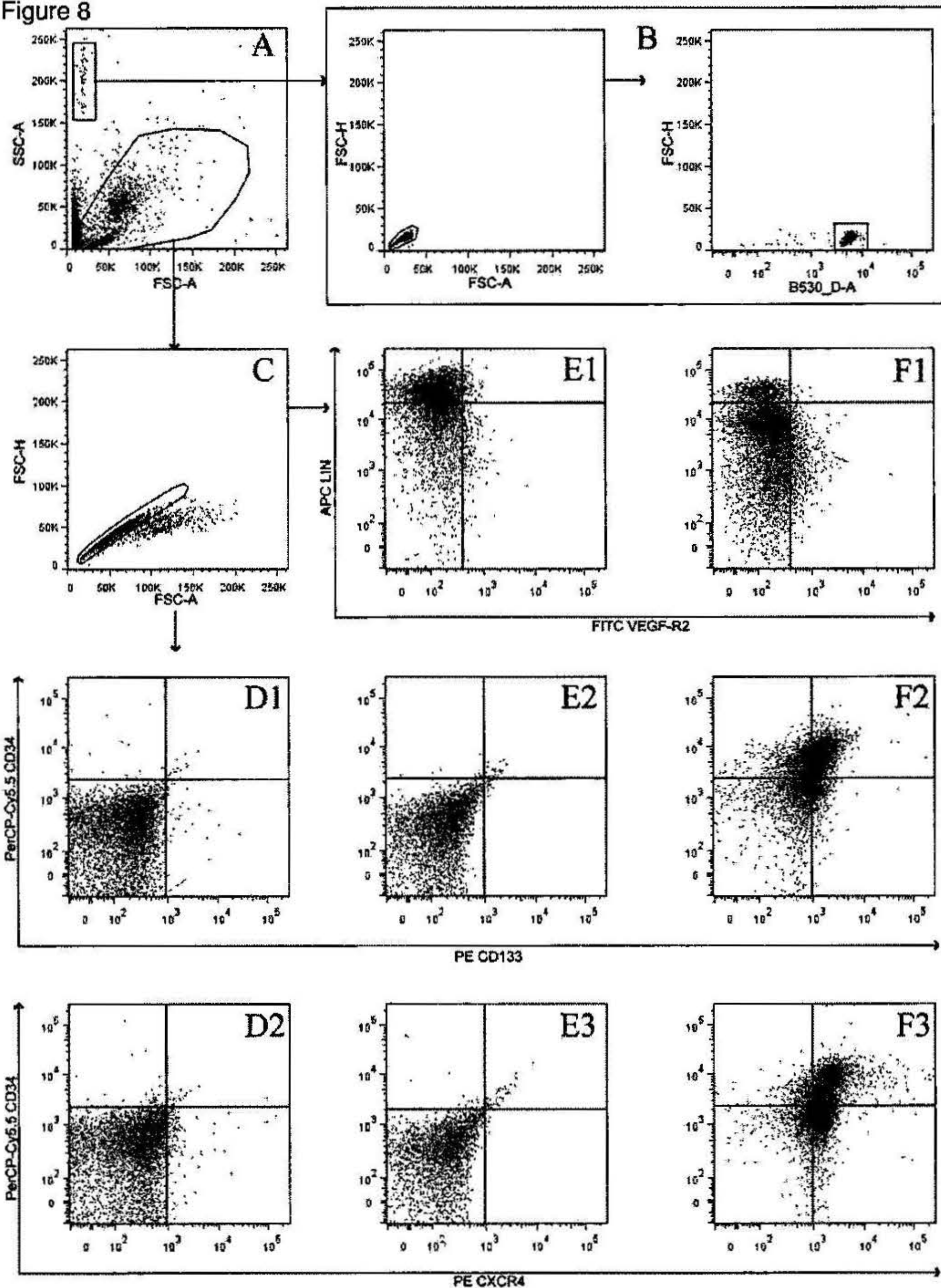

Figure 8: Flow cytometry analysis. Panel A shows the first gating step using FSC-A vs. SSC-A to select nucleated cells. The next step, gating on the singlet population using FSC-A vs. FSC-H, is shown in panel B. The third step is the selection of the $Lin^{-}/VEGFR2^{+}$ populating in the APC and FITC channels, shown in panel C. Panel D shows the population selected in panel C using the channels for PE (in this example CD133) and PerCp-Cy5.5 (CD34). Gates can be set to identify $CD34^{+}$, $CD34^{+}/CD133^{+}$ and $CD34^{+}/CXCR4^{+}$ populations. Results are shown in Table 6.

Compensation tubes were prepared using anti-mouse BD CompBeads (BD, Cat no 552843). Appropriate isotype controls and fluorescence-minus-one tubes were used to define positive populations using biexponential transformation. Flow cytometry absolute count beads standard (Bangs Laboratories, Cat no 580) were used as an internal counting standard for EPC enumeration on the FACSCanto-II. Beads were mixed with samples at a concentration of 10^5 beads per sample. The beads were then gated on the FSC-A vs. SSC-A, then FSC-A vs. FSC-H and then using channels 530 vs. FSC-H. Details of the antibodies and isotype controls are shown in Table 4.

Table 4: List of antibodies and isotype controls used in the flow cytometry analysis.

Antibody	Clone	Source	Catalogue number
Purified NA/LE Rat Anti-Mouse CD16/CD32	2.4G2	BD	553140
APC Mouse Lineage Antibody Cocktail, with Lineage Isotype Control Cocktail that contains equivalent concentrations of isotype-matched negative-control immunoglobulin.	CD3e (145-2C11); CD11b (M1/70); CD45R/B220 (RA3-6B2); Ly-76 (TER-119); Ly-6G and Ly-6C (RB6-8C5)	BD	558074
FITC Rat Anti-Mouse Flk-1	Avas 12alpha1	BD	560680
FITC Rat IgG2a, κ Isotype Control	R35-95	BD	553929
PE Rat Anti-Mouse Prominin-1 (CD133)	MB9-3G8	Miltenyi Biotec	130-092-334
PE Hamster IgG1 κ Isotype Control	A19-3	BD	553972
PerCP/Cy5.5 Anti-Mouse CD34	HM34	Biolegend	128608
PerCP/Cy5.5 Armenian Hamster IgG Isotype Control	HTK888	Biolegend	400932
PE Rat Anti-Mouse CD184	2B11/CXCR4	BD	551966
PE Rat IgG2b, κ Isotype Control	A95-1	BD	553989

3.2.10. Statistics

Data are presented as mean ± standard deviation (SD) for normally distributed data and as mean [interquartile range] when non-normally distributed. Normality was

assessed using the Shapiro-Wilk test and the D'Agostino and Pearson Omnibus normality tests. Differences in variances of normally distributed data were assessed using Levene's test. Differences between groups were either assessed using ANOVA (normally distributed data) or the Kruskal-Wallis test (non-normally distributed data). In cases of significant test results, posthoc comparison using Bonferroni correction (normal distribution) or Dunn's multiple comparison test (non-normal distribution) was performed. For statistical comparisons of percentage values from flow cytometry readings, percentage values were transformed computing their arcsine/square-root values. Statistical significance was defined as $p<0.05$ (two-tailed).

3.3 Results

3.3.1. Animal bodyweight and blood glucose levels

Diabetes was established in 16.7% mice on day 7 and in 100% mice on day 14 after the first injection of STZ (Fig 9). STZ-treated mice stopped gaining weight and were hyperglycaemic throughout the study (Fig 9). Average bodyweight in all mice at 4-weeks of age before induction of diabetes was 23.8±1.1 g. At 22-weeks of age, control mice had an average bodyweight of 32.6±1.5 g whereas diabetic animals had a bodyweight of 23.3±2.6 g (Fig 9A). While control animals gained weight ($p<0.0001$), diabetic animals did not ($p=0.59$). Diabetic mice were lighter than controls at the end of the study ($p<0.0001$) where no significant differences in bodyweight were found between CNTX (32.0±2.0 g) and CTX (33.0±1.1 g) or between DNTX (23.1±2.4 g) and DTX (23.4±2.8 g). The mean random blood glucose level of all mice at the start of the experiment (4-weeks of age) was 8.8±1.4 mmol/l. At 22-weeks of age, blood glucose in control animals remained similar to levels at the start at 8.6±1.1 mmol/l ($p=0.58$) whereas in diabetic animals a significant increase to 31.5±3.2 mmol/l was observed ($p<0.0001$ compared to start; $p<0.0001$ compared to controls; Fig 9B). No significant differences were found between CNTX (8.7±1.3 mmol/l) and CTX (8.5±1.1 mmol/l) or between DNTX (30.2±4.2 mmol/l) and DTX (32.6±1.8 mmol/l). At the end of the experiment the animal numbers in the four experimental groups were: CNTX n=7, CTX n=10, DNTX n=7 and DTX n=9.

Figure 9

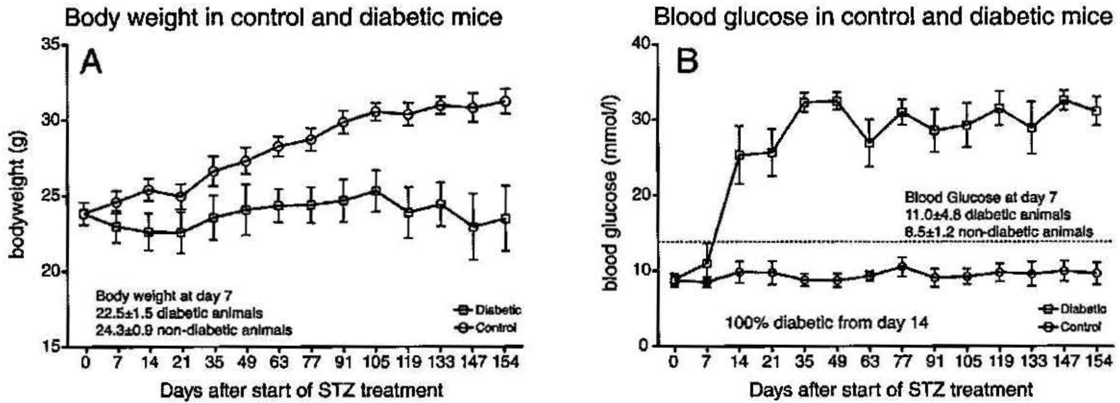

Figure 9: The development of bodyweight (panel A) and blood glucose (panel B) in control and diabetic mice. Circles and squares denote the mean while whiskers denote the 95% confidence interval. The dashed line in panel B marks the limit (13.3 mmol/l) to identify diabetic mice.

3.3.2. Breakdown of the blood-retinal barrier (BRB) revealed by FFA

FFA was conducted to monitor changes in the BRB at different intervals of diabetes (Fig 10). In non-diabetic mice, FFA showed sharp and well-defined retinal vasculature with lack of fluorescein leakage (Fig 10A). Slight and diffuse fluorescein leakage was observed 5 and 16 weeks after the development of hyperglycaemia (Fig 10, B and C), indicating breakdown of the BRB in diabetic mice. Retinal vascular leakage became more intense after one year of diabetes (Fig 10D). This observation is consistent with previous reports, which demonstrated that BRB breakdown in diabetic models, either chemically-induced or genetically modified, is most likely attributed to loss of vascular endothelial cells/pericytes and changes in molecules associated the BRB such as reduced expression of tight junction proteins and increased expression in angiogenic factors, rather than proliferation of abnormal vessels in the retina (Antonetti *et al.*, 1998, Barber *et al.*, 2000, Barber *et al.*, 2005, Feit-Leichman *et al.*, 2005, Ishida *et al.*, 2003a).

