On the stimulatory effect of microglial cells on angiogenesis

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Cover picture: An aortic ring in the 3-dimensional culture system.

To Lars; my partner and great love in life

To my dear children, Irma, Embla and LarsLove

To all of you that have stood by my side during this journey

ABSTRACT

Angiogenesis, the process by which new vessels sprout from pre-existing vessels, is fundamental to development, tissue growth and repair. Many factors affect angiogenesis, acting either as inhibitors or activators of the process. The main aim of this thesis was to investigate the role of microglia on angiogenesis. To facilitate the study, we applied the *ex vivo/in vitro* aortic ring model. The model, originally established for rat, was adapted to mouse and developed to function without excess addition of growth factors in a 3-D environment that mimics the *in vivo* situation, while disconnecting angiogenic sprouting from potential systemic confounding factors. In paper I, we showed that ablation of microglia in the retina leads to a poorly developed vascular network. The aortic ring model supplied with microglia showed that microglia has a positive effect on angiogenic sprouting. This effect was mediated by a soluble factor(s), and cell-cell contacts were not required. We also showed that the microglia-derived angiogenic factor(s) is distinct from vascular endothelial growth factor-A. Moreover, the sprouting aortic ring induces oriented migration of microglia towards the aortic ring. In paper II, we analysed the microglia transcriptome. We found that microglia express known activators and inhibitors of angiogenesis that might have a role in retinal blood vessel development. The aortic ring system was also used as a complement to *in vivo* analyses to address the function of sphingosine-1-phosphate receptor 1 ($S1P_1$) on angiogenesis (paper III). The results indicated that $S1P_1$ is required in endothelial cells to control vascular sprouting and tip cell formation. $S1P_1$-deficient mice display endothelial hyper-sprouting, and $S1P_1$ counteracts VEGF-A-induced sprouting. In paper IV, mice that express Cre from the endothelial/hematopoietic Tie2 promoter were used to mark transplanted bone marrow cells. The study aimed to address if grafted bone marrow cells can differentiate into pancreatic β-cells. The major part of the thesis concerns the establishment and use of the mouse aortic ring as a model for angiogenesis. The results indicated that the system can be used to identify and test putative modifiers of angiogenesis. Importantly, application of the aortic ring system enabled us to identify a positive effect of microglia on angiogenesis, which we could further pursue by microarray analyses. The presented work might therefore provide a platform for the identification of molecules that regulate angiogenesis.

Key words: microglia, angiogenesis, aortic ring.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their roman numerals.

I. **Simin F. Rymo**, Holger Gerhardt, Fredrik Wolfhagen Sand, Richard Lang, Anne Uv, Christer Betsholtz.
    A Two-Way Communication between Microglial Cells and Angiogenic Sprouts Regulates Angiogenesis in Aortic Ring Cultures.
    PLoS One. 2011 Jan 10;6(1):e15846.

II. **Simin F. Rymo**, Zulfeghar A. Syed, Anne Uv and Christer Betsholtz.
    A transcriptional profiling approach to identify microglia-derived factors that stimulate angiogenesis in aortic ring cultures.
    Manuscript

III. Konstantin Gaengel, Kazuhiro Hagikura, Colin Niaudet, Lars Muhl, Staffan Nyström, **Simin F. Rymo,** Bàrbara Laviňa Siemsen, Jennifer Hofmann, Lwaki Ebarasi, Long Long Chen, Karin Strittmatter, Guillem Genove, Pernilla Roswall, Peter Lönneberg, Per Uhlen, Anne Uv, Arindam Majumdar, Richard L. Proia and Christer Betsholtz.
    $S1P_1$ is a critical regulator of angiogenesis.
    Manuscript

IV. Anders H. Rosengren, Jalal Taneera, **Simin Rymo**, and Erik Renström.
    Bone marrow transplantation stimulates pancreatic β-cell replication after tissue damage.
    Islets. 2009 Jul-Aug;1(1):10-8.

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ABBREVIATIONS

AJ	adherence juntions
Ang	angiopoietin
BM	basement membrane
bFGF	basic fibroblast growth factor
BMP4	bone morphogenic protein 4
BMT	bone marrow transplantation
CFS-1	growth factor colony stimulating factor 1
CNS	central nervous system
Dll4	delta like 4
ECM	extra cellular matrix
Flk1	fetal liver kinase 1
Flt	fms-like tyrosine kinase
HIF	hypoxia inducible factor
LADMAC	mouse bone marrow cell line producing CSF-1
MEF cells	mouse embryonic fibroblasts
Np	neuropilin
Pecam	platelet/endothelial cell adhesion molecule
PDGF	platelet-derived growth factor
PIGF	placental growth factor
S1P	sphingosine-1-phosphate
Shh	sonic hedgehog
SMC	smooth muscle cell
STZ	streptozotocin
SphK 1 and 2	sphingosine kinases 1 and 2
S1P1 to 5	sphingosine-1-phosphate receptors sphingosine-1-phosphate
TGF-α	transforming growth factor alfa
Tie	tyrosine kinase with immunoglobulin- and epiderma llike domains
Tj	tight junctions
Tsp-1	thrombospondin
VEGFR-1, -2, -3	vascular endothelial growth factor receptors-1 (Flt-1), -2 (KDR), -3
VEGF-A, -B, -C	vascular endothelial growth factors -A, -B, -C
VSMC	vascular smooth muscle cell

INTRODUCTION

The cardiovascular system is the first functional organ system of the vertebrate embryo (Coultas et al., 2005b; Flamme et al., 1997; Palis et al., 1995; Risau, 1995; Rossant and Howard, 2002; Strilic et al., 2010). When multicellular organisms grow beyond a few millimetres and pass the diffusion limit, they need to establish a vascular system to transport oxygen and liquids, remove waste products and provide the possibility for communication between organs (Risau, 1997; Schmidt and Carmeliet, 2010; Strilic et al., 2010).

Blood vessel formation during embryonic development occurs by two different mechanisms: vasculogenesis from endothelial precursor cells and angiogenesis from pre-existing vessels. The former mechanism implies *de novo* formation of blood vessels from angioblasts. The latter mechanism involves at least two ways of vessel growth, i.e. angiogenic sprouting (Adams and Alitalo, 2007; Jain and Chan, 2003; Jain, 2003; Risau and Flamme, 1995) and intussusceptive angiogenesis (Burri and Tarek, 1990; Hansen-Smith et al., 1996). Furthermore, angiogenesis is a complex multistep process comprising different stages that involves migration, specification and proliferation of endothelial cells together with maturation and stabilization of blood vessels. A number of growth factors and their receptors mediate the formation of these stages during embryonic development, including FGF/FGFR, VEGF-A/VEGFR-1 and -2, Angiopoetin1/Tie2, PDGFB/PDGFRβ and Dll4/Notch (Betsholtz, 1995; Betsholtz and Raines, 1997; Ferrara, 2002; Flamme et al., 1995; Flamme et al., 1997; Folkman, 1992; Folkman and Shing, 1992; Gerhardt et al., 2003; Leveen et al., 1994).

In the adult organism, normal angiogenesis only occurs in the ovarian cycle, during wound healing and in special cases of growing tissues, such as muscle and fat. Nevertheless, angiogenesis in adults contributes to pathogenesis, such as tumour growth, diabetic retinopathy, proliferative retinopathy, and arthritis (Folkman, 2007; Folkman and Shing, 1992). A number of angiogenic factors, including members of the VEGF and FGF gene families, stimulate both physiological and pathological angiogenesis. Inhibitors of angiogenesis, such as endostatin, thrombospondin-1 (Tsp-1), and tumstatin (Fernando et al., 2008; Folkman, 2007; Nyberg et al., 2005) participate in the regulation of angiogenesis. Under normal conditions, the

balance between stimulatory and inhibitory factors are strictly controlled and adapted to the needs of the organisms (Carmeliet and Jain, 2000). However, disturbances of the balance between these factors may lead to angiogenic complications of a variety of ischemic and proliferative disorders.

There are other mechanisms than soluble ligand/receptor-interactions that play crucial roles in the progress of angiogenesis. Cadherins and integrins are membrane-bound proteins with influence on angiogenesis through their cell-cell or cell-matrix contacts. Integrins are heterodimeric membrane-bound molecules that act as receptors on endothelial cells for extracellular matrix-components, like fibronectin, collagen, laminin and glycosaminoglycans. More than 20 integrins have been identified. *In vitro* studies have shown that $\alpha_v\beta_3$ integrin mediates cell migration and is, during wound-healing processes, transiently localized on the tip-cell of angiogenic sprouts (Papetti and Herman, 2002).

VE-cadherin is a single transmembrane glycoprotein on endothelial cells that localizes at the adherens junctions. Junction connections are formed by Ca^{+2}-dependent binding of a cadherin molecule on an endothelial cell to the same kind of cadherin molecule on another endothelial cell. The intracellular part of cadherins is attached to catenins, thereby mediating adherens junction-signalling. An essential function of blood vessels is to be a barrier between the blood and the environment and, at the same time, to allow inter- and intracellular diffusion and transport of molecules, particles and cells. Adherens junctions play important roles in this vascular permeability (Cavallaro and Dejana, 2011).

