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STUDIES ON PERICYTES IN HEALTH AND DISEASE

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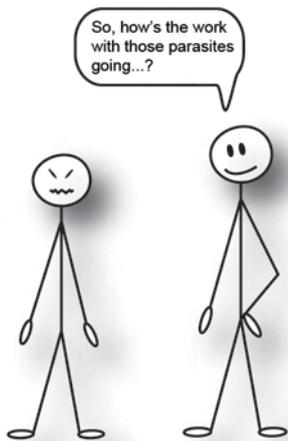
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To my boys



ABSTRACT

Pericytes are the perivascular mural cells of microvessels. They are intimately associated with endothelial cells and communicate with them via direct physical contact or through paracrine signaling pathways. These interactions are important for blood vessel maturation, remodeling, and maintenance. Pericytes are versatile and their varying morphological characteristics and distribution make them difficult to study. The lack of universal pericyte markers is a major problem. Recently, regulator of G-protein signaling 5 was discovered to be a novel pericyte gene. In paper I we studied the role of RGS5 *in vivo* by analyzing transgenic mice in which the gene has been knocked out. The vasculature of these mice appeared to develop normally with proper pericyte coverage, and the gene inactivation seemed to have no major influence on pathological angiogenesis. However, a significant decrease in blood pressure was observed, indicating a functional effect of RGS5 on the regulation of vascular tone.

A number of different functions have been attributed to pericytes, and in some organs they have more specific roles. In the brain, for instance, pericytes are part of the neurovascular unit together with specialized endothelial cells and astrocytic end-feet. Brain blood vessels have unique characteristics that restrict the passage of molecules between the brain and the blood (the blood-brain barrier), and this is critical for proper neuronal function. It has been postulated that endothelial tight junctions and astrocytic end-feet are the main players in maintaining blood-brain barrier integrity, but in paper III, we show, for the first time *in vivo*, the important contribution of pericytes. In pericyte-deficient mouse models, we show that blood-brain barrier function is impaired, as injected tracers extravasate via macromolecular transcytosis.

The role of pericytes in tumor vessels is debated, but a common belief is that pericytes contribute to stability, and that they might protect the vessels from anti-angiogenic therapy such as VEGF-A targeting. In paper II, however, we show that the absence of pericytes in experimental tumor models does not increase tumor sensitivity to VEGF-A withdrawal. This finding has consequences for the design of anti-angiogenic therapy, and for some of the anticancer strategies in use today.

PDGF-B and $-R\beta$ mutant mice display a severe reduction in pericytes due to a failure in their recruitment, and they suffer from a wide range of defects, ultimately leading to death *in utero*. The cardiac abnormalities of these animals are studied in detail in paper IV. Several of the malformations observed point towards an involvement of PDGF-B/ $-R\beta$ signaling in the contribution of epicardium-derived cells and cardiac neural crest cells to the primitive heart.

Studies included in this thesis have investigated the role of the pericyte-specific gene RGS5 *in vivo*, and also used various pericyte deficient mouse models to study the role of pericytes in the blood-brain barrier and in tumor vasculature. The cardiac defects in PDGF-B/ $-R\beta$ mutants were also analyzed in depth. To summarize, these studies have revealed novel functions for pericytes and confirmed the multifaceted nature of these cells.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

- I. **Generation and characterization of rgs5 mutant mice.**
Nisancioglu MH, Mahoney WM Jr, Kimmel DD, Schwartz SM, Betsholtz C, Genové G.
Mol Cell Biol. 2008 Apr;28(7):2324-31.
- II. **The absence of pericytes does not increase the sensitivity of tumor vasculature to VEGF-A blockade.**
Nisancioglu MH, Betsholtz C, Genové G.
Cancer Res. In press.
- III. **Pericytes regulate the blood-brain barrier.**
Armulik A, Genové G, Mäe M, Nisancioglu MH, Wallgard E, He L, Norlin J, Lindblom P, Strittmatter K, Johansson B.R., Betsholtz C.
Submitted.
- IV. **PDGF-B signaling is important for murine cardiac development: its role in developing atrioventricular valves, coronaries, and cardiac innervation.**
Van den Akker NM, Winkel LC, Nisancioglu MH, Maas S, Wisse LJ, Armulik A, Poelmann RE, Lie-Venema H, Betsholtz C, Gittenberger-de Groot AC.
Dev.Dyn. 2008 Feb;237(2):494-503.