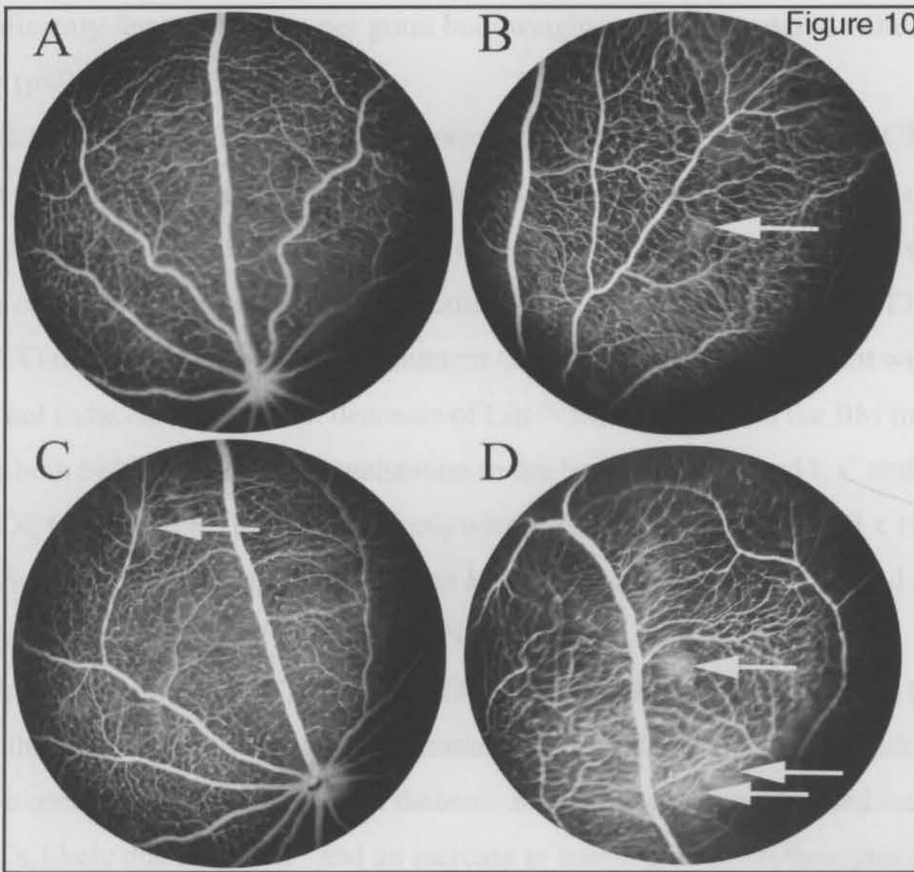

Figure 10: Breakdown of the blood-retinal barrier (BRB) in diabetic mice. Fundus fluorescein angiography (FFA) was conducted in non-diabetic controls (CNTX group, A, 22 weeks of age) and diabetic mice (DNTX group, B-D) 5 weeks (B), 16 weeks (C) and 1 year (D) after the onset of hyperglycaemia. Note: slight and diffuse fluorescein leakage (arrows) was detected in (B) and (C) and retinal vascular leakage became more intense 1 year after diabetes (D, arrows).

3.3.3. Cell numbers in the BM and the PB

The total number of BM cells isolated from each mouse was similar among the 4 groups but non-diabetic mice in CNTX and CTX groups seemed to have fewer BM cells compared with DNTX and DTX groups after normalizing to bodyweight (Fig 11, A and B). Overall, after red blood cell lysis approximately $5.8 \pm 1.1 \times 10^7$ cells were obtained from the BM from each mouse. CNTX and DNTX groups had slightly higher cell numbers compared with CTX and DTX groups respectively, but no statistically significant difference was found among these four groups. There were

significantly fewer BM cells per gram bodyweight in CTX compared with DNTX mice ($p<0.01$, Fig 11B).

Magnetic bead isolation yielded approximately $1.3\pm0.8 \times 10^6$ $Lin^-/VEGFR2^+$ cells from the BM of each mouse, representing ~2.1% of the total number cells isolated. The overall numbers of $Lin^-/VEGFR2^+$ cells and those normalized to bodyweight were compared between diabetic and non-diabetic mice both before (CNTX vs. DNTX) or after (CTX vs. DTX) treatment (Fig 11, C and D). Treatment with the cocktail induced a significant decrease of $Lin^-/VEGFR2^+$ cells in the BM in control mice both before and after normalization to the body weight (Fig 11, C and D): CNTX, 6.1 [3.9 to 8.7] x 10^4 per g bodyweight vs. CTX 2.1 [1.9 to 2.9] x 10^4 per g bodyweight, $p<0.05$, (Fig 11D), a much less profound effect was observed in diabetic animals after the cocktail treatment (DNTX vs. DTX, $p>0.05$, Fig 11D).

Untreated animals (CNTX and DNTX) had fewer PB white blood cells (WBCs) than those receiving the cocktail treatment (CTX and DTX) (Fig 11E). Administration of the cocktail in non-diabetic and diabetic mice resulted in an expected increase in WBCs, likely due to G-CSF, and an increase in haemoglobin, erythrocytes and haematocrit which was likely due to the effects of EPO. However, a statistically significant increase in WBC, erythrocyte count, haematocrit and haemoglobin was observed only in diabetic mice (DNTX vs. DTX) but not in non-diabetic mice after the cocktail treatment (Fig 11, E and F; Table 5).

Figure 11

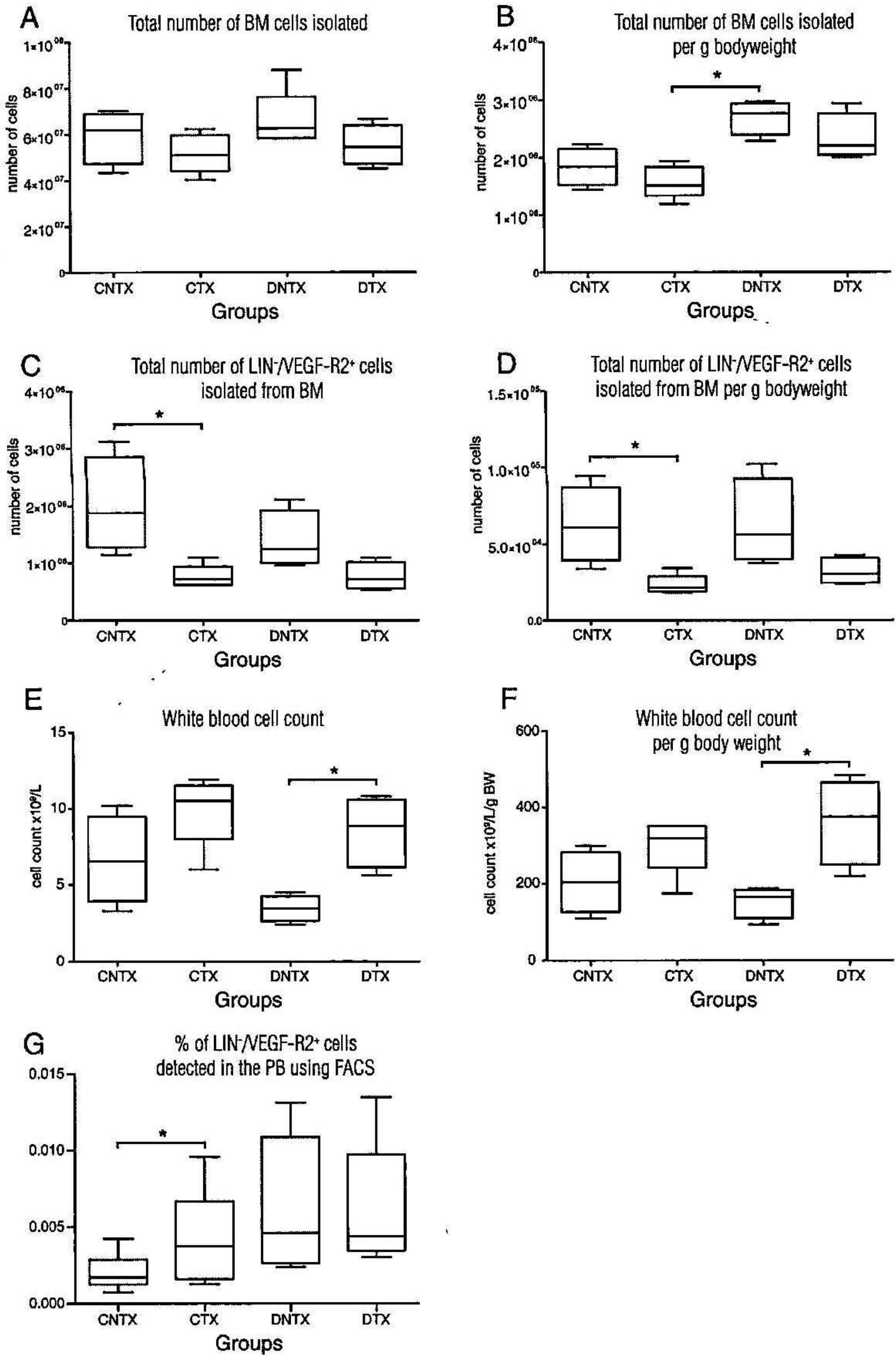

Figure 11: Boxplots showing the number of cells identified. Panel A shows the total number of mononuclear cells isolated from the bone marrow per g bodyweight. Whereas the number of $Lin^-/VEGFR2^+$ cells per g bodyweight is displayed in panel B. The concentration of white blood cells per litre of PB and per g bodyweight is shown in panel C. Horizontal bars with an asterisk indicate groups that show statistically significant differences. Panel G shows the percentage of $Lin^-/VEGFR2^+$ cells identified in the PB using flow cytometry. Boxplots are displaying the 25th percentile, median (50th percentile), 75th percentile as well as the minimum and maximum.

Table 5: Analysis of red blood cells, haemoglobin and haematocrit from peripheral blood. Significant changes (indicated by an asterisk) for the parameters analysed were only found in diabetic mice (DNTX vs. DTX), IQR: interquartile range.

	Peripheral Blood							
	CNTX		DNTX		CTX		DTX	
	median	IQR	median	IQR	median	IQR	median	IQR
red blood cell count (10^{12} cells/l)	7.675	7.473-8.103	7.730	7.420-8.335	9.070	7.855-9.190	9.975*	9.865-10.670
haemoglobin (g/l)	113.5	111.0-118.3	112.0	108.5-120.5	137.0	120.5-140.5	146.5*	144.5-155.3
haematocrit (fraction of 1)	0.3865	0.3775-0.4068	0.3920	0.3790-0.4235	0.4960	0.4235-0.5045	0.5500*	0.5483-0.5825

3.3.4. Flow cytometry analysis

Approximately 2.8% of the mononuclear BM cells detected by flow cytometry were Lin^- with no significant difference between CNTX and DNTX (Table 6). This percentage increased to ~4.9% after mice were treated with the drug cocktail, again no significant difference was found between control and diabetic animals (Table 6). Data for the analysis of PB Lin^- were only available for CTX and DTX groups. The CTX group had a higher proportion of Lin^- cell in PB than the DTX group, however this difference was not significant ($P>0.05$) (Table 6).

Most importantly, in non-diabetic animals (CNTX vs. CTX) the drug cocktail caused a significant decrease in $Lin^-/VEGFR2^+$ cells from ~0.05% to ~0.006% in the BM while in diabetic animals (DNTX vs. DTX) the change of $Lin^-/VEGFR2^+$ cell number was much less profound (Table 6). Similar changes were observed in the PB: a significant increase in $Lin^-/VEGFR2^+$ cells was observed in control animals (CNTX

vs. CTX) but not in diabetic animals (DNTX vs. DTX) after the cocktail treatment (Table 6).

