

Regulation of Endothelial Cell Growth by Tetrahydrobiopterin Bioavailability

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A thesis submitted for the degree of Doctor of Philosophy

University of Oxford

Trinity Term 2011

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Abstract

The vascular endothelium plays a critical role in the regulation of vascular homeostasis. Endothelial cell injury, loss, survival and regeneration are important aspects in the response to vascular injury and pathogenesis of vascular disease. Re-endothelialization has been shown to be a key event in vascular repair after injury.

Nitric oxide (NO) produced by endothelial nitric oxide synthase (eNOS) is a key regulator of endothelial cell biology. Loss of NO bioavailability in endothelial dysfunction states is an early step in the development of atherosclerosis. The cofactor tetrahydrobiopterin (BH4) is essential for eNOS catalytic activity; when BH4 is limiting eNOS becomes enzymatically uncoupled, leading to the generation of superoxide instead of NO.

eNOS function has been shown to be an important regulator of endothelial cell proliferation and survival as well as endothelial progenitor cell function. There is increasing evidence that the essential eNOS cofactor BH4 may enhance endothelial cell proliferation and endothelial progenitor function, both of which have the potential of enhancing vascular repair.

The aim of this thesis is to evaluate the effect of BH4 bioavailability on endothelial cell growth in the absence and presence of vascular disease using primary endothelial cells from mice with genetic modifications in BH4 availability. Endothelial progenitor cell availability in lungs and bone marrow was investigated to address a second source for endothelial repair and potential therapeutic target.

The duration of culture proved to be a major determinant of the biological phenotype of primary endothelial cells. Transgenic BH4 augmentation improved *in vitro* growth of primary endothelial cells in the presence but not in the absence of vascular disease. Endothelial progenitor cell content in lung tissue and bone marrow did not appear to be affected by transgenic BH4 augmentation. This thesis provides important new knowledge about the use of primary mouse endothelial cells in vascular research and insight into the role of BH4 availability on endothelial growth in a vascular disease setting.

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List of Abbreviations

7-AAD	7-Amino-Actinomycin
Akt	serine/threonine protein kinase (protein kinase B)
APC	Allophycocyanin
ApoE-KO	Apolipoprotein E knockout mouse
BAEC	Bovine aortic endothelial cells
BCA	Bicinchoninic Acid
bEND.3	Murine endothelial cell line (brain endothelioma)
BH2	7, 8-dihydrobiopterin
BH4	Tetrahydrobiopterin
BM	Bone marrow
BMMNC	Bone marrow mononuclear cells
bp	Base pairs
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CPRG	Chlorophenol red- β -D-galactopyranoside
Ct	Threshold cycle
DAHP	2, 4-Diamino-6-hydroxypyrimidine
DAPI	4',6-diamidino-2-phenylindole
DHFR	Dihydrofolate reductase
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxyribonucleotides (dATP, dCTP, dGTP and dTTP)
DOCA	Deoxycorticosterone acetate
DTE	Dithioerythritol
DTT	Dithiothreitol
EC	Endothelial cell
EDRF	Endothelium-derived relaxing factor
EDTA	Ethylenediaminetetraacetic acid
eEND.1	Murine endothelial cell line (embryonic endothelioma)
eNOS	Endothelial nitric oxide synthase
FACS	Fluorescence-activated cell sorting

FAD	Flavin adenine dinucleotide
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FMN	Flavin mononucleotide
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCH1	Guanosine triphosphate cyclohydrolase I (gene)
GTP	Guanosine triphosphate
GTPCH	Guanosine triphosphate cyclohydrolase I (protein)
H5V	Murine endothelial cell line (heart capillary endothelioma)
HAEC	Human aortic endothelial cells
HCAEC	Human coronary artery endothelial cells
hEGF	Human epidermal growth factor
hFGF-B	Human basic fibroblast growth factor
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HPEAC	Human pulmonary artery endothelial cells
hph-1	Mouse with systemically reduced GCH1 expression
HPLC	High performance liquid chromatography
HUVEC	Human umbilical vein endothelial cells
ICAM-2	Intercellular adhesion molecule 2
iNOS	Inducible nitric oxide synthase
KHB	Krebs-HEPES buffer
L-NAME	L-NG-Nitroarginine methyl ester
LacZ	Gene coding for beta-galactosidase
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
MACS	Magnetic cell sorting
NADPH	Nicotinamide adenine dinucleotide phosphate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
nor-NOHA	N ^ω -hydroxy-nor-Arginine
NOS	Nitric oxide synthase
ONOO⁻	Peroxynitrite
PAEC	Porcine aortic endothelial cells
PBS	Phosphate buffered saline
PBS-T	PBS containing 0.1% Tween-20
PCR	Polymerase chain reaction
PE	Phycoerythrin

PE-Cy7	tandem conjugate that combines PE and a cyanine dye (Cy7)
PECAM-1	Platelet endothelial cell adhesion molecule 1
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PMSF	Phenylmethylsulfonyl fluoride
PS	Phosphatidyl serine
R3-IGF-1	Human recombinant insulin-like growth factor-I
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
Sca-1	Stem cell antigen-1
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
sEnd.1 cells	Murine endothelial cell line (subcutaneous endothelioma)
SR	Sepiapterin reductase
SV40	Simian virus 40
TAE	Tris-acetate-EDTA buffer
tEND.1	Murine endothelial cell line (thymic endothelioma)
TNF-α	Tumour necrosis factor- α
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells
WT	Wild type
X-Gal	5-bromo-3-indoyl- β -D-galactopyranoside

Chapter 1:

Introduction

1 Introduction

1.1 The endothelium: A regulator of vascular homeostasis

The vascular endothelium forms a monolayer of cells that line the interior surface of blood vessels and plays a critical role in the regulation of blood pressure (Luscher, 1990), blood coagulation (Becker *et al.*, 2000; Sagripanti and Carpi, 2000), atherogenesis (Kinlay *et al.*, 2001; Luscher and Noll, 1995) and angiogenesis (Isner and Asahara, 1999). Endothelial cells produce a variety of factors affecting vascular tone and platelet aggregation. Endothelin-1 is a potent vasoconstrictor and contributes to the physiological maintenance of vascular tone but also plays a role in the development of cardiovascular disease (Haynes and Webb, 1994; Luscher and Barton, 2000; Yanagisawa *et al.*, 1988). The endothelium is further a site of eicosanoid formation (Schorr, 1985). Thromboxane A₂ acts as a vasoconstrictor and promotes platelet aggregation whereas prostacyclin acts as a vasodilator and inhibits platelet aggregation; both factors are also involved in the pathogenesis of vascular disease (Yuhki *et al.*, 2010). A major vasodilative compound released by endothelial cells is nitric oxide (NO) (Ignarro, 2002). NO, which is produced in the endothelium by endothelial nitric oxide synthase (eNOS), is not only a vasodilator but mediates many of the vasoprotective, antiatherogenic and antithrombotic properties of the endothelium. It inhibits leucocyte adhesion to endothelial cells and migration into the vascular wall, proliferation and migration of vascular smooth muscle cells as well as platelet aggregation and adhesion (Förstermann and Münzel, 2006). Not all vasoactive factors released by the endothelium have been fully identified yet. Endothelium-derived hyperpolarizing factor (EDHF) causes

vasodilation even in the presence of inhibitors for eNOS and cyclooxygenase but its function can be inhibited by potassium channel blockers (Chen *et al.*, 1988; Garland *et al.*, 2010). The contribution of EDHF to endothelium-dependent vasorelaxation seems to be greatest in small-diameter vessels and microvessels, whereas the contribution of NO appears to be greatest in large-diameter vessels (Nagao *et al.*, 1992).

1.2 The endothelium and vascular disease

Cardiovascular disease is a major cause of death in the western population and accounts for one in three of all deaths in the United Kingdom. Coronary heart disease accounts for 46 % and stroke for 23% of all deaths due to cardiovascular disease (British Heart Foundation Statistics Database, www.heartstats.org). Atherosclerosis is the primary cause of coronary heart disease and stroke and is characterized by inflammation, lipid deposition in the arterial wall and atherosclerotic plaque formation (Libby, 2002; Lusis, 2000). Atherosclerotic plaque growth causes narrowing of the arterial lumen leading to symptoms such as angina pectoris and *claudicatio intermittens*. Atherosclerotic plaque rupture can cause obstruction or occlusion of vessels resulting in myocardial infarction, stroke or limb ischemia.

Endothelial dysfunction with loss of NO bioavailability leads to impairment of the antiatherogenic, anti-inflammatory and antithrombotic properties of the endothelium described above and is an important step in the initiation and progression of atherosclerosis (Davignon and Ganz, 2004; Landmesser *et al.*, 2004). The endothelium-dependent dilatation of coronary arteries is impaired even in early

stages of atherosclerosis (Vita *et al.*, 1990; Zeiher *et al.*, 1991) and corresponds to the number of cardiovascular risk factors present in the patient (Vita *et al.*, 1990). Indeed, typical risk factors for cardiovascular disease including hypercholesterolemia (Creager *et al.*, 1990; Vita *et al.*, 1990), arterial hypertension (Panza *et al.*, 1990), smoking (Heitzer *et al.*, 1996), diabetes (Hink *et al.*, 2001), male gender (Vita *et al.*, 1990), old age (Vita *et al.*, 1990) and family history (Vita *et al.*, 1990) are all associated with endothelial dysfunction. Endothelial dysfunction in turn is associated with an increased risk of future cardiovascular events (Schachinger *et al.*, 2000; Suwaidi *et al.*, 2000) and restenosis after percutaneous coronary intervention (Munk *et al.*, 2011).

1.3 Endothelial repair in vascular disease

Endothelial cell injury, loss, survival and regeneration are important aspects in the pathogenesis of both native atherosclerosis and neointimal hyperplasia and accelerated atherosclerosis after vascular injury (Ehsan *et al.*, 2002; Fingerle *et al.*, 1990; Stefanec, 2000; Stoneman and Bennett, 2004). Re-endothelialization has been shown to be a key event in vascular repair after vascular injury (Hui, 2008), vein or arterial grafting (Allaire and Clowes, 1997; Feng *et al.*, 2011) and stenting (Rogers *et al.*, 1996; Tanous *et al.*, 2006), reducing the risk of thrombosis and the development of neointimal hyperplasia. Conversely, accelerated endothelial cell apoptosis and endothelial denudation are linked to the formation of coronary thrombotic atherosclerotic plaques that can cause acute coronary syndrome and myocardial infarction (Mallat and Tedgui, 2001; Xu *et al.*, 2009). Proatherosclerotic factors such as oxidized low-density lipoproteins, high glucose concentrations, angiotensin II and reactive oxygen species are not only known to induce endothelial

dysfunction but also promote endothelial cell apoptosis (Dimmeler *et al.*, 1998). However, both apoptosis and hyperproliferation seem to be involved in the response of endothelial cells to atherogenic factors (Apostolov *et al.*, 2007), leading to increased endothelial cell turnover. Disturbed flow has been shown to contribute to the formation of atherosclerotic lesions, neointimal hyperplasia and in-stent restenosis and to increase endothelial cell turnover (Chiu and Chien, 2011; Zeng *et al.*, 2009). Alterations in endothelial cell viability have also been described to be part of the pathogenic mechanisms that lead to pulmonary hypertension. Again, both increased apoptosis (Guerard *et al.*, 2006) and hyperproliferation (Levy *et al.*, 2007; Sakao *et al.*, 2005; Taraseviciene-Stewart *et al.*, 2006) seem to be involved in the pathophysiology.

A further group of cells that have been described to play a key role in endothelial repair after vascular injury are endothelial progenitor cells (EPCs). EPCs are a heterogeneous group of precursor cells that can be found resident in bone marrow (Asahara *et al.*, 1999; Loomans *et al.*, 2006), circulating in peripheral blood (Iwasaki *et al.*, 2007; Shi *et al.*, 1998) or resident in various tissues including vascular wall (Ingram *et al.*, 2005; Yao *et al.*, 2009; Zengin *et al.*, 2006), lung (Alvarez *et al.*, 2008) and adipose tissue (Planat-Benard *et al.*, 2004). Bone marrow derived EPCs have been shown to contribute to postnatal neovascularization, both in physiological and pathophysiological settings (Asahara *et al.*, 1999) and play an important role in re-endothelialization of blood vessels after vascular injury (Kaushal *et al.*, 2001; Shi *et al.*, 1998; Xu *et al.*, 2003). Administration of circulating EPCs has been demonstrated to be an effective therapeutic strategy for improving neovascularization in response to ischemia in animal models (Kalka *et*

et al., 2000; Kawamoto *et al.*, 2001; Kocher *et al.*, 2001; Urbich *et al.*, 2003) and there is some evidence that it may provide benefits in limb ischemia in humans (Lenk *et al.*, 2005; Tatenos *et al.*, 2006). A low number of circulating EPCs is associated with an increased risk of future cardiovascular events (Schmidt-Lucke *et al.*, 2005; Werner *et al.*, 2005). EPC function is impaired in patients with cardiovascular disease and correlates inversely with the number of risk factors (Vasa *et al.*, 2001b), EPC senescence in turn is associated with in stent restenosis (Matsuo *et al.*, 2006). As for endothelial cells, eNOS and NO have been shown to be key regulators of EPC biology (Aicher *et al.*, 2003; Thum *et al.*, 2007; Urao *et al.*, 2006).

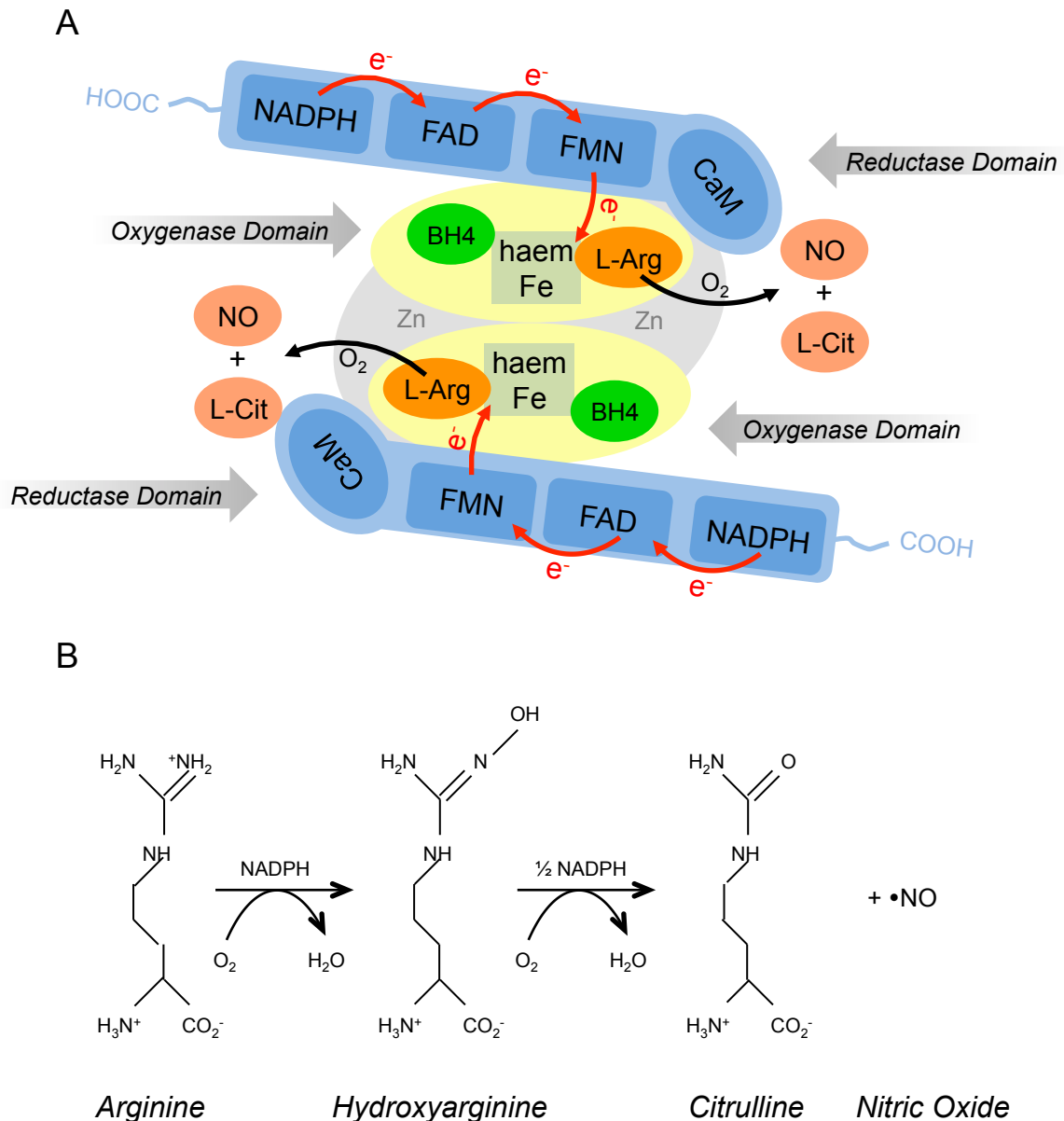
1.4 Nitric oxide and nitric oxide synthases

In 1980 Furchgott and Zawadzki found that blood vessels with intact endothelium relax in response to acetylcholine stimulation whereas vessels with damaged or missing endothelium contract due to the direct action of acetylcholine on vascular smooth muscle cells. They called the then unknown chemical compound that caused the relaxation “endothelium-derived relaxing factor” (EDRF) (Furchgott and Zawadzki, 1980). Seven years later both Palmer and colleagues (Palmer *et al.*, 1987) and Ignarro and colleagues (Ignarro *et al.*, 1987) almost simultaneously identified EDRF as the free radical nitric oxide (NO).

NO is produced in endothelial cells and other cell types such as thrombocytes, neuronal cells und macrophages by nitric oxide synthase (NOS) (Förstermann *et al.*, 1994a). In 1988 Palmer *et al.* identified L-arginine as the substrate for NO synthesis and L-citrulline as a co-product. There are three isoforms of NOS: neuronal NOS (nNOS or NOS I), inducible NOS (iNOS or NOS II) and endothelial NOS (eNOS or

NOS III) named by their main cell type or according to the order of their first purification and isolation of their cDNAs (Förstermann *et al.*, 1991). nNOS and eNOS are constitutively expressed and produce NO in response to increased intracellular calcium levels mediated by binding of calcium to calmodulin within the enzyme (Schmidt *et al.*, 1992). iNOS is usually not constitutively expressed but can be induced in macrophages and other cell types in response to inflammatory stimuli such as bacterial lipopolysaccharide and/or cytokines (Stuehr *et al.*, 1991). The activity of iNOS is calcium independent; the binding of calmodulin is so tight, even at subnanomolar levels of calcium, that it can be considered a subunit rather than a cofactor (Cho *et al.*, 1992). All three isoforms are active as homodimers, which catalyze oxidation of its substrate L-arginine to produce L-citrulline and NO in equimolar concentrations (Stuehr *et al.*, 2001). Each monomer of NOS consists of a C-terminal reductase domain, containing binding sites for NADPH, FAD, FMN and calmodulin and an N-terminal oxygenase domain containing an iron haem group and binding sites for L-arginine and tetrahydrobiopterin (BH₄) (Knowles and Moncada, 1994). NO biosynthesis takes place in the oxygenase domain. The three isoforms share 50-60% homology, with the highest degree of concordance in the C-terminal reductase domain and significant differences in the N-terminal part of the enzyme. All NOS isoforms catalyze the flavin-mediated electron transfer from C-terminal bound NADPH to the haem group in the active site of the oxygenase domain (Bredt *et al.*, 1992) (Figure 1.1 A). NOS enzymes hydrolyze L-arginine to N-hydroxy-L-arginine and then oxidize N-hydroxy-L-arginine to L-citrulline and NO (Stuehr *et al.*, 2001) (Figure 1.1 B).

Figure 1.1

**Figure 1.1: NOS catalysis.**

A: All three isoforms of NOS function as homodimers. Each monomers consists of a reductase domain with binding sites for NADPH, FAD, FMN and calmodulin (CaM) and an oxygenase domain containing an iron haem group and binding sites for L-arginine (L-Arg) and BH4. Starting from NADPH electrons flow to the flavins FAD and FMN of the reductase domain, to the iron of the haem in the oxygenase domain. CaM regulates electron flow between the reductase and oxygenase domain. BH4 seems to be essential to donate electrons to the haem group in the oxygenase domain in order to oxidize L-arginine.

B: NOS catalyzes oxidation of L-arginine to L-citrulline in order to generate NO, forming L-hydroxyarginine as an intermediate.

Adapted from Stuehr *et al.*, 2001.

1.4.1 eNOS and properties of endothelial NO

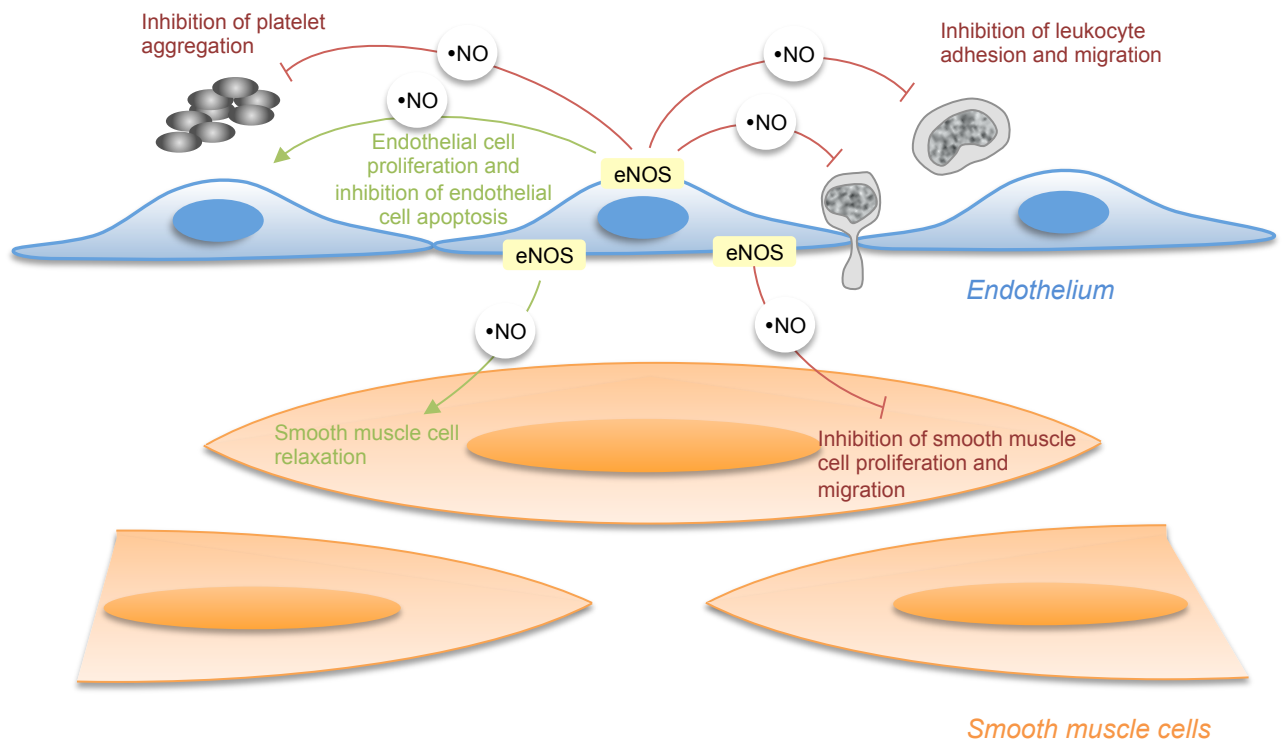
eNOS is predominantly expressed in vascular endothelium (Förstermann *et al.*, 1991; Pollock *et al.*, 1991; Pollock *et al.*, 1993) but also occurs in a variety of other cell types including cardiomyocytes (Balligand *et al.*, 1995), platelets (Sase and Michel, 1995; Wallerath *et al.*, 1997), neurons (Dinerman *et al.*, 1994) and kidney tubular epithelial cells (Tracey *et al.*, 1994). eNOS was first identified and isolated from bovine aortic endothelial cells (Förstermann *et al.*, 1991; Pollock *et al.*, 1991) and the amino acid sequence is highly conserved between mammalian species (approximately 94% identity) (Li *et al.*, 2002a).

eNOS is predominantly plasma membrane bound (Förstermann *et al.*, 1991) and found in invaginations of the membrane called caveolae. In basal conditions eNOS is tonically inhibited by its interactions with caveolin-1. Upon stimulation by agonists such as bradykinin and mobilization of intracellular Ca^{2+} , eNOS undergoes calcium-calmodulin binding, displacement from caveolin-1 protein and translocates to structures in the cytosol (Prabhakar *et al.*, 1998). There are several endogenous receptor-mediated agonists that stimulate eNOS via an increase in intracellular Ca^{2+} including acetylcholine, bradykinin, ATP, ADP, substance P, serotonin and histamine (Furchgott, 1983; Furchgott *et al.*, 1984). In addition, eNOS can be activated by mechanical stimuli such as unidirectional laminar shear stress (Buga *et al.*, 1991; Corson *et al.*, 1996) and cyclic strain (Awolesi *et al.*, 1995). However continuous oscillatory shear stress stimulates production of reactive oxygen species (ROS), which can scavenge NO and therefore reduce bioavailability (De Keulenaer *et al.*, 1998). Vascular endothelial growth factor (VEGF), estrogen, sphingosine 1-

phosphate and bradykinin stimulate the phosphoinositide 3-kinase (PI3K) pathway and consequently cause Akt-mediated phosphorylation of serine 1177, enhancing the activity of eNOS (Chen *et al.*, 1999; Dimmeler *et al.*, 1999).

As mentioned above, endothelial NO plays an important role in the regulation of vasotone. It diffuses into the vessel wall and leads to relaxation of vascular smooth muscle cells through stimulation of soluble guanylate cyclase (Moro *et al.*, 1996). Endothelial NO is further an important mediator of the vasoprotective, antiatherogenic and antithrombotic properties of the endothelium. It inhibits some of the key steps of atherogenesis including endothelial cell apoptosis (Dimmeler *et al.*, 1997; Rossig *et al.*, 1999) and leucocyte adhesion to endothelial cells and migration into the vascular wall (Kubes *et al.*, 1991; Lindemann *et al.*, 2000; Zeiher *et al.*, 1995) as well as proliferation and migration of vascular smooth muscle cells (Gorog and Kovacs, 1998; Tanner *et al.*, 2000) and platelet aggregation and adhesion (Cerwinka *et al.*, 2002; Radomski *et al.*, 1987). These vasoprotective effects of endothelial NO are lost after endothelial denudation during balloon angioplasty promoting neointimal hyperplasia (Von der Leyen *et al.*, 1995). Figure 1.2 provides an overview over vasoprotective effects of endothelial NO.

Figure 1.2

**Figure 1.2: Antiatherogenic and antithrombotic properties of endothelial NO.**

NO is produced in the endothelium by endothelial nitric oxide synthase (eNOS). It diffuses into the vessel wall and leads to relaxation of vascular smooth muscle cells and also inhibits proliferation and migration of smooth muscle cells. On the luminal surface of the blood vessel NO inhibits leucocyte adhesion to endothelial cells and migration into the vascular wall as well as platelet aggregation and adhesion. NO further mediates VEGF induced endothelial cell proliferation and plays a critical role in the prevention of endothelial cell senescence and apoptosis.

1.4.2 iNOS and NO in host defense and sepsis

iNOS can be induced in macrophages and various other cell types in response to inflammatory stimuli such as bacterial lipopolysaccharide and/or cytokines (Gross *et al.*, 1991; Stuehr *et al.*, 1991) and has been shown to play a major role in host defense due to the antimicrobial properties of NO (Nathan, 1997; Nathan and Hibbs, 1991). iNOS is a predominantly soluble enzyme and is activated in an apparently Ca^{2+} independent manner due to its tight interaction with calmodulin (Hevel *et al.*, 1991; Stuehr *et al.*, 1991; Yui *et al.*, 1991). Studies with iNOS knockout mice have shown that iNOS activation may have a critical role in the defense of infections with *Mycobacterium tuberculosis* (MacMicking *et al.*, 1997). However, immunostimulant-induced increases in iNOS activity and vascular NO have been demonstrated to be accountable for the severe and potentially lethal hypotension associated with septic shock, which leads to multi-organ failure (Kilbourn *et al.*, 1990; Titheradge, 1999).

1.4.3 nNOS

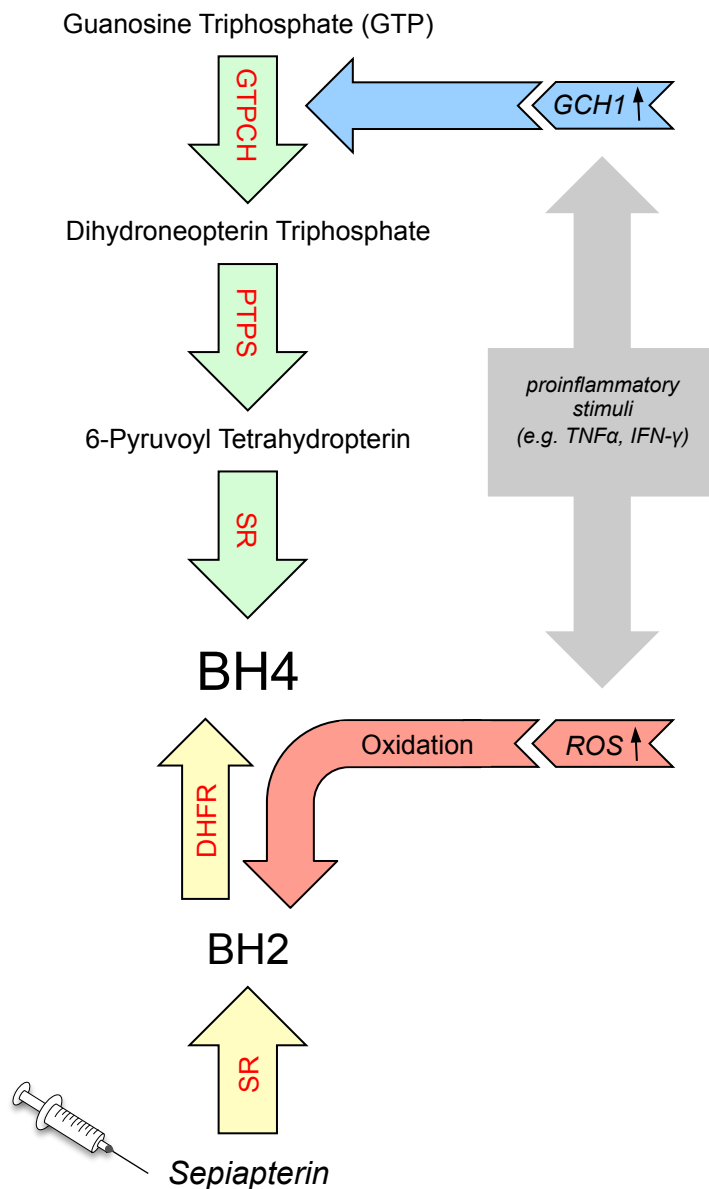
nNOS is constitutively expressed in neuronal tissue (Bredt *et al.*, 1990; Bredt and Snyder, 1990; Mayer *et al.*, 1990) and some non-neuronal cells including cardiac (Xu *et al.*, 1999) and skeletal myocytes (Kobzik *et al.*, 1994). nNOS is a predominantly soluble enzyme and is activated in a calcium-calmodulin dependent manner. NO produced by nNOS in the central nervous system appears to play a role in long term potentiation of synaptic transmission (Malen and Chapman, 1997; Schuman and Madison, 1991). nNOS derived NO also takes part in the regulation of vascular tone by reducing vascular sympathetic tone in the central nervous

system (Li *et al.*, 2001b; Salas *et al.*, 2003; Togashi *et al.*, 1992) and reducing smooth muscle cell tone via its function as a peripheral neurotransmitter in nitrenergic nerves (Seddon *et al.*, 2009; Seddon *et al.*, 2008; Toda and Okamura, 2003). nNOS expressed in intracardiac cholinergic ganglia enhances the release of acetylcholine (Herring and Paterson, 2001) and vagal response after exercise training (Danson *et al.*, 2004; Danson and Paterson, 2003), thus reducing the heart rate. nNOS localized in cardiomyocytes has been shown to regulate cardiac contractility by reducing the inotropic response to beta-adrenergic stimulation (Ashley *et al.*, 2002; Sears *et al.*, 2003) and endothelin-1 (Zhang *et al.*, 2009).

1.5 Tetrahydrobiopterin: Biosynthesis and role as a cofactor for NOS enzymes and aromatic amino acid hydroxylases

BH4 is produced from the sequential action of three enzymes (Figure 1.3); the initial and rate-limiting reaction is catalyzed by GTP cyclohydrolase 1 (GTPCH), a homodecameric enzyme with 28kD subunits (Auerbach and Nar, 1997). GTPCH, encoded by the GCH1 gene, catalyzes the conversion of guanosine triphosphate (GTP) to 7,8-dihydroneopterin triphosphate, which is subsequently converted to 6-pyruvoyl tetrahydropterin by 6-pyruvoyl tetrahydropterin synthase (PTPS). The final reaction in the pathway is catalyzed by sepiapterin reductase (SR) to produce BH4 (Thony *et al.*, 2000). BH4 is sensitive to oxidation by reactive oxygen species, forming BH2. However, BH2 can be recycled to BH4 via the salvage pathway in a reaction that requires NADPH and is catalyzed by dihydrofolate reductase (DHFR). BH4 can also be synthesized from the exogenous synthetic pterin sepiapterin, which is first metabolized to BH2 by sepiapterin reductase and then to BH4 by dihydrofolate reductase.

Figure 1.3

**Figure 1.3: BH4 biosynthesis and metabolism.**

De novo synthesis of BH4 occurs from GTP via a three-step enzymatic pathway (green arrows). GTPCH, encoded by the GCH1 gene, is the first and rate-limiting enzyme. BH4 can also be synthesized from the exogenous synthetic pterin sepiapterin, which is first metabolized to BH2 by sepiapterin reductase (SR) and then to BH4 by dihydrofolate reductase (DHFR) (yellow arrows). BH4 is sensitive to oxidation by reactive oxygen species (ROS), forming BH2. Inflammation can lead to up-regulation of GCH1 and increase in BH4 synthesis (blue arrows) but at the same time it can lead to oxidative loss of BH4 via increased production of ROS (red arrows).

GTPCH: GTP cyclohydrolase-1

PTPS: 6-pyruvoyl-tetrahydropterin synthase

SR: sepiapterin reductase

DHFR: dihydrofolate reductase

BH4 functions as a critical cofactor in a number of enzymes, including all three isoforms of NOS (Cosentino and Luscher, 1999; Kwon *et al.*, 1989; Mayer *et al.*, 1990). It is located close to the active site of the enzyme and acts as an intermediate electron donor to the haem group in the oxygenase domain, coupling oxygen reduction to L-arginine oxidation during NO synthesis (Stuehr *et al.*, 2001).

BH4 is also a necessary cofactor for phenylalanine-, tryptophan- and tyrosine-hydroxylases (Thony *et al.*, 2000). Deficiency in BH4 therefore may result in hyperphenylalaninaemia and decreased synthesis of the neurotransmitters dopamine, norepinephrine, epinephrine and serotonin. Mutations in genes encoding enzymes involved in BH4 synthesis result in a heterogeneous group of rare diseases characterized by varying degrees of hyperphenylalaninaemia and dystonia (Hanihara *et al.*, 1997; Ichinose *et al.*, 1995; Ichinose *et al.*, 1994), which may respond to BH4 therapy.

1.6 Oxidative stress and eNOS uncoupling in vascular disease

Oxidative cell damage by reactive oxygen species plays an important role in the pathogenesis of vascular disease (Sugamura and Keaney, 2011). Reactive oxygen species are highly reactive chemical species derived from molecular oxygen comprising both free radicals such as superoxide and non-radicals such as hydrogen peroxide. Superoxide production and endothelial dysfunction are functionally important because they are strongly related to clinical risk factor profile (Guzik *et al.*, 2000) and future risk of adverse cardiovascular events (Heitzer *et al.*, 2001). Superoxide and NO react at near diffusion rate forming peroxynitrite (OONO^-) (Huie and Padmaja, 1993), a strong oxidant, and depriving the vessel of NO

bioactivity. Reactive oxygen species are also involved in the activation of important intracellular signaling molecules, such as mitogen-activated protein kinases (MAPKs), that regulate numerous signaling pathways involved in cell growth, gene expression and apoptosis (Li *et al.*, 2004; Ushio-Fukai *et al.*, 1998). One of the sources of reactive oxygen species in the vascular wall are NADPH oxidases (Griendling *et al.*, 2000; Li *et al.*, 2002b) and animal models show evidence for activation of NADPH oxidases in the presence of cardiovascular risk factor such as hypertension (Li *et al.*, 2006; Rajagopalan *et al.*, 1996), diabetes mellitus (Hink *et al.*, 2001) and hypercholesterolemia (Warnholtz *et al.*, 1999). Further sources of reactive oxygen species in cardiovascular disease are xanthine oxidase (Harrison, 2002), lipoxygenases (Kuhn *et al.*, 1997; Zhao and Funk, 2004), myeloperoxidase (Eiserich *et al.*, 2002; Fu *et al.*, 2001) and NOS enzymes.

An important aspect of endothelial dysfunction states is a reduction of BH4 availability. Peroxynitrite causes rapid oxidation of BH4 to the inactive dihydrobiopterin (BH2), which is incapable of acting as a cofactor for eNOS (Milstien and Katusic, 1999). eNOS consequently becomes dysfunctional and L-arginine oxidation becomes enzymatically ‘uncoupled’ from oxygen reduction, leading to generation of superoxide rather than NO (Vasquez-Vivar *et al.*, 2003; Vasquez-Vivar *et al.*, 1998; Xia *et al.*, 1998). However, eNOS uncoupling is not just due to BH4 deficiency, but also results from a reduced ratio of BH4 to BH2 because the inactive BH2 competes for binding to the active site of eNOS (Crabtree *et al.*, 2008; Crabtree *et al.*, 2009). Oxidation of BH4 and eNOS uncoupling may therefore be both a consequence and contributor to endothelial dysfunction and vascular oxidative stress, as demonstrated in major cardiovascular risk factors such

as diabetes mellitus (Bitar *et al.*, 2005; Cai *et al.*, 2005; Guzik *et al.*, 2002), hypertension (Landmesser *et al.*, 2003) and hypercholesterolemia (Laursen *et al.*, 2001).

1.7 BH4/eNOS interactions mouse models of vascular disease

BH4 supplementation has been found to restore endothelial function in animal models of atherosclerosis (Hattori *et al.*, 2007), arterial hypertension (Hong *et al.*, 2001) and diabetes mellitus (Pieper, 1997) as well as in patients with coronary artery disease (Maier *et al.*, 2000) or cardiovascular risk factors such as hypercholesterolemia (Stroes *et al.*, 1997), diabetes mellitus (Heitzer *et al.*, 2000b), arterial hypertension (Higashi *et al.*, 2002) and smoking (Heitzer *et al.*, 2000a; Ueda *et al.*, 2000).

To investigate the impact of endothelial BH4 bioavailability on eNOS regulation and endothelial function in vascular disease states, a mouse line overexpressing human GCH under the control of the Tie-2 promoter and intronic enhancer was generated in-house by Dr. Nicholas Alp (Alp *et al.*, 2003).

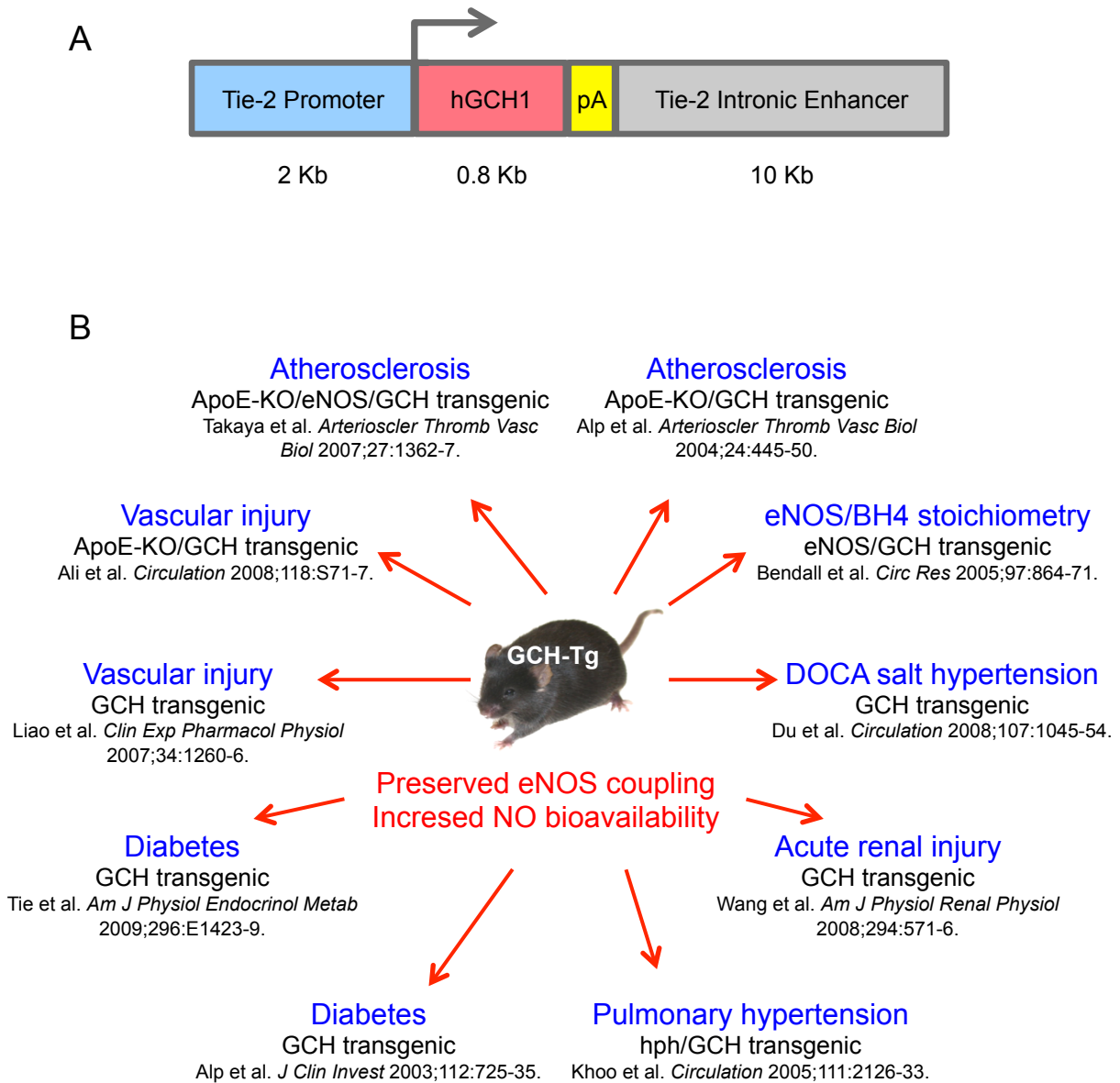
Endothelial specific GCH overexpression in these GCH transgenic mice has been shown to be sufficient to restore eNOS coupling and NO bioavailability in various vascular disease states including diabetes (Alp *et al.*, 2003; Tie *et al.*, 2009), arterial hypertension (Du *et al.*, 2008) and hypercholesterolemia (Alp *et al.*, 2004). GCH transgenic mice exhibit reduced neointima formation after wire injury of the carotid artery (Liao *et al.*, 2007) and hypercholesterolemic ApoE-KO that overexpress human GCH in the endothelium show a reduction in atherosclerosis (Alp *et al.*, 2004; Takaya *et al.*, 2007) and also reduced neointimal hyperplasia after vascular

injury (Ali *et al.*, 2008). BH4 deficiency in the hph-1 mouse on the other hand has been shown to lead to endothelial dysfunction and pulmonary hypertension, which can be rescued by endothelial specific GCH overexpression (Khoo *et al.*, 2005).

eNOS coupling seems to be directly related to eNOS/BH4 stoichiometry even in the absence of vascular disease. Mice with transgenic eNOS overexpression are healthy animals without vascular disease but have eNOS uncoupling that can be rescued by BH4 supplementation or transgenic GCH overexpression (Bendall *et al.*, 2005).

Figure 1.4 provides an overview over publications showing restored eNOS coupling due to endothelial BH4 augmentation in the GCH transgenic mouse in various vascular disease and injury settings.

Figure 1.4

**Figure 1.4: The GCH transgenic mouse.**

A: GCH transgene construct. The GCH transgenic mouse overexpresses human GCH1 under the control of the Tie-2 promoter and intronic enhancer specifically in endothelial cells.

B: Endothelial specific GCH overexpression in the GCH transgenic mouse has been shown to be sufficient to restore eNOS coupling and NO bioavailability in various vascular disease states.

1.8 Role of NO and BH4 in endothelial cell proliferation and survival

Re-endothelialization has been shown to be a key event in vascular repair after vascular injury (Allaire and Clowes, 1997; Hui, 2008; Rogers *et al.*, 1996), reducing the risk of thrombosis and the development of neointimal hyperplasia whereas accelerated endothelial cell apoptosis and endothelial denudation are linked to the formation of thrombotic atherosclerotic plaques (Mallat and Tedgui, 2001; Xu *et al.*, 2009).

eNOS and NO have been shown to mediate VEGF induced endothelial cell proliferation (Morbideilli *et al.*, 1996; Papapetropoulos *et al.*, 1997) and angiogenesis (Murohara *et al.*, 1998; Papapetropoulos *et al.*, 1997) and to play a critical role in the prevention of endothelial cell senescence (Hayashi *et al.*, 2006; Vasa *et al.*, 2000) and apoptosis (Dimmeler *et al.*, 1997; Kang-Decker *et al.*, 2007; Rossig *et al.*, 2000; Wright *et al.*, 2010).

There is increasing evidence that BH4, as an essential eNOS cofactor, may improve endothelial cell proliferation (Chen *et al.*, 2010; Marinos *et al.*, 2001; Michaelis *et al.*, 2007; Shimizu *et al.*, 1999) and tube formation (Chen *et al.*, 2010; Michaelis *et al.*, 2007). *In vitro* supplementation with the BH4 precursor sepiapterin has been shown to enhance proliferation in coronary endothelial cell from diabetic rats (Marinos *et al.*, 2001), bovine aortic endothelial cells (BAEC) (Shimizu *et al.*, 1999) and HUVEC (Chen *et al.*, 2010). These effects may be mediated either by a direct antioxidant effect of BH4 or/and improved eNOS function with increased NO availability and reduced production of reactive oxygen species.

In the GCH transgenic mouse, endothelial BH4 augmentation reduces neointima formation induced by vascular injury with endothelial denudation (Liao *et al.*, 2007). It is possible that this effect may be mediated by improved endothelial survival and/or proliferation but the impact of transgenic GCH overexpression on endothelial cell proliferation and survival has yet to be determined.

1.9 Role of NO and BH4 in endothelial progenitor cell expansion and mobilization

EPCs are a heterogeneous group of precursor cells that participate in neo-endothelialization and neovascularization. They can be found resident in bone marrow (Asahara *et al.*, 1999; Loomans *et al.*, 2006), circulating in peripheral blood (Iwasaki *et al.*, 2007; Shi *et al.*, 1998) or resident in various tissues including vascular wall (Ingram *et al.*, 2005; Yao *et al.*, 2009; Zengin *et al.*, 2006), lung (Alvarez *et al.*, 2008) and adipose tissue (Planat-Benard *et al.*, 2004).

eNOS and NO have been shown to play a critical role in EPC expansion, mobilization and function (Aicher *et al.*, 2003; Thum *et al.*, 2007; Urao *et al.*, 2006). eNOS-KO mice have been reported to have fewer EPCs in bone marrow and peripheral blood (Laufs *et al.*, 2004) and to show reduced VEGF induced EPC mobilization into the blood stream (Aicher *et al.*, 2003). eNOS uncoupling has been shown to induce EPC senescence (Lemarie *et al.*, 2011) and to impair EPC mobilization and migration (Thum *et al.*, 2007). Both EPC senescence and impaired migration due to eNOS uncoupling could successfully be rescued by BH4 augmentation (Lemarie *et al.*, 2011; Thum *et al.*, 2007). BH4 augmentation in the

GCH transgenic mouse has been shown to preserve the number and function of EPCs in hypertension (Xie *et al.*, 2010). Hypercholesterolemic ApoE-KO mice show a reduction in circulating EPC in comparison to WT mice (Xu *et al.*, 2003) but the impact of transgenic GCH overexpression on EPC content in ApoE-KO mice has yet to be determined.

1.10 Endothelial cells in vascular research

The use of primary endothelial cells or endothelial cell lines allows investigation of endothelial biology within a highly defined system, without confounding influences of other cell types, and using analytical approaches that are not possible in intact tissues.

There are several endothelial cell lines that are commonly used in cardiovascular research and commercially available. The murine endothelial cell lines sEND.1, tEND.1, eEND.1, bEND.3 and H5V are derived from subcutaneous, thymic, embryonic, brain and heart capillary endothelioma respectively, induced by infection with a retrovirus expressing polyoma middle T oncogene (Garlanda *et al.*, 1994; Montesano *et al.*, 1990; Williams *et al.*, 1988). Examples of immortalized human endothelial cell lines are HPMEC-ST1.6R cells, which are human pulmonary microvascular endothelial cells cotransfected with a plasmid encoding the catalytic component of telomerase (hTERT) and a plasmid encoding the simian virus 40 (SV40) large T antigen (Krump-Konvalinkova *et al.*, 2001), and EaHy.926 cells, a hybridoma of HUVEC and the permanent human cell line A549 (Edgell *et al.*, 1983). Two of the major advantages of using immortalized endothelial cell lines are the availability of large sample sizes and the small biological variation.

In contrast to endothelial cell lines, primary endothelial cells, which are commercially available as human endothelial cells and derived from umbilical vein (HUVEC), aorta (HAEC), coronary arteries (HCAEC) and pulmonary arteries (HPAEC) or as bovine and porcine endothelial cells and derived from aorta (BAEC and PAEC respectively), to name just a few, are more expensive, grow more slowly and can only be used for the first few passages. However, primary endothelial cells also have advantages over immortalized endothelial cell lines; they resemble the physiological properties of endothelial cells *in vivo* more closely, particularly concerning cell proliferation and apoptosis.

An advantage of using primary endothelial cells from mice instead of commercially available human, bovine or porcine endothelial cells is the availability of murine models for vascular disease with endothelial targeted genetic alterations, abolishing the need for transfections *in vitro*. In this thesis, endothelial cells from mice with genetic modifications in GCH1 and/or eNOS expression are of particular interest.

Murine endothelial cells have been isolated from various organs including heart (Li *et al.*, 2001a; Marelli-Berg *et al.*, 2000), aorta (Kevil and Bullard, 2001; Kobayashi *et al.*, 2005), vena cava inferior (Bucciarelli *et al.*, 2009), kidney (Rops *et al.*, 2004), retina (Matsubara *et al.*, 2000; Su *et al.*, 2003), brain (Weidenfeller *et al.*, 2005) and particularly lungs (Dong *et al.*, 1997; Fehrenbach *et al.*, 2009; Fleming *et al.*, 2005; Khan *et al.*, 2004; Kuhlencordt *et al.*, 2004; Lim *et al.*, 2003; Marelli-Berg *et al.*, 2000; Schniedermann *et al.*, 2010), as they are rich in microvascular endothelial cells.

A variety of cell surface markers are used for endothelial cell identification during isolation and for confirming endothelial cell phenotype in culture. CD31 (PECAM-1) and CD102 (ICAM-2) are the markers most commonly used for primary endothelial cell isolation (Dong *et al.*, 1997; Fehrenbach *et al.*, 2009; Fleming *et al.*, 2005; Kuhlencordt *et al.*, 2004; Lim *et al.*, 2003; Marelli-Berg *et al.*, 2000; Schniedermann *et al.*, 2010). Additionally, expression of eNOS (Demeule *et al.*, 2001), Tie-2 (Lim *et al.*, 2003), VE Cadherin (CD144) (Dong *et al.*, 1997; Fehrenbach *et al.*, 2009; Lim *et al.*, 2003) and Factor VIII-related antigen (King *et al.*, 2004), binding of Isolectin B4 (Fehrenbach *et al.*, 2009; Laitinen, 1987; Marelli-Berg *et al.*, 2000) and the uptake of acetylated LDL (Fehrenbach *et al.*, 2009; King *et al.*, 2004; Kuhlencordt *et al.*, 2004; Voyta *et al.*, 1984) are used as markers of endothelial identity. However both binding of Isolectin B4 and uptake of acetylated LDL have been shown to be not entirely selective to endothelial cells (Fehrenbach *et al.*, 2009; Marelli-Berg *et al.*, 2000).

Although several published studies have used primary mouse endothelial cells before, isolation and culture protocols vary significantly. The limited amount of endothelial cells, which can be isolated per animal, contamination with non-endothelial cells and the potential loss of endothelial cell phenotype and biological function over time represent potential limitations for the experimental use of primary mouse endothelial cells. To obtain reproducible and valid results using primary mouse endothelial cells in vascular research, optimal isolation and culture conditions need to be defined and the isolated and cultured cells require systematic characterization regarding endothelial phenotype and transgene expression.

1.11 Summary and potential clinical relevance of BH4 augmentation as a therapeutic strategy

The vascular endothelium plays a critical role in the regulation of vascular homeostasis. Endothelial cell injury, loss, survival and regeneration are important aspects in the pathogenesis of both native atherosclerosis and neointimal hyperplasia and accelerated atherosclerosis after vascular injury. Re-endothelialization has been shown to be a key event in vascular repair after vascular injury, reducing the risk of thrombosis and the development of neointimal hyperplasia. NO, produced in the endothelium by eNOS, is a key regulator of endothelial cell biology and mediates many of the vasoprotective, antiatherogenic and antithrombotic properties of the endothelium. Loss of NO bioavailability in endothelial dysfunction states is an early step in the development of atherosclerosis. The cofactor BH4 is essential for eNOS catalytic activity; when BH4 is limiting eNOS becomes enzymatically uncoupled, leading to the generation of superoxide instead of NO. NO has been shown to mediate endothelial cell proliferation and to prevent endothelial apoptosis and also plays a critical role in EPC expansion, mobilization and function. There is evidence that BH4, as an essential eNOS cofactor, may improve endothelial cell proliferation as well as number and function of bone marrow derived EPCs. Both improved endothelial proliferation and EPC number and function have the potential of enhancing re-endothelialization after vascular injury.

Pharmacological supplementation with BH4 has been shown to be a promising therapeutic strategy in vascular disease and has been addressed both in animal models (Hattori *et al.*, 2007; Hong *et al.*, 2001; Shinozaki *et al.*, 2000) and clinical

studies (Eskurza *et al.*, 2005; Higashi *et al.*, 2002; Maier *et al.*, 2000; Setoguchi *et al.*, 2001). Endothelial specific over-expression of GTPCH, the rate-limiting enzyme in the *de novo* synthesis of BH4 and encoded by the GCH1, has been shown to be sufficient to restore eNOS coupling and NO bioavailability in various vascular disease states. The work in this thesis addresses the impact of BH4 augmentation by endothelial specific transgenic over-expression of GTPCH on primary endothelial cell growth, both in the absence and presence of vascular disease. The results of this study will be important for further understanding the role of BH4 in endothelial regeneration, helping to determine whether supplementation with BH4 may represent a therapeutic strategy for improving vascular repair.

1.12 Hypothesis

The central hypothesis is that endothelial bioavailability of BH4, which is essential for eNOS catalytic activity, is a critical determinant of endothelial cell growth and regeneration in vascular disease states.

1.13 Aims

Using primary endothelial cells from mice with genetic modifications in GCH and/or eNOS expression, the aims of this thesis are:

- 1) To determine the optimal selection conditions and time scale to achieve a pure primary endothelial cell culture that provides sufficient material for biochemical analysis and displays the biological phenotype and functionality of the donor animal.
- 2) To evaluate the effects of genetic alterations in GCH expression on BH4 availability in primary endothelial cells and to determine whether these effects are sustained in tissue culture.
- 3) To investigate the effect of BH4 bioavailability on endothelial cell growth in the absence and presence of vascular disease.
- 4) To investigate the effect of transgenic GCH overexpression under the control of the Tie-2 promoter on endothelial progenitor cell content in lungs and bone marrow.

Chapter 2:

Methods

2 Methods

This chapter contains all techniques used in this thesis. Some techniques are outlined in principle and described in more detail in subsequent chapters. Chemicals were purchased from Sigma-Aldrich Inc. (UK), Fisher Scientific (UK) or VWR international (UK).

2.1 Genetic mouse models

Genetically modified mice were used to investigate changes in BH4 and eNOS biology in primary endothelial cells as a consequence of the isolation and culturing procedure and to investigate the influences of endothelial BH4 on primary endothelial cell growth.

All animal studies were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986 (HMSO, UK) under Project Licenses 30/2077 and 30/2562, and Personal License 30/7927.

Mice were housed in individually ventilated cages in a Specific-Pathogen-Free (SPF) environment in the Functional Genetics Facility at the Wellcome Trust Centre for Human Genetics, Oxford, UK. They were kept under a 12 hour light/dark cycle and controlled temperature of 20-22°C and given free access to a normal chow diet (2016 Teklad Global 16% Protein Rodent Diet; Harlan, Shaw's Farm, Blackthorn, Bicester, UK) and water *ad libitum*.

All mice were crossed onto a C57BL/6 background and kept in breeding colonies such that the appropriate wild type and/or non-transgenic littermates are produced in equal numbers as controls. C57BL/6 mice were originally obtained from Harlan UK Limited, Bicester, UK. All animals included into experiments were aged between 12 and 20 weeks.

2.1.1 eNOS transgenic mice

eNOS transgenic mice (eNOS mice) overexpress bovine eNOS under the control of the murine preproendothelin-1 promoter (Ohashi *et al.*, 1998). Heterozygote eNOS transgenic mice were mated with heterozygote GCH transgenic mice in an established breeding colony.

2.1.2 GCH transgenic mice

GCH transgenic mice (GCH mice) overexpress human GCH under the control of the Tie-2 promoter and intronic enhancer specifically in endothelial cells. This line was generated in-house by Dr. Nicholas Alp (Alp *et al.*, 2003).

Heterozygote GCH transgenic mice were bred with heterozygote eNOS transgenic mice. The offspring of this cross is expected to consist of 25% wild type mice (WT), 25% GCH mice, 25% eNOS mice and 25% eNOS/GCH double transgenic mice.

2.1.3 GCH/ApoE-KO/LacZ mice

To study the influence of endothelial BH4 levels in a vascular disease setting, the GCH transgenic mouse was crossed onto ApoE-Knockout (ApoE-KO) background.

ApoE-KO mice were originally purchased from Jackson Laboratories, USA on a C57Bl/6 background. These mice carry a targeted deletion of the Apolipoprotein E gene. Homozygote ApoE-KO mice have high levels of plasma cholesterol, endothelial dysfunction and develop aortic atherosclerosis (Zhang *et al.*, 1992).

GCH/ApoE-KO mice are heterozygote for the human GCH transgene and homozygote ApoE-KO.

These mice were further crossbred with transgenic Tie-2 LacZ mice (Jackson Laboratories, USA), which had been crossed onto ApoE-KO background. Tie-2 LacZ mice (LacZ mice) express β -galactosidase in the nucleus of endothelial cells (Kisanuki *et al.*, 2001) enabling the visualization and quantification of endothelial cells in situ. ApoE-KO/LacZ mice are homozygote ApoE-KO and heterozygote for the LacZ transgene.

Breeding GCH/ApoE-KO with ApoE-KO/LacZ mice produces litters, which are expected to consist of 25% ApoE-KO mice, 25% GCH/ApoE-KO mice, 25% ApoE/LacZ mice and 25% GCH/ApoE-KO/LacZ double transgenic mice.

2.1.4 hph-1 mice

Hph-1 mice carry a point mutation leading to underexpression of GTP cyclohydrolase I (Khoo *et al.*, 2004) with an 85% reduction in tissue BH4 levels (Khoo *et al.*, 2005) and were originally obtained from Dr. Simon Heales, National Hospital for Neurology and Neurosurgery, Queen Square, London, UK (Bode *et al.*, 1988). Heterozygote hph-1 mutation mice were bred with each other to produce

litters expected to consist of 25% hph-1 homozygotes (hph mice), 50% hph-1 heterozygotes and 25% wild type.