Structure and function of blood vessels

Blood vessels are tube-like structures, which are organized into arteries, veins, arterioles, venules and capillaries. Two different types of cells constitute the fundamental structure of blood vessels: endothelial cells and mural cells (Fig. 1) (Gerhardt and Betsholtz, 2003; Hellstrom et al., 1999). Endothelial cells line the inner wall of the tube and are connected through junctional complexes of three types: gap junctions, tight junctions (TJs) and adherens junctions (AJs). Gap junctions are specialized complexes that allow the passage of small molecules, water and ions from one cell to

another. The tight and adherens junctions are responsible for the cell-to-cell adhesion between the endothelial cells (Bazzoni and Dejana, 2004).
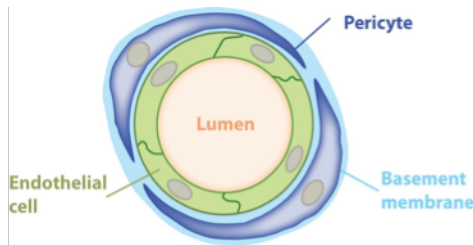
Fig 1. *Typical structure of a blood vessel.* Endothelial cells (green) encircle the lumen and are surrounded by a BM (pale blue). The pericytes (dark blue) are embedded in the BM.

Mural cells surround maturated blood vessels. They commonly entail vascular smooth muscle cells and pericytes (Gaengel et al., 2009; Gerhardt and Betsholtz, 2003). Vascular smooth muscle cells surround the larger vessels and are often organized into multilayers, whereas pericytes are associated with smaller vessels and are found either solitary or in a discontinuous monolayered manner. RGS5 is a pericyte marker (Cho et al., 2003), and actin is widely used as a marker of vSMCs. The morphology of pericytes and the degree to which they cover the endothelial tube vary between different tissues (Betsholtz et al., 2005; Bondjers et al., 2006; Gaengel et al., 2009; Gerhardt and Betsholtz, 2003; Lindahl et al., 1997; von Tell et al., 2006).

Blood vessels are embedded in a specialized extracellular matrix (ECM) called the basement membrane (BM). BM consists of different components that are highly structured and organized including collagen IV, fibronectin, laminin, heparansulfate and proteoglycans and, in minor amounts, collagen I, III and V. The BM provides attachment possibility for the cells and regulates cell survival. The organized structure of BM plays an important role in the selective permeability barrier in the retinal vasculature (Roy et al., 2010).

Recent observations have shown that the vascular system is not just a simple tubular network with a restricted task in metabolic exchange, but also serves to deliver signals to neighbouring cells, thereby playing a role in inductive signalling in development (Cleaver and Melton, 2003). Besides the reciprocal signalling between the endothelium and the mural cells within the vascular wall, endothelium-derived paracrine signals seem to play crucial roles in organogenic processes, such as the formation of the pancreas and the liver (Cleaver and Melton, 2003; Lammert et al., 2003).

Blood vessel formation

In vertebrates, vascular development occurs by a series of sequential genetic and morphological events that give rise to the formation and specification of blood and vasculature. The term vasculogenesis describes the *de novo* formation of vessels from vascular endothelial precursor cells (angioblasts) (Rogers et al., 1998). During vasculogenesis, angioblasts, in response to local signals such as growth factors (bFGF, VEGF) (Carmeliet et al., 1996; Ferrara et al., 1996; Shalaby et al., 1995) and the extracellular matrix, undergo specification, proliferation, migration, differentiation and finally coalescence to form the lining of nascent vessels (Lin et al., 2007; Risau and Flamme, 1995). Angiogenesis is the development of blood vessels from an already existing vascular bed (Folkman and Shing, 1992).

In mice, it is believed that blood cells and endothelial cells stem from common precursor cells that are called haemangioblasts. Haemangioblasts are mesodermal cells that are thought to arise in the posterior primitive streak in the early embryo. They represent a subpopulation of Brachyury positive cells, and are positive also for Flk-1 (VEGFR-2) (Eichmann et al., 2002; Lin et al., 2007; Vogeli et al., 2006). The haemangioblasts migrate into both extra-embryonic and intra-embryonic sites. Once they leave the primitive streak, they are committed to become either angioblasts or haematopoetic progenitors. Angioblasts generate vascular endothelial cells, whereas the pluripotent haematopoietic cells produce the various blood cells and lymphocytes. Evidence for the common origin of angioblasts and haematopoietic cells stems from studies of mice that lack *Flk-1*. In such mutant animals, there is a loss of both vascular and haematopoietic cells, suggesting that haemangioblast is a common progenitor for endothelial and haematopoietic cells (Ema and Rossant, 2003). Moreover, it has been shown that Flk-1-positive cells sorted from differentiating embryonic stem (ES) cells can give rise to single-cell-derived blast colonies (BL-CFCs) with ability to produce both endothelial and haematopoietic cells (Choi, 2002; Chung et al., 2002; Coultas et al., 2005b). The haemangioblasts that invade the yolk sac give rise to haematopoietic progenitors and angioblasts. The angioblasts appear to be specified as either venous or arterial already at this stage, but this specification might be dynamic during development. Clusters of angioblasts and haematopoietic cells aggregate to form blood islands, in which the haematopoietic cells become sheathed by endothelial cells. The blood islands then remodel to form the primary plexus (Fig. 2).
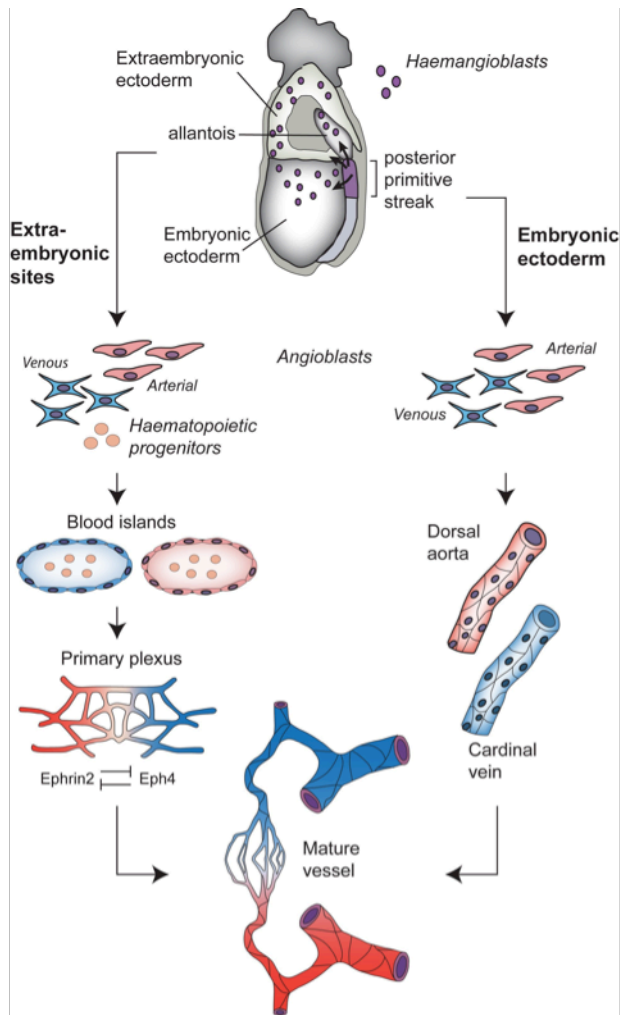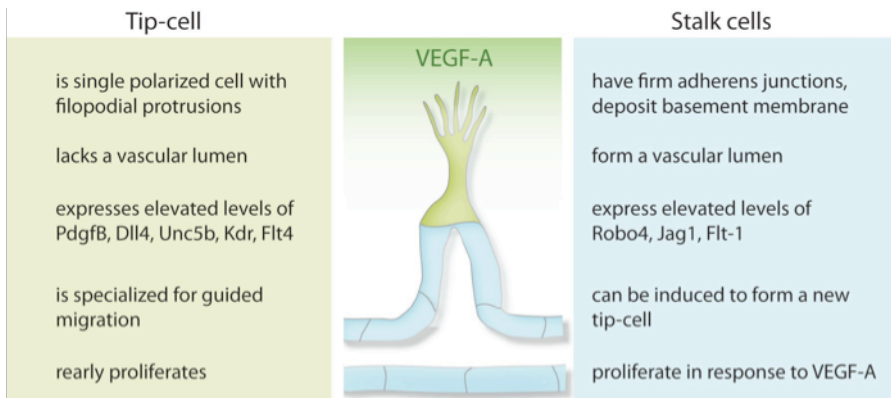
Fig 2. *Development of the circulatory system*. Haemangioblasts appear in the posterior primitive streak (top) and move to extra-embryonic sites (left) and intra-embryonic sites (right). Extra-embryonic vessel development: Angioblasts and haematopoietic cells form endothelial-lined blood islands in the yolk sac, which fuse to generate a primary capillary plexus. Intra-embryonic development: Angioblasts migrate and coalesce into cords with a lumen, and form the dorsal aorta and cardinal vein. The primary vessels remodel, together with the extra-embryonic plexus, to form a mature vasculature.

Modified from (Coultas et al., 2005b)

The haemangioblasts that enter the embryo are restricted to the angiogenic fate and migrate to different sites. Some of the angioblasts amass along the anterior/posterior body axis and form the dorsal aorta and cardinal vein. These primitive vessels are formed directly without intermediate steps of blood islands or primary plexus formation. The extra-embryonic plexus and the embryonic primary vessels then remodel and connect to form the mature circulatory system. Mural cells (pericytes and smooth muscle cells) are recruited to vessels by PDGF secreted by endothelial cells. Vascular development then proceeds mainly through angiogenesis.