Other publications not discussed in the thesis:

Peripheral mural cell recruitment requires cell-autonomous heparan sulphate.
Stenzel D, Nye E, Nisancioglu MH, Adams RH, Yamaguchi Y, Gerhardt H.
Blood. 2009 Jul 23;114(4):915-24.

Identification of a core set of 58 gene transcripts with broad and specific expression in the microvasculature.
Wallgard E, Larsson E, He L, Hellström M, Armulik A, Nisancioglu MH, Genove G, Lindahl P, Betsholtz C.
Arterioscler Thromb Vasc Biol. 2008 Aug;28(8):1469-76.

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ACKNOWLEDGEMENTS – Thank you!

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LIST OF ABBREVIATIONS

| | |
|--------------|--|
| ABC | ATP-binding cassette |
| α SMA | Alpha smooth muscle actin |
| Ang | Angiotensin |
| AQP | Aquaporin |
| AR | Adrenergic receptor |
| AV | Atrioventricular |
| BBB | Blood-brain barrier |
| BP | Blood pressure |
| cDNA | Complementary deoxynucleic acid |
| CNS | Central nervous system |
| DCC | Dystrophin-dystroglycan complex |
| EB | Evans blue |
| EC | Endothelial cell |
| ECM | Extracellular matrix |
| EMT | Epithelial-to-mesenchymal transformation |
| EPDC | Epicardium-derived cells |
| FGF | Fibroblast growth factor |
| GAP | GTPase activating protein |
| GFAP | Glial fibrillary acidic protein |
| GLUT1 | Glucose transporter 1 |
| GPCR | G protein-coupled receptor |
| GTP | Guanosine triphosphate |
| HSPG | Heparan sulfate proteoglycan |
| JAM | Junctional adhesion molecule |
| cNCC | Cardiac neural crest cells |
| NG2 | Neuron-glia 2 |
| NO | Nitric oxide |
| OAP | Orthogonal array of particles |
| OIR | Oxygen-induced retinopathy |
| PCR | Polymerase chain reaction |
| PDGF | Platelet-derived growth factor |
| PDGFR | Platelet-derived growth factor receptor |
| PEO | Pro-epicardial organ |
| Pgp | p-glycoprotein |
| RGS | Regulator of G-protein signaling |
| RTK | Receptor tyrosine kinase |
| RT-PCR | Reverse transcriptase PCR |
| S1P | Sphingosine-1-phosphate |
| VEGF | Vascular endothelial growth factor |
| VSD | Ventricular septal defect |
| vSMC | Vascular smooth muscle cell |

PERICYTES – AN INTRODUCTION

Normal tissue function depends on an adequate supply of oxygen and nutrients, and blood vessels constitute the elaborate transport system by which these necessities are delivered throughout our bodies. The vascular network also functions as a waste-handling system that removes waste products such as carbon dioxide and metabolites. Blood vessels are composed of two or three different cell types, depending on the type of vessel and its size. All vessels have an inner lining of endothelial cells surrounded by perivascular mural cells (pericytes and vascular smooth muscle cells, vSMC). The smallest-diameter vessels, i.e. arterioles, venules, and capillaries, are associated with solitary pericytes, while multiple concentric layers of vSMCs surround larger vessels. The largest arteries have a third outer layer of adventitial fibroblasts.