To obtain hph/GCH mice, which are homozygote for hph-1 mutation and heterozygote for the GCH transgene as well as hph controls, homozygote hph-1 mice were bred with hph/GCH mice leading to litters consisting of 50% hph and 50% hph/GCH mice.

2.1.5 Procedure for harvesting mouse organs

Animals were sacrificed by overdose of inhaled isoflurane and cervical dislocation or exsanguination. Following death the thoracic cavity was opened and both the systemic and pulmonary circulation were flushed with ice-cold PBS via the heart. When lungs were harvested for endothelial cell isolation, the lungs were removed before the infusion with PBS. Micro-dissection of the descending aorta was performed using a binocular microscope (Nikon SMZ1000/SMZ800, Nikon Europe BV, The Netherlands).

2.2 Genotyping

Mice were ear punched for identification purposes and the notches were used for DNA extraction.

2.2.1 DNA preparation for genotyping

Tissue was digested for a minimum of 3-4 hours at 55°C in 500µl of DNA lysis buffer (10mM Tris pH 8.5, 0.45% Non-idet P40, 0.45% Tween-20, 0.2mg/ml freshly added Proteinase K). DNA was purified by extraction with 500µl of

phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma-Aldrich, UK). The digest was mixed vigorously and centrifuged at 16,000 g for 15 minutes. The upper layer (~450µl) was transferred into a new Eppendorf tube and 50µl of 3M sodium acetate (pH 5.2) and 1ml of chilled 100% ethanol were added. The mixture was cooled for 30 minutes at -80°C, and then centrifuged at 16,000 g for 10 minutes to precipitate the DNA. The supernatant was discarded, and the DNA pellets washed with 1ml of 70% ethanol and centrifuged at 16,000 g for 10 minutes. After repeating the washing step once more the DNA pellet was resuspended in 40µl TE buffer (1mM Tris pH 8.0, 1mM EDTA).

2.2.2 Polymerase Chain Reactions for genotyping

The following PCR reactions were used for genotyping for eNOS, GCH and LacZ transgenes, hph-1 gene mutation and ApoE gene knockout. Products were run on a 3% agarose gel for hph-1 and on 1.5% agarose gels for all other reactions. Agarose was dissolved in 1xTAE (2M Tris-acetate and 100 mM EDTA, pH 8.3) by heating and the gel was cooled to < 60°C before the addition of ethidium bromide. After letting the gel set for 30 minutes, PCR products were run in the gel at 100-120V in 1x TAE. DNA bands were visualized using UV illumination.

2.2.2.1 eNOS-Tg genotyping PCR

Primers:

eNOSBov1572F:	5'-CTGATGGCCAAGCGAGTGAAAGCA-3'
eNOSbov1869R:	5'-GGATCTTGTAAGTCTTGTGCTGT-3'
D14MIT141-Forward	5'-CCAGCATTCCGAAGTCATTT-3'
D14MIT141-Reverse	5'-AGGGAAAGAAGACAGCACGA-3'

Reaction mix (25 µl):

13 µl	H ₂ O
2.5 µl	GeneAmp® PCR Buffer II (Applied Biosystems, USA)
2.5 µl	25 mM MgCl ₂ (Applied Biosystems, USA)
1.5 µl	8 mM dNTPs (Bioline, UK)
0.3 µl	each primer (100 pmol/µl) (Sigma-Aldrich, UK)
0.3 µl	5u/µl AmpliTaq Gold® DNA Polymerase (Applied Biosystems, USA)
4 µl	DNA (100 ng/µl)

Thermocycle sequence:

94°C for 15 min

then 35 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min

then 10 °C for 10 min

Expected Products:

WT: 150 bp

eNOS: 150 and 300 bp

2.2.2.2 GCH-Tg genotyping PCR

Primers:

Tie2F	5'-GGGAAGTCGCAAAGTTGTGAGTT-3
RH14	5'-GAACCCATTGCTGCACCTGG-3
D14MIT83-Forward	5'-AACACAAAAGTGTACCGTCCG-3'
D14MIT83-Reverse	5'-CTCCAAGTCCCAGCTACTCG-3'

Reaction mix (25 µl):

8.5 µl	H ₂ O
12.5 µl	BioMix™ Red (Bioline, UK)
0.5 µl	each primer (100 pmol/µl) (Sigma-Aldrich, UK)
2 µl	DNA (100 ng/µl)

Thermocycle sequence:

95°C for 15 min

then 26 cycles of 94°C for 1 min, 64°C for 1 min and 72°C for 5 sec

then 72°C for 10 min

then 10°C for 1 min

Expected Products:

WT: 202 bp (190 bp on hph-1 background)

GCH: 150 and 202 bp (150 and 190 bp on hph-1 background)

2.2.2.3 LacZ genotyping PCR*Primers:*

Tie2-LacZ-Forward	5'-ATCCTCTGCATGGTCAGGTC-3'
Tie2-LacZ-Reverse	5'-CGTGGCCTGATTCATTCC-3'
D14MIT83-Forward	5'-AACACAAAACGTACCGTCCG-3'
D14MIT83-Reverse	5'-CTCCAAGTCCCAGCTACTCG-3'

Reaction mix (12 µl):

3.92 µl	H ₂ O
6 µl	BioMix™ Red (Bioline)
0.24 µl	D14MIT83-Forward and -Reverse (100 pmol/µl) (Sigma-Aldrich, UK)
0.3 µl	Tie2-LacZ-Forward and -Reverse (100 pmol/µl) (Sigma-Aldrich, UK)
1 µl	DNA (100 ng/µl)

Thermocycle sequence:

94°C for 3 min

then 13 cycles of 94°C for 30 sec, 64°C (-0.5°C per cycle) for 30 sec and 72°C for 35 sec

then 26 cycles of 94°C for 20 sec, 58°C for 30 sec and 72°C for 35 sec

then 72°C for 2 min

then 20°C for 1 min

Expected Products:

WT: 202 bp

LacZ: 315 bp

2.2.2.4 hph-1 genotyping PCR*Primers:*

D14MIT60 Forward	5'-AGGCTGCCCATAAAAGGG-3'
D14MIT60 Reverse	5'GTTTGTGCTAATGTTCTCATCTGG-3'
D14MIT83 Forward	5'-AACACAAAACGTACCGTCCG-3'
D14MIT83 Reverse	5'-CTCCAAGTCCCAGCTACTCG-3'

Reaction mix (25 µl):

14 µl	H ₂ O
2.5 µl	GeneAmp® PCR Buffer II (Applied Biosystems, USA)
1.5 µl	25 mM MgCl ₂ (Applied Biosystems, USA)
1.5 µl	8 mM dNTPs (Bioline, UK)
0.5 µl	each primer (100 pmol/µl) (Sigma-Aldrich, UK)
0.5 µl	5u/µl AmpliTaq Gold® DNA Polymerase (Applied Biosystems, USA)
3 µl	DNA (100 ng/µl)

Thermocycle sequence:

95°C for 15 min

then 35 cycles of 94°C for 20 sec, 58°C for 1 min and 72°C for 45 sec

then 72°C for 10 min

then 25°C for 1 min

Expected Products:

WT: 202 and 136 bp

hph-1 heterozygote: 202, 190, 136 and 108 bp

hph-1 homozygote (hph): 190 and 108 bp

2.2.2.5 ApoE-KO genotyping PCR*Primers:*

IMR0180	5'-GCCTAGCCGAGGGAGAGCCG-3'
IMR0181	5'-TGTGACTTGGGAGCTCTGCAGC-3'
IMR0182	5'-GCCGCCCGACTGCATCT-3'

Reaction mix (25 µl):

16.5 µl	H ₂ O
2.5 µl	GeneAmp® PCR Buffer II (Applied Biosystems, USA)
1 µl	25mM MgCl ₂ (Applied Biosystems, USA)
1 µl	8 mM dNTPs (Bioline, UK)
0.5 µl	each primer (100 pmol/µl) (Sigma-Aldrich, UK)
0.5 µl	5u/µl AmpliTaq Gold® DNA Polymerase (Applied Biosystems, USA)
2 µl	DNA (100 ng/µl)

Thermocycle sequence:

94°C for 15 min

then 28 cycles of 94°C for 20 sec, 68°C for 40 sec, and 72°C for 2 min

then 25°C for 1 min

Expected Products:

WT: 155 bp

ApoE-KO heterozygote: 155 bp and 245 bp

ApoE-KO homozygote (ApoE-KO): 245 bp

2.3 Cell culture

All cell culture experiments took place under sterile conditions using laminar flow hoods and sterile disposable plastic ware. Culture media and supplements were purchased from PAA Laboratories, Sigma-Aldrich and Lonza. Tissue culture treated plastic ware was purchased from Greiner Bio-One, Corning and Appleton Woods. Cell cultures were kept in CO₂ incubators at 37°C with a humidity saturated atmosphere containing 5% CO₂.

2.3.1 sEND.1 cell culture

The sEND.1 mouse endothelioma cell line was originally derived from subcutaneous haemangioma (Williams *et al.*, 1988).

sEND.1 cells were grown in Complete Medium (DMEM high glucose containing 10% FCS, 100U/ml penicillin, 0.1mg/ml streptomycin and 2mM L-glutamine). When confluent, cells were detached using 0.05% Trypsin EDTA and passaged 1 in 10 or plated out in the desired density.

2.3.2 Primary endothelial cell isolation and culture

Primary endothelial cells provide a useful *in vitro* model in cardiovascular research, bearing closer resemblance to *in vivo* endothelial cell physiology than immortalized cell lines. Using mice as donor animals is particularly useful because of the availability of genetic modifications in this species. Primary murine endothelial cells have been used for various research applications and several different protocols have been deployed for their isolation and culture, but in comparison to immortalized cell lines and commercially available endothelial cells of human or bovine origin, primary mouse endothelial cell culture is a poorly defined system.

In this thesis, CD31 immunomagnetic bead selection was used for isolation and purification of primary endothelial cells from mouse lungs. Isolation and culture techniques are described in principle in the following paragraphs. Detailed experimental protocols can be found in subsequent chapters.

Endothelial cells were grown in gelatine coated dishes or flasks using EC medium (EGM®-2-MV supplemented with 5% FCS, hEGF, VEGF, hFGF-B, R³-IGF-1, hydrocortisone, Gentamycin/Amphotericin-B and ascorbic acid; Lonza, product code CC-3202).

2.3.2.1 Enzymatic digestion of mouse lungs

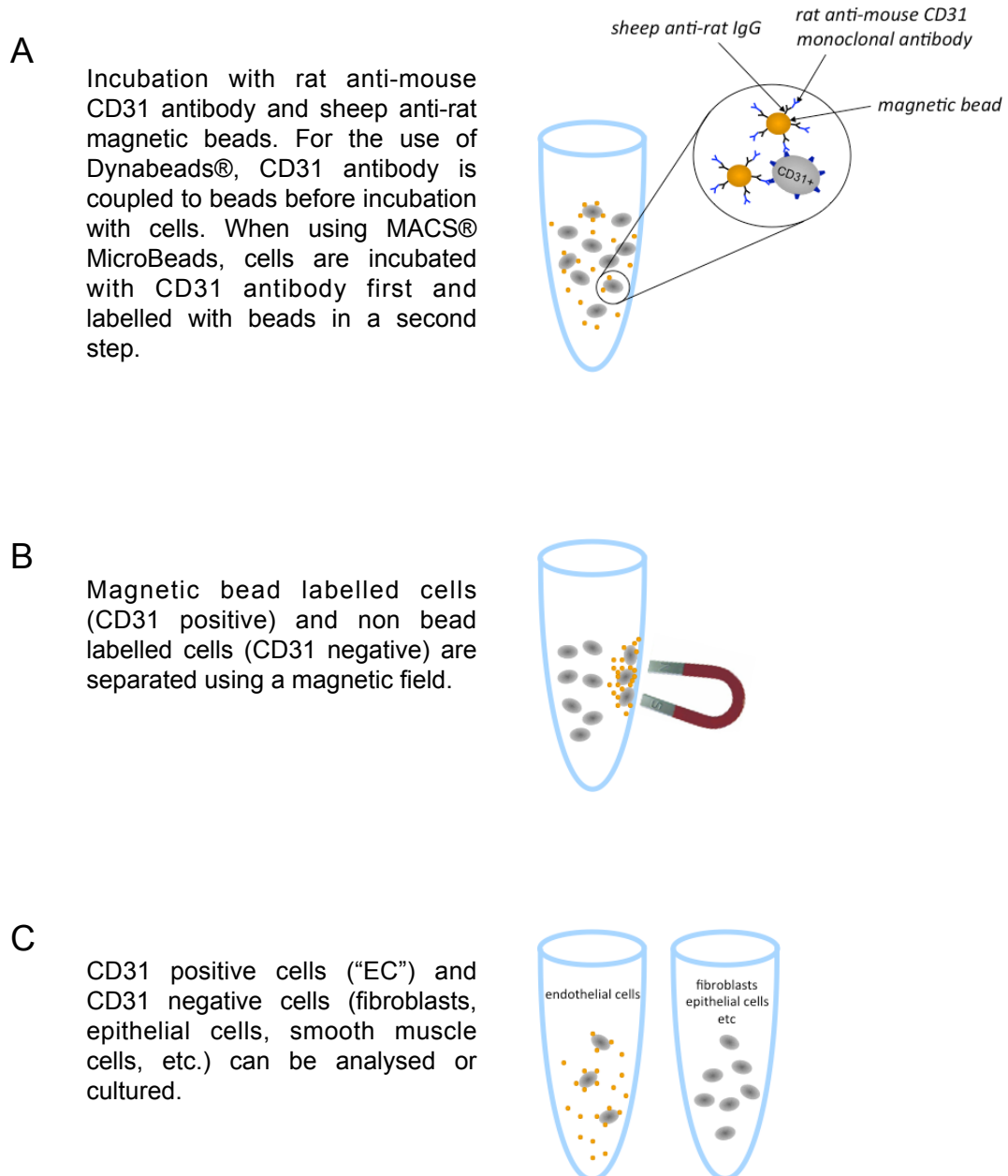
Animals were sacrificed by overdose of inhaled isoflurane and exsanguination. Following death, lungs were harvested into ice-cold DMEM. Lungs were finely minced and digested in DMEM containing 0.18 U/ml Liberase Blendzyme 3 (Roche) and 0.1 mg/ml DNase I (Roche) for 60 minutes at 37°C with gentle

agitation. After gently sheering the remaining visible tissue pieces by trituration through a 5 ml pipette, the digest was passed through a 100µm cell strainer (BD Biosciences), washed with PBS containing 10% FCS for Dynabead® selection or MACS buffer (PBS containing 0.5% BSA and 2mM EDTA) for MACS® MicroBead selection, respectively, and centrifuged at 250 g and room temperature for 5 minutes. For Dynabead® selection, the cell pellet was washed with 10 ml PBS/10%FCS, centrifuged at 250 g and room temperature for 5 minutes, resuspended in 1 ml PBS/10%FCS and transferred into a 1.7 ml Eppendorf tube for bead binding. For MACS® MicroBead selection, the cell pellet was resuspended in 10 ml MACS buffer, passed through a 40µm cell strainer (BD Biosciences), centrifuged at 250 g and room temperature for 5 minutes and resuspended in 500µl MACS buffer for CD31 antibody binding.

2.3.2.2 CD31 immunomagnetic bead selection

Immunomagnetic bead selection can be used for specific cell isolation or depletion. In this thesis the cell surface antigen CD31 (PECAM-1) was chosen to separate endothelial cells from non-endothelial cells. Figure 2.1 illustrates the principles of this procedure. Two different types of magnetic beads were used for endothelial cell isolation. Dynabeads® Sheep anti-Rat IgG (Invitrogen, product code 110.35), referred to as Dynabeads® in the following, were used in the earlier experiments of this thesis. MACS® Goat anti-Rat IgG MicroBeads (Miltenyi Biotech, product code 130-048-501), referred to as MACS® MicroBeads in the following, are flow cytometry compatible and were used in the later experiments of this thesis. Rat anti-mouse CD31 monoclonal antibody (MEC 13.3, BD Pharmingen) was used with both types of magnetic beads.

Figure 2.1

**Figure 2.1: Isolation of primary EC by anti CD31 immunomagnetic bead selection.**

Immunomagnetic bead selection can be used for specific cell isolation or depletion. The surface antigen CD31 (PECAM-1) was chosen to separate EC from non EC after enzymatic digest of mouse lungs.

CD31 immunomagnetic bead selection using Dynabeads® Sheep anti-Rat IgG:

Dynabeads® were coated with CD31 antibody by incubating 100µl (4×10^7 beads) of washed Dynabeads® with 12µl (6 µg) of rat anti-mouse CD31 monoclonal antibody in 300µl PBS/10%FCS at 4°C with gentle agitation over night. Dynabeads® were washed 3 times with 1 ml PBS/10%FCS using a magnet to remove unbound CD31 antibody and were resuspended in the initial volume of 100µl. Conjugated Dynabeads® were kept in a sterile tube in the fridge for up to 4 weeks.

2.5µl of CD31 conjugated Dynabeads® (10^6 beads) were added to the 1 ml of lung cells (Chapter 2.3.2.1) and incubated with gentle agitation at 4°C for 20 minutes. Using a magnet, the bead-bound cells were washed 4 times with 1 ml PBS/10%FCS. Bead-bound (CD31 positive) and non bead-bound (CD31 negative) cells were plated into 1 well of a gelatine coated 6 well dish (9.6 cm² growth area) each with EC medium. Alternatively, cells from 2 mice were plated into 1 T25 flask.

CD31 immunomagnetic bead selection using MACS® Goat anti-Rat IgG***MicroBeads:***

Lung cells (Chapter 2.3.2.1) were incubated in 500µl MACS buffer containing 1 µg/ml rat anti-mouse CD31 monoclonal antibody for 15 minutes at 4°C. 500µl MACS buffer was added to the sample and the cells were centrifuged at 300 g for 5 minutes at room temperature. The cell pellet was resuspended in 500µl MACS buffer and 70µl MACS® MicroBeads were added. After 15 minutes incubation at 4°C the sample was centrifuged at 300 g for 5 minutes at room temperature. The

cell pellet was resuspended in 500µl MACS buffer and added onto a MACS® MS Cell Separation Column in a magnetic field. The cells were allowed to pass through the column and the effluent (unlabeled cells, CD31 negative) was collected. After washing the column 3 times with 500µl MACS buffer each, MicroBead labeled cells (CD31 positive) were eluted from the column by removing it from the magnetic field and washing out the cells using MACS buffer. The cells were centrifuged at 300 g for 5 minutes at room temperature, resuspended in 500µl EC medium and plated into 1 well of a gelatine coated 12 well dish (3.8 cm² growth area).

2.3.2.3 Reselection of endothelial cell cultures using Dynabeads®

Three CD31 Dynabead® selections were necessary to obtain pure endothelial cell cultures. For the first reselection after isolation, the EC medium was replaced with 1 ml DMEM containing 2.5µl (1x10⁶) CD31-conjugated Dynabeads® for 1 well of a 6 well dish or with 2 ml DMEM containing 7.5µl (3x10⁶) CD31-conjugated Dynabeads® for a T25 flask. The cells are incubated for 20-30 minutes at 37°C, excess beads were washed off with PBS and cells were detached using Accutase™ (PAA Laboratories). Bead-bound and non-bead-bound cells were separated using a magnet and plated into 1 well (previously grown in 1 well of a 6 well dish) or 2 wells (previously grown in a T25 flask) of a gelatine coated 6 well dish. For endothelial cell culture the twice CD31 positive selected cells were kept and for non-endothelial cell culture the twice CD31 negative selected cells were kept. “Positive/negative” and “negative/positive” cells were discarded.

For the second reselection 3.75µl (1.5×10^6) CD31- conjugated Dynabeads® were used per well and the cells were plated back into 1 well of a 6 well dish.

2.3.3 Primary vascular smooth muscle cell isolation

Vascular smooth muscle cells (VSMC) were isolated from mouse thoracic aortas. Animals were sacrificed by overdose of inhaled isoflurane and cervical dislocation. Following death the thoracic cavity was opened and both systemic and pulmonary circulation was flushed with ice-cold PBS via the heart. The descending aorta was dissected, opened lengthways and placed in ice cold DMEM. The endothelial cell layer was removed using a cell scraper and the aorta was cut into 1-2 mm wide segments which were placed into 1 well of a gelatine coated 6 well dish containing 0.5 ml of Complete Medium. After 24 hours a further 0.5 ml medium was carefully added to the well. After approximately 7 days when the VSMC growing out of the aorta became confluent, the cells were detached using 0.05% Trypsin EDTA and plated into 2 wells of a gelatin coated 6 well dish. The cells were harvested and frozen after an additional 7 days when they had reached confluence again.

2.4 RNA techniques

RNA from endothelial cell (EC), non-endothelial cell (non-EC) and vascular smooth muscle cell (VSMC) samples was quantified using a NanoDrop 2000 (Thermo Scientific, UK). Gene expression was measured by synthesizing complementary DNA (cDNA) using SuperScript™ II Reverse Transcriptase (Invitrogen, UK) and performing real time RT-PCR using TaqMan® probes (Applied Biosystems, USA).

2.4.1 RNA extraction

Total RNA was extracted from cells by adding 1 ml of Trizol (Sigma Aldrich, Inc) directly into the well and subsequent freezing at -80°C. After thawing 200µl chloroform was added and the samples were mixed and centrifuged at 16,000 g and 4°C for 20 minutes. The aqueous phase (~700µl) was removed and 700µl of 70% ethanol was added. Samples were transferred to RNeasy columns (Qiagen, UK), where RNA binds to the membrane of the column and contaminants, including DNA, are washed through. Due to small sample size RNeasy micro kits (for purification of up to 45µg RNA; Qiagen, UK) were used for EC and non-EC; for VSMC RNeasy mini kits (for purification of up to 100 µg RNA; Qiagen, UK) were used. Because of potential residual genomic DNA contamination an on-column DNase digestion was performed before the final washing steps.

2.4.2 Complementary DNA synthesis

cDNA was synthesized using SuperScript™ II Reverse Transcriptase (Invitrogen, UK).

2µl random hexamers (500µg/ml) were added to 20µl extracted RNA containing a maximum of 2.5µg RNA. The mixture was incubated in a PCR machine at 65°C for 5 minutes before chilling on ice and addition of 18µl mastermix (8µl 5X First-Strand Buffer, 2.5µl 8mM dNTP mix, 4 µl 0.1 M DTT, 3µl H₂O and 0.5µl SuperScript™ II). The mixture was placed back in the PCR machine and incubated at 42°C for 1 hour. To inactivate the reaction the mixture was heated at 70°C for 5 minutes before storing synthesized cDNA at -20°C until required for RT-PCR.

2.4.3 Real time RT-PCR using TaqMan® probes

The first step of real time RT-PCR is reverse transcription of RNA into cDNA. This is followed by cDNA amplification in a PCR reaction. 25ng cDNA was used per sample and samples were run in duplicates or triplicates.

RT-PCR using TaqMan® gene expression assays were performed with an iCycler IQ real-time detection system (BioRad Laboratories, USA) in 96-well optical plates (Applied Biosystems, USA). For each 25µl reaction, 11.25µl of cDNA (25ng cDNA) was mixed with 12.5µl TaqMan® Universal Master Mix and 1.25µl TaqMan® gene expression assays. The reaction sequence was: 50°C for 2 minutes, then 95°C for 10 minutes; then 37 cycles of 95°C for 15 seconds and 60°C for 1 minute.

All TaqMan® probes were designed and optimized by Applied Biosystems, USA. Assay details are shown in Table 2.1. All primers and probes spanned two introns to avoid amplification of genomic DNA.

Gene	Applied Biosystems product number
mouse GTP cyclohydrolase 1	Mm00514993_m1
human GTP cyclohydrolase 1	Hs00609198_m1
mouse GAPDH	4352932E
mouse Pecam1 (CD31)	Mm00476702_m1
mouse smooth muscle myosin heavy chain	Mm00443013_m1

Table 2.1: TaqMan® Gene Expression Assays.

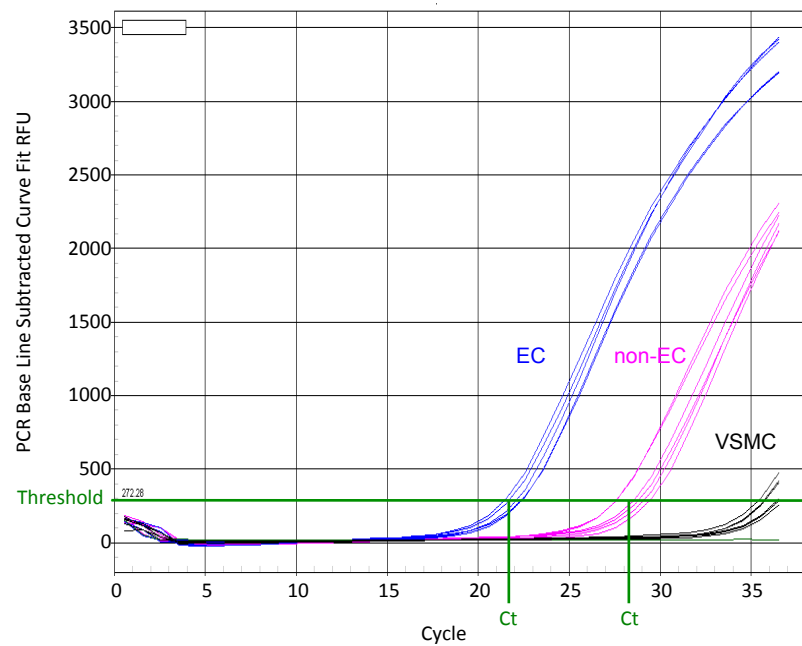
TaqMan® gene expression assays used are listed with Applied Biosystems product number. These assays are commercial products and sequences are not available.

TaqMan® probes (Applied Biosystems, USA) are oligonucleotides labeled with a reporter fluorophore at the 5' end and a quencher fluorophore at the 3' end and designed to specifically anneal within a DNA region amplified by a specific set of primers. During the PCR reaction Taq polymerase extends the primer. When reaching the TaqMan ® probe, the 5' to 3' exonuclease activity of the Taq polymerase cleaves the probe and releases the reporter fluorophore away from the close vicinity of the quencher. As a result the fluorescence intensity increases with each cycle of the PCR. As the reaction components become limiting, the rate of accumulation of released fluorophore decreases until a plateau is reached. The threshold cycle (Ct) value corresponds to where a detectable increase in fluorescence crosses an arbitrary threshold, and is inversely proportional to the

amount of initial PCR template. The threshold level is set to be above the baseline but sufficiently low to be within the exponential growth region of an amplification curve. To control for any inter sample variability in initial cDNA content, house-keeping genes are run for each sample on the same plate as the target gene. In these experiments GAPDH was used as a house-keeping gene. Target gene Ct values were corrected for inter-sample variability by expressing them as the delta Ct (Δ Ct). The Δ Ct was calculated as the target gene Ct value minus the house-keeping gene Ct value.

Figure 2.2 shows an amplification plot of CD31 in EC, non-EC and VSMC. EC have a significantly higher starting template than non-EC as the product can be detected earlier at a lower cycle number. With a Ct value larger than 35, there is no specific product detectable in VSMC.

Figure 2.2

**Figure 2.2: Amplification plot of CD31 in EC, non-EC and VSMC.**

This plot demonstrates increased fluorescence in EC (blue) in comparison to non-EC (pink). EC therefore have a significantly higher starting template than non EC as the product can be detected earlier at a lower cycle number. With a Ct value larger than 35, there is no specific product detectable in VSMC (black).

2.5 Determination of protein content using BCA™ Protein Assay Kit

Sample protein content was measured using the reducing agent compatible BCA™ protein assay (Pierce, USA). The Bicinchoninic Acid (BCA) protein assay is a highly sensitive colorimetric assay that is compatible with detergent solubilized protein solutions, but is dramatically affected by the presence of reducing agents such as DTT, DTE or β -mercaptoethanol. The assay solution contains Cu^{2+} ions which are reduced to Cu^+ by peptide bonds in the protein sample. The amount of Cu^{2+} being reduced is proportional to the amount of protein present in the solution. BCA chelates Cu^+ forming a purple-colored product that strongly absorbs light at a wavelength of 562 nm. Preincubation with iodoacetamide (10mg/ml final concentration) inactivates reducing agents and allows accurate protein concentration measurements in the presence of up to 5mM DTT or 35mM β -mercaptoethanol. For the generation of a standard curve, Protein standards ranging from 0.125 to 2 mg/ml were prepared from bovine serum albumin (BSA) (Sigma Aldrich, Inc) using the same buffer as was used for sample preparation (e.g. RIPA for western blotting, Resuspension Buffer for bioppterin analysis and lysis buffer for β -galactosidase activity assay).

In short, for measuring samples in duplicate, 12 μ l of iodoacetamide (20mg/ml in 0.4% NaOH) was added to 12 μ l sample or standard and incubated for 15 minutes at 37°C. 480 μ l of working reagent was added and the samples were incubated at 37°C for 30 minutes. 2 times 240 μ l per sample or standard was transferred into a 96 well plate and read in a microplate reader at 562 nm.

2.6 Western Blotting

Western blotting was used for quantification of specific proteins. Cell pellets were lysed in RIPA buffer (Tris 50mM, NaCl 150 mM, SDS 0.1%, deoxycholate salt 0.5%, Nonidet P40 1%, PMSF 1mM, DTT 1mM) containing protease inhibitors (Complete, Mini, EDTA-free. Roche, UK). To promote lysis, cell pellets in RIPA buffer were freeze thawed once on dry ice. The protein concentration of each sample was determined using the BCA™ protein assay as described before (Chapter 2.5). NuPAGE® LDS Sample Buffer and NuPAGE® Reducing Agent (Invitrogen, UK) were added to the lysates and the samples were heated at 70°C for 10 minutes right before gelelectrophoresis. 2-10µg protein per sample were resolved by SDS-PAGE using NuPAGE® Novex 4-12% Bis-Tris Gels and NuPAGE® MOPS SDS Running Buffer (Invitrogen, UK).

After transferring the resolved protein from the gel to a polyvinylidene difluoride membrane, the membrane was blocked over night at 4°C using 6% non-fat milk in PBS containing 0.1% Tween-20 (PBS-T). Membranes were then incubated with primary antibody in PBS-T with 6% milk for 1 hour at room temperature, washed with PBS-T and incubated with secondary antibody in PBS-T with 6% milk for 1 hour at room temperature. Table 2.2 provides an overview over all primary and secondary antibodies used. Secondary HRP coupled antibodies were purchased from Promega, USA and used at 1/5000 dilution for Goat Anti-Rabbit and Goat Anti-Mouse and at 1/10,000 dilution for Donkey Anti-Goat. Protein bands were visualized by chemiluminescence (Supersignal Pierce, USA). Quantification of band density in samples was calculated using AlphaEaseFC (FluorChem 8800) Software, USA.

Antibody	Target species	Reactivity	Working concentration	Company	Product code
Goat polyclonal CD102	mouse	mouse	0.2 µg/ml	R&D Systems	AF774
Goat polyclonal Tie2	mouse	mouse human	0.2 µg/ml	R&D Systems	AF762
Mouse monoclonal eNOS	human	human mouse rat	1/5000 (0.05 µg/ml)	BD Transduction Laboratories	610296
Mouse monoclonal GAPDH	rabbit	multi species including mouse	1/5000 (0.2 µg/ml)	Millipore, USA	MAB374
Mouse monoclonal α-smooth muscle actin	synthetic peptide of highly conserved region	multi species including mouse	1/10 000	Sigma-Aldrich	C6198
Rabbit polyclonal β-tubulin	human	multi species including mouse	1/10 000	Abcam	ab6046
Mouse monoclonal α-actinin	bovine	multi species including mouse	1/2000	Sigma-Aldrich	A5044
Rabbit polyclonal nNOS	human	human mouse rat	1/1000	BD Transduction Laboratories	610310
Mouse monoclonal iNOS	mouse	human mouse rat	1/10 000	BD Transduction Laboratories	610431
Mouse monoclonal GTPCH	human	human	1/2000 (0.25 µg/ml)	AbD Serotec	Mca3138z
Rabbit polyclonal GTPCH	human	human	1/2000	Kindly provided by Gabriele Werner-Felmayer, Inst. for Medical Chemistry and Biochemistry, Austria	
Rabbit polyclonal GTPCH	rat	rat mouse human	1/10 000	Kindly provided by Prof. Steven S. Gross, Cornell University, USA	

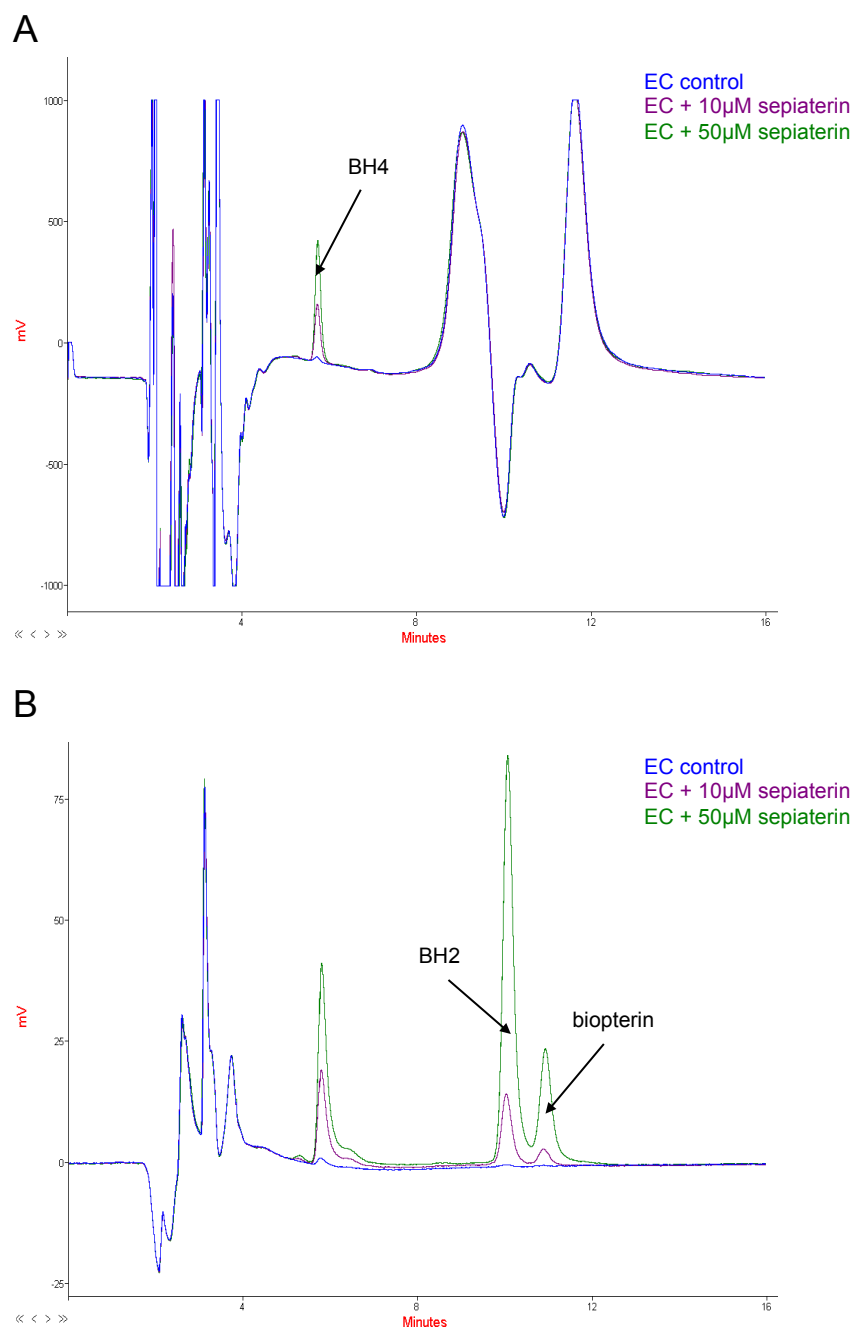
Table 2.2: Primary antibodies used for Western Blotting. Antibodies are listed with host and target species, reactivity, working concentrations and origin.

To re-probe membranes with a different primary antibody, membranes were stripped for 30 minutes at 50°C in 6.25mM Tris (pH 6.8) with 2% SDS and 100mM β -mercaptoethanol, blocked in PBS-T with 6% milk and incubated with antibodies as described above.

2.7 Measurement of BH4, BH2 and biopterin

BH4, BH2 and biopterin were measured by electrochemical and fluorescence detection respectively, following sample separation by HPLC (Heales and Hyland, 1989; Howells *et al.*, 1986). Frozen cell pellets were thawed in Resuspension Buffer (PBS pH 7.4 containing 1mM DTE and 0.5 μ M EDTA) and freeze-thawed once to promote cell lysis. The cell lysates were centrifuged for 5 minutes at 16,000 g and 4°C to remove any debris and the supernatants were transferred into new Eppendorf tubes. Protein was precipitated with 10X Precipitation Buffer (1mM phosphoric acid, 2 M trichloroacetic acid and 1mM DTE) followed by a 5 min spin at 16,000 g and 4°C. Samples were injected onto an isocratic HPLC system and quantified using sequential electrochemical detection (Coulochem III, ESA Inc., UK) and fluorescence (JASCO, UK) detection. HPLC separation was performed using a 250 mm ACE C-18 column (Hichrom, UK) and mobile phase comprising of 50mM sodium acetate, 5mM citric acid, 48 μ M EDTA and 160 μ M DTE pH 5.2 (all chemicals ultrapure electrochemical HPLC grade) at a flow rate of 1.3 ml/min. Background currents of +500 to +600nA and -50 to -60nA were used for the detection of BH4 on electrochemical cells E1 and E2, respectively. Quantification of BH4, BH2 and biopterin was completed by comparison with external standards after normalizing for sample protein. Representative chromatograms showing BH4, BH2 and biopterin peaks in primary endothelial cells are shown in Figure 2.3.

Figure 2.3

**Figure 2.3: Representative biopterin chromatogram traces.**

A: Electrochemical detection. **B:** Fluorescent detection. The traces show BH4 (A), BH2 (B) and biopterin (B) peaks in primary EC with and without sepiapterin treatment.

2.8 Measurement of eNOS enzymatic activity

eNOS enzymatic activity in cells was quantified by using ^{14}C -L-arginine to ^{14}C -L-citrulline radiochemical HPLC (de Bono *et al.*, 2007).

All experiments were performed in the presence of the arginase inhibitor nor-NOHA (Calbiochem, UK). Arginase activity is an important confounding factor in the measurement of NOS activity because it breaks down arginine to ornithine and urea. Ornithine can be converted to citrulline by ornithine transcarbamylase. $5\mu\text{M}$ nor-NOHA was found to be the optimal concentration to sufficiently inhibit arginase activity without inhibiting NOS activity in these experimental settings (de Bono *et al.*, 2007). For each sample experiments were undertaken in the absence and presence of the NOS inhibitor L-NAME (Sigma-Aldrich, UK) to identify residual unspecific, NOS independent L-citrulline production.

Endothelial and non-endothelial cells were grown in 6 well culture plates. Prior to incubation with ^{14}C -L-arginine the culture media was removed, the cells rinsed with 37°C warm Krebs HEPES buffer (KHB, consisting of NaCl 99mM/l , KCl 4.7mM/l , MgSO_4 1.2mM/l , KH_2PO_4 1.0mM/l , CaCl_2 1.9mM/l , NaHCO_3 25mM/l , glucose 11.1mM/l , and Na-HEPES 20mM/l) and pre-incubated with KHB ($\pm 1\text{mM}$ L-NAME) containing $5\mu\text{M}$ nor-NOHA for 60 minutes at 37°C . The pre-incubation buffer was then replaced by $600\mu\text{l}$ KHB ($\pm 1\text{mM}$ L-NAME) containing $1\mu\text{M}$ calcium ionophore A23187 (Sigma-Aldrich, UK), $5\mu\text{M}$ nor-NOHA and $2\mu\text{l}$ ^{14}C -labelled L-arginine (1.85MBq/ml , Amersham Biosciences UK Ltd., Chalfont St. Giles, UK) per well. The amount of ^{14}C -L-arginine added to each well was chosen

to be in excess to avoid arginine levels becoming limiting under stimulation with calcium ionophore. After 60 minutes incubation at 37°C the supernatant was transferred into an Eppendorf tube, 300µl water added per well and the plate was placed on dry ice for 10 minutes to promote cell lysis. The resulting lysate was added to the Eppendorf tube containing the supernatant and protein was precipitated by the addition of 300µl 10% trichloroacetic acid followed by 5 min centrifugation at 16,000 g.

Samples were injected onto a gradient HPLC system and quantified using a Lablogic β-RAM Model 3 continuous flow liquid scintillation detector (Lablogic Systems Ltd, Sheffield, UK). HPLC separation was performed using a 250 mm x 4.6 mm Supelcosil LC-SCX 5 cation-exchange column (Sigma-Aldrich, UK). Running conditions for elution at a rate of 1 ml per minute were as follows:

- Buffer A: distilled water
- Buffer B: 200mM sodium citrate pH 3.0
- 100% buffer A for 5 minutes
- linear gradient from 100% buffer A to 100% buffer B over 10 minutes
- then 100% buffer B for 15 minutes

The column was regenerated after each sample for 3 minutes using 100% buffer A. Scintillant fluid (Lablogic) was mixed in line at a ratio of 0.5:1 after elution from the column, before passage through the detector. To determine elution times for ¹⁴C-L-arginine, ¹⁴C-L-Citrulline and ¹⁴C-L-ornithine ¹⁴C-labelled standards (all from Amersham Biosciences UK Ltd.) were run for each experiment. Results were expressed as proportion of ¹⁴C-L-arginin converted into ¹⁴C-L-citrullin. Because the ¹⁴C-L-arginin used contained a small amount of contamination with ¹⁴C-L-

citrulline (~0.2%) background levels arginine “conversion” were deducted from the samples. Figure 3.7A shows a representative chromatogram for arginine to citrulline conversion in primary endothelial cells and non-endothelial cells.

2.9 β -Galactosidase activity assay

The High Sensitivity β -Galactosidase Assay Kit from Stratagene, USA, was used to measure β -galactosidase activity in primary endothelial cells and lung tissue.

This kit utilizes the conversion of the yellow-orange substrate chlorophenol red- β -D-galactopyranoside (CPRG) into galactose and the chromophore chlorophenol red. The absorbance maximum of chlorophenol red is 570 nm and the amount chlorophenol red can be determined using a microplate reader at 570-595 nm. Depending on the level of β -galactosidase activity in the sample, incubation times from 30 minutes to 72 hours can be used which gives the assay a broad dynamic range.

Cell pellets were lysed in 50 μ l lysis buffer followed by freeze-thawing, lungs were homogenized in 800 μ l lysis buffer and samples were centrifuged at 12,000 g and 4°C for 5 minutes. Duplicates of 20 μ l lysate per sample was transferred into a 96 well plate and 130 μ l 1X CPRG substrate was added to each sample. The plates were incubated at 37°C for 30 - 40 minutes until the samples had turned red. The reaction was then terminated by the addition of 80 μ l stop solution and the plate was read at 590 nm. Concentration of chlorophenol red formed and β -galactosidase activity [chlorophenol red formed/minute] was calculated via the optical density. The results were expressed as total β -galactosidase activity per endothelial cell

sample or whole lung respectively or as specific β -galactosidase activity [β -galactosidase activity/mg protein].

2.10 X-Gal staining

X-Gal staining was performed on endothelial cell cultures for *in situ* visualization and quantification of cells expressing the LacZ transgene. β -galactosidase catalyzes the hydrolysis of X-Gal (5-bromo-3-indoyl- β -D- galactopyranoside) to galactose and 5-bromo-4-chloro-3-hydroxyindole which is then oxidized into 5,5'-dibromo-4,4'-dichloro-indigo, forming an insoluble blue precipitate. Because β -galactosidase activity of cells from Tie-2 LacZ mice is mainly localized in the nucleus, the blue staining has the maximum intensity there.

Cells were fixed with 2% formalin and 0.2% glutaraldehyde in PBS for 10 minutes at room temperature. After rinsing the cells with PBS, 37°C warm staining solution (see Table 2.3) was added and the cells were incubated at 37°C for 16-24 hours. The staining solution was washed off and cells were imaged using a wide-field inverted Nikon TE200U fluorescence microscope with colour camera.

	Staining solution
Tris buffered Magnesium Saline (1 mM MgCl ₂ , 15 mM NaCl, 50 mM Tris pH 8.0)	10 ml
250 mM Potassium ferrocyanide K ₄ [Fe(CN) ₆]	200 μ l
250 mM Potassium ferricyanide K ₃ [Fe(CN) ₆]	200 μ l
X-Gal (50 mg/ml in dimethylformamide)	200 μ l

Table 2.3: Composition of X-Gal staining solution.

2.11 Eosin staining

Flow sorted cells were cytopun onto poly-L-lysine slides at low speed for 2 minutes, dried and stained with X-Gal (Chapter 2.10). After thoroughly washing the slides with PBS the slides were rinsed with 70% ethanol and stained with eosin solution (125ml 1% aqueous eosin, 375ml 80% ethanol, 2.5ml acetic acid) for 10 seconds before rinsing in 70% ethanol for 3 minutes twice, in 95% ethanol for 3 minutes, in 100% ethanol for 3 minutes and finally rinsing in Histo-Clear® (National Diagnostics) for 5 minutes before mounting in Neo-Mount® (Merck).

2.12 eNOS immunostaining

Endothelial cells and non-endothelial cells, which had been grown on coverslips, were fixed with 4% paraformaldehyde (PFA) in PBS for 30 minutes at room temperature. The cells were washed thoroughly with PBS, permeabilized with PBS containing 0.5% Triton X-100 for 30 minutes at room temperature and blocked with blocking buffer (PBS containing 0.1% Triton X-100 and 10% goat serum) for 30 minutes at room temperature. Incubation with mouse monoclonal eNOS antibody (1:400, BD Transduction Laboratories) took place over night at 4°C in blocking buffer. The cells were then rinsed with blocking buffer and incubated with biotinylated goat anti-mouse secondary antibody (1: 200, Vector Laboratories) in blocking buffer for 1 hour at room temperature. After rinsing the cells with PBS containing 0.1% Triton X-100, cells were incubated with Fluorescein Avidin D (1:200, Vector Laboratories) for 1 hour at room temperature. The coverslips were then rinsed with PBS containing 0.1% Triton X-100 and mounted using VECTASHIELD® containing DAPI (Vector Laboratories). Cells were imaged

using a wide-field inverted Nikon TE200U fluorescence microscope with B&W fluorescence camera.

2.13 Flow cytometry

Flow Cytometry was used for the identification and further analysis of cell populations in various different applications in this thesis. The principle of flow cytometry is to create a thin stream of fluid so that the suspended cells can pass one or more laser beams individually. Light scattering and fluorescence emission provides information about the cells properties such as approximate cell size and complexity/granularity. Fluorescent chemical compounds or fluorescent-labeled antibodies can be used to simultaneously analyze multiple characteristics of hundreds to thousands of particles per second.

Data was acquired using a CyAn™ ADP flow cytometer (Beckman Coulter) and Summit™ software and analyzed using FlowJo software. Flow sorting was performed using a MoFlo™ cell sorter (Beckman Coulter). The principles of the procedures used for flow cytometry and flow sorting of cells are outlined in the following paragraph and described in more detail in the subsequent chapters. Table 2.4 provides an overview over antibodies and isotype controls used.

In general, up to 5×10^5 cells were incubated in 100µl FACS wash (0.2% BSA in PBS) or MACS buffer containing a 1/100 dilution of Purified Rat Anti-Mouse CD16/CD32 (Fc-block) for 15 minutes on ice. 100µl FACS wash containing double the working concentration of fluorochrome conjugated antibody was added to the samples, the tubes were briefly vortexed and incubated for a further 15 minutes on

ice in the dark. After the antibody incubation the tubes were topped up with FACS wash and centrifuged at 250 g and 4°C for 5 minutes. The supernatant was poured off and the cells were fixed with 200µl of 2% formaldehyde (methanol-free, Thermo Scientific, USA) in PBS. The samples were kept in the dark at 4°C until they were analyzed by flow cytometry.

Antibody	clone	Working concentration	Company	Product code
FITC Rat Anti-Mouse CD31	MEC13.3	1/200	BD Pharmingen	553372
APC Rat Anti-Mouse CD31	MEC13.3	1/200	BD Pharmingen	551262
Alexa Fluor® 488 Rat Anti-Mouse CD102	3C4 (MIC2/4)	1/500	BioLegend	105609
PE Rat anti-Mouse CD34	RAM34	1/100	BD Pharmingen	551387
APC Rat anti-Mouse Flk-1	Avas 12alpha1	1/100	BD Pharmingen	560070
PE Rat Anti-Mouse Sca-1	E13-161.7	1/100	BD Pharmingen	553336
PE-Cy7 Rat Anti-Mouse CD45	30-F11	1/200	BD Pharmingen	552848
FITC anti-BrdU (part of FITC BrdU Flow Kit)		1/50	BD Pharmingen	559619
Isotype controls		clone	Company	Product code
FITC Rat IgG2a, κ Isotype Control		R35-95	BD Pharmingen	553929
PE Rat IgG2a κ Isotype Control		R35-95	BD Pharmingen	553930
APC Rat IgG2a κ Isotype Control		R35-95	BD Pharmingen	553932
PE-Cy7 Rat IgG2b κ Isotype Control		A95-1	BD Pharmingen	552849
Alexa Fluor® 488 Rat IgG2a K Isotype Control		RTK2758	eBioscience	53-4321-80
Fc-Blocking		clone	Company	Product code
Purified Rat Anti-Mouse CD16/CD32		2.4G2	BD Pharmingen	553142

Table 2.4: Flow cytometry antibodies and isotype controls. Antibodies were used in the stated working concentrations. Isotype controls were used in the same concentration as the corresponding antibody.

2.14 BrdU incorporation assay

Endothelial cells were isolated from mouse lungs by CD31 MACS® MicroBead isolation and cultured in gelatine coated wells of a 12 well dish for five days in 1 ml EC media. On day four 10µl BrdU (1mM) was added to each ml of culture media obtaining a final concentration of 10µM. The cells were harvested 16 hours later using Accutase™, passed through a 70µm cell strainer and washed with MACS buffer. Flow cytometry staining was performed in two steps: staining for CD31 and CD45 followed by cell permeabilization and staining for BrdU using the FITC BrdU Flow Kit (BD Pharmingen™). The cells were resuspended in 100µl MACS buffer with 1/100 Fc-block and incubated for 15 minutes on ice. 100µl MACS buffer containing APC Rat Anti-Mouse CD31 and PE-Cy7 Rat Anti-Mouse CD45 (final concentration 1/200) was added, the cells were incubated for 15 minutes on ice in the dark and washed with MACS buffer. For fixing and permeabilization the cells were resuspended in 100µl of BD Cytofix/Cytoperm buffer and incubated for 15 minutes on ice before washing with BD Perm/Wash buffer. The cells were then incubated with 100µl of BD Cytoperm Plus buffer, incubated for 10 minutes on ice, washed with BD Perm/Wash buffer and re-fixed with 100µl of BD Cytofix/Cytoperm buffer for 5 minutes on ice. After washing with BD Perm/Wash buffer the cells were incubated with 100µl DNase (300 µg/ml in PBS) for 1 hour at 37°C to expose incorporated BrdU. The cells were washed with BD Perm/Wash buffer and incubated with 50µl BD Perm/Wash buffer containing FITC anti-BrdU antibody (1/50) for 20 minutes at room temperature in the dark. After a final wash with BD Perm/Wash buffer the cells were resuspended in 200µl MACS buffer and stored at 4°C in the dark prior to flow cytometry.

2.15 *In vivo* Matrigel™ plug assay

In vivo angiogenesis was investigated using the Matrigel™ plug assay. BD Matrigel™ Basement Membrane Matrix is a gelatinous protein mixture extracted from the Engelbreth-Holm-Swarm mouse sarcoma and serves as a substrate for *in vitro* endothelial cell invasion and tube formation assays. When mixed with angiogenic factors such as FGF or VEGF and injected subcutaneously *in vivo*, endothelial cells amongst other cells migrate into the Matrigel™ plug and form vessels. Matrigel™ is liquid at 4°C and quickly forms a gel at 37°C.

After clipping the hair and disinfecting the injection site with 70% ethanol, 0.5 ml sterile ice-cold Matrigel™ (Phenol Red-Free, BD Biosciences, UK) containing 0.5 µg/ml bFGF (PeproTech, USA) was injected subcutaneously into the left flank of ApoE-KO and GCH/ApoE-KO mice under general anesthesia with isoflurane. After 6 days the animals were euthanized and the matrigel plugs removed.

For flow cytometry analysis the plugs were digested with 3U/ml Dispase II (Roche), 2.8 U/ml Liberase Blendzyme 3 (Roche), 38U/ml hyaluronidase (Sigma-Aldrich, UK) and 100U/ml DNase (Sigma-Aldrich, UK) in PBS. The liberated cells were strained through a 40µm strainer, washed with PBS containing 0.2% BSA and stained with CD31 APC and CD45 PE-Cy7 or the corresponding isotype controls.

2.16 Isolation of bone marrow and bone marrow mononuclear cells from mice

Animals were sacrificed by overdose of inhaled isoflurane and cervical dislocation. Following death, skin and muscle were dissected from thigh and calf and the leg was collected into PBS. After cleaning femur and tibia from remaining muscle

tissue using a scalpel, the epiphyses were cut off and bone marrow (BM) was flushed out into a petri dish using a FACS wash filled syringe with G25 needle. BM was then passed through a 70µm strainer to remove bone fragments and cell clumps, centrifuged at 250 g and 4°C for 5 minutes and resuspended in FACS wash.

To isolate bone marrow mononuclear cells (BMMNC), 1 ml of BM cell suspension was carefully layered over 3 ml of LSM 1077 (PAA, Austria) and centrifuged at room temperature for 10 minutes at 800 g. BMMNC were collected from the interphase and washed with FACS wash.

2.16.1 Lineage depletion of bone marrow mononuclear cells

Lineage negative cells were enriched from BMMNC using a lineage cell depletion kit (Miltenyi Biotec, Germany). BMMNC (maximum of 10^7 cells) from one mouse leg were washed with cold MACS buffer, centrifuged at 300 g and 4°C for 5 minutes and resuspended in 40µl MACS buffer. 10µl of Biotin-Antibody Cocktail was added to the sample, vortexed briefly and incubated on ice for 10 minutes. 30µl of MACS buffer plus 20µl of Anti-Biotin MicroBeads were added to the mix, vortexed briefly and incubated for an additional 15 minutes on ice. The cells were then washed by the addition of 1 ml MACS buffer and centrifuging at 300 g and 4°C for 5 minutes. The cell pellet was resuspended in 500µl MACS buffer and applied onto a MACS® MS Cell Separation Column in a magnetic field. The cells were allowed to pass through the column and the effluent (unlabeled cells, lineage negative) was collected. The column was washed 3 times with 500µl MACS buffer each and the effluent was collected in the same tube. MicroBead labeled, lineage positive cells were retained in the column. The collected lineage depleted cells were

centrifuged at 300 g and 4°C for 5 minutes and either washed with PBS and frozen for biopterin measurement or labeled with antibodies for flow cytometry analysis.

2.16.2 Flow cytometry staining of lineage negative enriched BMMNC

Lineage depleted BMMNC were resuspended in 100µl staining buffer (PBS containing 0.5% BSA, 2mM EDTA and 10% horse serum) with 1/100 Fc-block and incubated for 30 minutes on ice. 100µl staining buffer containing APC Rat anti-Mouse Flk-1 and PE Rat Anti-Mouse Sca-1 antibodies or isotype controls (see Table 2.4) (final concentration 1/100) was added and, after a brief vortex, the cells were incubated for 15 minutes on ice in the dark, washed with MACS buffer and fixed in 300µl 2% formaldehyde in PBS.

2.17 Statistics

Data were analyzed using Microsoft Excel (Microsoft, USA) and GraphPad Prism (USA) and expressed as the mean $\pm$ standard error of the mean (SEM).

Between group comparisons of normally distributed measurements were made using a two-tailed Student's t-test or, where the data was not normally distributed by applying a Mann-Whitney test. To test for outliers the Grubb's test (extreme studentized deviate method) was used. Power calculations were performed with PS Power and Sample Size Calculation Software. P values of less than 0.05 were regarded as statistically significant.



Chapter 3:

Development and Evaluation of Primary Mouse Endothelial Cell Culture



3 Development and Evaluation of Primary Mouse Endothelial Cell Culture

3.1 Introduction

Primary endothelial cells provide a useful *in vitro* model in cardiovascular research and offer many advantages over immortalized endothelial cell lines. They resemble the physiological properties of endothelial cells *in vivo* more closely than immortalized cell lines, which may have non-physiological characteristics, particularly concerning cell proliferation and apoptosis. At present, the cell types most routinely used in studies investigating endothelial cell biology and also commercially available are human umbilical vein endothelial cells (HUVEC) or endothelial cells from large conductance vessels like bovine aortic endothelial cells (BAEC). An advantage of using primary endothelial cells from mice instead of HUVEC, BAEC or immortalized cell lines is the availability of murine models for vascular disease with endothelial targeted genetic alterations, abolishing the need for transfections *in vitro*.

Murine endothelial cells have been isolated from various organs including heart (Li *et al.*, 2001a; Marelli-Berg *et al.*, 2000), aorta (Kevil and Bullard, 2001; Kobayashi *et al.*, 2005), vena cava inferior (Bucciarelli *et al.*, 2009), kidney (Rops *et al.*, 2004), retina (Matsubara *et al.*, 2000; Su *et al.*, 2003), brain (Weidenfeller *et al.*, 2005) and lung (Dong *et al.*, 1997; Fehrenbach *et al.*, 2009; Fleming *et al.*, 2005; Khan *et al.*, 2004; Kuhlencordt *et al.*, 2004; Lim *et al.*, 2003; Marelli-Berg *et al.*,

2000; Schniedermann *et al.*, 2010). Lungs are rich in microvascular endothelial cells and have been used for primary endothelial isolation from mice in a variety of studies. Table 3.1 provides an overview over some of the published protocols for primary endothelial cell isolation from mouse lungs.

Most protocols for the isolation of primary endothelial cells involve treatment of vessels or minced organs with digestive enzymes. The methods used to separate endothelial cells from other cells vary from gradient centrifugation (Li *et al.*, 2001a) to Fluorescence Activated Cell Sorting (FACS) (Fehrenbach *et al.*, 2009; Kevil and Bullard, 2001) or, most commonly used, immunomagnetic bead selection (Dong *et al.*, 1997; Fleming *et al.*, 2005; Khan *et al.*, 2004; Kuhlencordt *et al.*, 2004; Lim *et al.*, 2003; Marelli-Berg *et al.*, 2000). For gradient centrifugation to effectively isolate endothelial cells from a cell suspension, the other cells have to significantly differ from endothelial cells in their physical density (e.g. lipocytes or cardiomyocytes). However, there are too many cell types of similar density present in the lung for gradient centrifugation to be sufficient. A variety of cell surface markers are used for endothelial cell identification during flow sorting or immunomagnetic bead selection. CD31 (PECAM-1) and CD102 (ICAM-2) are the markers most commonly used for endothelial cell isolation from mouse lungs. For this thesis CD31-immunomagnetic bead selection was chosen as the primary method for endothelial cell isolation from mouse lungs, allowing higher throughput and being more cost-efficient than flow sorting.

Publication	Isolation method	EC marker (antibody clone)	Lung digestion method	Number of selections	Age of mice	Culture information
Dong <i>et al.</i> , 1997	Dynabeads®	CD31 (MEC 13.3)	Collagenase A (1mg/ml)	2 magnetic bead selections	Adult (18-20g females)	2 nd selection after 10 days in culture, several passages.
Marelli-Berg <i>et al.</i> , 2000	MACS® MicroBeads	CD31 (MEC 13.3) CD105 (MJ7/18) Islectin B4	Collagenase (0.5 mg/ml)	1 magnetic bead selection	6-8 weeks	Confluent after 10-14 days, several passages
Lim <i>et al.</i> , 2003	Dynabeads®	CD31 (MEC 13.3) CD102 (3C4)	Collagenase Type I (180-200U/ml)	2 magnetic bead selections (CD31 followed by CD102)	8-10 weeks	Cultured for up to 3 passages.
Kuhlencordt <i>et al.</i> , 2004	Dynabeads®	CD102 (3C4)	Collagenase A (0.1%)	2 magnetic bead selections	3-4 months	First bead selection after 2-4 days, on average 10 days in culture.
Khan <i>et al.</i> , 2004	MACS® MicroBeads	CD102	Collagenase A (400 U/ml)	1 magnetic bead selection	5-7 days	5-6 days in culture.
Fleming <i>et al.</i> , 2005	Dynabeads®	CD144	Dispase (5U/ml)	3 magnetic bead selections	6 weeks	3 selections and plus 1 additional passage in total.
Fehrenbach <i>et al.</i> , 2009	Flow sorting	CD102 (3C4)	Collagenase A (1mg/ml)	1 flow sort	7-14 days	Flow sorting after 2-3 days in culture, grown for up to 10 passages.
Schniedermann <i>et al.</i> , 2010	Dynabeads® and flow sorting	CD31 (MEC 13.3)	Collagenase A	1 magnetic bead selection followed by 1 flow sort	NA	Flow sorting after 8-10 days in culture followed by several passages.