Angiogenic sprouts consist of specialized endothelial cells

Angiogenesis, the formation of new vessels from pre-existing vessels, is a process that occurs during both embryogenesis and adult life. The process is essential to prevent or reverse tissue hypoxia (low oxygen) and, consequently, to reverse tissue ischemia. Several types of specialized endothelial cells are required to build a functional vessel branch during the sprouting process (Horowitz and Simons, 2008). The following model has been introduced for the functional specialization of endothelial cells during sprouting angiogenesis: each sprout is headed by an endothelial "tip cell", which is morphologically and functionally distinct from the endothelial "stalk cells" that follow behind the tip cell and extend the sprout stalk. A key feature of the endothelial tip cells is to be a pathfinder for the growing sprouts. The tip-cells are equipped with numerous filopodia at their leading edge, which participate in the navigation of the growing capillary by probing the environment to perceive the presence of attracting or rejecting signals (Huber et al., 2003). An attracting signal will induce F-actin polymerization and extend filopodia, whereas a rejecting signal will induce de-polymerization and generate retraction. Furthermore, filopodia adhere to form contact points and thereby connect the cell cytoskeleton to the ECM (Defilippi et al., 1999). In this way, the actin/myosin filaments can pull the cell towards these contact points and move the cell forward. The tip-cells do not form a lumen, and proliferate only sparsely (Gerhardt, 2008; Gerhardt and Betsholtz, 2003; Yana et al., 2007). Stalk cells, on the other hand, proliferate, form junctions, produce extracellular matrix, and form a lumen.
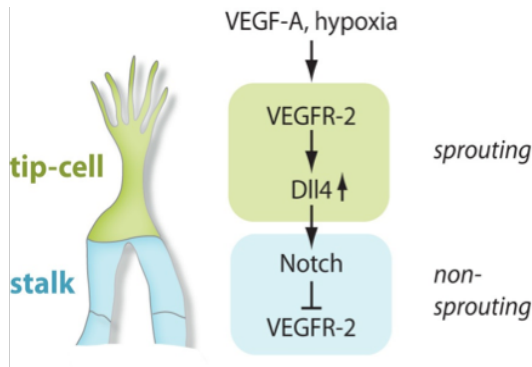
Tip-cell		Stalk cells
is single polarized cell with filopodial protrusions	VEGF-A	have firm adherens junctions, deposit basement membrane
lacks a vascular lumen		form a vascular lumen
expresses elevated levels of PdgfB, Dll4, Unc5b, Kdr, Flt4		express elevated levels of Robo4, Jag1, Flt-1
is specialized for guided migration		can be induced to form a new tip-cell
rearly proliferates		proliferate in response to VEGF-A

Fig 3. *Phenotypic and molecular differences between endothelial tip and stalk cells.* Tip cell (green), VEGF-A gradient (green), stalk cells (blue).

Modified from (Phng and Gerhardt, 2009).

Induction of tip cell formation by VEGFR2 and Dll4

Tip-cells have a specific phenotype in association with a characteristic expression of VEGF receptor-2 (VEGFR2), VEGFR3, platelet-derived growth factor (PDGF)-BB, Unc5B, Dll4, neuropilin-1 (NRP1) (Gerhardt et al., 2003; Suchting et al., 2007). The cells detect and react on gradients of angiogenic factors and translate their message into directional migration of the cell. Recent studies have demonstrated that a VEGF gradient is important in the selection and induction of the tip cell (Gerhardt et al., 2003). Binding of VEGF to VEGFR2 induces a signal that randomly appoints one endothelial cell to be the leader and become a tip cell. The neighboring endothelial cells are discouraged from doing so by a specific mechanism called *lateral inhibition* and consequently develop into stalk cells (Fig 4). Endothelial cells express various Notch receptors (Notch1, 3, 4) and ligands (Dll1, Dll4, Jagged1, Jagged2) (Roca and Adams, 2007). Notch is cleaved intra-cellularly to generate the NICD fragment that acts as a transcriptional regulator. Tip-cells are exposed to the highest levels of VEGF, which induces the expression of Dll4 in these cells (Roca and Adams, 2007). Dll4 binds to Notch on neighboring cells and down-regulates VEGFR2 signalling, and this leads to a reduced VEGF-induced

expression of Dll4 in these cells. In this way, the tip-cells strengthen their own position by establishing a self-reinforcing feedback loop that allows the leading cell to gain and retain its tip position, while preventing the neighboring cells from leaving their position in the stalk. Thus, Notch regulates endothelial cell specification by actively suppressing the tip-cell phenotype in stalk cells (Hellstrom et al., 2007).


Fig 4. *Notch-mediated regulation of endothelial cell specification.* The Dll4/Notch signalling between the tip-cell and stalk cells prevents the latter from becoming tip-cells.

Modified from (Adams and Alitalo, 2007).

VEGF/VEGF Receptors

Vascular endothelial growth factor (VEGF-A) and its receptor VEGFR2/Flk1 are certainly the most discussed and documented ligand/receptor pair involved in angiogenesis. VEGF-A, commonly called VEGF, is the most powerful pro-angiogenic factor known, and studies have shown that VEGF has a crucial role both in vasculogenesis and angiogenesis. VEGF is a member of the PDGF/VEGF growth factor family that includes placenta growth factor (PLGF), VEGF-B, VEGF-C and VEGF-D (Ferrara et al., 2003). There are two other structurally related proteins to VEGFs, VEGF-E that is found in parapoxviruses (Takahashi and Shibuya, 2005) and VEGF-F that is found in snake venoms (Suto et al., 2005). VEGF induces proliferation, sprouting and tube formation of endothelial cells (Carmeliet et al., 1996; Conway et al., 2001; Flamme et al., 1995; Gerhardt et al., 2003; Olsson et al., 2006; Ruhrberg et al., 2002). VEGFs can bind and signal via three different kinase tyrosine receptors,

VEGFR-1/Flt-1, VEGFR-2/KDR/Flk-1, and VEGFR-3. Receptor activation can also be assisted by co-receptors, such as heparan sulfate proteoglycans and neuropilins (Olsson et al., 2006). VEGF-A, -B, -F and PLGF bind to VEGFR1, VEGF-A and -E bind to VEGFR2, and VEGF-C and -D bind to VEGFR3 (Fig 5). Proteolytically cleaved products of the human VEGF-C and -D bind to VEGFR3 and also to VEGFR2, but with lower affinity (Olsson et al., 2006).
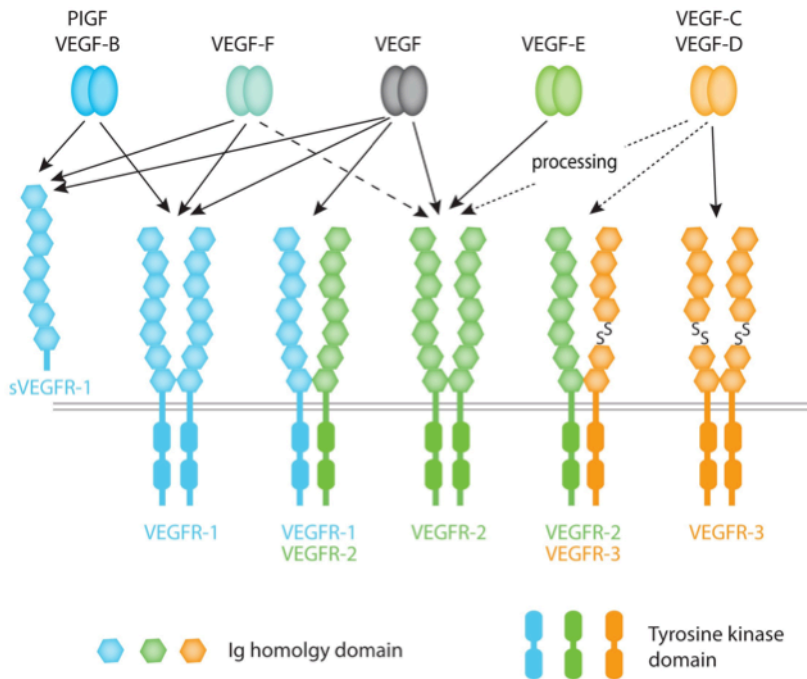
Fig. 5 *Interactions of VEGFs and their receptors (VEGFRs).* The VEGFs bind their receptors with different specificities (shown with arrows). Binding of dimeric VEGF ligands stimulates receptor dimerisation and leads to receptor auto-phosphorylation that activates downstream signalling events.

Modified from (Lohela et al., 2009)

VEGF-A in mice exists as three (most common) different isoforms that are the products of alternative splicing of the mRNA. The difference in these

isoforms lie in their differential inclusion of exons that encode charged domains that mediate binding to glycosaminoglycans and heparan sulphate components of the extra cellular matrix. The VEGF 188 isoform contains two heparan sulphate-binding sites and is sequestered on the cell surface or in the extra cellular matrix. VEGF 164 has only one heparan sulphate-binding site and has intermediate matrix-binding properties. Interestingly, mice that express only the VEGF 164 form normal vessels, indicating that this is the major form of VEGF that ensures proper vessel formation and patterning (Ruhrberg et al., 2002). The VEGF 120 isoform lacks the heparan sulphate-binding domain and is freely diffusible. Transgenic mice that express only the VEGF 188 isoform develop thin and highly branches vessels, whereas mice that only express VEGF 120 have been shown to develop a vascular phenotype with large vessels and few branches.

The hypoxia that builds up inside a growing tumour induces the formation of new blood vessels from the existing vasculature. This tumourogenic angiogenesis appears to occur in a process similar to that of normal angiogenesis. VEGF secreted from the tumour cells binds VEGFR2 and the co-receptor neuropilin-1 (NRP1) on the endothelial cell membrane of existing vessels and induces vascular leakage, endothelial cell proliferation and migration. A tumour-established VEGF gradient leads the migration of sprouting vessels towards the tumour. Tumour-produced VEGF might also act as an autocrine signal that contributes to tumour invasiveness by promoting the migration and survival of tumour cells themselves (Ferrara et al., 2003; Olsson et al., 2006). Strategies to prevent tumour-associated angiogenesis might therefore inhibit tumour growth. Notably, an anti-VEGF monoclonal antibody (Bevacisumab, Avastin) has been approved by the U.S. Food and Drug Administration for the treatment of metastasizing renal cell carcinomas, breast cancers, non-small cell lung cancers, or previously untreated metastatic colorectal cancers. The treatment with antibodies has been combined with chemotherapy.

Tie receptors and angiopoetin ligands

Four different angiopoetins (Ang1-4) have been identified. Angiopoetins are secreted growth factors, which bind to Tie receptors. There are two different Tie receptors, Tie-1 and Tie-2. All angiopoetins can bind to Tie-2. Angiopoetin 1 and 4 also bind to Tie-1, which was an orphan receptor for a long time (Gaengel et al., 2009; Saharinen et al., 2005). The activities of

Ang-3 and -4 are not well known, but they have been reported to have antagonistic (Ang-3) and agonistic (Ang-4) effects (Valenzuela et al., 1999) on angiogenesis. After the VEGF-VEGFR signalling system, angiopoetin and their tyrosine kinase receptors are the second endothelial-specific signalling system (Augustin et al., 2009; Dumont et al., 1992; Maisonpierre et al., 1993; Partanen et al., 1992; Sato et al., 1993; Thomas and Augustin, 2009). *In vivo* studies have shown that Ang-1 is a main agonistic ligand for the Tie-2 receptor (Klagsbrun and Moses, 1999; Red-Horse et al., 2007; Suri et al., 1996), while Ang-2 acts as an antagonist to Tie2 in most cases (Maisonpierre et al., 1997). The angiopoetin/Tie-signalling system is not required in the early stages of vascular formation, but is essential during the later events, such as vessel remodelling and smooth muscle cell and pericyte recruitment (Augustin et al., 2009; Ferrara, 2009). Tie-2 is expressed in the embryonic endothelium, in the endothelium of quiescent vasculature in the adult (Dumont et al., 1992; Gaengel et al., 2009; Wakui et al., 2006) and in bone marrow–derived monocytes/macrophages (De Palma et al., 2005). Tie-1 is expressed slightly later in the embryonic endothelium and its expression persists during adulthood (Gaengel et al., 2009). Mural cells express Ang-1, which binds to Tie-2 on endothelial cells and improve the interactions between the two cell types. Embryonic Tie-2 knockout mice have reduced number of perivascular cells and develop cardiovascular defect and die *in utero* between day E9.5-E10.5 (Sato et al., 1995). Tie-1 and Ang-1 are also essential for normal vascular development, whereas mice lacking Ang-2 may survive to adulthood.


Hypoxia

Tissue hypoxia is a condition with decreased physiological oxygen pressure in a tissue. Mammals have oxygen-sensing mechanisms that help them to handle the situation through adapting quickly to hypoxia. Such adaptation includes increased respiration, blood flow, cell survival responses and, in case of a persistent insufficient supply of oxygen, additional mechanisms to bring back oxygenation and help the body to adapt to hypoxia (Eltzschig and Carmeliet). Hypoxia regulates many transcription factors. One of the most important is the hypoxia-inducible factor (HIF). HIF is a heterodimeric transcription factor that consists of two subunits, HIF-1α or HIF-2α and HIF-1β (aryl hydrocarbon receptor nuclear translocator [ARNT] protein). HIF-1α is ubiquitous, whereas HIF-2α is restricted to

certain tissues (Eltzschig and Carmeliet). HIF-activation relies on the oxygen-sensing prolyl hydroxylases (PHDs). Under well-oxygenated conditions, PHDs hydroxylate conserved prolyl residues of the HIF-α subunits, thereby generating a binding site for the von Hippel-Lindau tumour suppressor protein, which targets HIF-α for proteosomal degradation. When oxygen levels decrease, the hydroxylase activity of PHDs is reduced, leading to HIF-1α accumulation. On binding to HIF-1β, the HIF complex translocates to the nucleus and activates transcription of several genes, including VEGF and Ang-2 (Mandriota and Pepper, 1998; Oh et al., 1999).

Retinal angiogenesis

The vertebrate retina is the light sensitive part of the eye and lines the inner surface of the eye. When light reaches the eye, it passes through the pupil and hits the cornea and lens, where it becomes focused and also inverted before being projected onto the retina at the back of the eye (Fig. 6).
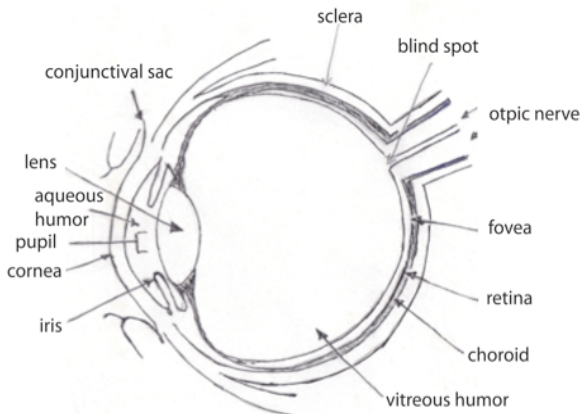
Fig 6. *A schematic cross section of the eye.* The eye is made up of three basic layers: the outer *corneoscleral layer,* the intermediate *uveal layer* (*uveal tract*) and the inner *retinal layer.*

The retina translates the visual information to a neural signal. The retinal tissue is organized as a multi-layer that consists of cells and cellular processes. Ganglion cells in the retina transmit the neural signal to the

brain, but the actual photoreceptors are the cones and rods. The axons of the ganglion cells form the optic nerve that delivers the information to the brain. Beside the ganglion and photoreceptor cells, there are different integration neurons (bipolar, horizontal and amacrine cells), Müller cells (support cells), astrocytes, microglia and endothelial cells in the retina. The outermost layer of the retina consists of pigmented epithelial cells that form a single layer resting on Bruch's membrane that separates retina from the choroid. The inner limiting membrane (ILM) represents the basement membrane of Müller cells that rest on the vitreous body. Just below the ILM run the axons of retinal ganglion cells (RGC) and the network of astrocytes. The first blood vessel plexus forms in the ILM.

Vascular growth in the retina

The brain is mainly vascularised by angiogenesis of leptomeningeal vessels (Herken et al., 1989; Kurz et al., 2001; Risau and Lemmon, 1988; Marin-Padilla, 1985). Likewise, the deeper networks of the retinal vasculature forms by sprouting angiogenesis (Fruttiger, 2007; Gariano et al., 1994; Gerhardt et al., 2003). There are publications claiming that the primary inner vascular plexus is formed by vasculogenesis from angioblasts that were found in the retina (Ashton, 1970; Chan-Ling et al., 2004; Chan-Ling et al., 1990; Flower et al., 1985; Hughes et al., 2000). However, the existence of such cells is still questionable. Angioblast differentiation is induced by endoderm, and vasculogenesis occurs in tissues containing endoderm. The retina, on the other hand, is a neuroectodermal extension of the diencephalons, the posterior division of the forebrain that connects the cerebral hemispheres with the mesencephalon (Morse and McCann, 1984), and is therefore related, from a developmental perspective, to the brain. Thus, the developing retina appears to lack endoderm needed for induction of vasculogenesis. Nevertheless, it cannot be excluded that endothelial precursor cells might exist in retinal vasculature and are involved in the formation of retinal vasculature (Otani et al., 2002). In general, the development of the retinal vasculature in mice and humans is very similar. The first sprouting vessels enter the retina at the optic nerve head. The vasculature spreads over the inner surface in a radially manner, from the centre of the retina to the periphery, to form a solid network (Fruttiger, 2002; Gariano et al., 1994). The arteries and veins of the retinal vasculature both enter and exit through the optic nerve (Fig 6). Astrocytes migrate into

the retina before the presence of a vasculature (Stone and Dreher, 1987; Watanabe and Raff, 1988). Astrocytes, like endothelial cells, emerge from the optic nerve head (Chan-Ling and Stone, 1991; Connolly et al., 1988; Dorrell et al., 2002; Fruttiger, 2002; Fruttiger, 2007; Gariano et al., 1996; Kopatz and Distler, 2000; Ling et al., 1989; Provis et al., 1997; Schnitzer, 1988; Stalmans et al., 2002; Stone and Dreher, 1987) and spread as a proliferating cell population in a radial manner from the middle point of the retina to the periphery of the tissue. Astrocytes form a network that functions as a scaffold for the growing vasculature. Astrocytes sense the hypoxia in the avascularized retina and express high levels of VEGF before they become covered by blood vessels (West et al., 2005). After one week, when the vascular network has reached the periphery of the retina and covers the whole retina, proliferation of astrocytes terminates and the expression of VEGF becomes down-regulated. After the vascular network has reached the periphery of the retina, the vessels start to sprout downwards into the inner plexiform layer, where they form a second vascular network parallel to the first one (Fruttiger, 2002; Fruttiger, 2007; Gerhardt et al., 2003; Stone et al., 1995). In mice, the primary vessel plexus at the inner retinal layer develops after birth, and the vessels at the growing edge of the vascular network are less mature than the more central vessels (Fruttiger, 2007). It is therefore possible to observe different stages of vascular differentiation in a spatial separation in a single retina (Fruttiger, 2002; Gerhardt, 2008). Thus, the retina is a good model for angiogenic studies.

Pathological vessel growth in the retina

Neovascularization in the adult retina is a major concern. For instance, neovascularization in diseases like diabetic retinopathy or age related macular degeneration leads to blindness. Thus, our understanding of the biological mechanisms that regulate blood vessel growth in the retina has important clinical implications.

Oxygen-induced retinopathy (OIR)

Oxygen-induced retinopathy is a pathological condition that is characterized by the growth of leaky vessels that form tuft-like structures towards the vitreous (Fruttiger, 2007). There is a mouse model for oxygen-

induced retinopathy. The principle for this model is that exposure of mouse pups to high oxygen pressure (hyperoxia) causes degeneration of retinal capillaries, and successive normalization of oxygen levels causes pathological reformation of the retinal vasculature. This mouse model is often used because it shows different stages of pathological neovascularization in the retina, i.e. vessel regression, phathological rebuilding of vessels and tuft formation.

Diabetic Retinopathy/Proliferative Retinopathy

More or less all patient with diabetes suffer from diabetic retinopathy that is a chronic microvascular complication and a side effect of their main illness. Diabetic retinopathy is characterized by gradual progressive changes in the retinal microvasculature, resulting in appearance of nonperfused areas in the retina and, consequently, pathologic intraocular proliferation (Calcutt et al., 2009; Frank, 2004; Gariano and Gardner, 2005). A sign of diabetic retinopathy is acellular capillaries associated with the appearance of microaneurysms that cluster around the areas of acellular capillaries. Animal studies have shown that pericytes disappear before leaving the area without blood flow (Hammes et al., 2004). The areas with non-functional capillaries will experience hypoxia, resulting in neovascularization to attempt to restore the oxygen level. This leads instead to proliferative diabetic retinopathy.

Microglial cells

Different types of cells are housed in the brain: cells that belong to the vascular structure, neurons and glial cells. There are two types of glial cells, macroglia (astroglia and oligodendroglia) that are derived from neuroectoderm and microglia. Microglial cells are of mesodermal origin and are found in the brain and the spinal cord. Microglial cells migrate to the central nervous system early during development before the onset of blood vessels, but it is now accepted that they can also be blood derived in the adult under certain pathological conditions. Retinal microglia comprise a heterogeneous population of cells that is believed to be of haemangioblastic mesodermal origin (Streit, 2001). It has been shown that the absence of microglia correlates with the formation of sparser network

formation in the developing retina (Checchin et al., 2006; Rymo et al.).

Methods

The Cre-lox system

Bacteriophage P1 uses the Cre-lox system to insert its own DNA into the genome of the host bacteria, so that it in this way becomes replicated. Cre recombinase is a phage enzyme that catalyzes the recombination between two loxP sequences. Cre can recombine its DNA with that of the host at specific loxP sequences (Hoess and Abremski, 1985). The Cre-lox system has been widely used in genetic manipulation in mice, in yeasts and also in plants, by using different promoters that control the expression of the Cre enzyme (Araki et al., 1997). The lox sequence consists of two 13 bp inverted sequences that flank the core sequence. The location and direction of the flanking loxP sequences decide the result of the recombination, which can be a chromosomal translocation (Fig 7a) an inversion (Fig 7b) or a deletion (Fig 7c).
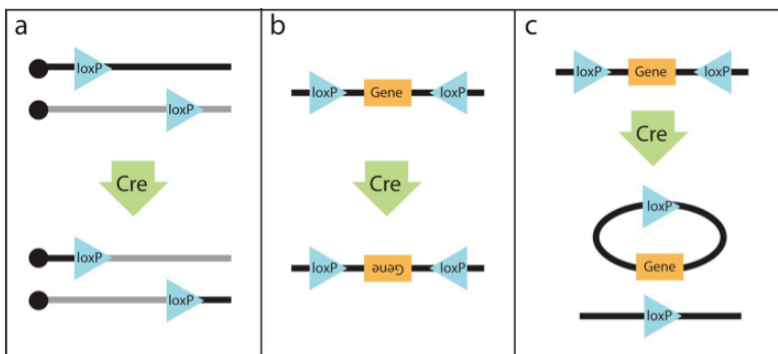
Fig 7. *Localisation and direction of the loxP sequence decide the outcome of the Cre-lox recombination.*

The *Cre* gene and the loxP sequences do not naturally exist in the mouse genome, but can be introduced by transgenic technology. The Cre and loxP-flanked strains are generated separately, and the mouse carrying the

Cre recombinase will be crossed with a mouse carrying the loxP construct to produce a Cre-lox strain. In order to generate a total or conditional knockout mouse with the Cre-lox system, Cre is placed under the control of a general or cell specific (conditional) promoter. In addition, modified Cre transgenes have been generated, which produce a dysfunctional Cre that needs an inducer, e.g. tamoxifen, to become active. This modification gives the opportunity to knockout genes at any wanted time point by administration of the inducer. Floxed strains are generated by flanking the gene of interest, or a critical part of it, by loxP sites. Well-used Cre reporter strains have loxP sites in combination with a marker, for example a visible fluorescent marker that gives the opportunity to trace Cre recombination status and also obtain information about the Cre recombination efficiency. Today, there are many different Cre-lox strains commercially available.

Mouse ex vivo aortic ring in 3-D culture system

More than a quarter century ago, Nicosia and Leighton cultured aortic rings from rat and reported the capacity of aortic rings in 3-D cultures to generate vessels *in vitro (Nicosia et al., 1982)*. Since then the model has been modified and used in many angiogenic studies, because it provides opportunity to investigate different steps of the angiogenic process from induction of endothelial sprouting to the recruitment of pericytes and regression of the vessels.

In our study the rat aortic ring culture system described by Nicosia (Nicosia and Ottinetti, 1990) is adapted to the mouse. This assay reproduces several aspects of angiogenesis *in vivo*, with the important difference that it disconnects the angiogenic sprouting process from potential systemic confounding factors such as blood flow, blood pressure and haemostatic regulation (Nicosia and Ottinetti, 1990) which is needed in some studies. Mouse aortic rings cultures mostly have collagen gels as the matrix and are supplemented with serum, but not with additional growth factors.

AIMS OF THIS STUDY

The main aim of the present thesis is to adapt and use the aortic ring system in the mouse to investigate regulatory factors of angiogenesis. Specifically, the system was used to study the role of microglial cells (papers I and II) and $S1P_1$ (paper III) on angiogenesis.

Another aim was to study the effect of endothelial-specific ablation of PDGFB, using Tie2-Cre mice. However, Tie2-Cre was found not to be endothelial-specific, and in paper IV it was used to mark transplanted bone marrow-derived cells to study their effect on insulin-producing β-cells.

A two-way communication between microglial cells and angiogenic sprouts regulates angiogenesis in aortic ring cultures (Paper I)

Background

The retina is an organ, in which too many or too few vessels are associated with pathology. Microglia are a type of glial cell found in the central nervous system, but they are not functionally connected via gap junctions. Microglia comprise a heterogeneous population of cells that share phenotypic characteristics and lineage properties with bone marrow-derived monocytes/macrophages, and are thus considered to be specialized local immuno-competent cells. However, recent work has unveiled a potential role for microglia also in retinal blood vessel formation. Retinal microglia populations are recruited at different developmental time points. The first population of microglial cells migrate to the central nervous system (CNS) and the mouse retina before the onset of blood vessel formation. Microglia can also invade the retina after it is vascularised, often in response to inflammation. The role of the microglia present in the early retina before vascularisation is obscure. During the first postnatal week of development, the microglia are distributed as singular cells in a regular density at the retinal surface, and ahead of the growing vascular plexus. These resident microglia are restricted to the *inner retinal region*, in close proximity to vascular sprouts and become intimately associated with the growing vasculature. When the resident microglia and systemic macrophages were depleted in neonatal rats, a pronounced decrease in retinal vascular area and density was observed. This phenotype was rescued by intraviteral injection of microglia, indicating an important role for microglia in normal retinal blood vessel formation (Checchin et al., 2006). In paper I we investigated this possible effect of microglia on angiogenesis.

Results and Discussion

Absence of microglia correlates with the formation of a sparser network of vessels in the developing retina

We used two different transgenic mice models to investigate whether microglial cells affect blood vessels formation and fusion during early postnatal retinal development. One was Macrophage colony stimulating factor/colony stimulating factor-1 (M-CSF/CSF-1) animals that lack CSF-1 protein and, as a consequence, lack or have fewer microglial cells in the retina (Kubota et al., 2009). The M-CSF/CSF-1 knockout mice developed a significantly sparser vessel network compared to control animals. The other model was PU.1 knockout mice that lack monocyte/macrophage cells, including the monocyte-derived retinal microglia (Fantin et al., 2010). PU.1 knockout mice also developed a sparser retinal vessel network than the controls (this paper).

Microglial cells associate with vessel sprout anastomoses during developmental angiogenesis in the mouse retina

Our experiments, using co-staining of microglia and endothelial cells, showed that microglial cells are commonly associated with endothelial tip-cells at sites where two tip-cells are contacting each other through filopodia. These are the sites of the future sprout anastomosis. In addition, our *in vivo* observations established a correlation between the presence of microglia and the formation of vessel anastomoses and secondary angiogenic sprouting in the developing mouse retina.

At this stage, we realized that it would be a great advantage to use an *in vitro* angiogenic model system that would allow us to perform analyses that were not doable in the *in vivo* system. Initially, we aimed to establish an *in vitro* retinal organ culture system, but due to technical difficulties we could not obtain analytical reproducibility. We were more successful in adapting the rat aortic ring culture system to the mouse. Combination of the aortic ring culture system with live microscopy studies turned out to be a good model to study angiogenesis. This model gave us the opportunity to investigate the influence of microglial cells on angiogenesis in a step-by-step manner, from the beginning of the sprouting process to fusion of the

branches, network formation and maturation of the vessels by recruitment of pericytes.

Microglia stimulate vessel sprouting and branching in vitro

To test the ability of microglial cells to induce vessel branching during angiogenesis, microglial cells were deposited locally into the 3-D gel that supports the aortic rings. In the presence of microglia, the aortic rings began to sprout earlier compared to cultures without added microglia. Microscopy on a daily basis demonstrated formation of a denser vessel network, and also longer branches, in cultures with microglial cells as compared to the controls. By counting the number of branches and fusions points and the length of the vessels each day for one week, we could show that there was a steady increase in branch number and branch length with time. Statistical analysis of the response peaks showed that vessel branch number peak was considerably higher in the presence than in the absence of microglia (p ≤0.0001). The difference in response in terms of peak branch length was not statistically significant. Together, our results suggest that microglial cells have a stimulatory effect on angiogenic sprout formation, branching and fusion in the *in vitro* mouse aortic ring model system.

Angiogenic effect of microglia in the aortic ring system is cell type-specific

We next asked whether the angiogenic effect of microglia in the aortic ring model was cell type-specific or could be mimicked by other mesenchymal cells. We therefore compared the effect of microglial cells on branching of aortic ring vessels with that of mouse embryonic fibroblasts (MEF). Statistical analysis showed that microglia induced a 5-10 fold increase in branch number. In contrast, the addition of MEF did not cause an increase in the number of branches. In fact, addition of MEF cells to the cultures caused a decreased branch number in comparison to aortic ring cultures with no cells added. The results support the notion that the stimulatory effect of microglia on vessel branching is specific for this cell type.

Direct cell-to-cell contact is not essential for the angiogenic stimulatory effect of microglial cells

In our aortic ring cultures, the applied microglial cells spread from their site of injection and finally infiltrated the endothelial network. An important question was therefore whether microglia stimulate vessel branching directly through physical contacts with the vascular network, or indirectly via soluble factors, or both. By mixing microglial cells with collagen prior to their injection into the gel of the aorta ring culture, we could immobilize the cells to a high degree and study their effects on aortic ring cultures before they made physical contact with the growing vessels. When comparing aortic rings cultured with or without such immobilized microglia, it was obvious that microglia induced sprouting long before the cells made physical contact with the growing vessel network. The analysis also showed that the angiogenic effect of microglial cells on vessel branching is dose-dependent. From these experiments we conclude that microglial cells release a soluble factor(s) that stimulates sprouting from the aortic rings.

Aortic ring explants induce secretion of stimulatory angiogenic factors from microglial cells and directed migration of the cells towards the explants

We consistently observed that microglia exhibited directed migration towards the aortic rings, even when the cells were retarded by suspension in collagen gel before injection. Parallel aorta ring cultures, in which MEFs replaced the microglia, showed a strikingly different pattern of cell migration. In contrast to the oriented migration exhibited by microglia, the MEFs spread radially from the site of injection. Moreover, when they came close to the aorta rings, they changed their direction of migration and turned away from the vessels. Random migration is a common feature of many cell types. Microglia, in the absence of an aortic ring, also show a random migration pattern. This supports the notion that the induction of directed migration of microglial cells towards the aortic ring explant is cell-type specific.

To address the question whether the aortic rings not only induced an oriented migration of microglial cells, but also influenced the release of

angiogenesis stimulatory factor(s) from the microglial cells, we compared the angiogenic effect of conditioned medium from separately cultured microglia with the angiogenic effect caused by the co-culture of microglia with aortic rings. In this setting, we also cultured aortic rings with only culture medium as control. We found large differences in the number of vessel sprouting in cultures with embedded microglial cells compared to cultures supplemented with conditioned medium collected from cultured microglial cells. Furthermore, there was a significant increase in vessel sprouting in aortic ring cultures that were supplemented with conditioned medium from cultured microglia cells compared to those that were cultured with control medium.

Taken together, the results suggest that the aortic ring induces oriented microglial migration. Moreover, microglia inherently secrete angiogenic factor(s), and this secretion appears to be stimulated by co-culture with aortic rings.

The microglia-derived angiogenic factor(s) is distinct from VEGF-A

The most pivotal regulator of blood vessel formation *in vivo* is VEGF-A, which is produced by many cell types, including myeloid cells. Therefore, VEGF-A became our first candidate to test as a mediator of the microglial cell effect on angiogenesis.

We added VEGF-A plus or minus a soluble chimeric VEGF receptor (VEGFR-1) that acts as an extracellular trap for VEGF-A, VEGF-B and PIGF, to aortic ring cultures. Addition of VEGF-A induced formation of vessels in a dose-dependent manner. VEGF-induced cultures showed a thick and solid vessel phenotype, which was different from the thin and branched vessels obtained in cultures in the presence of microglia. The effect of VEGF-A was inhibited by the simultaneous addition of the inhibitor (soluble VEGFR-1). The addition of the inhibitor alone to cultures with microglia had no effect on vessel phenotype. These results suggests that the angiogenic factor(s) released from microglial cells is distinct from VEGF-A, VEGF-B and PlGF. Our findings are consistent with previous reports showing that microglial cells in the developing mouse CNS do not express detectable levels of VEGF-A mRNA (Gerhardt et al., 2003).

The results from this study indicate that microglia have a direct positive effect on angiogenesis. Moreover, this effect appears to rely on soluble

factor(s) other than VEGF-A. The observation that microglia exhibit oriented migration towards the aortic ring further suggests that there is a two-way communication between microglia and the explant. The latter might contribute to the observed proximity of microglia and vascular sprouts *in vivo*.

Conclusions

- Microglial cells associate with vessels sprout anastomoses during developmental angiogenesis in the mouse retina.

- Absence of microglia correlates with the formation of a sparser network of vessels in the developing retina.

- Microglia stimulate vessel sprouting and branching in aortic cultures *in vitro*.

- The angiogenic effect of microglia does not depend on direct cell-to-cell contact.

- Aortic ring explants stimulate the angiogenic effect of microglia, and induces directed migration of microglia towards to explants**.**

- The microglia-derived angiogenic factor(s) is distinct from VEGF-A.

Identification of microglia-derived candidate regulatory factors with putative role in vessel formation by transcriptional profiling of microglia (Paper II)

Background

Microglia, a macrophage-related cell type in the central nervous system (CNS), is known to stimulate developmental and pathological angiogenesis. The nature of the angiogenic effect of microglia is unclear and appears different from vascular endothelial growth factor (VEGF-A). Addition of microglial cells to the aortic ring system is sufficient to stimulate vessel sprouting and has indicated that the angiogenic effect depends on secreted components. Moreover, the sprouting aortic ring induces oriented migration of microglia towards the explant. In paper II, we analysed the microglia transcriptome to identify potential secreted components that might mediate their pro-angiogenic effect.

Results and Discussion

Microarray analyses

In order to further elucidate the pro-angiogenic effect of microglia, the transcriptomes of microglial cells and MEF were compared. Messenger RNA from each cell line (microglia versus MEF) was subjected to transcription profiling using Affymetrix 1.0 microarrays. The array hybridization and initial processing of the data were performed in collaboration with the core facility for Bioinformatics and Expression analysis at the Department of biosciences and nutrition at Novum, Karolinska Institute, Stockholm, Sweden. Statistical processing of the data sets to detect differentially expressed genes, revealed 2578 genes that were up-regulated in microglial cells compared to MEFs (fold-change ≥ 2). Conversely, 2222 genes were found to be down-regulated (fold-change ≥ 2).

Microglia express genes associated with developmental process

The functional properties of the various up- and down-regulated gene products in microglial cells according to their representation in biological pathways were categorized using the Ingenuity Systems Pathway (IPA) database. The top functions in <u>Physiology and System Development</u> found for genes that were up-regulated in microglia included *tissue and organ development*. Down-regulated genes, on the other hand, contributed mainly to *hematological system development*. These differences in gene expression became even more evident by analyzing the top functions according to <u>Diseases and Disorders</u>. Genes up-regulated in microglia are associated mainly with *cancer, genetic disorders* and *developmental disorders*, while the down-regulated genes primarily relate to *immunological diseases*.

Microglia express potential angiogenic factors

Since microglia can stimulate angiogenesis in the absence of direct contact with the sprouting vessels, the pro-angiogenic effect of microglia is expected to rely on a secreted extracellular signal protein(s). Among the up-regulated genes in microglia, 220 were predicted to encode secreted proteins. Gene ontology clustering showed that the secreted proteins also exhibited high enrichment scores for *developmental processes* and *biological adhesion*, and that the most highly enriched molecular function was *binding*. Twelve of the extracellular genes were annotated as being involved in *angiogenesis*. They showed a fold-change ranging from 2 to 70. VEGF-A was included in the list, but with a moderate 5-fold up-regulation. Thus, microglial cells express a number of plausible candidate factors that might underlie the stimulatory effect of microglia on angiogenesis.

Stimulation of microglia by aortic ring explant

We previously noticed that microglial cells stimulate vessel sprouting in the aortic ring culture system to a greater extent than microglia-conditioned medium (Rymo et al., 2011). This might reflect a specific up-regulation of expression and release of pro-angiogenic factors from the microglia in response to the aortic ring. To address this question, two sets of aortic ring

cultures were set up, one with and one without an aortic ring explant, and microglial cells were added to all cultures. After incubation for 5 days, the microglia-containing part of the gel was excised and RNA was extracted. The transcription profile of microglia co-cultured with aortic rings compared to microglia grown in the absence of aortic rings revealed 33 genes with a fold-change ≥1.3. Gene ontology clustering according to biological processes revealed highest enrichment score for *response to stimulus*, while clustering according to *cellular component* produced the highest score for *extracellular gene products*.

Cluster analysis according to *molecular function* revealed high scores for *binding* and *chemorepellant activity*. The Ingenuity System Pathway (IPA) indicated that the network with the highest ranking was *infection mechanisms*, *infection disease*, *inflammatory disease*. Sixteen of the 33 up-regulated genes were, according to IPA, grouped under *inflammatory response*. The transcriptome analysis provided evidence that microglial cells become activated when cultured together with the aortic ring.

Microglia have a high functional plasticity. For example, while the activity of microglia in immunological disease stages is in line with a pathological role as macrophages, recent studies have indicated that microglia have important functions in promoting neurogenesis and angiogenesis in non-pathological situations. Our results showed that microglia in culture express a large number of known pro-angiogenic factors, as well as other secreted factors involved in organ development. Our results thereby provide a platform for further investigation of microglia-derived factors that mediate the stimulatory effect of these cells on angiogenesis *in vivo*. Together, our findings support the view that microglia have important functions in development processes, and demonstrate that this cell type secretes a number of pro-angiogenic factors that might explain their stimulatory role in angiogenesis.

Conclusions

- Analysis of the microglial transcriptome revealed that microglia in culture shows prominent expression of genes associated with *developmental process* and *biological adhesion*.

- Among these genes are known activators and inhibitors of angiogenesis that might have a role in retinal blood vessel development.

- Microglia co-cultured with aortic rings appear to switch on moderate expression of inflammatory response genes.

S1P$_1$ is a critical regulator of angiogenesis (Paper III)

Background

The bioactive lipid sphingosine-1-phosphate (S1P) has many functions in development and adult physiology. In mammals, two different kinases (SphK1 and SphK2) produce S1P from sphingosine (Kohama et al., 1998). A specific sphingosine phosphatase de-phosphorylate S1P back into sphingosine (Mandala et al., 1998), and an S1P-specific lyase catalyzes S1P degradation (van Veldhoven and Mannaerts, 1993; Zhou and Saba, 1998).

S1P has an important role during vascular maturation and is critical for vascular integrity (Allende and Proia, 2002; Hla, 2003). Platelets and other hematopoietic cells are major sources of S1P (Yang et al., 1999), leading to high concentrations of S1P in plasma and serum (Yatomi et al., 1997; Yatomi et al., 2001), while interstitial concentrations are low. Platelets lack S1P lyase, and are therefore unable to degrade S1P. Once activated, platelets release high amounts of S1P to serum (Yatomi et al., 1997). S1P binds to and activates a family of G protein–coupled receptors, called sphingosine phosphate receptor 1 to 5 (S1P$_1$ to S1P$_5$), formerly known as endothelial differentiation gene (Edg) receptors (Hla et al., 1999). Endothelial cells express S1P$_1$, S1P$_2$ and S1P$_3$ (Kono et al., 2004).

Complete S1P depletion through genetic ablation of both SphKs in mice results in severe neurological and vascular defects (Mizugishi et al., 2005). SphK1/2 double knockout embryos die between embryonic days (E) 11.5 and 13.5 and display widespread haemorrhages. The defects of these mice involve an abnormally developed vasculature, including vessel dilation and oedema. In paper III, we re-analysed the vascular phenotype of S1P$_1$$^{-/-}$ mice.

Results and Discussion

S1P$_1$$^{-/-}$ mice display endothelial hypersprouting

Most S1P$_1$$^{-/-}$ embryos appear grossly normal around E11.5 but develop severe internal bleeding and oedema around E12.5. This phenotype progressively worsens until death, which usually occurs around E14. It has previously been reported that S1P$_1$$^{-/-}$ show defects in pericyte recruitment (Liu et al., 2000). We analyzed S1P1$^{-/-}$ embryos ranging from ages that preceded the internal bleedings (E11.5) until shortly before death and failed to observe any delay in pericyte recruitment at any stage analyzed. We were also unable to detect reduced pericyte coverage in these embryos, but small pericyte detachment was observed at late stages, when the animals were close to death. On the other hand, we noticed that the vasculature in S1P$_1$$^{-/-}$ embryos was hypersprouting, as endothelial cells extended an increased number of filopodia. In addition, vessels often appeared dilated and abnormal in shape, and the vascular plexus was denser than in control animals.

Pharmacological modulation of S1P$_1$ signalling regulates angiogenesis

We used the selective S1P$_1$ agonist SEW2871, and the S1P$_1$ inhibitor W146 (Rosen et al., 2009; Sanna et al., 2004; Sanna et al., 2006) to examine the mechanism by which S1P$_1$ inactivation leads to endothelial hypersprouting and hyperplasia. Based on the phenotype of S1P$_1$$^{-/-}$ embryos, we expected that pharmacological inactivation of S1P$_1$ would create endothelial hypersprouting, whereas activation might produce opposite effects. We started by testing the biological activity of both compounds in a reliable and reproducible assay, the aortic ring sprouting assay (Nicosia and Ottinetti, 1990; Rymo et al., 2011; Zhu et al., 2003). The effects of pro- or anti-angiogenic factors have previously been successfully established using the aortic ring assay (Kruger et al., 2001). We treated aortic rings with either the S1P$_1$ agonist (SEW2871) or the antagonist (W146). As predicted from our *in vivo* data, pharmacological inhibition of S1P$_1$ caused hypersprouting from the aortic explants. Sprouts were increased in number and formed a dense network, but they appeared thin and discontinuous. In some instances, the leading cells of the sprouts had detached from the

sprout. In contrast, activation of $S1P_1$ signalling by addition of the agonist produced reduced network density and number of sprouts. The sprouts that formed were thicker, longer, continuous and more uniform than those in the control culture, and cells that had lost contact from the sprout were rarely observed.

To test if the $S1P_1$ agonist and antagonist were able to modulate angiogenesis *in vivo,* we analysed their effect on retinal angiogenesis. The $S1P_1$ activator, inhibitor, or vehicle only, was injected into mouse pups between P3-P5, and their retinas were analysed at P5. We found that the antagonist-treated animals displayed an overall denser retinal vasculature, and an increase in endothelial filopodia and tip cell number. The agonist-treated animals displayed a reduced vascular density in many areas of the retina. Thus, both genetic and pharmacological inactivation of $S1P_1$ causes endothelial hypersprouting and endothelial hyperplasia, whereas activation of $S1P_1$ negatively regulates endothelial sprouting.

Endothelial hyperplasia and disintegration of the aorta in $S1P_1^{-/-}$ mice

Embryos that lack $S1P_1$ were stained with various endothelial cell markers, which revealed a remarkable endothelial cell hyperplasia in the aorta. The endothelial hyperplasia seemed to arise around E11.5, and suggests that vasculogenesis initiates and proceeds normally in $S1P_1^{-/-}$ embryos. At this point however, endothelial cells started to lose contact with the aortic wall and formed aberrant branches that led into adjacent lumenized vessel aggregates of highly abnormal morphology. This "disintegration" of the aorta is likely to cause the severe internal bleedings and death of $S1P_1^{-/-}$ embryos. In addition, the vasculature in $S1P_1^{-/-}$ embryos showed increasing amounts of collagen IV and fibronectin, indicating an excessive extracellular matrix production by the hyperplastic endothelium. Despite the endothelial hyperplasia, pericytes were associated with the ectopic vessel networks. The small pericyte detachment observed at later stages seemed to be caused by proliferation of endothelial cells, which physically displace and disrupt the vSMCs coat. We propose that this disruption happens after the aorta is completely covered by vSMC, and that the disruption itself is a consequence of the hyperplastic endothelial cells expansion, occurring initially on the dorsal side of the aorta.

S1P₁ is required in endothelial cells to control vascular sprouting and tip cell formation

S1P$_1$ has been reported to be expressed in the developing vasculature, the forebrain and the heart between E9.5 and E13.5 (Liu et al., 2000). Thus, it is possible that the vascular defects in S1P$_1^{-/-}$ embryos could be a secondary consequence of cardiac dysfunction and subsequent circulation defects. To answer this question, we inactivated S1P$_1$ function after birth, and analysed the developing mouse retina. Using the S1P$_1$ lacZ reporter (Liu et al., 2000), we found that S1P$_1$ seems to be expressed exclusively by the endothelium in the developing retina. Selective ablation of S1P$_1$ in the retinal endothelium resulted in vascular hypersprouting with an increased number of filopodia and tip cells. Thus, it seems that S1P$_1$ is required cell-autonomously in endothelial cells to suppress their sprouting.

S1P inhibits VEGFR-2 phosphorylation and AKT activation

Two defects in S1P$_1$ $^{-/-}$ embryos suggested that S1P$_1$ could interfere with VEGF-A signalling: First, the ectopic branching from the aortic wall initially appeared at the dorsal side, suggesting that the dorsal cells responds to a signal that initially is not present on the ventral side, such as VEGF-A (Coultas et al., 2005a). Second, the hypersprouting phenotype has previously been reported in cases where VEGF-A is over-expressed or aberrantly distributed (Gerhardt et al., 2003). To address if the S1P$_1^{-/-}$ phenotype is related to VEGF-A signalling, we used a spheroid bead-sprouting assay using human umbilical vein endothelial (HUVEC) cells. Addition of VEGF-A to the medium caused an increase in tip cell formation. Addition of S1P activator caused a marked reduction in VEGF-induced tip cell number, whereas S1P$_1$ inhibition enhanced tip cell formation. S1P$_1$ inhibition also caused an increase in detached cells from the extending sprouts. Thus, in this assay, activation of S1P$_1$ signalling counteracts VEGF-A-induced endothelial sprouting and tip cell formation.

We next asked if S1P could affect the activation of VEGFR-2. Thus, primary human brain microvascular endothelial cells (HBMEC) were treated with S1P prior to stimulation with VEGF-A. This resulted in reduced VEGFR-2-phosphorylation, suggesting that S1P$_1$ counteracts VEGF-A signalling at the receptor level. Moreover, such pre-treated cells also displayed reduced phosphorylation of the downstream effector AKT.

In summary, we provide evidence that S1P via $S1P_1$ is a critical negative regulator of angiogenic sprouting in the developing mouse embryo. Based on the results, we propose that S1P, via its receptor $S1P_1$, negatively regulates VEGF-A/VEGFR-2 signalling. The developing vascular network might therefore be stabilized through the blood-borne factor S1P, which would be achieved by the inhibition of angiogenic responses in stalk cells. The stalk cells of sprouting vessels form a lumen that is in contact with blood (and hence exposed to S1P), while the tip cells do not contact the lumen and therefore would be less exposed to S1P. The suppression of tip cell characteristics by S1P, through $S1P_1$-mediated negative regulation of VEGFR-2, suggests that $S1P/S1P_1$ signalling might be fundamental to prevent stalk cells from responding to VEGF-A and form new tip-cells.

Conclusions

- $S1P_1$-deficient mice display endothelial hypersprouting.

- $S1P_1$ is required within endothelial cells to control vascular sprouting and tip cell formation.

- Pharmacological modulation of $S1P_1$-signalling regulates angiogenesis *in vitro* and *in vivo*.

- $S1P_1$ counteracts VEGF-A induced sprouting.

- $S1P_1$ can inhibit VEGFR2 and Akt phosphorylation.

Bone marrow transplantation stimulates pancreatic β-cell replication after tissue damage (Paper IV)

Background

Type I diabetes is a non-curable metabolic disease caused by autoimmune destruction of the insulin-producing β-cells in the pancreas. Attempts to establish reproducible methods for stable therapeutic β-cell reconstitution have so far been unsuccessful. Although transplantation of β-cell islets have yielded promising results, the long-term outcome is poor, and extensive use of the method is difficult due to by the shortage of donor islets (Shapiro et al., 2006). An alternative source of β-cells is the trans-differentiation of adult stem cells, but the low frequency of such differentiation events has so far prevented a wider use of this method (Gubbins et al., 2006; Schroeder et al., 2006). Another strategy is to use the endogenous sources of β-cells that already exist in the adult patient. New β-cells are formed throughout the life to compensate for apoptotic loss. β-cells have been shown to regenerate to some degree following β-cell destruction by partial pancreatectomi or streptozotocin (STZ) treatment (Bonner-Weir et al., 1993; Guz et al., 2001). However, the mechanisms for adult β-cell neoformation remain elusive. A number of studies claim that pancreatic progenitor cells can give rise to β-cell neogenesis *in vivo* and *in vitro* (Bonner-Weir et al., 1993; Bonner-Weir et al., 2000; Guz et al., 2001; Lardon et al., 2004; Zulewski et al., 2001). By contrast, recent reports suggest that β-cells in adult mice can be renewed by duplication of pre-existing β-cells (Dor et al., 2004; Nir et al., 2007). Additional studies claim that bone marrow cells can transdifferentiate to pancreatic β-cells after bone marrow transplantation (BMT) (Ianus et al., 2003). Finally. it has been proposed that bone marrow cells can adopt an endothelial cell fate and enhance tissue regeneration through neo-vascularisation (Hess et al., 2003). In paper IV, the capacity of transplanted bone marrow cells to engraft into the pancreas, to adopt an endothelial cell phenotype and to stimulate β-cell regeneration after islet damage, was investigated.

Results and Discussion

Characterization of Tie2-Cre/ZEG mice

The Tie2-Cre/ZEG transgenic mouse strain was generated by crossing Tie2-Cre mice (Kisanuki et al., 2001) with ZEG mice, where the *GFP* (green fluorescent protein) gene is preceded by floxed translation stop codons (Novak et al., 2000). Thus, cells that activate the Tie2 promoter will express Cre recombinase, resulting in removal of the translation stop signals and permanent GFP expression. FACS analysis of bone marrow cells in Tie2-Cre/ZEG mice showed that 70% of the cells were GFP$^+$. Of these, 97% expressed CD45 (a panhematopoietic marker) and 37% co-localized with expression of PECAM-1, an antigen shared by both endothelial and hematopoietic cells. The vast majority of PECAM-1$^+$ cells expressed CD45, demonstrating their hematopoietic origin. Only 3% of the GFP$^+$ bone marrow cells were PECAM-1$^+$ CD45$^-$, suggestive of an endothelial phenotype. These cells may represent mature endothelial cells from contaminated blood vessels in the bone marrow extract, or endothelial progenitor cells, as previously suggested (Rafii and Lyden, 2003).

When blood vessels were examined for the expression of the endothelial antigen von Willebrand´s factor (vWf), numerous GFP$^+$ cells were found to be located outside the vessels, scattered as rounded single cells in the parenchyma. These cells were CD45$^+$vWf $^-$, suggesting a hematopoietic cell fate. Thus, Tie2 is also present on blood cells, supporting previous data (De Palma et al., 2005; Yano et al., 1997), and questioning the validity of Tie2 as a specific marker for endothelium. Therefore, in this study, the GFP$^+$/vWf$^+$/CD45$^-$ phenotype in combination with morphological assessment was used as a criterion for transplant-derived endothelium.

Bone marrow transplantation lowers hyperglycemia

It is well established that BMT normalizes hyperglycemia after β-cell damage. To investigate this ability in hyperglycemic mice, β-cell damage was induced by intra-peritoneal injection of streptozotocin (STZ), and the mice then received intravenous injection of unfractionated bone marrow cells from Tie2-Cre/ZEG. At 4 weeks post-transplantation, recipient mice were sacrificed and examined for donor reconstitution and blood glucose.

FACS analysis revealed that 90% of peripheral blood cells in the recipient mice were donor-derived cells, as they expressed GFP. In non-transplanted control mice treated with low dose STZ, blood glucose remained slightly elevated, and mice treated with intermediate and high doses remained hyperglycemic. In bone-marrow transplanted mice, a reduction of blood-glucose concentration was observed in a dose-dependent manner with increasing levels of STZ treatment. The data demonstrated that bone-marrow transplantation improves hyperglycemia, but cannot cause full normalization.

Pancreatic engraftment of bone marrow-derived GFP⁺ cells

Pancreatic engraftment of bone marrow-derived GFP$^+$ cells

The ability of bone marrow engrafted cells to give rise to pancreatic β-cells was investigated four weeks after transplantation. No engrafted GFP+ cells that expressed insulin was found. Although plenty of engrafted donor-derived GFP$^+$ cells were observed in pancreatic sections, 99% of these cells were CD45$^+$. Moreover, only 2-5% of the engrafted GFP$^+$ cells co-expressed the endothelial antigen vWf. The vWf$^+$/GFP$^-$ cells were found mainly in conjunction with vessel structures, but almost all expressed CD45, suggesting a haematopoietic rather than an endothelial differentiation fate.

B-cell proliferation is stimulated by BMT

B-cell proliferation is stimulated by BMT

The insulin-positive islet area was drastically reduced following STZ treatment, both in transplanted and non-transplanted mice. However, bone marrow transplantation appeared to have beneficial effects on islet size. To examine whether the increased insulin-positive islet area arose from enhanced β-cell proliferation, BrdU was administered before sacrificing the mice. BrdU incorporation increased substantially in BMT-treated mice receiving STZ compared to normoglycemic transplanted mice. The analyses indicated that β-cell proliferation is related to the degree of islet damage and can be stimulated by the transplantation procedure.

BMT promotes endogenous angiogenesis after tissue damage

The number of recipient endothelial cells (GFP$^-$/vWF$^+$) in the pancreas parenchyma was examined. In transplanted mice, GFP$^-$/vWf$^+$ cell numbers increased by 20% after STZ treatment. In addition, a 50% enhancement in endothelial cell proliferation was demonstrated by vWf$^+$/BrdU$^+$ co-staining. In contrast, in the non-transplanted group there was no change in the number of endothelial cells in STZ-treated compared with normoglycemic animals. This indicates that bone marrow transplantation may promote angiogenesis after tissue damage.

The study demonstrates that bone marrow transplantation stimulates β-cell replication and islet regeneration in proportion to the degree of β-cell injury. The effect appears attributed to bone marrow-derived hematopoietic cells engrafted into the pancreas. The findings represent a novel mechanism by which β-cell replication can be adjusted to altered demands, and may in principle be exploited for therapeutic purposes. The concept is supported by a recent demonstration that bone marrow transplantation has strong beneficial effects on β-cell function in newly diagnosed type 1 diabetes patients (Voltarelli et al., 2007).

Conclusions

- Bone marrow transplantation stimulates proliferation of pancreatic β-cells, and does so in proportion to on the degree of pancreatic tissue damage.

- Bone marrow transplantation promotes endogenous angiogenesis after tissue damage. Only a minute fraction, if any, of the transplanted bone marrow-derived cells acquire an endothelial nature.

- Bone marrow-derived cells have limited, if any, capacity to differentiate into pancreatic β-cells.

- The majority of bone marrow-derived cells engrafted in the damaged tissue maintain their hematopoietic phenotype.

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