Pericytes, the perivascular cells adjacent to microvessels, were discovered for the first time over 100 years ago by Eberth and Rouget. Initially, these cells were referred to as ‘Rouget’ cells after their discoverer, but the more suitable term pericyte (peri; around, cyte; cell) was coined by Zimmerman in the early 1920s, as the cells were found to wrap themselves around and along the vessel. Pericytes were reported by Zimmerman to be the supporting and contractile cells of microvessels as they possess contractile fibers, and today it has indeed been shown that pericytes can contribute to vasoconstriction and vasodilatation in the regulation of blood flow [1]. With time, a number of other functions have been attributed to pericytes, and it seems like we are now only starting to understand the complexity of this versatile, controversial, and highly intriguing cell type.

MORPHOLOGY AND DISTRIBUTION

Pericytes have a prominent nucleus and limited amount of perinuclear cytoplasm, which extends processes that wrap around the abluminal wall of microvessels (Fig.1). The cells are embedded within the basement membrane which surrounds capillary tubes, and *in vitro* studies have shown that pericytes and endothelial cells contribute to the formation of this basement membrane [2]. Both fenestrated and non-fenestrated vessels interact with pericytes, and the cells’ morphological characteristics might vary from one tissue to another, within the same tissue, or even within the same capillary bed. Pericyte density also varies between different areas of the body, and it has been

shown to depend partly on blood pressure levels [3]. In humans, for instance, pericytes are more abundant further down the torso and legs where high blood pressure is needed to pump the blood upwards through the body. When it comes to the distribution of pericytes, this can even vary between different areas in a particular capillary bed, with higher densities often observed at vessel branch points [4]. Pericyte coverage of the vessel wall is another property that varies between the different tissues of the body. For example, in cardiac muscles of the rat the coverage is 11% while in the retina it is 41% [5]. This variation likely reflects the different functions of microvessels in different organs. In some organs, pericytes have been assigned special additional names as they have been shown to perform specific functions (see *tissue-specific functions* below).

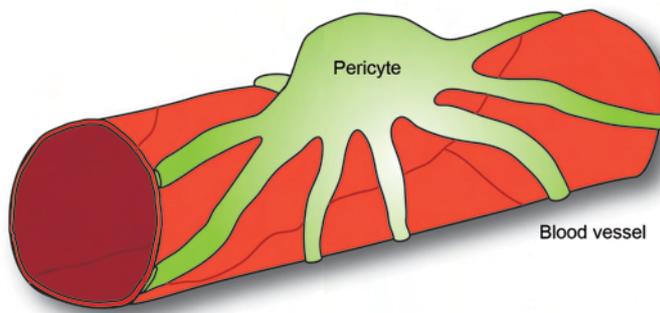


Fig.1. **The pericyte.** Pericytes are in close association with the abluminal side of microvessel endothelial cells. Protruding cytoplasmic processes wrap themselves around the vessel wall.

Pericytes are intimately associated with the endothelial surface and their cytoplasmic protrusions contact several endothelial cells along the length of the vessel. It has even been shown that pericytic processes can contact endothelial cells on neighboring vessels. These interactions with the endothelium are important for maturation, remodeling, and maintenance of the vascular system. Endothelial cells and pericytes communicate with each other via direct physical contact and paracrine signaling pathways (see *vascular development* below). Adhesion plaques mechanically link the cells to each other and gap junctions provide direct contacts between the cytoplasm of the two cells, allowing for exchange of ions and small molecules. In some tissues there are also tight junctions between the pericytes and endothelial cells. Another type of association is through the so-called peg-and-socket contact, where the basement membrane is absent and invaginations from one cell extend into the other. These junctional complexes have been reported to facilitate the transmission of contractions from pericytes to the endothelium [6, 7].