By flow cytometry, approximately 70% of the BM Lin$^-$/VEGFR2$^+$ cells were also positive for CD34 and a small portion, around 8%, was positive for CD133. Further analysis of Lin$^-$/VEGFR2$^+$/CD34$^+$ cells showed no differences between untreated (CNTX vs. DNTX) and treated (CTX vs. DTX) animals. Similar to the Lin$^-$/VEGFR2$^+$ population, a significant decrease in Lin$^-$/VEGFR2$^+$/CD34$^+$ cells was found in the BM only in control animals (CNTX vs. CTX), which was accompanied by a significant increase of Lin$^-$/VEGFR2$^+$/CD34$^+$ cells in the PB (CNTX vs. CTX, Table 6).

Interestingly, changes in Lin$^-$/VEGFR2$^+$ and Lin$^-$/VEGFR2$^+$/CD34$^+$ cells after the cocktail treatment were not found when analysing the numbers of Lin$^-$/VEGFR2$^+$/CD34$^+$/CD133$^+$ cells: neither control nor diabetic animals showed similar changes (decrease in BM, increase in PB) in Lin$^-$/VEGFR2$^+$/CD34$^+$/CD133$^+$ cells after treatment (Table 6).

As CXCR4 is the receptor for stromal derived factor-1 (SDF-1) and it acts as one of the important chemokines in mobilizing EPCs from the BM to the PB (Asahara *et al.*, 1999b, Heissig *et al.*, 2002, Moore *et al.*, 2001), we also evaluated changes in Lin$^-$/VEGFR2$^+$/CD34$^+$/CXCR4$^+$ cells. Treatment with the drug cocktail appeared to result in a decrease of Lin$^-$/VEGFR2$^+$/CD34$^+$/CXCR4$^+$ cells in the BM and an increase in the PB, the changes were however not statistically significant in control and diabetic groups (Table 6).

Flow cytometry showed the numbers of Lin$^-$/VEGFR2$^+$/CD34$^+$/CXCR4$^+$ and Lin$^-$/VEGFR2$^+$/CD34$^+$/CD133$^+$ cells from the BM were greater in diabetic animals than those of non-diabetic controls both before and after treatment, but the differences were not statistically significant (Table 6). Detailed changes in the percentages of the different cell populations in different groups are shown in Table 6.

Table 6: Analysis of flow cytometry data of cells isolated from the bone marrow (upper panel) and the peripheral blood (lower panel). Details are given in the text; significant changes upon treatment are indicated by an asterisk (CNTX vs. CTX), IQR: interquartile range. For Lin$^-$ in CNTX and DNTX no results are available due to a technical problem.

	Bone Marrow							
	CNTX		DNTX		CTX		DTX	
	median	IQR	median	IQR	median	IQR	median	IQR
LIN$^-$	2.63100	2.556-3.631	2.48800	1.801-3.295	4.90700	4.154-5.056	4.46800	4.035-5.289
LIN$^-$/VEGF-R2$^+$	0.05220	0.0280-0.0898	0.05295	0.02783-0.07101	0.00573*	0.004435-0.007544	0.01094	0.01058-0.01541
Lin$^-$/VEGF-R2$^+$/CD34$^+$	0.03391	0.01945-0.06110	0.03432	0.02121-0.05415	0.00536*	0.003357-0.006704	0.00999	0.009282-0.01468
Lin$^-$/VEGF-R2$^+$/CD34$^+$/CD133$^+$	0.00425	0.003573-0.005370	0.01031	0.004885-0.01318	0.00453	0.002646-0.005504	0.00834	0.006359-0.01336
Lin$^-$/VEGFR2$^+$/CD34$^+$/CXCR4$^+$	0.00835	0.008225-0.01375	0.01333	0.006469-0.02189	0.00569	0.002765-0.005844	0.00898	0.008014-0.01282

	Peripheral Blood							
	CNTX		DNTX		CTX		DTX	
	median	IQR	median	IQR	median	IQR	median	IQR
LIN$^-$	.	.	.	.	5.41300	2.969-7.225	3.34200	3.066-3.576
LIN$^-$/VEGF-R2$^+$	0.00170	0.001251-0.002871	0.00480	0.001703-0.009564	0.00829*	0.002717-0.02368	0.00439	0.003447-0.009735
Lin$^-$/VEGF-R2$^+$/CD34$^+$	0.00138	0.0008752-0.002382	0.00139	0.0006154-0.008325	0.00588*	0.002582-0.01279	0.00286	0.002025-0.005293
Lin$^-$/VEGF-R2$^+$/CD34$^+$/CD133$^+$	0.00163	0.0006317-0.002513	0.00163	0.0005714-0.02530	0.00170	0.0009646-0.003974	0.00236	0.001770-0.003698
Lin$^-$/VEGF-R2$^+$/CD34$^+$/CXCR4$^+$	0.00113	0.0008627-0.001427	0.00068	0.0004184-0.002667	0.00180	0.001254-0.006406	0.00157	0.001364-0.004207

All values of the respective population are given as percentage of the mononuclear fraction of bone marrow cells.

3.3.5. Cell culture, colony forming and immunocytochemistry

Isolated Lin$^-$/VEGFR2$^+$ cells started to attach approximately 24 hours after seeding into flasks coated with collagen-I. Cobble-stone shaped colonies started to form after 3 to 4 weeks but cell expansion and colony growth were very slow. Colonies from P0 cells were monitored until day 73 after seeding. No differences in growth rate and colony morphology were observed between cells isolated from control and diabetic mice (Fig 12). Passaging cells resulted in death of the cells after a few days, hence only unpassaged cells from both control and diabetic animals were studies over an extended period of time. All cells in culture took up Dil-acLDL and lectin on immunohistochemical analysis. Furthermore all cells in the culture dish were positive for CD34 and VEGFR2 (Fig 7).

Figure 12

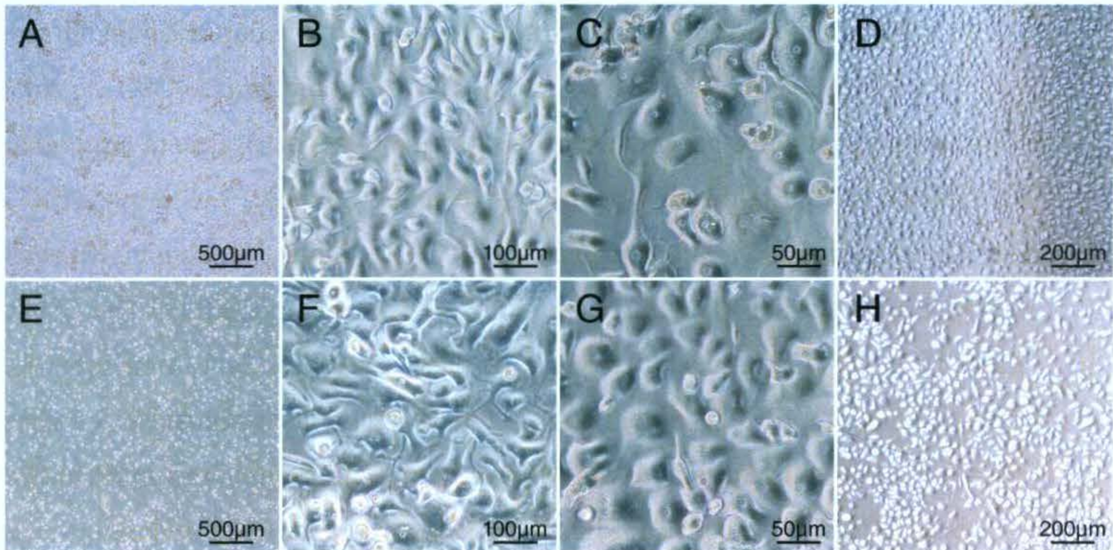

Figure 12: Panels A-D show cultured $Lin^-/VEGF\text{-}R2^+$ cells from control mice at day 0, day 7, day 35 and day 73, respectively. Similarly, cultured $Lin^-/VEGF\text{-}R2^+$ cells from diabetic mice are shown in panels E-H.

3.4 Discussion

This study compared the mobilization capacity of $Lin^-/VEGFR2^+$ progenitor cells from the BM to PB between diabetic mice with early diabetic retinopathy and non-diabetic controls after systemic administration of a cocktail consisting of different reagents. Three significant observations were made: First, 16 weeks of diabetes did not significantly affect the number of $Lin^-/VEGFR2^+$ in the BM and PB in either diabetic or non-diabetic mice, although breakdown of the BRB had been observed 16 weeks after diabetes. Second, mobilization of BM-EPCs from BM to PB was observed in non-diabetic animals but not in diabetic mice following treatment with a cocktail of G-CSF, EPO and BH4. The number of $Lin^-/VEGFR2^+$ in the BM decreased significantly in concert with an increase of $Lin^-/VEGFR2^+$ cells in the PB after the cocktail treatment in non-diabetic mice, while little change was observed in diabetic mice. Third, there was no difference in colony formation capability, colony growth pattern and immunocytochemical phenotypes of $Lin^-/VEGFR2^+$ isolated from control and diabetic animals.

The substances that we used in the cocktail had been individually shown to improve the mobilization and function of certain subsets of EPCs by different mechanisms. EPO acts by binding to the EpoR (Heeschen *et al.*, 2003), thereby stimulating the Janus kinase (JAK)/signal transducer and the activator of transcription (STAT) (Ribatti *et al.*, 1999) as well as the phosphatidylinositol-3 kinase (PI3K)/AKT pathways (Mahmud *et al.*, 2002). EPO has been reported to mobilize human $CD133^+$, murine $CD34^+/VEGFR2^+$, canine $CD34^+$ and murine $SCA1^+$ EPC subsets (Heeschen *et al.*, 2003, Hirata *et al.*, 2006, McVicar *et al.*, 2010, Urao *et al.*, 2006). G-CSF acts indirectly by releasing vascular endothelial growth factor (VEGF) (Ohki *et al.*, 2005), which stimulates EPC migration (Asahara *et al.*, 1999b, Hattori *et al.*, 2001). G-CSF has been reported to mobilize murine $CD34^+/VEGFR2^+$, murine $VEGFR2^+$ and murine $SCA1^+$ EPC subsets (Ohki *et al.*, 2005, Takahashi *et al.*, 1999, Yoshioka *et al.*, 2006). BH4 is supposed to improve eNOS function by preventing uncoupling of eNOS in EPCs (Thum *et al.*, 2007, Tiefenbacher, 2001). BH4 has been shown to improve human late outgrowth EPC function (He *et al.*, 2011). While this cocktail did mobilize $Lin^-/VEGFR2^+$ from the BM to PB in non-diabetic animals, it was ineffective in diabetic animals suggesting that early diabetes impairs the mobilization of BM $Lin^-/VEGFR2^+$.

Impaired mobilization of BM $Lin^-/VEGFR2^+$ may contribute to the pathogenesis of the complications of diabetes, such as the breakdown of the blood-retinal barrier that occurs early in diabetic retinopathy. The blood-retinal barrier, an extensive network of endothelial cells, depends on tight junctions between vascular endothelial cells. Retinal vascular endothelial dysfunction is an early feature of diabetic retinopathy. As the diabetic retinopathy develops, damage to EPC mobilization from the BM to PB could lead to the failure in retinal vascular repair, thus contributing to breakdown of the blood-retinal barrier.

Many studies have characterized the function of various types of EPCs isolated from the PB of diabetic individuals that were defined using different markers (Asahara *et al.*, 1997, Friedrich *et al.*, 2006, Heeschen *et al.*, 2003, Tepper *et al.*, 2002, Thum *et al.*, 2007). A reduction in the number of EPCs in the PB of diabetic individuals has been reported repeatedly in humans (Fadini *et al.*, 2005, Schmidt-Lucke *et al.*, 2005, Tepper *et al.*, 2002, Werner *et al.*, 2005). Recent studies, however, have identified conditions in people with diabetes, such as diabetic retinopathy, where EPCs in PB are increased rather than reduced (Brunner *et al.*, 2009, Lee *et al.*, 2006a).

Our study did not find any differences between diabetic and control mice either in numbers of Lin$^-$/VEGFR2$^+$ in the BM and PB. Another effect of diabetes that has been thoroughly investigated is the impaired capability of diabetic EPCs from human PB to form colonies and to proliferate (Loomans *et al.*, 2004, Schatteman *et al.*, 2000, Tepper *et al.*, 2002, Waltenberger, 2001). EPCs isolated from the PB of diabetic humans showed impaired proliferation, colony formation and integration into tubular networks compared with control EPCs (Loomans *et al.*, 2004, Tepper *et al.*, 2002). A different approach using EPCs isolated from human PB via centrifugation followed by CD34$^+$ selection resulted in very similar findings when placed in culture medium containing 30 mM glucose compared with cells cultured in normal glucose (5 mM) medium (Schatteman *et al.*, 2000). This difference in cell growth potentials between EPCs under diabetic and non-diabetic environments could not be observed in our study. While it is well known that the complications of diabetes take years to develop (Nathan *et al.*, 2009), the present study examined changes after 16-weeks of diabetes only, by which time the duration of hyperglycaemia might not be sufficient to compromise the number and function of Lin$^-$/VEGFR2$^+$ cells. This hypothesis is supported by a recent report that changes in EPCs function were closely associated with the duration of diabetes in humans (Fadini *et al.*, 2010). This may explain the comparable numbers of cells isolated from the BM between control and diabetic mice and also the similar growth pattern and cell phenotypes in this study. Advanced glycation end products, which accumulate over years in people with diabetes (Negrean *et al.*, 2007), have been reported to impair the function and induce apoptosis in EPCs (Chen *et al.*, 2009a).

Although there are clearly long-term effects of diabetes on EPCs, short-term effects are also important. Our results indicate that a short-term effect of diabetes in mice is impairment of mobilization of BM Lin$^-$/VEGFR2$^+$ to the PB. Further research is warranted to elucidate the underlying mechanisms of impaired EPC mobilization in diabetes.

CHAPTER 4

DIFFERENTIAL GENE EXPRESSION IN LIN$^-$/VEGFR2$^+$ BONE MARROW-DERIVED PROGENITOR CELLS ISOLATED FROM DIABETIC MICE

4 Differential gene expression in Lin⁻/VEGFR2⁺ bone marrow-derived progenitor cells isolated from diabetic mice

4.1 Introduction

The inner lining of blood vessels, the endothelium, is made up of a single layer of endothelial cells (ECs) (Cines *et al.*, 1998), which acts as a barrier between the blood and the surrounding tissue. It prevents inflammatory cell infiltration, modulates vascular tone and controls smooth muscle cell proliferation (Gimbrone *et al.*, 1997, Gimbrone *et al.*, 2000, Traub *et al.*, 1998). Damage to the endothelium can be repaired by proliferation and migration of nearby mature ECs (Hristov *et al.*, 2007, Miller-Kasprzak *et al.*, 2007), which have limited regenerative capacity (Ballard *et al.*, 2007, Werner *et al.*, 2006).

Endothelial progenitor cells originating from the BM (Asahara *et al.*, 1997) can migrate to the PB (Asahara *et al.*, 1997, Werner *et al.*, 2005) and repair injured endothelium (Rookmaaker *et al.*, 2002, Walter *et al.*, 2002, Werner *et al.*, 2002). These EPCs play an important role in regenerating the endothelium through migration, proliferation, differentiation and by secreting pro-angiogenic cytokines (Fadini *et al.*, 2006c, Mukai *et al.*, 2008). EPCs express a range of cell surface markers, among them stem cell markers (CD34, CD133) and endothelial markers (CD146, vWF, VEGFR2) (Fadini *et al.*, 2008b, Urbich *et al.*, 2004). As no single or unique marker for EPCs has been be identified, researchers use a range of markers and phenotypical properties to define them (Bellik *et al.*, 2005, Critser *et al.*, 2011, Friedrich *et al.*, 2006, Ingram *et al.*, 2004, Ishikawa *et al.*, 2004, Medina *et al.*, 2010c, Murohara *et al.*, 2000, Walenta *et al.*, 2005). Regardless of this lack of accuracy, the term "EPC" is used and it is acknowledged that "EPC" refers to a heterogeneous population of cells rather than a single population (Barber *et al.*, 2006b, Hirschi *et al.*, 2008, Medina *et al.*, 2010c).

Diabetes mellitus, characterized by chronic hyperglycaemia (Alberti *et al.*, 1998), is a metabolic condition that strongly affects EPCs. Diabetic patients have reduced numbers of EPCs (Awad *et al.*, 2005, Loomans *et al.*, 2004) in the PB and the function of such EPCs isolated from PB with respect to proliferation, tube formation and adhesion is impaired (Segal *et al.*, 2006, Tepper *et al.*, 2002). Most importantly,

EPCs from diabetic individual are less competent in repairing vascular injuries (Awad *et al.*, 2006, Jiang *et al.*, 2004, Schatteman, 2004, Stepanovic *et al.*, 2003, Tepper *et al.*, 2002). Not only impaired function but also reduced mobilization from the BM to the PB has been documented (Awad *et al.*, 2005, Segal *et al.*, 2006) in diabetic patients. Both the decreased number of EPCs and their impaired function have been proposed to be involved in the pathogenesis of vascular complications in diabetes (Ding *et al.*, 2005, Loomans *et al.*, 2004, Tepper *et al.*, 2002).

Since most studies to elucidate molecular mechanisms of EPC impairment in diabetes have been conducted in EPCs isolated from the PB in humans with a long history of diabetes, little is known about the changes occurring in EPCs located within the BM in the early stages of diabetes. A subset of BM derived EPCs, which are phenotypically characterized as $Lin^-/VEGFR2^+$ cells, were recently described (Barthelmes *et al.*, 2012a, Barthelmes *et al.*, 2012b). These $Lin^-/VEGFR2^+$ progenitor cells have typical properties of EPCs such as formation of cobblestone-shaped colonies, Dil-acLDL uptake, lectin binding, expression of typical EPC markers such as VEGFR2 and CD34, lack of expression of CD31, CD45, CD14 and CD115 and incorporation into damaged blood vessels *in vivo* (Barthelmes *et al.*, 2012a, Barthelmes *et al.*, 2012b).

We have recently shown (Barthelmes *et al.*, 2012a, Barthelmes *et al.*, 2012b) that BM-derived $Lin^-/VEGFR2^+$ progenitor cells isolated from diabetic mice showed neither functional differences nor reduced proliferative capacity compared with such cells isolated from non-diabetic mice. However, we found a distinct defect in mobilization of $Lin^-/VEGFR2^+$ progenitor cells from BM to the PB in diabetic mice. To explore the molecular mechanisms underlying this defect in mobilization of $Lin^-/VEGFR2^+$ bone marrow derived progenitor cells in spontaneously diabetic mice, the current study aimed to evaluate differential gene expression of 35 genes that were reported to be closely involved in EPC mobilisation and EPC function (see Table 7).

4.2 Methods

4.2.1. Animals

All animal studies were conducted in accordance with the New South Wales Animals Act (1985). Approval was issued by The University of Sydney Animal

Ethics Committee. The animals, $Ins2^{Akita}$ mice (Akita mice), were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). The Akita mouse carries a dominant point mutation in the Insulin 2 gene on chromosome 7 resulting in the development of diabetes at approximately 4 weeks after birth (Yoshioka *et al.*, 1997) with almost 100% penetrance (Wang *et al.*, 1999). As female mice develop diabetes more slowly and less stably compared with males (Yoshioka *et al.*, 1997), only male mice heterozygous for the $Ins2^{Akita}$ allele (diabetic group) as well as male mice homozygous for the wild type Ins2 allele (non-diabetic mice) were used in this study. Presence of the $Ins2^{Akita}$ allele or the wild type Ins2 gene was confirmed by RFLP analysis (Wang *et al.*, 1999). Once diabetes was established (blood glucose level>13.3mmol/L), mice were monitored weekly for changes in bodyweight and blood glucose levels for 18 weeks. The blood glucose level was measured using Accu-Chek Performa (Roche, Germany). No supplemental insulin was given. Only mice having blood glucose levels consistently $\geq$ 13.3 mmol/L were used in this study. Eight diabetic and 8 non-diabetic mice were used.

4.2.2. BM collection

After euthanizing mice with CO_2, the femora and tibiae of both legs were immediately excised and the diaphyses flushed using a 25g needle and 8 ml of IMag™ buffer (BD, Cat no 552362). The collected cells were placed on ice. After centrifugation (400 rcf, 5 min), the cell pellet was resuspended in 2 ml red cell lysing buffer (Sigma, Cat no R7757). After 5 min incubation and centrifugation (400 rcf, 5 min), the cell pellet was resuspended in 10 ml of IMag™ buffer and washed twice. Cells were eventually filtered using a 40 μm nylon cell strainer (BD, Cat no 352340), centrifuged (400 rcf, 5 min) and resuspended in 2 ml of IMag™ buffer. After cell counting (TC10, BioRad) and viability assessment using the trypan blue exclusion assay, cells were placed on ice for the isolation of $Lin^-/VEGFR2^+$ progenitor cells.

4.2.3. Immunomagnetic bead separation of BM $Lin^-/VEGFR2^+$

BM cells were incubated with NA/LE rat anti-mouse CD16/CD32 (Fc-block, 1 $\mu g/10^6$ cells, BD, Cat no 553140) for 15 min. After FC-block, BM cells were

incubated with a solution containing an APC mouse lineage antibody cocktail (BD, Cat no 558074) and a FITC rat anti-mouse Flk-1/VEGF-R2 antibody (BD, Cat no 560680). After 20 min incubation, cells were centrifuged (400 rcf, 5 min) and washed twice using cold IMag™ buffer. Cells were incubated with magnetic beads for 30 min at 4°C (APC magnetic particles-DM; BD, Cat no 557932). The Lin$^+$ depletion was conducted using a Dynal MPC-S magnetic separator (Invitrogen, Cat no 12020D). The Lin$^-$ fraction was further incubated with anti-FITC beads (Miltenyi Biotec, Cat no 130-048-701) for 30 min at 4°C. The fraction of Lin$^-$/VEGFR2$^+$ progenitor cells was obtained via a positive selection step using the magnetic separator. For RNA isolation, fractions of both Lin$^+$ and the Lin$^-$/VEGFR2$^+$ cells were collected, washed in PBS and centrifuged. Cell pellets were re-suspended in 100µl of RNA later solution (Qiagen, Cat no 76104) and snap-frozen in liquid nitrogen. Cells destined to undergo protein analysis were directly snap-frozen and stored at -80°C for further use.

4.2.4. Group design

Four experimental groups were established: (1) Lin$^+$ cells from non-diabetic animals, (2) Lin$^+$ cells from diabetic animals, (3) Lin$^-$/VEGFR2$^+$ cells from non-diabetic animals and (4) Lin$^-$/VEGFR2$^+$ from diabetic animals. The Lin$^+$ cells were used as an internal reference to identify differential gene expression occurring not exclusively in Lin$^-$/VEGFR2$^+$ cells. This setup allowed us to distinguish differential gene expression which specifically occurred in diabetic BM derived Lin$^-$/VEGFR2$^+$ progenitor cells from that which may also occur in other phenotypes of BM cells. Hence, only significant changes in gene expression observed in diabetic vs. non-diabetic Lin$^-$/VEGFR2$^+$ progenitor cells that did not occur in the Lin$^+$ population were considered in the final analysis.

4.2.5. RNA isolation

RNA isolation was performed at room temperature using the RNeasy Mini Kit (Qiagen, Cat no 74104) according to the manufacturer's instructions. Isolated RNA was snap-frozen and stored at -80°C for further use. RNA concentration was measured using a Nanodrop 1000 (Nanodrop Products, DE, USA), integrity was

assessed using the BioRad Experion automated electrophoresis system (BioRad, CA, USA) on a RNA StdSens Chip (BioRad Cat no 700-7159).

4.2.6. Reverse transcription

Reverse transcription was done using the iScript cDNA synthesis kit (BioRad, Cat no 170-8890). In brief, 4 µl of 5x iScript reaction buffer, 1 µl iScript reverse transcriptase, 500 ng RNA and water up to a total reaction volume of 20 µl were mixed. The reverse transcription program was designed as follows: 25°C for 5 min, 42°C for 60 min, 85°C for 5 min followed by 4°C at a hold step. Reactions were performed in a PCR machine (HBPX220, Hybaid, UK). The finial 20µl cDNA product was diluted into 160 µl total volume using MQ water.

4.2.7. Real Time PCR

Primer sequences for RT-PCR were obtained from http://pga.mgh.harvard.edu/primerbank and from http://primerdepot.nci.nih.gov/. *In silico* analyses were performed to identify the amplicon size and suitability of the primer pairs. An overview of genes tested and primers used is shown in Table 7. All primers had a melting temperature of approximately 61°C and were tested before RT-PCR using gel electrophoresis to visualize amplicons. For testing primers, a total reaction volume of 10 µl comprised of 5 µl Super Mix (SsoFast EvaGreen Supermix, BioRad, Cat no 172-5200), 1 µl of 4 µM forward and reverse primer mixture, 1 µl of cDNA and 3 µl water. PCR steps used were similar to the RT-PCR program used later: 95°C for 30s, 40 cycles of 95°C for 5s then 60°C for 20s. This was followed by a melting curve step starting from 65°C to 95°C each step lasting 30s, ramp rate was 0.5°C/s. PCR products were analysed in 2% agarose (in TBE buffer) gels to verify amplicon size.

Table 7: Overview of the 35 genes tested, allocated to either EPC mobilisation and/or EPC function, the primers used for RTPCR and references.

Functional Group	Gene Symbol	Gene name	Forward / Reverse Primer	References
mobilisation	MMP2	matrix metalloproteinase 2	CAAGTTCCCCGGCGATGTC TTCTGGTCAAGGTCACCTGTC	(Cheng *et al.*, 2007, Egeblad *et al.*, 2002, Libby *et al.*, 2000)
	MMP9	matrix metalloproteinase 9	CTGGACAGCCAGACACTAAAG CTCGCGGCAAGTCTTCAGAG	(Hattori *et al.*, 2002, Heissig *et al.*, 2002)
	CXCR4	chemokine receptor type 4	GAAGTGGGGTCTGGAGACTAT TTGCCGACTATGCCAGTCAAG	(Askari *et al.*, 2003, Kijowski *et al.*, 2001, Kollet *et al.*, 2003, Lapidot *et al.*, 2005, Neuhaus *et al.*, 2003, Petit *et al.*, 2007, Zheng *et al.*, 2007)
	CAV1	Caveolin 1	TGTATGACGCGCACACCAA TGGTTCTGCAATCACATCTTCAA	(Albrecht *et al.*, 2003, Kaplan *et al.*, 2007, Sbaa *et al.*, 2006)
	SDF-1	stromal cell-derived factor-1	TGCATCAGTGACGGTAAACCA TTCTTCAGCCGTGCAACAATC	(Lee *et al.*, 2006b)
	eNOS	endothelial nitric oxide synthase	GGTCCTGTGCATGGATGAG GTTGTACGGGCCTGACATTT	(Aicher *et al.*, 2003, Albrecht *et al.*, 2003, Chong *et al.*, 2002, Dernbach *et al.*, 2004, He *et al.*, 2004a, Heeschen *et al.*, 2003)
	ICAM1	intercellular adhesion molecule 1	GTGATGCTCAGGTATCCATCCA CACAGTTCTCAAAGCACAGCG	(Lee *et al.*, 2006b, Yoon *et al.*, 2006)
	VEGFR1 Flt1	vascular endothelial growth factor receptor 1	CCACCTCTCTATCCGCTGG ACCAATGTGCTAACCGTCTTATT	(Grant *et al.*, 2002, Hattori *et al.*, 2002, Luttun *et al.*, 2002)
	VEGFR2 Flk1	vascular endothelial growth factor receptor 2	TTTGGCAAATACAACCCTTCAGA GCAGAAGATACTGTCACCACC	(Fong *et al.*, 1999, Zachary *et al.*, 2001)
	HIF1A	Hypoxia-inducible factor-1	ACCTTCATCGGAAACTCCAAAG CTGTTAGGCTGGGAAAAGTTAGG	(Bernaudin *et al.*, 2002, Cai *et al.*, 2003, Ceradini *et al.*, 2005, Grimm *et al.*, 2002, Ruscher *et al.*, 2002)
	VCAM 1	vascular cell adhesion protein 1	AGTTGGGGATTCGGTTGTTCT CCCCTCATTCCTTACCACCC	(Jin *et al.*, 2006b)
	IL6	interleukin 6	GCCACCGTTACCCTGATTTG CCAGAGTACACCCAGTGAATGG	(Hazra *et al.*, Mobius-Winkler *et al.*, 2009, Moldenhauer *et al.*, 2008)
	EPOR	erythropoietin receptor	CAACAGCGGACACATCGAGTT TGCGGTGATAGCGAGGAGA	(Satoh *et al.*, 2006)
	EPO	erythropoietin	AGGAATTGATGTCGCCTCCA AGCTTGCAGAAAGTATCCACTGTG	(Chong *et al.*, 2002, Heeschen *et al.*, 2003, Urao *et al.*, 2006)
	PTPN11	protein tyrosine phosphatase SHP2	ATGACATCGCGGAGATGGTTT GGGTTACTCTTACTGGGCCTT	(Schroder *et al.*, 2009, Trop *et al.*, 2008)
	VEGFA	vascular endothelial growth factor A	GCACATAGAGAGAATGAGCTTCC CTCCGCTCTGAACAAGGCT	(Asahara *et al.*, 1999b, Grunewald *et al.*, 2006, Young *et al.*, 2002)
	SELE	E-Selectin	ATGCCTCGCGCTTTCTCTC GTAGTCCCGCTGACAGTATGC	(Nishiwaki *et al.*, 2007, Oh *et*

				al., 2007)
	AKT	protein Kinase B	GCACCTTTATTGGCTACAAGGA GGGGACTCTCGCTGATCCA	(Ackah *et al.*, 2005, Chen *et al.*, 2009a, Li *et al.*, 2009, Urbich *et al.*, 2004)
	BDNF	brain-derived neurotrophic factor	TCATACTTCGGTTGCATGAAGG GTCCGTGGACGTTTACTTCTTT	(Chen *et al.*, 2005, He *et al.*, 2012, He *et al.*, 2004b, Kermani *et al.*, 2007, Liu *et al.*, 2010)
function	CASP9	Caspase 9	TCCTGGTACATCGAGACCTTG AAGTCCCTTTCGCAGAAACAG	(Gerber *et al.*, 1998b, Valle *et al.*, 2005)
	HSPD1	heat shock protein 1	CACAGTCCTTCGCCAGATGAG CTACACCTTGAAGCATTAAGGCT	(Amberger *et al.*, 1997)
	PIK3R1	phosphatidylinositol 3-kinase regulatory subunit alpha	CCCACTACTGTAGCCAACAAC CGTACCAAAAAGGTCCCATCA	(Ackah *et al.*, 2005, Chen *et al.*, 2007, Dimmeler *et al.*, 2000, Fulton *et al.*, 1999, Ho *et al.*, 2006, Kim *et al.*, 2000, Madeddu *et al.*, 2008, Wilson *et al.*, 2001, Xia *et al.*, 2008).
	GATA 2	GATA binding protein 2	CACCCCGCCGTATTGAATG CCTGCGAGTCGAGATGGTTG	(Khandekar *et al.*, 2007)
	eNOS	endothelial nitric oxide synthase	GGTCCTGTGCATGGATGAG GTTGTACGGGCCTGACATTT	(Aicher *et al.*, 2003, Albrecht *et al.*, 2003, Chong *et al.*, 2002, Dernbach *et al.*, 2004, He *et al.*, 2004a, Heeschen *et al.*, 2003)
	VEGFR2 Flk1	vascular endothelial growth factor receptor 2	TTTGGCAAATACAACCCTTCAGA GCAGAAGATACTGTCACCACC	(Fong *et al.*, 1999, Zachary *et al.*, 2001)
	CLDN5	Claudin 5	GCAAGGTGTATGAATCTGTGCT GTCAAGGTAACAAAGAGTGCCA	(Madlambayan *et al.*, 2009, Medina *et al.*, 2010c)
	CDH5	vascular endothelial cadherin	AGGACAGCAACTTCACCCTCA AACTGCCCATACTTGACCGTG	(Hristov *et al.*, 2003a, Hur *et al.*, 2004, Murasawa *et al.*, 2005)
	IL6	interleukin 6	GCCACCGTTACCCTGATTTG CCAGAGTACACCCAGTGAATGG	(Hazra *et al.*, Mobius-Winkler *et al.*, 2009, Moldenhauer *et al.*, 2008)
	Tie-2	angiopoietin receptor 2	CGGCCAGGTACATAGGAGGAA TCACATCTCCGAACAATCAGC	(Asahara *et al.*, 1997, Kim *et al.*, 2006a, Murasawa *et al.*, 2005, Nowak *et al.*, 2004)
	FN1	Fibronectin	ATGTGGACCCCTCCTGATAGT GCCCAGTGATTTCAGCAAAGG	(Real *et al.*, 2011, Satoh *et al.*, 2006)
	P53	protein 53	GCGTAAACGCTTCGAGATGTT TTTTTATGGCGGGAAGTAGACTG	(Rosso *et al.*, 2006, Zampetaki *et al.*, 2008)
	PKC	protein kinase C	GTTTACCCGGCCAACGACT GGGCGATGAATTTGTGGTCTT	(Thum *et al.*, 2007, Xu *et al.*, 2008)
	NANOG	homeobox protein NANOG	TCTTCCTGGTCCCCACAGTTT GCAAGAATAGTTCTCGGGATGAA	(Romagnani *et al.*, 2005)
	FGF1	heparin-binding growth factor 1	CAGCTCAGTGCGGAAAGTG TGTCTGCGAGCCGTATAAAAG	(Carmeliet, 2000a, Chen *et al.*, 2009b, Takahashi *et al.*, 2012)
	IGF1	insulin-like growth factor 1	CTGGACCAGAGACCCTTTGC GGACGGGGACTTCTGAGTCTT	(Fleissner *et al.*, 2008, Humpert *et al.*, 2008)

	AKT	protein Kinase B	GCACCTTTATTGGCTACAAGGA GGGGACTCTCGCTGATCCA	(Ackah *et al.*, 2005, Chen *et al.*, 2009a, Li *et al.*, 2009, Urbich *et al.*, 2004)
	HOXA9	homeobox protein Hox-A9	GCCACACGAACCAAGAGGAC CGGGTGCGTACATAGAGCATAA	(Pirro *et al.*, 2007, Rossig *et al.*, 2005, Zampetaki *et al.*, 2008)
	BDNF	brain-derived neurotrophic factor	TCATACTTCGGTTGCATGAAGG GTCCGTGGACGTTTACTTCTTT	(Chen *et al.*, 2005, He *et al.*, 2012, He *et al.*, 2004b, Kermani *et al.*, 2007, Liu *et al.*, 2010)
	IL11	Interleukin 11	GGCCAGATAGAGTCGTTGCC GGGATCGGGTTAGGAGAACAG	(Li *et al.*, 2011, Mohri *et al.*, 2009)
	OCLN	Occludin	TTGAAAGTCCACCTCCTTACAGA CCGGATAAAAAGAGTACGCTGG	(Zengin *et al.*, 2006, Zhang *et al.*, 2005b)

RT-PCR was performed on a LightCycler 480 (Roche, Switzerland) using 384 well plates. Each group included seven individual samples (from 6 litters), each individual sample was replicated once (technical replicate). The program was as follows: 95°C for 5 min, 40 cycles of 95°C for 10s, 60°C for 20s and 72°C for 20s. Ramp rate was 4.8°C/s. Each well contained 5 µl Express Sybr Green (Invitrogen, Cat no 10000162), 0.5 µl water, 0.5 µl of 4 µM forward and reverse primer mixture and 4 µl of the diluted sample cDNA. Mouse glyceraldehyde-3-phosphate dehydrogenase (mGAPDH) and 18S ribosomal RNA (18srRNA) were used as reference genes. CT-values were computed using the 2nd order derivation method, CT values ≥ 35 were excluded from the analysis. Data analysis was performed using the RT^2 profiler PCR array data analysis available on http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php.

4.2.8. Protein isolation and Western Blot

For western blot analysis, 8 biological samples (8 different mice) from each group were used. The isolated cells were incubated and lysed for 30 min at 4°C in RIPA buffer (Sigma, Cat no 127K6009) containing protease inhibitor (Complete mini; Roche, Cat no 046931240010; 1 tablet per 10 ml RIPA buffer). Buffer volume was adjusted to a concentration of 5x10^4 cells/µl RIPA buffer. The lysed cells were

centrifuged at 12,000 rcf for 20 min at 4°C. The supernatant containing the protein was aliquoted (26 μl) and stored at -20°C for further use.

Gel-electrophoresis to separate proteins according to their size was done using 2,2-Bis(hydroxymethyl)-2,2',2"-nitrilotriethanol (Bis-Tris) polyacrylamide gels with a gradient from 4 to 12% under denaturing conditions (Nupage, Invitrogen, Cat no NP0335) using 2-(N-morpholino)ethanesulfonic acid sodium dodecyl sulfate (MES-SDS, Invitrogen, Cat No NP0002) as running buffer. Before loading the gel wells, 26 μl protein sample, 10 μl loading buffer (Invitrogen, Cat no NP0007), 4 μl 500 mM DL-dithiothreitol (DTT; Sigma, Cat no D9779-10G) were mixed and kept at 70°C for 10 min to denature the protein and after this kept on ice for 5 min. After gel-electrophoresis, gels were removed from the running chamber and placed on a 0.2 μm polyvinyl difluoride (PVDF) membrane (Invitrogen, Cat no LC2002). The protein transfer was done using a wet transfer system (BioRad Mini Trans-Blot, Cat no 170-3930). After the transfer, the PVDF membranes were washed for 5 min using water and then for 10 min using TBST: tris (hydroxymethyl) aminomethane (TRIS) buffer, 150 mM sodium chloride and 0.1% polyoxyethylene (20) sorbitan monolaurate (TWEEN 20). A blocking step followed using 5% bovine serum albumin (BSA, Sigma, Cat no 9418) in TBST and incubating the PVDF membrane for 1 h at room temperature. After washing the membrane twice in TBST, the incubation with the primary antibody (SDF-1; 1:2000, Abcam, Cat no ab25117) in TBST and 1% BSA followed over night at 4°C. The next day the primary antibody solution was removed and the PVDF membrane washed 3 times in TBST for 5 min. Exposure to the secondary antibody HRP-goat anti-rabbit IgG (H+L) conjugate (horseradish peroxidase coupled; Zymed, Cat no 81-6120) followed for 2 h at room temperature. After washing 3x for 5 min with TBST, the PVDF membrane was washed twice with TBS and then incubated for 5 min with the chemoluminescent agent (Millipore, Cat no WBKLS0500). Immediately after this, the chemoluminescent agent was removed and the PVDF membrane was analysed using a digital imaging system (G:Box, Syngene, MD, USA). The imaging software used to record chemiluminescence on the PVDF membranes produced images showing white bands on black background (no luminescence). After recording, the PVDF membrane was stripped of the antibodies by incubating for 5 min at room temperature with a western blot stripping buffer (Thermo Scientific, Cat no 46430). After washing the membrane in TBST, the incubation with the next primary antibody followed (E-Selectin, 1:2000, Abcam, Cat

no ab18981) and the procedure of overnight incubation, secondary antibody incubation and imaging was repeated.

4.2.9. Statistics

Data are presented as mean ± standard deviation (SD) for normally distributed data and as mean [interquartile range] when non-normally distributed. Normality was assessed using the Shapiro-Wilk test and the D'Agostino and Pearson Omnibus normality tests. Differences in variances of normally distributed data were assessed using Levene's test. Differences between two groups were either assessed using a student's t-test (normally distributed data) including Welch correction in case of unequal variances or Mann-Whitney test (non-normally distributed data). Statistical significance was defined as $p<0.05$.

4.3 Results

4.3.1. Animal body weight and blood glucose levels

All heterozygous male Akita mice had blood glucose levels ≥13.3 mmol/l from 4 weeks of age. At the time of analysis, mice 22 weeks of age had a bodyweight of 24.5±2.8 g and 36.0±2.8 g for diabetic and non-diabetic mice respectively ($p<0.0001$). Mean blood glucose at 22 weeks of age was and 33.4 [28.2-33.4] mmol/l in diabetic animals and 8.9 [7.8-10.2] mmol/l in non-diabetic ($p<0.0001$, Fig 13).

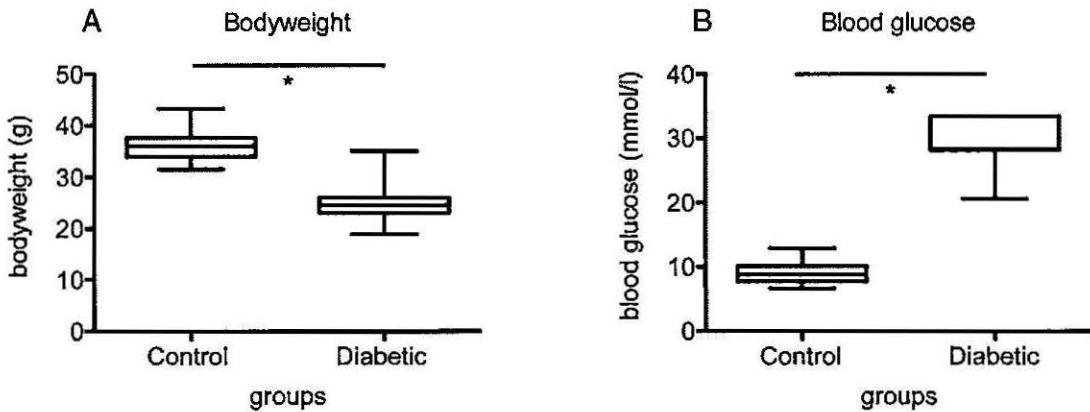

Figure 13: Bodyweight (panel A) and blood glucose levels (panel B) in non-diabetic and diabetic mice at 22 weeks of age (18 weeks of diabetes). Asterisk denotes statistical significance.

4.3.2. Cell numbers in the bone marrow

Overall an absolute number of 3.33 [2.93-3.98] $x10^7$ nucleated cells were isolated from the BM of each mouse. On average, 5.4±2.3% of the BM nucleated cells were $Lin^-/VEGFR2^+$ cells and 82.3±4.4% were Lin^+. The remaining $Lin^-/VEGFR2^-$ cells made up ~12%. While in non-diabetic mice 9.37 [8.61-10.75] $x10^5$ cells/g bodyweight were isolated, from diabetic mice 1.37 [1.10-1.69] $x10^6$ cells/g bodyweight could be obtained ($p<0.0001$). Similarly, the number of $Lin^-/VEGFR2^+$ progenitor cells in the BM was significantly greater in diabetic than non-diabetic animals after adjusting for bodyweight: 7.56 [6.20-8.37] $x10^4$ cells/g bodyweight vs. 4.24 [3.44-5.08] $x10^4$ cells/g bodyweight ($p<0.0001$). There were 1.17 [0.94-1.39] $x10^6$ Lin^+ cells/g bodyweight in diabetic animals and 7.58 [6.85-8.71] $x10^5$ Lin^+ cells/g bodyweight in non-diabetic animals ($p=0.074$).

4.3.3. RNA quality and quantity

From the Lin^+ fraction, 253.5±117.3 ng/µl RNA could be isolated from diabetic animals and 234.0±98.1 ng/µl RNA from non-diabetic animals ($p=0.64$). The $Lin^-/VEGFR2^+$ progenitor cells fraction yielded 135.3±68.1 ng/µl RNA from diabetic mice and 88.0±53.3 ng/µl RNA from non-diabetic mice ($p=0.05$). The integrity of

RNA, expressed as RNA quality indicator (RQI) was ≥7 in all samples, indicating intact RNA (see BioRad tech note 5761 Rev B; http://www.biorad.com).

4.3.4. Gene expression changes

Of the 35 genes studied, HSPD1, SDF-1 and SELE showed significant changes between non-diabetic and diabetic Lin$^-$/VEGFR2$^+$ progenitor cells. While SDF-1 was downregulated about 0.3-fold, SELE was upregulated 2.4-fold in diabetic compared to non-diabetic Lin$^-$/VEGFR2$^+$ progenitor cells. No significant changes in SDF-1 and SELE expression was found between non-diabetic and diabetic Lin$^+$ cells, whereas HSPD1 showed a significant change and a similar fold-change in the diabetic Lin$^+$ cells compared to the non-diabetic Lin$^+$ cells. Therefore, we considered that the differential expression of SDF-1 and SELE genes was specific for Lin$^-$/VEGFR2$^+$ progenitor cells. SDF-1 gene expression was decreased 3-fold while SELE gene expression was increased 2.4-fold in diabetic Lin$^-$/VEGFR2$^+$ progenitor cells compared with those isolated from non-diabetic mice. Unfortunately, no analysis could be performed for 6 genes including CAV1, eNOS, CLDN5, NANOG, OCLN and BDNF due to CT values ≥35. A detailed overview of the results of the gene-expression analysis is shown in Table 8.

Table 8: Overview of the RT-PCR analysis, only SDF-1 and SELE show significant changed in the Lin$^-$/VEGFR2$^+$ PCs while showing no significant changes in the Lin$^+$ group. Each group included seven individual samples, each individual sample was replicated once (technical replicate).

	Diabetic Lin$^+$ vs. Non-Diabetic Lin$^+$			Diabetic Lin$^-$/VEGFR2$^+$ vs. Non-Diabetic Lin$^-$/VEGFR2$^+$		
Gene	Fold-change	95% CI of fold-change	p-value	Fold-change	95% CI of fold-change	p-value
AKT	1.04	(0.84, 1.24)	0.7622	0.34	(0.00, 1.16)	0.9111
BDNF	-	-	-	-	-	-
CASP9	0.77	(0.54, 1.00)	0.1172	0.79	(0.55, 1.03)	0.1151
CAV1	-	-	-	-	-	-
CDH5	0.84	(0.62, 1.07)	0.1977	0.81	(0.30, 1.32)	0.8824
CLDN5	-	-	-	-	-	-
CXCR4	0.73	(0.50, 0.96)	0.0800	0.80	(0.54, 1.06)	0.1993

eNOS	-	-	-	-	-	-
EPO	1.06	(0.62, 1.51)	0.8700	1.16	(0.68, 1.64)	0.5543
EPO-R	1.21	(0.80, 1.61)	0.1860	0.90	(0.68, 1.13)	0.5005
FGF1	0.73	(0.08, 1.38)	0.3329	0.76	(0.17, 1.35)	0.2288
FN1	1.24	(0.72, 1.77)	0.5181	1.62	(0.94, 2.29)	0.0628
GATA2	0.73	(0.47, 0.99)	0.1182	0.62	(0.32, 0.93)	0.0534
HIF1A	0.52	(0.35, 0.69)	0.0019	0.78	(0.35, 1.20)	0.3114
HOXA9	1.29	(0.90, 1.68)	0.0890	0.65	(0.00, 1.72)	0.4628
HSPD1	0.51	(0.35, 0.68)	0.0005	0.59	(0.32, 0.86)	0.0371
ICAM1	0.95	(0.67, 1.22)	0.6373	0.70	(0.39, 1.01)	0.0997
IGF1	0.89	(0.18, 1.60)	0.6833	1.02	(0.14, 1.90)	0.9718
IL11	1.48	(0.74, 2.23)	0.1445	1.70	(0.64, 2.77)	0.1572
IL6	1.04	(0.84, 1.25)	0.7680	1.33	(0.90, 1.75)	0.1526
MMP2	0.88	(0.38, 1.38)	0.5561	0.88	(0.35, 1.41)	0.5012
MMP9	0.78	(0.40, 1.17)	0.4096	1.05	(0.76, 1.34)	0.6447
NANOG	-	-	-	-	-	-
OCLN	-	-	-	-	-	-
P53	0.92	(0.79, 1.05)	0.2973	1.05	(0.89, 1.22)	0.6169
PIK3R1	0.80	(0.56, 1.04)	0.2263	0.81	(0.60, 1.02)	0.1029
PKC	1.04	(0.77, 1.30)	0.7644	1.08	(0.82, 1.33)	0.6184
PTPN11	0.94	(0.77, 1.11)	0.5361	1.14	(0.96, 1.32)	0.1280
SDF-1	**0.48**	**(0.16, 0.80)**	**0.0891**	**0.32**	**(0.04, 0.60)**	**0.0149**
SELE	**1.92**	**(0.24, 3.60)**	**0.1913**	**2.41**	**(1.42, 3.39)**	**0.0005**
Tie2	2.28	(0.89, 3.68)	0.0059	1.22	(0.69, 1.75)	0.5184
VCAM1	1.22	(0.85, 1.60)	0.2085	0.62	(0.25, 0.98)	0.0687
VEGFA	1.08	(0.00, 3.69)	0.9400	1.12	(0.83, 1.41)	0.4433
VEGFR1	1.03	(0.70, 1.35)	0.8699	0.59	(0.25, 0.93)	0.0729
VEGFR2	0.56	(0.26, 0.86)	0.0721	0.55	(0.14, 0.96)	0.2806

4.3.5. Protein level changes

As only SDF-1 and SELE showed specific changes in Lin⁻/VEGFR2⁺ progenitor cells in diabetic animals compared with non-diabetics, validation of differential expression at the protein level was performed only for these two genes. For the two genes studied, western blot showed that only SDF-1 was significantly down-regulated in diabetic BM- Lin⁻/VEGFR2⁺ progenitor cells, while no significant change was found for SELE in western blot analysis (see Fig. 14 and 15, Table 9).

Figure 14

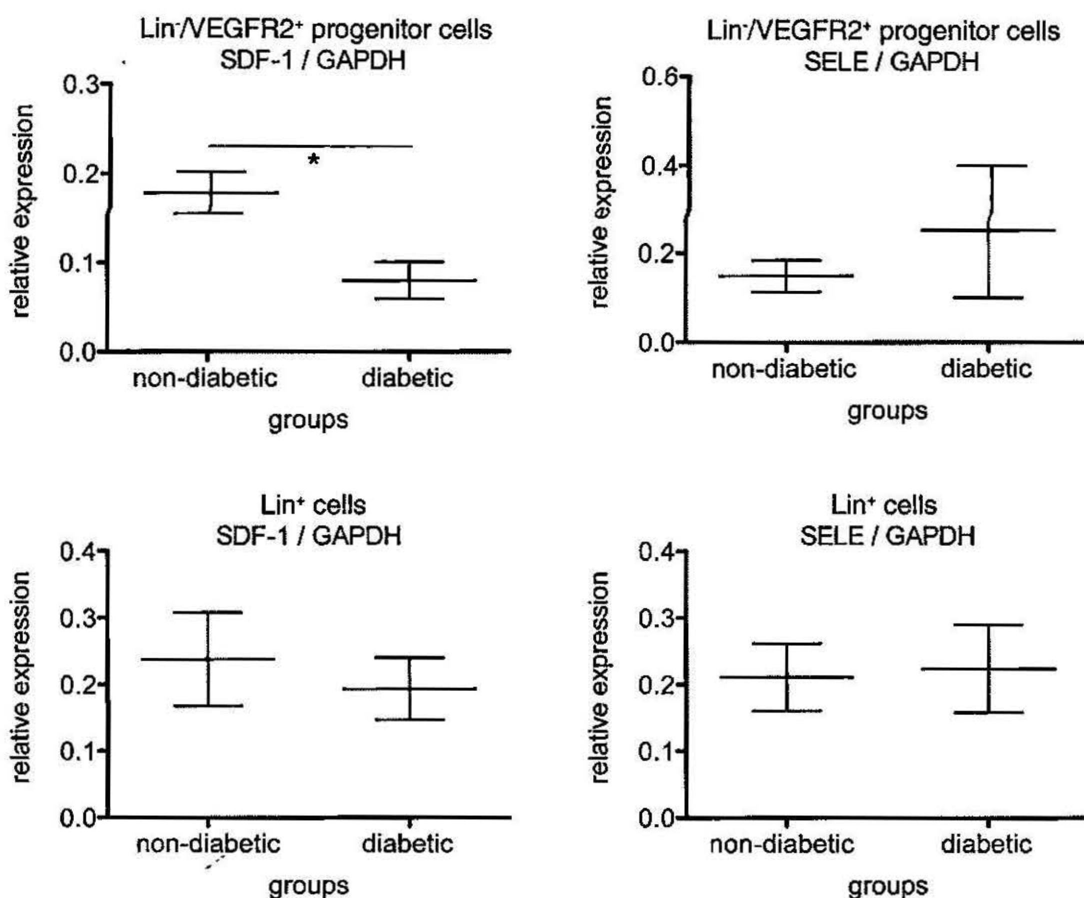

Figure 14: Relative expression of SDF-1 and SELE in relation to GAPDH expression in Lin-/VEGFR2$^+$ and Lin$^+$ cells. The central bar indicates the mean, whiskers the ± 1 SD interval. Asterisk denotes statistical significance. Only for SDF-1 a significant change at the protein level can be detected. Mean and SD were calculated based on the 8 biological samples from individual mice in each group.

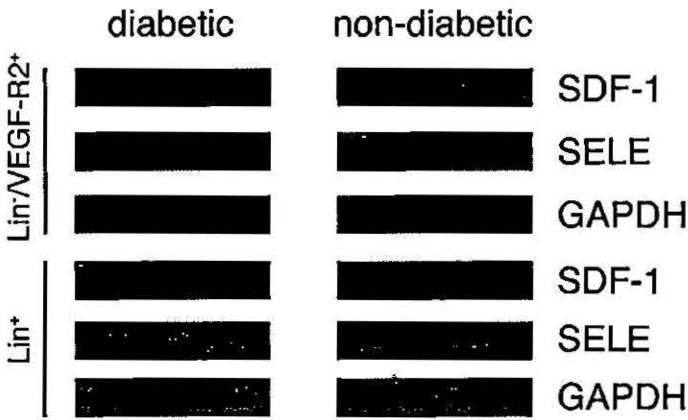

Figure 15: Western blot membrane imaging. While Lin^+ cells do not show obvious changes, $Lin^-/VEGFR2^+$ progenitor cells show changes regarding SDF-1 and SELE expression, though only changes observed in SDF-1 are statistically significant. Two lanes per group are shown to demonstrate interindividual sample variability.

Table 9: Overview of the western blot analysis, only SDF-1 shows significant changes on the protein level while the up-regulation of SELE is not significant. Analysis was done using 8 biological replicates per group.

	Lin^-		$Lin^-/VEGFR2^+$	
	Diabetic vs. Non-Diabetic		Diabetic vs. Non-Diabetic	
	Fold change	p-value	Fold change	p-value
SELE	1.06	0.676	1.69	0.2136
SDF-1	0.86	0.1645	0.45	**0.0114**

4.4 Discussion

The present study analysed differential gene expression in freshly isolated $Lin^-/VEGFR2^+$ progenitor cells obtained from murine BM in the early stages of diabetes (18 weeks). A total of 35 genes that were previously reported to be involved in EPC mobilization and EPC function (Ackah *et al.*, 2005, Aicher *et al.*, 2003, Albrecht *et al.*, 2003, Chen *et al.*, 2009a, Chong *et al.*, 2002, Dernbach *et al.*, 2004, Fleissner *et al.*, 2008, Fong *et al.*, 1999, Grant *et al.*, 2002, Hattori *et al.*, 2002, Hazra *et al.*, He *et*

al., 2004a, Heeschen *et al.*, 2003, Humpert *et al.*, 2008, Lee *et al.*, 2006b, Li *et al.*, 2009, Luttun *et al.*, 2002, Mobius-Winkler *et al.*, 2009, Moldenhauer *et al.*, 2008, Urao *et al.*,. 2006, Urbich *et al.*, 2004, Zachary *et al.*, 2001) were tested to see whether the reported diabetes-related changes observed mainly in EPCs from PB of diabetic humans could also be found in BM-derived progenitor cells from mice with early diabetes. There were three main findings. Firstly, the number of Lin$^-$/VEGFR2$^+$ progenitor cells /g bodyweight within the BM was significantly higher in diabetic than non-diabetic animals. Secondly, SDF-1 and SELE were significantly differentially expressed in diabetic Lin$^-$/VEGFR2$^+$ progenitor cells but not in diabetic BM Lin$^+$ cells, indicating that the differential expression of SDF-1 and SELE are specific for BM Lin$^-$/VEGFR2$^+$ progenitor cells in diabetic mice. The changes observed at the mRNA level were confirmed by western blot analysis only for SDF-1. Thirdly, 6 genes including CAV1, eNOS, CLDN5, NANOG, OCLN and BDNF showed such low levels of expression that no comparison could be made. These results demonstrate differential expression of one gene – SDF-1 – of BM derived progenitor cells in diabetic mice that may contribute to their dysfunctional mobilization from the BM to the PB and that Lin$^-$/VEGFR2$^+$ progenitor cells may be very early progenitor cells.

The observation of higher numbers of Lin$^-$/VEGFR2$^+$ progenitor cells /g bodyweight found in diabetic mice compared with non-diabetic animals is consistent with previous findings in a streptozotocin-induced diabetic mouse model (Barthelmes *et al.*, 2012b). This may indicate that in early stages of DM progenitors are "trapped" inside the BM. The reduced expression of SDF-1, one of the key factors involved in EPC mobilisation may partially contribute to this finding. Based on the current results, it seems in early diabetes mainly impacts mobilisation rather than cell genuine progenitor cell function – which would tally reports that reduction of EPCs in the peripheral blood in patients with diabetes is not necessarily coupled to an impaired function (Fadini *et al.*, 2006b).

The ability of EPCs to produce SDF-1 has been shown in EPCs isolated from the BM (Yun *et al.*, 2003) as well as in those isolated from the PB (Urbich *et al.*, 2005a). As EPCs express both SDF-1 and its receptor (CXCR4) (Yamaguchi *et al.*, 2003), an autocrine/paracrine regulation loop for SDF-1 (Yin *et al.*, 2007) within EPCs has been proposed. The functional interaction between SDF-1 and CXCR4 in EPCs within the BM is still unclear. However, recent studies indicate that the interaction between

SDF-1 ligand and its CXCR4 receptor appears to play important roles in both mobilization of EPCs from the BM to the PB and EPC maturation (De Falco *et al.*, 2009, Yin *et al.*, 2007). Our finding of down-regulation of SDF-1 in diabetic BM Lin$^-$/VEGFR2$^+$ progenitor cells indicates that reduced expression of SDF-1 may contribute to the impaired mobilization of Lin$^-$/VEGFR2$^+$ progenitor cells observed in diabetic mice. A recent study proposed that high glucose leads to reduced expression of HIF1α which in turn results in a lower level of SDF-1 expression (Thangarajah *et al.*, 2009). The interactions between HIF1α expression, regulation of SDF-1 expression and mobilization of BM-EPCs in diabetic retinopathy warrant further research.

Expression of SELE (CD62E) in circulating EPCs has been described (Chen *et al.*, 2006, Janic *et al.*, 2010). Several recent studies suggest that SELE expression is a sign of EC or EPC activation (Avci-Adali *et al.*, 2009, Dignat-George *et al.*, 2011, Nguyen *et al.*, 2009) because unstimulated BM-derived EPCs do not express SELE (Reyes *et al.*, 2002). The up-regulation of SELE in Lin$^-$/VEGFR2$^+$ progenitor cells in diabetic BM may be attributed to increased production of inflammatory cytokines such as interleukin 1 and tumour necrosis factors caused by the diabetic condition (Navarro-Gonzalez *et al.*, 2011).

Many studies have linked the EPC function to eNOS, which is considered one of the cardinal enzymes involved in mobilization of EPCs from the BM to the PB as well as maintaining the normal function of EPCs in physiological conditions (Aicher *et al.*, 2003, Albrecht *et al.*, 2003, Dimmeler *et al.*, 2000, Gallagher *et al.*, 2007b, Heissig *et al.*, 2002, Palmer *et al.*, 1988, Palmer *et al.*, 1987, Thum *et al.*, 2006, Urbich *et al.*, 2002b). In the present study we found that eNOS expression in BM Lin$^-$/VEGFR2$^+$ progenitor cells was very low. This is in accordance with a number of observations showing that eNOS expression is absent in immature EPCs but its level is increased when EPCs become more mature (Dernbach *et al.*, 2004, He *et al.*, 2004a, Thum *et al.*, 2006). The low level of eNOS as well as CAV1, CLDN5, NANOG, OCLN and BDNF expression in BM Lin$^-$/VEGFR2$^+$ progenitor cells may indicate that the Lin$^-$/VEGFR2$^+$ population consists mainly of immature or very early progenitor cells.

This study has some limitations. Mobilisation of progenitor cells from the BM is complex and based on the interplay of different factors – results from testing 35 genes for differential expression can explain only partially observed effects of DM on BM progenitor cells. Further studies may choose broader approaches. Another limitation

is that gene expression was tested only at one time point and hence no information on the timing of the SDF-1 expression changes can be monitored – or whether other genes may be differentially expressed in the course of DM impacting mobilisation and/or function.

Overall, the present study indicates that a short period of diabetes is sufficient to induce reduced expression of SDF-1, which could be one of the predominant reasons to account for the dysfunctional mobilization of BM-EPCs from the BM to PB.

These findings shed new light on the cellular and molecular mechanisms of retinal vascular dysfunction in early diabetic retinopathy. As endothelial dysfunction is an early feature of diabetic retinopathy, failure to repair injured retinal microvasculature by BM-EPCs will contribute to breakdown of the blood-retinal barrier, thus causing diabetic macular oedema and impairing vision in the early stages of the disease. Future research on regulation of SDF-1 expression in BM-EPCs may lead to novel therapeutic strategies for treatment of early diabetic retinopathy.

CHAPTER 5

CONCLUSIONS

5 Chapter 5 - Conclusions

Diabetes Mellitus (DM) is a metabolic disorder characterized by chronic hyperglycaemia (Alberti *et al.*, 1998). DM leads to microvascular and macrovascular complications which are the predominant causes of morbidity and mortality in people with DM (Alberti *et al.*, 1998, Cade, 2008). The commonest microvascular complication of DM is Diabetic Retinopathy (DR), which is also the leading cause of blindness in the working population of the developed world (Ciulla *et al.*, 2003, Foster *et al.*, 2005, Klein *et al.*, 1998).

A major feature and an early step towards the development of vascular complications is the marked impairment of Endothelial Precursor Cell (EPC) functions by DM (Caballero *et al.*, 2004, Ellis *et al.*, 2005b, Grant *et al.*, 2002, Segal *et al.*, 2006, Sengupta *et al.*, 2003). People with diabetes have both reduced numbers of EPCs in their peripheral blood (PB) and reduced function of EPCs, especially their ability to repair vascular damage (Awad *et al.*, 2006, Jiang *et al.*, 2004, Schatteman, 2004, Stepanovic *et al.*, 2003, Tepper *et al.*, 2002). As well as their functional impairment, impaired EPC mobilisation from the bone marrow (BM) has been reported in diabetic individuals (Awad *et al.*, 2005, Segal *et al.*, 2006). These functional impairments together with reduced mobilisation are thought to be major factors causing diabetic vascular complications (Ding *et al.*, 2005, Loomans *et al.*, 2004, Tepper *et al.*, 2002). Taking into account the therapeutic options and potential benefits arising from a transplantation of EPCs (Jung *et al.*, 2008, Rafii *et al.*, 2003, Sekiguchi *et al.*, 2009, Tateishi-Yuyama *et al.*, 2002), strategies to transplant EPCs to treat DR are being developed.

The vast majority of studies available today on EPC impairment in DM were done using EPCs from the PB of humans. Since these experiments were performed using material from patients who are likely to have had diabetes for years, the results and conclusions may not apply to earlier phases of the disease process, where the most effective interventions might need to be applied. Animal models are suited to study early diabetic changes in a controlled environment and to establish concepts for early interventions in DM.

This thesis describes a series of experiments that aimed to study changes due to DM occurring in EPCs still located within the BM and to explore whether EPCs from the BM might be a good resource for transplantation of EPCs to treat retinal vascular damage such as occurs in DR.

Aim I of the thesis was to isolate EPCs from the BM of mice, to characterise their ability for tube formation *in vitro,* to evaluate their potential to contribute to repair of damaged retinal vessels *in vivo* and to compare the findings with EPCs isolated from PB. The experiments to achieve aim I showed that EPCs from the BM of mice were capable of repairing vascular damage, however to a lesser extent than was described by studies using human EPCs from the PB. This may be because EPCs within the BM are less mature. The process to fully develop the repair potential of EPCs may only be activated by the translocation of EPCs from the BM to the PB.

The second aim of this thesis was to study changes in EPC numbers both in PB and BM in early diabetes (18 weeks) in mice, to characterise phenotypical differences of EPCs isolated from the BM of diabetic and non-diabetic mice *in vivo* and to evaluate an *in vitro* approach to increase the mobilization of EPCs from the BM of diabetic and non-diabetic mice to the PB using a drug cocktail. *In vitro* experiments showed that diabetic mice had significantly more EPCs in the BM per kg bodyweight compared to their non-diabetic littermates, while EPC numbers in the PB were comparable. The *in vivo* experiments showed no difference in the growth behaviour and phenotypical properties between diabetic and non-diabetic EPCs. Failure of mobilization from the BM to the PB of EPCs was found after 18 weeks of diabetes, which is an early stage in what is usually a life-long disease. These results indicate that, in the very early stages of DM, the observed impairment of EPCs is more a problem of mobilisation than function. This is a new and very important finding, as previous reports from human EPCs isolated from the PB of patients with established DM showed clear functional deficits (Capla *et al.*, 2007, Chen *et al.*, 2007, Fadini *et al.*, 2005, Fadini *et al.*, 2006a, Fadini *et al.*, 2006c, Gallagher *et al.*, 2007a, Hill *et al.*, 2003, Loomans *et al.*, 2004, Tepper *et al.*, 2002).

Based on findings from experiments of aim II, studies for aim III included identification of early diabetes-related changes in EPCs from diabetic mice which may be responsible for their impaired function and mobilisation from the BM. To

investigate further the effect of diabetes on EPCs isolated from the BM, real-time PCR and western blot analysis were performed. These experiments found that in this model of early diabetes none of the genes previously described as being crucial for EPC function (e.g. eNOS) and being decreased in DM (Aicher *et al.*, 2003, Ding *et al.*, 2000, Guo *et al.*, 2000, Lloyd *et al.*, 2001) was actually changed. This is in accordance with the findings from the experiments for aim II – where no differences between diabetic and non-diabetic EPCs were found in the *in vitro* experiments. The studies also showed that the changes seen at genomic and proteomic levels were related to the mobilisation deficit which was observed in the diabetic mice. Among them was the significant downregulation of SDF-1. This change was exclusively observed in EPCs, not in other cell populations from the BM of diabetic mice.

Overall the studies reported have shed further light on the potential of EPC therapy for DR. Contrary to expectations, the BM seemed to be an inferior source of EPCs for transplantation than the PB, which is more accessible. The studies also suggested that loss of function of EPCs in early DM is mainly due to impaired mobilisation rather than functional defects, which presumably develop later.

Some limitations of this thesis need to be mentioned. Although most changes seen in early diabetes in humans are found in animal models used in this thesis, confirmation of the findings in studies using human material are required as lack of direct comparison of EPCs from the BM of mice and of humans makes it difficult to estimate whether changes observed in the experiments described here are found in humans too or whether there are great differences between species. Such differences might affect applicability of the research findings when discussing DM related EPC changes in humans. Based on the findings presented in this thesis, a microarray based analysis of gene expression changes in EPCs from diabetic vs. non-diabetic mice had been planned. Due to technical and administrative issues, the analysis could not be finished in time.

The results from this thesis and new findings regarding the changes of EPCs in early DM are the starting point for further research on how to pharmacologically modulate EPCs to overcome the early diabetes-induced mobilisation deficit. Furthermore strategies will be evaluated to improve the repair potential of EPCs isolated from the BM to expand them *in vitro* and prepare them for transplantation.

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APPENDIX

7 Appendix

- supplementation of EGM-2 medium with 20%FCS according to (Friedrich *et al.*, 2006, Sun *et al.*, 2009b, Thum *et al.*, 2006, Thum *et al.*, 2007, Wang *et al.*, 2008, Wang *et al.*, 1998)

- RNA quality assessment and usage for RTPCR according to (Fleige *et al.*, 2006)

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