Table 3.1: Comparison of published protocols for primary endothelial cell isolation from mouse lungs. Most protocols use traditional Collagenase for tissue digestion and immunomagnetic bead selection with CD31 or CD102 for endothelial cell isolation. However, the protocols vary widely regarding the total number of selection procedures and time spent in culture before the cells are included into experiments.

Although several published studies including those cited above have used primary mouse endothelial cells, these cells are notoriously troublesome to employ in experiments. Common problems include poor reproducibility and inconsistency due to low cell viability, cell senescence, slow cell proliferation and contamination with non-endothelial cells. Even an initially low percentage of contaminating fibroblasts can overgrow endothelial cell populations due to a shorter generation time. The very limited number of cells which can be isolated from each mouse and potential loss of endothelial cell phenotype and biological function over time also represent potential limitations for the use of primary endothelial cells as a model.

Using CD31 immunomagnetic bead selection for the isolation of primary endothelial cells from mouse lungs, the specific experimental aims of this chapter are:

- To determine the optimal isolation and culture conditions to reproducibly obtain viable and pure endothelial cells.
- To systematically characterize cultured primary endothelial cells with regard to morphology, expression of endothelial cell markers and endothelial targeted GCH, LacZ and eNOS transgenes and eNOS activity.
- To evaluate the influence of the selection procedure and time spent in culture on expression of endothelial cell markers and the endothelial targeted GCH transgene.
- To characterize subpopulations of CD31 immunomagnetic isolated cells with regard to expression of endothelial cell markers, morphology, proliferation in culture, expression of endothelial targeted GCH and LacZ transgenes and endothelial progenitor markers.

3.2 Optimization of isolation and culture conditions

The conditions for lung tissue digestion and magnetic bead isolation were optimized to ensure good reproducibility of the procedure and maximize cell yield while minimizing the impact of the isolation procedure on cell viability. This chapter describes the rationale behind finding optimal conditions; tissue digestion and magnetic bead isolation procedures are described in detail in Chapters 2.3.2.1 and 2.3.2.2 and the reselection procedure is described in further detail in Chapter 2.3.2.3.

3.2.1 Tissue digestion

Most published protocols for the isolation of primary endothelial cells from lungs use Collagenase A or Collagenase Type I for tissue digestion. Traditional collagenase preparations from *clostridium histolyticum* culture supernatants can vary significantly in enzymatic activity from batch to batch and bear a high risk of contamination with endotoxin. In this thesis Liberase Blendzymes (Roche) were chosen for tissue digestion in place of traditional collagenase in order to maximize reproducibility and minimize the risk of contaminating the cultures, particularly with endotoxin. Liberase Blendzymes are blends of purified collagenase isoforms I and II and the neutral protease dispase (Blendzyme 1) or thermolysin (Blendzyme 2, 3 and 4) with Blendzyme 1 containing the lowest and Blendzyme 4 the highest neutral protease activity. When choosing the digestive enzyme concentration two factors have to be considered: ensuring a thorough digestion for maximum cell yield and avoiding over-digesting and damaging cell functionality. Liberase Blendzyme 3 at the medium concentration of 0.18U/ml was found to produce the best results and increasing the Blendzyme 3 concentration to 0.28U/ml, the top of

the recommended concentration range, did not further improve tissue digestion in our protocol whereas lower concentrations led to incomplete digestion. Liberase Blendzyme 3 was used for all lung digestion in this thesis but Roche has since replaced their Liberase Blendzymes by the second generation of Liberase purified Enzyme Blends in order to increase reproducibility even further.

The addition of DNase (0.1 mg/ml DNase I, Roche) proved to be essential to prevent cell clumping due to DNA released from cells damaged during tissue processing.

60 minutes enzymatic digestion at 37°C with gentle agitation was found to sufficiently degrade minced lung tissue. Reducing digestion time to 45 minutes led to incomplete digestion and increasing digestion time to 75 minutes did not improve digestion any further.

3.2.2 CD31 immunomagnetic bead selection

CD31 Immunomagnetic bead selection was used to separate endothelial cells from non-endothelial cells after lung digest. Figure 2.1 illustrates the principles of this procedure.

Dynabeads® Sheep anti-Rat IgG (Invitrogen) were used in the earlier experiments of this thesis because they allow fast and cost efficient isolation of primary endothelial cells. However, due to strong auto fluorescence Dynabeads® interfere with flow cytometry, particularly when only part of the endothelial cell population has Dynabeads® bound to the membrane. MACS® Goat anti-Rat IgG MicroBeads

(Miltenyi Biotech) are flow cytometry compatible and were used in the later experiments of this thesis.

To ensure isolation of the same population of cells out of the lung digest, the same rat anti-mouse CD31 monoclonal antibody (MEC 13.3, BD Pharmingen) was used with both types of magnetic beads.

3.2.2.1 Dynabeads® Sheep anti-Rat IgG

Dynabeads® Sheep-anti Rat IgG are uniform, superparamagnetic polystyrene beads (4.5µm diameter) coated with polyclonal sheep anti-rat IgG antibodies and bind monoclonal rat IgGs. For primary endothelial isolation they were coated with rat anti-mouse CD31 monoclonal antibody.

When incubating lung cells with CD31 conjugated Dynabeads®, the magnetic beads bind to CD31 positive cells. CD31 positive and CD31 negative cells are separated from each other by placing the sample in a magnetic field. For a positive cell selection it is crucial not to overload the cells with Dynabeads®, with the aim of binding no more than 4 Dynabeads® to each endothelial cell. Not all beads will bind to cells, therefore greater than four times the estimated number of target cells are required. Varying quantities of Dynabeads® per sample were tested, starting with 5×10^6 beads (12.5µl) and reducing in units of 2.5µl down to 2.5µl. The optimal quantity of Dynabeads® was found to be 1×10^6 beads (2.5µl) per sample, as this quantity neither limits the availability of beads nor overloads the cells. Figure 3.1 illustrates the effects of Dynabead® overloading and phagocytosis.

Figure 3.1

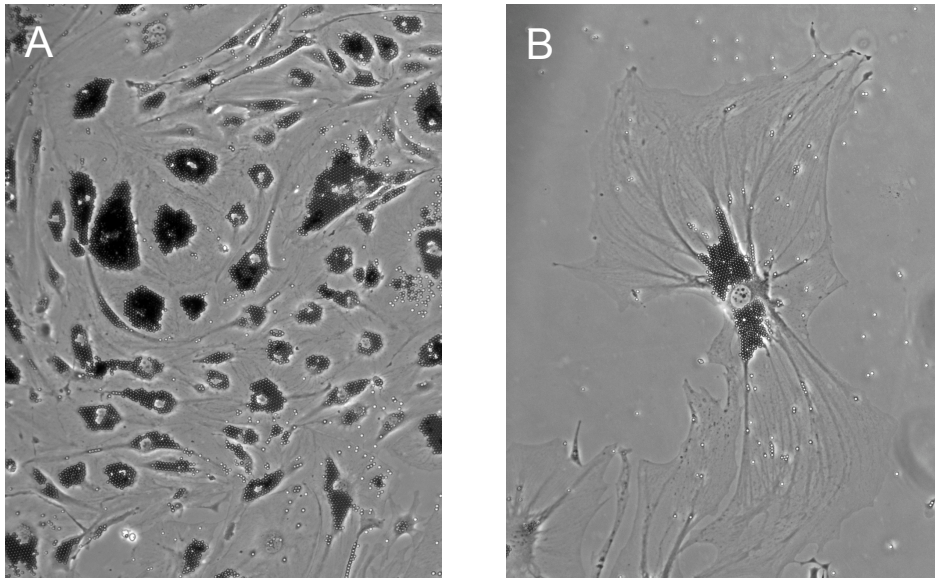


Figure 3.1: Determining the optimal quantity of Dynabeads® used for isolation and reselection of EC.

After 2 Dynabead® selections with 5×10^6 and 2×10^6 beads respectively parts of the CD31 positive culture are overloaded with beads (**A**) and some CD31 positive cells have phagocytized large amounts of Dynabeads® (**B**). Because of the weight of Dynabeads®, overloaded cells tend to settle in the centre of the well (**A**). The pictures above were taken during the set up phase for finding the correct quantity of Dynabeads®. Determining the correct quantity of Dynabeads® is crucial to ensure efficient EC isolation without overloading the cells with beads. Bead binding for reselection of the displayed cells took place after cell detachment. In later experiments this step was replaced by a Dynabeads® incubation directly in the well to further reduce the risk of coating the entire cell surface with beads.

Binding too many Dynabeads® to endothelial cells, as demonstrated in Figure 3.1 A, reduces cell viability and the ability of cells to attach to the dish after bead selection. Endothelial cells are also known to phagocytize Dynabeads® (Conrad-Lapostolle *et al.*, 1996; Jackson *et al.*, 1990; Ryan, 1986); Figure 3.1 B shows a senescent cell after phagocytosis of a large number of Dynabeads®. To minimize phagocytosis of Dynabeads® the bead incubation times were kept as short as possible (20-30 minutes) and the first bead selection took place at 4°C. For reselection incubating the cells with CD31 conjugated Dynabeads® directly in the well before detaching the cells with Accutase™ produced better results than detaching the cells and then incubating with beads. Binding Dynabeads® to cells directly in the well, means beads can only bind to the exposed “luminal” surface of the cell and also offers the opportunity to directly observe the bead binding process under a microscope.

Some protocols include a Trypsin EDTA incubation step after Dynabead® binding to release Dynabeads® from the cell surface (Dong *et al.*, 1997). However, in these experiments Trypsin EDTA did not effectively shed Dynabeads® from the cell surface but reduced cell viability. The observation that Trypsin EDTA might not be suitable for removal of Dynabeads® from the cell surface is supported by the fact that other protocols first bind Dynabeads® to endothelial cells and then detach the cells for magnetic separation using Trypsin EDTA (Kuhlencordt *et al.*, 2004).

3.2.2.2 MACS® Goat anti-Rat IgG MicroBeads

MACS® Goat anti-Rat IgG MicroBeads are very small (50 nanometers diameter) superparamagnetic particles conjugated to goat anti-rat IgG antibodies. They are composed of a biodegradable matrix and there is no need to remove them after the

separation process. For primary endothelial cell isolation, the cells are labeled with rat anti-mouse CD31 monoclonal antibody first. Subsequently the cells are labeled with Goat anti-Rat IgG MicroBeads and the cell suspension is loaded onto a column which is placed in a magnetic field. Magnetically labeled cells (CD31 positive) are retained within the column and unlabeled cells (CD31 negative) run through. This method is very sensitive and only minimal labeling with MACS® MicroBeads is needed.

Advantages of using MACS® MicroBeads instead of Dynabeads® are flow cytometry compatibility, reduced risk of cell senescence due to bead overloading and reduced contamination with fibroblasts after only one selection. Disadvantages of MACS® MicroBeads compared to Dynabeads® are the considerably higher cost per sample and the slightly more time-consuming isolation procedure. Table 3.2 provides an overview over the properties of MACS® Goat anti-Rat IgG MicroBeads in comparison to Dynabeads® Sheep-anti Rat IgG.

	MACS® Goat anti-Rat IgG MicroBeads		Dynabeads® Sheep-anti Rat IgG	
Size (diameter)	50 nm	↑	4.5 µm	↓
Biodegradable	yes	↑	no	↓
Flow cytometry compatible	yes	↑↑	no*	↓↓
Cost per sample	high	↓↓	low	↑↑
Cell separation speed	+ 1 hour	↓	fast	↑

Table 3.2: Comparison of MACS® Goat anti-Rat IgG MicroBeads and Dynabeads® Sheep-anti Rat IgG. Relative advantages are indicated by “↑” and relative disadvantages are indicated by “↓”. *Dynabeads® are very autofluorescent but cells labeled with Dynabeads® can be analyzed by flow cytometry under specific conditions (Chapter 5.2.3.2).

3.2.3 Choosing culture ware surface area

In relation to cells Dynabeads® are heavy and quickly settle at the bottom of the tube or culture dish. This leads to accumulation of Dynabead® bound cells in the centre of the well. Because this effect gets more pronounced with smaller surface areas of the culture wells, lung endothelial cells from 1 mouse had to be seeded into 1 well of a 6 well dish (9.6 cm²) or 1 T25 flask (25 cm²) for the endothelial cells from 2 mice respectively. However, the surface area of 1 well of a 6 well dish is large in relation to the amount of endothelial cells harvested later. This can produce technical problems when having to incubate living cells in the well for certain techniques such as arginine to citrulline conversion, diluting the assay product too much.

In contrast to Dynabeads®, MACS® MicroBeads are very small and light and do not affect the way cells settle in the culture dish. Seeding CD31 positive cells isolated from the lungs of 1 mouse into 1 well of a 12 well dish yielded the most reproducible results.

3.2.4 Cell detachment

Accutase™ was used in place of traditional Trypsin EDTA to detach primary endothelial cells for reselection or cell harvest. Primary endothelial cells proved to attach strongly to gelatine coated dishes and using Trypsin EDTA resulted in poor cell detachment and reduced viability due to long incubation times. Accutase™ was more successful in detaching endothelial cells and had little influence on cell viability. After detachment with Accutase™ to passage endothelial and non-

endothelial cells, the cells very quickly re-attached to the gelatine coated dishes and only very few floating cells could be observed.

3.2.5 Purification of primary lung endothelial cells

A minimum of two CD31 Dynabead® selections were necessary to obtain pure primary lung endothelial cell cultures without contaminating areas of fibroblast growth. Because endothelial cells have a longer generation time than fibroblasts, even a small amount of residual fibroblasts after the first or second magnetic bead selection can lead to significant fibroblast contamination after only a short growth period. Three CD31 Dynabead® selections proved to consistently produce primary lung endothelial cell cultures without significant contamination with fibroblasts as assessed by cell morphology, CD31 Dynabead® binding ability and eNOS immunofluorescence. Figure 3.2 illustrates the process of isolating and reselecting primary endothelial cells to obtain pure cultures. After approximately 16 days in culture, it was possible to obtain 2 wells of a 6 well dish of endothelial cells from the lungs of 1 mouse and 4 wells of a 6 well dish from 2 mice respectively. After isolating endothelial cells with CD31-conjugated Dynabeads® as described above, the cells were plated into gelatine coated wells containing EC medium. After 4 to 5 days the cells were incubated with CD31-conjugated Dynabeads®, detached using Accutase™ and bead-bound and non-bead-bound cells were separated using a magnet. After a further 4 days in culture the cell were bead selected for the third time. 4 days later the cells were passaged 1 in 2 and were ready to be used for experiments after approximately 16 days in culture. Table 3.3 gives detailed information on culture surface area and quantity of Dynabeads® used depending on the number of lungs used per endothelial cell isolate.

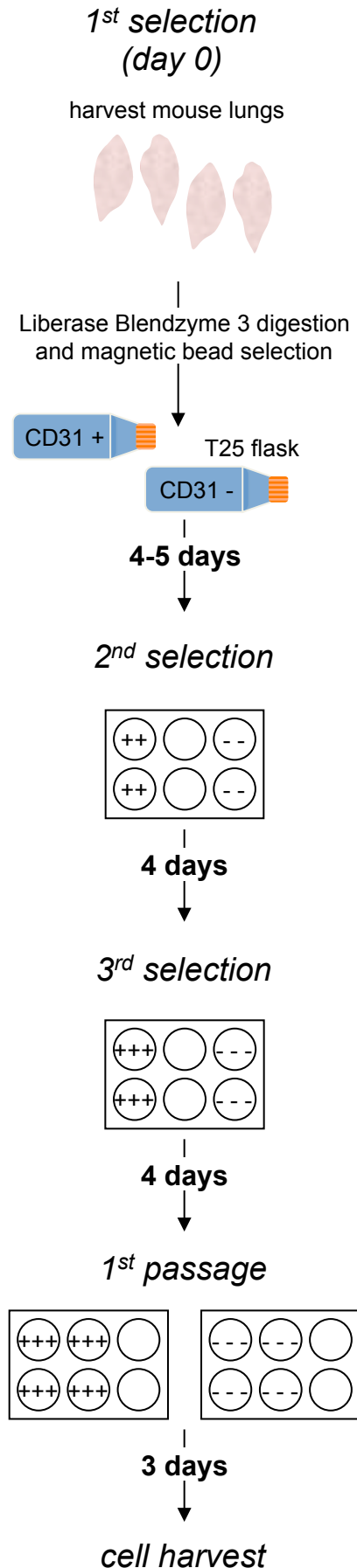


Figure 3.2: Isolation and culture of primary endothelial cells from mouse lungs.

Lungs from 2 mice are harvested, finely minced and digested in DMEM containing 0.18U/ml Liberase Blendzyme 3 (Roche) and 0.1 mg/ml DNase I (Roche) for 60 minutes at 37°C. The digested tissue is filtered through a 100 µm cell strainer and centrifuged at 200 g for 8 minutes. The cell pellet is washed with PBS/10% FCS, centrifuged, resuspended in 1 ml PBS/10%FCS and incubated with 1×10^6 CD31-conjugated Dynabeads® per lung at 4°C for 30 minutes. Bead-bound cells are separated from non-bead-bound cells using a magnet. Bead-bound (CD31 positive) and non-bead-bound cells are resuspended in 2 ml EC medium (EGM-2-MV supplemented with hEGF, hydrocortisone, VEGF, hFGF-B, R3-IGF-1, ascorbic acid, gentamicin, amphotericin-B and 5% FCS) and plated in gelatine coated T25 flasks.

For reselection after 4-5 days the EC medium is replaced with 2 ml DMEM containing 3×10^6 CD31-conjugated Dynabeads® and the cells are incubated for 30 minutes at 37°C. Excess beads are washed off with PBS and cells are detached using Accutase™. Bead-bound and non-bead-bound cells are separated using a magnet and plated into 2 wells of a gelatine coated 6 well dish.

After a further 4 days in culture the cell are bead selected for the third time using 1.5×10^6 CD31-conjugated Dynabeads® per well. After magnetic separation the cells are plated back into 2 well of a fresh gelatine coated dish.

A 1:2 passage follows after a further 4 days in culture.

3 rounds of immunoselection proved to produce the most reliable results regarding purity of endothelial cells. After approximately 16 days in cell culture 4 wells of a 6 well dish of endothelial cells from 2 mice are ready to be harvested.

		Lungs from 1 mouse	Lungs from 2 mice
1st selection	Dynabeads®	1×10^6	2×10^6
	Initial culture area	1 well of 6 well dish	T25 flask
2nd selection	Dynabeads®	1×10^6 (per well)	3×10^6 (per flask)
	New culture area	1 well of 6 well dish	2 wells of 6 well dish
3rd selection	Dynabeads®	1.5×10^6 (per well)	1.5×10^6 (per well)
	New culture area	1 well of 6 well dish	2 wells of 6 well dish

Table 3.3: Quantity of CD31-conjugated Dynabeads® and culture surface area used for each step of isolating and purifying primary endothelial cells from mouse lungs.

The growth surface area of 1 well of a 6 well dish is 9.6 cm^2 and that of a T25 flask is 25 cm^2 . For the first selection, CD31-conjugated Dynabeads® were bound to cells in suspension. For the second and third selection, bead binding took place directly in the well or flask before cell detachment.

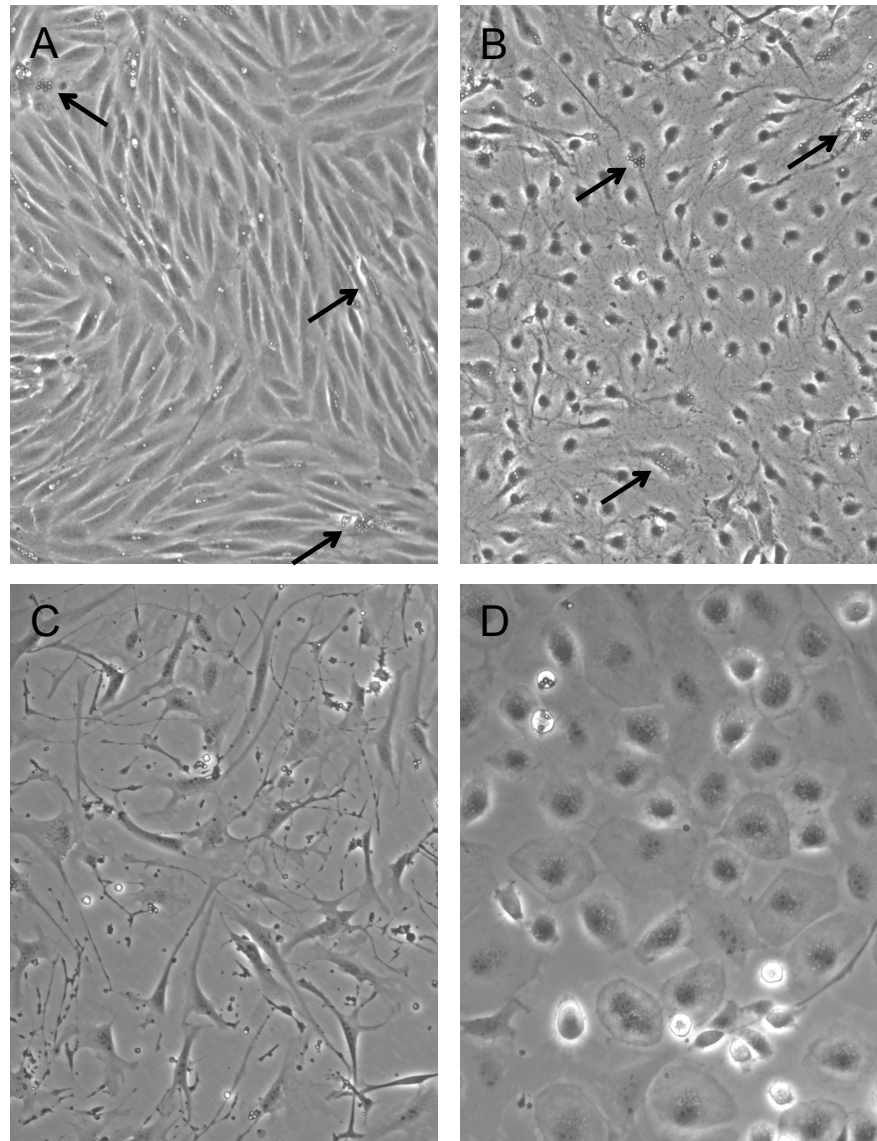
3.3 Characterization of primary endothelial cells from mouse lungs

To confirm selectivity of the magnetic bead selection and endothelial identity of the isolated cells, CD31 positive and CD31 negative selected cells were characterized with regard to expression of endothelial cell markers, NOS activity and expression of endothelial specific transgenes.

3.3.1 Morphology of CD31 positive and CD31 negative isolated cells

CD31 positive selected cells (endothelial cells) proliferated in tissue culture forming several individual islands of cells that later merged into a strictly contact inhibited cell monolayer. Two main morphologies of endothelial cells could be distinguished: “streamlined” and “round” (Figure 3.3 A-B). Cells of both morphologies displayed the ability to bind CD31 Dynabeads® during reselection.

Figure 3.3

**Figure 3.3: EC and non-EC morphologies.**

EC and non-EC were isolated by CD31 Dynabead® isolation and cultured on gelatine coated plates for 7 days.

A+B: CD31 positive selected cells (EC). Two main morphologies can be distinguished in CD31 positive selected cells: “streamlined” (**A**) and “round” (**B**). Residual Dynabeads® can be found attached to the cells (→).

C+D: CD31 negative selected cells (non-EC). Non-EC are a mixture of different cell types which occur in the lung. In culture they mainly display the morphology of fibroblasts (**C**) and epithelial cells (**D**).

CD31 negative cells expectedly displayed a mix of morphologies of cells occurring in the lung, e.g. fibroblasts, epithelial cells and smooth muscle cells, and continued to proliferate beyond confluence. Figures 3.3 and 3.4 show examples of endothelial and non-endothelial cell morphologies. The morphology of isolated endothelial and non-endothelial cells was independent from the type of magnetic beads used.

Figure 3.4

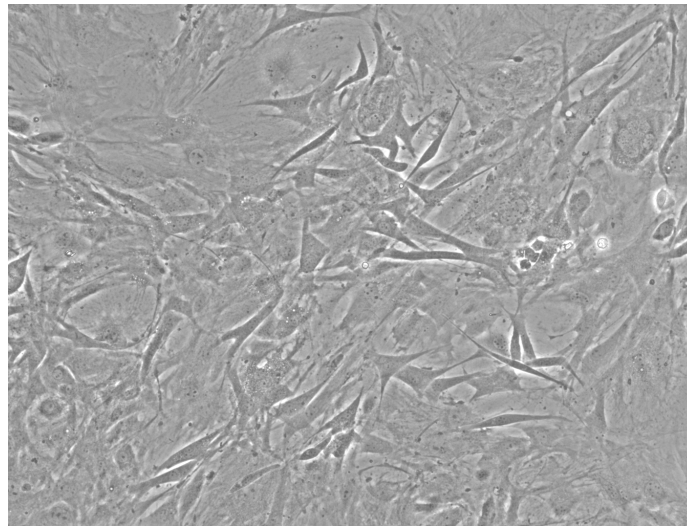


Figure 3.4: CD31 negative cells lack contact inhibition.

CD31 negative cells (non-EC) 10 days after CD31 Dynabead® selection. After initially displaying fibroblast and epithelial cell like morphologies, non-EC continued to proliferate rapidly and beyond confluence. Despite looking very large and irregular when over confluent, these cells were viable and continued to grow after passaging.

3.3.2 Expression of endothelial cell markers in CD31 positive and negative cells after three selections and one passage

After 3 selections with CD31-conjugated Dynabeads® and 1 passage, CD31 positive selected cells showed the morphology of microvascular endothelial cells (Figure 3.5 A) and expressed eNOS, detected by immunofluorescence and western blotting (Figure 3.5 C and E), and also showed expression of the endothelial cell markers CD102 and Tie-2 (Figure 3.5 E). CD31 negative selected cells were a mix of different cell types displaying the morphology of fibroblasts, smooth muscle cells and epithelial cells (Figure 3.5 B). They were negative for the endothelial cell markers eNOS, CD102 and Tie2 (Figure 3.5 D and E).

Figure 3.5

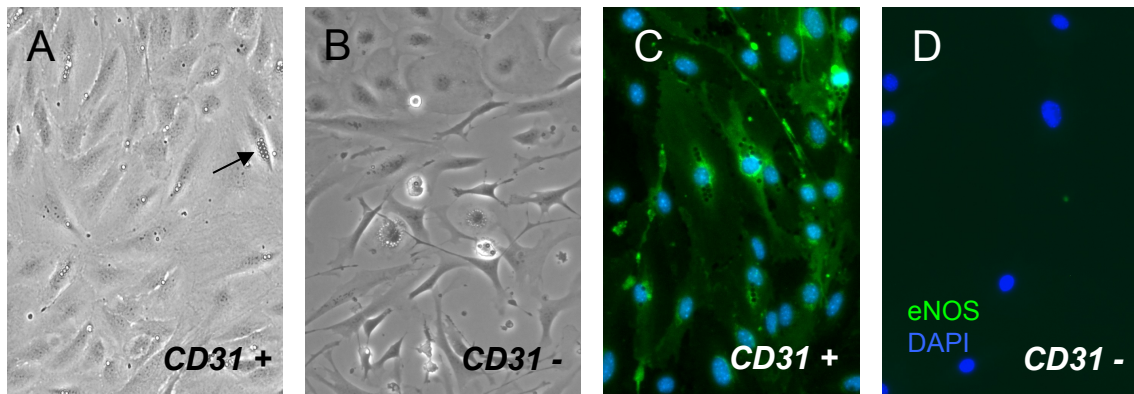
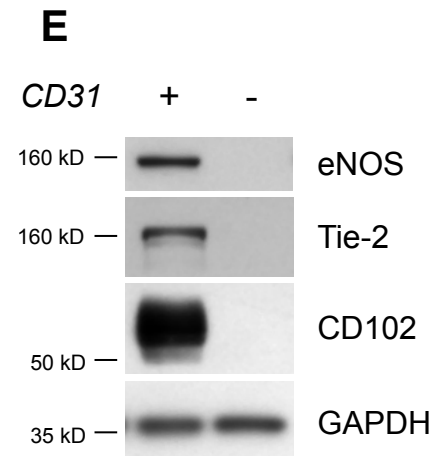


Figure 3.5: Endothelial cell characterization after 3 selections and 1 passage.

EC were isolated from mouse lungs by CD31-Dynabead® selection and underwent 3 selections and 1 passage (16-18 days in culture).

A and B: Phase contrast microscopy. CD31 positive selected cells (**A**) show the typical morphology of microvascular endothelial cells. The arrow indicates residual magnetic beads. CD31 negative selected cells (**B**) are a mix of cells which display the morphology of fibroblasts, smooth muscle cells and epithelial cells.

C and D: Immunofluorescent detection of eNOS (green), counterstained with DAPI (blue). CD31 positive selected cells (**C**) show strong eNOS immunofluorescence whereas CD31 negative cells isolated from the same lung do not show any eNOS fluorescence (**D**).



E: Western blotting shows expression of eNOS, Tie-2 and CD102 in CD31 positive selected cells, whereas these endothelial cell markers are not detectable in CD31 negative cells. GAPDH was used as a loading control.

3.3.3 Expression of NOS isoforms

eNOS is a key regulator of endothelial cell biology. To detect potential protein expression of NOS isoforms other than eNOS in isolated primary endothelial and non-endothelial cells, western blotting was performed. Three isoforms of NOS have been described in mammals: nNOS (neuronal NOS, NOS I), iNOS (inducible NOS, NOS II) and eNOS (endothelial NOS, NOS III) (Förstermann *et al.*, 1994b; Mungrue *et al.*, 2003).

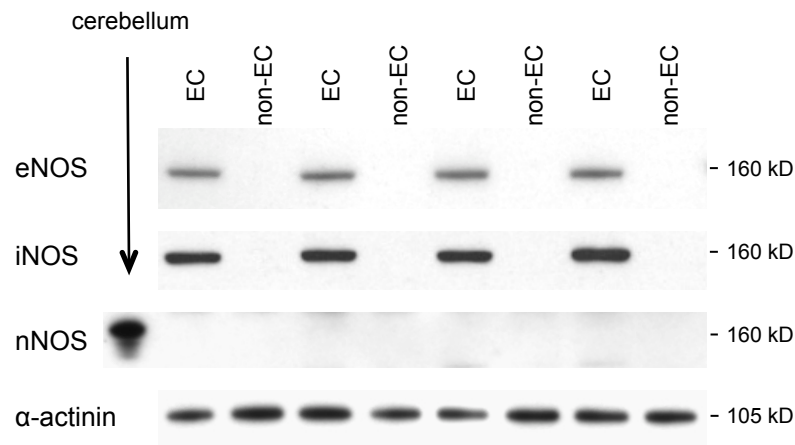
nNOS is constitutively expressed neuronal tissue (Bredt *et al.*, 1990; Bredt and Snyder, 1990; Mayer *et al.*, 1990) and some non-neuronal cells including cardiac (Xu *et al.*, 1999) and skeletal myocytes (Kobzik *et al.*, 1994) and travels at 150-160 kD on SDS-PAGE (Bredt and Snyder, 1990; Schmidt *et al.*, 1991).

iNOS is induced by cytokines and lipopolysaccharide (LPS) mainly in macrophages (Hauschildt *et al.*, 1990; Stuehr *et al.*, 1991) but also other cell types such as vascular smooth muscle cells (Busse and Mulsch, 1990) and travels at approximately 130 kD on SDS-PAGE (Xie *et al.*, 1992). Endothelial cells do not appear to generally express iNOS even after stimulation. Human intestinal microvascular endothelial cells and bovine pulmonary endothelial have been reported to express iNOS after stimulation with TNF- α and LPS (Binion *et al.*, 1998; Chicoine *et al.*, 2011) whereas in HUVEC and bEND.3 cells TNF- α /LPS stimulation did not induce iNOS expression (Binion *et al.*, 1998; Kacimi *et al.*, 2011).

eNOS is constitutively expressed and predominantly but not exclusively found in endothelial cells (Li *et al.*, 2002a; Pollock *et al.*, 1991; Pollock *et al.*, 1993) and travels at approximately 140 kD on SDS-PAGE (Sessa *et al.*, 1992). Amongst other cell types, eNOS expression has been detected in cardiomyocytes (Balligand *et al.*, 1995), kidney tubular epithelial cells (Tracey *et al.*, 1994) and platelets (Sase and Michel, 1995; Wallerath *et al.*, 1997).

Western blotting of primary endothelial cells that had been selected with CD31-conjugated Dynabeads® three times and passaged once showed expression of eNOS whereas non-endothelial cells did not express eNOS (Figure 3.6). Probing the membrane with mouse monoclonal iNOS antibody (BD Transduction Laboratories) resulted in bands with a molecular weight of approximately 140 kD in endothelial but not in non-endothelial cells. However, as most antibodies against iNOS, this antibody is known to cross-react with eNOS so the detected bands are likely to be due to eNOS expression. There was no expression of nNOS detectable in either endothelial or non-endothelial cells.

Figure 3.6

**Figure 3.6: Detection of NOS isoforms in endothelial and non-endothelial cells.**

EC were isolated from mouse lungs by CD31-Dynabead® selection and underwent 3 selections and 1 passage (16 days in culture).

Western blotting showed eNOS expression in endothelial cells but not non-endothelial cells.

Probing the membrane with mouse monoclonal iNOS antibody resulted in approximately 140 kD bands in EC but not in non-EC. However, this antibody is known to cross-react with eNOS so the detected bands are likely to be due to eNOS expression.

There was no expression of nNOS detectable in either EC or non-EC. Cerebellum lysate was used as a positive control for nNOS.

α-actinin was used as loading control.

3.3.4 NOS activity in primary mouse endothelial cells

BH4 is an essential cofactor for NOS catalytic activity. Hph mice carry a point mutation leading to underexpression of GTP cyclohydrolase I (Khoo *et al.*, 2004) with an 85% reduction in tissue BH4 levels (Khoo *et al.*, 2005). When quantified by the conversion of C14-arginine to C14-citrulline, NOS activity in lung homogenates from hph mice is reduced by 35% compared to WT (Khoo *et al.*, 2005).

Primary mouse endothelial and non-endothelial cells were isolated from WT and hph mouse lungs by CD31 Dynabead® selection and NOS enzymatic activity was quantified by the conversion of C14-arginine to C14-citrulline in order to test whether:

- a) eNOS activity in cultured primary endothelial cells can be assessed by C14-arginine to C14-citrulline conversion.
- b) There is a significant difference in NOS activity detectable between endothelial and non-endothelial cells.
- c) This method is sensitive enough to detect a significant reduction in NOS activity in hph endothelial cells.

C14-arginine to C14-citrulline conversion experiments were undertaken in the presence of the arginase inhibitor nor-NOHA. Experiments were also undertaken in the absence and presence of the NOS inhibitor L-NAME. The L-NAME inhibitable fraction of arginine conversion is due to NOS activity whereas residual L-citrulline production in the presence of L-NAME is NOS independent. A detailed description of the method can be found in Chapter 2.8.

CD31 Dynabead® isolated cells were reselected twice and passaged once and analyzed for C14-arginine to C14-citrulline conversion as one batch after 16-18 days in culture. The results of this experiment are shown in Figure 3.7. Endothelial cells (CD31 positive cells) showed detectable arginine to citrulline conversion that was L-NAME inhibitable. As reported above, non-endothelial cells did not express eNOS protein and as expected there was no L-NAME inhibitable arginine to citrulline conversion detectable. However, in contrast to previously published data from hph and WT lung homogenates, reduced BH4 bioavailability in hph endothelial cells did not alter arginine conversion in comparison to WT (Khoo et al., 2005). One reason for this finding might be that BH4 levels in hph endothelial cells after 16-18 days in culture are in fact high enough to maintain normal eNOS function. As shown in Figure 3.8, directly after the initial isolation endothelial cells from hph mice showed a 77 % reduction in BH4 content compared to WT (1.3 ± 0.2 pmol/mg protein (SEM) versus 5.6 ± 0.5 pmol/mg protein respectively). After 17 days in culture endothelial cells from hph mice still showed the same relative reduction in BH4 content compared to WT (11.0 ± 0.4 pmol/mg protein versus 51.0 ± 9.6 pmol/mg protein), but the BH4 content had increased both in endothelial cells from WT and hph. After this culture period hph endothelial cells had reached double the BH4 content of WT endothelial cells after the initial isolation. It is therefore possible that despite being BH4 deficient relative to WT, hph endothelial cells after 3 selections are not BH4 deficient anymore in absolute terms. Detailed results of biopterin measurements in endothelial cells from different genetic backgrounds are shown in Chapter 4.

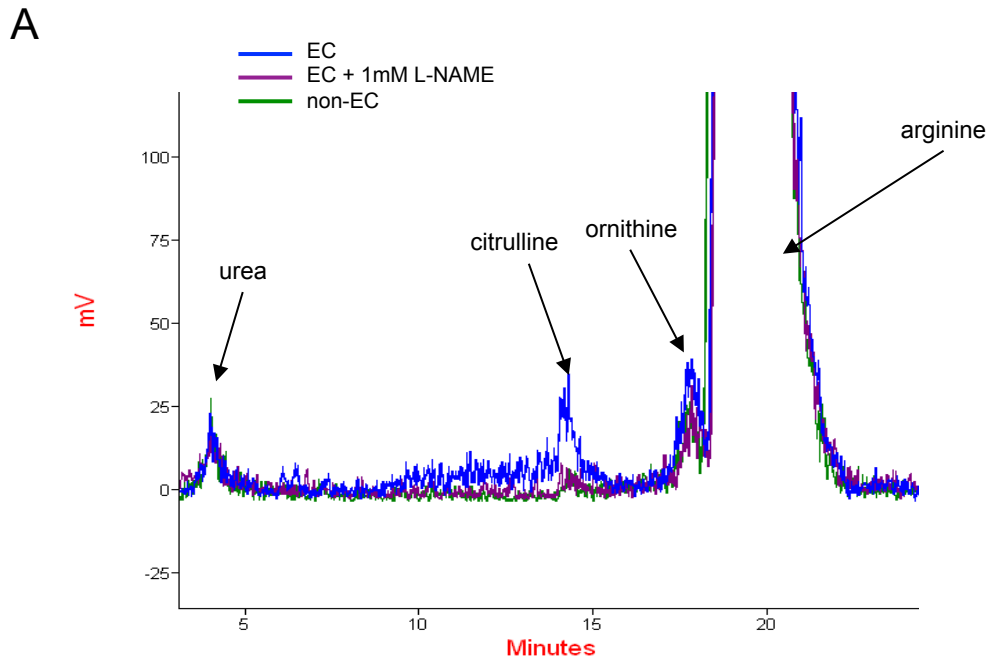
Figure 3.7: Quantification of eNOS enzymatic activity in cultured primary EC by arginine to citrulline conversion and radiochemical HPLC.

Primary mouse EC were isolated by CD31 Dynabead® selection from hph-1 mice and WT littermates and eNOS enzymatic activity was quantified by the conversion of C14-arginine to C14-citrulline. All experiments were carried out in the presence of 5µM NorNOHA.

A: Chromatogram for C14-arginine to C14-citrulline HPLC. To display the differences in the citrullin peak between EC (blue), EC after incubation with 1mM L-NAME (purple) and non-EC (green) (zoomed in).

B: Each culture underwent 3 magnetic bead selections and 1 passage and the cells were analyzed 16-18 days after the initial isolation. EC showed detectable arginine to citrulline conversion that was inhibitable with 1mM L-NAME. No difference in arginine conversion between WT and hph EC was detectable. There was no significant difference in arginine to citrulline conversion detectable between non-EC with and without L-NAME. n=6 per group.

C: EC after 5 days in culture. Arginine to citrulline conversion was too close to the detection limit and no significant difference could be detected between WT with and without 1mM L-NAME or hph with and without 1mM L-NAME respectively. n=3-4 per group.



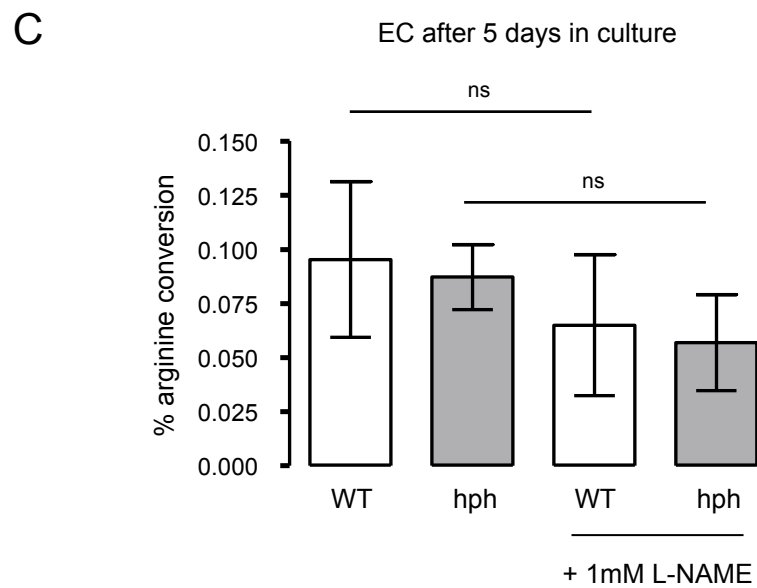
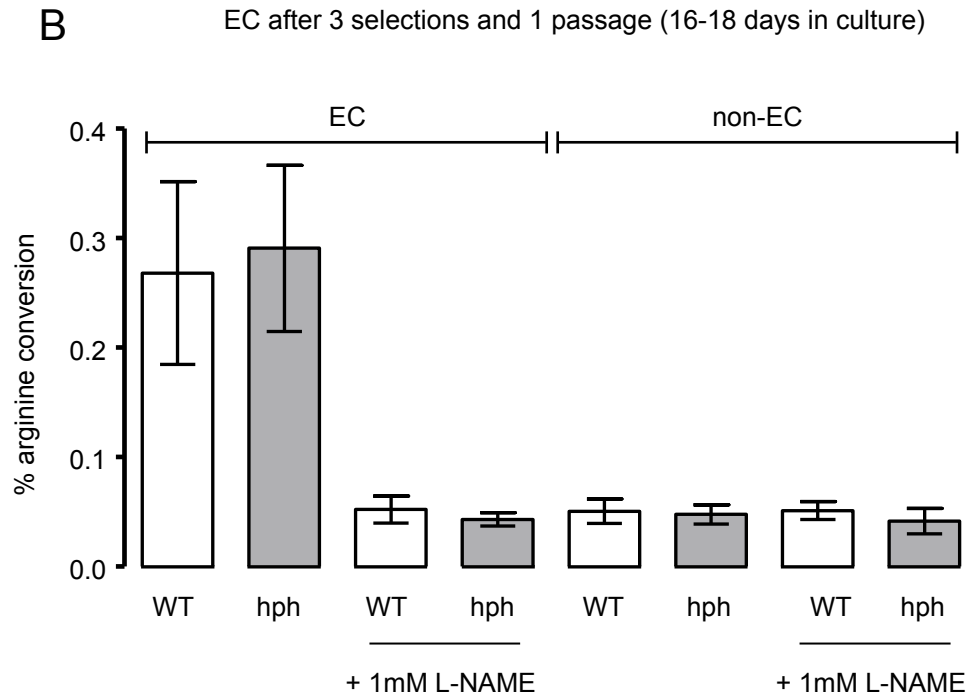


Figure 3.8

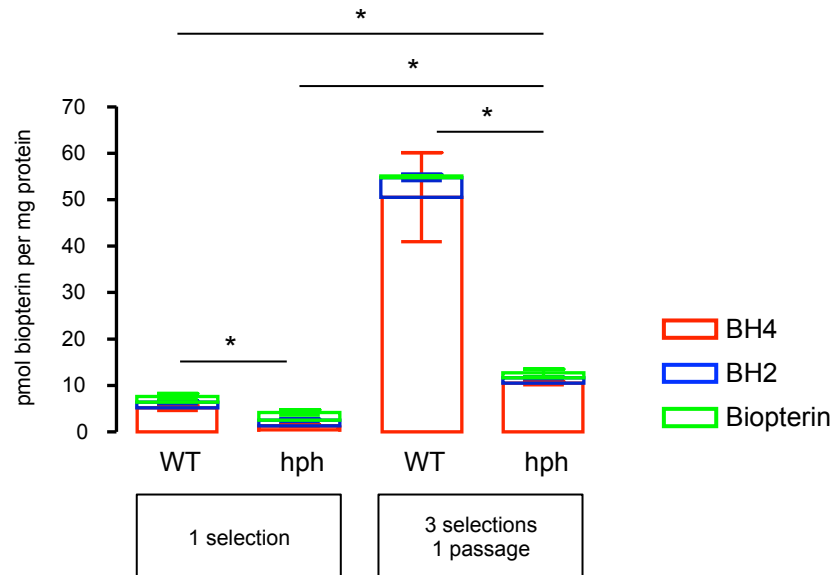


Figure 3.8: Bipterin levels in primary WT and hph EC after one and after 3 CD31 Dynabead® selections.

Primary mouse EC were isolated by CD31 Dynabead® selection from mouse lungs and either immediately snap frozen for later analysis (1 selection) or plated into culture, reselected twice and passaged once (3 selections 1 passage) before analysis of bipterin content.

Directly after the initial isolation, EC from hph mice showed a 77 % reduction in BH4 content compared to WT (1.3 ± 0.2 pmol/mg protein (SEM) versus 5.6 ± 0.5 pmol/mg protein respectively).

After 3 CD31 Dynabead® selections and 1 passage (17 $\pm$ 0.6 days (SEM) days in culture) EC from hph mice still showed the same relative reduction in BH4 content compared to WT (11.0 ± 0.4 pmol/mg protein versus 51.0 ± 9.6 pmol/mg protein).

However, after 17 days in culture BH4 content had increased both in EC from WT and hph with hph EC reaching almost double the BH4 content of WT EC after the initial isolation.

n=5-9 per group. *p<0.05.

To test whether a difference in NOS activity between hph and WT endothelial cells could be detected after a shorter culture period, endothelial cells were isolated from WT and hph mouse lungs by CD31 Dynabead® selection and analyzed for C14-arginine to C14-citrulline conversion after 5 days in culture (Figure 3.7 C). The number of endothelial cells after only 5 days in culture is very low ($< 5 \times 10^4$ cells) and arginine to citrulline conversion was very low and too close to the detection limit to confidently evaluate NOS activity. The calculated sample size needed to detect a difference in arginine conversion between WT with and without L-NAME with a beta error level of 50% is $n=21$ per group. This would require the use of 42 mice per genotype, which is not feasible. Plating the cells into a smaller culture well could potentially improve the sensitivity of the assay because the experiment could be performed in a smaller volume of buffer of which a larger proportion could be injected onto the HPLC system leading to larger citrulline peaks. However, as mentioned above, CD31 Dynabead® isolated endothelial cells do not grow well in 12 well dishes due to cell accumulation in the centre of the well, which would undermine the benefits of using a smaller well size. To confidently measure NOS activity in primary endothelial cells it might therefore be necessary to explore alternative methods to the C14-arginine to C14-citrulline conversion assay, which are sensitive enough to analyze small cell samples. Fluorescent detection of NO with diaminofluoresceins (Kojima *et al.*, 1998) for example might also allow simultaneous analysis of endothelial marker expression and NO synthesis by flow cytometry.

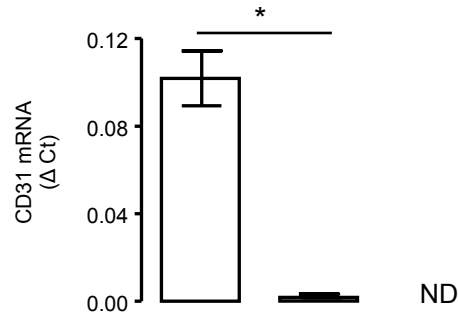
3.3.5 Expression of endothelial targeted transgenes in cultured primary endothelial cells

The expression of endothelial targeted transgenes in cultured endothelial (CD31 positive) and non-endothelial (CD31 negative) cells was evaluated by RT-PCR and western blotting. Endothelial and non-endothelial cells were isolated from mouse lungs by CD31 MACS® MicroBead isolation as described in Chapter 2.3.2 and cultured for 5 days before being used for further analysis. VSMC were cultured from aortas as described in Chapter 2.3.3 and used as a further negative control next to non-endothelial cells from lungs.

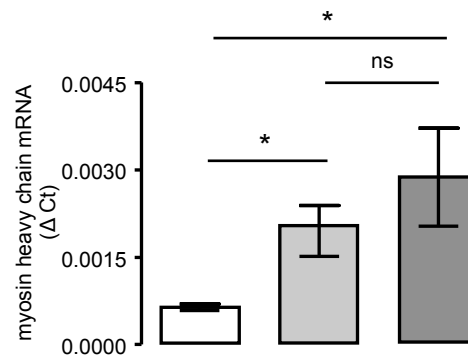
3.3.5.1 Validation of isolated cell types: Expression of CD31, myosin heavy chain and mouse GCH1 mRNA in cultured endothelial cells, non-endothelial cells and VSMC

CD31, smooth muscle myosin heavy chain and mouse GCH1 mRNA levels were quantified by RT-PCR and corrected for the level of the housekeeping gene GAPDH (Figure 3.9).

Figure 3.9 A



B



C

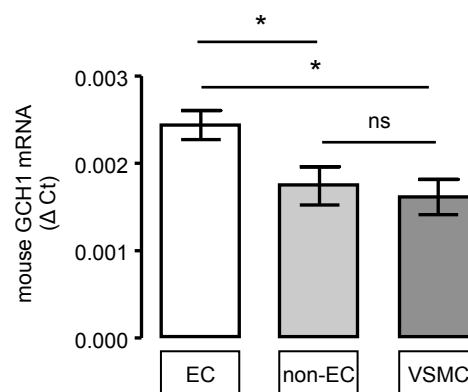


Figure 3.9: CD31, myosin heavy chain and mouse GCH1 mRNA expression in mouse EC, non-EC and VSMC.

EC and non-EC were isolated from mouse lungs by CD31 MACS® MicroBead isolation and cultured for 5 days. VSMC were cultured from mouse aortas after the removal of the endothelium.

CD31, smooth muscle myosin heavy chain and mouse GCH1 mRNA levels were quantified by RT-PCR and corrected for the level of the housekeeping gene GAPDH.

A: The expression of CD31 was specific to EC (ND = non detectable).

B: The expression of smooth muscle myosin heavy chain mRNA was significantly lower in EC than in non-EC or VSMV. Next to epithelial cells and fibroblasts, non-EC contain airway smooth muscle cells which explains the medium level of smooth muscle myosin heavy chain mRNA in non-EC.

C: Mouse GCH1 mRNA expression was significantly higher in EC than in non-EC or VSMC.

n=5-8 per group, *p<0.05.

The expression of CD31 was specific to endothelial cells. There was no CD31 expression detectable in VSMC and CD31 mRNA levels in non-endothelial cells were reduced by 98-99% compared to endothelial cells. mRNA of the smooth muscle cell marker myosin heavy chain was detectable in endothelial cells but at a significantly lower level than in non-endothelial cells or VSMV. Expression of smooth muscle myosin heavy chain is not entirely specific to smooth muscle cells and has been described in other cell types including endothelial cells (Azuma *et al.*, 2009; Cevallos *et al.*, 2006). The medium level of smooth muscle myosin heavy chain mRNA present in non-endothelial cells can be explained by the presence of airway smooth muscle cells in the non-endothelial cell culture. All evaluated cell types expressed mouse GCH1 mRNA with endothelial cells expressing significantly higher levels than non-endothelial cells or VSMC.

These results confirm a successful separation of endothelial and non-endothelial cells by CD31 MACS® MicroBead selection.

3.3.5.2 Expression of human GCH1 and LacZ mRNA in cultured endothelial cells, non-endothelial cells and VSMC from ApoE-KO, GCH/ApoE-KO and ApoE-KO/LacZ mice

Both the GCH and the LacZ transgene are expressed under the control of the Tie2 promoter. mRNA expression of these transgenes is therefore only expected in endothelial cells but not non-endothelial cells or VSMC.

Human GCH1 and LacZ mRNA levels in endothelial cells, non-endothelial cells and VSMC were quantified by RT-PCR and corrected for the level of the housekeeping gene GAPDH (Figure 3.10).

Figure 3.10

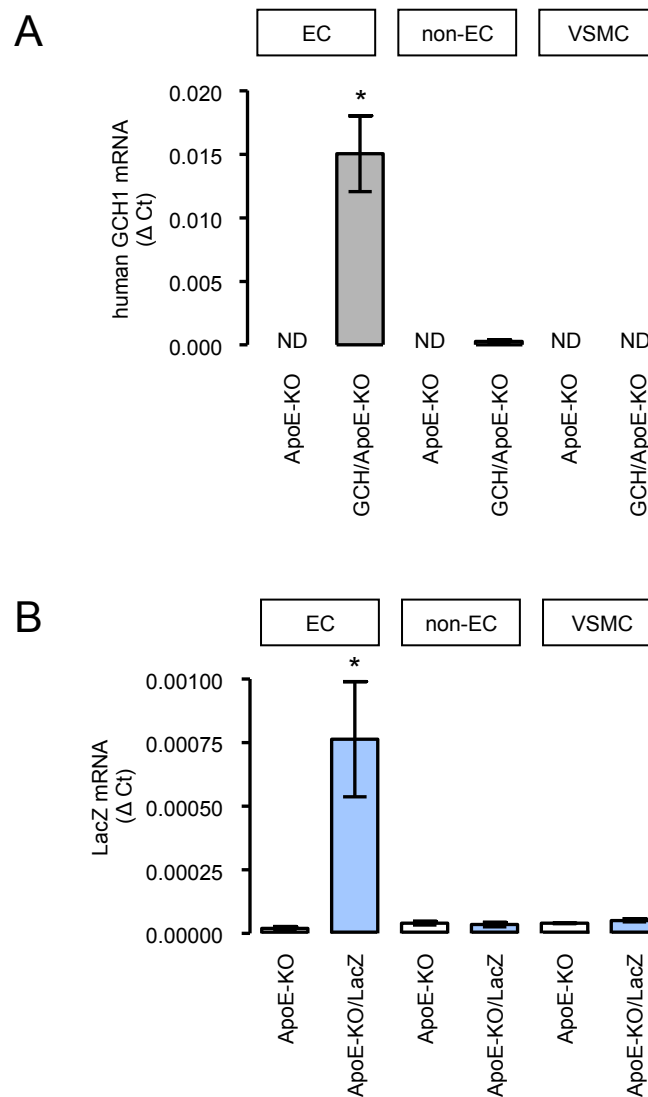


Figure 3.10: Human GCH1 and LacZ mRNA expression in EC, non-EC and VSMC from ApoE-KO, GCH/ApoE-KO and ApoE-KO/LacZ mice.

EC and non-EC were isolated from mouse lungs by CD31 MACS® MicroBead isolation and cultured for 5 days. VSMC were cultured from mouse aortas after the removal of the endothelium.

Human GCH1 and LacZ mRNA levels were quantified by RT-PCR and corrected for the level of the housekeeping gene GAPDH.

The expression of both human GCH1 (**A**) and LacZ (**B**) under the control of the Tie-2 promoter was specific to transgenic EC (ND = non detectable).

n=3-4 per group, *p<0.05.

The expression of human GCH1 and LacZ was specific to transgenic endothelial cells. There was no human GCH mRNA detectable in non-transgenic (ApoE-KO) endothelial or non-endothelial cells and VSMC of all genotypes. Corresponding to the CD31 mRNA results, expression of human GCH1 was reduced by 98-99% in GCH transgenic non-endothelial cells compared to GCH transgenic endothelial cells. A very small proportion (1-2%) of endothelial cells present in the non-endothelial cell culture could explain the low level expression of both CD31 and human GCH1. LacZ mRNA could not be detected in non-EC and VSMC.

3.3.5.3 Protein expression of transgenic human GTPCH in cultured endothelial cells, non-endothelial cells and VSMC from ApoE-KO, GCH/ApoE-KO and ApoE-KO/LacZ mice

After confirming that mRNA of the human GCH transgene is specifically expressed in endothelial cells after 5 days in culture, endothelial cells, non-endothelial cells and VSMC were evaluated for transgenic human GCH protein content by western blotting. The endothelial cell markers CD102, Tie-2 and eNOS were used to validate endothelial phenotype and VSMC phenotype was confirmed by the expression of α smooth muscle actin.

Endothelial and non-endothelial cells were isolated from lungs from ApoE-KO and GCH/ApoE-KO mice by CD31 MACS[®] MicroBead isolation and cultured for 5 days before being lysed for Western blotting analysis. VSMC were cultured from ApoE-KO and GCH/ApoE-KO aortas after the removal of the endothelium. Western blotting results for the expression of endothelial and smooth muscle cell markers and transgenic human GTPCH (hGTPCH) are shown in Figure 3.11.

Figure 3.11

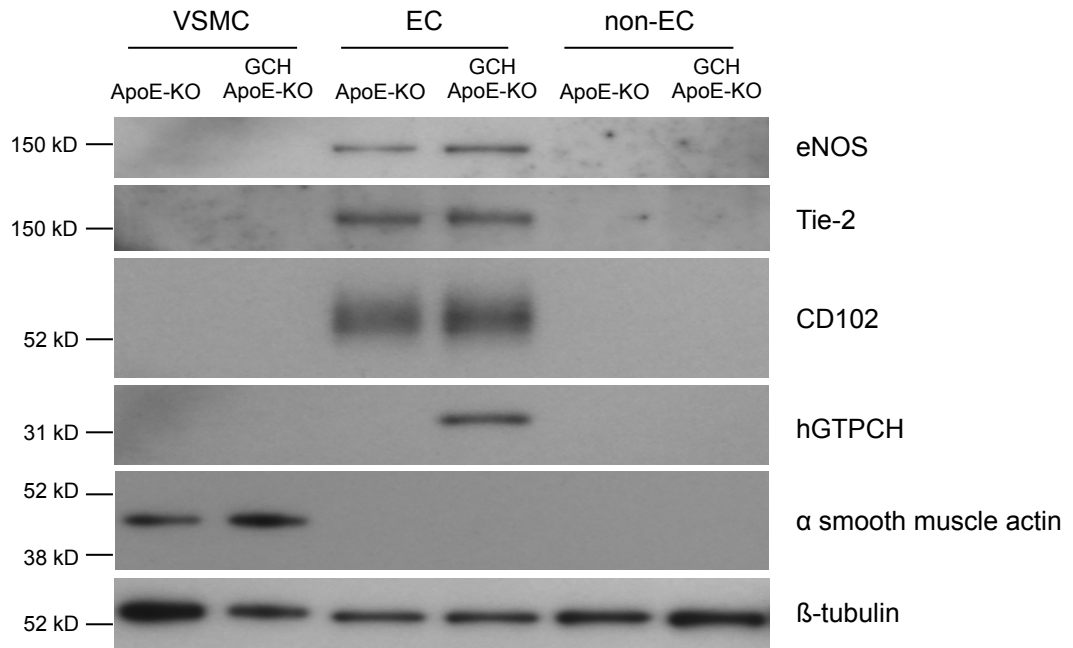


Figure 3.11: Protein expression of endothelial cell markers and transgenic human GTPCH in EC, non-EC and VSMC.

EC and non-EC were isolated from lungs from ApoE-KO and GCH/ApoE-KO mice by CD31 MACS® MicroBead isolation and cultured for 5 days before being lysed in RIPA buffer for Western blotting analysis. VSMC were cultured from ApoE-KO and GCH/ApoE-KO aortas after the removal of the endothelium.

Western blotting showed expression of the endothelial cell markers eNOS, Tie-2 and CD102 exclusively in CD31 positive selected cells (EC).

Human GTPCH (hGTPCH) protein was only detectable in EC from GCH transgenic mice.

The VSMC phenotype was confirmed by the expression of α smooth muscle actin, which could not be detected in EC nor non-EC.

β-tubulin was used as a loading control.

As before, expression of the human GTPCH protein was specific to endothelial cells from GCH transgenic mice. Expression of the endothelial cell markers eNOS, Tie-2 and CD102 was only detected in endothelial cells and α -smooth muscle actin was only expressed in VSMC but not in endothelial or non-endothelial cells.

3.3.5.4 Protein expression of endothelial targeted transgenes in cultured endothelial and non-endothelial cells from double transgenic eNOS/GCH mice

Both GCH and eNOS transgenes are endothelial targeted. However, eNOS transgenic mice overexpress bovine eNOS under the control of the murine preproendothelin-1 promoter (Ohashi *et al.*, 1998) whereas GCH transgenic overexpress human GCH under the control of the Tie-2 promoter and intronic enhancer (Alp *et al.*, 2003).

In Chapters 3.3.5.2 and 3.3.5.3, RT-PCR and western blotting had demonstrated endothelial specificity of transgenic human GCH expression in GCH/ApoE-KO mice. In order to test endothelial specificity of eNOS overexpression, endothelial and non-endothelial cells were isolated from WT, GCH, eNOS and eNOS/GCH mouse lungs by CD31 MACS[®] MicroBead isolation and cultured for 5 days. The expression of eNOS, endogenous and transgenic GTPCH and the endothelial cell markers Tie-2, CD102 was analyzed by western blotting (Figure 3.12).

Again, Tie-2 and CD102 protein was exclusively detected in endothelial cells. As on ApoE-KO background, human GTPCH protein was only detectable in endothelial cells from GCH transgenic mice (GCH and eNOS/GCH), whereas

endogenous GTPCH was detectable in endothelial cells and non-endothelial cells from all genotypes. The rabbit anti-rat GTPCH antibody used detects both mouse GTPCH (mGTPCH) and the marginally larger hGTPCH protein, leading to detection of a double band in GCH transgenic endothelial cells.

eNOS protein was strongly overexpressed in endothelial cells from eNOS transgenic mice (eNOS and eNOS/GCH). As expected WT and GCH non-endothelial cells did not show expression of eNOS protein. However, there were eNOS bands detectable in non-endothelial cells from eNOS transgenic mice. Since western blotting is not as sensitive as RT-PCR detecting transgene expression from a few residual endothelial cells within the non-endothelial cell culture, this is most likely due to the non-endothelial cell culture containing airway epithelial cells. It has been shown that transgenes under the control of the preproendothelin-1 promoter, as used for overexpression of bovine eNOS in eNOS transgenic mice, are predominantly but not exclusively expressed in the endothelium but also in airway epithelium (Harats *et al.*, 1995).

Figure 3.12

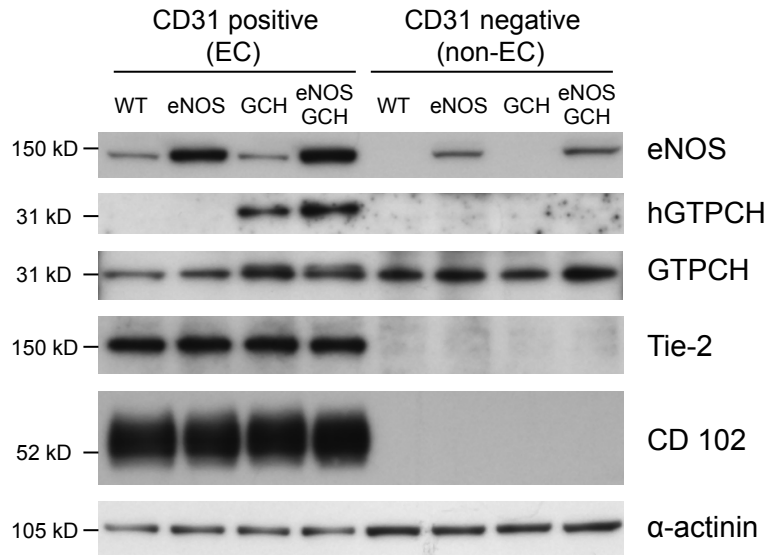


Figure 3.12: Protein expression of endothelial cell markers and endothelial targeted transgenes in EC and non-EC from eNOS/GCH transgenic mice after 5 days in culture.

EC and non-EC were isolated from mouse lungs by CD31 MACS® MicroBead isolation and cultured for 5 days before being lysed in RIPA buffer for Western blotting analysis. α-actinin was used as a loading control.

Western blotting showed expression of the endothelial cell markers Tie-2 and CD102 exclusively in CD31 positive selected cells (EC).

hGTPCH protein was only detectable in EC from GCH transgenic mice (GCH and eNOS/GCH), whereas endogenous GTPCH was detectable in EC and non-EC from all genotypes. The rabbit anti-rat GTPCH antibody detects both mGTPCH and the marginally larger hGTPCH protein, leading to detection of a double band in GCH and eNOS/GCH EC.

eNOS protein was strongly overexpressed in EC from eNOS transgenic mice (eNOS and eNOS/GCH). As expected WT and GCH non-EC did not show expression of eNOS protein but there were eNOS bands detectable in eNOS transgenic non-EC. This is most likely due to “non-EC” containing airway epithelial cells. In eNOS transgenic mice, bovine eNOS is overexpressed in the endothelium under the control of the preproendothelin-1 promoter. However, it has been shown that transgenes under the control of the preproendothelin-1 promoter are predominantly but not exclusively expressed in the endothelium but also in airway epithelium.

3.4 Biological changes in cultured primary endothelial cells over time

The results presented above show that after 16-18 days in culture CD31 Dynabead® isolated endothelial cells express the endothelial cell markers CD102, Tie-2 and eNOS (Chapter 3.3.2) and also show eNOS activity (Chapter 3.3.4.). However, primary cells are susceptible to undergo changes over time in culture. To evaluate whether significant changes in expression of endothelial cell markers and endothelial targeted transgenes occur in primary mouse endothelial cells during the time needed to achieve pure cultures time course experiments were performed.

3.4.1 Long term culture of CD31 Dynabead® isolated endothelial cells

Being able to grow primary endothelial cells over a longer period of time and passage them repeatedly would reduce the number of mice needed and potentially allow the use experimental methods that require a larger endothelial cell sample than can be isolated from one mouse. Because endothelial cells form a strictly contact inhibited monolayer and grow in the form of cell islands, cells in the periphery of the island keep dividing until islands merge while cells in the centre of the island are confluent already and have stopped dividing. As a result primary mouse endothelial cells achieve a monolayer that covers the whole well only very slowly. Passaging endothelial cells before coverage of the well can improve overall proliferation.

The experiment presented in Figure 3.13 was carried out during the method development phase in order to assess the consequences of prolonged endothelial cell culture. Endothelial cells and non-endothelial cells after two CD31 Dynabead®

selections were left to proliferate in culture over increasing periods of time and passaged up to 3 times. While the cells retained their morphology even after 7 weeks in culture, the expression of endothelial cell markers determined by western blotting decreased over time. Interestingly the expression of eNOS and Tie-2 appeared to decrease more rapidly than the expression of CD102.

Figure 3.13

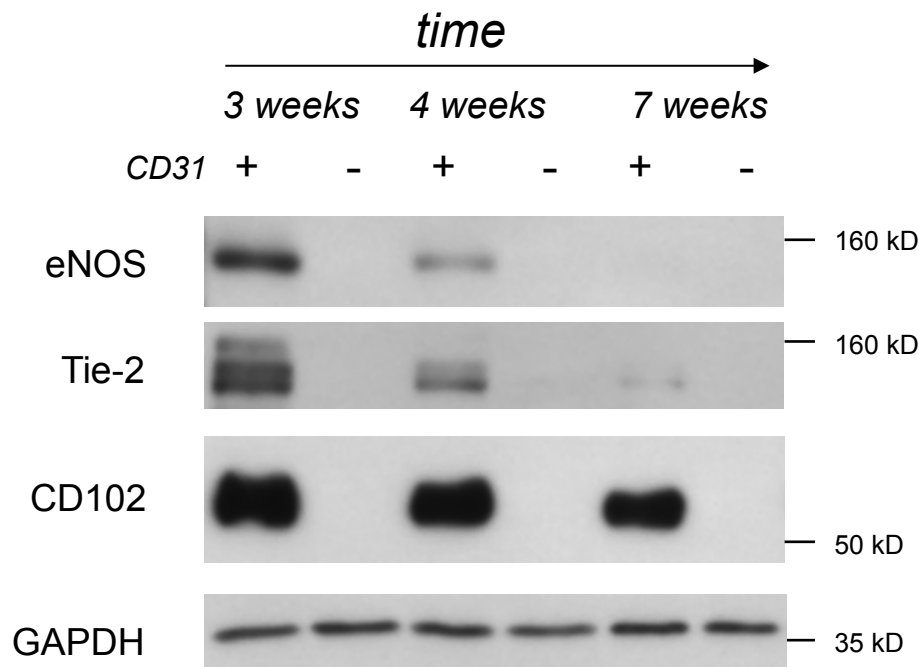


Figure 3.13: Time-dependent expression of endothelial cell markers in cultured endothelial cells.

EC were isolated from mouse lungs by CD31 Dynabead® selection. CD31 positive and negative cells were reselected with anti CD31 beads once and subsequently kept in tissue culture over increasing periods of time. CD31 positive selected cells (EC) show expression of the endothelial cell markers eNOS, Tie-2 and CD102 by Western Blotting but the level of expression of these markers decreases over time. CD31 negative selected cells (non-EC) do not show expression of endothelial cell markers. GAPDH was used as a loading control.

3 weeks: 2 selections and 1 passage
 4 weeks: 2 selections and 1 passage
 7 weeks: 2 selections and 3 passages

3.4.2 Protein expression of endothelial cell markers and transgenic human GTPCH in primary endothelial cells after different stages of reselection

To evaluate potential changes in expression of endothelial cell markers and endothelial targeted transgenes in primary mouse endothelial cells during the three CD31 Dynabead® selections, part of the cells was harvested for western blot analysis during each reselection step (Figure 3.14).

BH4 is an essential cofactor for eNOS and eNOS transgenic mice exhibit eNOS uncoupling due to a stoichiometric deficiency of BH4, which can be rescued by transgenic GCH overexpression (Bendall *et al.*, 2005). Modifications in endogenous GTPCH expression or eNOS expression may influence the cellular requirements for additional BH4 in cultured primary endothelial cells and affect regulation of the expression of transgenic GCH via a feedback mechanism. Therefore changes in transgenic human GTPCH over time in culture were investigated on WT, BH4 deficient hph and eNOS transgenic background.

Western blotting showed a rapid down-regulation of the endothelial cell markers Tie-2 and CD102 and human GTPCH from the initial (SI) to the third Dynabead® selection (SIII), by which time the cells had spent 8-9 days in culture. This phenomenon was observed on WT (Figure 3.14 B), hph (Figure 3.14 C) and eNOS transgenic (Figure 3.14 D) background. No down-regulation of eNOS was observed on WT or hph background with or without transgenic GCH overexpression. In eNOS transgenic endothelial cells there appeared to be a down-regulation of eNOS

from SII to SIII, though the results were not as clear as the down-regulation of Tie-2, CD102 and hGTPCH.

Figure 3.14: Expression of endothelial cell markers and transgenic human GTPCH in primary EC after different stages of reselection.

Primary EC were isolated from mouse lungs by CD31 Dynabead® selection. During each reselection step a part of the cells was harvested to be analysed for expression of endothelial cell markers by western blotting.

A: The lungs from 6 or 8 mice were harvested and underwent Liberase Blendzyme digestion. The strained cells were mixed together and divided into aliquots equalling the cells from two lungs for bead binding. The equivalent to the cells from 2 mice each were plated into T25 flasks and the remaining cells (equivalent to the cells from 2 mice) were frozen.

For reselection, the cultures were incubated with CD31 conjugated Dynabeads®. Part of the reselected cells was plated into 6 well dishes (equivalent of the cells of 1 mouse per well) and the other part was frozen for western blotting analysis.

To take the cells through to 3 bead selections, 6 mice were needed in the beginning. To take the cells through to 3 bead selections plus 4-5 days in culture (usual point of the 1st passage) 8 mice were needed.

B-D: Western blotting of EC after different stages of reselection. **B:** EC from WT and GCH transgenic mice. **C:** EC from hph and hph/GCH mice. **D:** EC from eNOS and eNOS/GCH transgenic mice.

Western blotting showed a rapid down-regulation of Tie-2, CD102 and human GTPCH from SI to SIII. The polyclonal rabbit anti-rat GTPCH antibody cross-reacts both with mGTPCH and hGTPCH which leads to the formation of a double band in GCH transgenic EC with hGTPCH being marginally larger than mGTPCH (GTPCH, **B-D**). The mouse monoclonal anti-human GTPCH antibody specifically detects hGTPCH in GCH transgenic EC (**D**) and also shows a down regulation of hGTPCH from SI to SIII.

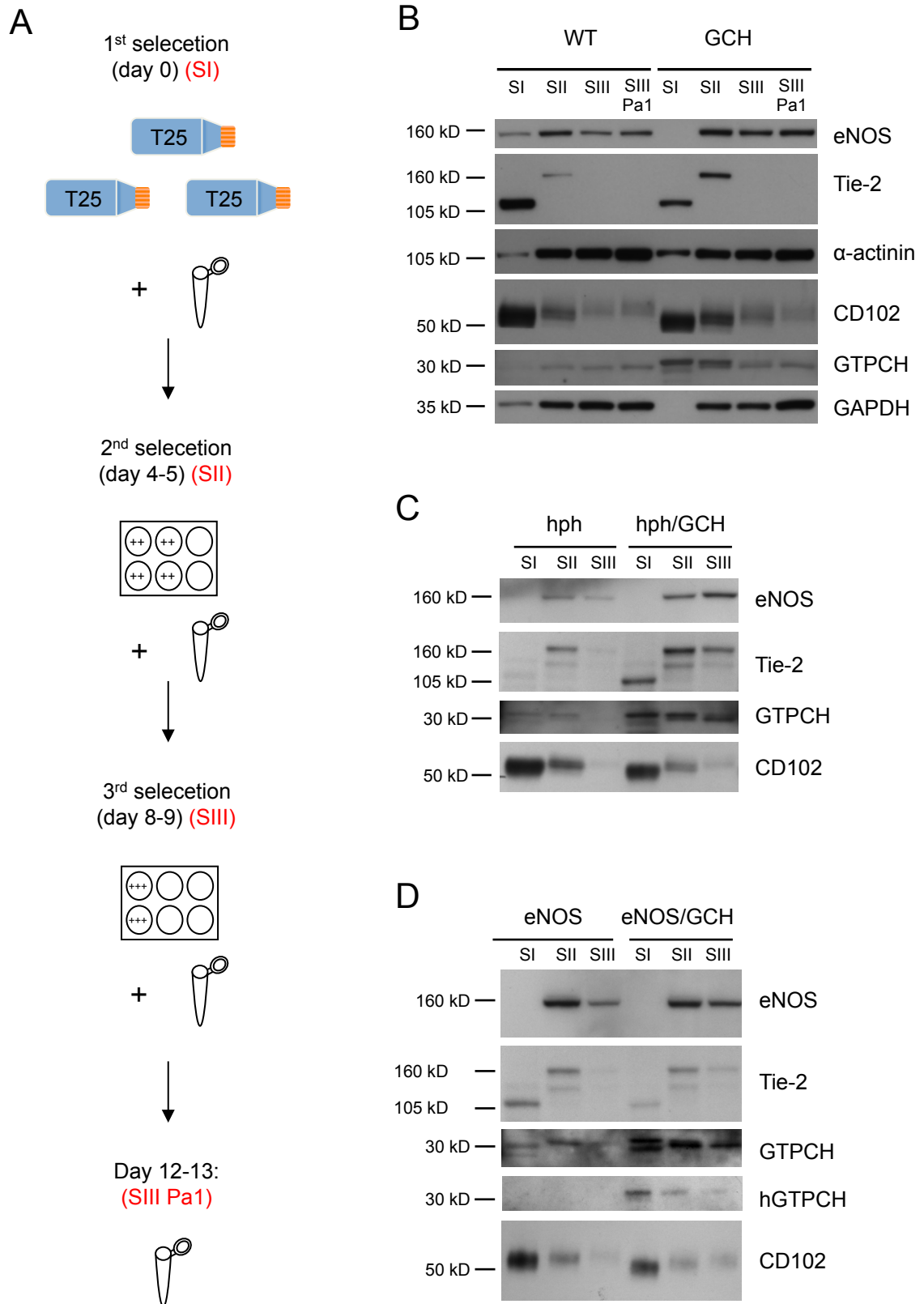
There was also no or only a weak 135kD band for eNOS detectable after the initial isolation, which might be due to protein degradation. There appeared to be a down-regulation of eNOS from SII to SIII in eNOS transgenic EC (**D**), though the results are not as clear as the down-regulation of Tie-2, CD102 and hGTPCH

Despite equal protein loadings, EC directly after the first selection (SI) showed weak and variable bands for the housekeeping proteins GAPDH and α -actinin (**B**). This might be due to proteolytic cleavage of these proteins during the initial enzymatic tissue digest, or even changes in expression of these house-keeping proteins during the time in tissue culture.

Both Tie-2 and CD102 experienced a shift in apparent size from SI to SIII. CD102 is a heavily glycosylated protein that might get partially deglycosylated during the enzymatic digest or alternatively even stronger glycosylated during the time in culture. The shift in apparent size of Tie-2 might also, at least in part, be due to deglycosylation and/or protein degradation during the isolation procedure.

Overall the time point of the second selection (4 to 5 days after the initial isolation) seems to be suitable to analyze primary endothelial cells because full size eNOS protein has been regenerated and there is still strong expression of GCH and eNOS transgenes.

Figure 3.14



Western blotting also revealed the loss of full size eNOS protein in SI, potentially due to protein degradation during the isolation procedure. Both Tie-2 and CD102 experienced a shift in apparent size from SI to SIII. CD102 is a heavily glycosylated protein that might get partially deglycosylated during the enzymatic digest or alternatively even stronger glycosylated during the time in culture. The shift in apparent size of Tie-2 might also, at least in part, be due to deglycosylation and/or protein degradation during the isolation procedure.

Despite equal protein loadings, endothelial cells directly after the first selection showed weak and variable bands for the housekeeping proteins GAPDH and α -actinin. Proteolytic cleavage of these proteins during the initial enzymatic tissue digest or changes in expression of these house-keeping proteins during the time in tissue culture are possible explanations for this finding.

Overall the time point of the second selection (SII, 4 to 5 days after the initial isolation) seems to be suitable to analyze primary endothelial cells because full size eNOS protein has been regenerated and there is still strong expression of GCH and eNOS transgenes.

3.4.3 Influences of enzymatic tissue digest on eNOS, Tie-2 and CD102 protein

The time course experiments described above revealed loss of full size eNOS protein in endothelial cells directly after the initial CD31 Dynabead® selection. If eNOS protein is indeed degraded in freshly isolated endothelial cells, these cells

cannot be used for experiments relying on eNOS activity immediately but have to be cultured until full size eNOS has been regenerated.

To assess whether protein degradation had lead to a loss of full size eNOS in freshly isolated endothelial cells, western blotting was performed on primary endothelial cells directly after the initial CD31 Dynabead® selection and after 3 days in culture (Figure 3.15). The mouse monoclonal eNOS antibody detected two fragments of approximately 60kD and 28kD respectively in endothelial cells immediately after the initial magnetic bead selection, whereas after 3 days in culture the antibody only detected one band of approximately 135-140kD, corresponding to the size of eNOS protein. Degradation of eNOS with the formation of a 60kD fragment has been described before in association with caspase-3 induction during apoptosis (Tesauro *et al.*, 2006). In addition, caspase-3 has been described to cleave α -actinin (Communal *et al.*, 2002). Induction of apoptosis in endothelial cells during the isolation procedure could potentially be a reason for eNOS degradation and weak western blotting bands for α -actinin. However, as shown in Chapter 5.2.3.2 (Figure 5.5) approximately 64% of all endothelial cells isolated by CD31 Dynabead® selection are viable as assessed by Annexin-V/7-AAD staining and flow cytometry.

Directly after the first CD31 Dynabead® selection the goat polyclonal Tie-2 antibody routinely used in this thesis detected an approximately 110kD band instead of the expected 160kD (Figure 3.15). A second Tie-2 antibody (Santa Cruz Biotechnology, sc324, rabbit polyclonal), raised against a different immunogen, was used to confirm that the 110kD band was related to Tie-2. The apparent shift in molecular weight of Tie-2 could be due to protein degradation; Deglycosylation of

Tie-2 during the isolation procedure might also be a possible explanation. The calculated molecular weight of mouse Tie-2 is 125.8kD. Due to glycosylation it travels at 140-160kD on SDS-PAGE under reducing conditions. Deglycosylation of cultured endothelial cell lysates led to detection of a Tie-2 band in the region of the predicted molecular weight (Figure 3.16). It is therefore unlikely that deglycosylation is the sole reason for the shift in apparent size of Tie-2 from 160 to 110kD after the initial Dynabead® selection and both deglycosylation and protein degradation during lung digest could play a role.

CD 102 is a heavily glycosylated protein and also undergoes a slight shift in apparent size from 50-55kD after the initial isolation to 55-60kD after the third Dynabead® selection (Figure 3.16). The predicted molecular weight for CD102 is 31.4kD. After deglycosylation the CD102 band was detected at approximately 33-34kD. It is unclear whether the subtle change in apparent size of CD102 with time in culture is caused by partial deglycosylation during tissue digest or increasing glycosylation during the time in cell culture, or a combination of both.

Figure 3.15

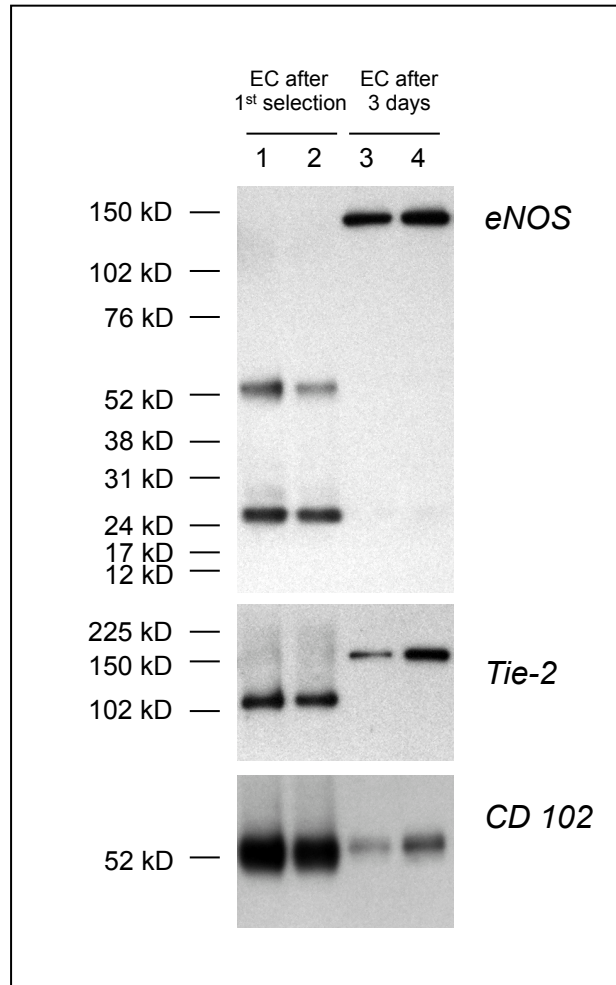


Figure 3.15: EC after enzymatic tissue digest and one CD31 Dynabead® selection or after 3 days in culture.

Primary EC were isolated from mouse lungs by CD31 Dynabead® selection. After the bead selection the cells were either snap frozen for later analysis (columns 1 and 2) or plated into gelatine coated dishes, grown for 3 days and lysed with RIPA buffer for western blot analysis (columns 3 and 4). n=4 per group.

Western blotting showed degradation of eNOS protein in EC immediately after the initial bead selection (columns 1 and 2). The mouse monoclonal eNOS antibody detected two fragments of approximately 60 kD and 28 kD respectively. After 3 days in culture full size eNOS had been regenerated and degradation products were undetectable (columns 3 and 4).

Directly after the first selection (columns 1 and 2) the goat polyclonal Tie-2 antibody detects an approximately 110 kD band instead of the expected 160 kD (columns 1 and 2). This might be due to protein degradation and/or deglycosylation of Tie-2 during the isolation procedure. The calculated molecular weight of mouse Tie2 is 125.8 kD but due to glycosylation it travels at 140-160 kD on SDS-PAGE under reducing conditions.

CD 102 is a heavily glycosylated protein and also undergoes a slight shift in apparent size after the initial isolation.

Figure 3.16

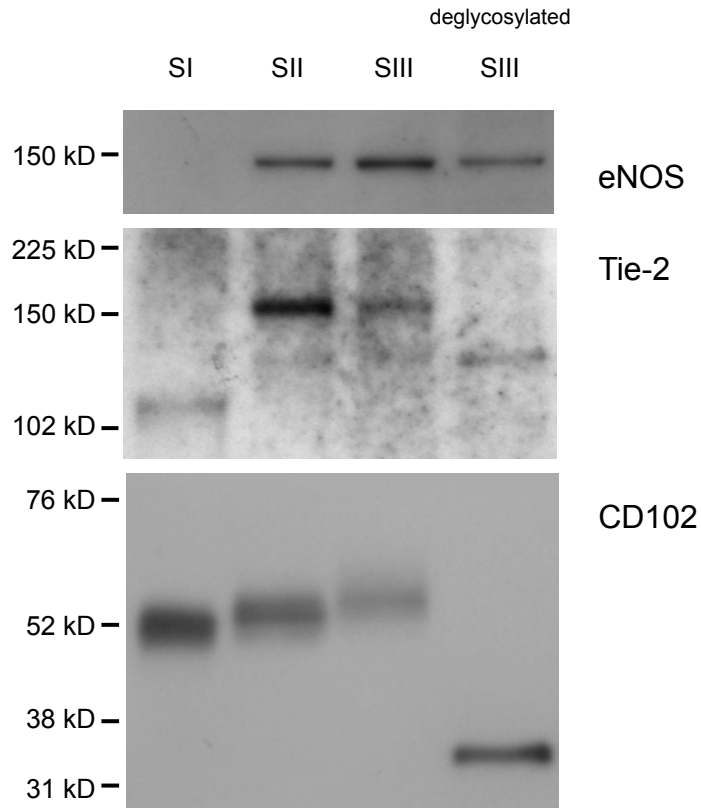


Figure 3.16: Shift in apparent size of Tie-2 and CD102 in EC after different stages of resection.

SI: 1st selection (day 0)
 SII: 2nd selection (day 4)
 SIII: 3rd selection (day 8-10)

Primary EC were isolated from mouse lungs by CD31 Dynabead® selection and analyzed by western blotting after different stages of reselection. To examine the impact of Tie-2 and CD102 glycosylation on the travelling behaviour of these proteins during SDS-PAGE, EC after 3 selections were deglycosylated with PNGase F.

There was no full size eNOS detectable in SI as described before. PNGaseF treatment had no effect on eNOS whereas both Tie-2 and CD102 travelled at significantly lower molecular weights after deglycosylation. The predicted molecular weight for Tie-2 is 125.8 kD. After deglycosylation the Tie-2 band was detected in the region of the predicted molecular weight. Deglycosylation therefore is unlikely to be the sole reason for the shift in apparent size of Tie-2 to 110kD in SI and protein degradation during lung digest might also play a role.

The predicted molecular weight for CD102 is 31.4 kD. After deglycosylation the CD102 band was detected at approximately 33-34 kD. It is unclear whether the subtle change in apparent size of CD102 from SI to SIII is caused by partial deglycosylation during tissue digest or increasing glycosylation during the time in cell culture, or a combination of both.

3.5 Analysis of subpopulations of CD31 MACS® MicroBead selected cells

CD31 is a well-recognized marker for endothelial cells. However, CD31 is also expressed in platelets, monocytes, granulocytes and lymphocyte subsets. EC medium that was used to grow CD31 positive selected cells is optimized to support proliferation of endothelial cells. However, the presence of CD31 positive non-endothelial cells such as monocytes/macrophages cannot be excluded. The pan-leukocyte marker CD45 was used to identify leukocytes within the CD31 positive lung cell isolate. CD31 magnetic bead isolation was performed using MACS® MicroBeads to allow flow cytometry analysis and cell sorting. An overview over antibodies used for flow cytometry can be found in Table 2.4.

CD31 MACS® MicroBead isolated cells were analyzed by flow cytometry and classed into subpopulations with regard to the level of CD31 and CD45 expression. Expression of endothelial cell markers, endothelial progenitor markers and hGCH/LacZ transgenes in the subpopulations were assessed by flow cytometry or western blotting and X-Gal staining respectively. To investigate proliferation potential of the subpopulations in culture, flow sorted cells were plated into gelatine coated dishes containing EC medium.

3.5.1 Assessment of CD31 MACS® MicroBead isolation specificity

Cells were isolated from mouse lungs by CD31 MACS® MicroBead selection. CD31 APC antibody was used for stained samples and unconjugated CD31 antibody was used for unstained control samples or isotype controls. For assessment

of CD31 MACS® MicroBead isolation specificity, cells were incubated with APC isotype control in place of CD31 APC antibody before MicroBead incubation.

Bead bound cells were eluted from the columns, washed with MACS buffer and blocked with rat anti-mouse CD16/CD32 (Fc-block) 1/100 in MACS buffer for 15 minutes on ice. MACS buffer containing CD45 PE-Cy7 or isotype control were added, achieving a final concentration of 1/200 for CD45 PE-Cy7 or isotype control. After 15 minutes incubation on ice in the dark the samples were washed with MACS buffer, fixed with 2% formaldehyde and analyzed by flow cytometry.

With regard to the level of CD31 and CD45 expression there were four subpopulations of CD31 MACS® MicroBead isolated cells detectable (Figure 3.17 D): CD31 high/CD45 dim, CD31 low/CD45 negative, CD31 dim/CD45 positive and CD31 negative/CD45 negative. The CD31 high/CD45 dim population was subsequently found to express various endothelial cell markers and to proliferate in culture exhibiting endothelial cell morphology (Chapters 3.5.2 to 3.5.7).

To test the specificity of CD31 MACS® MicroBead selection, APC isotype control was used for isolation in place of CD31 APC antibody. Only 1.9×10^4 cells were eluted from the column using isotype control, equaling 4% of the average total number of cells isolated from 1 mouse lung using CD31 antibody. About half of the eluted cells were CD45 positive. 30% of all cells were CD45 positive and had bound APC isotype control. However, 70 % of all unspecifically isolated cell were negative for APC which leads to the conclusion that they had been retained in the column unspecifically without MicroBead binding. There were no unspecifically isolated cells detectable within the CD31 high/CD45 dim gate (Figure 3.17).

Figure 3.17

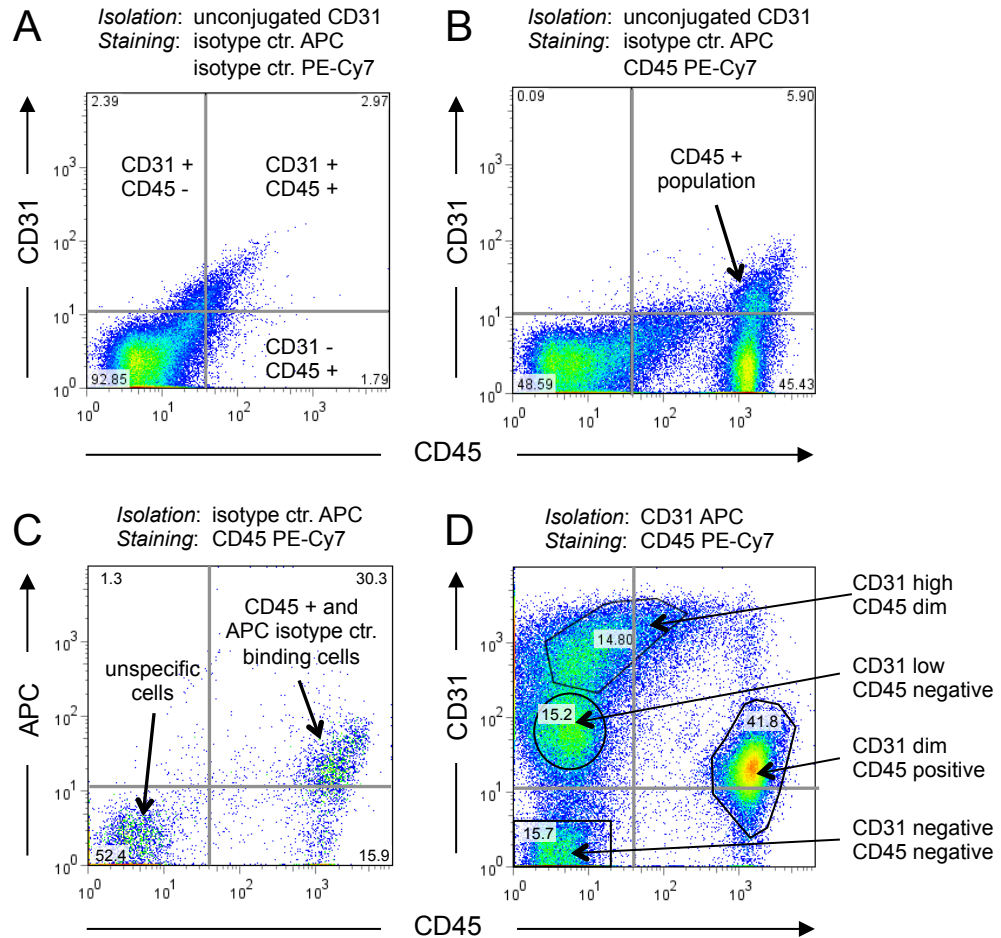


Figure 3.17: Flow cytometry of CD31 MACS® MicroBead selected cells. Assessment of isolation specificity.

Cells were isolated from mouse lungs by CD31 MACS® MicroBead selection. After Fc blocking the cells were stained for flow cytometry analysis. To assess the specificity of CD31 MACS® MicroBead isolation, cells were incubated with APC isotype control in place of CD31 APC antibody before MicroBead incubation.

A: Cells isolated with unconjugated CD31 antibody and stained with APC and PE-Cy7 isotype control. After Fc blocking there was no binding of APC isotype control and only minimal binding of PE-Cy7 isotype control detectable in comparison to unstained control. The gates for APC negative and PE-Cy7 negative are set to a 95% confidence limit.

B: Cells isolated with unconjugated CD31 antibody and stained with APC isotype control and CD45 PE-Cy7. The CD45 positive population is more fluorescent in the APC channel than the CD45 negative/dim population.

C: Cells isolated with APC isotype control and stained with CD45 PE-Cy7. If CD31 MACS® MicroBead isolation was 100% specific, no cells would be isolated with isotype control. However, a total of 1.9×10^4 cells are eluted from the column, equaling 4% of the average total number of cells isolated from 1 mouse lung using CD31 antibody. About half of the cells are CD45 positive of which two thirds bind APC isotype control. APC negative cells represent cells unspecifically retained in the column without antibody binding during isolation.

D: Cells isolated with CD31 APC and stained with CD45 PE-Cy7. There are 4 populations detectable regard to CD31 and CD45 expression. CD31 high/CD45 dim cells were subsequently identified as EC.

Some degree of unspecific isolation of CD45 positive cells had to be expected because the protocol for CD31 MACS® MicroBead selection does not contain an Fc-blocking step before incubation with CD31 antibody. Due to CD16/CD32 antibody having been produced in rat, Fc-blocking with this antibody cannot take place before incubation with MACS® Goat anti-Rat IgG MicroBeads. Overall the number of isotype control “isolated” cells is small (< 5%) in comparison to using CD31 antibody.

3.5.2 CD31 and CD102 cell surface marker expression in CD31 MACS® MicroBead isolated cells

The expression of CD102 in CD31 MACS® MicroBead isolated cells was assessed by flow cytometry (Figure 3.18). Cells were isolated from mouse lungs by CD31 MACS® MicroBead selection using CD31 APC or CD31 FITC antibody. Isolated cells were blocked with Fc-block 1/100 in MACS buffer for 15 minutes on ice. MACS buffer containing CD45 PE-Cy7 only or both CD45 PE-Cy7 and CD102 AlexaFluor 488 was added to CD31 FITC or CD31 APC isolated samples respectively, achieving a final concentration of 1/200 for CD45 PE-Cy7 or 1/500 for CD102 AlexaFluor 488. After 15 minutes incubation on ice in the dark the samples were washed with MACS buffer, fixed with 2% formaldehyde and analyzed by flow cytometry.

Figure 3.18

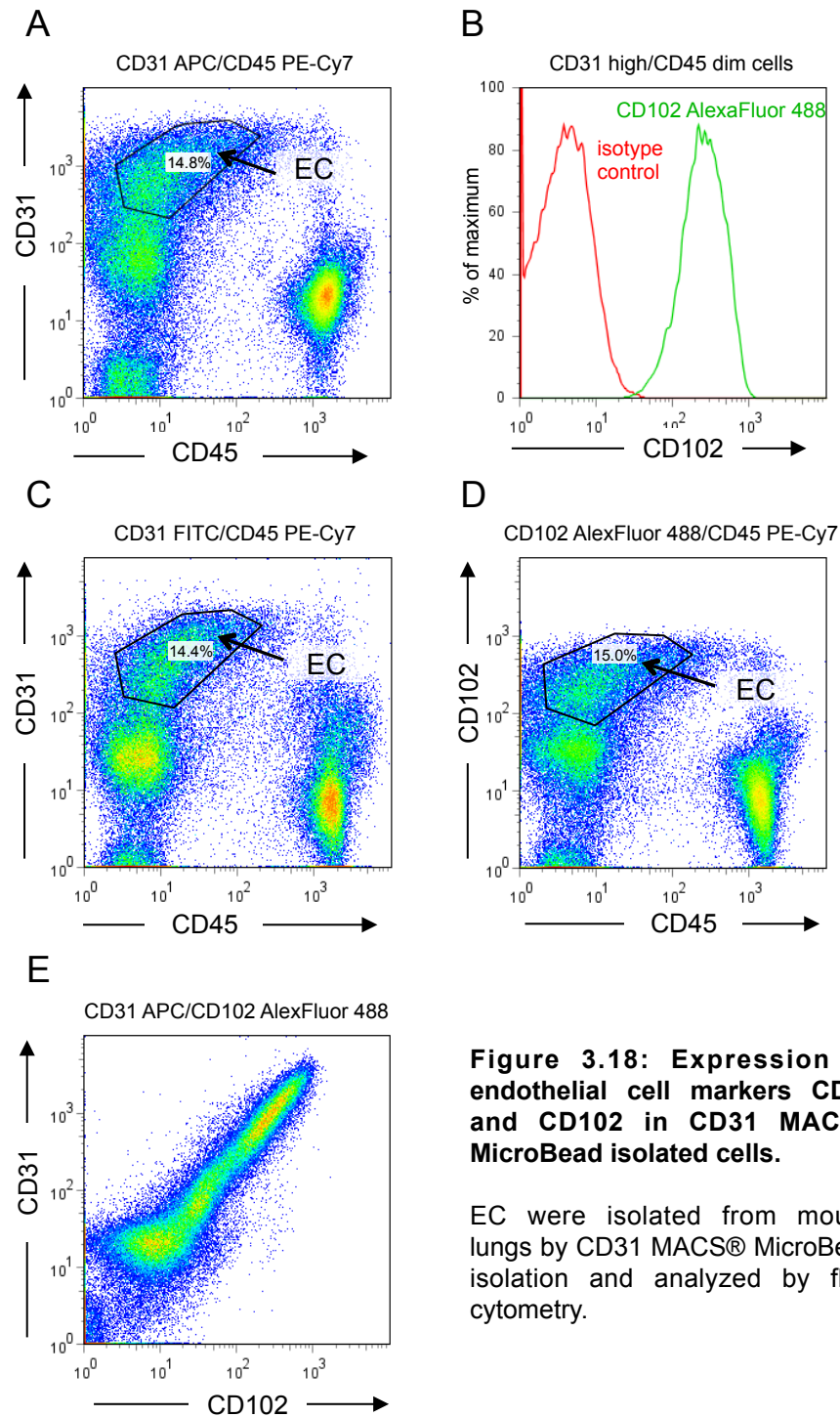


Figure 3.18: Expression of endothelial cell markers CD31 and CD102 in CD31 MACS® MicroBead isolated cells.

EC were isolated from mouse lungs by CD31 MACS® MicroBead isolation and analyzed by flow cytometry.

Figure 3.18:

A+B: CD31 APC was used for the MicroBead isolation and the cells were stained with CD45 PE-Cy7 and CD102 AlexaFluor 488 APC antibodies or the corresponding isotype controls. A gate was applied around the EC population (CD31 high/CD45 dim) (**A**) and cell within this gate were analyzed for CD102 expression. All EC were positive for CD102 (**B**).

C: Cells isolated with CD31 FITC and stained with CD45 PE-Cy7. FITC is less bright compared to APC, however the same populations of cells were identified.

D: Cells isolated with CD31 APC and stained with CD45 PE-Cy7 and CD102 AlexaFluor 488 APC antibodies or the corresponding isotype controls. Using CD102 AlexaFluor 488 to identify EC led to the same results as CD31 APC or CD31 FITC.

E: There is a linear relationship between CD31 APC and CD102 AlexaFluor 488 fluorescence in CD31 MACS® MicroBead isolated cells.

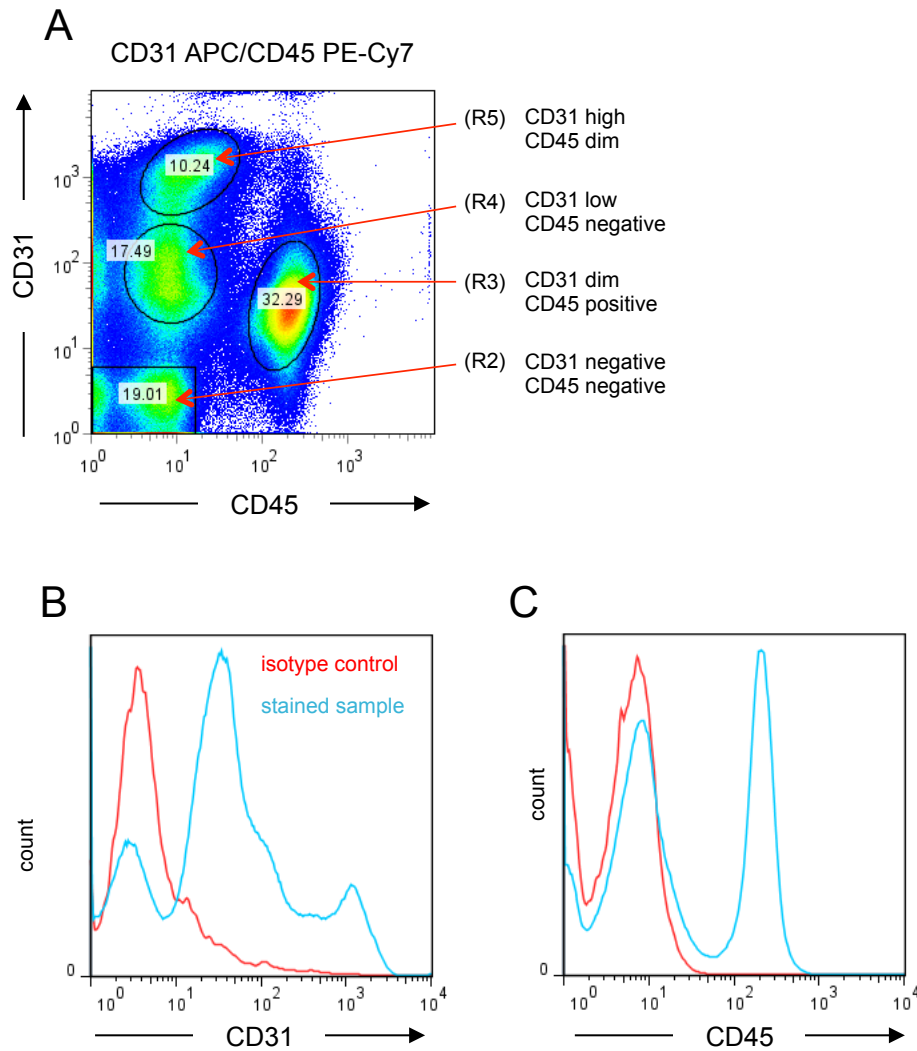
These results show that both CD31 and CD102, conjugated with different fluorochromes, can interchangeably be used to identify EC.

APC has got a better stain index than FITC or AlexaFluor 488 (www.bdbiosciences.com/colors). However, both CD31 APC and CD31 FITC detected the same cell populations (Figure 3.18). All CD31 high/CD45dim cells (endothelial cells) were CD102 positive. Indeed, there was a linear relationship between CD31 APC and CD102 AlexaFluor 488 fluorescence in CD31 MACS® MicroBead isolated cells. These results show that both CD31 and CD102, conjugated with different fluorochromes, can interchangeably be used to identify endothelial cells.

3.5.3 Morphology of flow sorted subpopulations of CD31 MACS® MicroBead selected cells

Cells were isolated from mouse lungs by CD31 MACS® MicroBead isolation using CD31 APC. After Fc blocking the cells were stained with CD45 PE-Cy7 as described above and subjected to flow cytometry analysis and sorting (Figure 3.19). Flow sorted cells were plated into gelatine coated wells and cultured in EC media.

Figure 3.19

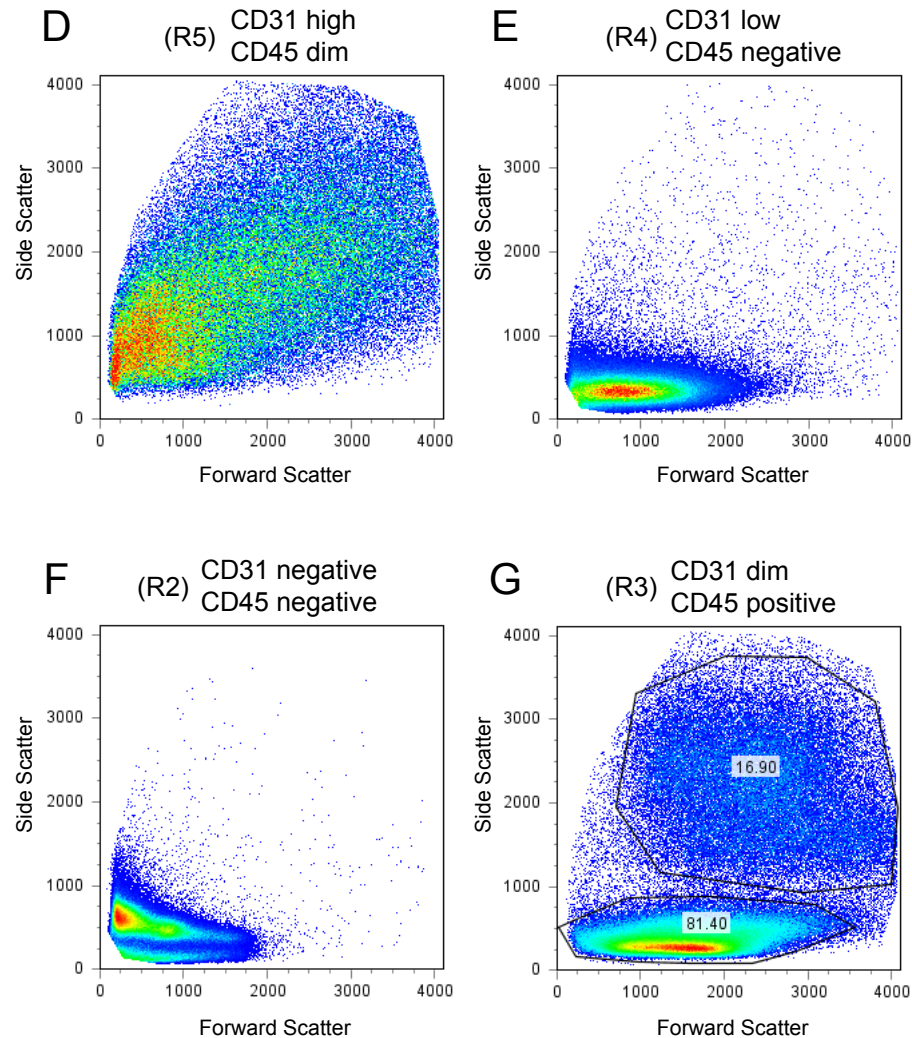
**Figure 3.19: Flow sorting of CD31 MACS® MicroBead selected cells.**

Cells were isolated from mouse lungs by CD31 MACS® MicroBead isolation using CD31 APC. After Fc blocking the cells were stained with CD45 PE-Cy7 and subjected to flow cytometry analysis and sorting.

A: With regard to CD31 and CD45 expression there were 4 populations detectable which were sorted and collected for later analysis.

B-C: Comparison of fluorescence signals of isotype control (red) and antibody stained (blue) samples. **B:** Histograms for CD31 APC fluorescence. **C:** Histograms for CD45 PE-Cy7 fluorescence.

Figure 3.19



D-G: Forward and side scatter plots for the different populations of CD31 MACS® MicroBead isolated cells.

D: CD31 high/CD45 dim cells showed a more heterogenous and more complex morphology than the other populations.

E-F: CD31 low/CD45 negative cells and CD31negative/CD45 negative cells were smaller and less complex than CD31 high/CD45 dim cells.

G: The CD31 dim/CD45 positive population consisted of 2 main subpopulations with relatively small, not very complex cells (e.g. lymphocytes) forming the majority and larger, more complex cells (e.g. granulocytes) the minority population.

Forward and side scatter plots of flow cytometry data suggest that the four subpopulations of CD31 MACS® MicroBead isolated cells differ in relative cell size and complexity (Figure 3.19). CD31 high/CD45 dim cells showed a more heterogeneous and more complex morphology than the other populations. CD31 low/CD45 negative cells and CD31negative/CD45 negative cells were smaller and less complex than CD31 high/CD45 dim cells. The CD31 dim/CD45 positive population consisted of two main subpopulations with relatively small, not very complex cells (e.g. lymphocytes) forming the majority and larger, more complex cells (e.g. granulocytes) the minority population.

One day after plating flow sorted cells into culture, all populations had settled on the bottom of the well. Phase contrast microscopy revealed morphological differences between the populations that matched the flow cytometry data (Figure 3.20 A). CD31 low/CD45 negative cells and CD31negative/CD45 negative cells appeared as homogenous populations of small cells, whereas the CD31 dim/CD45 positive population also contained some larger cells. CD31 high/CD45 dim cells displayed a more heterogeneous morphology with less regularly shaped cells than the other populations.

3.5.4 Culture characteristics of flow sorted subpopulations of CD31 MACS® MicroBead selected cells

Cells were isolated from mouse lungs by CD31 MACS® MicroBead isolation using CD31 APC antibody. The isolated cells were then stained with CD45 PE-Cy7 and subjected to flow sorting. The sorted cells were plated into gelatin coated wells and cultured in EC medium. The experiment was repeated 5 times, on average 8.9 ± 2.4

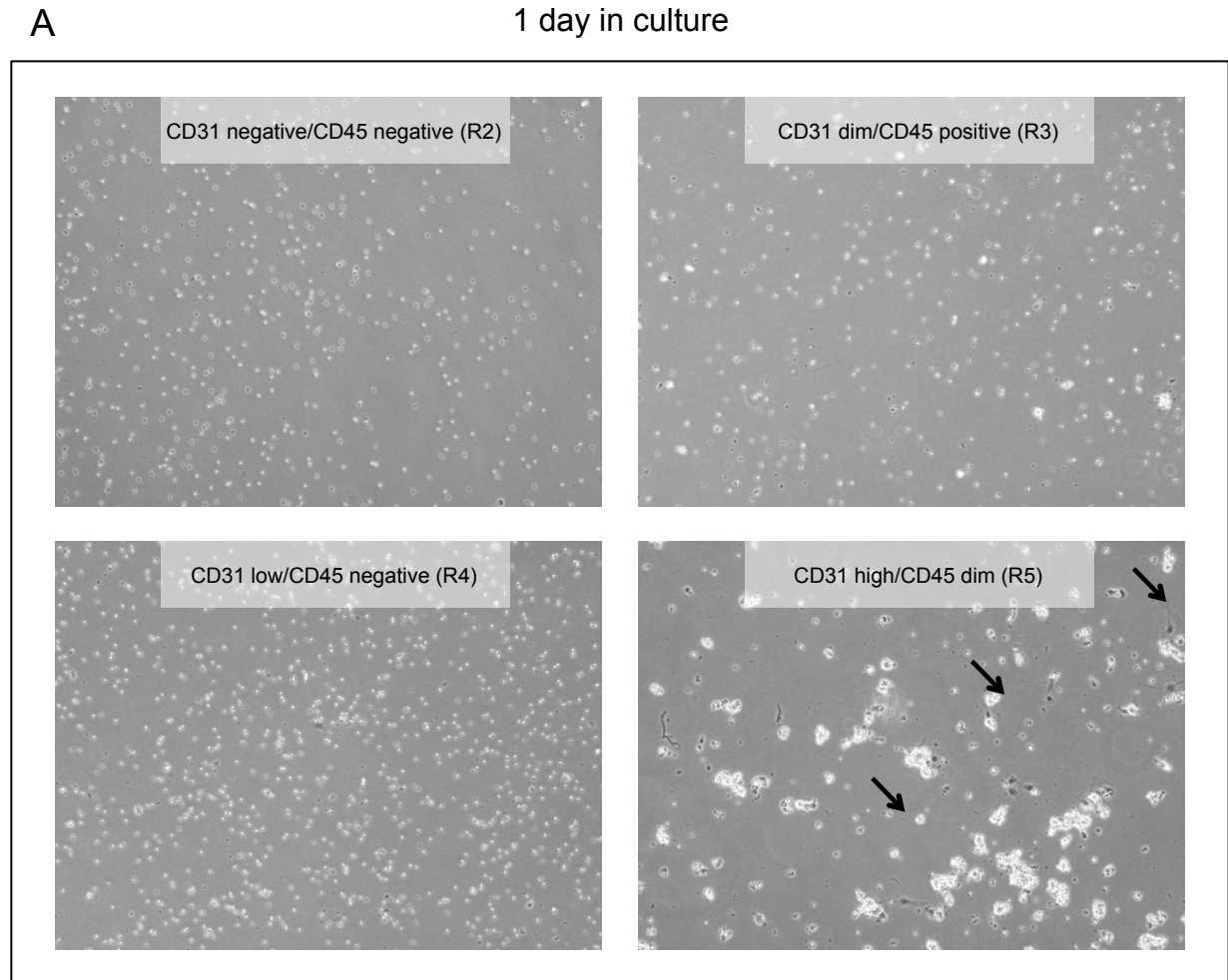
$\times 10^4$ CD31 negative/CD45 negative, $2.2 \pm 0.2 \times 10^5$ CD31 dim/CD45 positive, $7.8 \pm 0.4 \times 10^4$ CD31 low/CD45 negative and $6.7 \pm 0.7 \times 10^4$ CD31 high/CD45 dim cells were obtained per mouse lung. 2.4×10^5 to 3.8×10^5 cells per population were plated into 1 well of a 12 well dish.

24 hours after flow sorting all populations of CD31 MACS® MicroBead isolated cells had settled on the bottom of the wells but the majority of cells had not attached. Only in CD31 high/CD45 dim cultures there were clear signs of attachment with cells starting to spread and proliferate (Figure 3.20 A-B).

After 3 days in culture clear formation of colonies was observed in the CD31 high/CD45 dim population. All other populations only showed very few scattered cells (Figure 3.20 C).

After 6 days in culture large islands of cells had formed in the CD31 high/CD45 dim cell population (Figure 3.20 D), displaying the two phenotypes “streamlined” and “round” which had been observed in cultures of Dynabead® isolated endothelial cells (Figure 3.3) before. All other cell populations still only displayed very few cells attached to the well without formation of closely clustered colonies as in CD31 high/CD45 dim cells.

Figure 3.20

**Figure 3.20: Flow sorted cells in culture. Phase contrast microscopy.**

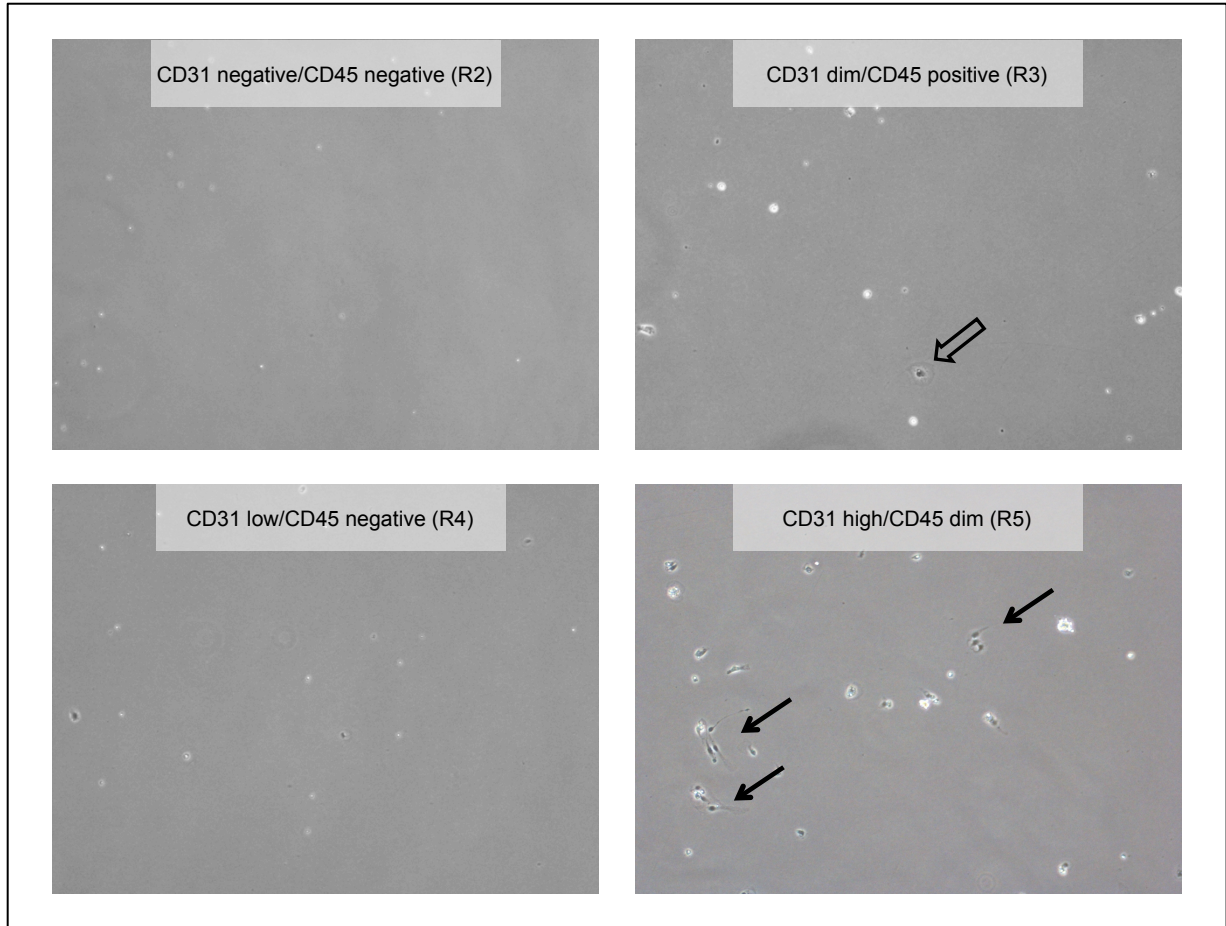
Cells were isolated from mouse lungs by CD31 MACS® MicroBead isolation using CD31 APC antibody. The isolated cells were then stained with CD45 PE-Cy7 and subjected to flow cytometry analysis and sorting. The sorted cells were plated into gelatine coated wells and cultured in EC medium.

A: 1 day after flow sorting. The cells have settled on the bottom of the wells but the majority does not appear to have attached. Only in CD31 high/CD45 dim (R5) cultures there are clear signs of attachment with cells starting to spread (→). R2-R5 also differ morphologically. R2 and R4 are homogenous populations of consist of very small cells, whereas R3 also contains some larger cells. R5 displays a more heterogenous morphology with less regularly shaped cells.

Figure 3.20

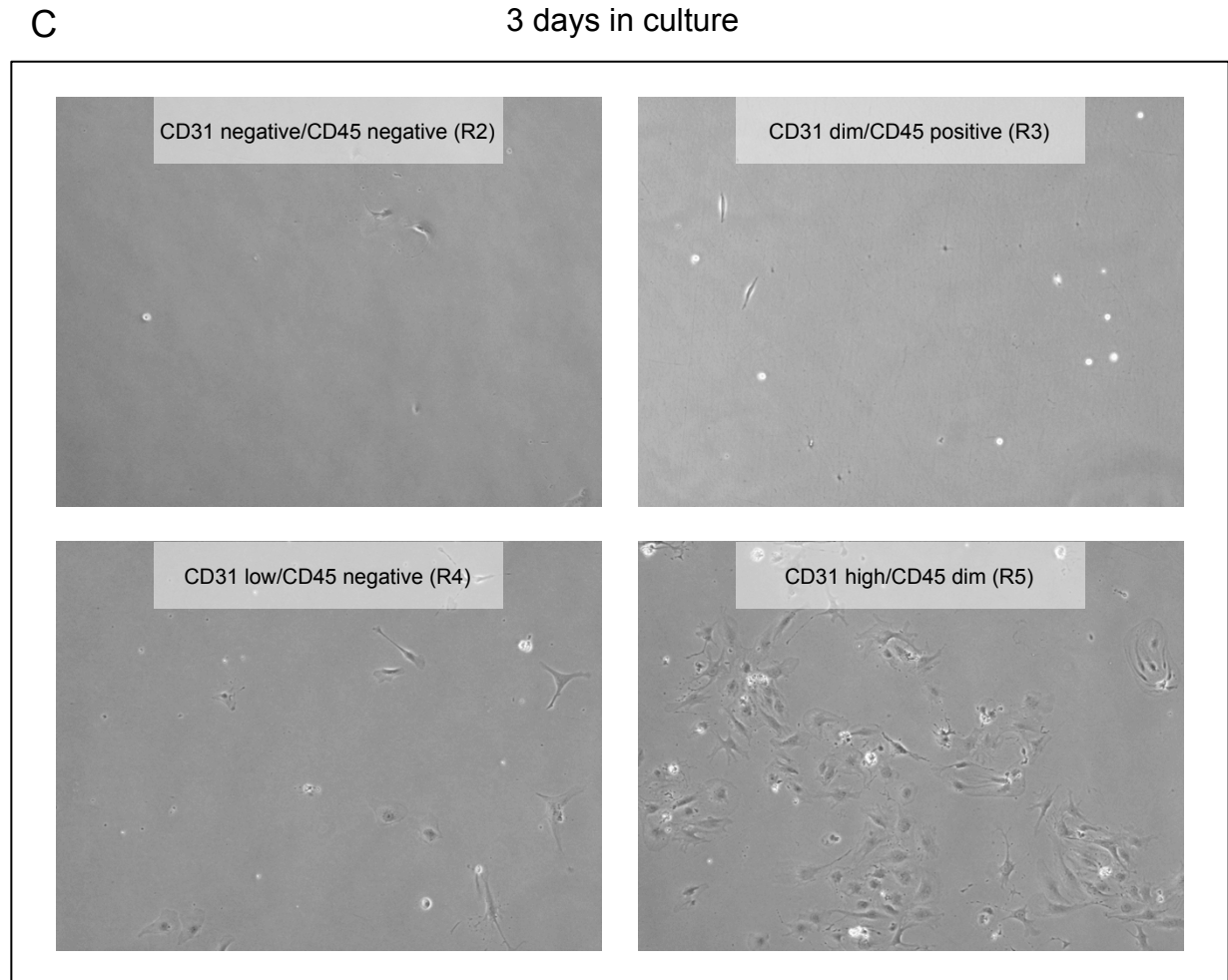
B

1 day in culture (after 2 washes with PBS)



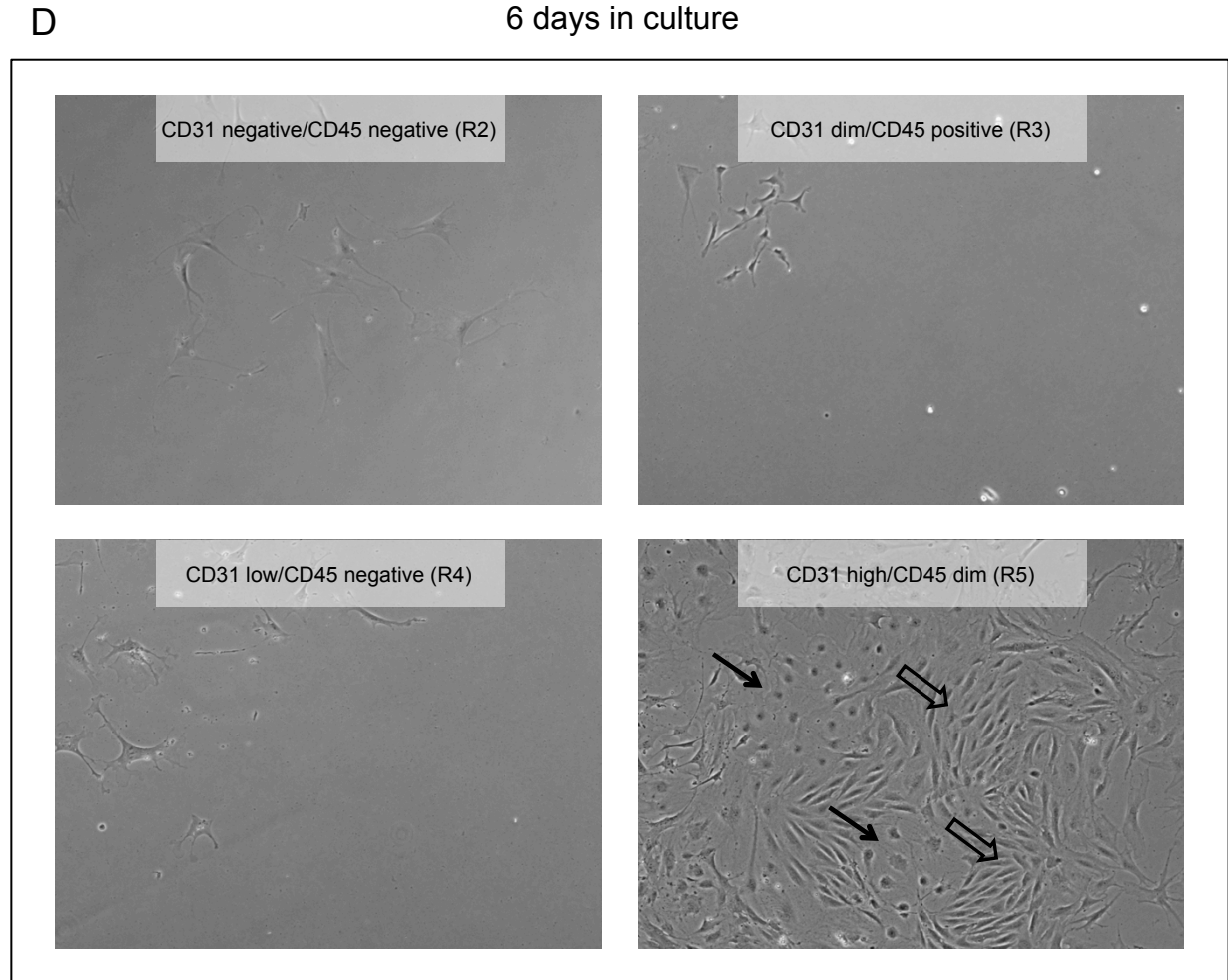
B: The cultures were washed twice with PBS to remove unattached cells. There are no clearly attached cells visible in R2 and R4 and only very few isolated attached cells (⇨) in R3. R5 however shows the first signs of cell proliferation (→).

Figure 3.20



C: After 3 days in culture clear formation of colonies can be observed in R5. R2, R3 and R4 only show very few scattered cells.

Figure 3.20



D: After 6 days in culture large islands of cells have formed in R5, displaying the two phenotypes - “streamlined” (⇨) and “round” (➡) – which have been observed in cultures of Dynabead® isolated EC before. R2, R3 and R4 still only show very few scattered cells.

The CD31 high/ CD45 dim population continued to proliferate in culture. After 21 days the cells were completely confluent and still displayed “streamlined” and “round” morphologies (Figure 3.21 A-B). The cells were harvested using Accutase™, washed with MACS buffer, Fc blocked (1/100 in MACS buffer) on ice for 15 minutes and stained with CD31 APC and CD45 PE-Cy7 or isotype controls (all 1/200 in MACS buffer) for 15 on ice in the dark. Flow cytometry of the formerly CD31 high/ CD45 dim population now showed two main subpopulations of cells: CD31 high/CD45 dim and CD31 negative/CD45 negative (Figure 3.21 C-D). There are two possible explanations for the occurrence of CD31 negative/CD45 negative cells. Either they originate from a small number of contaminating CD31/CD45 negative cells, which might have proliferated in the presence of CD31 high/CD45 dim cells, or part of the CD31 positive population has de-differentiated in culture and lost CD31 expression.

Figure 3.21

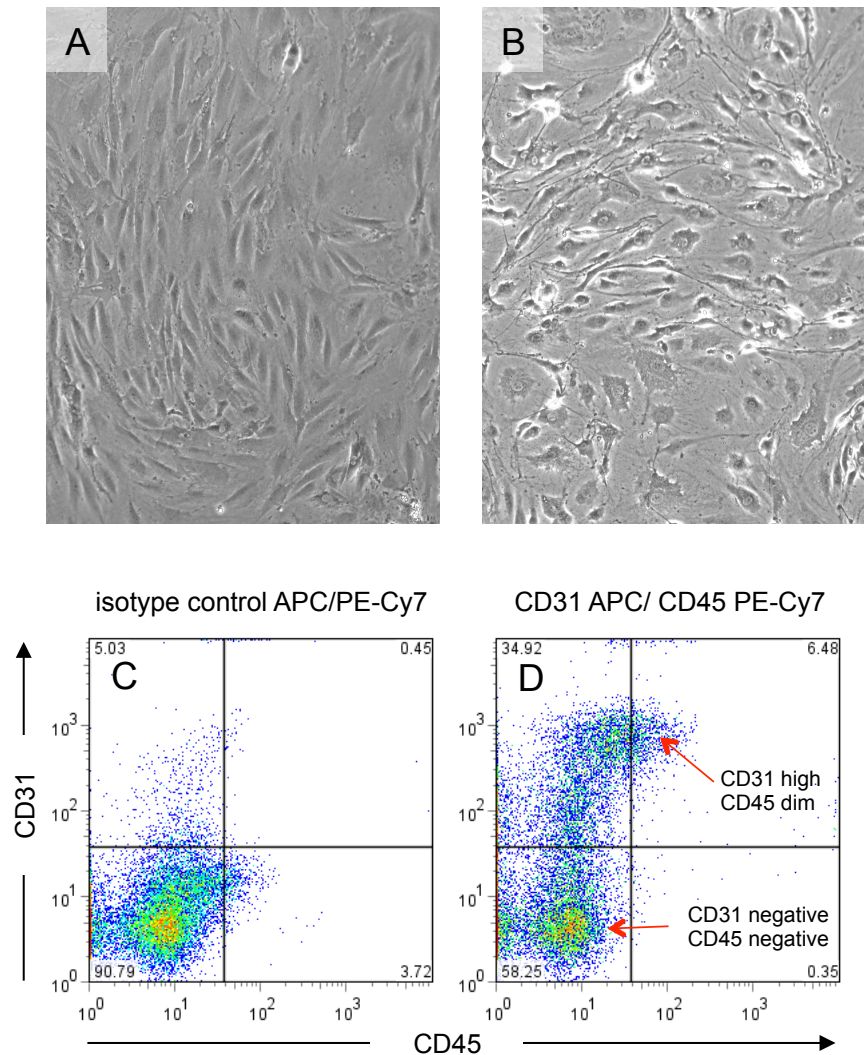


Figure 3.21: Flow sorted CD31 high/CD45 dim (R5) cells after 21 days in culture.

Cells were isolated from mouse lungs by CD31 MACS® MicroBead isolation using CD31 APC antibody. The isolated cells were then stained with CD45 PE-Cy7 and subjected to flow cytometry analysis and sorting. The sorted cells were plated into gelatine coated wells and cultured in EC medium. The CD31 high/CD45 dim (R5) population continued to proliferate in culture. After three weeks, when the cells were completely confluent, they were harvested and stained with CD31 APC and CD45 PE-Cy7 for flow cytometry analysis.

A-B: Phase contrast microscopy. The cells displayed the same two phenotypes ("streamlined" (A) and "round" (B)) which were also observed in culture from CD31 Dynabead® selected EC (Figure 3.3).

C-D: Flow cytometry. **C:** Isotype controls for APC and PE-Cy7. **D:** CD31 APC/CD45 PE-Cy7 stained cells. There were two main subpopulations of cells detectable: CD31 high/CD45 dim and CD31 negative/CD45 negative. There are two possible explanations for the occurrence of CD31 negative/CD45 negative cells. Either they originate from a small number of contaminating CD31/CD45 negative cells, which proliferate rapidly in the presence of CD31 high/CD45 dim cells, or part of the CD31 positive population de-differentiates in culture and loses CD31 expression.

3.5.5 Expression of endothelial cell markers and transgenic human GTPCH protein in flow sorted subpopulations of CD31 MACS® MicroBead selected cells

To identify the GCH transgene expressing population, cells were isolated from mouse lungs by CD31 MACS® MicroBead isolation and flow sorted as described above. The sorted cells were lysed at a concentration of 1.9×10^5 cells in 40µl in RIPA buffer and analyzed by western blotting (Figure 3.22).

Expression of eNOS and Tie-2 could only be detected in CD31 high/CD45 dim cells. CD102 expression also was the strongest in CD31 high/CD45 dim cells with only weak bands detectable in CD31 low/CD45 negative and CD31 dim/CD45 positive. Human GTPCH was only detectable in CD31 high/CD45 dim cells from GCH transgenic animals. These results directly correlate with the flow cytometry data for CD31 and CD102 expression in flow sorted CD31 MACS® MicroBead isolated cells (Chapter 3.5.2).

Figure 3.22

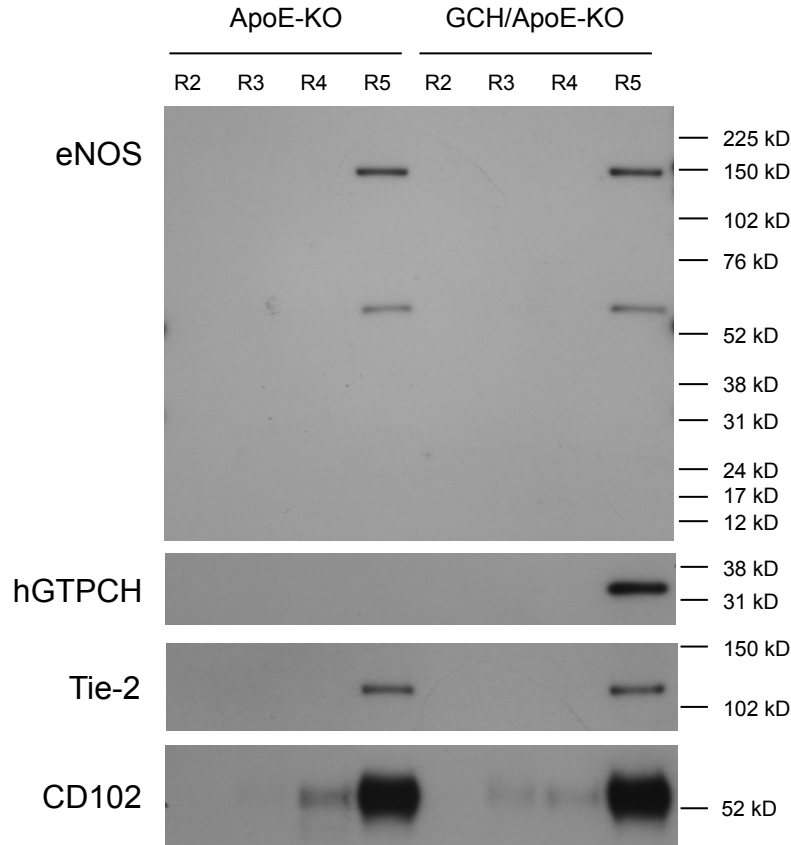


Figure 3.22: Expression of endothelial cell markers and hGTPCH in flow sorted CD31 MACS® MicroBead selected cells.

Cells were isolated from mouse lungs by CD31 MACS® MicroBead isolation using CD31 APC antibody. The isolated cells were then stained with CD45 PE-Cy7 and subjected to flow cytometry analysis and sorting. The sorted cells were lysed at a concentration of 1.9×10^5 cells in 40 μ l in RIPA buffer and analyzed by western blotting.

Expression of eNOS and Tie-2 could only be detected in CD31 high/CD45 dim cells (R5). eNOS was partly degraded as described before (Figure 3.15). CD102 expression also was the strongest in CD31 high/CD45 dim cells (R5) with only weak bands detectable in R4 > R3. hGTPCH was only detectable in CD31 high/CD45 dim cells (R5) from GCH transgenic animals.

These results directly correlate with the flow cytometry data for CD31 and CD102 (Figure 3.18).

3.5.6 X-Gal staining of flow sorted subpopulations of CD31 MACS® MicroBead selected cells

To identify the LacZ transgene expressing population of cells, cells were isolated from ApoE-KO and ApoE-KO/LacZ mouse lungs by CD31 MACS® MicroBead isolation and flow sorted as described above. Approximately 8×10^4 cells of each population were cytopspun onto poly-L-lysine coated slides at 20 g for 2 minutes (StatSpin Cytofuge 2). The slides were air dried, fixed and stained with X-Gal. Before cover-slipping with Neo-Mount® the cells were lightly stained with eosin. Only CD31 dim/CD45 positive and CD31 high/CD45 dim cells stuck permanently to the slides, staying in place during the X-gal staining procedure. CD31 negative/CD45 negative cells and CD31 low/CD45 negative cells are significantly smaller and it is possible that they might require significantly faster and longer spinning than the other populations to adhere to the slides.

There were no blue cells detected in CD31 dim/CD45 positive cells from Tie-2 Lac Z positive and Tie-2 LacZ negative mice (Figure 3.23 A and C) or CD31 high/CD45 dim cells from Tie-2 LacZ negative mice (Figure 3.23 D). The only population that showed blue cells was CD31 high/CD45 dim from Tie-2 Lac Z positive animals (Figure 3.23 B). Interestingly not all LacZ positive CD31 high/CD45 dim cells stained blue. Primary endothelial cell isolated from mouse lungs are a mixture of makro- and microvascular, arterial and venous endothelial cells. However, heterogeneity of Tie-2 expression across the vasculature has been reported before (Anghelina *et al.*, 2005) and might be a reason for the lack of blue staining in a large proportion of CD31 high/CD45 dim cells.

Figure 3.23

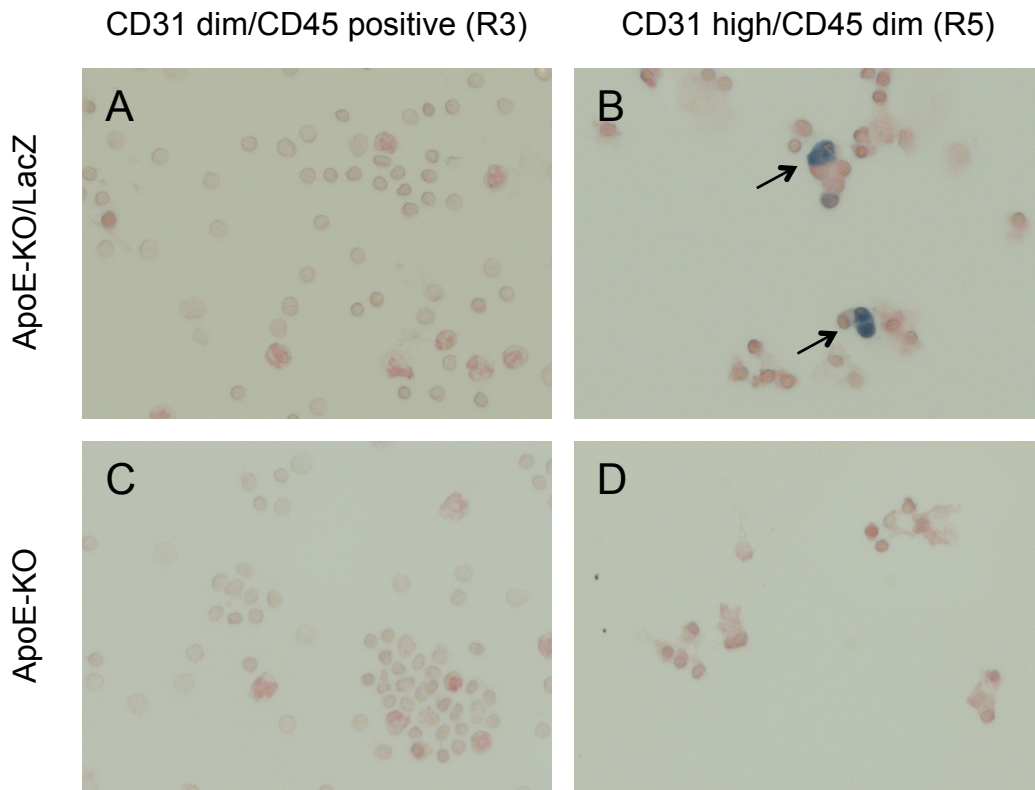


Figure 3.23: Flow sorted CD31 MACS® MicroBead selected cells. X-Gal staining of cytopun cells.

Cells were isolated from ApoE-KO and ApoE-KO/LacZ mouse lungs by CD31 MACS® MicroBead isolation using CD31 APC antibody. The isolated cells were then stained with CD45 PE-Cy7 and subjected to flow cytometry analysis and sorting. The sorted cells were cytopun onto poly-L-lysine coated slides, fixed, stained with X-Gal and lightly stained with eosin before cover-slipping with Neo-Mount®.

Only CD31 dim/CD45 positive (R3) and CD31 high/CD45 dim (R5) cells adhered permanently to the slides, staying in place during the x-gal staining procedure. It is possible that R2 and R4 might require significantly higher cytopinning speeds than R3 and R5 because of the small cell size. There were no blue cells detected in CD31 dim/CD45 positive (R3) cells from Tie-2 Lac Z positive and Tie-2 LacZ negative mice (**A** and **C**) or CD31 high/CD45 dim (R5) cells from Tie-2 LacZ negative mice (**D**). The only population that showed blue cells was CD31 high/CD45 dim (R5) from Tie-2 Lac Z positive animals (**B**).

When the flow sorted populations were plated onto gelatine coated plates and cultured in EC media, cell islands formed in the CD31 high/CD45dim population. After X-Gal staining on day 6, blue cell islands were exclusively observed in CD31 high/CD45dim flow sorted cells from Tie2-LacZ positive mice (Figure 3.24).

Figure 3.24

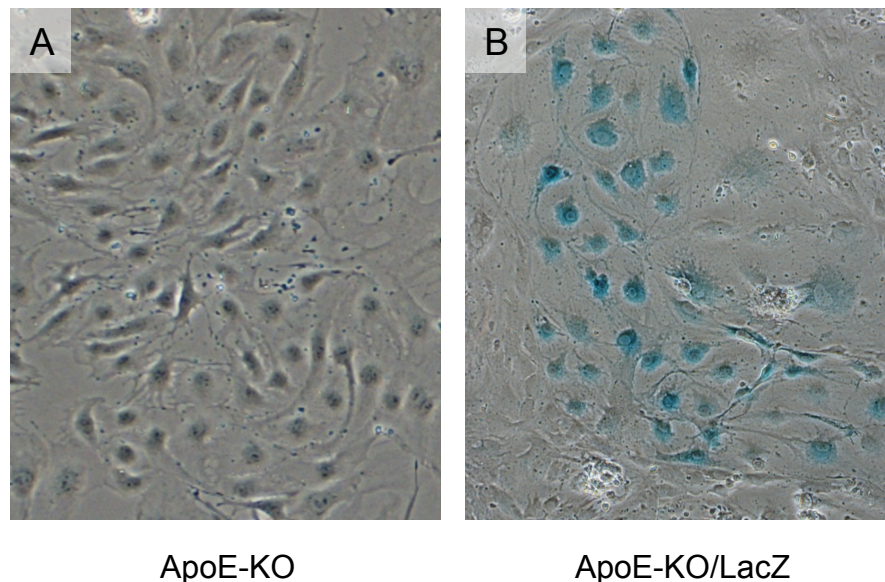


Figure 3.24: Flow sorted CD31 MACS® MicroBead selected cells. X-Gal staining of CD31 high/CD45 dim cells after 6 days in culture.

Cells were isolated from ApoE-KO and ApoE-KO/LacZ mouse lungs by CD31 MACS® MicroBead selection using CD31 APC antibody. The isolated cells were then stained with CD45 PE-Cy7 and subjected to flow cytometry analysis and sorting. The sorted cells were plated into gelatine coated wells and cultured in EC medium. After 6 days the cells were fixed and stained with X-Gal.

Blue staining was only observed in CD31 high/CD45dim flow sorted cells from Tie2-LacZ positive mice (B). However, not all CD31 high/CD45 dim cells stained blue.

3.5.7 Expression of CD34 and Flk-1 in subpopulations of CD31 MACS® MicroBead isolated cells

Endothelial progenitor cells are precursor cells with a high proliferative behavior that participate in neoendothelialization and neovascularization. Because of the striking differences in proliferation of cultured subpopulations of CD31 MACS® MicroBead isolated cells (Figure 3.20) the expression of endothelial progenitor markers CD34 and Flk-1 was assessed by flow cytometry (Figure 3.25).

Cells were isolated from mouse lungs by CD31 MACS® MicroBead isolation using CD31 FITC antibody. Isolated cells were blocked with Fc-block 1/50 in MACS buffer containing 10% rat serum (Sigma-Aldrich, UK) for 15 minutes on ice and stained with CD45 PE-Cy7, CD34 PE and Flk-1 APC antibodies (1/200, 1/100 and 1/100 in MACS buffer containing 10% rat serum) or the corresponding isotype controls. After 15 minutes incubation on ice in the dark the samples were washed with MACS buffer, fixed with 2% formaldehyde and analyzed by flow cytometry.

The CD31 high/CD45 dim population was the only population containing cells expressing both progenitor markers CD34 and Flk-1 (Figure 3.25 B). There was CD34 expression detectable in the CD31 low/CD45 negative population but no significant expression of Flk-1 (Figure 3.25 C). No significant expression of CD34 or Flk-1 could be detected in CD31 negative/CD45 negative cells (Figure 3.25 D). There was no Flk-1 expression detectable in CD31 dim/CD45 positive cells, either. However, PE fluorescence was heterogeneous within this population so it cannot be

excluded that a subpopulation of CD31 dim/CD45 positive cells expresses CD34 (Figure 3.25 E).

Figure 3.25: Expression of CD34 and Flk-1 in CD31 MACS® MicroBead isolated cells.

EC were isolated from mouse lungs by CD31 MACS® MicroBead isolation. CD31 FITC was used for the isolation and the cells were stained with CD45 PE-Cy7, CD34 PE and Flk-1 APC antibodies or the corresponding isotype controls for flow cytometry analysis.

A: With regard to CD31 and CD45 expression there were 4 populations identified, which were further analyzed for CD34/Flk-1 expression (**B-E**).

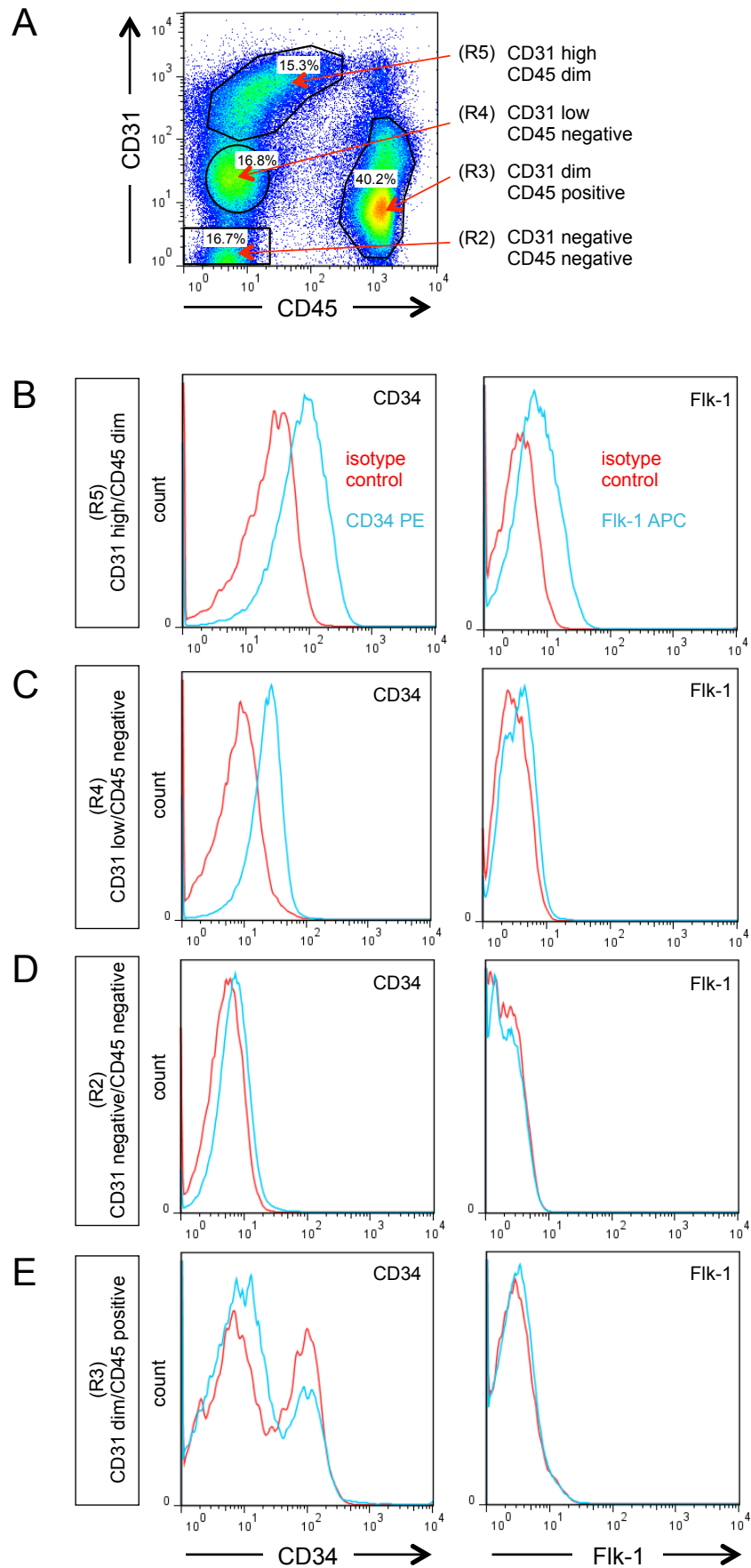
B: The CD31 high/CD45 dim population was the only population containing CD34/Flk-1 positive cells.

C: The CD31 low/CD45 negative population contained CD34 positive cells but did not show significant expression of Flk-1.

D: The CD31 negative/CD45 negative population did not show significant CD34 or Flk-1 expression.

E: There was no Flk-1 expression detectable in CD31 dim/CD45 positive cells. PE fluorescence was inhomogeneous within this population. It therefore cannot be excluded that a subpopulation of CD31 dim/CD45 positive cells expresses CD34.

Figure 3.25



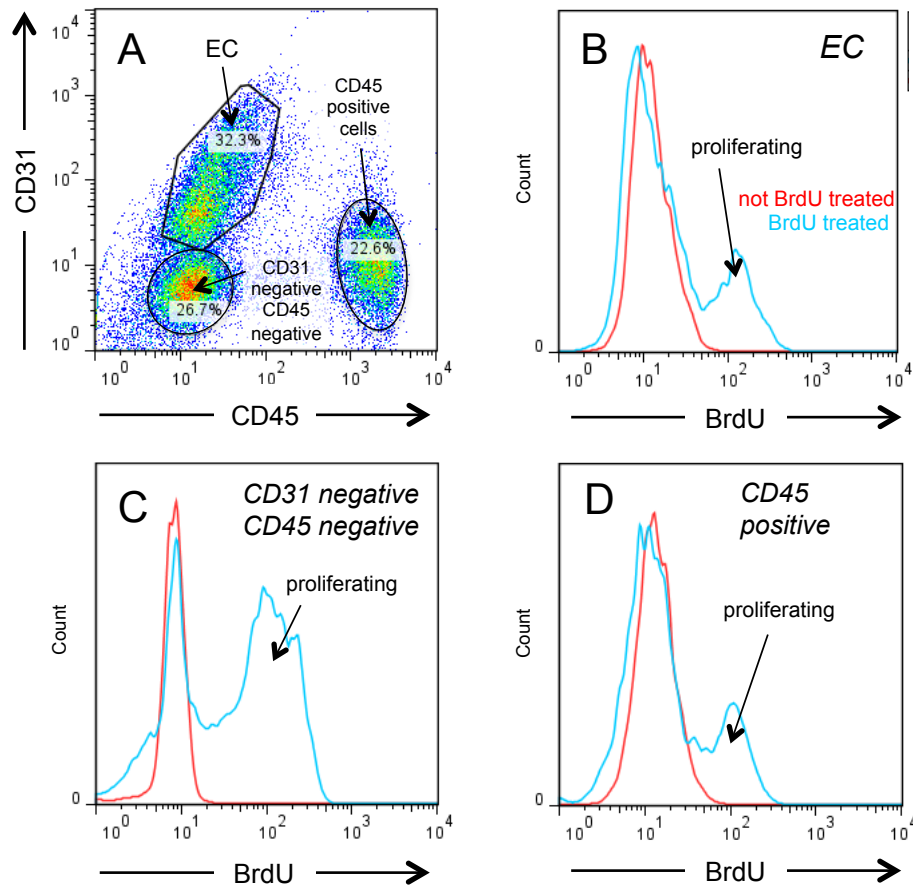
3.5.8 BrdU incorporation into cultured CD31 MACS® MicroBead selected cells

In order to monitor proliferation of CD31 MACS® MicroBead isolated cells a bromodeoxyuridine (BrdU) incorporation assay was performed. BrdU is an analog of the DNA precursor thymidine and is incorporated into newly synthesized DNA. Incorporated BrdU can be stained with fluorescent antibodies and quantified by flow cytometry.

Endothelial cells were isolated from mouse lungs by CD31 MACS® MicroBead isolation and cultured for five days. On day four BrdU was added to the culture media and the cells were harvested for flow cytometry analysis 16 hours later. A detailed protocol for the BrdU incorporation assay can be found in Chapter 2.14. In short, after Fc-blocking the cells were stained with CD31 APC and CD45 PE-Cy7 antibodies, followed by permeabilization and staining with BrdU FITC antibody. Cells that had not been treated with BrdU but had been stained with BrdU FITC antibody were used as a negative control.

With regard to CD31 and CD45 expression, there were three distinctive populations detectable after five days in culture: CD31positive/CD45 dim cells, CD45 positive cells and CD31negative/CD45 negative cells (Figure 3.26 A). In comparison to CD31 MACS® MicroBead isolated cells that had not been cultured, CD31 expression was lower and it was not possible to discriminate between CD31 high and CD31 low expressing populations.

Figure 3.26

**Figure 3.26: BrdU incorporation into cultured primary endothelial cells.**

EC were isolated from mouse lungs by CD31 MACS® MicroBead isolation and cultured for 5 days. On day 4 BrdU was added to the culture media and the cells were harvested 16 hours later for flow cytometry analysis.

A: CD31/CD45 staining revealed 3 distinctive cell populations: CD31positive/CD45 dim (EC), CD45 positive and CD31negative/CD45 negative.

B-D: Analysis of BrdU incorporation into the 3 populations.

All populations showed BrdU incorporation. In contrast to the CD31 high/CD45 dim cells being the only proliferating population in culture after flow sorting, it appears that in presence of all CD31 MACS® MicroBead isolated cells all populations have the ability to proliferate in culture.

All populations showed BrdU incorporation (Figure 3.26). This finding is unexpected after discovering that the CD31 high/CD45 dim population is the only population that significantly proliferates in culture after flow sorting (Chapter 3.5.4, Figure 3.20). The appearance of CD31 negative/CD45 negative cells in flow sorted CD31 high/CD45 dim cells after three weeks in culture (Figure 3.21) suggests that loss of CD31 expression in culture might at least contribute to the formation of a larger CD31 negative/CD45 negative population. It is also possible that CD31 dim/CD45 positive and CD31 negative/CD45 negative cells have the ability to proliferate in culture in the presence of CD31 high/CD45 dim cells but not separately.

3.6 Isolation of primary endothelial cells from mouse hearts

To investigate whether CD31 MACS® MicroBead selection can be used to isolate endothelial cells from other mouse organs than lungs, cells were isolated from mouse hearts by CD31 MACS® MicroBead isolation as described for lungs in Chapter 2.3.2. The isolated cells were cultured on gelatine coated dishes in EC media (1 well of a 24 well dish per heart). After 6 days in culture large islands of cells had formed which could morphologically not be distinguished from lung endothelial cells, displaying “streamlined” and “round” morphologies (Figure 3.27).

To determine whether CD31 MACS® MicroBead selected cells from hearts also express the endothelial cell progenitor markers CD34 and Flk-1, cells were isolated using CD31 FITC antibody and stained with CD45 PE-Cy7, CD34 PE and Flk-1 APC antibodies or the corresponding isotype controls as described above (Chapter 3.5.7). Flow cytometry plots of heart endothelial cells are shown in Figure 3.28.

Figure 3.27

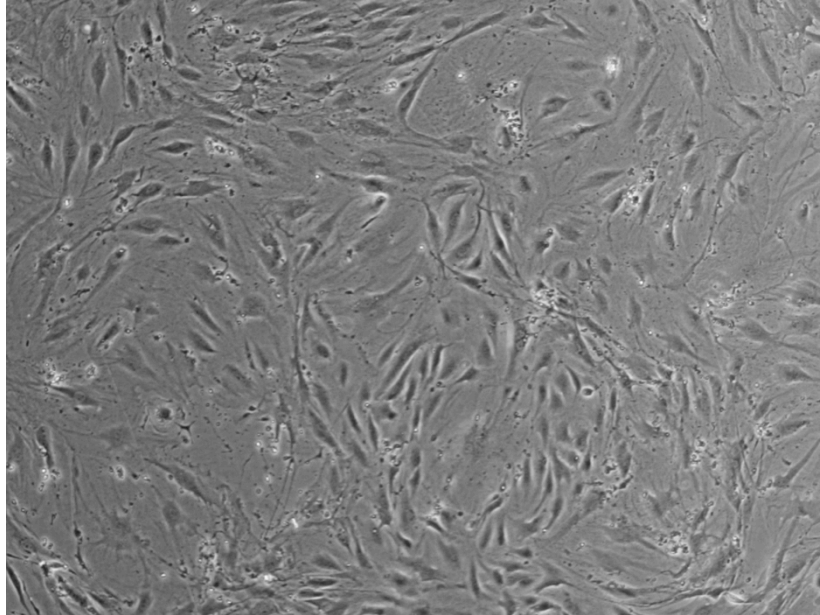


Figure 3.27: Phase contrast microscopy of EC isolated from mouse hearts after 6 days in culture.

EC were isolated from mouse hearts by CD31 MACS® MicroBead isolation and cultured on gelatine coated dishes in EC media for 6 days. The growing cells could morphologically not be distinguished from lung EC.

Figure 3.28

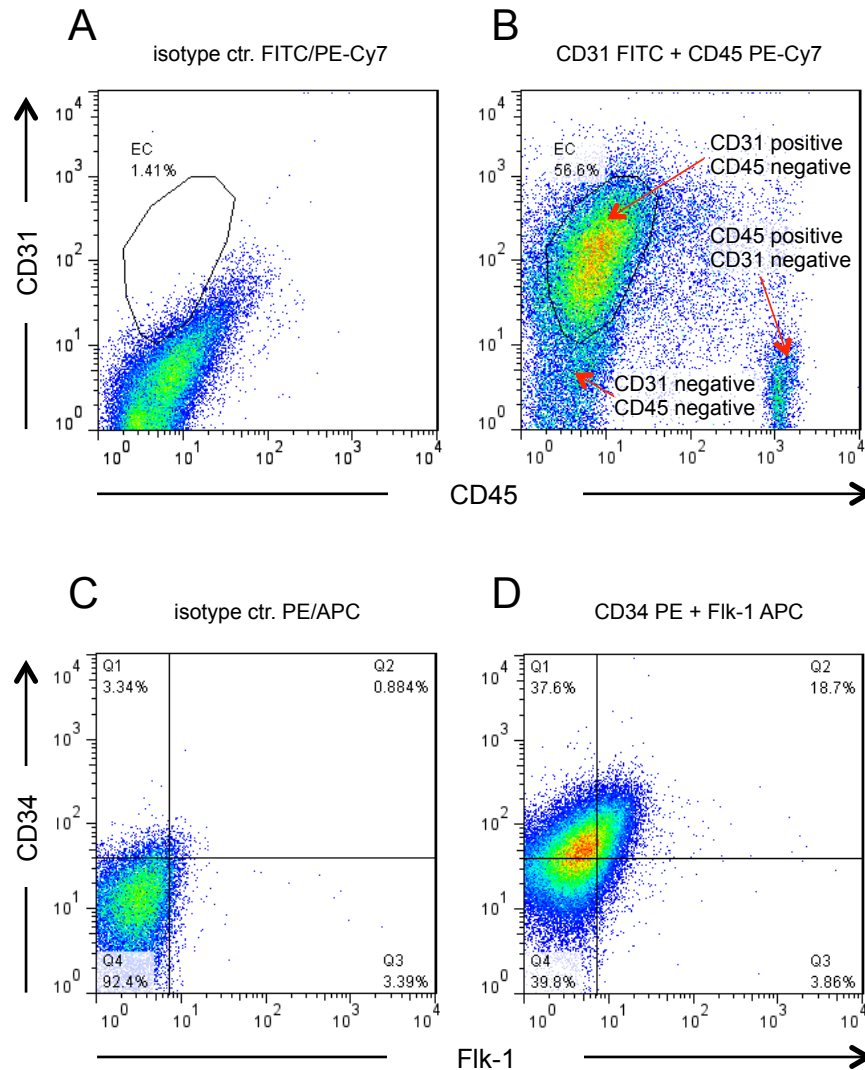


Figure 3.28: Expression of CD34 and Flk-1 in CD31 MACS® MicroBead isolated cells from mouse hearts.

EC were isolated from mouse hearts by CD31 MACS® MicroBead isolation. CD31 FITC was used for the isolation and the cells were stained with CD45 PE-Cy7, CD34 PE and Flk-1 APC antibodies or the corresponding isotype controls for flow cytometry analysis.

A-B: For EC quantification a gate was applied around the CD31 positive/CD45 negative population.

C-D: Flk-1 and CD34 positive cells were quantified within the EC gate.

In comparison to CD31 MACS® MicroBead isolated cells from lungs, the cell isolates from hearts contain fewer CD45 positive and fewer CD31 negative/CD45 negative cells (Figure 3.25). Also, the CD31 positive/CD45 negative population (EC) from hearts appears to have slightly lower CD31 and CD45 fluorescence than the EC population from lungs (CD31 high/CD45 dim).

In hearts, the content of Flk-1/CD34 positive cells within the EC gate is similar to lungs (Figure 5.6).

With regard to CD31 and CD45 expression, there were three populations detectable: CD31 positive/CD45 negative cells that formed the largest population, CD31 negative/CD45 negative cells and CD45 positive/CD31 negative cells. In comparison to CD31 MACS® MicroBead isolated cells from lungs, the cell isolates from hearts contained fewer CD45 positive and fewer CD31 negative/CD45 negative cells (Figure 3.25). Also, the endothelial cell population (CD31 positive/CD45 negative) from hearts appeared to have slightly lower CD31 and CD45 fluorescence than the corresponding population from lungs (CD31 high/CD45 dim).

Gating on the CD31 positive/CD45 negative population of CD31 MACS® MicroBead isolated heart cells, the content of CD34 positive/Flk-1 positive cells within the CD31 positive/CD45 negative gate from hearts was similar to the content of Flk-1/CD34 positive cells within the CD31 high/CD45 dim gate from lungs (Figure 5.6).

In conclusion, CD31 MACS® MicroBead selection can be used to isolate endothelial cells from mouse hearts. These cells proliferate in culture and a proportion of the isolated endothelial cells also expresses the endothelial progenitor markers CD34 and Flk-1. However, the yield of endothelial cells per mouse is considerably lower using the heart as a source compared to the lungs and proliferation of the isolated heart endothelial cells in culture is slower than proliferation of lung endothelial cells. Further investigation regarding expression of transgenes and endothelial cell markers in cultured heart endothelial cells would be necessary to routinely use these cells for experiments.

3.7 Summary and Discussion

Primary endothelial cells provide a useful *in vitro* model in cardiovascular research, bearing closer resemblance to *in vivo* endothelial cell physiology than immortalized cell lines such as sEND-1 cells. Using mice as donor animals is particularly useful because of the availability of genetic modifications in this species. Primary murine endothelial cells have been used for various research applications and several different protocols have been deployed for their isolation and culture. However, in comparison to immortalized cell lines and commercially available endothelial cells of human or bovine origin, primary mouse endothelial cell culture is still a poorly defined system. Potential limitations of this model are the very limited amount of cells that can be isolated from each mouse and loss of endothelial cell phenotype and biological function over time in cell culture.

The aim of this chapter was to determine the optimal selection conditions and time scale to achieve a pure endothelial cell culture that provides sufficient material for biochemical analysis and displays the biological phenotype and functionality of the donor animal.

The key findings of this chapter are:

- Primary endothelial cells can be reproducibly isolated from mouse lungs by CD31 immunomagnetic bead selection with Dynabeads® or MACS® MicroBeads
- The isolated primary endothelial cells display typical microvascular endothelial cell morphology, express the endothelial cell markers CD31, CD102, Tie-2 and eNOS and have detectable eNOS activity.
- GCH and LacZ transgenes under the control of the Tie-2 promoter are specifically expressed in primary endothelial cells but not in non-endothelial cells or vascular smooth muscle cells.
- More than one immunomagnetic bead selection is necessary to obtain a pure endothelial cell culture prolonging total culture time. However, time in cell culture is a major determinant of the biological phenotype of endothelial cells.
- A subpopulation of CD31 immunomagnetic isolated cells expresses the endothelial progenitor cell markers CD34 and Flk-1. This subpopulation is also the only population expressing GCH and LacZ transgenes and the only population proliferating in culture after flow sorting.

The results presented in this chapter demonstrate that although endothelial cells can be reproducibly isolated from mouse lungs by CD31 immunomagnetic bead selection, the isolation and culture conditions are crucial determinants of the biological phenotype of the isolated cells. Several protocols for the isolation of primary endothelial cells from mouse lungs have been published (Table 3.1) and

immunomagnetic bead selection with CD31 or CD102 is the most commonly used method. However, the protocols vary widely with regard to the total number of selection procedures and time endothelial cells spend in culture before they are included into experiments. This study found that a single magnetic bead selection is not sufficient to obtain a pure endothelial cell culture due to contamination with rapidly growing non-endothelial cells, a finding that is supported by the majority of the published studies (Dong *et al.*, 1997; Fleming *et al.*, 2005; Kuhlencordt *et al.*, 2004; Lim *et al.*, 2003; Schniedermann *et al.*, 2010). The data presented in this chapter also indicates that the time consuming process of purifying primary endothelial cells by reselection leads to significant changes in phenotype with some endothelial cell markers (e.g. Tie-2) being down-regulated more rapidly than others (e.g. eNOS). Most importantly, the expression of the GCH transgene is also down-regulated during the reselection process. Fujikawa *et al.* have previously shown that the level of Tie-2 expression in endothelial cells depends on cell density *in vitro* and *in vivo* with lower levels of Tie-2 protein present at a low cell density *in vitro* and during the repopulation phase after endothelial denudation *in vivo* (Fujikawa *et al.*, 1999). The regulatory mechanisms leading to a reduction in Tie-2 expression at low cell density are unknown but it is possible that they also influence the expression of the GCH transgene, which is under the control of the Tie-2 promoter and intronic enhancer. Due to the small number of endothelial cells isolated per mouse lung most published studies on primary mouse endothelial cells reselect and passage the cells over a considerable period of time (Dong *et al.*, 1997; Fehrenbach *et al.*, 2009; Fleming *et al.*, 2005; Lim *et al.*, 2003; Marelli-Berg *et al.*, 2000; Schniedermann *et al.*, 2010). However, despite qualitatively maintaining expression of endothelial cell markers, purifying and passaging primary mouse lung endothelial cells may not be

applicable depending on the scientific question. For experiments relying on the expression of Tie-2 driven transgenes it may therefore be advisable to carry out experiments between days three and five after the initial isolation to avoid significant changes in biological phenotype of cultured primary endothelial cells. At this time there is still strong expression of endothelial cell markers and GCH transgene detectable and full size eNOS protein, which was lost after the initial selection, has been regenerated. Alternatively CD31 Immunomagnetic bead selection with MACS® MicroBeads followed by flow sorting achieves an endothelial cell population that produces large endothelial cell islands within four to six days in culture without the need of reselection. However, this procedure requires a large number of mice (4 donor mice per 1 well of a 12 well dish) and is time consuming, cost intensive and not suitable for large batched experiments.

It has further been confirmed in this chapter that GCH and LacZ transgenes under the control of the Tie-2 promoter are expressed specifically in primary endothelial cells. Previously, endothelial specificity of human GCH expression had not been shown directly but biopterin, GCH mRNA and GTPCH protein were measured in whole organs or aorta (Alp *et al.*, 2004; Alp *et al.*, 2003). Endothelial localization of β -galactosidase activity in Tie-2 LacZ transgenic mice on the other hand had been visualized by X-Gal staining, immunostaining or *in situ* hybridization in organs and blood vessels (Anghelina *et al.*, 2005; Feng *et al.*, 2011; Schlaeger *et al.*, 1997). Here endothelial specificity of both human GCH and LacZ expression is verified directly for the first time using primary endothelial cells.

Flow cytometry analysis of CD31 immunomagnetic isolated cells from lungs showed that the CD31 high/CD45 dim subpopulation is the only population expressing human GCH and LacZ transgenes and also the only population capable of proliferating in culture after flow sorting. Further analysis revealed that this subpopulation contains CD31^{high}/CD45^{dim}/CD34⁺/Flk-1⁺ cells, suggesting endothelial progenitor properties within this population. Table 3.4 provides an overview over the properties of the different subpopulations of CD31 MACS® MicroBead isolated cells.

		Subpopulations			
		R2	R3	R4	R5
Flow cytometry	CD31	negative	dim	low	high
	CD102	negative	dim	low	high
	CD45	negative	positive	negative	dim
	CD34 ⁺ /Flk-1 ⁺ cells	no	no	no	yes
Western blotting	eNOS	-	-	-	+
	CD102	-	(+)	(+)	+
	Tie-2	-	-	-	+
	hGCH	-	-	-	+
X-Gal staining	Blue cells	NA	no	NA	yes
Flow sorting	Proliferation in culture	no	no	no	yes

Table 3.4: Subpopulations of CD31 MACS® MicroBead isolated cells. CD31 high/CD45 dim cells (R5) express high levels of the endothelial cell markers CD102, eNOS and Tie-2. This subpopulation is the only population that expresses the hGCH and LacZ transgenes, contains cells that express both CD34 and Flk-1 and is also the only subpopulation that proliferates in culture after flow sorting.

Endothelial progenitor cells (EPCs) are a heterogeneous group of precursor cells that participate in neoendothelialization and neovascularization. They can be found resident in bone marrow (Asahara *et al.*, 1999; Loomans *et al.*, 2006), as bone marrow derived circulating EPCs in peripheral blood (Iwasaki *et al.*, 2007; Shi *et al.*, 1998) and resident in the vascular wall (Ingram *et al.*, 2005; Yao *et al.*, 2009; Zengin *et al.*, 2006) and lung tissue (Alvarez *et al.*, 2008). There is no single conclusive marker to define EPCs; therefore a combination of several endothelial and progenitor markers is commonly used to measure EPC content. Table 3.5 provides an overview over cell surface markers used for the identification of EPCs with examples from the literature. Iwasaki and colleagues identified a population of CD31^{high}/CD45^{dim}/CD34⁺ cells from human peripheral blood as EPCs that improved neovascularization in rats after myocardial infarction (Iwasaki *et al.*, 2007). These EPCs exhibit a similar profile of cell surface markers to the cells isolated from mouse lungs in this thesis. Alvarez and colleagues described resident microvascular endothelial progenitor cells cultured from rat lungs that express the endothelial cell markers CD31, CD144 and eNOS and the progenitor markers CD34 and Flk-1 (Alvarez *et al.*, 2008). Finally Schniedermann *et al.* investigated cultured endothelial cells with a high proliferative potential that had been isolated from mouse lungs by CD31 Dynabead® isolation and found expression of the endothelial cells markers CD31, CD102, CD105 and MECA-32 and progenitor markers CD34, Flk-1 and CD133 (Schniedermann *et al.*, 2010). This study was published in July 2010 shortly after the flow cytometry experiments in this thesis investigating CD34 and Flk-1 expression in CD31 MACS® MicroBead isolated cells had been initiated. Although this affects the novelty of the findings regarding EPC content in CD31 immunomagnetic isolated cells from mouse lungs, the results of this thesis and

Schniedermann *et al.* are complementary. Unlike the other publications investigating cultured EPCs from mouse or rat lungs (Alvarez *et al.*, 2008; Schniedermann *et al.*, 2010) the experiments presented herein have been undertaken on freshly CD31 immunomagnetic isolated cells. The use of multiplex flow cytometry allows quantitative analysis of subpopulations of CD31 immunomagnetic isolated cell from mouse lungs (Chapter 5.2.3.3). In this current study CD31^{high}/CD45^{dim}/CD34⁺/Flk-1⁺ cells (endothelial cells expressing progenitor markers) have also been detected in CD31 MACS® MicroBead isolated cells from mouse hearts, suggesting that the described population of resident EPCs is not exclusive to lung.

Publication	Endothelial progenitors	Endothelial markers								Progenitor markers					Pan-leukozyte marker
		CD31	CD102	CD105	CD144	Tie-2	eNOS	MECA-32	vWf	CD34	Flk-1	CD133	Sca-1	CD117	CD45
Asahara <i>et al.</i> , 1997	Circulating, human peripheral blood	+				+	+			+	+				+
Iwasaki <i>et al.</i> , 2007	Circulating, human peripheral blood	+								+	+				(+)
Peichev <i>et al.</i> , 2000	Circulating, human peripheral blood									+	+				
Ceradini <i>et al.</i> , 2004	Circulating, human peripheral blood	+									+				
Ingram <i>et al.</i> , 2005	Resident, human aorta	+		+	+			+	+	+	+				-
Ingram <i>et al.</i> , 2004	Circulating, human umbilical cord blood	+		+	+			+	+	+	+				-
	Circulating, human peripheral blood	+		+	+			+	+	+	(+)				-
Urao <i>et al.</i> , 2006	Circulating, human umbilical cord blood	+		+	+			+	+	+	(+)				-
	Circulating, mouse peripheral blood									+	+		+		(+)
Laufs <i>et al.</i> , 2004	Circulating, mouse peripheral blood									+	+	+	+		
	Resident, mouse bone marrow									+	+	+	+		
Schroder <i>et al.</i> , 2009	Circulating, mouse peripheral blood									+	+	+	+		
Xie <i>et al.</i> , 2010	Circulating, mouse peripheral blood									+	+	+	+		
Zhang <i>et al.</i> , 2006	Resident, mouse bone marrow												+		
Zengin <i>et al.</i> , 2006	Resident, human vascular wall (IMA)	-		-	-	+		+	+	+	+				
Alvarez <i>et al.</i> , 2008	Resident microvascular, rat lung	+			+		+	+	+	+	+	-			-
Schniedermann <i>et al.</i> , 2010	Resident microvascular, mouse lung	+	+	+					+	+	+	+			-
Current study	Resident microvascular, mouse lung	+	+			+	+		+	+	+				(+)

Table 3.5: Markers most commonly used to identify EPCs and examples of publications. “+”, “(+)” and “-” indicate “expression”, “low expression” and “no expression” respectively. There is no single conclusive marker to define EPCs.

Chapter 4:

Characterization of Genetic Modifications in BH4 Synthesis using Primary Murine Endothelial Cells

4 Characterization of Genetic Modifications in BH4 Synthesis using Primary Murine Endothelial Cells

4.1 Introduction

Tetrahydrobiopterin (BH4) is an essential cofactor for all three isoforms of NOS (Cosentino and Luscher, 1999; Kwon *et al.*, 1989; Mayer *et al.*, 1990). It is located close to the active site of the enzyme and acts as an intermediate electron donor to the haem group in the oxygenase domain, coupling oxygen reduction to L-arginine oxidation during NO synthesis (Stuehr *et al.*, 2001). An important aspect of endothelial dysfunction states is a reduction of BH4 availability, resulting in eNOS uncoupling, whereby oxygen reduction becomes enzymatically ‘uncoupled’ from L-arginine oxidation and eNOS generates superoxide rather than NO (Vasquez-Vivar *et al.*, 2003; Vasquez-Vivar *et al.*, 1998; Xia *et al.*, 1998). BH4 supplementation has been found to restore endothelial function in animal models of atherosclerosis (Hattori *et al.*, 2007), arterial hypertension (Hong *et al.*, 2001) and diabetes mellitus (Pieper, 1997) as well as in patients with coronary artery disease (Maier *et al.*, 2000) or cardiovascular risk factors such as hypercholesterolemia (Stroes *et al.*, 1997), diabetes mellitus (Heitzer *et al.*, 2000b), arterial hypertension (Higashi *et al.*, 2002) and smoking (Heitzer *et al.*, 2000a; Ueda *et al.*, 2000).

The initial and rate-limiting reaction of the *de novo* synthesis of BH4 is catalyzed by GTP cyclohydrolase 1 (GTPCH), encoded by the GCH gene (Thony *et al.*,

2000). It has been shown that endothelial specific GCH overexpression in the GCH transgenic mouse (GCH mouse), which overexpresses human GCH under the control of the Tie-2 promoter, is sufficient to restore eNOS coupling and NO bioavailability in various vascular disease states including diabetes (Alp *et al.*, 2003; Tie *et al.*, 2009), arterial hypertension (Du *et al.*, 2008) and hypercholesterolemia (Alp *et al.*, 2004). In hypercholesterolemic ApoE-KO mice transgenic endothelial BH4 augmentation reduces atherosclerosis (Alp *et al.*, 2004; Takaya *et al.*, 2007) and neointimal hyperplasia after vascular injury (Ali *et al.*, 2008). BH4 deficiency in the hph-1 mouse on the other hand has been shown to lead to endothelial dysfunction and pulmonary hypertension, which can be rescued by endothelial specific GCH overexpression (Khoo *et al.*, 2005).

The effects of genetic modifications in GCH expression on BH4 bioavailability and endothelial cell function have only been investigated in whole organs or blood vessels so far and the focus of this chapter is to determine the effects of genetic alterations of GCH expression on BH4 bioavailability directly in primary endothelial cells of various genetic backgrounds.

Hypothesis:

The central hypothesis of this chapter is that genetic modifications in GCH expression will lead to sustained changes in BH4 availability in cultured primary mouse endothelial cells.

Aims:

Using primary lung endothelial cells isolated from mice with and without genetic alterations in GCH expression, the experimental aims of this chapter are:

- To investigate the effect of genetic modifications in GCH expression on BH4 levels in primary endothelial cells.
- To determine the relationship of GTPCH protein content to BH4 availability in cultured primary endothelial and non-endothelial cells with and without genetic modifications in GCH expression.
- To evaluate the influence of time spent in culture on BH4 bioavailability in primary endothelial cells.

4.2 Biopterin levels in primary endothelial cells

Primary mouse endothelial cells were isolated by CD31 Dynabead® selection from mouse lungs after enzymatic tissue digest and snap frozen. BH4, BH2 and biopterin were measured by electrochemical and fluorescence detection respectively, following sample separation by HPLC.

4.2.1 Primary endothelial cells with and without transgenic overexpression of eNOS and/or GCH

GCH transgenic mice overexpress human GCH under the control of the Tie-2 promoter specifically in endothelial cells (Alp *et al.*, 2003) (Chapter 3.3.5) and eNOS transgenic mice overexpress bovine eNOS under the murine preproendothelin-1 promoter. Transgenic overexpression of eNOS had no significant effect on BH4 content in primary endothelial cells. Overexpression of

GCH resulted in a greater than 10-fold increase in BH4 levels in primary endothelial cells on WT or eNOS background (Figure 4.1 A).

In comparison to BH4 levels measured in aortas from WT or eNOS transgenic mice (Bendall *et al.*, 2005), primary endothelial cells contained higher levels of BH4. The relative increase in BH4 levels due to transgenic GCH overexpression was much greater in primary endothelial cells compared to whole aorta (>10-fold versus 2 to 3-fold respectively). In aorta, the endothelial cell monolayer only accounts for a small proportion of the total vessel wall mass, “diluting” the effect of endothelial specific GCH overexpression. In contrast to eNOS transgenic aortas, which had significantly reduced BH4 levels in comparison to WT (Bendall *et al.*, 2005), no significant difference in BH4 content between primary endothelial cells from WT or eNOS transgenic mice could be detected.

4.2.2 Primary endothelial cells from WT and hph-1 mice with and without transgenic overexpression of GCH

Hph-1 mice carry a point mutation leading to underexpression of GTP cyclohydrolase I (Khoo *et al.*, 2004) and an 85% reduction in lung BH4 levels in homozygote hph-1 mice (hph mice) has been reported previously (Khoo *et al.*, 2005). In comparison to BH4 levels measured in lungs from WT and hph mice (Khoo *et al.*, 2005), primary endothelial cells contained higher levels of BH4. BH4 levels in primary lung endothelial cells from hph mice were reduced by 77% compared to WT (Figure 4.1 B).

Presence of the GCH transgene increased BH4 levels by more than 10-fold on WT background and more than 25-fold on hph background. The relative increase in BH4 levels due to transgenic GCH overexpression was much greater in primary endothelial cells compared to lungs, both on WT background (>10-fold versus 2-fold respectively) and hph background (>25-fold versus 6-fold). This is most likely due lung tissue containing a large proportion of non-endothelial cells, which do not express the GCH transgene. As observed in lungs (Khoo *et al.*, 2005), BH4 levels in hph/GCH endothelial cells were significantly lower than in GCH endothelial cells. The magnitude of reduction in BH4 content in hph/GCH endothelial cells compared to GCH however cannot simply be explained by the reduction in endogenous GTPCH activity in hph endothelial cells compared to WT.

4.2.3 Primary endothelial cells from ApoE-KO mice with and without transgenic overexpression of GCH

BH4 levels in primary endothelial cells from GCH/ApoE-KO mice were increased by approximately 5-fold compared to ApoE-KO (Figure 4.1 C). Again, the relative increase in BH4 levels due to transgenic GCH overexpression was greater in primary endothelial cells than reported for aorta previously (Alp *et al.*, 2004) (~5-fold versus ~3-fold respectively).

Figure 4.1

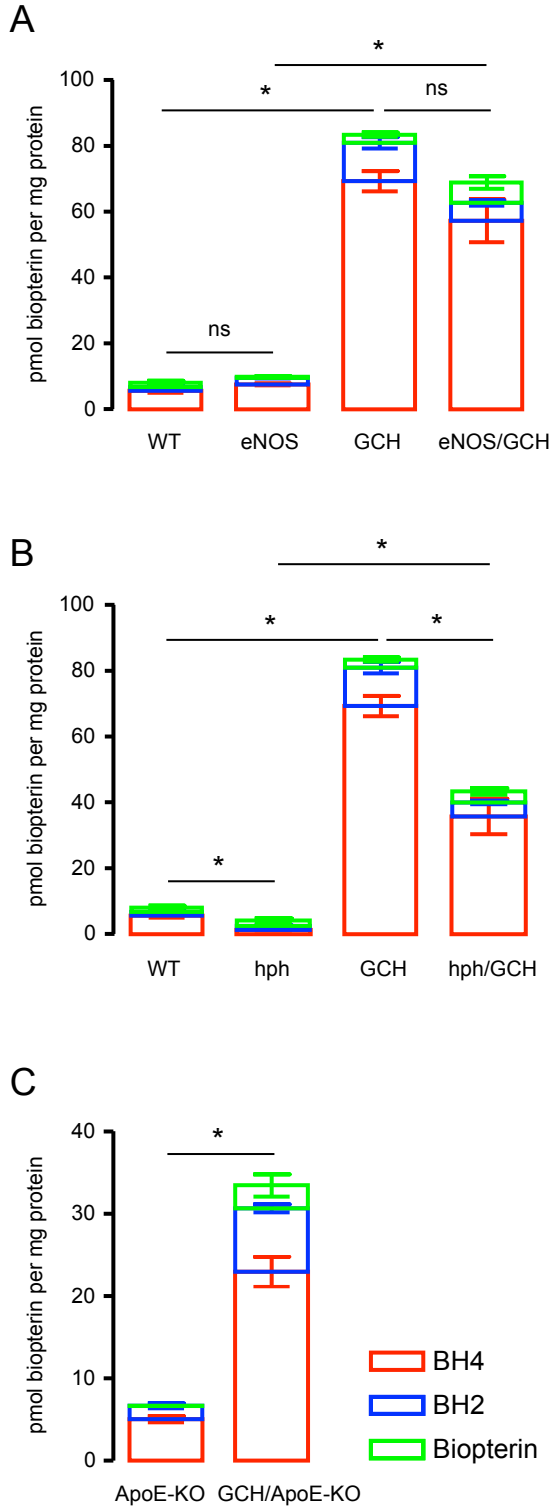


Figure 4.1: Biopterin levels in primary EC after one CD31 Dynabead® selection.

Primary mouse EC were isolated by CD31 Dynabead® selection from mouse lungs after enzymatic tissue digest, snap frozen and analyzed for bipterin content.

A: EC from WT, eNOS, GCH and eNOS/GCH mice. EC from GCH and eNOS/GCH mice showed an >10-fold increase in BH4 levels compared to WT and eNOS. There was no significant difference in BH4 content between WT and eNOS EC or GCH and eNOS/GCH EC respectively. n=3-8 per group.

B: EC from WT, hph, GCH and hph/GCH mice. EC from hph mice showed a 77% reduction in BH4 content compared to WT. Presence of the GCH transgene increased BH4 levels by > 10-fold on WT background and > 25-fold on hph background. However, EC from hph/GCH mice had significantly lower bipterin levels than GCH EC. n=5-9 per group.

C: EC from ApoE-KO and GCH/ApoE-KO mice. Biopterin levels in GCH/ApoE-KO EC were increased by approximately 5-fold compared to ApoE-KO. n=4 per group.

*p<0.05.

The increase in BH4 due to transgenic GCH overexpression was noticeably lower on ApoE-KO background than on WT background (Figure 4.1). GCH/ApoE-KO and GCH mice were not bred as littermates but in two separate colonies after the GCH transgene had been crossed onto ApoE-KO background. Therefore biopterin measurements can only be compared with reservations between WT and ApoE-KO or GCH and GCH/ApoE-KO. Oxidative degradation of BH4 to BH2 and biopterin in ApoE-KO mice could in part explain this finding but does not account for the noticeably lower levels of total biopterin in GCH/ApoE-KO endothelial cells compared to GCH.

In contrast to biopterin measurements from ApoE-KO and GCH/ApoE-KO aortas (Alp *et al.*, 2004), there was no sign of increased oxidative degradation of BH4 in ApoE-KO endothelial cells compared to GCH/ApoE-KO (BH4/BH2=3.3±0.6 versus 3.0±0.2 respectively).

4.3 GTPCH protein content and BH4 availability in cultured primary endothelial and non-endothelial cells with and without genetic modifications of GCH expression

To determine whether BH4 levels in cultured primary endothelial and non-endothelial cells are directly related to GTPCH protein content and whether genetic modifications in GCH expression lead to sustained changes in BH4 availability, endothelial and non-endothelial cells were isolated by CD31 Dynabead® selection from hph and GCH mice and their corresponding WT littermates. Each culture underwent three magnetic bead selections and one passage and the cells were harvested 17 ± 0.6 days (SEM) after the initial isolation. BH4, BH2 and biopterin

were measured by electrochemical and fluorescence detection respectively, following sample separation by HPLC. GTPCH protein content in cell lysates was determined by western blotting.

4.3.1 Cultured primary endothelial and non-endothelial cells from WT and hph-1 mice

Western blotting showed a significant reduction in GTPCH protein in hph endothelial cells compared to WT (Figure 4.2). There was significantly more GTPCH protein detectable in WT and hph endothelial cells compared to WT and hph non-endothelial cells respectively. There was no difference in the expression of eNOS or Tie-2 between the genotypes. BH4 levels were significantly reduced in hph endothelial cells compared to WT and biopterin levels were higher in endothelial cells than non-endothelial cells. There was a significant correlation between GTPCH content in endothelial and non-endothelial cells and BH4 levels ($r^2=0.94$, $p<0.0001$) with GTPCH protein appearing to directly determine cellular BH4 availability.

As shown before in Figure 3.8, in comparison to WT endothelial cells from hph mice after 17 days in culture still showed the same relative reduction in BH4 content as observed directly after the initial selection (78% reduction after 17 days in culture versus 77% reduction after the initial selection). However, BH4 content had increased by 9-fold in cultured endothelial cells from both WT and hph mice. After the 17-day culture period hph endothelial cells had reached double the BH4 content of WT endothelial cells after the initial isolation. These results suggest an up-regulation of GTPCH both in endothelial cells from WT and hph mice during culture.

Figure 4.2

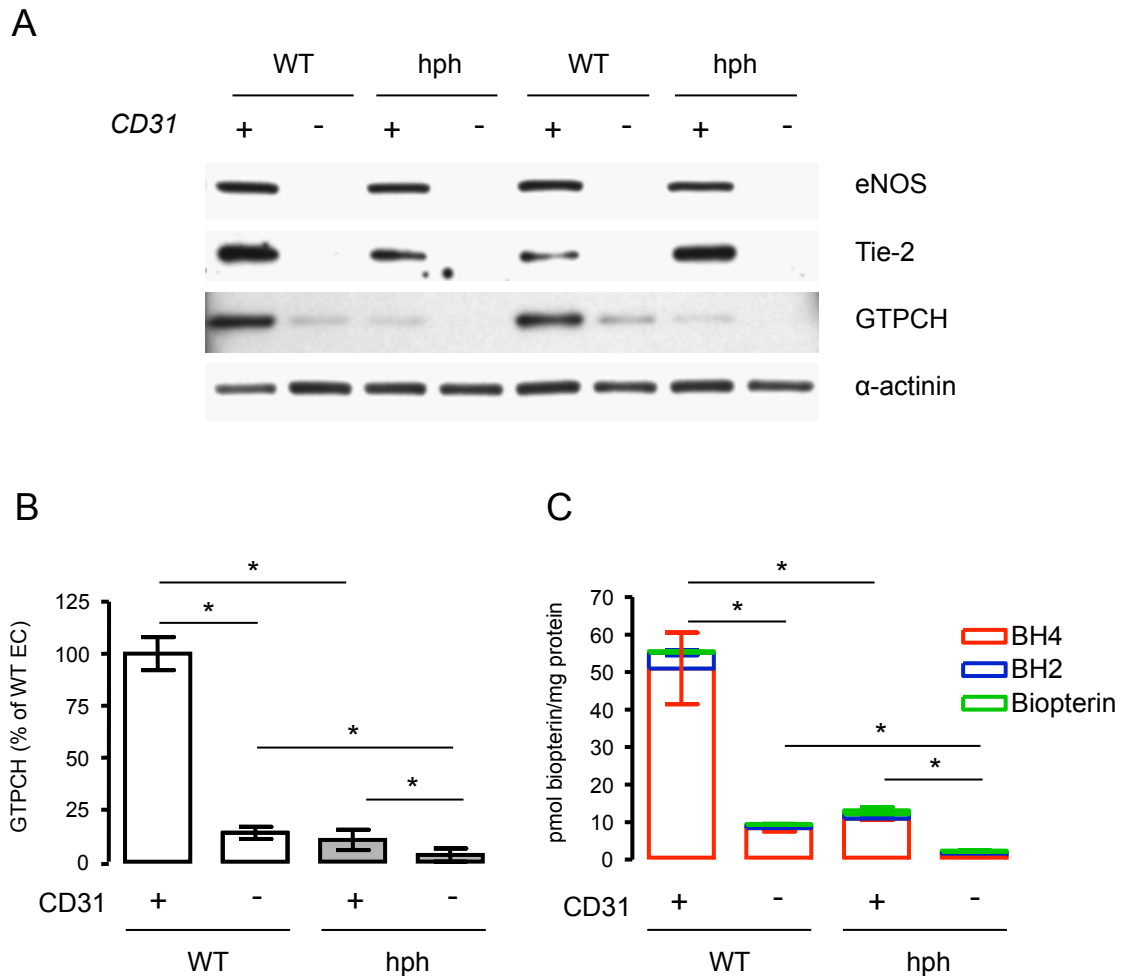


Figure 4.2: GTPCH protein and biotin levels in cultured EC and non-EC from WT and hph-1 mice.

Primary mouse EC were isolated by CD31 Dynabead® selection from hph mice and WT littermates. Each culture underwent 3 magnetic bead selections and 1 passage and the cells were harvested 17 ± 0.6 days (SEM) after the initial isolation.

A and **B**: Western blotting showed a marked reduction in GTPCH protein in hph EC (CD31+) compared to WT. There was significantly more GTPCH protein detectable in WT and hph EC compared to WT and hph non-EC (CD31-) respectively. There was no difference in the expression of eNOS or Tie-2 between the genotypes. α -actinin was used as a loading control. $n=3$ per group.

C: Primary EC from hph-1 mice showed considerably lower BH4 and total biotin levels than WT EC. Biotin levels were significantly higher in EC compared to non-EC. These results are directly in line with the results for GTPCH protein. $n=5$ per group.

* $p < 0.05$.

4.3.2 Cultured primary endothelial and non-endothelial cells from WT and GCH mice

Primary mouse endothelial cells were isolated by CD31 Dynabead® selection from GCH mice and WT littermates. Each culture underwent three magnetic bead selections and one passage and the cells were harvested 17 ± 0.6 days (SEM) after the initial isolation.

As described above for cultured endothelial cells from hph and WT mice, biopterin levels were significantly higher in endothelial cells than in non-endothelial cells (Figure 4.3). There was no significant difference in BH4 levels of cultured primary endothelial cells from GCH and WT mice detectable, suggesting that the expression of human GCH in endothelial cells from GCH transgenic mice is down-regulated in cell culture. Using the rabbit anti-rat GTPCH antibody, which detects both mouse and human GTPCH protein, western blotting did not show a significant difference in total GTPCH protein between WT and GCH endothelial cells. These findings are consistent with the results of the time course experiments presented in Chapter 3.4.2, which showed rapid down-regulation of hGTPCH protein expression in GCH transgenic endothelial cells in culture and suggest that after three selections and one passage the main determinant of biopterin content in GCH transgenic endothelial cells is endogenous murine GTPCH.

Figure 4.3

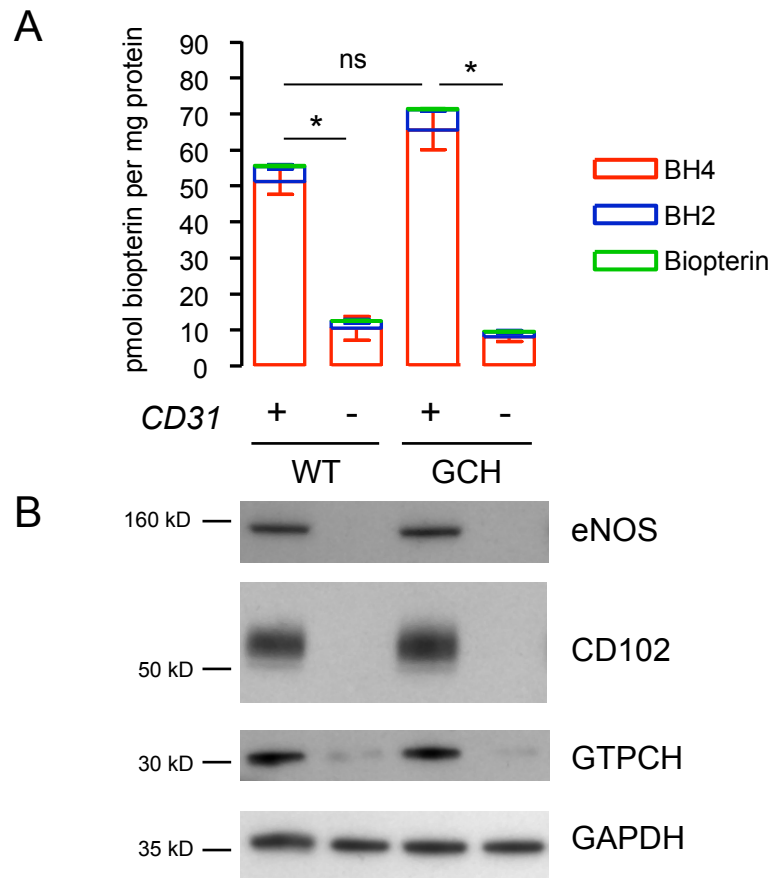


Figure 4.3: GTPCH protein and biopterin content in cultured EC from WT and GCH mice.

Primary mouse endothelial cells were isolated by CD31 Dynabead® selection from GCH mice and wild type (WT) littermates. Each culture underwent 3 magnetic bead selections and 1 passage and the cells were harvested 17 ± 0.6 days (SEM) after the initial isolation.

A: Biopterin levels were quantified using HPLC with electrochemical and fluorescent detection. There was no significant difference in biopterin levels of cultured primary EC (CD31+) from GCH and WT mice, suggesting that the expression of human GCH in EC from GCH transgenic mice is down regulated in cell culture. Both in WT and GCH biopterin levels were significantly higher in EC than in non-EC (CD31-).

B: Using the rabbit anti-rat antibody, which detects both mouse and human GTPCH protein, western blotting did not show any difference in total GTPCH protein between WT and GCH. There was no difference in eNOS or CD102 protein between WT and GCH EC, indicating that the selection and culturing procedures are reproducible. GAPDH was used as a loading control.

These results suggest that after selecting and culturing primary EC cells for an average of 17 days, murine GTPCH is the main determinant of biopterin content in GCH EC.

* $p < 0.05$, $n = 3$ per group.

4.4 Influence of culture duration on BH4 levels in primary endothelial cells

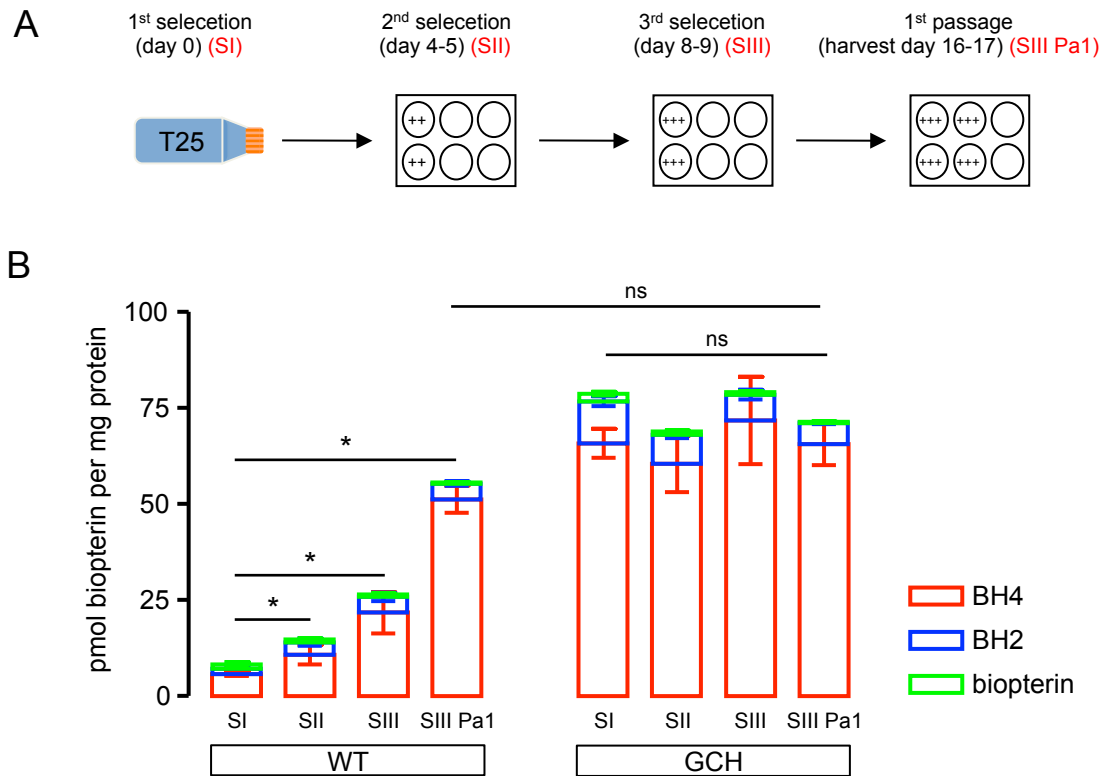
The results presented in Chapter 3.4.2 suggest that transgenic human GTPCH is down-regulated rapidly in cell culture and, as presented above, after three selections and one passage there is no significant difference in BH4 levels or GTPCH protein between WT and GCH endothelial cells detectable anymore.

To closely investigate the process of biopterin level changes in primary endothelial cells from WT and GCH transgenic mice in relation to duration of culture, endothelial cells were isolated by CD31 Dynabead® selection and analyzed for biopterin content after each step of reselection (Figure 4.4 A).

BH4 and total biopterin levels directly after the initial isolation were more than 10-fold higher in GCH endothelial cells compared to WT. BH4 and total biopterin levels in WT endothelial cells increased rapidly with each step of reselection. BH4 and total biopterin levels in WT endothelial cells after the second selection were already significantly higher than after the initial selection even though the cells had only been cultured for four to five days.

After three selections and one passage - a total of 16 to 17 days in culture – BH4 levels in WT endothelial cells were 9-fold higher than after the initial isolation and there was no significant difference between WT and GCH endothelial cells detectable anymore. However, there was no change in BH4 levels in GCH transgenic endothelial cells detectable within the 16 to 17 days of culture.

Figure 4.4

**Figure 4.4: Changes in bioppterin content in cultured primary EC over time.**

Primary mouse endothelial cells were isolated by CD31 Dynabead® selection from GCH mice and wild type (WT) littermates. To investigate changes in bioppterin content, EC after different stages of reselection (**A**) were analyzed.

B: BH4 and total bioppterin levels directly after the initial isolation (SI) were more than 10-fold higher in GCH EC compared to WT EC. BH4 and total bioppterin levels in WT EC increased rapidly in culture. Both BH4 and total bioppterin were significantly increased in WT EC after only 4-5 days in culture (SII) and continued to increase. After 16-17 days in culture BH4 levels in WT EC (SIII Pa1) were 9-fold higher than after the initial isolation (SI) and there was no significant difference between WT and GCH EC anymore. However, bioppterin content in GCH EC did not change significantly within 16-17 days of culture.

These results suggest that during culture of primary EC GTPCH activity increases in WT EC. Because bioppterin levels do not change in GCH EC despite down regulation of human GTPCH expression (see Figure 3.14), it is likely that mouse GTPCH is the main determinant of bioppterin levels in GCH EC after 16-17 days of culture.

* $p < 0.05$, $n = 3-10$ per group.

These results suggest that during culture of primary endothelial cells endogenous mouse GTPCH is up-regulated in WT endothelial cells. Because biopterin levels do not change in GCH endothelial cells despite down-regulation of human GTPCH expression (as shown in Chapter 3.4.2), it is likely that mouse GTPCH is the main determinant of biopterin levels in GCH endothelial cells after 16 to 17 days of culture.

4.5 Summary and discussion

GTPCH, encoded by the GCH gene, catalyzes the first and rate-limiting reaction in the *de novo* synthesis of BH4 (Thony *et al.*, 2000). The effects of genetic modifications in GCH expression on BH4 bioavailability and endothelial cell function have only been investigated in whole organs or blood vessels so far and the aims of this chapter were to evaluate the effects of genetic alterations in GCH expression on BH4 availability in primary endothelial cells and to determine whether these effects are sustained in tissue culture.

The key findings of this chapter are:

- Transgenic overexpression of human GCH leads to a pronounced increase in BH4 content in primary endothelial cells. The relative increase in BH4 levels due to transgenic GCH overexpression is higher in primary endothelial cells than previously shown in tissue or blood vessels.
- Cultured primary endothelial cells have significantly higher BH4 levels than non-endothelial cells.
- BH4 levels in primary endothelial and non-endothelial cells correlate with GTPCH protein content.

- BH4 levels in primary endothelial cells increase during time spent in culture.

The results presented in this chapter demonstrate that genetic modifications in GCH expression lead to alterations in BH4 levels in primary mouse endothelial cells, providing information about absolute and relative levels of endothelial BH4 in these mice for the first time.

In the *hph-1* mouse, systemic underexpression of GCH leads to a relative reduction in BH4 levels in primary endothelial cells very similar to the reduction observed in whole lung tissue (Khoo *et al.*, 2005) or liver (Tatham *et al.*, 2009). The relative reduction in *hph* BH4 levels compared to WT is sustained when primary endothelial cells are reselected and cultured. However, compared to freshly isolated endothelial cells, after three selections and one passage, absolute BH4 levels are significantly increased both in *hph* and WT endothelial cells. Although *hph* endothelial cells are still BH4 deficient relative to cultured WT endothelial cells, they might not be anymore in absolute terms having reached BH4 levels higher than in freshly isolated WT endothelial cells. This increase in BH4 during primary endothelial cell culture might compromise experiments relying on BH4 deficiency in *hph* cells.

In the GCH transgenic mouse, endothelial specific overexpression of GCH leads to increased levels of BH4 in freshly isolated primary endothelial cells. The effects of GCH overexpression on BH4 levels in the GCH transgenic mouse have previously only been shown in endothelial cell rich organs like lungs or heart and in aorta (Alp *et al.*, 2004; Alp *et al.*, 2003). Measuring BH4 levels in whole organs or blood

vessels however does not provide direct information about endothelial BH4 levels. It has been shown in this chapter that the increase in BH4 content due to transgenic GCH overexpression is greater in primary endothelial cells than previously reported in lung tissue or aorta (Alp *et al.*, 2004; Alp *et al.*, 2003) and that the relative increase seems to depend on the genetic background of the mouse with the largest relative but not absolute increase in BH4 on hph background. However, although the absolute levels of BH4 in primary endothelial cells from GCH mice do not change within the reported 17 days of culture, BH4 levels in WT endothelial cells increase and there is no significant difference between GCH and WT detectable anymore after this time period. This is most likely due to up-regulation of endogenous mouse GTPCH in cultured endothelial cells. EC medium contains bFGF, which has been shown to increase BH4 content and GTPCH mRNA in cultured mouse brain microvascular endothelial cells (Shimizu *et al.*, 2003). The concentration of bFGF in EC medium is 4ng/ml. Shimizu *et al.* found an increase in BH4 using bFGF concentrations of 10ng/ml or higher but not with 5ng/ml. Also, the increase in GTPCH mRNA and biopterin content was reported to reach its peak after 24 hours, which makes it unlikely that the increase in BH4 in WT endothelial cells over 16 days is solely due to bFGF in the culture medium.

As reported before in Chapter 3.4.2, human GTPCH is down-regulated in GCH transgenic endothelial cells in culture and it is likely that mouse GTPCH is the main determinant of BH4 levels in GCH transgenic endothelial cells after prolonged culture. These changes have to be accounted for when designing experiments using primary endothelial cells from GCH mice and maintaining GCH endothelial cells in culture for a prolonged period is not advisable.

It has also been shown in this chapter that cultured primary endothelial cells have significantly higher levels of GTPCH protein and BH4 than non-endothelial cells. In comparison to the mouse GCH mRNA levels in endothelial and non-endothelial cells presented in Chapter 3.3.5.1, GTPCH and BH4 levels are unexpectedly high in cultured endothelial cells compared to non-endothelial cells. However, cells were harvested for RNA extraction after only 5 days in culture at which point biopterin levels in endothelial cells were significantly lower than after 16-17 days in culture. It is possible that GTPCH is up-regulated preferentially in cultured endothelial but not non-endothelial cells. Western blotting results from endothelial and non-endothelial cells after 5 days in culture (Figure 3.12) would support this hypothesis but more detailed analysis would be necessary to answer this question.

As it has been reported before for organs, blood vessels and cell lines (Tatham *et al.*, 2009), BH4 levels in endothelial and non-endothelial cells correlate directly with GTPCH protein content, suggesting that GTPCH expression is the main determinant of BH4 levels in these cells.

In summary, the hypothesis that genetic modifications in GCH expression will lead to sustained changes in BH4 availability in cultured primary mouse endothelial cells has partly been disproven. Relative to WT the effect of the hph-1 mutation is indeed sustained but BH4 levels increase both in WT and hph endothelial cells during culture. Although absolute BH4 levels in GCH endothelial cells are sustained in culture, due to increasing BH4 levels in WT endothelial cells the relative effect diminishes over time in culture.

Chapter 5:

BH4 and Endothelial Cell Growth

5 BH4 and Endothelial Cell Growth

5.1 Introduction

The vascular endothelium plays a critical role in the regulation of blood pressure (Luscher, 1990), blood coagulation (Becker *et al.*, 2000), atherogenesis (Luscher and Noll, 1995) and angiogenesis (Isner and Asahara, 1999). Endothelial cell injury, loss, survival and regeneration are important aspects in the pathogenesis of both native atherosclerosis and neointimal hyperplasia and accelerated atherosclerosis after vascular injury (Ehsan *et al.*, 2002; Fingerle *et al.*, 1990; Stefanec, 2000; Stoneman and Bennett, 2004). Proatherosclerotic factors such as oxidized low-density lipoproteins, high glucose concentrations, angiotensin II and reactive oxygen species are not only known to induce endothelial dysfunction but also promote endothelial cell apoptosis (Dimmeler *et al.*, 1998). However, both proliferation and apoptosis seem to be involved in the response of endothelial cells to atherogenic factors (Apostolov *et al.*, 2007). Alterations in endothelial cell viability have also been described to be part of the pathogenic mechanisms that lead to pulmonary hypertension. Again, both hyperproliferation (Levy *et al.*, 2007; Sakao *et al.*, 2005; Taraseviciene-Stewart *et al.*, 2006) and increased apoptosis (Guerard *et al.*, 2006) seem to be involved in the pathophysiology, possibly depending on disease cause and stage.

eNOS and NO have been shown to mediate VEGF induced endothelial cell proliferation (Morbidelli *et al.*, 1996; Papapetropoulos *et al.*, 1997) and angiogenesis (Murohara *et al.*, 1998; Papapetropoulos *et al.*, 1997) and to play a

critical role in the prevention of endothelial cell senescence (Hayashi *et al.*, 2006; Vasa *et al.*, 2000) and apoptosis (Dimmeler *et al.*, 1997; Kang-Decker *et al.*, 2007; Rossig *et al.*, 2000; Wright *et al.*, 2010) as well as EPC mobilization and function (Aicher *et al.*, 2003; Thum *et al.*, 2007; Urao *et al.*, 2006).

There is increasing evidence that the essential eNOS cofactor BH4 improves endothelial cell proliferation (Chen *et al.*, 2010; Marinos *et al.*, 2001; Michaelis *et al.*, 2007; Shimizu *et al.*, 1999), EPC mobilization into the bloodstream (Xie *et al.*, 2010) and EPC function (Thum *et al.*, 2007). Table 5.1 provides an overview over publications investigating the effect of BH4 on endothelial cell proliferation or EPC mobilization and function. *In vitro* supplementation with the BH4 precursor sepiapterin has been shown to enhance proliferation in coronary endothelial cells from diabetic rats (Marinos *et al.*, 2001), bovine aortic endothelial cells (BAEC) (Shimizu *et al.*, 1999) and HUVEC (Chen *et al.*, 2010) whereas GTPCH overexpression in the GCH transgenic mouse preserved the number of circulating EPC during hypertension (Xie *et al.*, 2010). These effects may be mediated either by a direct antioxidant effect of BH4 or/and improved eNOS function with increased NO availability and reduced production of reactive oxygen species. Work from our group (carried out by Dr. Ziad Ali) that investigates the effects of GCH over-expression on vein graft re-endothelialization and has been presented but not published yet, shows that improved BH4 bioavailability in the GCH transgenic mouse might reduce endothelial cell loss after injury and promote endothelial cell repopulation in vein grafts (oral presentation at AHA Scientific Sessions 2008, Rinze *et al.*, 2008).

	<i>Publication</i>	<i>Cells studied</i>	<i>Method of BH4 delivery</i>	<i>Result</i>
Endothelial cells	Marinos <i>et al.</i> , 2001	Primary rat coronary endothelial cells	10µM Sepiapterin in culture media	- Enhanced proliferation of endothelial cells from spontaneous diabetic rats - Effect can be attenuated by NOS inhibition - Inhibition of BH4 synthesis by 10mM DAHP decreases endothelial cell proliferation
	Shimizu <i>et al.</i> , 1999	BAEC	10µM/50µM BH4 or 10-100µM Sepiapterin in culture media	- BH4 and Sepiapterin stimulate proliferation in BAEC - Proliferative effect of sepiapterin inhibitable by sepiapterin reductase inhibitor
	Michaelis <i>et al.</i> , 2007	HUVEC	10µM BH4 in culture media	Prevents ribavirin-induced tube formation inhibition
	Chen <i>et al.</i> , 2010	HUVEC	10µM Sepiapterin in culture media	- Increases cell proliferation, migration and tube formation - Effect inhibited by PI3K inhibitor
Endothelial progenitor cells	Xie <i>et al.</i> , 2010	Mouse circulating EPC (Sca-1/Flk-1 positive)	GCH transgenic mouse	- GTPCH overexpression preserves circulating EPC in DOCA-salt hypertensive mice - Effect eNOS independent (eNOS^{-/-}/GCH^{+/-} hybrid mice)
	Thum <i>et al.</i> , 2007	CFUs from human PBMCs	10µM BH4 in culture media	In vitro BH4 treatment improves glucose mediated EPC dysfunction (superoxide ↓ migratory capacity ↑)

Table 5.1: Examples of publications investigating the influence of BH4 on endothelial cell proliferation or EPC mobilization and function.

The focus of this chapter is to explore the effect of BH4 bioavailability on endothelial cell growth in the absence and presence of vascular disease. For this, the ApoE-KO mouse is used as a model for vascular disease and endothelial dysfunction. In 1992 the ApoE-KO mouse was generated in two separate laboratories through gene knockout of the apolipoprotein E (ApoE) gene (Piedrahita *et al.*, 1992; Plump *et al.*, 1992). ApoE-KO mice available today from Jackson Laboratories are descendants of the mouse created by Piedrahita and colleagues. ApoE, a glycoprotein expressed in various tissues throughout the body with the highest levels in liver and brain (Srivastava *et al.*, 1996), is found in all lipoproteins except low-density lipoproteins (LDL) and functions as a ligand for the lipoprotein receptor. It plays an important role in the metabolism of cholesterol and triglycerides by binding to receptors in the liver that clear chylomicrons and very low-density lipoprotein (VLDL) remnants from the bloodstream. Homozygote ApoE-KO mice have 5 times normal levels of plasma cholesterol and, in contrast to WT mice, spontaneously develop endothelial dysfunction and atherosclerotic lesions on normal chow diet with the first foam cell lesions detectable in the aorta by the age of 10 weeks (Nakashima *et al.*, 1994; Zhang *et al.*, 1992).

Hypothesis:

The central hypothesis of this chapter is that BH4 augmentation will improve endothelial cell proliferation in a vascular disease setting.

Aims:

The aim of this chapter is to determine the influence of modifications in BH4 availability on endothelial cell proliferation. More specifically:

- To evaluate the effect of endothelial specific GCH overexpression on growth potential of primary endothelial cells from ApoE-KO mice as a model of vascular disease.
- To investigate the effect of *in vitro* supplementation with the BH4 precursor sepiapterin on proliferation of primary endothelial cells from ApoE-KO mice.
- To evaluate the effect of endothelial specific GCH overexpression on primary endothelial cell growth in the absence of a vascular disease state.
- To determine the effect of inhibiting BH4 synthesis on proliferation of the mouse endothelioma cell line sEND.1
- To assess the effect of endothelial specific GCH overexpression on *in vivo* angiogenesis using a Matrigel plug assay.

5.2 Influence of transgenic GCH overexpression on growth potential of cultured primary endothelial cells from hypercholesterolemic ApoE-KO mice

It has been shown that endothelial specific GCH overexpression in the GCH transgenic mouse is sufficient to restore NO bioavailability and endothelial function in various vascular disease states including diabetes (Alp *et al.*, 2003; Tie *et al.*, 2009), arterial (Du *et al.*, 2008) and pulmonary hypertension (Khoo *et al.*, 2005). In ApoE-KO mice transgenic endothelial BH4 augmentation reduces endothelial dysfunction and atherosclerosis (Alp *et al.*, 2004; Takaya *et al.*, 2007) and neointimal hyperplasia after vascular injury (Ali *et al.*, 2008). To investigate the influence of GCH overexpression on primary endothelial cell proliferation *in vitro*,

lung endothelial cells were isolated from Apo-KO and GCH/ApoE-KO mice. All cell growth experiments were conducted blinded using strictly age and sex matched cohorts of mice.

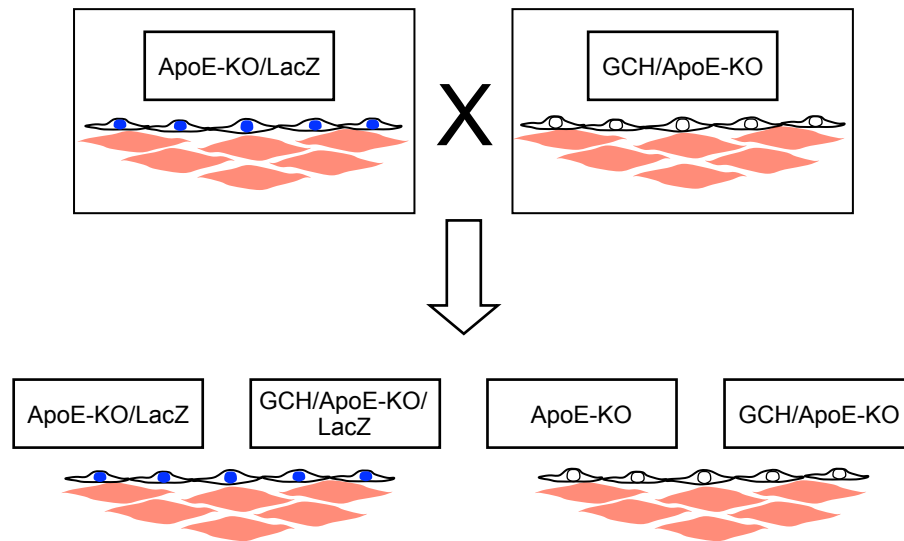
5.2.1 Analysis of ApoE-KO/LacZ and GCH/ApoE-KO/LacZ endothelial cell growth in culture by X-Gal staining

Primary endothelial cells were isolated from ApoE-KO/LacZ and GCH/ApoE-KO/LacZ mice by CD31 Dynabead® selection and plated into gelatine coated wells of a 6 well dish (1 well per mouse). The cells were cultured in EC medium for 3 or 4 days respectively before X-Gal staining (Chapter 2.10) was performed. To obtain the number of blue endothelial cells per sample, all blue colonies were photographed using a wide-field inverted microscope with colour camera and blue cells were counted subsequently. Both the 3-day and the 4-day growth experiment were performed in two batches each. To adjust for batch-to-batch variability, results for total endothelial cell number and number of colonies were adjusted to ApoE-KO mean of each batch.

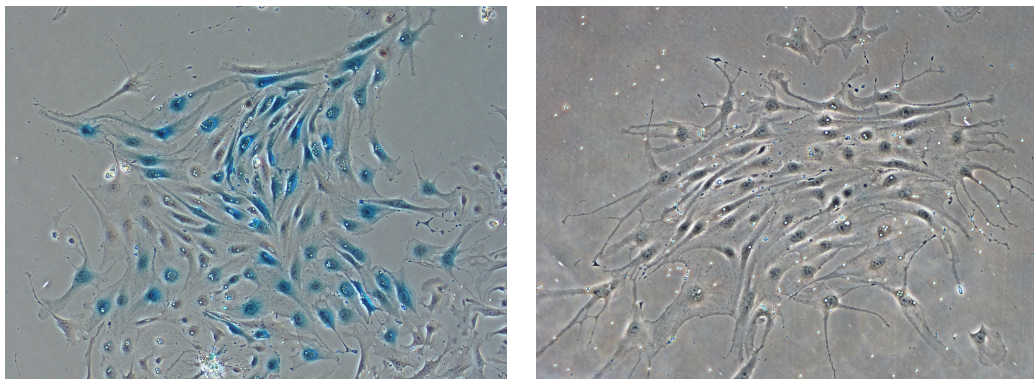
Figure 5.1 illustrates the breeding strategy to produce ApoE-KO and GCH/ApoE-KO littermates with and without expression of the Tie2-LacZ transgene. Blue X-Gal staining was specific to endothelial cells from LacZ positive mice, there was no blue staining observed in endothelial cells from LacZ negative mice or non-endothelial cells of either genotype. This observation goes directly in line with the LacZ mRNA data presented in Chapter 3.3.5.2.

Figure 5.1

A



B



LacZ positive

LacZ negative

Figure 5.1: EC growth in cell culture: ApoE-KO/LacZ vs. GCH/ApoE-KO/LacZ.

A: Breeding strategy to produce ApoE-KO and GCH/ApoE-KO animals with and without expression of the Tie2-LacZ transgene.

B: Primary endothelial cells were isolated by CD31 Dynabead® selection and grown on gelatine coated dishes in EC media for three days. The cells were subsequently fixed with 2% formalin / 0.2% glutaraldehyde for 10 minutes at room temperature and stained with X-Gal for 24 hours. Cultures from LacZ positive animals showed blue endothelial cell islands, there was no blue staining detectable in cultures from LacZ negative animals. To compare cell growth between ApoE-KO/LacZ and GCH/ApoE-KO/LacZ every blue island in each well was photographed and the total number of blue cells was counted.

The total number of endothelial cells, the number of endothelial cell colonies and average colony size were significantly increased in GCH/ApoE-KO cultures compared to ApoE-KO both after 3 and 4 days in culture (Figure 5.2). However, after 4 days in culture the increase in total endothelial cell number and particularly colony size in GCH/ApoE-KO cultures was more pronounced than after 3 days of culture, indicating not only sustained proliferation from day 3 to day 4 but also faster proliferation in GCH/ApoE-KO endothelial cells compared to ApoE-KO.

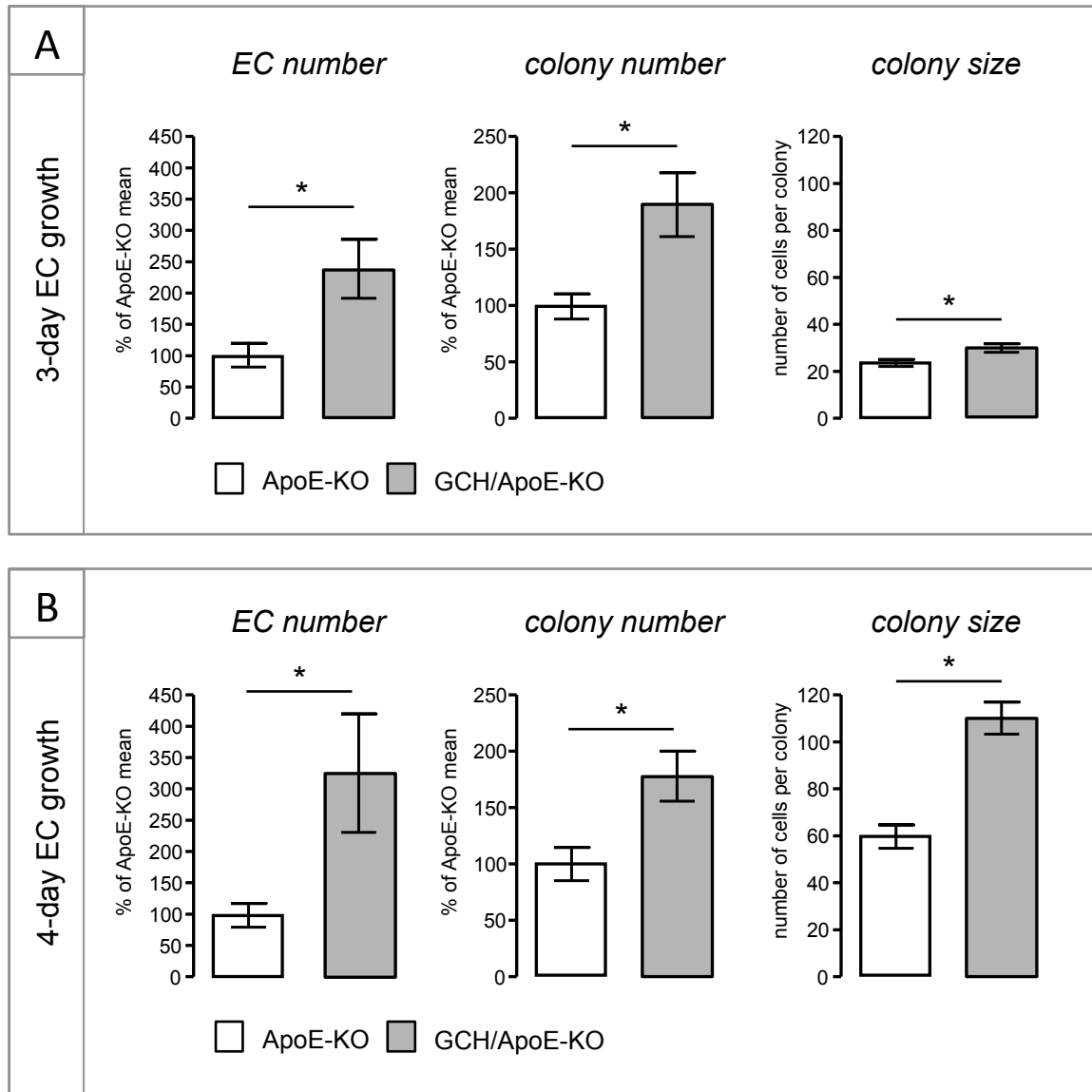


Figure 5.2: Analysis of ApoE-KO/LacZ and GCH/ApoE-KO/LacZ EC growth in culture by X-Gal staining.

Primary EC were isolated from ApoE-KO/LacZ and GCH/ApoE-KO/LacZ animals by CD31 Dynabead® selection and grown in tissue culture for 3 (**A**) or 4 (**B**) days respectively. The cells were subsequently fixed and X-Gal staining was performed to obtain the number of blue LacZ positive cells per sample. To adjust for batch-to-batch variability, results for total EC number and number of colonies were adjusted to ApoE-KO mean for each batch.

A: EC growth in culture over 3 days. Both number of ECs, number of colonies and the size of EC colonies were significantly increased in GCH/ApoE-KO cultures compared to ApoE-KO. In comparison to ApoE-KO, after 3 days in culture the total number of blue EC was increased by 135%, there were 95% more EC colonies and the colonies contained 30% more cells in GCH/ApoE-KO cultures. * $p < 0.05$, $n = 5$ per group.

B: EC growth in culture over 4 days. Both number of ECs, number of colonies and the size of EC colonies were significantly increased in GCH/ApoE-KO cultures compared to ApoE-KO. However, in comparison to the 3 day EC growth results, the increase in total EC number (+225%) and especially colony size (+85%) in GCH/ApoE-KO cultures was more pronounced. There were 80% more colonies in GCH/ApoE-KO cultures than in ApoE-KO; however, after 4 days in culture colonies have become so large that some start merging, potentially leading to a slight underestimation of colony number in GCH/ApoE-KO cultures. * $p < 0.05$, $n = 6$ per group.

5.2.2 Investigation of EC proliferation by BrdU incorporation and flow cytometry

Lung endothelial cells were isolated from ApoE-KO and GCH/ApoE-KO mice by CD31 MACS® MicroBead isolation and plated into gelatine coated wells of a 12 well dish (1 well per mouse). The cells were cultured in EC medium for 5 days in total. On day 4 BrdU was added to the culture media and the cells were harvested 16 hours later for flow cytometry analysis. To determine the total number of endothelial cells per sample and the number of proliferating endothelial cells, the cells were stained with CD31 APC, CD45 PE-Cy7 and BrdU FITC antibodies and analyzed by flow cytometry. Detailed methods describing the BrDU incorporation assay and flow cytometry staining can be found in Chapter 2.14. To adjust for batch-to-batch variability, results for total endothelial cell number and number of BrdU incorporating endothelial cells were adjusted to ApoE-KO mean of each batch.

Both the total number of endothelial cells and the number of endothelial cells that had incorporated BrdU were significantly increased in GCH/ApoE-KO compared to ApoE-KO cultures (Figure 5.3).

Figure 5.3

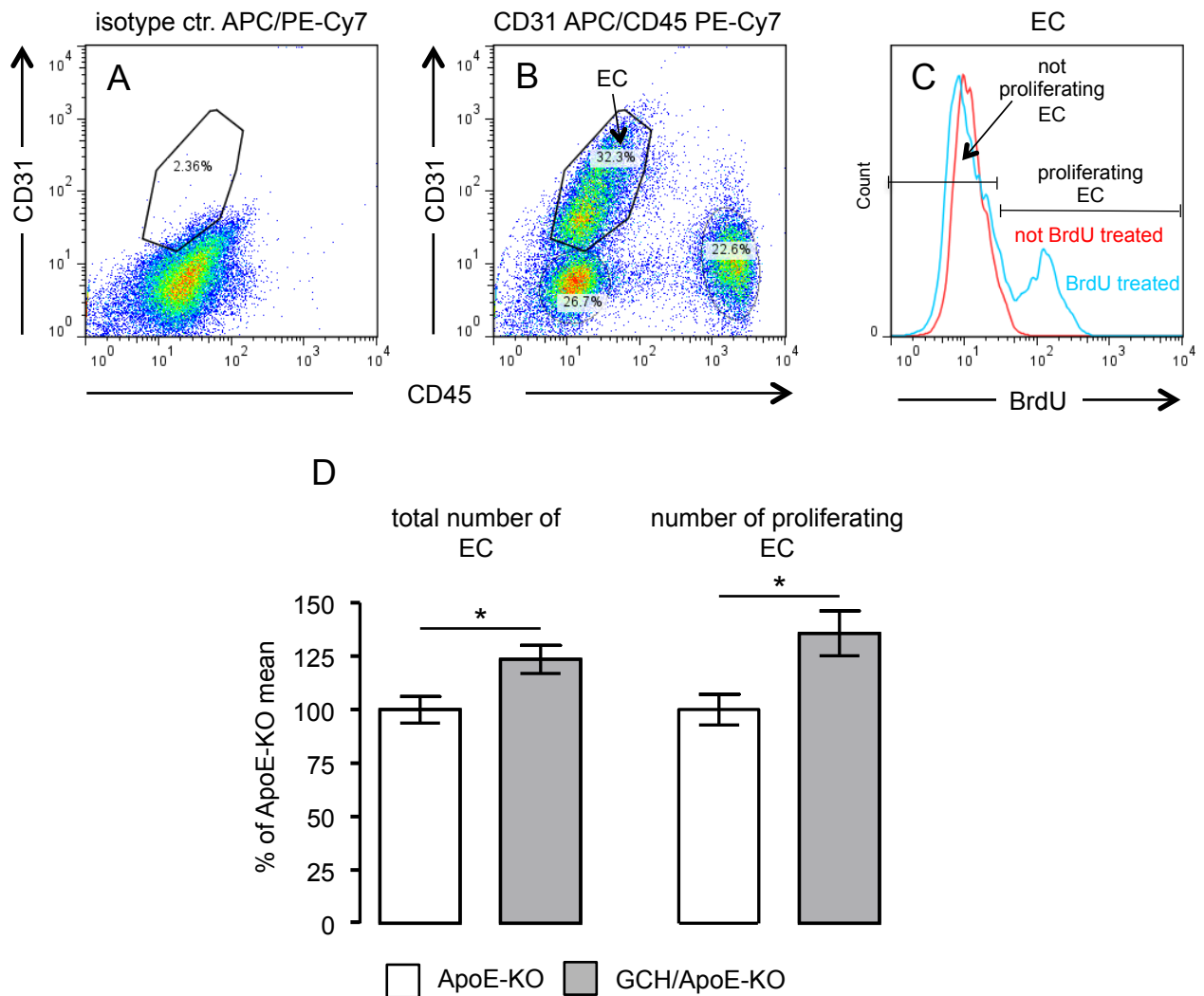


Figure 5.3: Investigation of EC proliferation over 5 days in culture by BrdU incorporation and flow cytometry. ApoE-KO v.s. GCH/ApoE-KO.

EC were isolated from mouse lungs by CD31 MACS® MicroBead isolation and cultured for 5 days. On day 4 BrdU was added to the culture media and the cells were harvested 16 hours later for flow cytometry analysis. After Fc blocking the samples were stained with CD31 APC, CD45 PE-Cy7 and BrdU FITC antibodies or the corresponding isotype controls. A defined number of FITC CaliBRITE™ beads was added to each sample to determine the total number of EC per sample.

A: Cells stained with isotype controls for APC and PE-Cy7.

B: Cells stained with CD31 APC and CD45 PE-Cy7 antibodies. The total number of EC was determined by gating on the EC population (CD31 positive/CD45dim).

C: Analysis of BrdU incorporation. Triple stained with CD31, CD45 and BrdU antibodies and gated on EC population. BrdU FITC antibody stained but not BrdU treated cells were used as a negative control to set the 95% confidence interval.

D: After 5 days in culture both total number of EC and number of proliferating EC were significantly increased in GCH/ApoE-KO compared to ApoE-KO. * $p < 0.05$ $n = 9$ per group.

However, the difference in proliferation between ApoE-KO and GCH/ApoE-KO endothelial cells over 5 days, determined by flow cytometry, was not as pronounced as over 3 or 4 days, determined by X-Gal staining. There are several possible explanations for this finding including technical reasons amongst the most likely. Using the same CD31 antibody for MACS® MicroBead and Dynabead® isolation minimizes the risk of selecting generally different populations of cells from the lung digest, although CD31 Dynabead® isolation might be less sensitive thus selecting fewer CD31 low expressing cells than MACS® MicroBead isolation. It cannot be excluded that using different magnetic beads may influence growth behavior in cell culture. Primary endothelial cells have the tendency to grow better in clusters. Dynabeads® are very large in comparison to MACS® MicroBeads and promote the formation of cell clusters towards the centre of the culture well, whereas MACS® MicroBead selected cells distribute more evenly. It also has to be considered that a 5-day growth period might be too long because the cells are getting confluent before the end of the experiment. Endothelial cells grow in a strictly contact inhibited manner, which means the sample growing more slowly might be able to catch up with the faster growing but more confluent sample. The growth period of 5 days was chosen to ensure enough cells for flow cytometry were obtained, particularly because the BrdU staining protocol involves several spinning steps. In addition, the more confluent primary endothelial cells are, the more difficult it is to obtain a single cell suspension when detaching the cells and neither the use of Accutase™ nor of Trypsin EDTA is sufficient to avoid larger cell clumps entirely. These clumps however will be lost when straining the cell suspension through a 70µm cell strainer and it is possible that a larger proportion of cells is lost from the more confluent culture.

5.2.3 Endothelial cell content in lungs from ApoE-KO and GCH/ApoE-KO mice

There are three main probable explanations for the increase in endothelial cell number in GCH/ApoE-KO cultures after 3,4 or 5 days:

1. A difference in number of endothelial cells that are isolated and plated into culture.
2. A difference in viability or proliferation potential of isolated endothelial cells.
3. A difference in proliferation rate.

To exclude a difference in endothelial cell content in lungs and a difference in the amount of isolated endothelial cells, β -galactosidase activity was measured as an indicator for endothelial cell content in lungs and endothelial cell isolates from Tie-2 LacZ positive ApoE-KO and GCH/ApoE-KO mice. The number and viability of CD31 Dynabead® isolated endothelial cells was further determined by flow cytometry. The total number of endothelial cells isolated from each lung by CD31 MACS® MicroBead selection was also determined by flow cytometry. To investigate whether endothelial cell isolates from GCH/ApoE-KO mice contain more immature endothelial cells that may have higher proliferation potential than mature endothelial cells, expression of the EPC markers CD34 and Flk-1 was investigated in MACS® MicroBead isolated cells.

5.2.3.1 β -Galactosidase activity in lungs and EC isolates

The expression of the LacZ gene under the control of the Tie-2 promoter is endothelial specific and β -galactosidase activity can be used as an indicator for endothelial cell content. β -galactosidase activity in lungs and CD31 Dynabead® selected endothelial cells from Tie-2 LacZ positive ApoE-KO and GCH/ApoE-KO mice was measured by the conversion of CPRG (red- β -D-galactopyranoside) to galactose and chlorophenol red (Chapter 2.9). Lungs and endothelial cells from Tie-2 LacZ negative mice were used as negative controls.

There was no significant difference in β -galactosidase activity detectable in lungs or freshly isolated endothelial cells from ApoE-KO/LacZ and GCH/ApoE-KO/LacZ mice (Figure 5.4). These results suggest that there is no significant difference in lung endothelial cell content between ApoE-KO and GCH/ApoE-KO mice, which could confound the results of primary endothelial cell growth experiments.

Figure 5.4

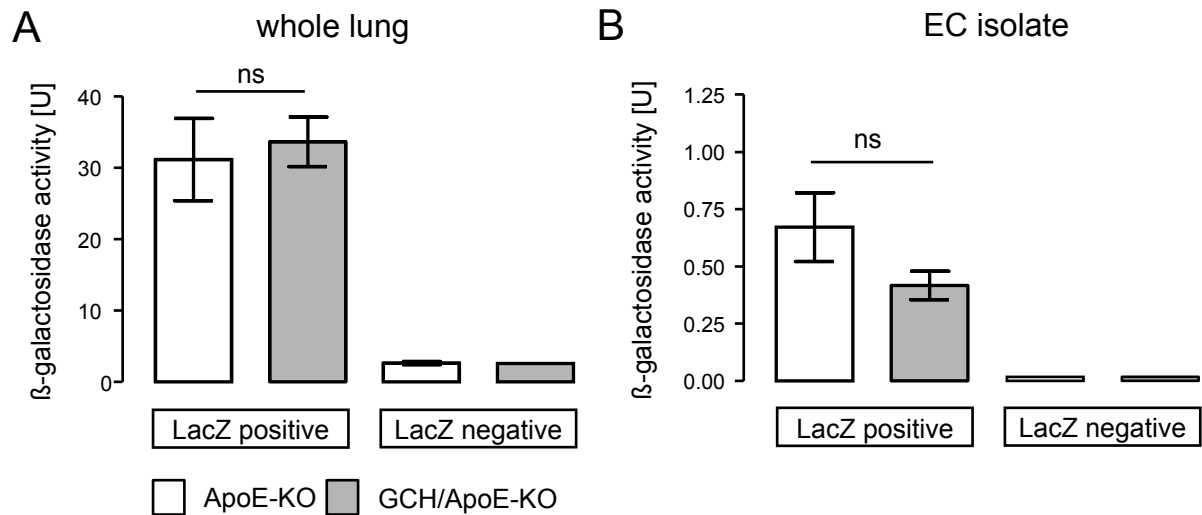


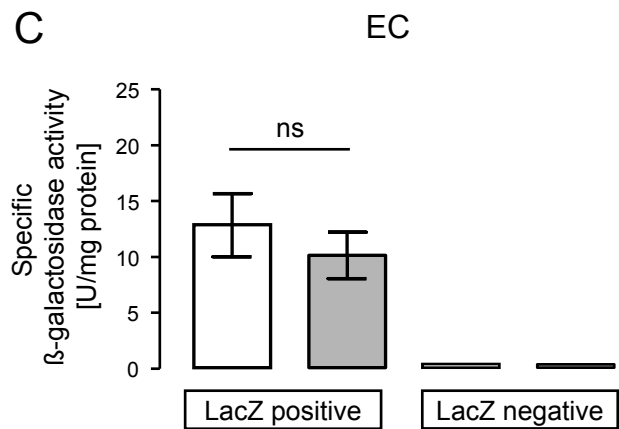
Figure 5.4: β -Galactosidase activity in lungs and EC isolates.

β -galactosidase activity in lungs and CD31 Dynabead® selected EC from Tie-2 LacZ positive ApoE-KO and GCH/ApoE-KO mice was measured by the conversion of CPRG (red- β -D-galactopyranoside) to galactose and chlorophenol red. β -galactosidase activity in lung tissue from Tie-2 LacZ positive mice can be used as an indicator for endothelial cell content.

A: β -galactosidase activity per whole lung.

B: β -galactosidase activity per EC isolate from 1 lung.

C: Specific β -galactosidase activity in EC.



There was no significant difference in β -galactosidase activity detectable in lungs (**A**) or primary EC isolates after the first magnetic bead selection from Tie-2 Lac Z positive ApoE-KO and GCH/ApoE-KO mice (**B+C**).

Lungs and EC from Tie-2 LacZ negative mice were used as negative controls.

n=4 per group.

5.2.3.2 Number and viability of CD31 Dynabead® selected endothelial cells

The PE AnnexinV Apoptosis Detection Kit I (BD Bioscience) was used to detect early and late apoptosis in CD31 Dynabead® isolated endothelial cells. This kit contains AnnexinV-PE, which detects early apoptosis and 7-Amino-Actinomycin (7-AAD), which detects late apoptosis or necrosis. In viable cells phosphatidyl serine (PS) is located on the cytoplasmic side of the cell membrane. As one of the earliest events in apoptosis PS is translocated to the cell surface and thus exposed to AnnexinV binding. 7-AAD is a fluorescent dye with a strong affinity to DNA. Cells with intact membranes exclude 7-AAD whereas the membranes of dead or late apoptotic cells are permeable to 7-AAD.

Endothelial cells were isolated from ApoE-KO and GCH/ApoE mouse lungs by immunoselection with Dynabeads® coated with FITC conjugated CD31 antibody. Beadbound cells from each mouse lung were washed twice with ice cold PBS and incubated in 200µl 1X Binding Buffer with 10µl AnnexinV-PE and 10µl 7-AAD for 15 minutes at room temperature in the dark. Subsequently 200µl 1X Binding Buffer was added and flow cytometry analysis was performed within 1 hour.

Two main cell populations could be distinguished in the Forward and Side Scatter plots, one consisting of larger and more complex cells than the other. The larger and more complex cells were identified as FITC positive, meaning these cells had strongly bound CD31-FITC coated Dynabeads® (Figure 5.5).

Figure 5.5

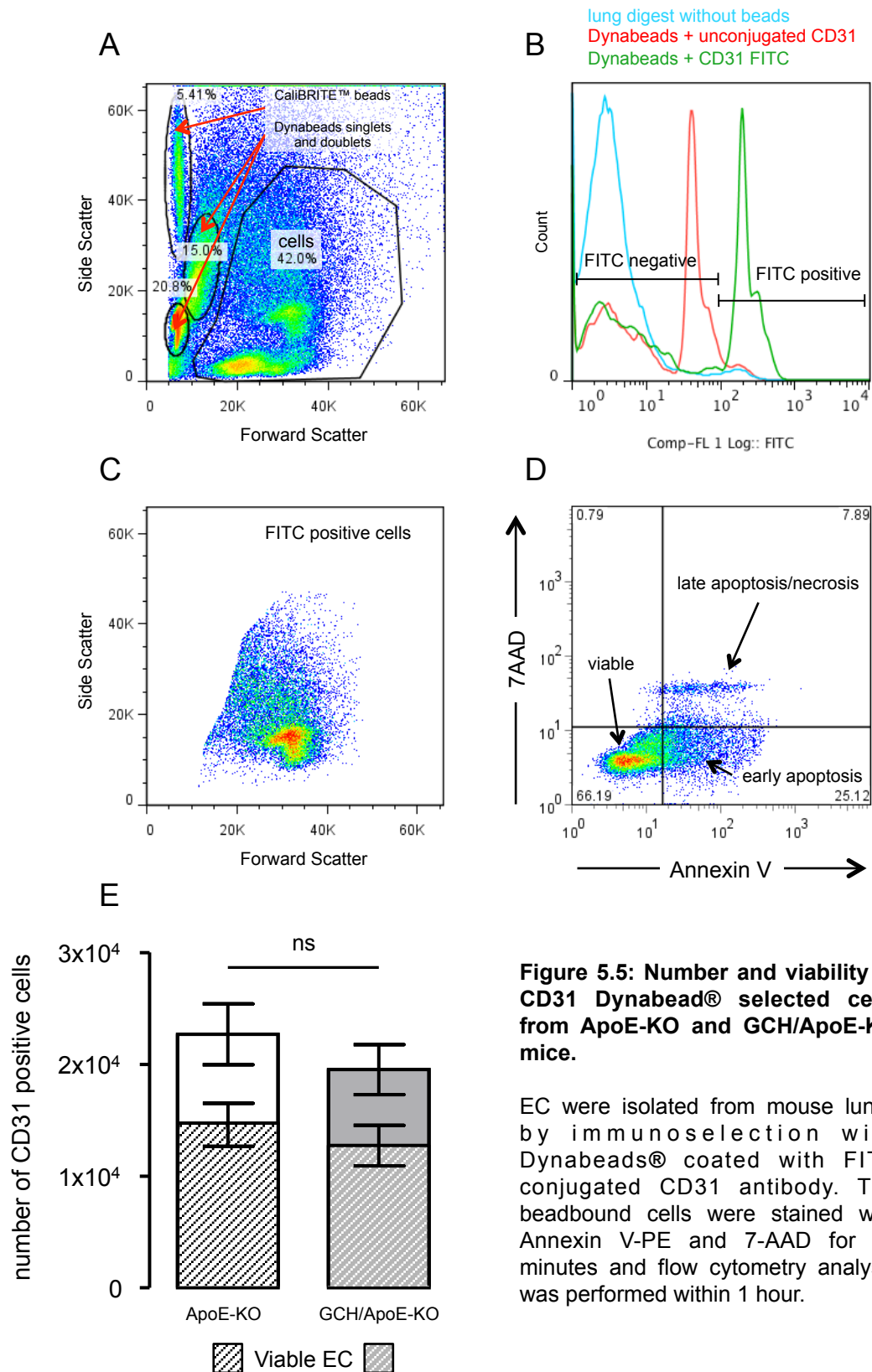


Figure 5.5: Number and viability of CD31 Dynabead® selected cells from ApoE-KO and GCH/ApoE-KO mice.

EC were isolated from mouse lungs by immunoselection with Dynabeads® coated with FITC conjugated CD31 antibody. The beadbound cells were stained with Annexin V-PE and 7-AAD for 15 minutes and flow cytometry analysis was performed within 1 hour.

A: Forward and side scatter plot for all events. A defined number of PE CaliBRITE™ beads was added to each sample to determine the total number of CD31 positive cells per endothelial cell isolate. A gate was drawn around all cells for further analysis. Morphologically it was possible to distinguish two main populations of cells with one consisting of larger and more complex cells than the other.

B: FITC fluorescence in “cells” gate. **Blue histogram:** Lung digest cells without magnetic bead selection. **Red histogram:** Cells isolated with magnetic beads coated with CD31 antibody without fluorochrome. This histogram was used to determine the 95% confidence interval for FITC negative cells and demonstrates the striking autofluorescence of Dynabeads® in comparison to the not bead-selected sample. **Green histogram:** Cells isolated with magnetic beads coated with CD31 FITC antibody. The fluorescence profile of contaminating non-beadbound cells in the Dynabead® selected samples corresponds to the fluorescence profile of not bead selected lung digest cells (blue histogram). All further analysis was performed on the FITC positive subpopulation (CD31 positive cells) of CD31 FITC isolated cells to exclude contaminating CD31 negative cells.

C: Forward and side scatter plot for FITC positive cells. The FITC positive (CD31 positive) subpopulation was identified as the population of larger, more complex cells.

D: AnnexinV-PE was used to detect early apoptosis and 7-Amino-Actinomycin (7-AAD) to detect late apoptosis or necrosis in CD31 positive cells. Approximately 64% of CD31 positive cells were viable (AnnexinV negative/7-AAD negative) and the rest either in early (AnnexinV positive/7-AAD negative) or late (AnnexinV positive/7-AAD positive) apoptosis.

E: There was no significant difference in the total number of CD31 positive cells or the number of viable CD31 positive cells from ApoE-KO and GCH/ApoE-KO mice.

n=5 per group.

Approximately 30% of all Dynabeads® isolated cells were FITC positive and there was no significant difference between the genotypes.

The FITC positive (CD31 positive) population was analyzed for AnnexinV-PE and 7-AAD binding. Approximately 64% of CD31 positive cells were viable (AnnexinV negative/7-AAD negative) and the rest either in early (AnnexinV positive/7-AAD negative) or late (AnnexinV positive/7-AAD positive) apoptosis. There was no significant difference in the total number of CD31 positive cells or the number of viable CD31 positive cells from ApoE-KO and GCH/ApoE-KO mice (Figure 5.5), leading to the conclusion that a difference in endothelial cells viability

directly after the isolation process is unlikely to be the cause of the observed increase in endothelial cell number in GCH/ApoE-KO cultures after 3,4 and 5 days.

5.2.3.3 Endothelial and endothelial progenitor cell content in CD31 MACS® MicroBead isolated cells

Endothelial cells were isolated from ApoE-KO and GCH/ApoE-KO mouse lungs by CD31 MACS® MicroBead isolation. CD31 FITC was used for the isolation and the cells were stained with CD45 PE-Cy7, CD34 PE and Flk-1 APC antibodies or the corresponding isotype controls as described in Chapter 3.5.7 and analyzed by flow cytometry.

As discussed in Chapter 3.5 four subpopulations of cells can be identified with regard to CD31 and CD45 expression. CD31 high/CD45 dim cells also express high levels of the endothelial cell markers CD102, Tie-2 and eNOS and they are the only cells that express the GCH and LacZ transgenes and proliferate in culture after flow sorting. This endothelial cell population was further analyzed for expression of the EPC marker CD34 and Flk-1 (Figure 5.6). There was no significant difference in the total number of endothelial cells between ApoE-KO and GCH/ApoE-KO detectable. Approximately 25% of all endothelial cells were also positive for CD34 and Flk-1 and there was no difference in the number and percentage of Flk-1/CD34 positive endothelial cells detectable between the genotypes. These results suggest that the observed increase in endothelial cell number in GCH/ApoE-KO cultures after 3,4 or 5 days is not driven by a larger percentage of highly proliferative endothelial progenitor cells in lung endothelial cell isolates from GCH/ApoE-KO mice.

Since neither a significant difference in number or viability of isolated endothelial cells nor in progenitor marker expression could be detected it is likely that the observed increase in endothelial cell number in GCH/ApoE-KO cultures can be explained by an increase in proliferation rate due to transgenic BH4 augmentation. To test whether in vitro BH4 supplementation has got the same effects on primary endothelial cell growth as GCH overexpression, primary endothelial cells from ApoE-KO mice were incubated with the BH4 precursor sepiapterin.

Figure 5.6: EC and EPC content in CD31 MACS® MicroBead isolated cells from ApoE-KO and GCH/ApoE-KO mice.

EC were isolated from mouse lungs by CD31 MACS® MicroBead isolation. CD31 FITC was used for the isolation and the cells were stained with CD45 PE-Cy7, CD34 PE and Flk-1 APC antibodies or the corresponding isotype controls for flow cytometry analysis. A defined number of FITC CaliBRITE™ beads was added to each sample to determine the total number of EC and EPC per sample.

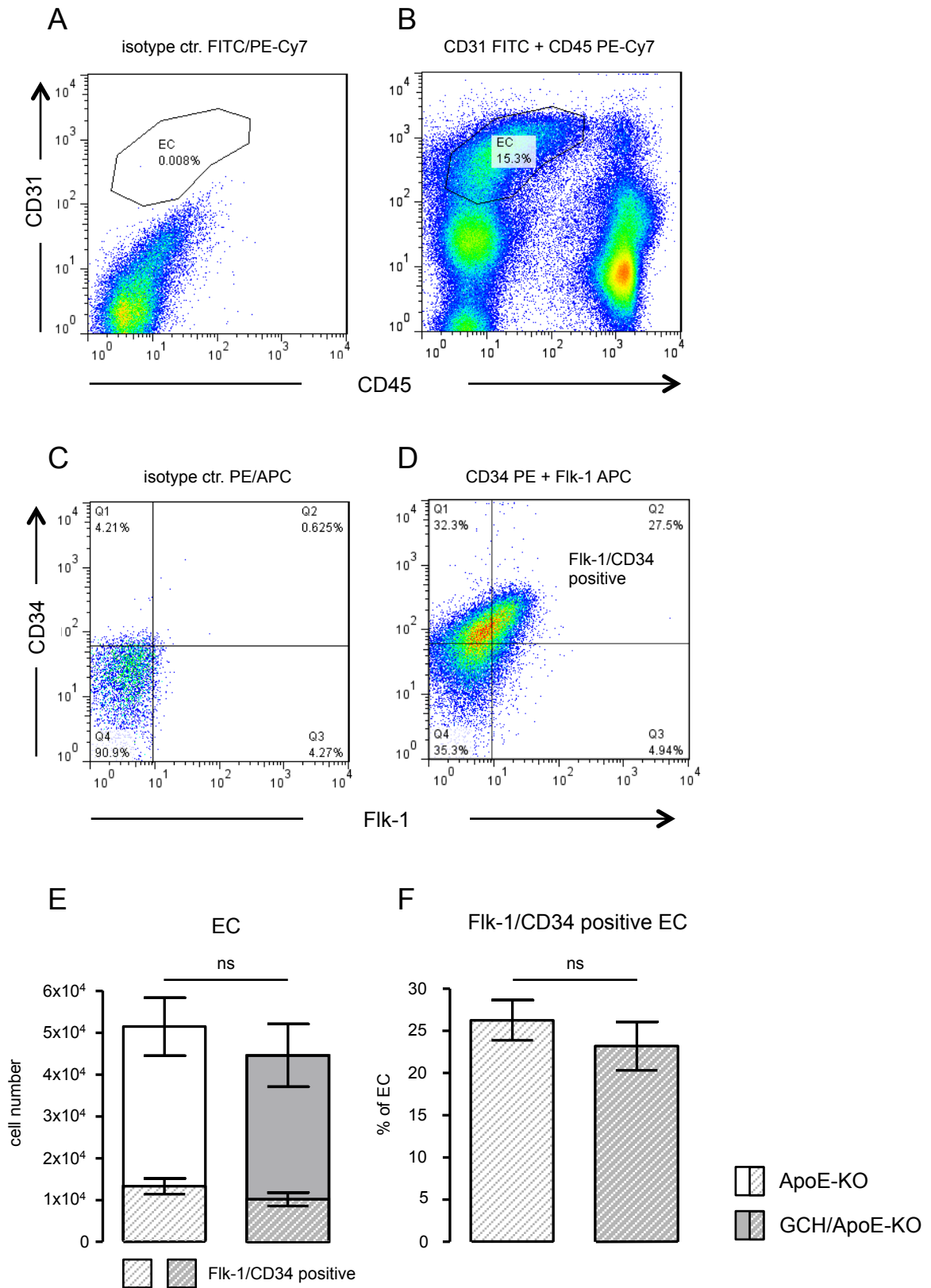
A-B: For EC quantification a gate was applied around the CD31 high/CD45 dim population.

C-D: Flk-1 and CD34 positive cells were quantified within the EC gate

E-F: There was no significant difference in the total number of EC or the number and percentage of Flk-1/CD34 positive EC between the genotypes.

n=5-7 per group.

Figure 5.6



5.3 Influence of *in vitro* Sepiapterin supplementation on growth of cultured ApoE-KO EC

In vitro BH4 supplementation with the precursor sepiapterin has been shown to enhance proliferation in coronary endothelial cell from diabetic rats (Marinos *et al.*, 2001), bovine aortic endothelial cells (BAEC) (Shimizu *et al.*, 1999) and HUVEC (Chen *et al.*, 2010). To investigate whether *in vitro* sepiapterin supplementation has the same effect on proliferation of primary endothelial cells from ApoE-KO mice as transgenic GCH overexpression, endothelial cells were isolated from mouse lungs by CD31 MACS[®] MicroBead isolation and cultured in EC medium with and without sepiapterin for four days before flow cytometry analysis.

5.3.1 Dose finding for *in vitro* sepiapterin supplementation

The aim was to identify the concentration of sepiapterin in the culture media, which would lead to a relative increase in BH4 in cultured primary endothelial cells similar to the 5-fold increase observed in GCH/ApoE-KO compared to ApoE-KO. The most commonly used concentration of sepiapterin is 10 μ M (Chen *et al.*, 2010; Marinos *et al.*, 2001; Shimizu *et al.*, 1999; Thum *et al.*, 2007) but the use of concentrations up to 100 μ M has been reported.

Endothelial cells were isolated from ApoE-KO mouse lungs by CD31 MACS[®] MicroBead isolation. The cells from 12 lungs were pooled and divided into 12 samples to be plated into wells with media containing 0 μ M, 5 μ M, 10 μ M or 50 μ M sepiapterin. Media was replaced every 24 hours and after 4 days cells were harvested for biopterin analysis.

Figure 5.7

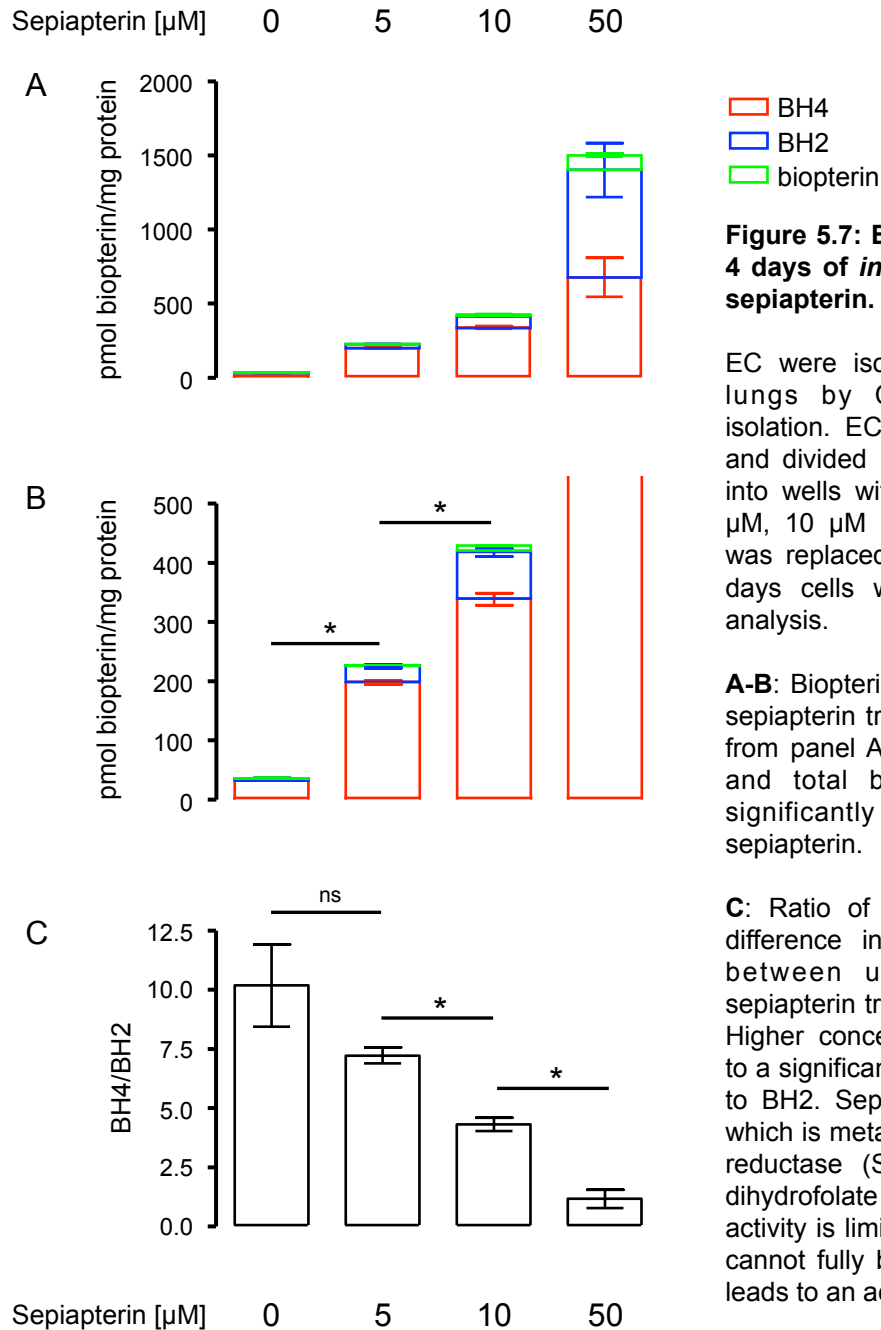


Figure 5.7: Biopterin content in EC after 4 days of *in vitro* supplementation with sepiapterin.

EC were isolated from ApoE-KO mouse lungs by CD31 MACS® MicroBead isolation. EC from 12 lungs were pooled and divided into 12 samples to be plated into wells with media containing 0 μ M, 5 μ M, 10 μ M or 50 μ M sepiapterin. Media was replaced every 24 hours and after 4 days cells were harvested for biopterin analysis.

A-B: Biopterin levels in EC after 4 days of sepiapterin treatment. Panel B shows data from panel A on an expanded scale. BH4 and total biopterin content increased significantly with increasing doses of sepiapterin.

C: Ratio of BH4 to BH2. No significant difference in the ratio of BH4 to BH2 between untreated cells and 5 μ M sepiapterin treated cells could be detected. Higher concentrations of sepiapterin lead to a significant decrease in the ratio of BH4 to BH2. Sepiapterin is a synthetic pterin, which is metabolized to BH2 by sepiapterin reductase (SR) and further to BH4 by dihydrofolate reductase (DFHR). If DFHR activity is limiting high doses of sepiapterin cannot fully be converted into BH4, which leads to an accumulation of BH2.

* $p < 0.05$, $n = 3$ per group.

Levels of BH4 and total biopterin content increased significantly with increasing doses of sepiapterin (Figure 5.7). 5 μ M Sepiapterin lead to a 6-fold increase in BH4 levels and was chosen as the concentration for the growth experiment. Although 10 μ M and 50 μ M sepiapterin significantly increased BH4 levels, these high concentrations also lead to a significant decrease in the ratio of BH4 to BH2. Sepiapterin is a synthetic pterin that is metabolized to BH2 by sepiapterin reductase and further to BH4 by dihydrofolate reductase (DFHR). If DFHR activity is limiting high doses of sepiapterin cannot fully be converted into BH4, which leads to an accumulation of BH2. The ratio of BH4 to BH2 is of importance because non-functional BH2 competes with BH4 for binding to the active site of eNOS, which can lead to eNOS uncoupling (Crabtree *et al.*, 2008; Crabtree *et al.*, 2009).

5.3.2 Influence of *in vitro* supplementation with 5 μ M sepiapterin on primary endothelial cells growth in culture

Endothelial cells were isolated from ApoE-KO mouse lungs by CD31 MACS® MicroBead isolation. For each sample the cells from 2 lungs were pooled and divided into 2 for plating into 2 wells containing either EC medium or EC medium with 5 μ M sepiapterin respectively. Media were replaced every 24 hours and after 4 days cells were harvested for flow cytometry analysis. Cells were stained with CD31 FITC, CD45 PE-Cy7, CD34 PE and Flk-1 APC antibodies as described before. The use of CD31 FITC or CD31 APC, as used for quantification of endothelial cells after 5 days in culture (Chapter 5.2.2), is compared in Figure 5.8. Although particularly CD45 positive cells are autofluorescent in the FITC channel, the combinations of CD31 FITC with CD45 PE-Cy7 or CD31 APC with CD45 PE-Cy7 antibodies lead to the same results when quantifying endothelial cells.

Figure 5.8

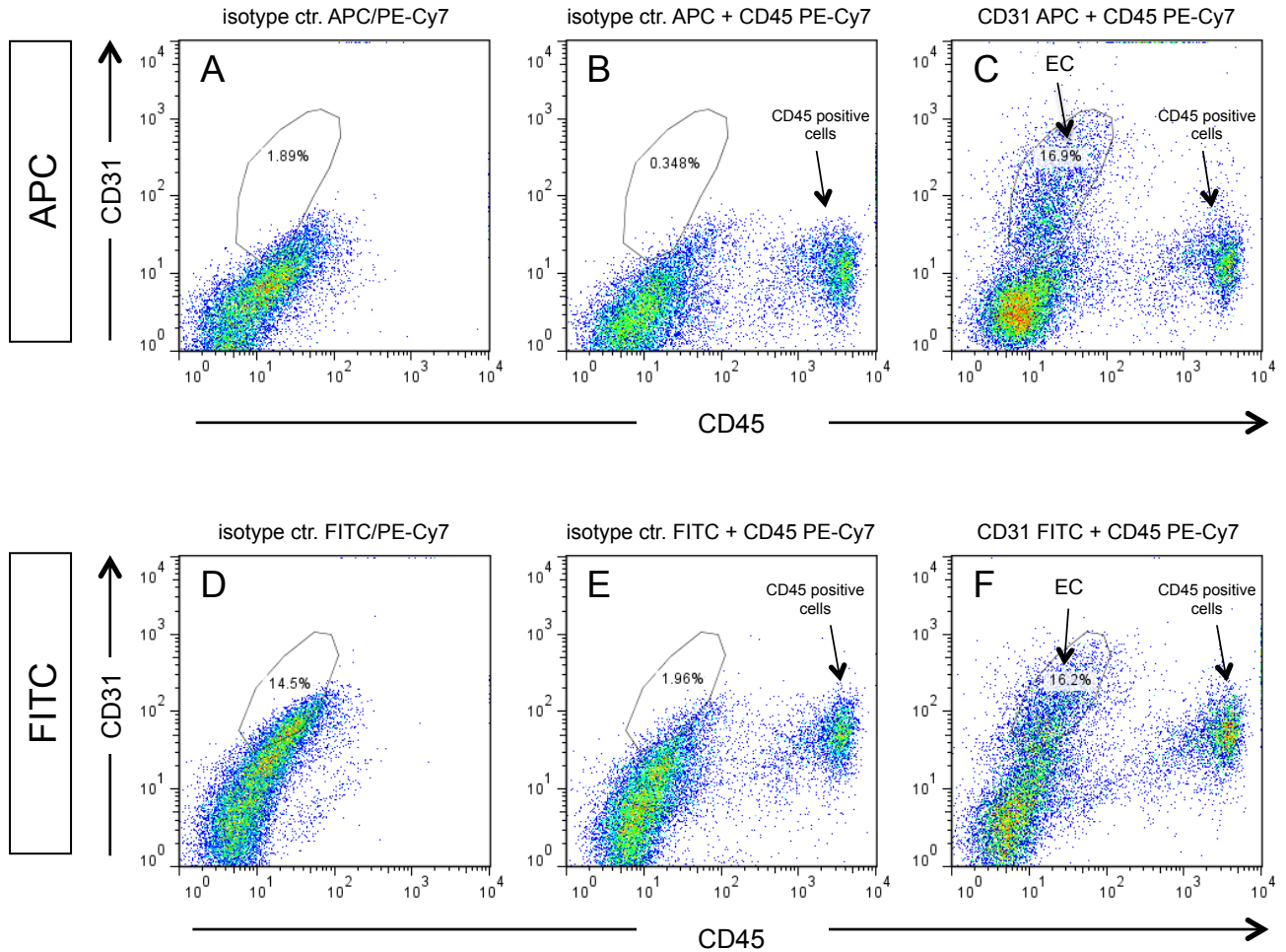


Figure 5.8: Quantification of EC by flow cytometry after 4 days in culture. Comparison of APC and FITC coupled CD31 antibody.

EC were isolated from mouse lungs by CD31 MACS® MicroBead isolation and cultured for 4 days before flow cytometry analysis. After Fc blocking the samples were stained with CD45 PE-Cy7 and CD31 FITC or CD31 APC or the corresponding isotype controls. To quantify EC a gate around the CD31 positive/CD45 dim population was applied.

A-C: Identification of EC with CD31 APC. **D-F:** Identification of EC with CD31 FITC. Both fluorochromes identified the same number of cells as EC (16.9% and 16.2% of all cells for CD31-APC and -FITC respectively) (**C** and **F**). However, the cells showed pronounced autofluorescence heavily overlapping with FITC detection (**D**) with CD45 positive cells being particularly fluorescent (**E**). Using CD31 FITC alone to identify CD31 positive cells would not be appropriate in these settings but using it in combination with CD45 PE-Cy7 allows the quantification and further analysis of EC.

Figure 5.9

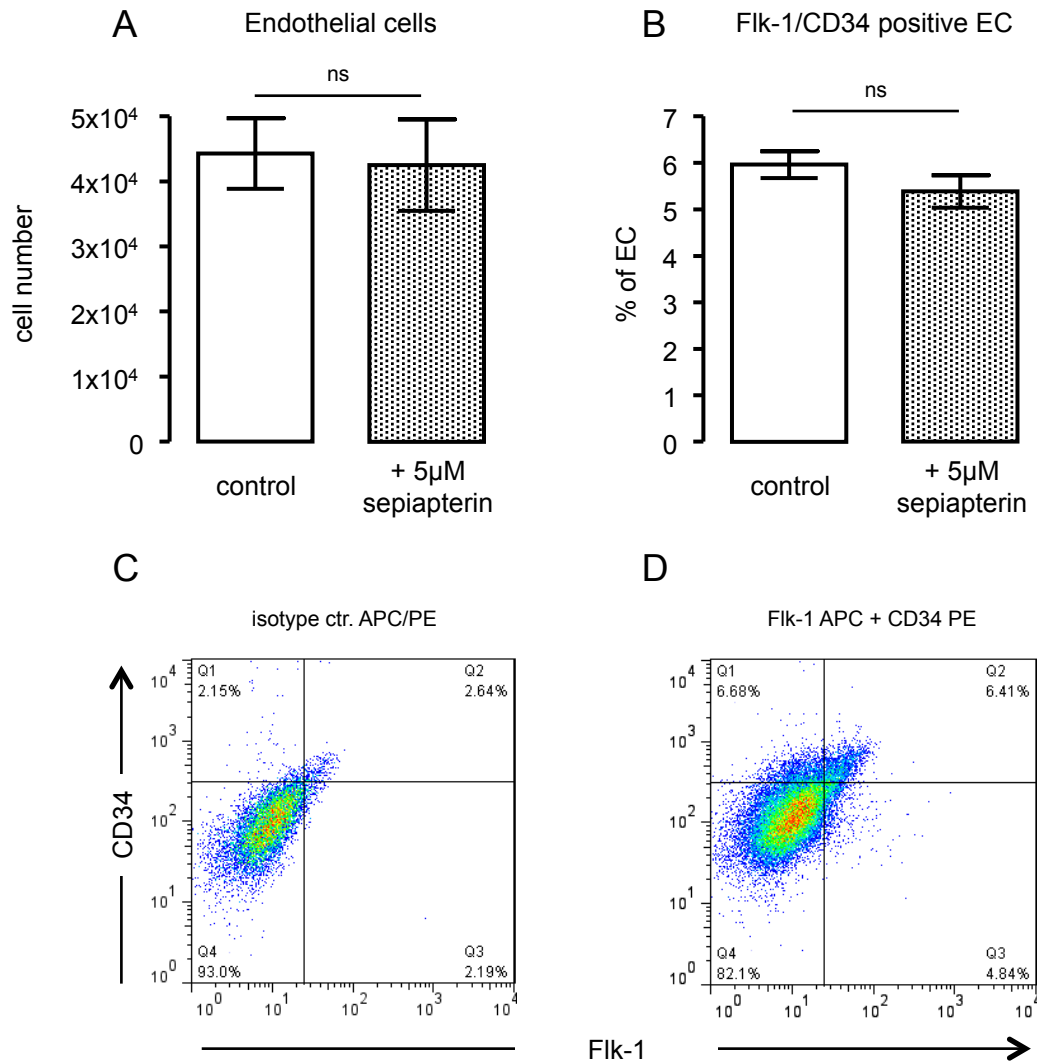


Figure 5.9: Influence of *in vitro* supplementation with 5µM sepiapterin on EC growth in culture.

EC were isolated from ApoE-KO mouse lungs by CD31 MACS® MicroBead isolation. For each sample EC from 2 lungs were pooled and divided into 2 for plating into 2 wells containing either EC medium or EC medium with 5µM sepiapterin respectively. Media were replaced every 24 hours and after 4 days cells were harvested for flow cytometry analysis. Cells were stained with CD31 FITC, CD45 PE-Cy7, CD34 PE and Flk-1 antibodies or the corresponding isotype controls. A defined number of FITC CaliBRITE™ beads was added to each sample to determine the total number of EC and EPC per sample. CD31 and CD45 were used to gate on EC and further analyze CD34 and Flk-1 staining in this gate.

A: Total number of endothelial cells after 4 days in culture. **B:** Proportion of EC, which are also CD34 and Flk-1 positive. **C-D:** Analysis of CD34 and Flk-1 within the EC gate.

5µM sepiapterin had no influence on the total number of EC after 4 days in culture or the proportion of EC positive for the EPC marker CD34 and Flk-1.

n=8 per group.

5 μ M sepiapterin did not influence the total number of endothelial cells after 4 days in culture. Both in control and sepiapterin treated samples 5-6% of endothelial cells were also CD34 and Flk-1 positive (Figure 5.9). This proportion of CD34 and Flk-1 double positive endothelial cells is lower than directly after MACS® MicroBead isolation (Figure 5.6), suggesting that a substantial proportion of endothelial cells has matured in culture, losing progenitor marker expression.

5.4 Influence of transgenic GCH overexpression on growth potential of cultured endothelial cells from a healthy background without vascular disease

In contrast to ApoE-KO mice, WT and GCH transgenic mice do not develop atherosclerosis on normal chow diet; eNOS transgenic mice are also healthy animals without vascular disease but show eNOS uncoupling that can be rescued by transgenic GCH overexpression (eNOS/GCH mice) (Bendall *et al.*, 2005). To investigate the effect of endothelial specific GCH overexpression on endothelial cell growth in the absence of a vascular disease state, primary endothelial cells were isolated by CD31 MACS® MicroBead selection from WT, GCH, eNOS and eNOS/GCH mouse lungs. The cells were cultured over 5 days before staining with CD31 APC and CD45 PE-Cy7 antibodies and flow cytometry analysis.

The results were adjusted to WT mean of each batch to adjust for batch-to-batch variability. There was no significant difference in total number of endothelial cells detectable between the genotypes (Figure 5.10). In contrast to ApoE-KO background, transgenic GCH overexpression does therefore not appear to affect *in vitro* growth of primary endothelial cells from healthy animals.

Figure 5.10

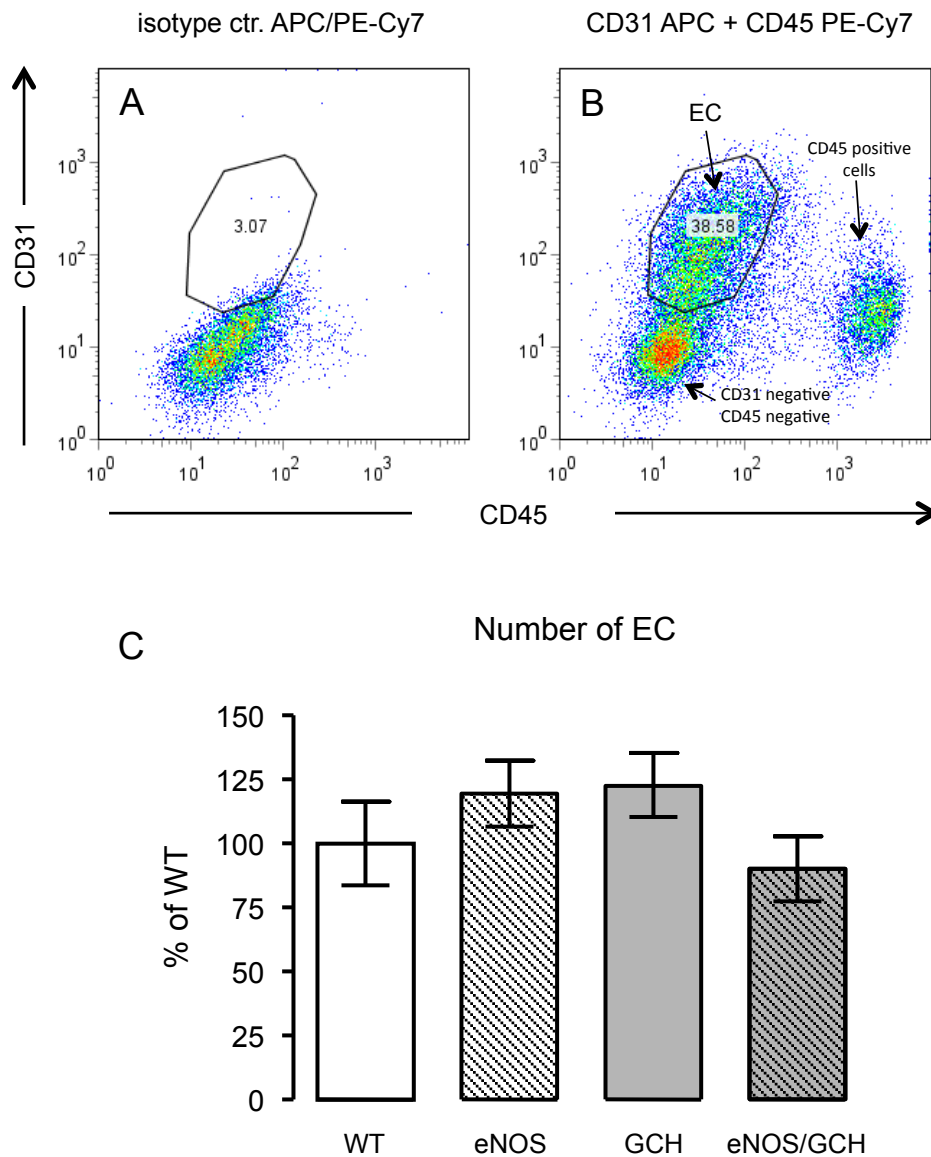


Figure 5.10: EC from WT, eNOS, GCH and eNOS/GCH mice. Comparison of EC growth over 5 days in culture.

EC were isolated from mouse lungs by CD31 MACS® MicroBead isolation and cultured for 5 days. The cells were then harvested for flow cytometry analysis and stained with CD31 APC and CD45 PE-Cy7 antibodies or the corresponding isotype controls. A defined number of FITC CalIBRITE™ beads was added to each sample to determine the total number of EC and EPC per sample.

A-B: Cells stained with isotype controls for APC and PE-Cy7 (**A**) and stained with CD31 APC and CD45 PE-Cy7 antibodies (**B**). There are three distinct populations detectable, CD31 positive/CD45dim (EC), CD45 positive and CD31 negative/CD45 negative cells.

C: There are no significant differences in number of EC between the genotypes.

n=4 per group.

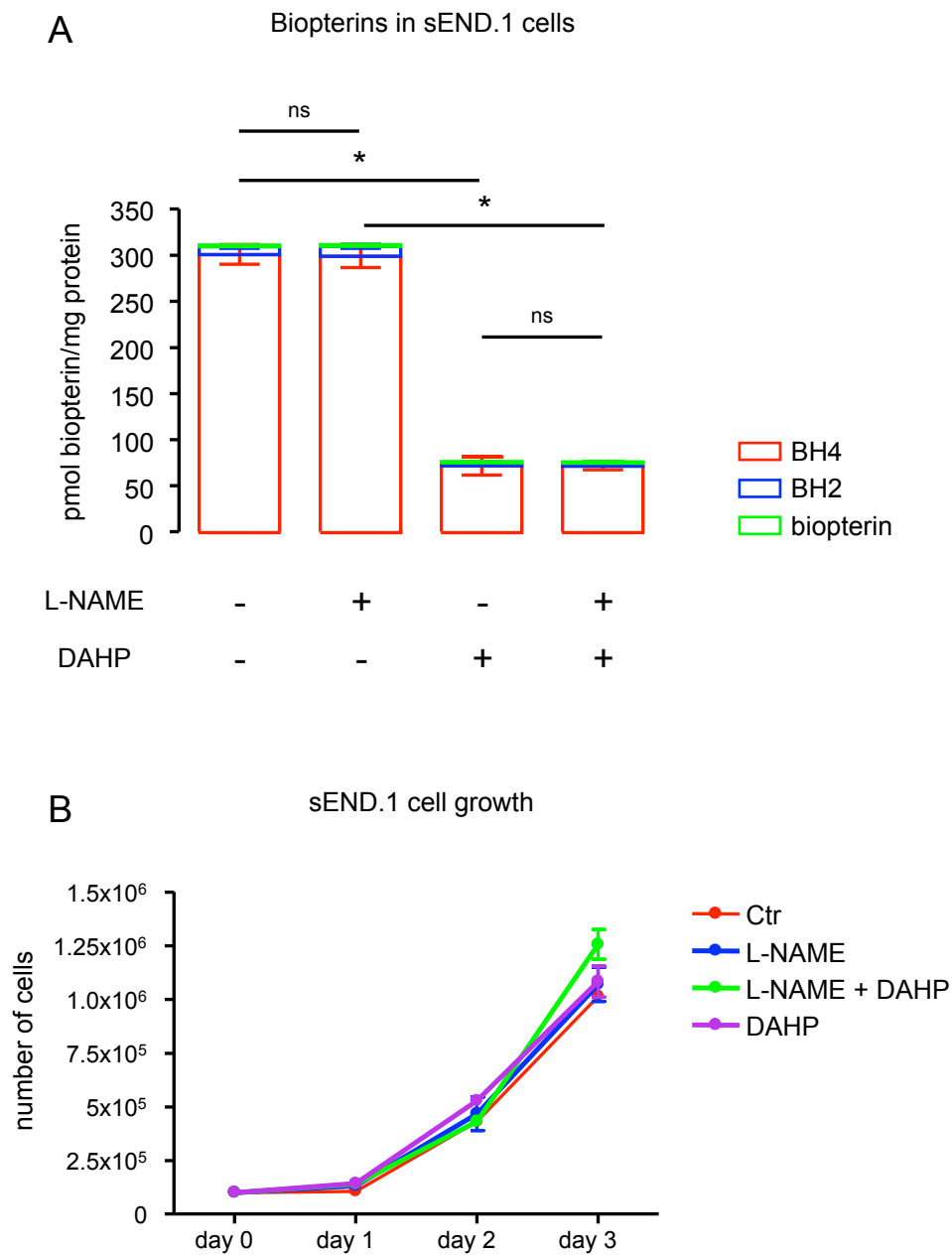
5.5 Influence of BH4 availability on sEND.1 cell growth

sEND.1 cells were originally derived from a mouse subcutaneous capillary endothelioma induced by infection with a retrovirus expressing polyoma middle T oncogene (Williams *et al.*, 1988). As an endothelial cell line they strongly express eNOS and GTPCH protein and exhibit high levels of BH4 and eNOS activity (Crabtree *et al.*, 2009; Ghigo *et al.*, 1995; Walter *et al.*, 1994). To study whether a reduction in BH4 bioavailability would affect sEND.1 cell proliferation, BH4 synthesis was inhibited by the addition of 1mM DAHP to the culture medium. 1mM L-NAME was used to inhibit eNOS to evaluate whether potential changes in proliferation with DAHP treatment may be eNOS dependent.

On day 0 sEND.1 cells were plated into 6 well dishes containing medium $\pm$ 1mM L-NAME $\pm$ 1mM DAHP at a density of 1×10^5 cells/well. Because DAHP was solubilized in DMSO, 1% DMSO was added to the medium of all groups. Every 24 hours 4 wells per group were harvested for cell count and biopterin measurements respectively and the medium in the remaining cells was replaced with fresh medium.

1mM DAHP reduced BH4 levels by 76%. L-NAME treatment did not alter BH4 levels. Neither eNOS inhibition by L-NAME nor GTPCH inhibition by DAHP had an influence on sEND.1 cell proliferation (Figure 5.11).

Figure 5.11

**Figure 5.11: Influence of BH4 availability on sEND.1 cell growth.**

On day 0 sEND.1 cells were plated into 6 well dishes containing medium $\pm$ 1mM L-NAME $\pm$ 1mM DAHP at a density of 1×10^5 cells/well. Every 24 hours 4 wells per group were harvested for cell count and biopterin measurements respectively.

A: Biopterin levels in sEND.1 cells after 24 hours of L-NAME and DAHP treatment. There was no significant difference in biopterin content in L-NAME treated cells compared to control. DAHP treatment reduced BH4 levels by 76% with or without L-NAME treatment
* $p < 0.05$, $n = 4$ per group.

B: L-NAME and DAHP treatment had no influence on sEND.1 growth. $n = 4$ per group.

It is possible that the concentration of DAHP and was chosen too low to affect sEND.1 cell proliferation. Marinos *et al.* used a 10-fold higher concentration of DAHP (10mM) to inhibit proliferation of rat coronary endothelial cells (Marinos *et al.*, 2001). However, in her publication 10mM DAHP only achieved a 68% reduction in BH4 levels compared to 1mM DAHP leading to a 76% reduction in BH4 in sEND.1 cells. Similar to the data presented in this chapter, 1mM DAHP has been reported to reduce BH4 levels in HUVEC by 75% before (Werner-Felmayer *et al.*, 1993). When testing the effect of 1mM DAHP on BH4 levels of primary lung endothelial cells from GCH transgenic mice, after 3 days in culture BH4 content was reduced by 68% in samples with 1mM DAHP. sEND.1 cells contain very high levels of BH4 and it is possible that after inhibiting *de novo* synthesis of BH4 with 1mM DAHP there might still be sufficient BH4 present to maintain normal eNOS function. However, inhibiting eNOS with L-NAME did not affect sEND.1 cell proliferation either. sEND.1 cells are tumor-derived and NO bioavailability might not have the same effect on these cells as reported for primary endothelial cells (Marinos *et al.*, 2001).

5.6 *In vivo* Angiogenesis: Quantification of EC migration into Matrigel™ plugs

Endothelial cell proliferation and migration is a key step in the initiation of angiogenesis, the formation of new blood vessels (Isner and Asahara, 1999). Physiological angiogenesis plays an important role in tissue growth, wound healing (Tonnesen *et al.*, 2000) and in the response to ischemia (Kersten *et al.*, 1999; Lloyd *et al.*, 2001). To investigate the effect of endothelial specific GCH overexpression on *in vivo* angiogenesis in ApoE-KO mice a Matrigel plug assay was performed.

The Matrigel plug assay was introduced by Passaniti and colleagues and is now commonly used for *in vivo* testing for angiogenesis (Passaniti *et al.*, 1992). Matrigel™ Basement Membrane Matrix is a gelatinous protein mixture extracted from the Engelbreth-Holm-Swarm mouse sarcoma, which is liquid at 4°C and quickly forms a gel at body temperature. When mixed with angiogenic factors such as FGF or VEGF and injected subcutaneously *in vivo*, endothelial cells amongst other cells migrate into the Matrigel plug and form vessels. Traditionally angiogenesis in the plug is assessed histologically or by measuring hemoglobin content. However, both methods only serve as an estimation of endothelial invasion and angiogenesis and histology in particular relies on evenly distributed vessels throughout the plug, which is not necessarily the case. In 2009 Adini *et al.* therefore published an alternative method for quantifying Matrigel plug angiogenesis more precisely by flow cytometry (Adini *et al.*, 2009). The method used here for quantification of endothelial cell content follows the method published by Adini and colleagues.

To assess the necessity of adding growth factors to Matrigel to stimulate cell invasion and angiogenesis, 0.5 ml ice-cold Matrigel™ without added growth factors was injected subcutaneously into the flank of anaesthetized ApoE-KO/LacZ mice. After 6 days the animals were euthanized, the Matrigel plugs removed, fixed and stained with X-Gal as described in Chapter 2.10. Blue endothelial staining showed small, newly developed vessels invading the Matrigel plugs and forming short side branches (Figure 5.12).

Figure 5.12

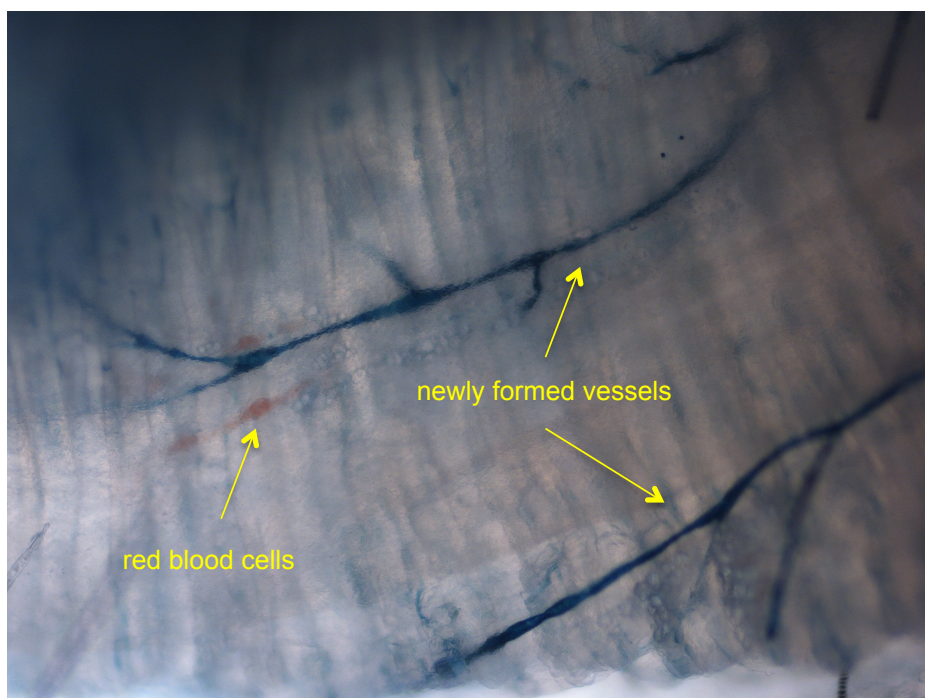


Figure 5.12: Matrigel™ plug, X-Gal staining of angiogenesis.

0.5 ml ice-cold Matrigel™ without added growth factors was injected subcutaneously into the flank of 3 anaesthetized ApoE-KO/LacZ mice. After 6 days the animals were euthanized, the Matrigel plugs removed, fixed and stained with X-Gal.

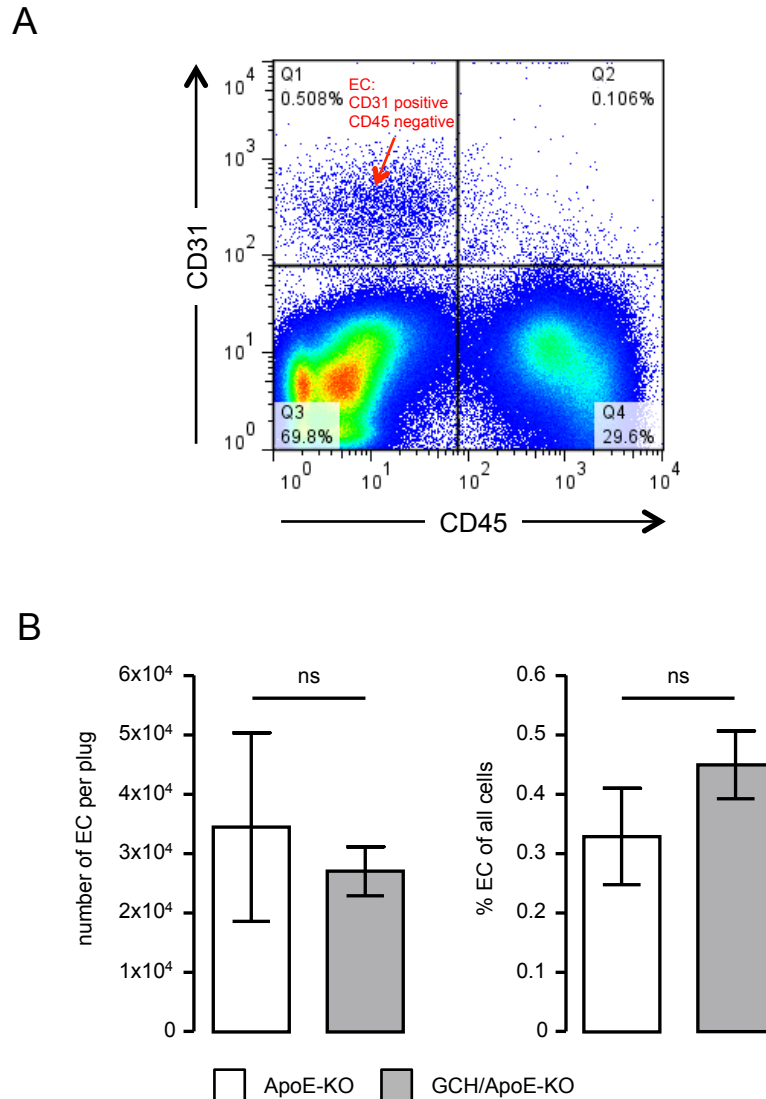
Blue endothelial staining showed newly developed small vessels invading the Matrigel plugs and forming short side branches. Angiogenesis was not evenly distributed throughout the plug but appeared to originate from few areas on the surface of the plug.

Angiogenesis was not evenly distributed throughout the plug but appeared to originate from only one or two areas on the surface of the plug. The addition of bFGF as described in Adini and colleagues (Adini *et al.*, 2009) was therefore deemed necessary in order to obtain sufficient cells for analysis.

ApoE-KO and GCH/ApoE-KO mice were anaesthetized and injected subcutaneously into the flank with 0.5 ml ice-cold Matrigel™ containing 0.5µg/ml bFGF. After 6 days the animals were euthanized and the Matrigel plugs removed. The plugs were then digested, stained with CD31 APC and CD45 PE-Cy7 antibodies as described in Chapter 2.15 and analyzed by flow cytometry.

The removed plugs appeared highly infiltrated with cells and vascularized, causing partial disintegration in some plugs. There was no significant difference in endothelial cell content in Matrigel plugs between ApoE-KO and GCH/ApoE-KO as assed by flow cytometry (Figure 5.13). However, despite pronounced cell infiltration into the plugs, the average percentage of endothelial cells was only 0.4%, more than 10-fold lower than reported for C57Bl/6 mice (Adini *et al.*, 2009). It is possible that this may be due to using ApoE-KO mice instead of WT and/or to the age of the injected mice. Adini *et al.* used 2-month-old mice whereas the mice used in the presented experiment were 4 to 5 months old.

Figure 5.13

**Figure 5.13: Quantification of EC migration into Matrigel™ plugs.**

ApoE-KO and GCH/ApoE-KO mice were anaesthetized and injected subcutaneously into the flank with 0.5 ml ice-cold Matrigel™ containing 0.5 µg/ml bFGF. After 6 days the animals were euthanized and the Matrigel plugs removed.

The plugs were then digested with 3U/ml dispase, 2.8 U/ml Liberase Blendzyme III, 38U/ml hyaluronidase and 100U/ml DNase in PBS. The liberated cells were strained through a 40µm strainer, washed with PBS containing 0.2% BSA and stained with CD31 APC and CD45 PE-Cy7 or the corresponding isotype controls.

A: To identify EC, the Matrigel plug cells were stained with CD31 APC and CD45 PE-Cy7. The gates were set to APC and PE-Cy7 isotype control and the CD31 positive/CD45 negative population (EC) was quantified.

B: There was no significant difference in number of Matrigel plug EC between ApoE-KO and GCH/ApoE-KO or the EC percentage of all plug cells.

n=5-9 per group.

A range of growth factor concentrations was tested during the setup period for the matrigel plug assay. As expected, there was only a minor angiogenic response in the negative control or in plugs containing 0.1µg/ml bFGF. bFGF concentrations of 0.2µg/ml, 0.4µg/ml and 0.5µg/ml resulted in variably infiltrated plugs, some of which were deep red as a sign of high hemoglobin content and started disintegrating, even with 0.2µg/ml bFGF. A cohort of ApoE-KO mice was also injected with Matrigel containing VEGF 100ng/ml. However, when removing the plugs after 6 days, the same problem of disintegrating deep red plugs was observed in 2 out of 6 of the mice. When analyzing the cells contained in the plugs, the endothelial cell content was very low with fewer than 10⁴ CD31 positive/CD45 negative cells per plug.

5.7 Summary and Discussion

Survival or loss of endothelial cells are important aspects of the response to injury or vascular disease, whereas endothelial cell proliferation is a critical step in endothelial regeneration, vascular repair and angiogenesis. eNOS and NO have been shown to be key regulators of endothelial cell proliferation (Morbidelli *et al.*, 1996; Papapetropoulos *et al.*, 1997), migration (Murohara *et al.*, 1999) and angiogenesis (Murohara *et al.*, 1998; Papapetropoulos *et al.*, 1997) and to play a critical role in the prevention of endothelial cell senescence (Hayashi *et al.*, 2006) and apoptosis (Dimmeler *et al.*, 1997; Kang-Decker *et al.*, 2007; Rossig *et al.*, 2000; Wright *et al.*, 2010). There is increasing evidence that the essential eNOS cofactor BH4 may enhance endothelial cell proliferation (Chen *et al.*, 2010; Marinos *et al.*, 2001; Michaelis *et al.*, 2007; Shimizu *et al.*, 1999). *In vitro* supplementation with the BH4 precursor sepiapterin has been shown to enhance

proliferation in coronary endothelial cell from diabetic rats (Marinos *et al.*, 2001), bovine aortic endothelial cells (BAEC) (Shimizu *et al.*, 1999) and HUVEC (Chen *et al.*, 2010). These effects may be mediated either by a direct antioxidant effect of BH4 or/and improved eNOS function with increased NO availability and reduced production of reactive oxygen species.

The aim of this chapter was to investigate the effect of BH4 bioavailability on endothelial cell growth in the absence and presence of vascular disease, using primary endothelial cells with and without transgenic GCH overexpression, the endothelial cell line sEND.1 and an *in vivo* Matrigel plug assay.

The key findings of this Chapter are:

- BH4 augmentation by transgenic endothelial specific GCH overexpression improves *in vitro* growth of primary endothelial cells from ApoE-KO mice.
- *In vitro* supplementation with the BH4 precursor sepiapterin does not significantly alter *in vitro* growth of primary endothelial cells from ApoE-KO mice.
- In the absence of vascular disease endothelial specific GCH overexpression does not appear to improve *in vitro* growth of primary endothelial cells.
- Inhibition of BH4 synthesis with 1mM DAHP and NO synthesis with 1mM L-NAME does not affect proliferation of sEND.1 cells.
- Endothelial specific GCH overexpression does not appear to improve *in vivo* Matrigel plug angiogenesis in ApoE-KO mice.

5.7.1 *In vitro* growth of primary mouse endothelial cells

The results presented in this chapter demonstrate for the first time that endothelial BH4 augmentation in the GCH mouse enhances *in vitro* growth of primary endothelial cells in a vascular disease setting. However, *in vitro* supplementation with the BH4 precursor sepiapterin did not improve *in vitro* growth of primary endothelial cells despite leading to a similar increase in BH4 levels as transgenic GCH overexpression. There was no evidence that transgenic GCH overexpression enhances *in vitro* growth of primary endothelial cells in the absence of vascular disease.