IDENTIFICATION OF PERICYTES

Studying pericytes is and has always been a challenge due to their diverse characteristics, functions, and location, and due to the difficulty of isolating pure primary pericytes, but mainly because of the lack of specific pan-pericyte molecular markers. It is questionable whether such markers will ever be discovered, considering the versatility of these cells. Pericytes are largely defined based on morphology and location, but they are also commonly identified using a handful of existing dynamic molecular markers. The problem with most of these is that pericytes are able to express different markers in different species, tissues, and developmental stages. Below, I describe a number of these pericyte markers. Regulator of G-protein signaling 5 (RGS5) and platelet-derived growth factor receptor β (PDGFR- β) will be discussed in depth as they are most relevant for my studies.

Regulator of G-protein signaling 5 (RGS5)

RGS5 is a member of the regulator of G-protein signaling family, which consists of more than 30 members divided into several subfamilies [8]. These proteins function as modulators of G-protein coupled receptors (GPCR), which are involved in a wide variety of essential biological functions. RGS5 acts as a GTPase-activating protein (GAP) for heterotrimeric G-protein α -subunits (G_{α}) [9] (Fig.2). G-protein heterotrimers are composed of a GDP-bound G_{α} subunit and a $G_{\beta\gamma}$ heterodimer. In the absence of an extracellular ligand the G-protein is GDP-bound, inactive, and attached to the intracellular surface of the 7-transmembrane GPCR. When a ligand binds to the receptor, a conformational change is induced in the G-protein and GDP is exchanged for GTP. The G_{α} subunit can dissociate from the $G_{\beta\gamma}$ heterodimer, and they are now both free to activate downstream signaling pathways [10]. The signaling is terminated when GTP is hydrolyzed back to GDP and the G-protein heterotrimer reforms and binds to its receptor. The intrinsic rate of GTP hydrolysis is quite slow, and it has been suggested that GAPs, such as RGSs, can increase the rate by up to 2000 times [11]. Thus, RGS proteins reduce GPCR-mediated signaling by either allowing for a more rapid termination of GPCR activity after removal of the ligand, or by decreasing GPCR ligand sensitivity as a higher concentration of ligand is needed to achieve the same degree of signaling.

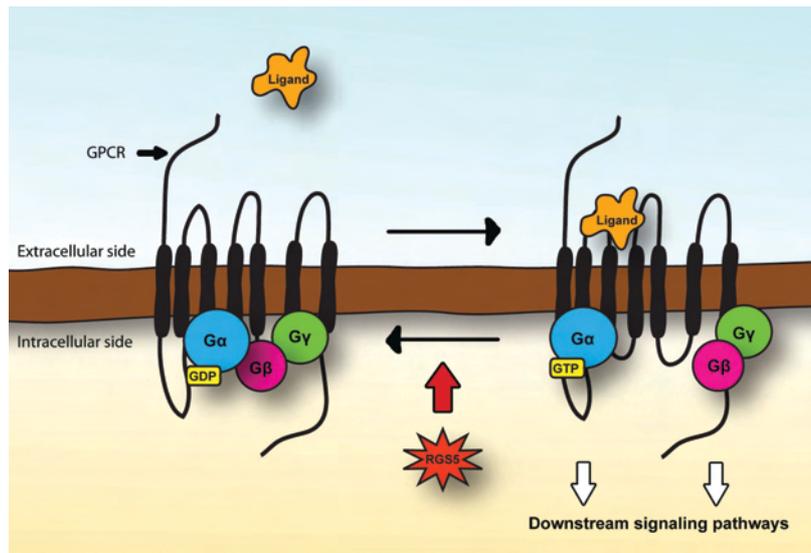


Fig.2. **The mechanism of action of RGS5.** RGS5 induces hydrolysis of GTP to GDP in G-protein α -subunits. This leads to termination of GPCR signaling and the concomitant downstream signaling activity.

All RGS protein family members have a conserved 120 amino-acid residue, the RGS domain, which is responsible for interacting with G_{α} subunits. RGS5, along with RGS4 and -16, is a member of the R4 subfamily of small RGS proteins. These RGSs are approximately 200 amino acid residues long and have short amino- and carboxyl-terminal residues [12, 13]. RGS4, -5, and -16 are highly homologous [9], but their expression patterns do not appear to overlap significantly, implying that the proteins have different biological roles. RGS4 is mainly expressed in the central nervous system (CNS) and heart and RGS16 in the heart, brain, liver, and hematopoietic cells [14]. RGS5 mRNA is present in all major organs of the body, excluding the liver and lungs, but highest expression is found in the CNS and in kidney blood vessels and mesangial cells of the glomerulus [15].

RGS5 was identified as a marker for brain pericytes by comparing the gene expression of microvascular fragments from wild-type mice and mice almost completely devoid of pericytes (platelet-derived growth factor B (PDGF-B) knockout mice). This was done using cDNA microarray technology, and RGS5 turned out to be the most downregulated gene in the brains of PDGF-B null animals [16]. Microarray analyses have also shown that RGS5 mRNA expression is much higher in arterial pericytes compared to veins [17]. As the other markers described, RGS5 has limited value as a pericyte marker (mostly due to the lack of a good antibody), and until recently the

possible functions of this gene have only been touched upon. Lately, however, a number of studies have emerged to shed light on the issue.

In 2005, Berger et. al. reported that RGS5 is upregulated during the ‘angiogenic switch’ in tumorigenesis, and during wound healing and ovulation [18]. There is also an upregulation of the gene in tumor vessels in human renal cell carcinoma [19] while angiogenic vessels in regressing tumors under therapy express reduced levels of RGS5 [20]. This indicates that there is a strong correlation between RGS5 expression and active vessel remodeling during neovascularization. However, studies of primates show that RGS5 is downregulated in smooth muscle cells of atherosclerotic plaques [21] and in the fibrous cap of advanced atherosclerotic plaques in humans [22]. As failed regulation of SMC growth in arteries contributes to atherosclerosis, these results might suggest that RGS5 is involved in maintaining normal peripheral artery function by inhibiting excessive G protein signaling.

RGS2, another member of the RGS-family, has previously been reported to regulate blood pressure (BP) [23]. Now a role for RGS5 in BP regulation has also been established as several reports point towards an association between RGS5 and hypertension in both humans and mice [24-26]. RGS5 has also been shown to be downregulated in cerebral capillaries of stroke-prone spontaneously hypertensive rats [27] and in resistance vessels (vessels involved in autoregulation of the blood supply to the vascular bed) of two hypertensive rat models [28]. Also, RGS5 reduces the activity of vasoconstrictors such as angiotensin II and endothelin-1, norepinephrine, and thrombin [15, 29, 30]. These studies are in conflict with experiments showing that the lack of RGS5 leads to hypotension in mice [24, 31](Paper I) and indicate a more complex role for this protein *in vivo*. In line with these studies are reports showing that RGS5 is one of several RGS proteins that are regulated by the so-called N-end rule pathway, which involves nitric oxide (NO)-dependent targeting of specific proteins for degradation [32-34]. Since NO is a known vasodilator, this implies that RGS5 attenuates vasodilatation signaling. Also, RGS5 has been shown to be upregulated in atria in response to overexpression of the β_2 -adrenergic receptor (β_2 AR) and to the chronic stimulation of β ARs [35]. Both β_1 AR and β_2 AR signaling is associated with vessel relaxation. As RGS2 is involved in attenuating vasoconstrictor GPCR signaling [23], and RGS5 has been implicated in the reduction of vasodilatation signaling, one might speculate that normal BP homeostasis depends on fine-tuning of GPCR-mediated

vasoactivity by RGS proteins. Developing methods to modulate the expression and function of RGS proteins could possibly be a strategy for treating and preventing hypertension and cardiovascular disease.

Recently, a new role for RGS5 in vascular remodeling has emerged. Hamzah and colleagues show that murine pancreatic tumors in an RGS5-deficient background display vessels with normalized morphology, improved blood flow, and reduced hypoxia and vascular permeability. Pericytes in the RGS5-deficient mice appear more mature than those in the wildtype situation, as they express low levels of PDGFR- β and high levels of α SMA and NG2 – an expression pattern believed to be reminiscent of mature pericytes [36]. Interestingly, the lack of RGS5 leads to an improved outcome of antitumor therapy as the normalized vasculature facilitates lymphocyte migration into the tumor tissue and subsequent tissue destruction [37]. This finding is of great importance as it makes RGS5 a potential candidate for pharmaceutical intervention.

Platelet-derived growth factor receptor- β (PDGFR- β)

PDGFR- β , along with PDGFR- α , is a cell surface tyrosine kinase receptor (RTK) for members of the PDGF family. These receptors are structurally related and upon ligand binding, they dimerize and trigger intracellular signaling cascades that ultimately lead to cellular responses such as proliferation and migration [38]. The PDGF family is a subfamily of the PDGF/vascular endothelial growth factor (VEGF) superfamily. Members of this family are structurally and functionally related and are conserved throughout the animal kingdom. They all contain cysteine knots, which are special stabilizing structural motifs characterized by eight conserved cysteine residues [39]. The PDGF subfamily consists of four members, PDGF-A, -B, -C, and -D, which dimerize with each other to form functional heterodimers, PDGF-AA, -BB, -AB, -CC, and -DD. PDGF-A and -B are secreted in their active form, while PDGF-C and -D must undergo extracellular proteolytic removal of their N-terminal CUB domain in order to become biologically active ligands [40]. The PDGF receptors are related in sequence and form both homo- and heterodimers that differ in their affinities for the various ligands. A number of PDGF-PDGFR interactions have been shown *in vitro*, but *in vivo* there is functional evidence for only a few of these, namely those of PDGF-AA and -CC via PDGFR- α , and PDGF-BB via PDGFR- β [41].

In the mouse embryo, PDGFR- β is expressed by vascular mural cell progenitors [42], while PDGF-B is mostly restricted to the developing vascular endothelium [43]. Targeted deletion of either PDGF-B or PDGFR- β in mice leads to a failure in the recruitment of pericytes to the developing vasculature, and the embryos die during late gestation due to a sudden onset of edema, dilation of the heart and large blood vessels, widespread capillary hemorrhage, and subsequent cardiovascular failure [42-45]. Histological examination of the embryos revealed additional defects such as abnormal kidney glomeruli [45], cardiac abnormalities [44] (paper IV), defective development of the labyrinthine layer of the placenta [46], and hematopoietic deficiencies [47]. These vascular deficiencies include endothelial hyperplasia, increased capillary diameter, and abnormal endothelial cell shape and ultrastructure, indicating a strict role for pericytes in regulation of the developing vasculature [44, 45, 48].

PDGFR- β is a widely used marker for pericytes, and there are several good antibodies available. However, as discussed for the other markers, the expression of this protein is not unique to pericytes. Other cells such as fibroblasts, neurons, and macrophages, to name a few, express PDGFR- β [38]. Also, the expression level of PDGFR- β is not constant. In pericytes of normal tissue, for example, the expression level is quite low, while it increases during inflammation [49]. Therefore, this marker should also be used with caution in the identification of pericytes.

Alpha smooth muscle actin (α SMA)

Actin is a highly conserved cytoskeletal protein found in all eukaryotic cells, and it participates in a variety of important cellular processes. In mammals, six isoforms of actins have been discovered, and these are divided into three subgroups; alpha, beta, and gamma actins. The beta and gamma isoforms are present in non-muscle cells where they serve as part of the cytoskeleton and mediate internal cell motility. Alpha actins are found in cells of the smooth-muscle lineages and are part of the contractile apparatus. α SMA is expressed by most pericytes and vSMCs, and it is probably the best characterized and most frequently used pericyte marker. However, it is not perfect. It has been shown, for example, that pericytes in the brain vasculature of chicken embryos express α SMA, while pericytes from the mouse, rat, and human brain do not [50]. