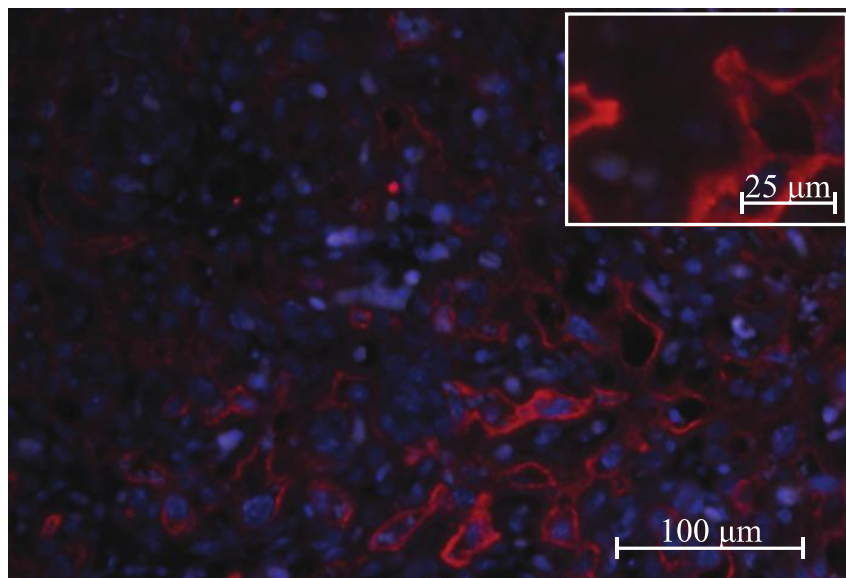UiT
THE ARCTIC
UNIVERSITY
OF NORWAY

Department of Medical Biology, IMB

Identification, isolation and characterisation of scavenging endothelial cells in placenta

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Nina Mjølsnes
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CSIRO · UNIVERSITY OF TROMSØ

Nina Mjølsnes

Abstract

Haemopoiesis occurs in the BM, where haemopoietic stem cells (HSC) reside in a three-dimensional "niche" and give rise to the millions of circulating blood cells required everyday of life. The precise regulation of this immense task occurs by interaction of HSC with other BM cells and extracellular matrix molecules. In addition, the BM vasculature has also now been identified as a key HSC regulator.

The most common use of HSC is in clinical transplantation for patients with haematological malignancies, immunologic diseases and other blood disorders. Recently, murine bone marrow (BM) scavenging endothelial cells (BMSEC) were identified, isolated and characterised. These cells were demonstrated to have transplant potential; homing to the BM and contributing to new blood vessel formation in irradiated recipients. In order to potentially use endothelial cells to improve HSC transplants a clinically applicable source of human endothelial cells needs to be identified and characterised.

Human placenta is a readily available tissue and human placental endothelial cells can be easily and non-invasively isolated. As human samples are extremely precious, a study of murine placental endothelial cells was used as a surrogate. For the first time, this study has identified, isolated and characterised placental scavenging endothelial cells (PSEC). These findings, together with functional assays, including homing and transplantation, provide evidence suggesting placenta SEC have the potential to home and engraft in a transplant recipient.

Contents

Abbreviations

A	Advenced glycation end product modified bovine serum albumin
BM	Bone marrow
BMSEC	BM scavenging endothelial cells
BSA	Bovine serum albumin
BT	Biotinyl Tyramide
CAV1	Caveolin-1
CK7	Cytokeratin-7
CFSE	5- (and 6-) Carboxyfluorescein Diacetate Succinimidyl Ester
CL-P1	Collectin placenta 1
CXCR-4	Chemokine receptor type-4
E	Embryonic
ESAM	Endothelial cell-selective adhesion molecule
EtOH	Ethanol
FA	FITC-AGE
FACS	Fluorescent Activated Cell Separation
FITC	Fluorecein isothiocyanate
FSA	Formaldehyde treated serum albumin
FSC	Forward scatter
FcγRllb2	Fc-gamma-receptor llb2
G	Gauge
HA	Hyaluronan/hyaluronic acid
HSC	Hemopoietic stem cells
KC	Kupffer cells
LSEC	Liver SEC
LRP-1	Low-density lipoprotein receptor related protein-1
NK	Natural killer cells
OPN	Osteopoetin
oxLDL	oxidized low-density lipoproteins
PECAM-1; CD31	Platelet/endothelial cell adhesion molecule 1
PDGFR-α/CD140a	Platelet-derived growth factor receptor-α
PDGFR-β/CD140b	Platelet-derived growth factor receptor-β
PI	Propidium iodide

PSEC	Placenta SEC
RBC	Red blood cells
RFP	Red Fluorescence Protein
SEC	Scavenging endothelial cells
SDF-1	Stromal cell derived factor-1
SNARF	Seminaphtorhodafluor-1-Carboxylic Acid Acetate Succinimidyl Ester
SSC	Side scatter
SR	Scavenging receptors
SR-B1	Scavenger receptors class B type 1
tmSCF	Membrane-bound stem cell factor
VCAM-1	Vascular cell adhesion molecule-1
VE-Cadherin	Vascular endothelial cadherin
WT	Wild type

Chapter 1

Introduction

1.1.0 Endothelial cells

Endothelial cells line all blood vessels in vertebrates and function as a selective barrier between the tissue and circulation. As part of this barrier, the endothelial cells are the main regulator of vascular homeostasis by interacting with circulating cells in the blood on one hand and cells in the vascular wall, such as smooth muscle cells, and tissue on the other (Reviewed by Aird (Aird 2007)). They adhere to each other through adhesive structures or cell-to-cell junctions (Reviewed by Michiels, (Michiels 2003)). Three types of junctions have been described in endothelial cells: tight junctions, adherent junctions and gap junctions (Dejana, Corada et al. 1995; Schnittler 1998). The adherent junctions, formed by vascular endothelial cadherin (VE-Cadherin) is present at the endothelial cell surface and are often used as a marker for identifying endothelial cells (Dejana, Corada et al. 1995). Platelet/endothelial cell adhesion molecule-1 (PECAM-1)/CD31, a member of the Ig superfamily, is another important marker used to identify endothelial cells, however this marker is also expressed on the surface of platelets, monocytes and neutrophils, where it is known to regulate leucocyte passage across the endothelium (Newman, Berndt et al. 1990), as well as haemopoietic stem cells (HSC) (van der Loo, Slieker et al. 1995) and haemopoietic precursors (Watt, Williamson et al. 1993).

Endothelial cell selective adhesion molecule (ESAM) was first described as a selective endothelial marker (Hirata, Ishida et al. 2001), however it is also strongly expressed by HSC (Yokota, Oritani et al. 2009), megakaryocytes and activated platelets (Nasdala, Wolburg-Buchholz et al. 2002). Another important endothelial marker is the embryonic endothelial marker endomucin, which is a sialomucin expressed in aortic endothelial cells, umbilical vein and microvascular endothelial cells (Brachtendorf, Kuhn et al. 2001; Liu, Shao et al. 2001), but also expressed on HSC (Matsubara, Iwama et al. 2005). However, none of these endothelial cell markers are specific and due to this lack of unique markers, the use of phenotype to isolate pure populations of these cells is highly challenging.

1.2.0 Scavenging endothelial cells

Studies from the early 1980s revealed that an extracellular matrix molecule, hyaluronan (HA), was eliminated from the circulation of rabbits and rats by liver endothelial cells (Eriksson, Fraser et al. 1983; Smedsrod, Pertoft et al. 1984). Furthermore, during the past few decades, extensive studies have identified these cells as a group of specialised endothelial cells, termed scavenging endothelial cells (SEC), that in addition to functioning as vascular lining, efficiently eliminate an array of circulating waste macromolecules by clathrin-mediated endocytosis (reviewed by Sorensen, McCourt (Sorensen, McCourt et al. 2012)).

All vertebrates contain populations of highly endocytically active endothelial cells. SEC are distributed in gills of Agnatha and Chondrichthyes, heart or kidney in

Osteichthyes and liver in Reptilia, Amphibia, Aves and Mammalia (Seternes, Sorensen et al. 2002).

1.2.1   Liver SEC

In mammals, SEC make up the entire hepatic sinusoidal endothelium in the liver and this population of liver LSEC (LSEC) is now known to serve as the most important site of elimination of circulating waste products (Smedsrod, Pertoft et al. 1990; Seternes, Sorensen et al. 2002). Together with Kupffer cells (KC), LSEC constitute the largest scavenger cell system in the body (Kawai, Smedsrod et al. 1998; Elvevold, Nedredal et al. 2004). Compared to KC, LSEC remove soluble macromolecules and colloids (<200 nm in diameter) via receptor-mediated endocytosis while KC phagocytose particulate matter (>200 nm in diameter) (Figure 1) (Seternes, Sorensen et al. 2002).

LSEC are involved in the clearance of many endogenous and exogenous soluble and colloidal substances. This very effective LSEC scavenging function is facilitated by the expression of 3 main categories of endocytosis receptors i) various scavenger receptors (SR), ii) mannose receptor and iii) Fc-gamma-receptor IIb2 (FcγRIIb2) (reviewed by Smedsrod (Smedsrod 2004))

SR expressed by LSEC are SR-A (macrophage SR) (Hughes, Fraser et al. 1995), SR-B (SRB1 and CD36) (Malerod, Juvet et al. 2002) and SR-H (stabilin 1/FEEL-1 and stabilin 2/FEEL-2/HARE) (McCourt, Smedsrod et al. 1999; Zhou, Weigel et al. 2000; Adachi and Tsujimoto 2002; Politz, Gratchev et al. 2002). Stabilin 1 and 2 mediate

endocytosis in LSEC of modified proteins and lipoproteins such as formaldehyde treated serum albumin (FSA), advanced glycation end products modified bovine serum albumin (A) and oxidized low-density lipoproteins (oxLDL) (Blouin, Bolender et al. 1977; Smedsrod, Melkko et al. 1997; Falkowski, Schledzewski et al. 2003; Hansen, Longati et al. 2005; Li, Oteiza et al. 2011). In addition, stabilin 2 mediate the LSEC uptake of extracellular matrix macromolecules such as HA, chondroitin sulfate and N-terminal propeptides of procollagen (I, III) (Smedsrod, Pertoft et al. 1984; Melkko, Hellevik et al. 1994; McCourt, Smedsrod et al. 1999; Harris, Weigel et al. 2008).

Studies in rats, mice and humans show that both stabilin 1 and stabilin 2 receptors are expressed on sinusoidal endothelial cells in liver, bone marrow, spleen, lymph nodes suggesting the presence of SEC in sinusoids in a variety of tissues (Goerdt, Walsh et al. 1991; Falkowski, Schledzewski et al. 2003; Martens, Kzhyshkowska et al. 2006; Du, Li et al. 2008; Qian, Johansson et al. 2009).
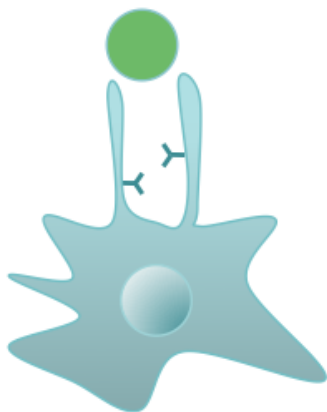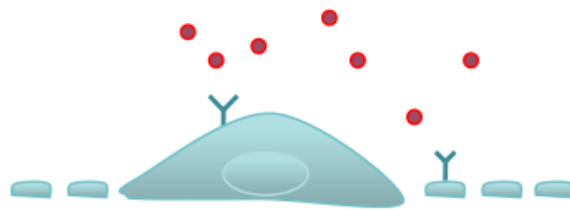
Kupffer cell   Liver sinusoidal endothelial cell

Figure 1: Kupffer cells and LSEC are the major scavenging cell system.

Schematic of the cells responsible for the removal of soluble and particulate waste. Drawing provided by Ana Oteiza.

As mentioned, other important LSEC endocytic receptors include the mannose receptor, a type 1 integral membrane protein (McGreal, Martinez-Pomares et al. 2004) that mediates uptake of endogenous glycoproteins and microbial glycans (Stahl and Ezekowitz 1998). LSEC mannose receptors ligands include C terminal propeptides of type I collagen (Smedsrod, Melkko et al. 1990), collagen α-chains (Malovic, Sorensen et al. 2007) and tissue plasminogen activator (Smedsrod, Einarsson et al. 1988). In addition, LSEC depends on this receptor to recruit lysosomal enzymes from the circulation in order to keep and effective degradation capacity (Elvevold, Simon-Santamaria et al. 2008).

Furthermore, FcγRIIb2 mediates clearance of circulating soluble IgG immune complexes (Mousavi, Sporstol et al. 2007). Besides LSEC, FcγRIIb2 has also been reported in placental endothelial cells {Lyden, 2001 #2061; Sedmak, Davis et al. 1991).

Although other receptors, like low-density lipoprotein receptor related protein-1 (LRP-1), are expressed on LSEC {Oie, 2011 #2090}, these three receptor groups constitute the main waste-clearing receptors in these cells (reviewed by Sorensen et al. (Sorensen, McCourt et al. 2012)).

1.2.2 Endocytosis of soluble waste as a functional marker

Several studies classify sinusoidal endothelial cells as SEC by using their endocytic function as a reliable functional marker (reviewed by Elvevold (Smedsrod, Einarsson et al. 1988; Smedsrod 2004; Elvevold, Smedsrod et al. 2008; Sorensen, McCourt et al. 2012). In these studies, modified proteins and lipoproteins such as FSA, oxLDL and A conjugated to fluorescent tags such as FITC (fluorecein isothiocyanate) have been used to delineate SEC both *in vivo* and *in vitro* (Hansen, Longati et al. 2005; Ito, Sorensen et al. 2007; Elvevold, Simon-Santamaria et al. 2008; Qian, Johansson et al. 2009; Li, Oteiza et al. 2011; Sorensen, McCourt et al. 2012). Recently, A fluorescently labelled with FITC (FA) has been successfully used as a specific marker for bone marrow (BM) SEC (BMSEC) (Figure 2, submitted manuscript). *In vitro* and *in vivo* incubations with FA enables BMSEC to endocytose it and appear fluorescent, making it possible to track the ligand in endocytic structures as well as identify the cells using both fluorescent microscopy and flow cytometry (submitted manuscript).
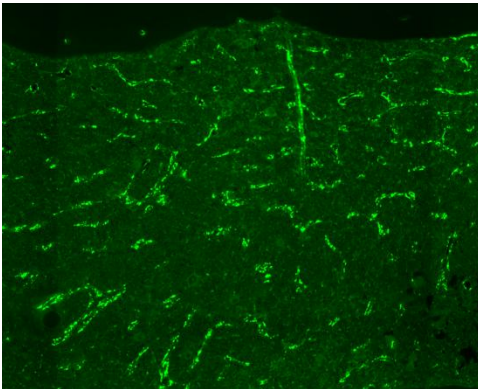
Figure 2: Endocytosis of FA by BMSEC: FA denotes sinusoidal uptake (green). Image taken by Peter McCourt.

1.2.3   Bone marrow SEC

BMSEC line sinusoids throughout the entire BM and have recently been shown to be able to be further sub-setted on the basis of their scavenger ability (Qian, Johansson et al. 2009) and the expression of CD31, B220, Gr-1, Mac-1, CD45, VE-Cadherin and ESAM (submitted manuscript). As mentioned above, CD31 is a known endothelial marker that is expressed on both LSEC and BMSEC (submitted manuscript; (Harb, Xie et al. 2009; Xie, Wang et al. 2010) that functions as a signalling and adhesion molecule (Mahooti, Graesser et al. 2000). B220 is a known pan-B marker (Coffman and Weissman 1981), but its expression is not restricted to B cells (Saga et al 1990). B220 is also expressed on a sub-population of endothelial cells (Li, Hu et al. 2009). In addition, Gr-1, is known to be expressed on monocytes, neutrophils (Ly-6G; neutrophils), T cells, plasmacytoid dendritic cells and subsets of macrophages and Mac-1 (CD11b; macrophages), known as a differentiation antigen present on macrophages (Springer, Galfre et al. 1979), have also been shown to be expressed on BM cells that scavenge the endothelial cell ligand Dil-AcLDL *in vitro* (Li, Hu et al. 2009). Endothelial cells have traditionally been phenotypically defined and isolated on the basis of a CD45$^-$ phenotype (Hooper, Butler et al. 2009; Butler, Nolan et al. 2010). CD45 is known a haemopoetic cell- surface glycoprotein with tyrosine phosphatase activity in its cytoplasmic domain (Tonks, Charbonneau et al. 1988; Kiener and Mittler 1989; Mustelin, Coggeshall et al. 1989; Ostergaard, Shackelford et al. 1989; Thomas 1989) which recently has been identified on LSEC sub-populations (Harb, Xie et al. 2009; Xie, Wang et al. 2010; Wang, Wang et al. 2012). Furthermore, both VE-Cadherin and ESAM have previously been determined to be present on

endothelial cells (Nasdala, Wolburg-Buchholz et al. 2002; Hooper, Butler et al. 2009; Butler, Nolan et al. 2010). BM endothelial cells have also recently been reported to be a critical component of the microenvironment, actively contributing to the regulation of HSC and their maintenance of blood cell production (as reviewed by Ellis and Nilsson (Ellis and Nilsson 2012)).

1.3.0 Haemopoietic stem cells

HSC, as other stem cells, have the capacity of population self-renewal and reside at the top of the well known hierarchically organised system, giving rise to all types of blood cells. Located in the BM they are an important part of the maintenance of homeostatic control of blood cell production. HSC differentiate to become multi potent progenitor (MPP) cells with limited capacity for population renewal. However, they retain full lineage potential, reconstituting more rapidly and are significantly more abundant. In turn MPP divide and differentiate into oligo-potent progenitors (OPP) that divide into two groups of developmentally restricted cells, named, common lymphoid progenitors (CLP) and common myeloid progenitors (CMP), which again can give rise to all types of blood cells (Reviewed by Bryder et al. (Bryder, Rossi et al. 2006)).

1.3.1 BM microenvironment

HSC interact with many different cell types within a dynamic three-dimensional space in BM: the stem cell niche. The regulation of HSC differentiation is dependent on the BM microenvironment; with the current understanding of this regulative role resulting from numerous studies over the last few decades. The BM microenvironment includes a variety of molecules, for example, stromal cell derived factor-1 (SDF-1), chemokine receptor type-4 (CXCR- 4) (Itkin and Lapidot 2011) and membrane-bound stem cell factor (tmSCF) (Driessen, Johnston et al. 2003) which have been shown to be important in the endosteal region (bone/bone marrow interface) by helping retain or anchor HSC, while osteopontin (OPN) (Nilsson, Johnston et al. 2005; Stier, Ko et al.

2005) and HA (Nilsson, Haylock et al. 2003) have been demonstrated to negatively regulate adult HSC. The term "haemopoietic inductive microenvironment" was first proposed by John Trentin in 1971 (Trentin 1971), where he demonstrated that HSC differentiation is regulated by stromal cells, and the term "niche" was first introduced by Schofield in 1978 (Schofield 1978) to describe the microenvironment. BM HSC and progenitors, phenotypically identical for up to least nine cell surface markers (CD3, B220, Gr-1, Mac-1, Ter119, Sca-1, c-Kit, CD150 and CD48), but residing in different regions of the BM have different haemopoietic potential, demonstrating that the microenvironment and their location significantly influences HSC properties (Grassinger, Haylock et al. 2010).

A close association between HSC and sinusoidal endothelial cells has previously been shown (Reviewed by Ellis and Nilsson (Ellis and Nilsson 2012)). Studies reveal that sinusoidal endothelium within BM is not only an important regulator of HSC but is also critical for HSC homing (Ellis et al. Blood 2011). Homing is the process where HSC attracted to the BM interact with BM vasculature to be able to migrate into extravascular space, similarly to that of leukocytes rolling to sites of inflammation (Lapidot, Dar et al. 2005; Chute 2006). HSC periodically move out of the BM by migrating through BM sinusoids and then return to the BM. Sinusoidal endothelial surface molecules and chemokine expression are critical for HSC to be able to home (reviewed by (Ellis and Nilsson 2012)). Examples of such surface molecules are vascular cell adhesion molecule-1 (VCAM-1), E-selectin and P-selectin (Frenette, Subbarao et al. 1998; Mazo, Gutierrez-Ramos et al. 1998; Vermeulen, Le Pesteur et al. 1998). Furthermore, HA expression on endothelial cells is important for HSC homing to the metaphyseal region (Ellis, Grassinger et al. 2011), whilst OPN is

important for homing of murine central HSC (Grassinger, Haylock et al. 2009). Whilst many haemopoietic cell types are readily transplantable and quickly home back to the BM microenvironment, whether this extends to endothelial cells and specifically SEC remains controversial.

1.3.2   Are endothelial cells and specifically SEC transplantable?

HSC are very important in clinical transplantation as a curative treatment in patients with haematological malignancies, immunologic diseases and other blood disorders (Reviewed by Appelbaum (Appelbaum 2003)). BM, umbilical cord blood and mobilised peripheral blood are all existing sources for HSC (Reviewed by Welniak et al. (Welniak, Blazar et al. 2007). However after preparative ablative treatments such as irradiation or chemotherapy, the whole BM needs to be reconstituted, including vasculature. Freshly isolated human or murine CD31$^+$ or BMSEC have been demonstrated to be transplantable, contributing to new blood vessel formation ((Kim, Wu et al. 2010), submitted manuscript)). However, other studies found no evidence of endothelial cells contributing to blood vessel formation post-transplant (Haruta, Nagata et al. 2001; Hooper, Butler et al. 2009).

BM transplantation is a very important treatment and the addition of transplantable endothelial cells has the potential to improve clinical outcome through the rapid regeneration of vessels. In order to reconstitute BM after ablative treatment the transplanted cells need to migrate and enter the BM through the vasculature, anchor themselves in the suitable niche and proliferate (reviewed by Nilsson et al. (Nilsson, Simmons et al. 2006). By helping build up the necessary niche for stem cells

endothelial cells would allow HSC to more rapidly generate the necessary BM cells and circulating blood. Rapid thrombopoiesis after ablative treatments is crucial for reconstitution and the interaction of megakaryocytes with the BM vasculature is required to reconstitute the platelet pool (Hamada, Mohle et al. 1998; Avecilla, Hattori et al. 2004). As a consequence, the development of methods to rapidly reconstitute BM vasculature post-transplant are of potential clinical valuable.

1.3.3 New applicable source of human SEC

In order to potentially use SEC to improve BM transplantation, a clinically applicable human source needs to be identified and characterised. As described above, the ability of endothelial cells to be transplanted and form functional blood vessels has only been assessed in murine models. Human BMSEC would be a very poor source of SEC for potential clinical use, because harvesting BMSEC from a patient would be an invasive and painful procedure. Human placenta, however, is a readily available tissue and human blood vessel SEC in the placenta can easily be non-invasively isolated. The placenta is routinely discarded after childbirth and the use of other placental derived cells in clinical medicine is already established (Barker and Wagner 2003; Grewal, Barker et al. 2003). A dramatic improvement of clinical BM transplantation and reduction in the time for BM reconstitution could be a reality if SEC isolated from human tissue have the same potential as mouse BMSEC. As human samples are extremely precious, a study of SEC in murine placenta can be used as a surrogate to identify whether to expect human placental endothelial cells to potentially be of clinical value.

Mouse and human placentas have analogous functions and for certain processes, such as the exchange of nutrients and waste products between mother and foetus, mouse is a good model. Although there are differences between the human and mouse placentas, the mouse placenta is an easy model to work with because it is small in size and its gestation time is short. The mouse labyrinth and the foetal part of the human placenta are functionally analogous (reviewed by Georgiades et al. (Georgiades, Ferguson-Smith et al. 2002). This is the area where foeto-maternal exchange of nutrients and waste products occurs, and endothelial cells that line the foetal capillaries in this area have a potential to be scavenging cells.

1.4.0 Placental components

As reviewed by multiple groups, the placenta provides nutrients and oxygen to the foetus; assists foetal waste products to be discarded; synthesises bioactive molecules such as hormones, growth factors and cytokines; protects the foetus against pathogens and forms an immunologic barrier between a mother and foetus (Rossant and Cross 2001; Georgiades, Ferguson-Smith et al. 2002; Fuchs and Ellinger 2004). The placenta develops continuously during a pregnancy, however this growth is more or less complete in the third trimester (Castellucci, Scheper et al. 1990). After birth the placenta is no longer needed and gets expelled from the body.

The placenta is a highly complex organ and consists of different regions that are anatomically and physiologically distinct. It can be divided into three layers, which have different terms in different species: uterine tissue (decidua basalis and

myometrium), basal plate (human)/junctional zone (mouse) and foetal placenta (human)/labyrinth (mouse) (Figure 3). (Reviewed by Georgiades, Ferguson-Smith et al. (Georgiades, Ferguson-Smith et al. 2002)).
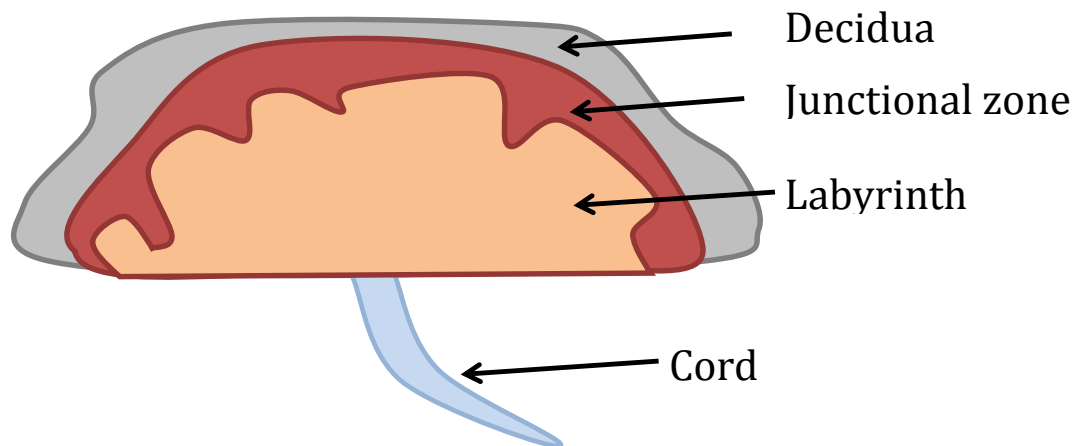
Figure 3: Mouse placenta: The decidua (grey, maternal derived) sits on the labyrinth (apricot, foetal derived) and is separated by the junctional zone (red, foetal derived). Drawing provided by Ana Oteiza..

As mouse was used as a model in this project, the different placental layers described below are from mouse placenta.

1.4.1   Uterine tissue/Decidua basalis and myometrium

The maternal side of the placenta is bordered with uterine tissue that changes dramatically during pregnancy and forms the decidua basalis. Within this, new blood vessels form and existing vessels dilate (Pijnenborg, Robertson et al. 1981; Cross, Hemberger et al. 2002); (Adamson, Lu et al. 2002). However, the vasculature in this

region is very unusual, as it is not lined by endothelial cells but with trophoblast cells, one of the most important cell types in the placenta (Cross, Hemberger et al. 2002). The trophoblast cells migrate from the foetal part of the placenta and invade the developing spiral arteries. By invading the arteries, the endothelial cells get eliminated via a mechanism not yet fully understood, however both VE-Cadherin and CD31 are present at the contact site between trophoblasts and endothelial cells suggesting that VE-Cadherin and CD31 are involved in transendothelial migration (Lambot, Lybaert et al. 2006). Maternal blood bathes the trophoblast cells to enhance exchange of nutrients and waste products. (Rossant and Cross 2001; Adamson, Lu et al. 2002). The trophoblast cells in this placental layer are called peri/endovascular trophoblast cells and glycogen trophoblast cells. Glycogen trophoblast cells are suggested to influence foetal-maternal interactions by secreting hormones such as insulin-like growth factor 2 (IGF2) (Reviewed by Fowden (Fowden 2003)) and they are thought to arise from spongiotrophoblast cells from the foetal part of the placenta. Other important cell types in the decidua basalis are uterine stromal epithelial cells, glandular epithelial cells, smooth muscle cells and natural killer (NK) cells.

1.4.2   Junctional zone

The junctional zone lines the foetal part of the placenta and is suggested to play an important role in hormone secretion, however its precise function is unknown. There is no foetal blood in this layer but there are trophoblast lined maternal arterial and venous vessels (Brosens, Robertson et al. 1967; Muntener and Hsu 1977; Pijnenborg,

Robertson et al. 1981; Redline and Lu 1989). The main cellular component of the junctional zone is cytotrophoblasts that synthesise secreted hormones such as prolactin-like protein-A (PLP-A) and PLP-B (Muller, Ishimura et al. 1998; Georgiades, Ferguson-Smith et al. 2002).

1.4.3 Labyrinth

The labyrinth is the foetal part of the placenta, where the foetus is connected to the placenta through the umbilical cord and the umbilical cord makes it possible for foetal blood to access the placenta to come in close contact to maternal blood via foetal arteries and veins (Rossant and Cross 2001; Adamson, Lu et al. 2002). The cells interacting in the foetal-maternal exchange of nutrients and waste products include foetal endothelial cells and trophoblasts. The ultrastructure of the labyrinth consists of three trophoblastic layers and one layer of foetal capillary endothelial cells which directly line the circulating foetal blood (Enders 1965) (Figure 4).
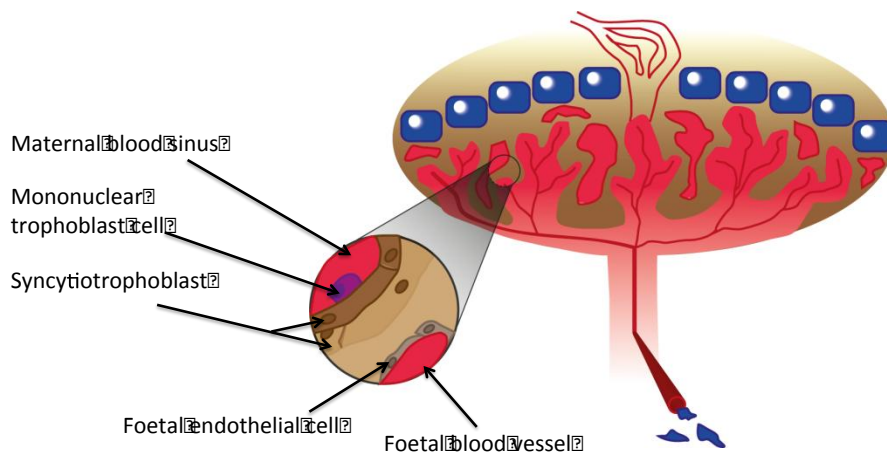
Figure 4: Mouse labyrinth. Schematic illustration of mouse placental labyrinth highlighting the layers of trophoblasts lining maternal blood sinusoids and endothelial cells lining foetal blood vessels.

The trophoblastic layer I line the maternal blood space and consist of mononuclear trophoblast cells, named cytotrophoblasts. Layer II and III are syncytiotrophoblastic (Enders 1965). Both layer I and II are in direct communication with maternal blood due to fenestrations that traverse layer I and hydrophilic molecules from the maternal circulation have been found within the space between layer I and II (Enders 1965; Takata and Hirano 1997). However hydrophilic substances do not cross the maternal surface of layer II and the foetal surface of layer III suggesting a barrier between the two circulations (Enders 1965). There is direct communication through gap-junctions between cytoplasma in layer II and III, which makes this one, functional structure (Enders 1965; Takata and Hirano 1997).

Paracrine signalling between trophoblasts and endothelium is suggested based on trophoblast giant cells expressing platelet-derived growth factor receptor (PDGFR)-β/CD140b but not the ligand PDGF-B, and PDGF-B expression by endothelium during mid/late gestation (Chhabra, Lechner et al. 2012). Other trophoblasts are found to express both the ligand and the receptor (Holmgren, Claesson-Welsh et al. 1992; Andrae, Gallini et al. 2008), however not much is known about the cellular origin and regulation of the different trophoblast sub-types. It has also been reported that human placental cytotrophoblasts express PDGFR-α/CD140a and CD140b (Holmgren, Claesson-Welsh et al. 1992). However, little is reported about CD140a expression on

other specific sub-groups of trophoblasts or the presence of this antigen in mouse placenta.

1.4.4   Placenta SEC

It is of interest to investigate if the endothelial cells in the foetal part of the placenta have scavenging functions and transplant potential. Northern blot analysis shows that collectin placenta 1 (CL-P1) RNA was found in murine placenta, heart and lung. CL-P1 is a scavenger receptor that is a membrane-type collectin from placenta and is found in most vascular endothelial cells in murine vessels (Ohtani, Suzuki et al. 2001), suggesting that placental endothelial cells have the ability to scavenge. Foetal endothelial cells also express FcRIIb2, which again suggests ability to endocytose. (Lyden, Robinson et al. 2001). Scavenger receptors class B type 1 (SR-B1) are reported on human syncytiotrophoblast membranes and on human primary trophoblasts and function to uptake and transport cholesterol across the syncytiotrophoblast layer (Alsat, Bouali et al. 1984; Malassine, Besse et al. 1987; Wadsack, Hammer et al. 2003), suggesting that trophoblasts also have a scavenging function.

To date, SEC have not been identified or characterised in placenta. However, the reported presence of scavenging receptors on placental endothelial cells suggests they play a scavenging role. SEC have previously been identified in mammal spleen, lymph node, liver and BM and this study will investigate SEC in placenta.

Chapter 2

2.0.0 Aims of study

The primary hypotheses for this project is that placental endothelial cells located in blood vessels in the foetal part of the placenta will have similar characteristics, scavenger properties and transplant potential to that seen in mouse BM sinusoidal endothelial cells. By identifying and characterising placental endothelial cells in mouse models data can then be translated into the human setting.

The aim for this study is to identify, isolate and characterise scavenging endothelial cells in mouse placenta and assess the potential of these cells to home to mouse BM post-transplant and furthermore assess their transplant ability.

Chapter 3

3.0.0 Materials and methods

3.1.0 Mice

3.1.1 Mouse strains

Adult C57 BL6/J mice aged 6-10 weeks were used as wild type (WT) controls and recipients. Red Fluorescent Protein (RFP) mice, expressing a pbActin-CMV-DsRed T3 transgene, kindly donated by Patrick Tam (Children's Medical Research Institute, New South Wales, Australia) were used as donors to assess transplant potential. Pregnant C57 BL6/J and RFP mice at the gestational ages of 14.5 and 16.5-18.5 were used to obtain placentas. All animal experiments were approved by the Monash Animal Research Platform (MARP) ethics committee (Ethics No: MAS/2010/14). Mice were housed at Monash Animal Service (MAS) and provided with mouse chow (Barastok, Victoria, Australia) and acidified water ad libitum.

3.1.2 Time mating

Mice were time mated to obtain placentas at specific gestational ages. Whilst the best mimic of available human placentas would be full-term murine placentas (embryonic (E) 21), this was not logistically feasible as mice eat their placentas very quickly after birth. In addition, mice are renown for delivering at E 19-E21. Consequently, to obtain reproducible gestationally aged matched placentas, they were harvested by E 18.5.

Time mating procedure:

- Females were housed together at 5 per cage to synchronise estrous cycle by dense co-hosting.

- Female mice were "teased" by placing dirty bedding from male cage into female's cage for 3-4 days prior to mating to cause the females to come into oestrus. As a consequence, the females will be highly receptive to the male and in turn will increase the chance of a successful pregnancy.

- 3-4 days post "teasing" a male was placed in the cage at 4-5 pm and then removed between 6-9 am the next day. This allows the gestational age to be accurately determined to within ~12 hours.

- Immediately after the removal of the male, the females were checked for vaginal plugs; a "plug" of sperm found at the vaginal opening. Mice with detected plugs were then designated as E 0.5. In addition, as the absence of a plug does not necessarily mean the mice are not pregnant, females were not re-mated until after a possible pregnancy was confirmed at E 14.5.

3.1.3 Irradiation

For long-term transplant assays, recipient mice were irradiated 24 hours prior transplantation with two opposing ^{137}Cs sources (Gammacell 40; Atomic Energy of Canada, Ontario, Canada), given as a split dose (2 x 4.5 Gy: total 9 Gy), 5 hours apart. This irradiation regime has previously been shown to be optimal because it gives minimum gut damage and best survival 2 weeks after irradiation before donor cell reconstitution occurs (Bradley and Hodgson 1979)**.** The recipients were injected with cells of interest, isolated as described in section (3.2.0 and 3.3.0), along with 200 000 irradiated BM filler cells (single dose of 15 Gy). After irradiation, the filler cells are viable and function, however they are unable to divide. Filler cells are injected for support to ensure short-term survival. At 14 days post transplant, the animals are at their critical point haematologically, with their BM at the nadir in terms of cellularity. All irradiations were performed at MAS. Post-transplant, recipient mice were kept on heat pads and fed with a 1:1 mix of water and powdered mouse chow (Barastoc, Ridely AgriProducts, Victoria, Australia) and Ensure (food supplement; Abbott Laboratories, Illinois, Unated States) daily, made into a slurry using antibiotic water (200 µl of the antibiotic agent, Oral Baytril 25, to 1 l autoclaved drinking water).

3.2.0 Harvesting murine placentas

Placentas were harvested from pregnant mice at E 14.5-E 18.5, placed in PBS-2 % serum, then dissected as previously published (Gekas, K et al. 2008). Specifically, the decidua (white capsule) was removed from the placenta (Figure 5) as it interferes with

single-cell preparation. This leaves mushroom top looking, discoid shaped placentas (Figure 5), which were washed in PBS-2 % serum.

Specific procedure:

- Pregnant mothers were euthanised by cervical dislocation.
- The uterus was removed.
- Individual embryo conceptus were isolated and the embryo immediately euthansied by decapitation.
- The placenta was carefully dissected from yolk sac.
- The decidua (white capsule) was separated from the placenta leaving a mushroom top looking, discoid shaped placenta (Figure 5).
- Placentas were collected and washed in PBS-2 % serum.
- RFP placentas were confirmed using fluorescent goggles.
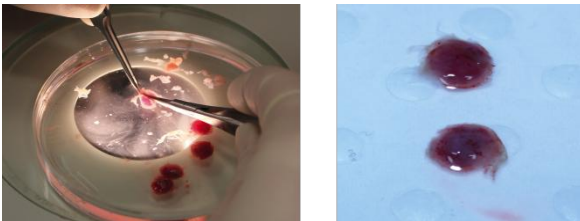
Figure 5: Placenta dissection

Dissection of E 14.5-E 18.5 murine placentas by removing the decidua leaving a discoid shaped placenta containing the labyrinth.

3.3.0 Preparation of single cell placenta suspensions

To prepare a single cell suspension, enzymatic dissociation of the placenta tissue was performed using 1 mg/ml Collagenase Type 1 (Worthington, Biochemical

Corporation, Lakewood) in PBS-2 % serum at 1 ml per placenta at 37 ^{o}C in an orbital shaker, 750 rpm. In this context, collagenase breaks the peptide bonds in collagen, which is a component in the placental extracellular matrix, helping to break down the tissue. This was done in combination with mechanical disruption by drawing the placental tissue gently up and down using different gauged (G) needles (16 G, 18 G and 21 G) and a 3-5 ml syringe every 5 minutes. Due to collagenase being known to cleave cell surface molecules, such as receptors (Haylock, Williams et al. 2007), the incubation time in collagenase was as short as possible to preserve as many of the characteristics of the cells as possible. The disaggregation stage was deemed to be complete when the single cell suspension was easy to draw up and down through the 21-gauge needle.

Specific procedure:

- 2-5 placentas from each mouse were pooled together in a 15 mL tube. Pooling more than 5 placentas made disaggregation very difficult.

- Placentas were incubated in 1 mg/ml Collagenase Type I in PBS-2 % serum at 1 ml per placenta at 37 °C in an orbital shaker at 750 rpm, for 5 minutes.

- After a 5 minute incubation, mechanical disruption was done by drawing the placental tissue gently up and down using a 16 G needle and a 3 or 5 ml syringe. Following a further 5 minute incubation at 37 °C in an orbital shaker at 750 rpm mechanical disruption was repeated using an 18 G needle. The process was then repeated, but with a 21 G needle. This resulted in 10-15 minutes total incubation time.

- As soon as it was easy to draw the suspension of single cells up and down through the 21 G needle the cells were washed by filling up the tube with PBS-2 % serum,

filtering it through a 40 μm nylon cell strainer into a 50 ml tube, then centrifuging

at 400 G for 5 minutes. The supernatant was removed and the cell pellet

resuspended in 10 ml PBS-2 % serum, then re-washed using centrifugation.

- The pellet was then resuspended in 4 ml PBS-2 % serum.

- Indication of cell viability was assessed using phase contrast microscope.

- The cellularity of each placenta was determined using an automated cell counter

  (Sysmex KX-21N, Sysmex Corporation, Kobe, Japan).

3.4.0 BM harvesting and cell isolation

BM from femurs, tibiae and iliac crests was harvested from mice for antibody

labelling controls in analysis experiments and to look for homing and long-term

reconstitution after transplantation. Three different methods were used: enhanced

method for the isolation of total BM, isolation of central BM and isolation of

endosteal BM as previously described (Williams and Nilsson 2009). For harvesting

central BM, the bones were flushed with PBS-2 % serum, and only the central BM

collected. Central BM was used as filler cells for support in transplantations.

Endosteal BM was used for compensation during flow cytometry and was harvested

from the flushed bones. The enhanced BM isolation method is both central and

endosteal BM extracted together.

Specific procedure:

3.4.1 Bone isolation:

- Bones were harvested (femurs, tibia and iliac crests) and adherent muscles and tissue removed using a surgical blade.

3.4.2 Enhanced method:

- Bones were collected into approximately 15 ml cold PBS-2 % serum in one 50 ml conical tube, then decanted into a sterile mortar.
- Bones were thoroughly ground with the pestle to expose the medullary cavity to enzymatic digestion, but without reducing the bone to powder.
- The cell supernatant was filtered through a 40 µm nylon cell strainer into a 50 ml conical tube and the cells washed by filling the tube with PBS-2 % serum and centrifugation (400 g, 5 minutes, 4 °C).
- The crushed bones were transferred into a 50 ml conical tube containing 1 mL of a solution of 4 mg/ml dispase (GIBCO, Invitrogen corporation, New Zealand) and 3 mg/ml collagenase I in PBS per 2 femurs, 2 tibiae and 2 iliac crests.
- The bone fragments were agitated for 5 minutes at 37 °C in an orbital shaker at 750 rpm.
- 10-15 ml PBS was added to the bone fragments and the tube vigorously shaken for 10 seconds.
- The cell suspension was filtered through a 40 µm nylon cell strainer into a 50 ml conical tube.

- A further 10-15 ml PBS was added to the bone fragments, the previous two steps repeated, before the 50 ml tube was filled with PBS-2 % serum and the cells washed using centrifugation (400 g, 5 minutes, 4 ^{O}C).

- The cell pellets from both the initial supernatant and the digestion were resuspended and pooled in PBS-2 % serum and a cell count performed.

3.4.3 Isolation of central marrow

- The metaphyseal regions of each of the femur and tibia were removed and transferred into a 50 ml tube containing 15 ml PBS-2 % serum for harvesting the endosteal sample. For the iliac crest, the acetubular notch was removed and also transferred to PBS-2 % serum (Figure 6).

- A 1 ml syringe attached to a 21 G (femurs and tibiae) or 23 G needle (iliac crests) was used to flush the bones with PBS-2 % serum into a 50 ml centrifuge tube.

- The femurs were flushed by inserting the 21 G needle into both ends (epiphysis) of the femoral shaft.

- The iliac crests were flushed by inserting a 23 G needle into the shaft of the exposed bone after acetabular notch was removed.

- Inserting a 21 G needle into the proximal end flushed the tibiae.

- The bones had a relatively clean translucent look to them when properly flushed.

- The flushed marrow was washed by filling the tube with fresh PBS-2 % serum and centrifugation (400 g for 5 minutes at 4 ^{O}C).

- The cells were resuspended in 20 ml PBS-2 % serum and filtered through a 40 µm nylon cell strainer into a 50 ml conical tube prior to the cellularity being determined.
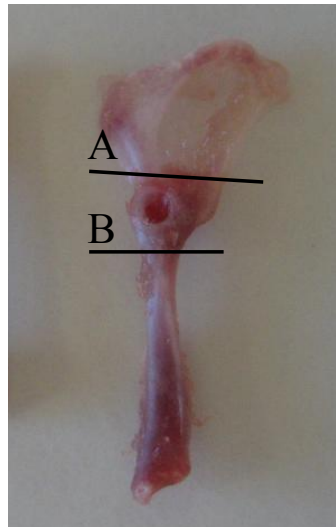
Figure 6: (A) The flat triangular piece of cartilage was removed (white line) from the iliac crest. (B) The acetabular notch was removed (white line) and is now ready to be flushed. Photo taken by Shen Heazlewood.

3.4.4 Isolation of endosteal marrow

- Flushed bones were collected into approximately 15 ml cold PBS-2 % serum and the procedure for enhanced method described above followed to isolate a single cell suspension of endosteal BM cells.

3.5.0 FA preparation

A (kindly provided by Peter McCourt, Vascular Biology Research Group, University of Tromsø, Norway), was conjugated to FITC (FA) for use as a soluble ligand in order to detect scavenging endothelial cells both *in vivo* and *in vitro*. In this context, scavenging endothelial cells, which express endocytic scavenger receptors, such as stabilin 1 and stabilin 2, rapidly and effectively endocytose FA, leaving them easily identifiable by both flow cytometry and immunohistochemistry. FA was dialysed in Slide-a-lyser dialysis cassettes (3500 MWCO, 3 ml capacity).

Specific procedure:

- 1 ml of 10.3 mg/ml A was thawed out and the volume was determined by using a pipette.
- The appropriate amount of FITC was weighed out into an eppendorf tube so that there was 1/5th by weight FITC to protein (e.g. 10 mg A required 2 mg FITC).
- The FITC was dissolved into 1/10th the A volume (e.g. 1 ml of A required 0.1 ml) of 1M Na_2CO_3 pH 10.
- The dissolved FITC was immediately transferred into the A and mixed with repeated pipetting up and down.
- Conjugation occurred by incubating the tube overnight light protected and at room temperature.
- The following day the reaction tube was vortexed and transferred to a pre-PBS-wetted Slide-a-lyser dialysis cassette. The volume was carefully recorded. This volume minus the added carbonate volume gave the exact volume of the A, and thus the exact amount (mg) of A.

- In order to remove the free FITC (FITC unbound to the protein), FA was dialysed using 4 changes of 500 ml PBS at 4 °C while stirring for various times:

    1. 1 hour

    2. 4 hours

    3. overnight

    4. 4 hours

- Post-dialysis, the FA was carefully retrieved from dialysis cassette and the volume collected carefully recorded.

- The FA was transferred to eppendorf tubes (approximately 1 ml in each).

- The post-dialysis protein concentration was determined (= mg start protein /post-dialysis volume) based on the assumption of no protein loss post dialysis. No good method of determining protein concentration is available as it is not detectable by NanoDrop and FITC interferes with spectroscopic methods and make them inaccurate. A method for FA concentration determination is yet to be established.

- A working solution of 0.375 mg/ml was generated such that dosing at 10 □l/g mouse body weight yields a dose of 3.75 mg/kg.

- FA aliquots were stored at -80 °C and only re-frozen once.

5.6.0 FA endocytosis

In order to allow FA to be endocytosed by the endothelial cells, placental single cell suspensions were "floated" in FA (2.5 µg/ml) in PBS-1.0 % bovine serum albumin (BSA) for 10-40 minutes at 37 °C on a rotator. Specifically, up to 10 million cells

were incubated in a minimum of 1 ml FA in PBS-1.0 % BSA. BSA was added to avoid FA unspecific binding. In addition, placenta explants were incubated in FA under the same conditions in one experiment in order to observe FA endocytosis in intact tissue. Initial experiments were designed to optimise the opportunity for placental endothelial cells to endocytose FA. As such, both single cell suspensions and explants were assessed. Explants are dissected placentas, with the decidua removed, leaving the exposed labyrinth. Explants were incubated in 2.5 μg/ml FA at 37 °C followed by copious washing in PBS-0.5 % BSA. The explants were enzymatically dissociated with collagenase Type I following FA incubation.

Specific procedure

A pellet of placental cells (isolated using the method described above) was resuspended in a minimum of 1 ml 2.5 μg/ml FA in PBS-1.0 % BSA per 10 million cells, or per individual explant.

The cells were incubated for 10-40 minutes at 37 °C on a rotator, then washed twice in PBS-2 % serum, by filling up the tube, centrifuge at 400 G for 5 minutes at 4 °C, discarding the supernatant and resuspending the pellet.

3.7.0 Enrichment strategies

2.7.1 Density Separation

Discontinuous density centrifugation using Nycoprep (1.077g/cm^3, 265mOsm, pH 6.9; made from Nycodenz, Axis-Shield, Oslo, Norway; Appendix B) was used to enrich for mononuclear cells. Cells from both the interface and pellet were collected to assess if this method would enrich for our scavenging endothelial cells of interest. Followed procedures were as previously described (Williams and Nilsson 2009).

Specific procedure:

- The cell suspension was diluted to 2×10^8 cells/20 ml and divided into 20 ml aliquots over an even number of 50 ml centrifuge tubes.
- Each tube was underlayed with 10 ml Nycoprep using a cannula attached to a 20 or 30 ml syringe to create a gradient.
- The gradients were centrifuged at 600 g for 20 minutes at room temperature without a brake.
- Mononuclear cells were collected from both the interface between the PBS layer and the Nycoprep solution and the pellet using cannulas attached to a 10 ml syringe, into a 50 ml centrifuge tube. The mononuclear cells from two gradients were collected into a 50 ml conical tube and washed with PBS-2 % serum using centrifugation (400 g for 5 minutes at 4 °C).
- The supernatant was decanted, the cell pellets and interface was resuspend in 50 ml PBS-2 % serum and counted.

2.7.2 RBC Lysis

Placental cell preparations are contaminated with red blood cells, which have high auto fluorescence and thereby can interfere with placental cell detection by flow cytometry. Red blood cells (RBC) can be eliminated by lysis using a lysis buffer (Appendix).

Specific procedure:

Stock lysis buffer was diluted 1:10 in PBS to make a fresh working solution immediately before use. The lysis solution needs to be made fresh before use because storing a solution at <10x concentration results in ammonium carbonate being formed, rendering the solution ineffective.

1 ml lysis buffer was added to the cells and incubated for 1-2 minutes.

Cells were washed with PBS-2 % serum using centrifugation (400 g for 5 minutes at 4 ^{O}C). The washing step was repeated.

The cells were resuspended as necessary for specific assays.

3.8.0 Fluorescent Activated Cell Separation (FACS)

This is a method used for both cell sorting and analysis. Flow cytometric analysis was performed using a LSRII analyser (Becton Dickinson, New Jersey, United States) and cell sorting was performed using an Influx (Becton Dickinson, New Jersey, United States). The LSRII is equipped with 7 solid-state lasers (355, 405, 488, 532, 561, 592 and 628 nm) and the Influx is equipped with 5 lasers (355, 405, 488, 561 and 635 nm). The excitation laser, detector and detector filter range for each fluorochrome are summarised in Table 1. For analysis typically $0.5-2x10^6$ events were recorded by analysing 10 000 events per second, except homing analysis where 40-60 $x10^6$ cells were recorded by analysing 30 000 events per second due to small number of donor cells in the recipient. To set all voltages unstained BM cells were used and single fluorochrome stained BM cells were used to compensate the fluorescence spectral to minimise overlap between dyes. When using RFP and/or BV650, these fluorochromes needed to be compensated first due to their relatively bright signal. Cell sorting of placenta was performed either one way or two way using a nozzle diameter of 100 μm and a sheath pressure of 20 psi. Only purity mode was used because population of interest is of high frequency, which results in the highest purity of cells of interest in the sorted population. However, this will also lower the yield of desired cells because only the desired cell in the middle drop is sorted if the side drops does not contain an unwanted cell. Cells can also be enriched for by using enriched mode where also side drops will be sorted even if they do not contain the desired cell, then all the desired cells will be sorted along with some other cells, but yield will increase. The decision on which mode to use is based on the total number of cells and the frequency of the cells of interest. For processing large numbers of cells containing a low frequency of

cells of interest to be sorted, an initial enrichment sort mode would be used to enrich

the proportion of the cells of interest at high speed (30 000 cells per second), with a

high yield but at a lower purity. Then, a purity sort would then follow.

Table 1: The excitation laser, detector and detector filter range for each fluorochrome used by

LSRII for cell analysis and Influx for cell sorting

	LSRII			Influx		
Flurochromes	**Laser**	**Detector**	**Detector range (nm)**	**Laser2**	**Detector2**	**Detector range (nm)2**
Foreward(scatter	,	FSC	,	,	FSC	,
Side(scatter	,	SSC	,	,	SSC	,
BV421	Violet	V450		Violet	[405]460/50	
BV650	Violet	V650		Violet	[405]610/20	
APC,Cy7	Red	R780	750,810	Red	[635]780LP	750,810
RFP	Yellow,green	YG610	600,620	Yellow,green		
PE	Yellow,green(	YG585	564,606	Yellow,green	[561]605/40	573,613
APC	Red	R660	650,670			
PB	Violet	V500	425,475	Violet	[405]660/20	425,475
FITC	Blue	B540	525,555	Blue	[488]528/38	509,547
V500(	Violet	V525	500,550	Violet	[405]550/50	
AF647	Red	R660	650,670	Red	[635]660/20	655,685
eFl660	Red	R660	650,670	Red		
AF594	Yellow,green	YG610				
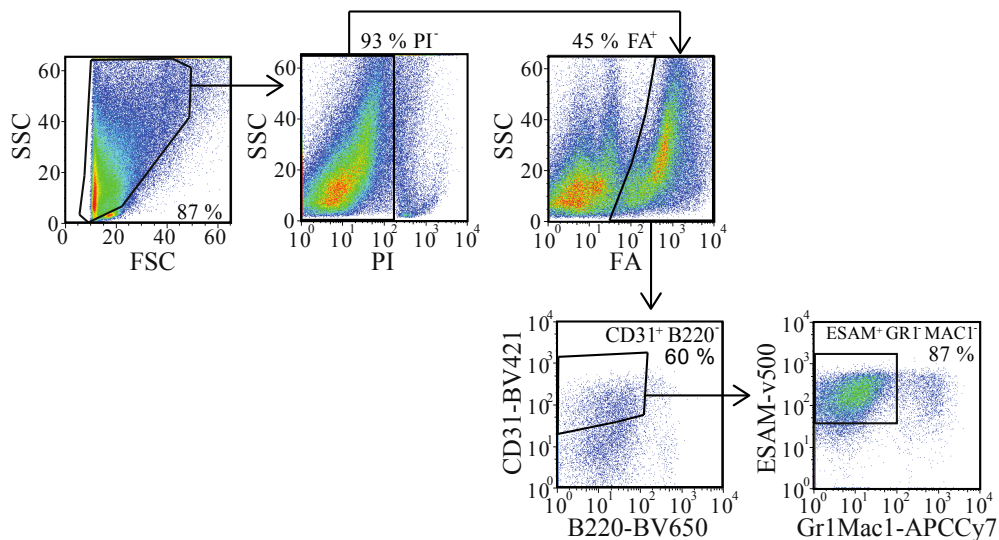
Figure 7: Gating strategy

Gating strategy for PSEC cell sorting by Influx sorter. SSC=side scatter,

FSC=forward scatter, PI= propidium iodide.

3.8.1 Antibody labelling for flow cytometry

Each antibody was titrated to find the optimal concentration: the concentration where the antibody specifically binds and provides the biggest difference from the appropriate isotype control, which itself shows very little evidence of non-specific binding compared to the unlabelled control. As a consequence, as each antibody has its own specific affinity to a particular antigen, it will have its own optimal titration. Too much antibody can decrease the ability to distinguish the positive population from the negative one because of non-specific binding of the isotype and hence the "background" will increase. Too little antibody will not allow "true positive" cells to be distinguished from negative cells.

Specific procedure:

- Cells were aliquoted into an appropriate sized tube.
- The cell suspension was washed in PBS-2 % serum and centrifuged at 400 g for 5 minutes at 4 °C.
- The supernatant was decanted to give a dry pellet, which was resuspended at 1×10^7 cells /100 µl in optimally pre-titrated antibody and a minimum of 50 µl antibody if the pellet contained less than 1×10^7 cells. Antibodies were briefly spun down in a microfuge before use to eliminate nonspecific background staining by any protein aggregates formed during storage.
- The cells were incubated with antibody for 20 minutes on ice protected from light.

- The cell suspension was washed in PBS-2 % serum and centrifuged at 400 g for 5 minutes at 4 °C.

- Where the primary antibody was unconjugated and required a secondary fluorescent label the cell pellet was resuspend at 1×10^7 cells/100 µl in optimally pre-titrated fluorescent labelled secondary antibody and incubated for a further 20 minutes.

- The cell suspension was washed in PBS-2 % serum and centrifuge at 400 g for 5 minutes at 4 °C.

- The cell pellet was resuspended in PBS-2 % serum in a volume of 100-200 µl.

- To analyse only viable cells Propidium iodide (PI) was added to the cell suspension. As PI will label free DNA in the suspension we will know that all cells that are not stained with PI are viable.

Table 2: Antibodies

Antibodies used for flow cytometry

Antigen	Fluorochrome	Isotype	Clone	Company	Cat#	mg/ml (stock)	Use at (µg/ml)
CD144 (VE-Cadherin)	AF594	Rat IgG1	B12-1	eBioscience	16-1441-85	1	1
CD31	AF647	Rat IgG2a	390	Biolegend	102416	0.5	0.5
CD31	BV421		390	Biolegend	102423	0.1	0.1
B220	APC-Cy7	Rat IgG2a	RA3_6B2	Pharmingen	552094	0.2	0.5
B220	BV650		RA3_6B2	BD Horizon	563893	0.2	5
GR1	PE	Rat IgG2b	RB6-8C5	Pharmingen	553128	0.2	0.1
GR1	APC-Cy7	Rat IgG2b	RB6-8C5	Pharmingen	557661	0.2	0.2
GR1	PB	Rat IgG2b	RB6-8C5	Biolegend	108430	0.5	0.5
MAC-1	PE	Rat IgG2b	M1/70	BD Pharmingen	553311	0.2	0.1
MAC-1	APC-Cy7	Rat IgG2b	M1/70	Pharmingen	557657	0.2	0.5
MAC-1	PB	Rat IgG2b	M1/70	Biolegend	101224	0.5	0.5
ESAM1	PE		1G8	Biolegend	136203	0.2	1
ESAM1	Biotin	Goat IgG		R&D	BAF2827	1	2.5
CD45	PB	Rat IgG2b	30-F11	Biolegend	103126	0.5	1.25
CD45	V500	Rat IgG2b	30-F11	BD Horizon	561487	0.2	1
CD45	APC-Cy7	Rat IgG2b	30-F11	Biosciences	557659	0.2	2
CD45	FITC	Rat IgG2b	30-F11	BD Pharmingen	553080	0.5	1
CD45	Biotin	Rat IgG2b	30-F11	BD Pharmingen	553078	0.5	2
CD45	Purified	Rat IgG2b	30-F11	BD Pharmingen	553076	0.5	2
CD140a	PE	Rat IgG2a	APA5	Pharmingen		0.2	1
CD140b	APC	Rat IgG2a, κ	APB5	Biolegend	136007	0.2	10
Streptavidin-V500				BD Horizon	561419	0.5	2
Endomucin	eFl660	Rat IgG2a	eBioV. 7C7	eBioscience	50585180	0.2	5

3.8.2 PSEC identification

PSEC were identified by staining the cells with a cocktail of antibodies followed by flow cytometric analysis (Table 2). PSEC populations were identified based on FA, CD31 and B220 expression and were further characterised based on Gr-1, Mac-1, ESAM, CD45 and endomucin expression following gating through a FSC-A versus FSC-H to ensure single cells. To eliminate contaminating trophoblasts, PSEC were negatively selected based on CD140-a/b expression. Viability was determined by PI labelling.

3.9.0 PSEC transplant

PSEC; $CD31^{hi}B220^-Gr-1^-Mac-1^-ESAM^+CD45^-$ (Table 3) isolated from RFP mice were transplanted into irradiated C57 BL6/J recipients. PSEC were transplanted along with 200 000 lethally ablated whole BM filler cells or in specific instances non-irradiated whole BM filler cells. The non-irradiated BM cells were used to help the recipient's BM to reconstitute, however this results in a competitive reconstitution assay between BM cells and PSEC being established. PSEC engraftment potential was analysed 4 weeks post-transplant

Table 3: PSEC antibodies and fluorophores used for FACS for re-vascularisation assays

P-ESC

Antibody	Fluorophores
CD31	AF647
B220	BV650
Gr-1	PB
Mac-1	PB
ESAM	V500
CD45	APC-Cy7

3.9.1 Analysis of PSEC engraftment potential

After 4 weeks, mice were euthanised and BM from two marylands (iliac crest, femur and tibia) was harvested followed by flow cytometric analysis and the determination of the proportion of donor (RFP^+) reconstitution. To ensure enough RFP^+ cells, 5-10 million whole BM cells were assessed.

3.10.0 PSEC homing

The engraftment potential of transplanted cells depends on their ability to home to the BM. A 15 hour non-ablated *in vivo* homing assay was done to determine how many cells of interest homed by labelling the cells with one of two tracking dyes: CFSE or SNARF. CFSE, 5- (and 6-) Carboxyfluorescein Diacetate Succinimidyl Ester (initially CFDA-SE, but becomes CFSE when incorporated, Molecular Probes, Oregon, United States) and SNARF, seminaphtorhodafluor-1-Carboxylic Acid Acetate Succinimidyl Ester (Invitrogen, California, United States), were used as previously described (Nilsson, Johnston et al. 2001; Grassinger, Haylock et al. 2010). These very bright dyes are especially suitable for tracking rare populations of transplanted donor cells because the positive events can always be detected at more than 3 logs above background (Figure 8). The 15 hour time point was chosen based on previous observations (Hendrikx, Martens et al. 1996; Nilsson, Dooner et al. 1997).
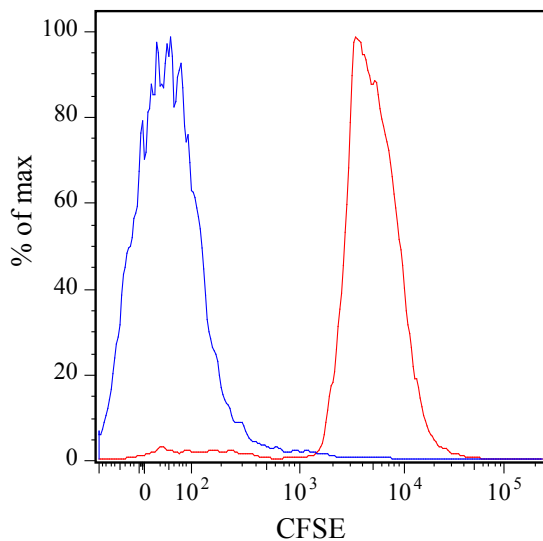
Figure 8: Flow cytometric histogram showing CFSE is detected 3 logs above background

Specific procedure for preparation of CFDA, SE stock:

- To make a 44.8 mM stock solution of CSFE 1 ml anhydrous DMSO was added to 25 mg vial.

- In a glass vial, a working solution of 5 mM CFDA, SE was made by adding a 100 µl aliquot of the 44.8 mM stock solution of CFDA, SE to 796 µl of DMSO. CFDA, SE was stored at -20 °C in a secondary container with desiccant.

Specific procedure for CSFE staining of cells:

- Cell population of interest was isolated by sorting; PSEC.

- Cells were resuspended at 5×10^6 cells/ml in PBS supplemented with 0.5 % heat inactivated serum, a minimum volume of 200 µl was used for small cell numbers.

- Cells were pre-warmed in a 37 °C water bath for 2 minutes.

- A 5 µM CFSE solution was prepared by diluting 1 µl of 5 mM CFDA, SE stock in 999 µl in PBS and kept light protected.

- 22.2 µl of the 5 µM CFDA, SE solution was added to each 200 µl of cell suspension to give a final concentration of 0.5 µM CFDA, SE.

- Cell suspensions were incubated in a 37 °C water bath for 10 minutes in the dark.

- After incubation 1 ml ice cold PBS supplemented with 20 % serum was added and gently mixed. This is to stop the reaction as CFSE is toxic to the cells over time.

- Cells were centrifuged at 400 g for 5 minutes and the supernatant decanted.

- The cell pellet was resuspended in 300 µl PBS-2 % serum.

- Cells were counted in hemocytometer or eosinophil chamber (depends on how many cells. With few cells an eosinophil chamber is suitable) and checked for positive staining in fluorescence microscope (Olympus BX51): FITC-488 filter.

- Cells were centrifuged at 400 g for 5 minutes and the supernatant decanted.

- Cells were resuspended in 200 µl PBS per intended recipient mouse; allowing 100 µl extra cell suspension for losses during injection.

Specific procedure for preparation of SNARF-1 stock:

- A 1 mM stock solution of SNARF-1 was made by adding 84.4 µl anhydrous DMSO to a 50 µg vial of SNARF-1. The solution was stored light protected at -20 ^{O}C. SNARF-1 Carboxylic acid, acetate, succinimidyl ester is susceptible to hydrolysis and must be protected from moisture during storage.

Specific procedure for SNARF-1 staining of cells

- Isolated cell population of interest; PSEC.

- Cells were resuspended at 5 x10^6 cells /ml in PBS supplemented with 0.5 % heat inactivated serum, a minimum volume of 200 µl for small cell numbers and staining was be carried out in a 1.5 ml eppendorf tube.

- Cells were pre-warmed in a 37 °C water bath for 2 minutes.

- A 10 µM SNARF-1 solution was prepared by diluting 1 µl of 1 mM SNARF-1 stock in 99 µl in PBS (1 in 100 dilution) and kept light protected.

- 22.2 µl of the 10 µM SNARF-1 solution was added to each 200 µl of cell suspension to give a final concentration of 1.0 µM SNARF-1 (1 in 10 dilution).

- The cell suspensions were incubated in a 37 °C water bath for 10 minutes in the dark.

- 1 ml ice cold PBS supplemented with 20 % serum was then added and mixed gently. This stoped the reaction.

- Cells were centrifuge at 400 g for 5 minutes. The supernatant was decanted.

- The cell pellet was resuspended in 300 µl PBS-2% serum.

- Cells were counted in hemocytometer or eosinophil chamber and checked for positive staining in fluorescence microscope (Olympus BX51): Texas-red- 594 filter.

- Cells were centrifuged at 400 g for 5 minutes and the supernatant decanted.

- Cells were resuspended in 200 µl PBS per intended recipient mouse; allowing 100 µl extra cell suspension for injection losses.

- Cells were counted using a hemocytometer or an eosinophil chamber (Thomas Scientific, New Jersey, United States).

Specific procedure for homing analysis:

Freshly isolated PSEC (CD31^{hi}B220$^-$Gr1$^-$Mac-1$^-$ESAM$^+$CD45$^-$) and

Endomucin$^+$CD140a^{+int}/CD140a$^-$, CFSE$^+$ or SNARF$^+$ from E 17.5 placentas (CFSE

and SNARF was swapped between the experiments to eliminate the possibility of the

dye having an impact on the experiment) were transplanted into non-ablated

recipients together with 200 000 BM filler cells (non-irradiated C57 non-fractionated

BM cells). Different numbers of each cell population of interest were injected

depending on how many cells were isolated (depending on how many placentas the

mum had on the day).

- Cell suspension was injected into non-ablated recipient mice via the lateral tail
  vein.
- The needle used to inject was rinsed gently with IMDM supplemented with
  1% BSA and L-Glutamine (2mmol/l), then placed in $5\%O_2 10\%CO_2$ in $85\%N_2$
  overnight as a positive control for flow cytometric analysis the next day.
- Cells were allowed to home for 15 hours.

3.10.1 Homing Analysis

15 hours post-transplant, mice were euthanised and BM harvested from 2 marylands

(iliac crest, femur and tibia) using the enhanced BM collection method (procedure

section above). CFSE$^+$/SNARF$^+$ cells were detected using a LSRII flow cytometer

and number of positive events quantitated. The calculation of the % donor cells that

homed to the BM was calculated as per table 4. Due to relatively small numbers of

PSEC (Figure 17,18 and 20 40-60 x 10^6 total processed events were assessed to

ensure enough positive cells were analysed. To keep the files manageable, a gate was

drawn around the cells not expressing CFSE and SNARF and only those events

occurring outside the gate were saved (Figure 17, 18 and 20). To determine the

proportion of nucleated cells, smaller files containing all events were collected.

Table 4: Homing calculation. The table shows one example on how the proportion of transplanted cells that homed was calculated.

Calculation	Example
Number of transplanted cells	1950
Processed events by Flow Cytometry	49480929
Proportion nucleated cells gated on Flow cytometer	73.5%
Measured number of CFSE$^+$ or SNARF$^+$ events	3
Number of cells harvested (count from Sysmex)	113000000
Total nucleated cell number processed for analysis (%nucleated cells x processed events/100)	36368483
Proportion of donor cells (positive events/total nucleated cells x 100)	0.000008%
Total number of donor cells* (%donor cells/100 x cells harvested/30** x 100)	31
Proportion of positive events from each transplant (total donor cells/transplanted cells x 100)	1.60%

*Assumes that cells from one femur, tibia, iliac crest represent approximately 15% of total BM (Briganti, Covelli et al. 1970; Boggs 1984).

** BM was harvested from two femurs, iliac crests and tibiae

3.11.0 Statistical analysis

Data are presented as mean±SEM. Differences between means were evaluated by

unpaired t-test or a one-way ANOVA where appropriate. One-way ANOVA on

Ranks or Mann-Whitney rank sum test was used when equal variance or normal

distribution failed. A p-value of ≤ 0.05 was considered significant. Statistical analysis

was performed using Sigma Plot 12.0 or Prism version 6 (GraphPad Inc., San Diego,

CA, USA). Flow Cytrometric analysis was performed using the MAC software

FlowJo.

3.12.0 Immunohistochemistry

3.12.1 Fixation

Whole placentas were immersion fixed in 4 % paraformaldehyde, 0.1 % glutaldehyde for 48 hours at room temp under vacuum. Fixed tissues were transferred into PBS 0.1 M Sorensens phosphate buffer (0.1 M Na_2HPO_4 in 0.1 M KH_2PO_4, pH 7.4) with 5 % sucrose prior to embedding in paraffin.

3.12.2 Embedding and sectioning

Transversely cut half placentas were dehydrated through increasing concentrations of ethanol (EtOH):

1. 70 % EtOH for 5 minutes, x2
2. 100 % EtOH for 5 minutes, x2
3. Xylene for 5 minutes, x2

Followed by embedding in paraffin wax at 60 ^{O}C and stored at room temperature. A microtome (RM2040, Leica Reichert Jung, Grale Scientific, Victoria, Australia) was used for sectioning ice-cold samples into 3 μm sections. A 37 ^{O}C water bath was used to float the sections to easily transfer them to glass slides (Superfrost Plus; Thermo Scientific, Braunschweig, Germany) (4 sections per slide). The slides were dried over night at 37 ^{O}C.

3.12.3 Staining

To examine the distribution of VE-Cadherin and FA within the placenta, paraffin wax embedded sections were dewaxed in Xylene and rehydrated through a graded series of ethanol to water. After rehydrating, proteins are exposed and can be immunolabelled. Antigen retrieval was done to achieve improved antigen access by braking protein crosslinks. To block endogenous peroxidase slides were washed in PBS and treated with 3 % hydrogen peroxide in PBS. Non-specific binding was then blocked (PBS 0.5 % BSA, 1 % CWFS, 10 μg/ml Donkey IgG (Abcam, Cambridge, United Kingdom)). Excess blocking buffer was removed and secondary antibody added, donkey-anti-rat biotin (2 μg/ml in PBS- 0.5 % BSA containing 10 μg/ml Donkey IgG) and incubated in the dark over night at room temperature. The next day sections were washed, endogenous biotin blocked using TSA (Biotin system, Perkin Elmer, NEL700), and stained with antibody, SA-HRP diluted 1/100 (TSA Biotin system, Perkin Elmer, NEL700). To amplify the signal sections were incubated in the amplification reagent, Biotinyl Tyramide (BT) (TSA Biotin system, Perking Elmer, NEL700). Stock was made up as 1/50 using 1x Amplification Diluent. The amplification reaction was stopped by washing followed by staining with the tertiary antibody, Streptavidin-Alexa 594 (Molecular Probes, 2 mg/ml), 2 μg/ml in PBS-0.5 % BSA containing 10 μg/ml Donkey IgG). For nuclear staining, sections were incubated with 1 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, California, United States) for 15 minutes (in the dark). Sections were mounted in Vectashield anti-fade mounting medium (H-1000; Vector Laboratories Inc. California, United States) and coversliped (Menzel-Glaser, Braunschweig, Germany). Coverslips were

sealed using nail polish and stored at 4 °C. To visualise the section, a fluorescence microscope (Olympus BX51) was used and fluorescence assessed using a triple band pass filter (DAPI+FITC+Texas Red). Images were taken using an Olympus DP70 camera and DPController (Olympus) software. The images were later processed with Adobe PhotoShop CS5.

Specific immunostaining procedure:

All washing steps were performed on an orbital mixer and all incubation/ blocking steps were performed in a humidified chamber.

- De-waxed the paraffin embedded sections.
a) Xylene 5 minutes x2
b) 100 % EtOH 5 minutes x2
c) 70 % EtOH 5 minutes x2
- Distilled H_2O for 5 minutes x1 and washed sections in PBS for 5 minutes x2.
- Antigen retrieval: Slides were heated in 10 mM citrate buffer pH 6.0 for 20 minutes. The slides were placed in the bottom of a black staining box in the buffer and the box was placed in the oven at 37 °C and the oven heated to 90 °C. Slides were removed when the oven reached 90 °C.
- Slides were cooled by removing the box from the oven and leaving it at room temperature for 20 minutes.
- Slides were washed twice in PBS for 5 minutes and the sections circled using a Pap pen between washes (making a hydrophobic ring).

- Sections were incubated in 50 mM glycine in PBS (pH 3.5) for 5 minutes.

- Sections were washed in PBS/0.3 % Triton-x for 15 minutes.

- Sections were washed twice in PBS for 5 minutes.

- Slides were treated with hydrogen peroxide in PBS (3 %) for 15 minutes in room temperature.

- Slides were washed in PBS/0.05 % tween-20 for 5 minutes x3.

- Slides were incubated in blocking buffer PBS 0.5 % BSA, 1 % CWFS, 10 μg/ml donkey IgG.

- The excess of blocking buffer was tipped off sections.

- Sections were incubated with secondary antibody donkey-anti-rat biotin (2 μg/ml PBS/0.5 % BSA containing 10 μg/ml donkey IgG) overnight.

- Sections were washed in tris buffered saline x3 for 5 minutes.

- Sections were blocked in TNB (TSA Biotin system, Perkin Elmer, NEL700) buffer for 30 minutes at room temperature.

- Sections were washed in tris buffered saline 5 minutes.

- Sections were incubated with SA-HRP diluted 1/100 (TSA Biotin system, Perkin Elmer, NEL700) in TNB buffer for 30 minutes at room temperature.

- Sections were washed in tris buffered saline x3 for 5 minutes.

- Sections were incubated in Biotinyl Tyramide (BT) (Amplification Reagent) (TSA Biotin system, Perking Elmer, NEL700) working solution for 6 minutes at room temperature. Stock made up 1/50 using 1x Amplification Diluent. The timer was started when BT was loaded on first section and noted the time when loading the last. Every section had to be incubated with BT for 6 minutes.

- Sections were washed in PBS/0.05 % tween-20 x3 for 5 minutes

- Sections were incubated in Streptavidin-Alexa 594 (Molecular Probes, 2 mg/ml) (2 µg/ml in PBS/0.5 % BSA containing 10 µg/ml donkey IgG) for 30 minutes at room temperature.

- Sections were washed in PBS/0.05 % tween-20 x3 for 5 minutes.

- Sections were counter stained with 0.25 µg/ml DAPI (in PBS) for 15 minutes (in the dark to prevent fading of fluorochrome).

- Sections were washed in PBS/0.05 % tween-20 x3 for 5 minutes.

- Sections were mounted in Vectashield with 22 x 60 coverslips and allowed to dry overnight covered in aluminium foil in 4 °C.

Specific haematoxylin and eosin staining procedure:

- Filtered approximately 250 ml Eosin, Haematoxylin and Scotts tapwater with vacuum filters.

- Eosin was acidified with 0.5 % Acetic acid.

- Sections were incubated in Haematoxylin for 2 minutes.

- Sections were washed by them dipping in H_2O.

- Sections were washed in Scotts tapwater for 10 seconds.

- Sections were washed by dipping in H_2O and touched against tissue paper to ensure minimal amounts of water on sections before incubation in Eosin

- Sections were incubated in Eosin for 4 minutes.

- Sections were dehydrated the by incubation in 3x 100 % EtOH for 5 minutes followed by 2x xylene for 5 minutes.

- Sections were mounted in Vectashield with 22 x 60 coverslips. Sections were dried overnight covered in aluminium foil in 4 °C

Chapter 4

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4.0.0 Results:

4.1.0 Optimising single cell placenta preparations

Placenta is a soft solid tissue. As such, it is necessary to generate a single cell preparation to be able to analyse the cells within it. The method was optimised using time titration of collagenase treatment, collagenase concentration titration and in the presence and absence of serum (Figure 9). No significant difference was evident in cell yields between the preparations obtained using the different conditions (Figure 9). As the single cell % did not significantly vary and the cell yield was not significantly different, the lowest incubation time of 10 minutes and the lowest collagenase concentration of 1 mg/ml were chosen to minimise any additional impact on the cells such as loss of cell surface receptors. It is also important to note that times of less than 10 minutes in collagenase failed to disaggregate the placenta to the point that it could be drawn through a 21-gauge needle.

A

C57 E14.5-18.5

18 G   21 G

16 G

Collagenase
PBS-2 % serum
10-90 min incubation

B

| E17.5, 90 min, -serum | E14.5, 30 min, +serum | E16.5, 20 min, +serum | E17.5, 10 min, +serum |

29 %   32 %   27 %   31 %

FSC-H

FSC-A

C **Single cell preparation of placenta with different collagenase incubation times and concentrations**

Time (min)	± serum	Concentration (mg/ml)	Gestation	% Single cells	Cells/Placenta
90	PBS -	3	E 17.5	28.7 ± 2	649999 ± 132287
30	PBS +	1	E 14.5	32.0 ± 0.4	625000 ± 47871
20	PBS +	1	E 16.5	28.6 ± 1.3	650000 ± 28868
10	PBS +	1	E 17.5	28.3 ± 0.9	511111 ± 11111

Figure 9: Optimising single cell preparation from placenta
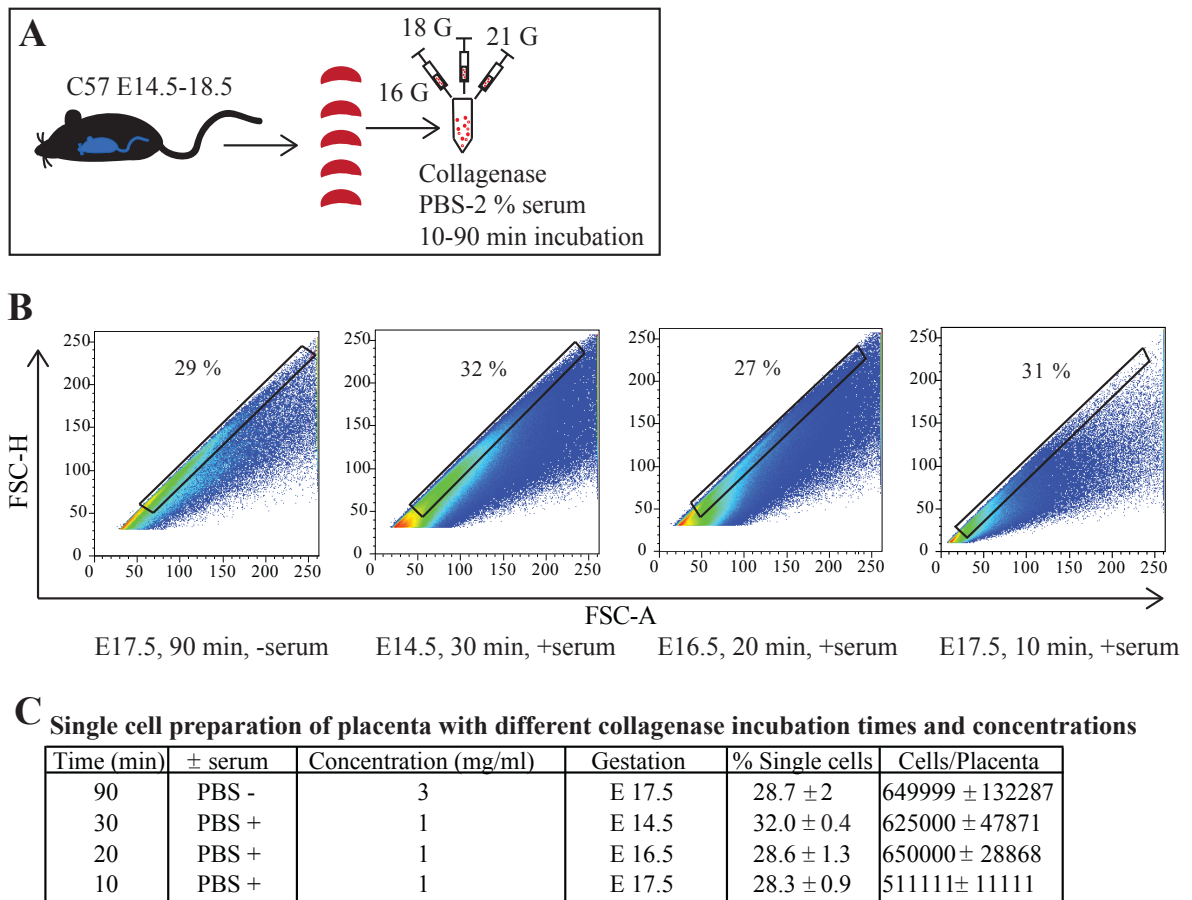
(A) Schematic of single cell preparation from placental labyrinths. **(B)** Representative flow cytrometric dot plots. **(C)** Calculation of nucleated cells per induvidual placenta * $p<0.05$ One way ANOVA on Ranks. 90 min; n=3, 30 min; n=4, 20 min; n=3, 10 min; n=3. Each biological repeat represent replicates of 2-5 placentas. Data is shown as mean $\pm$ SEM.

4.2.0 FA-endocytosis

4.2.1 Explants versus single cell preparations

It has previously been demonstrated that albumin does not cross the placental barrier after intravenous injection (Lambot, Lybaert et al. 2006) and therefore it was anticipated that following intravenous injection of FA into a mouse it would not be detected in the foetal portion of the placenta. This was confirmed using microscopic analysis of placental sections after FA injection (Figure 10). Both FA and anti VE-Cadherin were intravenously injected into the same pregnant recipient, but only VE-Cadherin was clearly evident in the placental tissue (Figure 10B). Whilst no FA specific staining was detected in sections of the placenta, opposed to the punctuated appearance that is normally evident when cells endocytose FA. In contrast FA was clearly evident in the BMSEC of the pregnant recipient (Figure 10A).
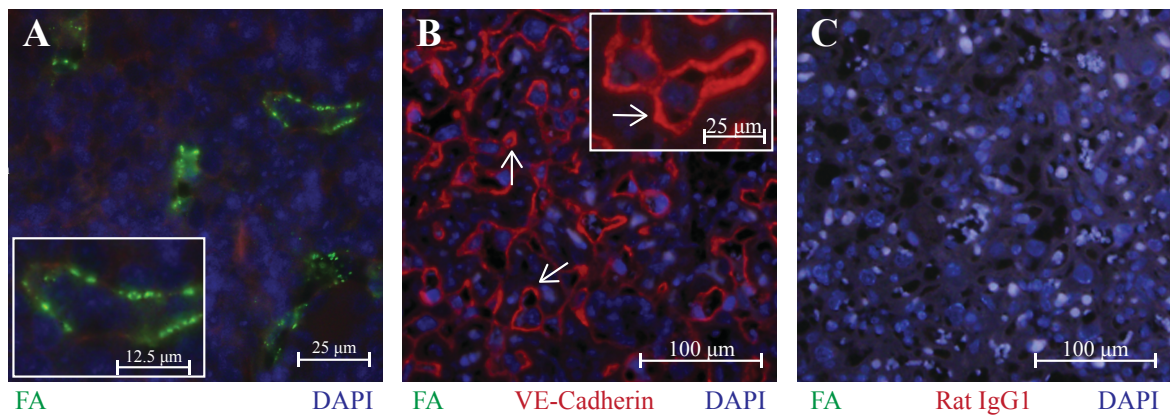
Figure 10: VE-Cadherin labelling and FA endocytosis in BM and placenta

(A) BM injected with FA and high resolution of sinusoid (insert) **(B)**

Placental explant injected with anti VE-Cadherin and high resolution of vessel (insert). **(C)** Placental explant injected with anti VE-Cadherin isotype. Arrows show VE-Cadherin[+] vessels in the labyrinth.

To investigate if placental endothelial cells had the capacity to endocytose FA, explants, pieces of placental tissue, were incubated in FA for 60 minutes. The incubation of whole tissue avoids the need for the initial collagenase treatment and hence the possibility of scavenging receptor internalised during this processing. The explants were initially dissected to remove the decidua, leaving the labyrinths (Chapter 3), which were incubated in 2.5 µg/ml FA in PBS-1 % BSA for 60 minutes at 37 °C. For comparison, a single cell preparation of equivalent labyrinth tissue post collagenase Type I digestion (Chapter 3) was incubated in FA under the same conditions (Figure 11). In addition, following FA incubation, the explants were processed into single cell suspensions as previously described (Chapter 3). Flow cytometric analysis revealed 30 077 ± 4477 FA$^+$ cells per placenta in single cell preparations incubated in FA and significantly fewer, 2646 ± 943, FA$^+$ cells from explants ($p < 0.01$) (Figure 11 C-G) suggesting that the incubation conditions are not optimal for endothelial cells to be exposed to sufficient FA to be able to identify them as SEC by their endocytic function.

The rapid endocytosis of FA by placental single cell suspensions were comparable to that evident in BM (Figure 13). This demonstrates that the disaggregation of placental cells with collagenase does not adversely affect scavenging receptors and the incubation of these cells provides sufficient opportunity for FA endocytosis.
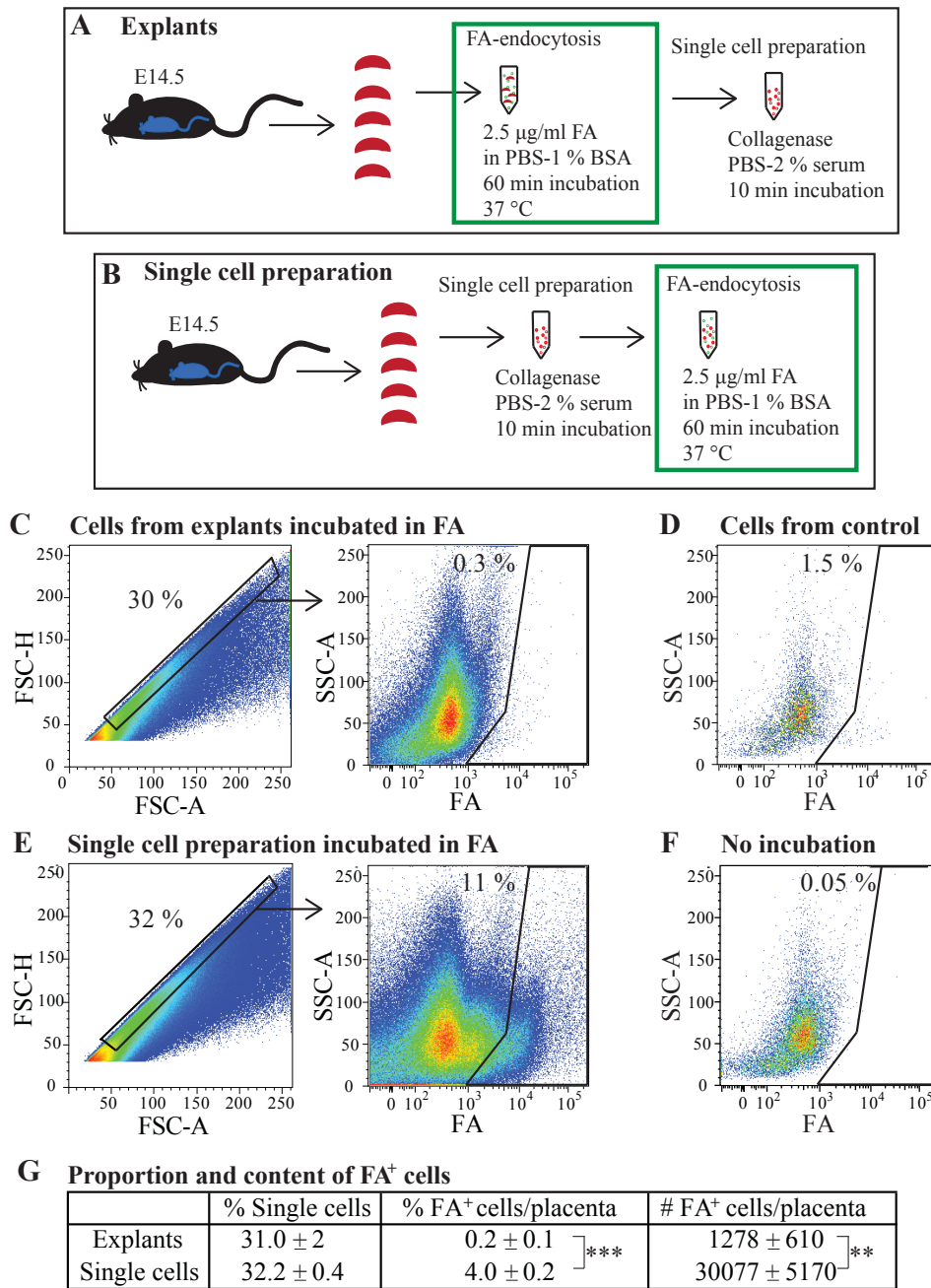
Figure 11: Analysis of PSEC FA endocytosis

(A) Schematic of FA endocytosis in placental explants and **(B)** single cell preparations. **(C)** Representative flow cytometric dot plots of single cells isolated from explants incubated in FA**. (D)** Representative dot plots of single cells isolated from explants incubated in PBS. **(E)** Representative dot plot of cell preparations incubated in FA. **(F)** Unincubated single cell preparation**. (G)** Proportion and content of FA+ cells in explants and single cell preparations. *** $p<0.001$ and ** $p<0.01$ by t-test. Explants n=3, single cell preparations n=4, with 2-5 placentas in each preparation. Data is shown in mean ± SEM.

4.2.2 Titration of FA incubation time

Placental endothelial cells incubated in FA as a single cell preparation clearly endocytosed FA (Figure 11). To optimise this process, a titration of FA incubation time was done. Placental labyrinth cells were incubated in 2.5 µg/ml FA in PBS-1 % BSA for 10, 20, 30 or 40 minutes at 37 °C (Figure 12). The resulting proportion of viable FA$^+$ cells was 2.3 %, 2.2 %, 2.0 % and 2.4 % respectively and the number of viable FA$^+$ cells per placenta was 11444, 10862, 15339 and 12230 respectively (Figure 12B-E). The difference in both of these parameters was not significant, so 10 minutes incubation time was chosen for FA endocytosis to minimise the period the disaggregated cells were incubated at 37 °C and hence reducing any possible negative impact on the cells. By adding PI, which labels dead cells, flow cytometric analysis revealed that 93±0.3 % of cells were viable (Figure 12B), again suggesting the FA incubation time chosen had minimal negative impact on the cells.
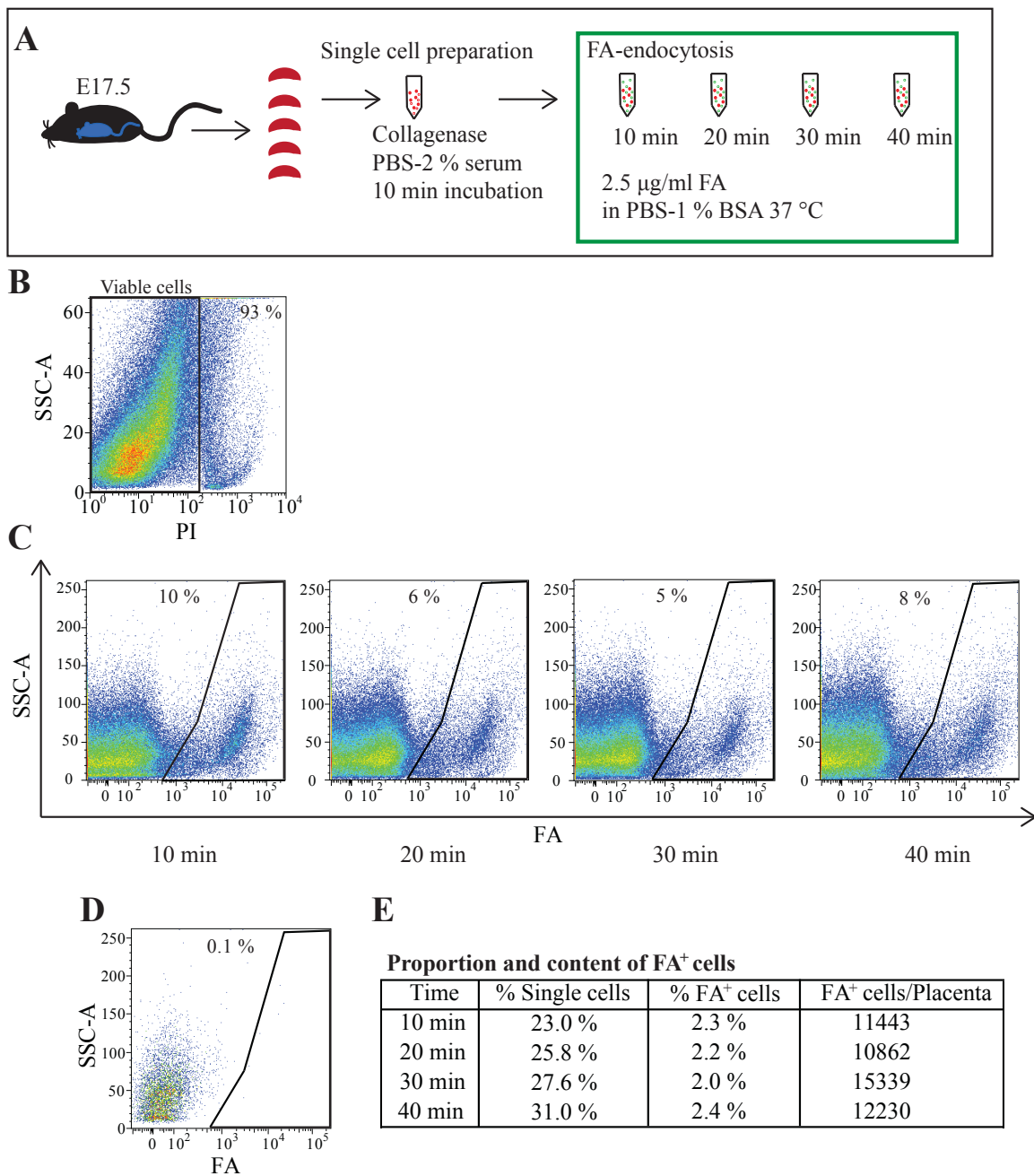
Figure 12: Titration of FITC-AGE incubation time

(A) Schematic illusrating FA endocytosis time titration. (B) Cell viability gated using propidium iodide (PI).

(C) Dot plots of individual viable single cell preparations. Unincubated placental single cell preparation

(D) was used to draw the FA gate. (E) Proportion and content of FA+ cells per placenta. n=1. Each biological

repeat represent replicates of 2-5 placentas pooled.

4.3.0 Identification of placental scavenging endothelial cells

PSEC were initially identified using their ability to endocytose FA, which has previously been used as a specific functional marker for BMSEC (Figure 10B), and then phenotypically characterised based on methods previously applied to BMSEC (Figure 13, submitted manuscript).

Figure 13: Isolation, identification and characterisation of BMSEC

(A) Scematic of BMSEC identification. **(B)** Representative flow cytrometric

 dot plots shows BMSEC sub-populations based on CD31, B220, GR1, MAC1 and ESAM expression.

Placental FA$^+$ cells were sub-fractionated based on their expression of cell surface markers. Using the endothelial marker CD31 an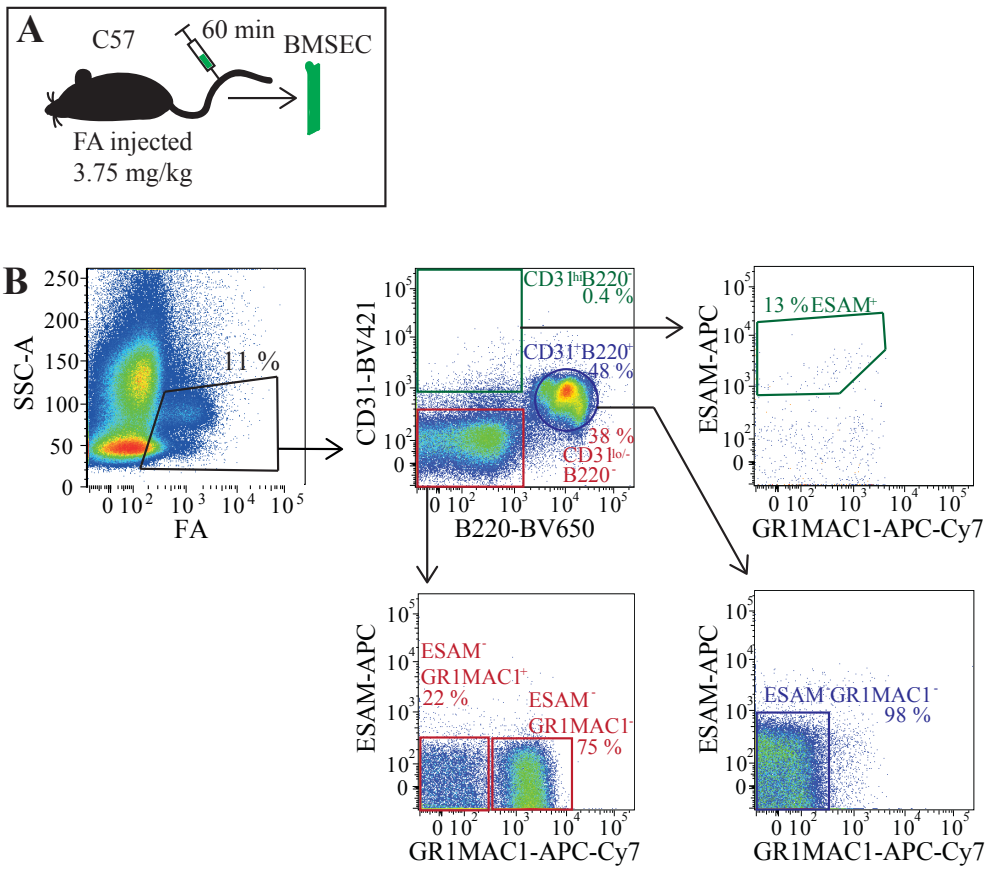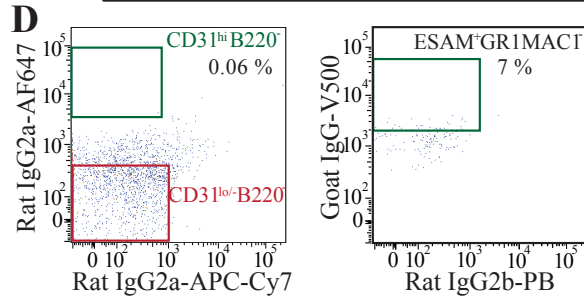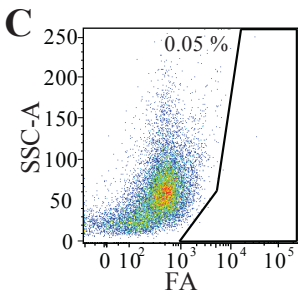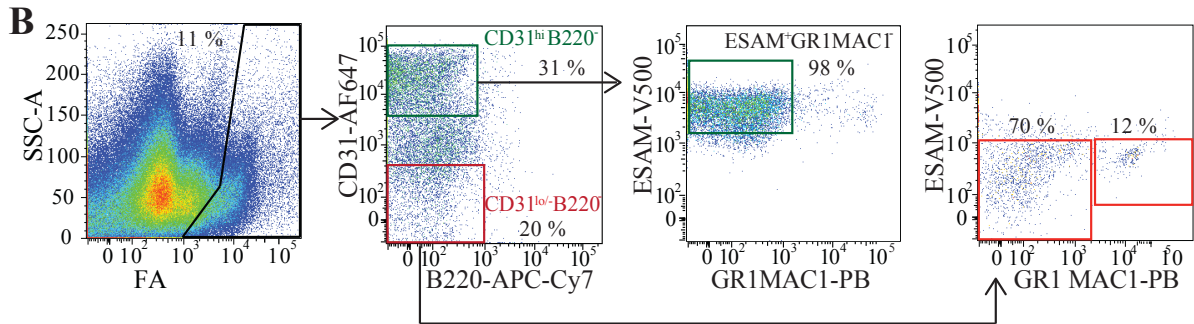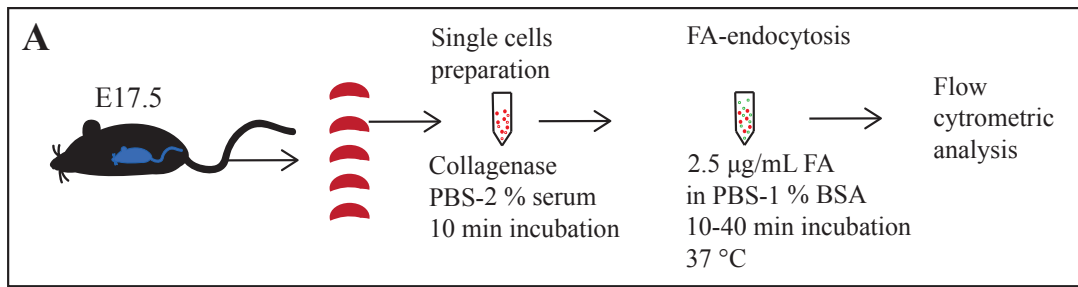d the pan-B cell marker B220, PSEC were sub-fractionated into two distinct FA$^+$ sub-populations: CD31^{hi}B220$^-$ and CD31$^{lo/-}$B220$^-$ (Figure 14A-F). Interestingly, in contrast to what is evident in the BM, a distinct population of CD31$^+$B220$^+$ cells was not revealed in the placenta (Figure 13B and 14B). Conversely, compared to BMSEC, PSEC have significantly more CD31^{hi}B220$^-$ cells (36 % ± 1.8 % of the FA$^+$ cells compared to ≤ 1 % in BMSEC, p<0.001) (Figure 14G). FA$^+$CD31^{hi}B220$^-$ cells isolated from BM have previously been shown to have revascularisation potential *in vivo* and therefore the phenotype and potential of this population in the placenta was investigated further.

To characterise the phenotype of PSEC (FA$^+$CD31^{hi}B220$^-$), Mac1, Gr1 and ESAM expression was assessed. Very few FA$^+$CD31^{hi}B220$^-$ cells expressed Gr1 and Mac1 (<2%, Figure 14B), whilst conversely, essentially all of this population expressed ESAM (92% ± 1.4 %) (Figure 14B). Again, this was in contrast to that evident in BMSEC, where Gr1 and Mac1 are known to subdivide the FA$^+$CD31^{hi} B220$^-$ cells and ESAM is only expressed on a small proportion of FA$^+$CD31^{hi}B220$^-$Gr1$^-$Mac1$^-$ cells (Figure 13B).

A population expressing Gr1 and Mac1 was detected in the FA$^+$CD31^{-lo}B220$^-$ sub-population, where only a small proportion of cells expressed ESAM (Figure 14B). This is very similar to what is evident in BM (Figure 13B). In addition, in the placenta the FA$^+$CD31^{hi}B220$^-$ sub-population was similar in size as the FA$^+$CD31^{-lo}B220$^-$ population (Figure 14F), which is also in contrast to that evident in BM (Figure 13B).

A E17.5 → Single cells preparation → FA-endocytosis → Flow cytometric analysis

Collagenase PBS-2 % serum 10 min incubation

2.5 μg/mL FA in PBS-1 % BSA 10-40 min incubation 37 °C

B

11 %

CD31^{hi}B220$^-$ 31 %

CD31$^{lo/-}$B220$^-$ 20 %

ESAM$^+$GR1MAC1$^-$ 98 %

70 % 12 %

C 0.05 %

D

CD31^{hi}B220$^-$ 0.06 %

CD31$^{lo/-}$B220$^-$

ESAM$^+$GR1MAC1$^-$ 7 %

E PSEC sub-populations per placenta

# Cells/placenta	649992±103829
# FA$^+$	16818±4169
# CD31$^+$ B220$^-$	5294±1112
# ESAM$^+$GR1MAC1$^-$	5171±1236
# CD31$^-$ B220$^-$	4376±915

F

G ****

H
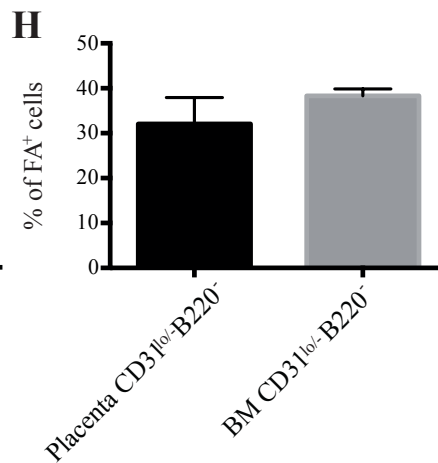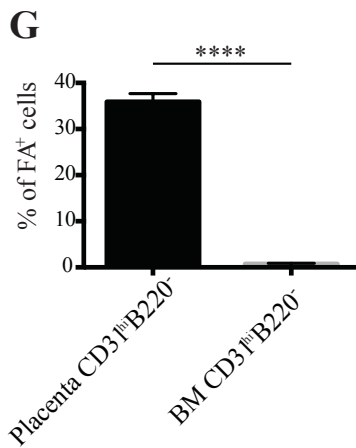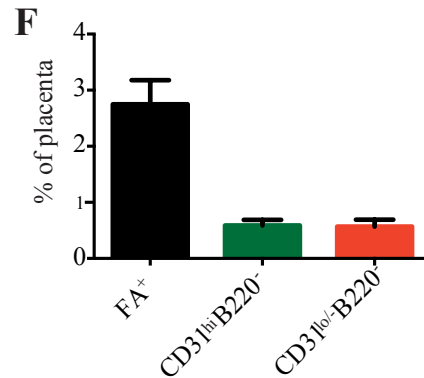
Figure 14: Identifiction and isolation of PSEC

(A) Schematic of PSEC identification. (B) Representative flow cytrometic dot plots of PSEC sub-populations. (C) Unstained placental single cell preparation to set the FA gate. (D) Isotypes for CD31 and B220 in first dot plot and isotypes for GR1, MAC1 and ESAM in second dot plot. (E) Number of cells within PSEC sub-populations per placenta. (F) Proportion of FA^+, $FA^+CD31^{hi}B220^-$ and $FA^+CD31^{lo/-}B220^-$ in each placenta. (G) Comparison of $CD31^{hi}B220^-$ cells in placenta and BM.****$p<0.0001$ by t-test. (H) Comparison of $CD31^{lo/-}B220^-$ cells in placenta and BM. Cells/placenta: n=7, FA^+: n=7, $CD31^{hi}B220^-$: n=7, $CD31^{lo/-}B220^-$: n=3, $ESAM^+GR1^-MAC1^-$: n=6. Each biological repeat represent replicates of 2-5 placentas pooled. Data is presented as mean±SEM.

However the placental and BM FA$^+$CD31^{-lo}B220$^-$ populations were equivalent (Figure 14H).

In agreement with other reports where BM sinusoidal endothelial cells are isolated on the presumption of a CD45$^-$ phenotype (Hooper, Butler et al. 2009; Butler, Nolan et al. 2010), no CD45 expression was detected on FA$^+$CD31^{hi}B220$^-$ (Figure 15A). However, almost all FA$^+$CD31^{hi} B220$^-$ ESAM$^+$ (91.8% ± 0.7%) expressed endomucin, a known embryonic endothelial marker (Figure 15B).

These findings demonstrate PSEC are heterogeneous, suggesting that the different populations may have different functions. However functions of any PSEC sub-populations are yet to be determined.
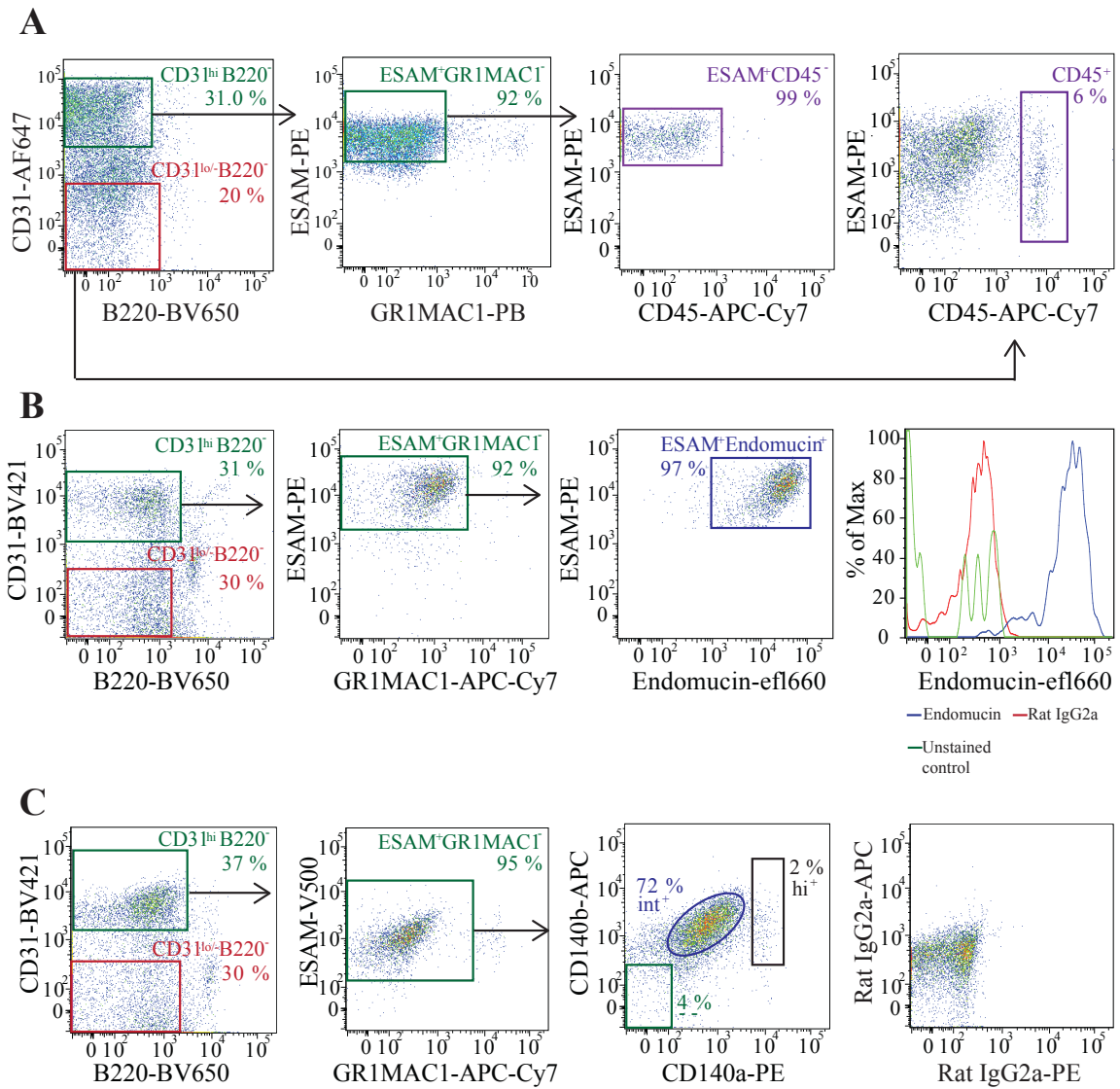
Figure 15: Characterisation of PSEC based on CD45, Endomucin, CD140a and CD140b expression
(A) Representative flow cytrometric dot plots of CD45 expression in PSEC. **(B)** Representative flow cytrometric dot plots of endomucin expression in PSEC and histogram of endomucin, isotype and unstained control. **(C)** Representative flow cytrometric dot plots of CD140a and CD140b exression and isotypes. int=CD140a^{+int}CD140b^{+int}, hi=CD140a^{+hi}CD140b^{+hi}, - -=CD140a$^-$ CD140b$^-$

4.4.0 PSEC transplantation analysis

Initially, 4000-20000 sorted $FA^+CD31^{hi}B220^-Gr1^-Mac1^-$ $ESAM^+CD45^-$ placental cells isolated from RFP donors were transplanted into C57 lethally irradiated mice to assess their transplant ability and engraftment (Figure 16A). However, none of the recipients survived the 4 week assay and therefore BMSEC in the transplant recipients was not assessed. Analysis of 4 recipients 14-17 days post-transplant revealed donor RFP^+ cells in 3 recipients, although these were at very low levels (Figure 16B-D).

Subsequently, a second transplant was initiated, mirroring the first except that half the recipients were transplanted with non-ablated total BM filler cells to ensure their survival as opposed to the normal lethally irradiated filler cells. 1000-10 000 sorted placental $FA^+CD31^{hi}B220^-Gr1^-Mac1^-$ $ESAM^+CD45^-$ cells were transplanted into each C57 lethally irradiated recipient and engraftment assessed after 4 weeks (Figure 16A). However, no recipients transplanted with irradiated filler cells survived and no RFP^+ cells were detected in any of the recipients transplanted with non-ablated filler cells (results not shown). In order to confirm that transplanted cells were homing to the BM as has been previously demonstrated with HSC (Nilsson, Johnston et al. 2001) and BMSEC (manuscript submitted), multiple homing assays were performed.

A C57 RFP E18.5 — FA incubated 40 min — RFP⁺FA⁺CD31ʰⁱB220⁻ GR1⁻MAC1⁻ESAM⁺CD45⁻ — Irradiated C57 — Fillers 200k — 4 wk TD — Analysis — BMSEC (FA⁺CD31ʰⁱB220⁻ GR1⁻MAC1⁻CD45⁻)
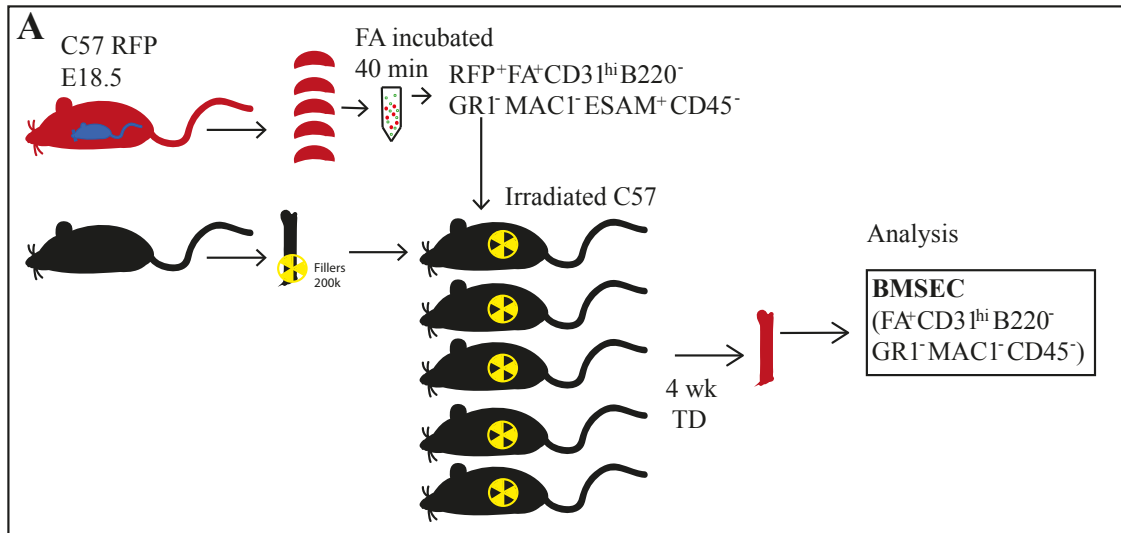
B Transplant analysis

	Cells transplanted	RFP⁺
Mouse # 1	20500	0
Mouse # 2	17000	764
Mouse # 4	4250	26
Mouse # 5	11000	5499
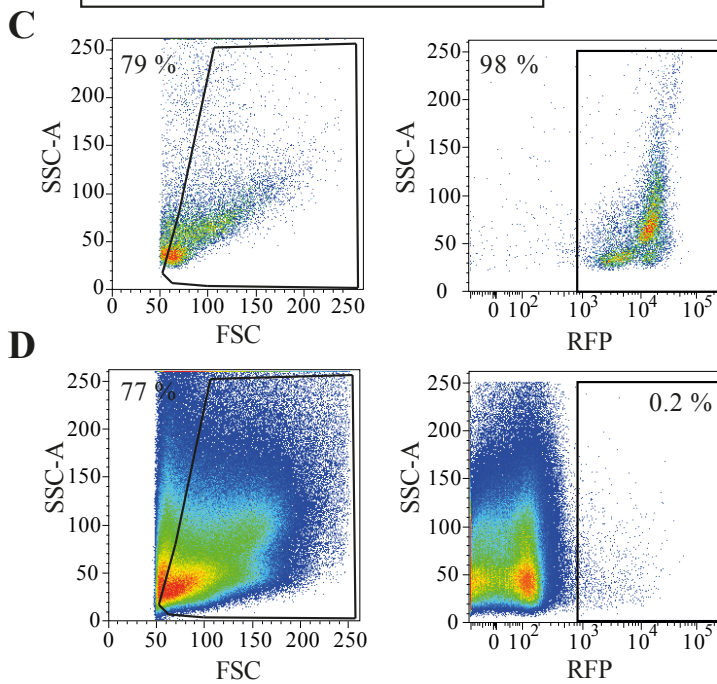
Figure 16: Transplant analysis

(A) Schematic of PSEC transplant **(B)** Transplant analysis. **(C)** Representative flow cytometric dot plots of negative control. **(D)** Representative dot plots of donor cells evident in recipient 4 weeks post transplant. TD=Take down.

4.5.0 Homing assay

Analysis of the potential of PSEC to home involved placental $FA^+CD31^{hi}B220^-Gr1^-$ $Mac1^-ESAM^+CD45^-$ cells being stained with CFSE prior to being transplanted into recipients together with non-ablated total BM filler cells (Figure 17A). After 15 hours, recipient BM was harvested and the presence of donor $CFSE^+$ cells assessed using flow cytometry. However, following the analysis of > 40 000 000 BM cells from each recipient no donor cells were detected (Figure 17B and C). This suggests that either the cells lacked the ability to home to adult BM, or the frequency of homed cells was too low to detect.

Therefore, an additional homing assay was performed using significantly higher numbers of transplanted cells (Figure 18A). Analysis of >70 000 000 marrow cells from each recipient revealed low numbers of homed cells (Figure 18B and C). However, the average proportion of cells homed (0.05%) is significantly lower than that previously demonstrated for HSC (Nilsson, Johnston et al. 2001). Within our laboratory it has been demonstrated that the ability for foetal HSC to home to adult BM is also significantly reduced (6-7 %, unpublished data) and BMSEC ability to home is 10 % (submitted manuscript). In addition, the ability of cells isolated from placenta to home in these assays has not previously been assessed.

Figure 17: Analysis of PSEC homing potential.

(A) Schematic of PSEC homing assay. TD = take down. **(B)** Representative flow cytometric dot plots of forward scatter, side scatter and CFSE in BM post homing. **(C)** Calculation of % donor cells homed to BM.

C

PSEC assessed in 5 recipients 15 hr post transplant
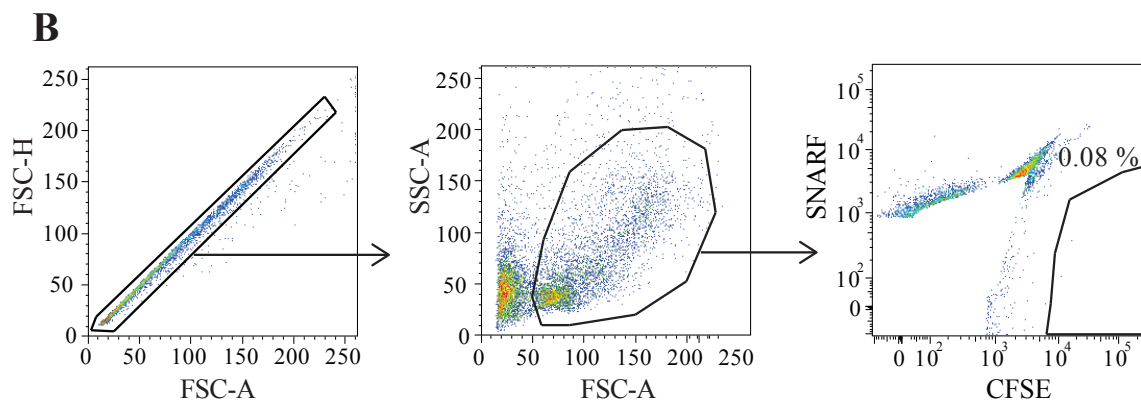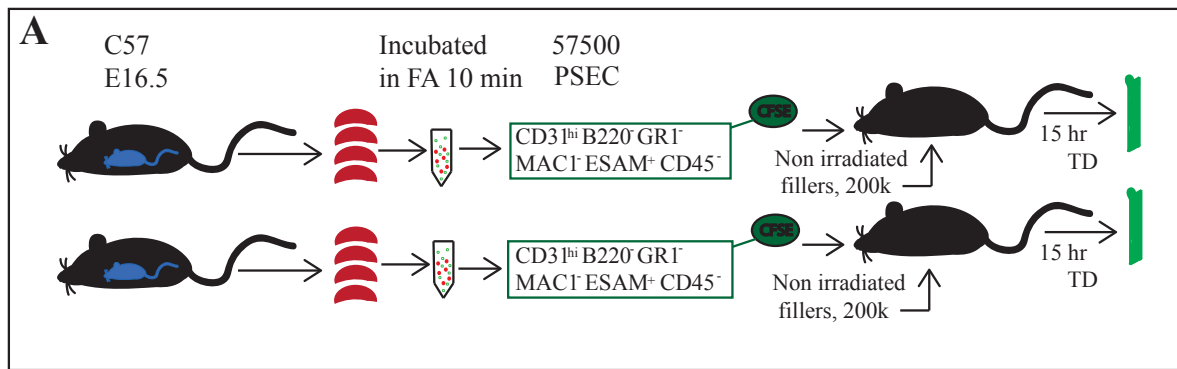
PSEC	Recipient 1	Recipient 2	Recipient 3	Recipient 4	Recipient 5
# cells transplanted	6300	10650	4500	3600	3300
Prosessed events	51433465	42625939	46507221	46496645	42986320
Nucleated cells	60.3 %	58.2 %	57.7 %	61.7 %	57.7 %
Positive events	0	0	0	0	0
Total # donor cells	0	0	0	0	0
% donor cells	0	0	0	0	0

Figure 18: Analysis of PSEC homing potential

(A) Schematic of PSEC isolation from placenta. TD = take down. **(B)** Representative flow cytometric dot plots of forward scatter, side scatter and CFSE in BM post transplant. **(C)** Calculation of the % PSEC homed to BM.

A potential explanation for the very low levels of homing and engraftment is a contamination of the transplanted SEC with trophoblast or other placental cells that also endocytose FA. Trophoblasts are known to express cell surface markers in common with endothelial cells, such as VE-Cadherin (Lambot, Lybaert et al. 2006) and the scavenging receptor SR-B1 (Alsat, Bouali et al. 1984; Malassine, Besse et al. 1987; Wadsack, Hammer et al. 2003) and therefore potentially endocytose FA. As a consequence, strategies were developed to distinguish and remove any residual trophoblasts.

4.6.0 Identification of Trophoblasts

4.6.1 Enrichment of PSEC using density separation

To identify and eliminate contaminating trophoblasts, different approaches were tested. Initially, a density gradient was used to determine if this could enhance the separation of trophoblasts from endothelial cells (Figure 19A). However, PSEC were not enriched using a density gradient. There was a significant loss in the recovery of all PSEC sub-populations (Figure 19B) and the proportion of $CD31^{hi}B220^{-}ESAM^{+}Gr1^{-}Mac1^{-}$ in the FA^{+} cells was not significantly higher following a density separation ($p>0.05$, Figure 19C).
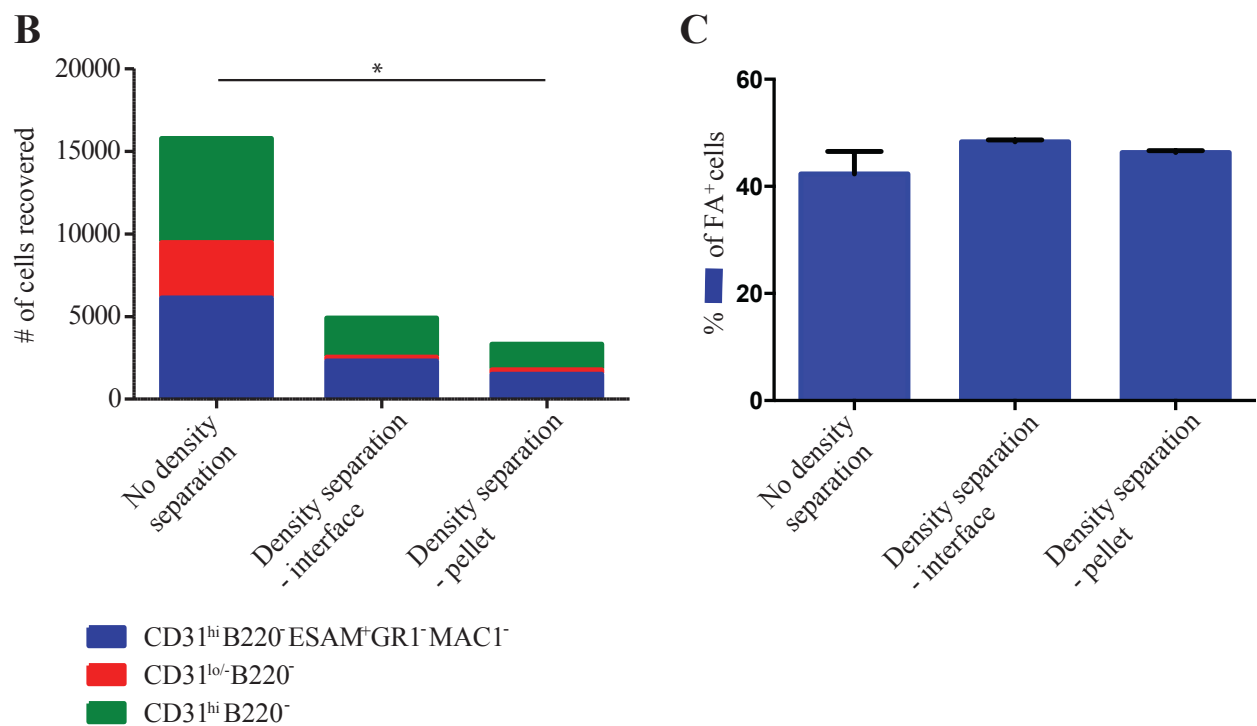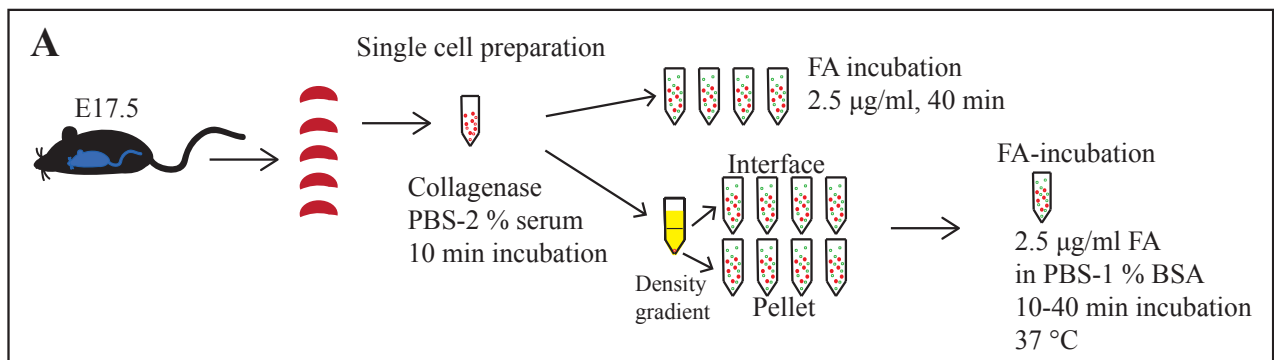
Figure 19: PSEC Enrichment using density separation.

(A) Schematic illustrating density separation. **(B)** Cell recovery post enrichment *p<0.05 following One Way ANOVA on Ranks. **(C)** Enrichment of $CD31^{hi}B220^{-}ESAM^{+}GR1^{-}MAC1^{-}$. Data is shown as mean±SEM, n=3.

4.6.2 Further subsetting of PSEC using CD140a and b

Previously, CD140a has been reported to be expressed by cytotrophoblasts (Holmgren, Claesson-Welsh et al. 1992) but its expression on endothelial cells has not been reported. We assessed the presence of CD140a on placenta cells in combination with the other PSEC markers and determined that FA^+ cells also expressed CD140a (Figure 15C). This suggests that either trophoblasts are also capable of endocytosing FA or PSEC express CD140a.

CD140b has also been reported to be expressed on trophoblasts. Flow cytometric analysis of placental $FA^+CD31^{hi}B220^-Gr1^-Mac1^-$ $ESAM^+CD45^-$ cells demonstrated these cells express both CD140a and CD140b, with the majority of the cells having intermediate expression of both markers and a small population with high expression of both markers, suggesting trophoblast contamination or that endothelial cells express both CD140 receptors (Figure 15C).

4.6.3 Further subsetting of PSEC using CD140a and Endomucin

Endomucin was found to be expressed on 92 ± 0.7 % of the $FA^+CD31^{hi}B220^-$ $ESAM^+Gr1^-Mac1^-$ population. In addition, approximately 15 % of this population do not express CD140a ($CD140a^-$), 60 % express intermediate levels ($CD140a^{+int}$) and 5 % high levels of CD140a ($CD140a^+$) (Figure 20A). The $Endomucin^+CD140a^-$ and $Endomucin^+CD140a^{+int}$ were transplanted to assess their homing ability (Figure 20B-D). One population was labelled with SNARF and one with CFSE (CFSE and SNARF were swapped between experiments to eliminate the possibility of the dye

having an impact). Between 2000 and 4650 FA$^+$CD31$^+$B220$^-$ESAM$^+$Gr1$^-$Mac1$^-$ Endomucin$^+$CD140a$^-$ donor cells were transplanted, but only one event was detected in one recipient 15 hours post-transplant (Figure 20D), suggesting either these cells do not home, or the level of homing is below that detectable in this homing assay. Conversely, when between 100 000 and 200 000 FA$^+$CD31$^+$B220$^-$ESAM$^+$Gr1$^-$Mac1$^-$ Endomucin$^+$CD140a^{+int} donor cells were transplanted. 0.16 % ± 0.08 % of cells homed to adult BM (Figure 20C and D). This provides evidence that this latter PSEC sub-population has homing potential, although it is very low.
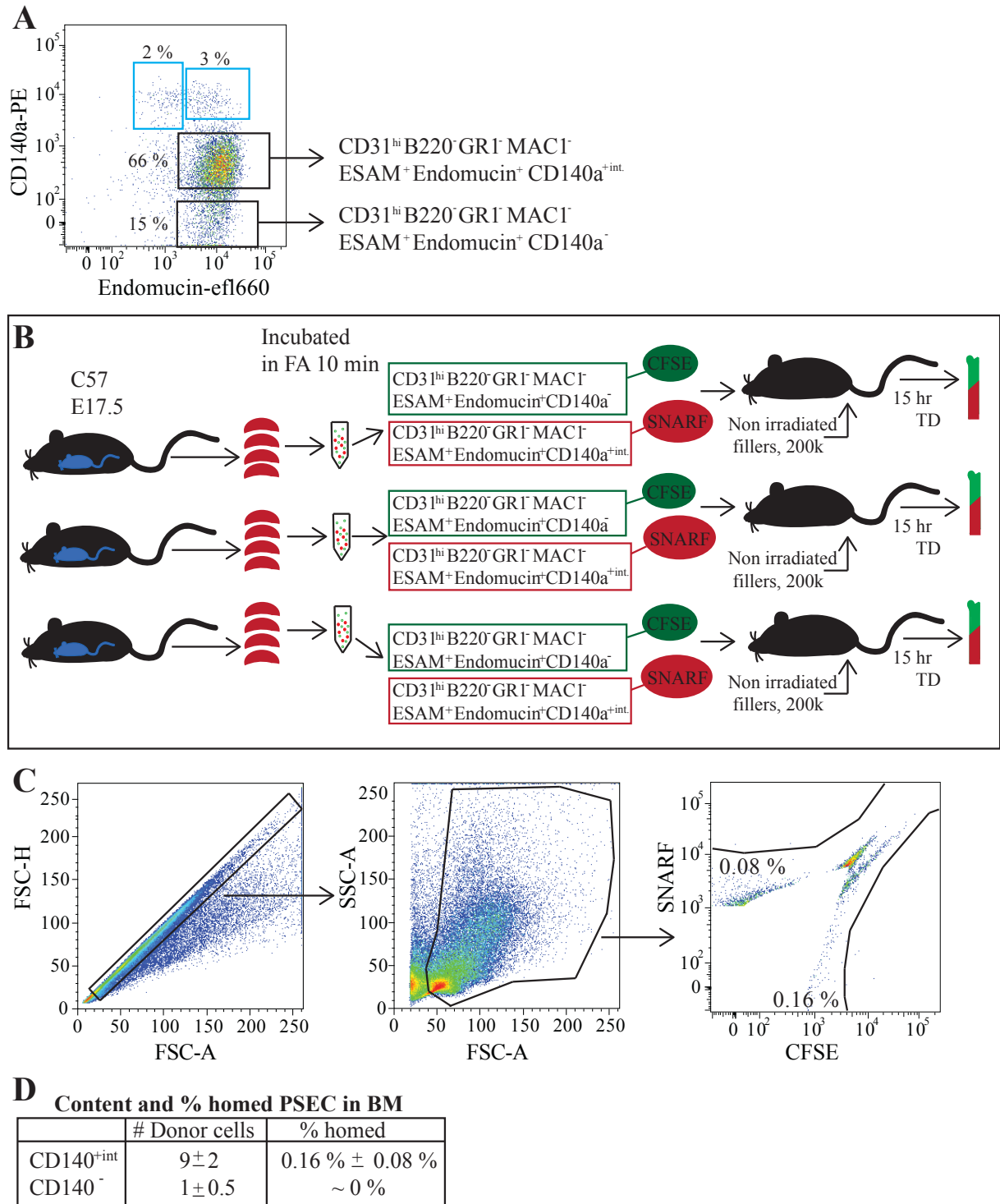
Figure 20: Analysis of homing potential of PSEC + endomucin$^+$ CD140a^{+int}/endomucin$^+$ CD140a$^-$.

(A) Representative flow cytrometric dot plot of four PSEC sub-populations based on CD140a and endomucin expression, where two of them were transplanted (B) Schematic of placental donor sub-populations in homing assay. (C) Representative flow cytometric dot plots of forward scatter, side scatter and CFSE and SNARF in BM post transplant. (D) Content and proportion of homed PSEC sub-populations to BM.

Chapter 5

5.0.0 Discussion

PSEC have been isolated, identified and characterised using immunohistochemistry and flow cytometry and their functional characteristics assessed using homing and transplant analysis. In order to isolate and identify cells using flow cytometry, viable single cell preparations needed to initially be prepared. Methods to prepare single cell placenta suspensions were optimised to achieve the best possible cell yield.

5.1.0 Preparation

Placenta is a highly vascular and complex tissue and it is challenging to isolate single homogenous cell populations. Previous studies have demonstrated that enzymatic processing both liver and BM using pronase and collagenase can result in the removal of cell surface proteins (Eskild, Kindberg et al. 1989; Haylock, Williams et al. 2007). Titrations for collagenase incubation were used to identify the lowest incubation time to minimise any additional impact on the cells such as loss of cell surface receptors. To prevent this, incubation time in collagenase Type I was optimised at 10 minutes, which is significantly less than other published procedures where the placenta was treated in collagenase Type I from 45 minutes to 1.5 hours (Ottersbach and Dzierzak 2005, Zuckermann and Head 1986, Gekas, Dieterlen-Lievre et al. 2005). It is also important to note that times of less than 10 minutes in collagenase failed to disaggregate the placenta to the point that it could be drawn through a 21-gauge needle. Other reports also use trypsin to dissociate the placenta, however using trypsin

has been reported to give lower cell recoveries (Gekas, Dieterlen-Lievre et al. 2005) and therefore it was not used in this study. In order to identify enothelial cells in the single cell placental preparations both phenotypical and functional characteristics of endothelial cells were used.

5.2.0 Endothelial identification

Placental endothelial cells were identified using surface markers previously shown to be associated with endothelial cells in other tissues, including VE-Cadherin, CD31 and ESAM. VE-Cadherin is often portrayed as a specific marker for endothelial cells, where it forms adherent junctions to adjacent cells through binding to itself (Lampugnani, Resnati et al. 1992). However, VE-Cadherin has also been described on other cell types, for example trophoblasts (Damsky and Fisher 1998) and therefore cannot be described as endothelial specific. CD31, a member of the Ig superfamily, contributes to endothelial cell-cell adhesion and is another important marker commonly used to identify endothelial cells (Watt, Gschmeissner et al. 1995; Ilan and Madri 2003; Feng, Nagy et al. 2004). However CD31 is also expressed on the surface of platelets, monocytes and neutrophils, where it regulates leucocyte passage across the endothelium (Newman, Berndt et al. 1990), as well as hemopoietic precursors and HSC (Watt, Williamson et al. 1993; van der Loo, Slieker et al. 1995). ESAM was originally described as an endothelial specific marker (Hirata, Ishida et al. 2001), however it is also expressed on haemopoietic cells including HSC (Ooi, Karsunky et al. 2009; Yokota, Oritani et al. 2009) megakaryocytes and activated platelets (Nasdala, Wolburg-Buchholz et al. 2002).

As a consequence of these markers not being specific for endothelial cells, PSEC were isolated using their ability to scavenge. This has previously been described as a reliable method for LSEC (Sorensen, McCourt et al. 2012) and BMSEC (submitted manuscript; (Qian, Johansson et al. 2009)), where a tagged soluble waste product, such as FA, was used as a ligand for SEC scavenger receptors such as stabilin 1 and stabilin 2 and (Miyazaki, Nakayama et al. 2002; Hansen, Longati et al. 2005). FcRIIb2 endocytic receptor is known to be expressed by placental endothelial cells (Lyden, Robinson et al. 2001) suggesting placenta endothelial cells will be similar to SEC in other tissues such as liver, with endocytosis being a primary function.

The current study using FA in placenta demonstrates that PSEC can also be isolated based on scavenging function and a combination of cell surface markers. However, besides endothelial cells, the placenta labyrinth contains a large proportion of trophoblasts (Enders 1965) and SR-B1 has been reported on human syncytiotrophoblasts and trophoblasts (Alsat, Bouali et al. 1984; Malassine, Besse et al. 1987; Wadsack, Hammer et al. 2003), suggesting that they also have an endocytic function. Studies performed in cell lines over-expressing SR-B1 have reported that this receptor may mediate the uptake of A (Miyazaki, Nakayama et al. 2002). However, besides LSEC, SR-B1 is expressed by macrophages and is also reported to participate in phagocytosis (Malerod, Juvet et al. 2002; Maderna and Godson 2003; Nakagawa, Shiratsuchi et al. 2005). As a consequence, possible contamination of trophoblasts when isolating endothelial cells from heterogeneous placenta preparations is an issue. In addition, as VE-Cadherin and CD31 are also both reported to be expressed by trophoblasts (Lambot, Lybaert et al. 2006), additional markers were used in the attempt of discriminate between PSEC and trophoblasts.

CD140a and CD140b are tyrosine kinase receptors that bind the PDGF protein (Yarden, Escobedo et al. 1986; Claesson-Welsh, Eriksson et al. 1989; Matsui, Heidaran et al. 1989) are expressed on cells of mesenchymal origin, such as fibroblasts, glia, epithelial and neuronal cells (Heldin 1988) and are both expressed on placental trophoblasts (Holmgren, Claesson-Welsh et al. 1992; Andrae, Gallini et al. 2008). In addition, there are no reports demonstrating CD140 expression on placental endothelial cells. However, capillary endothelial cells in rat brain express CD140-b and is suggested to have weak angiogenic activity (Smits, Hermansson et al. 1989). Based on the current findings, where both receptors were expressed on a high proportion of PSEC (70 %), either a large proportion of FA$^+$ trophoblasts contaminates the isolated cell preparation or placental endothelial cells also express CD140a and -b. As a consequence, the expression of endomucin was used in an attempt to positively select for PSEC.

Endomucin is a sialomucin expressed in aortic, umbilical vein and microvascular endothelial cells (Liu, Shao et al. 2001), as well as mouse embryo endothelium (Brachtendorf, Kuhn et al. 2001). In addition, there are no reports of endomucin expression on trophoblasts. In this study, essentially all PSEC (FA$^+$CD31^{hi}B220$^-$ ESAM$^+$) expressed endomucin (91.8% $\pm$ 0.7%) suggesting any contaminating trophoblasts also express endomucin. If trophoblasts and endothelial cells both endocytose FA as well as express VE-Cadherin, CD31, endomucin and CD140 receptors, other methods need to be identified to discriminate between these two populations.

In other studies trophoblast isolation has been done using a Percoll gradient centrifugation (Zuckermann and Head 1986), however various degrees of contamination by non-trophoblastic cells were observed (Zuckermann and Head 1986). In addition, in this study, density separation for PSEC enrichment was tested, but neither PSEC enrichment nor good cell recovery was observed. Several previous attempts to discriminate trophoblasts using other markers have been reported. These include an antibody to an apparent trophoblast specific antigen in murine placenta (MA_{21}) (Linnemeyer, Vernon et al. 1990). However, as the MA_{21} antibody is not commercially available it was not used in this study. In addition, caveolin-1 (CAV1), the first marker protein for caveolae (specialized plasma membrane microdomains) (Rothberg, Heuser et al. 1992), has been detected by immunohistochemistry on placental $CD31^+$ endothelial cells in both human and mouse (Mohanty, Anderson et al. 2010). However, CAV1 expression by trophoblasts remains controversial (Lyden, Anderson et al. 2002; Linton, Rodriguez-Linares et al. 2003) and as described above trophoblasts are known to express CD31 (Damsky and Fisher 1998; Lambot, Lybaert et al. 2006). In addition, the potential to use CAV1 to discriminate trophoblasts from endothelial cells is highly limited as CAV1 is an intracellular marker (Lambot, Lybaert et al. 2006) and therefore cannot be used to isolate viable populations for functional analysis.

Furthermore, in human placenta the intermediate filament cytokeratin-7 (CK7) has been reported to be highly expressed throughout the trophoblast lineage (Haigh, Chen et al. 1999; Blaschitz, Weiss et al. 2000) and CK7 is suggested to be used as a first choice intracellular marker for trophoblasts (Maldonado-Estrada, Menu et al. 2004). To date the ability of CK7 to discriminate trophoblasts and PSEC in mice has not

been tested, but as CK7 is also an intracellular marker, similarly to CAV1, its usefulness would be severely limited.

The endocytic receptor FcγRIIb2 is reported to be expressed on placental endothelial cells in human (Sedmak, Davis et al. 1991; Lyden, Robinson et al. 2001) and no reports identifying this receptor on trophoblasts have been published. Future investigations are required to determine if this receptor will be valuable in separating placental endothelial cells from contaminating trophoblasts.

There is also a possibility of macrophage contamination in placental sub-populations as they also express stabilin 1 (Palani, Maksimow et al. 2011). However, based on evidence of macrophages in the placenta expressing Mac1 (Chang, Pollard et al. 1993) they should be removed using Mac1 depletion. In addition, macrophages in mouse placenta also express F4/80 (Takahashi, Naito et al. 1991) and can be discriminated based on this marker. Not only macrophages but also trophoblasts are known to be phagocytic (Amarante-Paffaro, Queiroz et al. 2004). For future experiments this phagocytic function could possibly be used to distinguish between trophoblasts and endothelial cells.

5.3.0 PSEC function

To ultimately determine whether PSEC have the potential to re-vascularise BM it is crucial to assess their ability to home to the BM. Although the transplant potential of specific haemopoietic sub-populations of placental cells has been analysed (Gekas, Dieterlen-Lievre et al. 2005), the ability of cells isolated from placenta to home to BM has not previously been specifically assessed. In the current study, the homing

potential of PSEC was determined to be very low, with endomucin$^+$CD140a^{-int} PSEC having a homing efficiency of 0.16 %. This is significantly lower than that previously demonstrated for HSC (Nilsson, Johnston et al. 2001) where the homing ability was shown to be 30 %. However, based on findings in our laboratory, the ability of foetal HSC and BMSEC to home to adult BM are also significantly reduced compared to adult HSC (6-7 % for E 14.5 foetal liver HSC and 10 % for BMSEC), suggesting that the homing potential of PSEC could be expected to be extremely low.

To improve the ability to assess homing of PSEC, increased donor cell numbers could be used, the recipient marrow cellularity could be decreased by using irradiated recipients, which mirrors a clinical transplant, or the isolation a more defined PSEC population with increased homing potential. However, each of these options has challenges. Increasing the number of donor cells has practical issues that may question the feasibility of the use of this cell source in clinical applications. Transplanting into irradiated recipients, whilst mirroring the clinical setting, as ablation is required to remove diseased cells, removes the active components of homing, as the damaged vessels leads to cells "leaking" into the BM as opposed to them actively tethering and migrating through the endothelium (Paris, Fuks et al. 2001). Future studies focussing on further characterising PSEC subpopulations may provide a specific sub-population with increased homing potential.

The ultimate goal of this study is to determine the ability of PSEC to contribute to re-vascularisation after homing to the BM post-transplant. Currently, a transplant of PSEC in an irradiated model revealed very low levels of donor engraftment in comparison to that detected following a transplant of BMSEC (manuscript submitted).

A transplant of adult BM FA$^+$CD31^{hi}B220$^-$ cells revealed a 12 545 ± 1 288 fold expansion 4 weeks post-transplant (submitted manuscript) compared to PSEC where 0.2 % donor cells were detected 3 weeks post transplant. Based on the assumption that 0.16 % of PSEC have the ability to home and 5499 of 11000 transplanted donor cells were detected in one recipient 3 weeks post-transplant, this equates to a 300 fold expansion, suggesting that these cells engraft and potentially may have the ability to re-vascularise. Identifying a sub-population of PSEC with increased homing potential should lead to an increase in engraftment and the ability to assess re-vascularisation. Re-vascularisation is assessed by re-injecting recipients with FA 4 weeks post-transplant and determining the number and type of donor SEC using both immunohistochemistry and flow cytometry. Analysis of re-vascularisation was not possible in the current study, as transplanted recipients had to be euthanised prior to the 4 week assay end-point and were too ill for the re-injection of FA.

5.4.0 Translation into the human setting

To translate these findings into the human setting, PSEC need to be identified, isolated and characterised from human placenta. Mouse labyrinth and the human foetal part of the placenta are functionally analogous (Georgiades, Ferguson-Smith et al. 2002), they are morphologically distinct and the human foetal placenta may express different antigens to that evident in mouse placenta (Rossant and Cross 2001). However, the foetal blood circulation in human placenta is also lined by endothelial cells and it is possible that they have the same phenotypic characteristics to that evident in mouse placenta. As CD31, ESAM and VE-Cadherin are also expressed on endothelial cells in human tissue (Lampugnani, Resnati et al. 1992; Gurubhagavatula, Amrani et al. 1998; Hirata, Ishida et al. 2001), these may be useful to identify human

placental endothelial cells as well. As trophoblasts are also evident in human placenta (Rossant and Cross 2001) and they express CD140a and CD140b (Holmgren, Claesson-Welsh et al. 1992) the issues of trophoblast contaminations may be evident in human placenta as well. However, mouse PSEC were identified by negative selection for Gr1 and the pan-B marker B220, which although demonstrating various degrees of cross-reactivity with human tissue, are both mouse antigens. Consequently, other human markers need to be evaluated to specifically identify sub-populations of human PSEC. Furthermore, as described above, the FcγRIIb2 receptor is also reported to be expressed on human placental endothelial cells (Sedmak, Davis et al. 1991; Lyden, Robinson et al. 2001)and therefore may be useful in isolating and identifying PSEC in human placenta.

Chapter 6

6.0.0 Conclusion and future aspects

This project has resulted in the identification, isolation and characterisation of PSEC from the foetal part of mouse placenta. In addition, preliminary functional analysis of these cells demonstrates that PSEC sub-populations are able to home to and engraft in adult BM post-transplant. Moreover, PSEC shared similar characteristics to those identified on mouse BMSEC. Currently, the biggest challenge for isolating PSEC is the inability to readily discriminate these from trophoblasts, which have similar phenotypic characteristics.

For future experiments discrimination based on FcγRIIb2 expression, an endocytic receptor expressed on placental endothelial cells, needs to be investigated to determine if this receptor is also shared with trophoblasts. If purified, specific sub-populations of PSEC can successfully be isolated and then translated into the human setting, the potential use of human PSEC to improve BM transplants can be assessed.

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Appendix

Solutions

Single cell preparation and enrichment

Table 5: Phosphate-Buffered Saline (PBS)-310 mOsm

Milli-Q H2O	8 L
Glucose	2 g
KCL	2 g
KH_2PO_4	2 g
NaCl	80 g

Calibrate to pH 7.4, Osmotic pressure to 310 mOsm

Table 6: Red blood cell lysis buffer (10x stock)

Milli-Q H_2O	800 mL
1.5 M NH_4Cl	80.2 g
100 mM $NaHCO_3$	8.4 g
10 mM Disodium EDTA	3.7 g

Calibrate to pH 7.4 and add Milli-Q to achieve a final volume of 1 L

Table 7: Nycoprep (1.077 g/cm^3)

NycoPrep Universal stock solution*	300 mL
20 mM tricine-NaOH (pH 7.2)	300 mL
0.65 % NaCl	676.6 mL

Calibrate to pH 6.9, Osmotic pressure to 265mOsm

* *NycoPrep Universal stock solution (300ml Nycodenz: 60 % (w/v) in water, 1.310 g/cm³, 580 mOsm; Axis-Shield, Oslo, Norway)*

Immunohistochemestry

Table 8: PBS w/o (without CaCl and MgCl) -280 mOsm

Milli-Q H_2O	10 L
KCl	2 g
KH_2PO_4	2.4 g
Na_2HPO_4	11.5 g
NaCl	80 g

Calibrate to pH 7.4, osmotic pressure to 280 mOsm

Table 9: 10 mM citric acid (pH 6.0)

Citric acid, trisodium salt dehydrate (MW-294.1)	2.94 g

Calibrate to pH 6.0 and add Milli-Q water to achieve a final volume of 1 L

Table 10: 50 mM glycine in PBS (pH 3.5)

Glycine (MW-75.07)	3.75 g

Calibrate to pH 3.5 and add PBS w/o to achieve a final volume of 1 L

Table 11: PBS-0.3% Triton-X

Triton X-100	6 mL

Add PBS w/o to a final volume of 2 L

Table 12: PBS 0.05% tween-20

Tween-20	1 mL

Add PBS w/o to a final volume of 2 L

Table 13: 3 % hydrogen peroxide in PBS

30 % hydrogen peroxide	15 mL
PBS	135 mL

Table 14: Blocking buffer

10 % bovine serum albumin (BSA)	0.75 mL
100 % Cold Water Fish Skin (CWFS)	0.15 mL

Add PBS w/o to a final volume of 15 mL

Table 15: Tris buffered saline

1 M Tris-HCl	200 mL
Milli-Q H$_2$O	1800 mL
NaCl	17.5 g
Tween-20	1 mL