In contrast to the results presented above there are publications reporting enhanced endothelial cell proliferation after *in vitro* incubation with sepiapterin (Chen *et al.*, 2010; Marinos *et al.*, 2001; Shimizu *et al.*, 1999). Shimizu *et al.* (Shimizu *et al.*, 1999) used high concentrations of BH4 (10 μ M and 50 μ M) or sepiapterin (10 μ M and 50 μ M and 100 μ M) and found that both BH4 and sepiapterin increased proliferation and tube formation in BAEC in a concentration-dependent manner. Marinos *et al.* found a significant increase in proliferation of primary coronary endothelial cells from spontaneous diabetic rats in the presence of 10 μ M sepiapterin (Marinos *et al.*, 2001). In a separate publication, the same research group showed that endothelial cells from spontaneous diabetic rats have highly reduced levels of BH4, GTPCH activity (both 12% of normal) and GTPCH protein (Meininger *et al.*, 2000). The levels of BH4 in endothelial cells from non-diabetic rats were described to be similar to BH4 levels found in HUVEC (Meininger *et al.*, 2000; Werner-Felmayer *et al.*, 1993). Interestingly BH4 levels of cultured endothelial cells from spontaneous diabetic rats (passage 5-15) were equivalent to freshly isolated

endothelial cells despite not being exposed to the diabetic environment in the rat for a prolonged time. Chen and colleagues (Chen *et al.*, 2010) also reported a moderate increase in HUVEC proliferation and increased tube formation with 10 μ M sepiapterin. All these studies have in common that they are using a minimum of 10 μ M sepiapterin whereas the ApoE-KO endothelial cell growth experiment took place in the presence of 5 μ M sepiapterin. It is possible that the chosen concentration of 5 μ M sepiapterin was too low to enhance proliferation of primary endothelial cells from ApoE-KO mice. However, 5 μ M sepiapterin raised BH4 levels 6-fold compared to control, similar to the 5-fold increase in BH4 observed in GCH/ApoE-KO endothelial cells. It is also possible that endothelial cells that contain very low levels of BH4 like HUVEC (Aoki *et al.*, 2010; Chen *et al.*, 2010) benefit more from sepiapterin supplementation. The levels of BH4 reported for HUVEC by Chen and colleagues (Chen *et al.*, 2010) are only 14% of the BH4 levels in ApoE-KO endothelial cells. Biopterins also appear to be mainly oxidized in HUVEC with only 32% being BH4, whereas BH4 accounts for 75% of total biopterin in ApoE-KO endothelial cells. The magnitude of increase in endothelial cell proliferation with 10 μ M sepiapterin reported by Shimizu *et al.* (+20%), Marinos *et al.* (+40%) and Chen *et al.* (+20%) is similar to the 25-30% increase in proliferation of GCH/ApoE-KO endothelial cells after 5 days in culture. However, the proliferative effect of transgenic GCH overexpression was significantly more pronounced after a shorter growth period of 3 or 4 days. Interestingly, a significant proliferative effect of transgenic GCH overexpression could only be detected in the presence of vascular disease.

There was no evidence that a difference in lung endothelial cell content or viability of the isolated primary endothelial cells between ApoE-KO and GCH/ApoE-KO mice may have caused the observed differences in *in vitro* growth. The simultaneous increase in endothelial colony size and colony number in GCH/ApoE-KO cultures potentially indicates that GCH/ApoE-KO endothelial cell isolates may contain more cells with proliferative potential, which start forming endothelial cell islands and that these cells also proliferate at a faster rate forming larger islands. To investigate whether there may be a difference between ApoE-KO and GCH/ApoE-KO in content of mature and progenitor marker expressing endothelial cells, Flk-1 and CD34 positive endothelial cells were quantified in endothelial cell isolates. EC medium contains VEGF and enhanced expression of Flk-1 (VEGF receptor-2) could have caused enhanced endothelial cell survival (Gerber *et al.*, 1998) and proliferation (Morbideilli *et al.*, 1996; Papapetropoulos *et al.*, 1997). However, there was no difference in expression of the progenitor markers Flk-1 and CD34 in primary endothelial cells. Interestingly, these markers are down-regulated during 4 days in culture. The angiogenic and anti-apoptotic effect of VEGF is mediated by eNOS (Fukumura *et al.*, 2001; Gerber *et al.*, 1998; Morbideilli *et al.*, 1996; Papapetropoulos *et al.*, 1997), dependent on PI3K signaling (Gerber *et al.*, 1998; Papapetropoulos *et al.*, 1997). Sugiyama *et al.* (Sugiyama *et al.*, 2009) showed that knockdown of GTPCH1 attenuated VEGF induced eNOS activity and NO production and that these effects could be recovered by supplementation with BH4. It is therefore conceivable that conversely improved eNOS coupling due to endothelial BH4 augmentation by sepiapterin or GCH overexpression may enhance the proliferative and anti-apoptotic effect of VEGF.

Du *et al.* have shown that endothelial BH4 augmentation in the GCH transgenic mouse preserves phosphorylation of eNOS at the activating site serine 1177 in DOCA salt hypertension (Du *et al.*, 2008). Examining the effects of BH4 augmentation with sepiapterin in HUVEC, Chen *et al.* found an increase both in eNOS phosphorylation at serine 1177 and phosphorylated Akt. In addition, levels of GTP-bound wild-type RAS were increased and p85 α , a PI3K regulatory subunit, was up-regulated in sepiapterin treated cells. As an explanation for the interaction of BH4 augmentation and Akt/eNOS phosphorylation they suggested a positive feedback loop -initiated by improved eNOS function due to BH4 augmentation- of wild-type RAS activation leading to PI3K dependent Akt phosphorylation in turn leading to eNOS phosphorylation and activation (Chen *et al.*, 2010).

Activation of the PI3K/Akt signaling pathway due to improved eNOS coupling in the GCH transgenic mouse would both enhance endothelial cell proliferation and protect endothelial cells from apoptosis. Further work is needed to define the role PI3K signaling in mediating the effects of GCH overexpression on endothelial cell survival and proliferation in the absence and presence of vascular disease. Such experiments may include western blotting of endothelial cell lysates after a short period of growth to evaluate the effects of GCH overexpression on eNOS and Akt phosphorylation and the use of a PI3K inhibitor such as LY294002 during the growth period.

5.7.2 sEND.1 cell proliferation

Considering the role of NO in mediating endothelial cell proliferation and survival after apoptotic stimuli, modifying eNOS activity could be expected to have an

influence on proliferation in the endothelial cell line sEND.1. However, neither eNOS inhibition with L-NAME nor inhibition of GTPCH with DAHP influenced sEND.1 cell proliferation. It is possible though that oncogene expression in sEND.1 cells overrides physiological regulatory mechanisms of cell proliferation. sEND.1 cells are derived from mouse endothelioma induced by infection with a retrovirus expressing polyoma middle T oncogene (Williams *et al.*, 1988). There is evidence that polyoma middle T transformation of endothelial cells leads to activation of the PI3K/Akt pathway (Sausville *et al.*, 2008; Whitman *et al.*, 1985) and inhibiting eNOS activity with L-NAME or reducing the availability of the eNOS cofactor BH4 with DAHP may therefore not have a significant effect on cell proliferation, at least not in the absence of an apoptotic stimulus.

5.7.3 Matrigel plug angiogenesis assay

Similar to VEGF, bFGF has also been reported to stimulate *in vivo* angiogenesis and *in vitro* endothelial cell tube formation in an eNOS dependent manner (Babaei *et al.*, 1998; Sieber *et al.*, 2001). However, the signaling pathways involved in the proliferative and anti-apoptotic effects of VEGF on endothelial cells are more widely researched. Using Matrigel plugs containing bFGF for an *in vivo* angiogenesis assay, no difference between ApoE-KO and GCH/ApoE-KO could be detected. In both genotypes Matrigel plugs were strongly infiltrated with cells. However, endothelial cell content was lower than reported before for C57Bl/6 mice (Adini *et al.*, 2009). bFGF has been reported to stimulate BH4 synthesis in mouse brain microvascular endothelial cells, peaking at a concentration of 50ng/ml bFGF (Shimizu *et al.*, 2003), which is significantly lower than the bFGF concentration commonly used in Matrigel plugs in order to stimulate angiogenesis. In the

presented study, Matrigel plugs contained 500ng/ml bFGF and it cannot be excluded that BH4 synthesis was stimulated in endothelial cells, potentially confounding the results. Matrigel plugs containing VEGF were also tested, but similarly to using bFGF the plugs were highly infiltrated with cells and only contained a very small proportion of endothelial cells. Considering the technical difficulties in quantifying Matrigel plug angiogenesis and the potentially confounding influences of growth factors in the plug, an alternative and possibly pathophysiologically more relevant way of studying the influence of endothelial GCH overexpression on angiogenesis might be a vascular injury model such as hind limb ischemia.



Chapter 6:

Influence of GCH

Overexpression under the

Control of the Tie-2 Promoter on

Bone Marrow Biopterin Levels

and Endothelial Progenitor Cell

Content



6 Influence of GCH Overexpression under the Control of the Tie-2 Promoter on Bone Marrow Biopterin Levels and Endothelial Progenitor Cell Content

6.1 Introduction

Endothelial progenitor cells (EPCs) are a heterogeneous group of precursor cells that participate in neo-endothelialization and neovascularization. They can be found resident in bone marrow (Asahara *et al.*, 1999; Loomans *et al.*, 2006), as bone marrow derived circulating EPCs in peripheral blood (Iwasaki *et al.*, 2007; Shi *et al.*, 1998) or resident in the vascular wall (Ingram *et al.*, 2005; Yao *et al.*, 2009; Zengin *et al.*, 2006), lung tissue (Alvarez *et al.*, 2008) and adipose tissue (Planat-Benard *et al.*, 2004). Bone marrow derived EPCs have been shown to contribute to postnatal neovascularization, both in physiological and pathophysiological settings (Asahara *et al.*, 1999) and play an important role in re-endothelialization of blood vessels after vascular injury (Kaushal *et al.*, 2001; Shi *et al.*, 1998; Xu *et al.*, 2003). Administration of circulating EPCs may even present a therapeutic strategy for neovascularization in response to ischemia and has been shown to be effective in animal models (Kalka *et al.*, 2000; Kawamoto *et al.*, 2001; Kocher *et al.*, 2001; Urbich *et al.*, 2003) and there is some evidence that it may provide benefits in limb ischemia in humans (Lenk *et al.*, 2005; Tateno *et al.*, 2006). Number and function of EPCs are related to cardiovascular risk factors and cardiovascular events. Migration of EPCs isolated from patients with cardiovascular disease is impaired

and correlates inversely with the number of risk factors (Vasa *et al.*, 2001b), whereas a low number of circulating EPCs is associated with an increased risk of future cardiovascular events (Schmidt-Lucke *et al.*, 2005; Werner *et al.*, 2005). Both diabetes mellitus (Thum *et al.*, 2007) and arterial hypertension (Xie *et al.*, 2010) have been shown to impair function and number of circulating EPCs. Oxidized LDL has been shown to impair EPC proliferation (Wang *et al.*, 2004), migration (Wang *et al.*, 2004), differentiation (Imanishi *et al.*, 2003) and vasculogenic potential (Imanishi *et al.*, 2004; Wang *et al.*, 2004; Zhou *et al.*, 2007) and to induce EPC senescence (Imanishi *et al.*, 2004) in animal models. EPC senescence in turn has been shown to be associated with in stent restenosis (Matsuo *et al.*, 2006) in humans. Xu *et al.* (Xu *et al.*, 2003) reported that bone marrow EPCs contribute to re-endothelialization of vein grafts in mice and that ApoE-KO mice exhibit reduced graft re-endothelialization and reduced numbers of blood EPC in comparison to WT.

eNOS and NO play a key role in the expansion of EPC in the bone marrow and EPC mobilization into the blood stream. eNOS-KO mice have been reported to have fewer EPCs in bone marrow and peripheral blood (Laufs *et al.*, 2004) and to show reduced VEGF induced EPC mobilization into the blood (Aicher *et al.*, 2003). eNOS uncoupling has been shown to induce EPC senescence (Lemarie *et al.*, 2011) and to impair EPC mobilization and migration (Thum *et al.*, 2007). Both EPC senescence and impaired migration due to eNOS uncoupling could successfully be rescued by BH4 augmentation (Lemarie *et al.*, 2011; Thum *et al.*, 2007). Urao *et al.* showed that erythropoietin improves re-endothelialization after vascular injury and enhances EPC mobilization via Akt/eNOS activation (Urao *et al.*, 2006). Treatment

with statins (HMG-CoA reductase inhibitors) has also been reported to increase EPC content in bone marrow (Dimmeler *et al.*, 2001) and the number of circulating EPC mobilized from bone marrow (Llevadot *et al.*, 2001; Vasa *et al.*, 2001a) via PI3K/Akt signaling (Dimmeler *et al.*, 2001; Llevadot *et al.*, 2001). Phosphorylation of eNOS at serine 1177 by pAkt leads to eNOS activation and Aoki *et al.* demonstrated that fluvastatin increases eNOS activity through phosphorylation of eNOS via the PI3K/Akt pathway and via an increase in BH4 in endothelial cells (Aoki *et al.*, 2010). The importance of eNOS activity and BH4 availability for EPC expansion and function is further stressed by Xie *et al.* (Xie *et al.*, 2010), who found that BH4 augmentation in the GCH transgenic mouse preserves the number of circulating EPC and tube formation of bone marrow derived EPC in DOCA-salt hypertension.

ApoE-KO mice show a reduction in circulating EPC in comparison to WT mice (Xu *et al.*, 2003) but the impact of transgenic GCH overexpression on EPC expansion in bone marrow of ApoE-KO mice has yet to be determined. The focus of this chapter is to investigate the effect of transgenic GCH overexpression on BH4 levels in different populations of bone marrow cells and on EPC content in bone marrow of ApoE-KO mice.

Hypothesis:

The central hypothesis of this chapter is that GCH overexpression under the control of the Tie-2 promoter will increase BH4 levels in bone marrow EPCs and enhance bone marrow EPC expansion and therefore bone marrow EPC content in a vascular disease setting.

Aims:

Using bone marrow, bone marrow mononuclear cells (BMMNC) and lineage depleted BMMNC from mice with and without transgenic GCH overexpression, the aims of this chapter are:

- To evaluate the effect of transgenic GCH overexpression under the control of the Tie-2 promoter on BH4 content in bone marrow, BMMNC and lineage depleted BMMNC as fractions of bone marrow containing increasing proportions of EPCs.
- To determine the effect of transgenic GCH overexpression on EPC content in bone marrow of ApoE-KO mice.

6.2 Biopterin levels in different fractions of bone marrow from mice with and without transgenic GCH overexpression

Bone marrow is composed of stromal cells (fibroblasts, macrophages, osteoblasts and osteoclasts, adipocytes and sinusoidal endothelial cells) and mononuclear stem cells (hemangiogenic and mesenchymal stem cells). EPC can be identified in the in the mononuclear fraction of bone marrow (Loomans *et al.*, 2006; Zhang *et al.*, 2006) and only represent a very small proportion of whole bone marrow.

The GCH transgenic mouse overexpresses human GCH under the control of the Tie-2 promoter and intronic enhancer. Tie-2, a tyrosine kinase receptor for angiopoietins, is predominantly expressed in endothelial cells but also in hemangiogenic progenitor cells (Moore and Lemischka, 2004; Sato *et al.*, 1995;

Schlaeger *et al.*, 1997; Shaw *et al.*, 2004; Takakura *et al.*, 1998). If the GCH transgene is expressed in hemangiogenic progenitor cells in the bone marrow but not stromal cells or mesenchymal stem cells, the increase in BH4 levels due to GCH overexpression should more apparent in BMMNC than whole bone marrow. The percentage of EPC in the preparation can be further increased by lineage depletion and thus removing mature hematopoietic cells and their precursors.

To investigate the influence of transgenic GCH overexpression on BH4 content in fractions of bone marrow containing increasing proportions of EPCs in the absence of vascular disease, bone marrow was harvested from WT, eNOS, GCH and eNOS/GCH mice whereas ApoE-KO and GCH/ApoE-KO mice were used as a model of vascular disease.

The isolation of bone marrow, BMMNC and lineage depletion of BMMNC are described in detail in Chapter 2.16. In short, animals were sacrificed by overdose of inhaled isoflurane and cervical dislocation and bone marrow was collected from mouse femur and tibia and passed through a 70µm strainer to remove bone fragments and cell clumps. For the isolation of BMMNC, bone marrow cell suspension was carefully layered over 3 ml of LSM 1077 and centrifuged at room temperature for 10 minutes at 800 g. BMMNC were collected from the interphase. Lineage negative cells were enriched using a lineage cell depletion kit (Miltenyi Biotec).

6.2.1 Bone marrow and bone marrow mononuclear cells from mice with and without transgenic overexpression of eNOS and/or GCH

Bone marrow was collected from WT, eNOS transgenic, GCH transgenic and eNOS/GCH double transgenic mice and BMMNC were isolated by density gradient centrifugation with LSM 1077.

A 40-60% increase in biopterin levels was observed in bone marrow from mice carrying the GCH transgene (GCH and eNOS/GCH) compared to WT and eNOS respectively (Figure 6.1 A). There was no difference in BH4 or total biopterin levels in bone marrow between WT and eNOS or GCH and eNOS/GCH respectively. As hypothesized, the increase in BH4 levels due to GCH overexpression was more apparent in BMMNC than whole bone marrow. BH4 levels in BMMNC from GCH and eNOS/GCH mice were increased by 150% compared to WT and eNOS respectively. There was no difference between WT and eNOS or GCH and eNOS/GCH respectively (Figure 6.1 B).

Figure 6.1

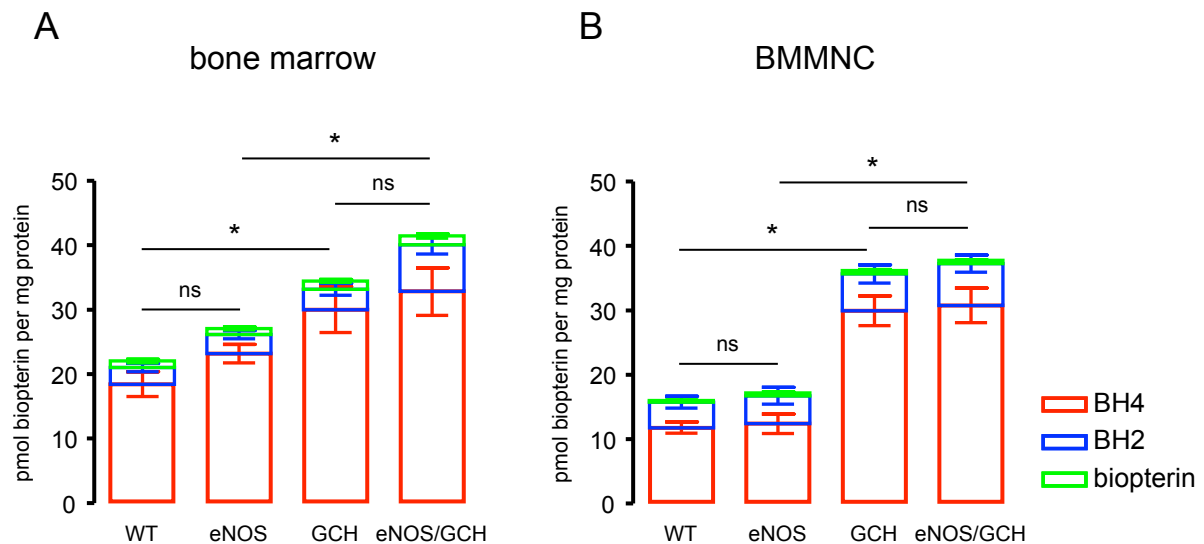


Figure 6.1: Biopterin levels in bone marrow and BMMNC from WT, eNOS, GCH and eNOS/GCH mice.

Bone marrow (BM) was collected from mouse femur and tibia and passed through a 70 μ m strainer to remove bone fragments and cell clumps. To isolate BMMNC, 1 ml of BM cell suspension was carefully layered over 3 ml of LSM 1077 and centrifuged at room temperature for 10 minutes at 800 g. BMMNC were collected from the interphase and washed with PBS before being frozen for bipterin analysis.

A: There was no difference in BH4 or total bipterin levels in BM between WT and eNOS or GCH and eNOS/GCH respectively. A small but significant increase in biopterin levels was observed in BM from mice carrying the GCH transgene (GCH and eNOS/GCH) compared to WT and eNOS respectively.

B: BH4 levels in BMMNC from GCH and eNOS/GCH mice were increased to 2.5 fold of WT and eNOS respectively.

In mixed BM only a small number of cells express the hGCH transgene which means only a small increase in bipterins can be detected in GCH and eNOS/GCH cells. By isolating BMMNC the proportion of hGCH transgene expressing cell is increased and the differences in bipterin values become more apparent.

n=6-8 per group, *p<0.05.

6.2.2 Bone marrow, bone marrow mononuclear cells and lineage depleted bone marrow mononuclear cells from ApoE-KO mice with and without transgenic overexpression of GCH

Bone marrow was collected from ApoE-KO and GCH/ApoE-KO mice and BMMNC were isolated by density gradient centrifugation with LSM 1077. To remove mature hematopoietic cells such as T cells, B cells, monocytes/macrophages and their committed precursors, BMMNC were magnetically labeled with a mix of antibodies against a panel of lineage antigens (CD5, CD45R, CD11b, Ly-6G/C, Ly-7/4 and Ter-119) and Anti-Biotin MicroBeads.

There was no difference in BH4 or total biopterin levels in bone marrow between ApoE-KO and GCH/ApoE-KO (Figure 6.2 A). BMMNC from GCH/ApoE-KO mice showed a 50% increase in BH4 levels compared to ApoE-KO (Figure 6.2 B) whereas BH4 levels in lineage negative BMMNC from GCH/ApoE-KO mice were increased 7-fold (Figure 6.2 C). Interestingly, biopterin values in lineage negative BMMNC were similar to biopterin values in ApoE-KO and GCH/ApoE-KO endothelial cells (Figure 4.1 C).

Figure 6.2

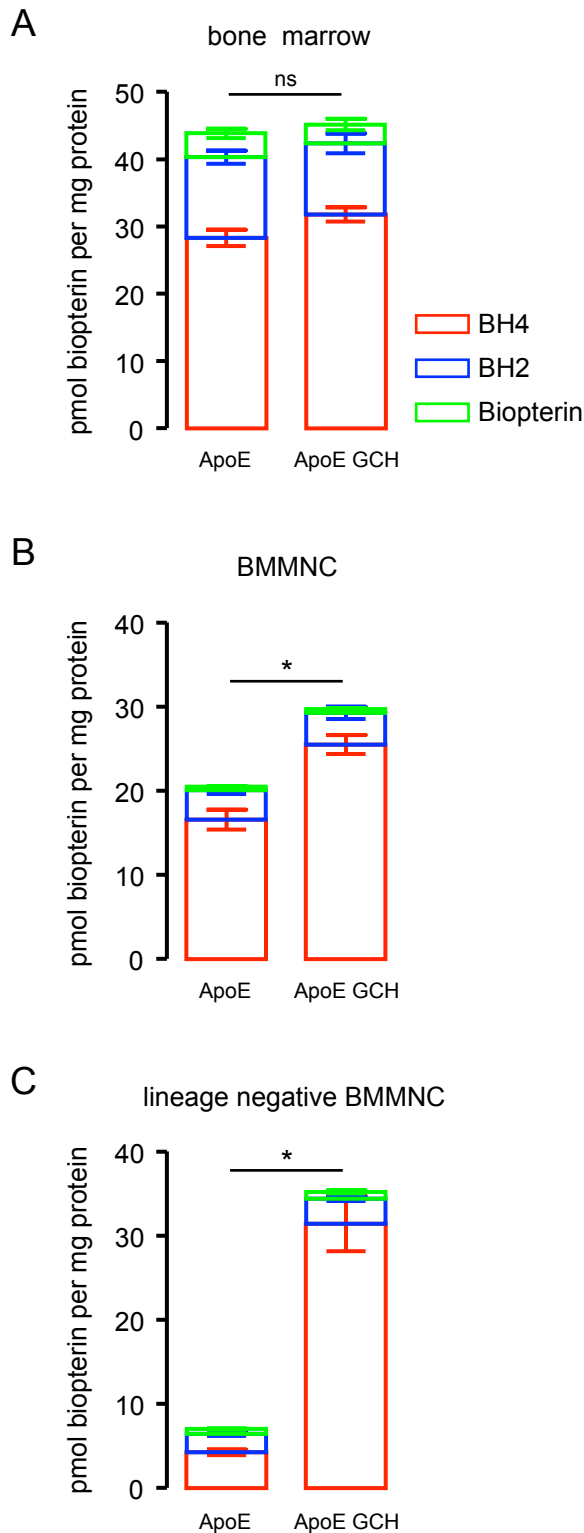


Figure 6.2: Biopterin levels in bone marrow, BMMNC and lineage depleted BMMNC from ApoE-KO and GCH/ApoE-KO mice.

Bone marrow (BM) was collected from mouse femur and tibia and passed through a 70 μ m strainer to remove bone fragments and cell clumps. To isolate BMMNC, 1 ml of BM cell suspension was carefully layered over 3 ml of LSM 1077 and centrifuged at room temperature for 10 minutes at 800 g. BMMNC were collected from the interphase and washed. Lineage negative cells were enriched using a lineage cell depletion kit. Samples of BM, BMMNC and lineage negative BMMNC were frozen for biopterin analysis.

A: There was no difference in BH4 or total biopterin levels in BM between ApoE-KO and GCH/ApoE-KO.

B: BMMNC from GCH/ApoE-KO mice showed a moderate increase in BH4 levels compared to ApoE-KO.

C: BH4 levels in lineage negative BMMNC from GCH/ApoE-KO mice were increased 7-fold compared to ApoE-KO.

EPC expressing the GCH transgene are the minority in mixed BM cells, which explains that there was no significant difference in biopterin levels in BM. The aim of isolating BMMNC and further lineage depleting BMMNC to eliminate hematopoietic cells was to increase the proportion of EPC within the sample.

Lineage negative BMMNC display biopterin values similar to ApoE-KO and GCH/ApoE-KO EC.

n=5-7 per group, *p<0.05.

Similar to the biopterin measurements in bone marrow and BMMNC from WT, GCH, eNOS and eNOS/GCH mice, the increase in BH4 levels due to GCH overexpression became more apparent the higher the expected percentage of EPCs in the sample (bone marrow < BMMNC < lineage depleted BMMNC). However, in contrast to the healthy WT or eNOS transgenic background, on ApoE-KO background mice carrying the GCH transgene did not show a significant increase in BH4 levels in bone marrow. The relative increase in BH4 levels in BMMNC due to GCH overexpression was also higher on WT and eNOS transgenic background than on ApoE-KO background. These observations go in line with BH4 measurements in primary endothelial cells also showing a higher relative increase in BH4 in GCH transgenic animals on WT or eNOS background than on ApoE-KO background (Figure 4.1) and could potentially be explained by increased inflammation and oxidative stress in ApoE-KO mice. However, GCH/ApoE-KO and eNOS/GCH are two separate colonies and littermate controlled studies would be necessary to allow valid conclusions.

6.3 Endothelial progenitor content in bone marrow mononuclear cells from ApoE-KO and GCH/ApoE-KO mice

There is no single conclusive marker to define EPCs, therefore a combination of several endothelial and progenitor markers is commonly used to measure EPC content. Table 3.5 provides an overview of cell surface markers used for the identification of EPCs with examples from the literature. In this study EPC were identified by Sca-1 and Flk-1 expression as published by Urao and colleagues (Urao *et al.*, 2006), Laufs and colleagues (Laufs *et al.*, 2004), Schroder and colleagues (Schroder *et al.*, 2009) and Xie and colleagues (Xie *et al.*, 2010). Stem cell antigen

1 (Sca-1) is expressed on multipotent hematopoietic stem cells but Sca-1 positive cells have also been shown to have the potential of developing into various types of mature cells other than blood cells including endothelial cells (Xiao *et al.*, 2006), smooth muscle cells (Xiao *et al.*, 2007) and cardiomyocytes (Gojo *et al.*, 2003). Flk-1 (VEGF receptor 2) is a marker of endothelial lineage (Millauer *et al.*, 1993; Yamaguchi *et al.*, 1993) and has been shown to be required for EPC proliferation and survival (Larrivee *et al.*, 2003; Shalaby *et al.*, 1997).

To quantify EPC content, bone marrow was collected from ApoE-KO and GCH/ApoE-KO mice. BMMNC were isolated from bone marrow by density gradient centrifugation with LSM 1077. Lineage negative cells were enriched using a lineage cell depletion kit, stained with Flk-1 APC and Sca-1 PE antibodies as described in Chapter 2.16 and analyzed by flow cytometry.

$4.6 \pm 0.2 \times 10^7$ bone marrow cells were isolated from 1 mouse leg. The number of BMMNC from 1 leg was approximately 25% of bone marrow and the proportion of lineage negative BMMNC was less than 1% of all bone marrow cells. There was no difference between the genotypes in the number of bone marrow cells, BMMNC or lineage negative BMMNC (Figure 6.3).

Figure 6.3

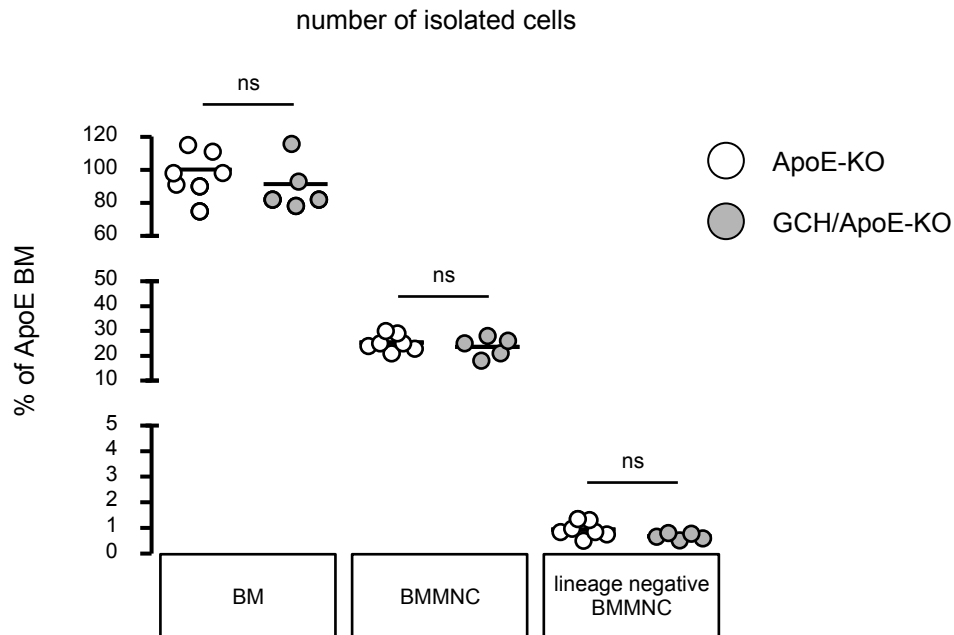


Figure 6.3: Numerical relationship between total bone marrow cell, BMMNC and lineage depleted BMMNC per mouse.

Bone marrow (BM) was collected from mouse femur and tibia and passed through a 70 μ m strainer to remove bone fragments and cell clumps. To isolate BMMNC, 1 ml of BM cell suspension was carefully layered over 3 ml of LSM 1077 and centrifuged at room temperature for 10 minutes at 800 g. BMMNC were collected from the interphase and washed. Lineage negative cells were enriched using a lineage cell depletion kit. The total number of cells in each group was determined by flow cytometry.

There was no difference in the amount of BM cells, BMMNC or lineage negative BMMNC, which were isolated from ApoE-KO mice compared to GCH/ApoE-KO.

$4.6 \pm 0.2 \times 10^7$ BM cells were isolated from 1 mouse leg. The number of BMMNC from 1 leg was approximately 25% of BM and the amount of lineage negative BMMNC was <1% of all BM cells.

n=5-7 per group.

2.25±0.40% of ApoE-KO and 3.27±0.58% of GCH/ApoE-KO lineage negative cells were positive for Flk-1 and Sca-1 (Figure 6.4). This difference in the percentage of Flk-1/Sca-1 positive lineage negative BMMNC is not statistically significant. Flk-1/Sca-1 positive cells are a minority population of BMMNC and the inter sample variation is high when quantifying these cells by flow cytometry, leading to a low sensitivity of detecting a potential difference between the genotypes. N=13 for each group are needed for a beta error level of 50% and for a beta error level of 20% n=29 of Apo-KO and GCH/ApoE-KO are required each. Experimental setups that might require fewer mice for confidently determining whether GCH overexpression affects EPC content in ApoE-KO mice are discussed below.

Figure 6.4

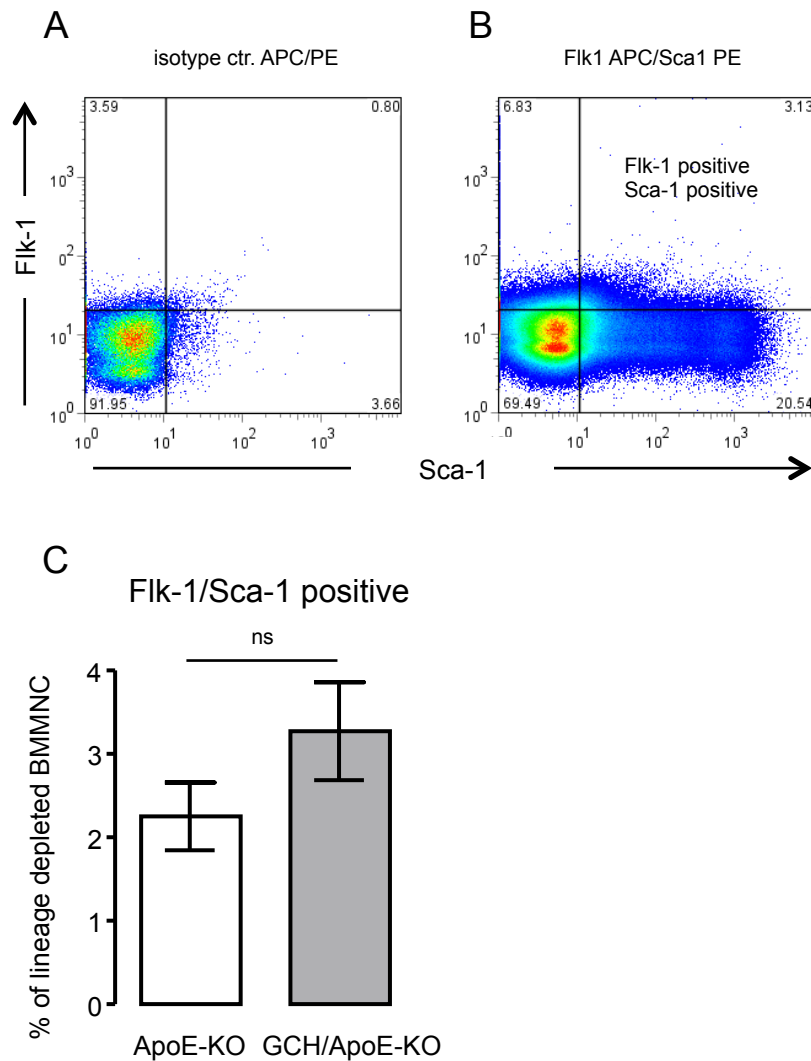


Figure 6.4: EPC content in lineage depleted BMMNC from ApoE-KO and GCH/ApoE-KO mice.

Bone marrow (BM) was collected from mouse femur and tibia and passed through a 70 μ m strainer to remove bone fragments and cell clumps. To isolate BMMNC, 1 ml of BM cell suspension was carefully layered over 3 ml of LSM 1077 and centrifuged at room temperature for 10 minutes at 800 g. BMMNC were collected from the interphase and washed. Lineage negative cells were enriched using a lineage cell depletion kit. Lineage negative cells were stained with Flk-1 APC and Sca-1 PE antibodies or the corresponding isotype controls to identify EPC by flow cytometry.

A-B: The gates for Flk-1 and Sca-1 negative were set using APC and PE isotype control stained lineage negative BMMNC (**A**). 2.3 to 3.3 % of the cells were positive for Flk-1 and Sca-1 (**B**).

C: There was no significant difference in the percentage of Flk-1/Sca-1 positive lineage negative BMMNC (EPC) between ApoE-KO and GCH/ApoE-KO detectable.

n=9-11 per group.

6.4 Summary and discussion

EPCs are a heterogeneous group of precursor cells that participate in neo-endothelialization and neovascularization. They can be found resident in bone marrow (Asahara *et al.*, 1999; Loomans *et al.*, 2006), in peripheral blood (Iwasaki *et al.*, 2007; Shi *et al.*, 1998) or resident in the vascular wall (Ingram *et al.*, 2005; Yao *et al.*, 2009; Zengin *et al.*, 2006) and various tissues including lung tissue (Alvarez *et al.*, 2008). Bone marrow derived EPCs have been shown to contribute to postnatal neovascularization (Asahara *et al.*, 1999) and play an important role in re-endothelialization of blood vessels after vascular injury (Kaushal *et al.*, 2001; Shi *et al.*, 1998; Urao *et al.*, 2006; Xu *et al.*, 2003).

eNOS and NO play a key role in the expansion of EPC in the bone marrow and EPC mobilization into the blood stream (Aicher *et al.*, 2003; Laufs *et al.*, 2004). eNOS uncoupling has been shown to induce EPC senescence (Lemarie *et al.*, 2011) and to impair EPC mobilization and migration (Thum *et al.*, 2007). Both EPC senescence and impaired migration due to eNOS uncoupling could successfully be rescued by BH4 augmentation (Lemarie *et al.*, 2011; Thum *et al.*, 2007). BH4 augmentation in the GCH transgenic mouse has been shown to preserve the number of circulating EPC and tube formation of bone marrow derived EPC in arterial hypertension (Xie *et al.*, 2010). ApoE-KO mice show a reduction in circulating EPCs in comparison to WT mice (Xu *et al.*, 2003) but the impact of transgenic GCH overexpression on EPC content in ApoE-KO mice has yet to be determined.

The aim of this chapter was to investigate the effect of transgenic GCH overexpression under the control of the Tie-2 promoter on BH4 levels in different

populations of bone marrow cells containing increasing proportions of EPCs and to determine the effect on EPC content in bone marrow of ApoE-KO and GCH/ApoE-KO mice.

The key findings of this chapter are:

- Transgenic GCH overexpression under the control of the Tie-2 promoter increases BH4 content in bone marrow, BMMNC and lineage depleted BMMNC.
- The higher the expected proportion of EPCs in the sample, the stronger the relative increase in BH4 levels due to GCH overexpression: Relative BH4 increase in lineage negative BMMNC is greater than in BMMNC, which in turn is greater than in whole bone marrow.
- The increase in BH4 in BMMNC due to transgenic GCH overexpression is lower on ApoE-KO background than on WT background.
- No significant difference in bone marrow EPC content could be detected between ApoE-KO and GCH/ApoE-KO mice.

The results presented in this chapter demonstrate that transgenic GCH overexpression under the control of the Tie-2 promoter increases BH4 levels in bone marrow cells in relation to EPC enrichment, suggesting expression of the GCH transgene in bone marrow EPCs. Therefore the GCH transgenic mouse provides a good *in vivo* model for investigating the influence of BH4 bioavailability on EPC expansion and function.

BH4 values and increase in BH4 due to GCH transgene expression in lineage negative BMMNC was comparable to primary endothelial cells. Xi *et al.* have also recently shown an increase in BH4 in EPCs from GCH transgenic mice (Xie *et al.*, 2010). In contrast to investigating BH4 content in lineage negative BMMNC, Xie *et al.* cultured EPCs from BMMNC. These cultured EPCs contained approximately 7pmol BH4 per mg protein for WT cells, similar to the 4pmol per mg protein found in lineage negative BMMNC from ApoE-KO mice. However, Xie *et al.* reported a 2-fold elevation of BH4 to 14pmol per mg protein in GCH transgenic EPCs whereas BH4 in lineage negative BMMNC from GCH/ApoE-KO mice was increased more than 7-fold to 31pmol per mg protein. Considering the results presented in Chapters 3 and 4 showing rapid changes in mouse and human GTPCH expression and as a result changes in BH4 levels in cultured primary endothelial cells, it is possible that cultured EPCs may undergo similar changes.

The results for bone marrow EPC content in ApoE-KO and GCH/ApoE-KO animals are inconclusive. Because the number of peripheral blood EPCs in ApoE-KO mice at basal level proved to be too low to be directly determined by flow cytometry, EPC content in bone marrow was investigated. However, the percentage of EPCs in lineage depleted BMMNC is also low and variation relatively high, which means a large number of mice would be required to detect a moderate difference. Xie *et al.* also reported that on WT background GCH overexpression only affects the number of EPCs in comparison to WT under DOCA salt treatment but not in controls (Xie *et al.*, 2010). Induction of DOCA salt hypertension or vascular injury might therefore be necessary to bring out or amplify a potential difference between ApoE-KO and GCH/ApoE-KO and also enable direct

measurement of circulating blood EPCs. It is also possible that EPC expansion in the bone marrow is not affected by BH4 availability in the same way EPC mobilization into the blood stream is.

Impaired vein graft re-endothelialization and increased vein graft atherosclerosis in ApoE-KO mice has been reported to be associated with reduced numbers of circulating EPCs (Xu *et al.*, 2003). BH4 augmentation due to transgenic GCH overexpression has been shown to reduce vein graft atherosclerosis in ApoE-KO mice (Ali *et al.*, 2008) and preliminary results from our lab show that GCH/ApoE-KO mice may also exhibit improved endothelial cell repopulation in vein grafts (Rinze *et al.*, 2008). Vein grafts and endothelial-denuded blood vessels are in part repopulated with bone marrow derived EPCs (Urao *et al.*, 2006; Xu *et al.*, 2003). eNOS signaling has been shown to play a key role in re-endothelialization of blood vessels with EPCs (Urao *et al.*, 2006) and improving eNOS coupling via BH4 augmentation has been shown to improve EPC mobilization and function (Lemarie *et al.*, 2011; Thum *et al.*, 2007). However, it is also possible that transgenic BH4 augmentation may improve EPC mobilization in an eNOS independent manner as Xi *et al.* showed in eNOS-KO mice (Xie *et al.*, 2010).

Further work is needed to define the influence of transgenic BH4 augmentation on EPC number and function in ApoE-KO mice. Both improved endothelial cell proliferation and EPC mobilization have the potential to improve re-endothelialization of vein grafts and to contribute to the observed reduction in vein graft atherosclerosis in GCH/ApoE-KO mice. Xu *et al.* showed that the endothelium in vein grafts is lost within the first 3 days following the procedure and

gradually repopulated with recipient derived endothelial cells over time (Xu *et al.*, 2003). Bone marrow transplant experiments (Tie-2 LacZ positive bone marrow into LacZ negative recipients) could be useful for differentiating bone marrow derived endothelial cells from other endothelial cells in repopulated vein grafts in ApoE-KO and GCH/ApoE-KO mice. Hind limb ischemia experiments in ApoE-KO and GCH/ApoE-KO may also provide insight into the influence of GCH overexpression on angiogenesis and revascularization and may define a potential therapeutic role of BH4 in vascular injury.

Chapter 7:

General Discussion

7 General Discussion

The vascular endothelium plays a critical role in the regulation of vascular homeostasis. Endothelial cell injury, loss, survival and regeneration are important aspects in the response to vascular injury and pathogenesis of vascular disease. Re-endothelialization has been shown to be a key event in vascular repair after injury, reducing the risk of thrombosis and the development of neointimal hyperplasia. Both differentiated endothelial cells and bone marrow derived endothelial progenitor cells have been shown to contribute to re-endothelialization of blood vessels after vascular injury. eNOS and NO are key regulators of endothelial cell proliferation and survival as well as endothelial progenitor cell expansion, mobilization and function. There is increasing evidence that the essential eNOS cofactor BH4 may enhance endothelial cell proliferation. The effect of BH4 may be mediated either by its direct antioxidant properties or/and improved eNOS function with increased NO availability and reduced production of reactive oxygen species. BH4 augmentation has further been shown to rescue endothelial progenitor senescence and impaired migration due to eNOS uncoupling and to preserve the number of circulating endothelial progenitor cells in arterial hypertension.

The hypothesis of this thesis therefore was that endothelial bioavailability of BH4, which is essential for eNOS catalytic activity, is a critical determinant of endothelial cell growth and regeneration in vascular disease states. The aim was to investigate the effect of BH4 bioavailability on endothelial cell growth in the absence and presence of vascular disease and on endothelial progenitor cell availability in lungs and bone marrow.

The majority of the experiments were conducted using primary endothelial cells from mice with genetic modifications in GCH and/or eNOS expression. The rationale behind the decision to use primary endothelial cells was that they would allow the effect of genetic modifications in BH4 availability to be investigated within a highly defined system, without confounding influences of other cell types, and using analytical approaches that are not possible in intact tissues. Using mice as donor animals is particularly useful because of the availability of genetic modifications in this species. However, in comparison to immortalized cell lines and commercially available endothelial cells of human or bovine origin, primary mouse endothelial cell culture is still a poorly defined system. An important potential limitation of this model is the possibility that primary mouse endothelial cells may lose endothelial cell phenotype and biological function over time in cell culture. It was therefore necessary to optimize selection and culture conditions in order to obtain a pure endothelial cell culture that would provide sufficient material for biochemical analysis and display the biological phenotype and functionality of the donor animal.

The key findings of this thesis are as follows:

- Primary endothelial cells can be reproducibly isolated from mouse lungs by CD31 immunomagnetic bead selection with Dynabeads® or MACS® MicroBeads.

- Transgenic overexpression of human GCH under the control of the Tie-2 promoter is specific to endothelial cells and leads to a significant increase in BH4 content in primary endothelial cells.
- The duration of culture is a major determinant of the biological phenotype of primary endothelial cells. The expression of Tie-2 and transgenic human GTPCH is down-regulated rapidly, whereas BH4 content due to endogenous mouse GTPCH increases over time.
- A subpopulation of isolated endothelial cells expresses the endothelial progenitor cell markers CD34 and Flk-1. This subpopulation also exclusively expresses the GCH and LacZ transgenes and is the only subpopulation of CD31 isolated endothelial cells, which proliferates in culture.
- BH4 augmentation by transgenic GCH overexpression improves *in vitro* growth of primary endothelial cells from atherosclerotic ApoE-KO mice. The observed difference in endothelial cell growth cannot be explained by a difference in the number or viability of isolated endothelial cells or a difference in the percentage of highly proliferative, endothelial progenitor marker expressing cells. In the absence of vascular disease, GCH overexpression does not appear to improve endothelial cell growth.
- Transgenic overexpression of human GCH under the control of the Tie-2 promoter increases BH4 content in different cellular fractions of bone

marrow to varying degrees. The strongest relative increase in BH4 levels due to GCH overexpression is observed in lineage depleted BMMNC, which are expected to contain the highest proportion of EPCs. GCH overexpression did not appear to affect percentage of EPCs in bone marrow from ApoE-KO mice.

Several protocols for the isolation of primary endothelial cells from mouse lungs have been published and immunomagnetic bead selection with CD31 or CD102 is the most commonly used method (Dong *et al.*, 1997; Khan *et al.*, 2004; Kuhlencordt *et al.*, 2004; Lim *et al.*, 2003; Marelli-Berg *et al.*, 2000; Schniedermann *et al.*, 2010). However, the protocols vary widely with regard to the total number of selection procedures and time endothelial cells spend in culture before they are included into experiments. Although it was possible to reproducibly isolate endothelial cells from mouse lungs by CD31 immunomagnetic bead selection, the isolation and culture conditions proved to be crucial determinants of the biological phenotype of the isolated cells. The time consuming process of purifying primary endothelial cells by reselection led to significant changes in phenotype with some endothelial cell markers (e.g. Tie-2) being down-regulated more rapidly than others (e.g. eNOS). Most importantly, the expression of the Tie-2 driven GCH transgene is also down-regulated during the reselection process. Due to the small number of endothelial cells isolated per mouse lung, most published studies on primary mouse endothelial cells include reselection and passaging of endothelial cells over a considerable period of time (Dong *et al.*, 1997; Fehrenbach *et al.*, 2009; Fleming *et al.*, 2005; Lim *et al.*, 2003; Marelli-Berg *et al.*, 2000; Schniedermann *et al.*, 2010). However, for experiments relying on the expression of

Tie-2 driven transgenes it may be advisable to avoid reselection procedures and carry out experiments between days three and five after the initial isolation, as in all endothelial cell growth experiments presented in this thesis.

For the first time, endothelial specificity of the Tie-2 driven GCH and LacZ transgenes has been verified directly using primary endothelial cells. It was also possible to identify a subpopulation of CD31 selected cells, which not only shows strong expression of the endothelial cell markers CD31, CD102 and Tie-2 but is the only subpopulation that expresses the GCH and LacZ transgenes. Interestingly this subpopulation of endothelial cells contained CD34 and Flk-1 positive cells and was the only population that significantly proliferated *in vitro* after flow sorting. In accordance with the published data of Schniedermann and colleagues (Schniedermann *et al.*, 2010), these findings indicate the presence of a group of endothelial progenitor cells with a high proliferative potential within mouse lungs.

After optimization of selection and culture conditions and characterization of primary mouse endothelial cells, the main focus of this thesis was to investigate the effects of BH4 bioavailability on endothelial cell growth in the absence and presence of vascular disease. The results presented in this thesis demonstrate for the first time that endothelial BH4 augmentation in the GCH transgenic mouse enhances *in vitro* growth of primary endothelial cells in a vascular disease setting. In contrast to previously reported findings (Chen *et al.*, 2010; Marinos *et al.*, 2001; Shimizu *et al.*, 1999), *in vitro* supplementation with the BH4 precursor sepiapterin did not improve *in vitro* growth of primary endothelial cells despite leading to a similar increase in BH4 levels as transgenic GCH overexpression. Possible

explanations for this apparent contradiction with the other studies may include the lower dose of sepiapterin used in this thesis and the higher cellular BH4 levels present in untreated primary mouse endothelial cells compared to the endothelial cell types used in the other studies. It is also possible that, in case of the ApoE-KO mouse, endothelial cells may have to be exposed to augmented BH4 levels *in vivo* over a longer period of time in order to effectively enhance *in vitro* proliferation of isolated endothelial cells. Interestingly, a significant proliferative effect of transgenic GCH overexpression could not be detected in the absence of vascular disease.

In addition to differentiated endothelial cells, endothelial progenitor cells also represent an important source for re-endothelialization. It was possible to identify a group of endothelial progenitor cells with a high proliferative potential within the CD31 positive cell fraction from mouse lungs. However, there was no difference in the percentage or total number of these CD34 and Flk-1 positive endothelial cells detectable between ApoE-KO and GCH/ApoE-KO. The observed improvement in primary endothelial cell growth in GCH/ApoE-KO cultures can therefore not be explained by a greater number of resident lung endothelial progenitor cells. Further work is needed to identify potential functional differences in these cells, which might affect viability and proliferation *in vitro* and *in vivo*.

Bone marrow derived endothelial progenitor cells also contribute to postnatal neovascularization and play an important role in re-endothelialization of blood vessels after vascular injury. eNOS function has been shown to be a key regulator of endothelial progenitor cell expansion, mobilization and function and the

deteriorative effects of eNOS uncoupling on endothelial progenitor viability and function can be rescued by BH4 augmentation (Lemarie *et al.*, 2011; Thum *et al.*, 2007). Xie *et al.* found that BH4 augmentation in the GCH transgenic mouse preserves the number of circulating endothelial progenitor cells in DOCA-salt hypertension (Xie *et al.*, 2010). The hypothesis in Chapter 6 therefore was that BH4 augmentation would improve bone marrow endothelial progenitor cell expansion and therefore content in a vascular disease setting. As in the endothelial cell growth experiments, the ApoE-KO mouse was used as a model of vascular disease. No significant difference in bone marrow endothelial progenitor content could be detected between ApoE-KO and GCH/ApoE-KO mice. However, the percentage of endothelial progenitor cells in the samples of lineage depleted bone marrow mononuclear cells is generally low and variation relatively high, which means a large number of mice are required to detect a moderate difference. It is possible that induction of vascular injury may bring out or amplify a potential difference in bone marrow endothelial progenitor cell content between ApoE-KO and GCH/ApoE-KO. It would also be of great interest to directly measure circulating endothelial progenitor cells in peripheral blood by flow cytometry without previous culture. However, the markedly low percentage of endothelial progenitor cells in peripheral blood and the small total blood volume of a mouse compromise direct measurements; in the ApoE-KO mouse, the number of circulating endothelial progenitor cells turned out to be too low to be determined reliably. Increasing endothelial progenitor mobilization by vascular injury may enable direct measurement of circulating blood endothelial progenitor cells in ApoE-KO and GCH/ApoE-KO mice and amplify potential differences.

7.1 Future Directions

The objective of this thesis was to elucidate the role of the essential eNOS cofactor BH4 in endothelial cells growth. Although this work has uncovered important new knowledge about primary mouse endothelial cell culture and the influence of genetic BH4 augmentation on primary endothelial cells growth, many questions remain unanswered and new questions arise.

Further investigation is needed to determine whether the influence of BH4 on primary endothelial cell growth is primarily mediated by improved eNOS function. Endothelial cell growth experiments using a NOS inhibitor such as L-NAME could be employed and fluorescent detection of NO with diaminofluoresceins (Kojima *et al.*, 1998) may allow simultaneous analysis of endothelial marker expression and NO synthesis by flow cytometry. There is increasing evidence that endothelial BH4 augmentation may lead to activation of the PI3K/Akt signaling pathway due to improved eNOS coupling (Chen *et al.*, 2010; Du *et al.*, 2008), which would both enhance endothelial cell proliferation and protect endothelial cells from apoptosis. Further work is needed to define the role PI3K signaling in mediating the effects of GCH overexpression on endothelial cell survival and proliferation in the absence and presence of vascular disease and short-term primary endothelial cell culture may provide a useful model. Short-term culture of primary mouse endothelial cells as opposed to reselection and cell passaging is necessary to avoid significant changes in biological phenotype including down-regulation of transgenic GCH. Disadvantages of short-term culture are remaining contamination with non-endothelial cells and the small cell yield per mouse. It may be worth exploring whether primary endothelial cells, which are grown in flow chambers, exposed to

laminar shear stress, undergo the same changes as endothelial cells grown under static conditions.

An interesting finding in this thesis was the occurrence of a subpopulation of CD31 selected cells, which expresses the Tie-2 driven GCH and LacZ transgenes, contains cells that are positive for the endothelial progenitor markers CD34 and Flk-1 and shows high proliferative potential *in vitro*. There was no difference between ApoE-KO and GCH/ApoE-KO in the percentage of total number of these cells; however, simultaneous staining with Annexin-V may provide insight into potential differences in cell viability of this subpopulation. Early addition of BrdU to these cells after flow sorting may also allow identification of the exact cell type exhibiting rapid *in vitro* proliferation.

In vitro BH4 augmentation with sepiapterin did not appear to improve *in vitro* growth of primary endothelial cells despite raising BH4 levels to a similar extent as GCH overexpression. It may therefore be beneficial to employ *in vivo* mouse models. When combined with a vascular injury model such as hind limb ischemia or vein grafting, treatment of ApoE-KO and GCH/ApoE-KO mice with the NOS inhibitor L-NAME and/or the GTPCH inhibitor DAHP would allow close investigation of the impact of BH4/eNOS stoichiometry on revascularization or re-endothelialization respectively. These experiments would also provide the possibility for further investigations into the influence of transgenic BH4 augmentation on endothelial progenitor cell expansion, mobilization and function in ApoE-KO mice. In addition, bone marrow transplant experiments (Tie2 LacZ positive bone marrow into LacZ negative recipients) could be useful for

differentiating bone marrow derived endothelial cells from proliferating and migrating resident endothelial cells in repopulated vein grafts or neovessels after hind limb ischemia.

7.2 Concluding Remarks

These findings provide important new knowledge about the use of primary mouse endothelial cells in vascular research and insight into the role of BH4 availability on endothelial growth in the presence and absence of vascular disease. The presented data directly verifies endothelial specificity of the Tie-2 driven GCH and LacZ transgenes for the first time using primary endothelial cells. As a subpopulation of primary endothelial cells, a population of lung endothelial progenitor cells has been identified. This subpopulation specifically expresses the GCH and LacZ transgenes and is the only population that significantly proliferates *in vitro* after flow sorting. The data in this work shows for the first time that genetic BH4 augmentation by transgenic GCH overexpression improves primary endothelial proliferation in a vascular disease setting; however further work is needed to establish the mechanisms by which BH4 improves endothelial cell growth and to identify the impact of BH4 availability on endothelial progenitor expansion, mobilization and function. The data presented in this thesis indicate a potential therapeutic role of BH4 in vascular injury states.

Chapter 8:

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8 References

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Chapter 9:

Appendices

9 Appendices

9.1 Timing and Venue of Research

The work described in this thesis was conducted between October 2007 to December 2010, in the British Heart Foundation (BHF) Molecular Cardiology Laboratory and Functional Genetics Facility, Department of Cardiovascular Medicine, Wellcome Trust Centre for Human Genetics, Oxford, UK.

9.2 Research Funding

The author was funded by a British Heart Foundation Clinical Research Training Fellowship. The laboratory of Professor Keith Channon is supported by a program grant from the British Heart Foundation.

9.3 Attributions

The author is directly responsible for the design, planning, execution and analyses of all experiments. The GCH transgenic mouse line was originally generated in-house by Dr. Nicholas Alp. The radiochemical HPLC system for the detection of arginine to citrulline conversion was set up by Dr. Joe De Bono, the HPLC system for the electrochemical detection of BH4 was set up by Dr. Mark Crabtree. Flow sorting of MACS® MicroBeads isolated cells was conducted by Andrew Worth.

9.4 Acknowledgements

After completing my Dr. med. at University of Hamburg I was welcomed by Professor Keith Channon into his research group as a post-doctoral research fellow. I would like to thank Professor Channon for the opportunity to pursue this DPhil, and to him and his entire research group for the help, support and friendship they have shown me over the last year. I owe special thanks to the Margarete-Waitz Foundation for the funding grant that made my first two years of research in Oxford possible before pursuing to a DPhil. I must also thank Professor Hugh Watkins for the opportunity to undertake a DPhil in the Department of Cardiovascular Medicine, Oxford.

My deepest gratitude is to the members of the Channon group. To Professor Channon personally for his invaluable guidance and encouragement towards my work, and the whole group for their helpful comments in lab meetings and advice with experimental work. In particular I wish to thank Dr. Eileen McNeill for proof reading my thesis and for her continued valuable advice. Dr. Mark Crabtree and Mr. Ashley Hale who have always been a great support throughout the years as were Dr. Jennifer Bendall, Dr. Gillian Douglas and Ms. Jyoti Patel. I would also like to thank all of them for their fantastic company. I thank both Dr. James Brown and Mr. Phil Townsend for their technical support in the lab and Ms. Carol Williams, Ms. Viv Clark and staff at the Functional Genetics Facility.

I would like to express my love and gratitude towards my family and friends for their understanding and support, particularly Rob, who had to endure a very stressed me throughout the final phases of the DPhil.

9.5 Publications arising from Research

Peer-reviewed articles:

Antoniades C, Shirodaria C, Crabtree M, **Rinze R**, Alp N, Cunnington C, Diesch J, Tousoulis D, Stefanadis C, Leeson P, Ratnatunga C, Pillai R, Channon KM. Altered plasma versus vascular biopterins in human atherosclerosis reveal relationships between endothelial nitric oxide synthase coupling, endothelial function, and inflammation. *Circulation*. 2007 Dec 11;116(24):2851-9.

Bendall JK, **Rinze R**, Adlam D, Tatham AL, de Bono J, Wilson N, Volpi E, Channon KM. Endothelial Nox2 overexpression potentiates vascular oxidative stress and hemodynamic response to angiotensin II: studies in endothelial-targeted Nox2 transgenic mice. *Circ Res*. 2007 Apr 13;100(7):1016-25.

Schulz E, Tsilimingas N, **Rinze R**, Reiter B, Wendt M, Oelze M, Woelken-Weckmüller S, Walter U, Reichenspurner H, Meinertz T, Münzel T. Functional and Biochemical Analysis of Endothelial (Dys)function and NO/cGMP Signaling in Human Blood Vessels With and Without Nitroglycerin Pretreatment. *Circulation*. 2002;105:1170-1175.

Abstracts at scientific conferences:

Ruth Rinze, Ziad A Ali, Gillian Douglas, Nicholas Alp, Keith M. Channon. Tetrahydrobiopterin (BH4) Improves Endothelial Cell Survival After Vascular Injury. AHA Scientific Sessions, New Orleans, November 2008 (oral presentation).

Ruth Rinze, Ziad A Ali, Eileen M^CNeill, Gillian Douglas, Nicholas J. Alp, Keith M. Channon. Tetrahydrobiopterin Improves Endothelial Cell Survival After Vascular Injury. AHA Scientific Sessions ATVB Early Career Networking Reception, New Orleans, November 2008 (poster presentation).

Ruth Rinze, Ziad A Ali, Eileen M^CNeill, Gillian Douglas, Nicholas Alp, Keith M. Channon. Tetrahydrobiopterin (BH4) improves endothelial regeneration in vitro and in vivo. European Meeting on Vascular Biology, Marseille, September 2009 (poster presentation).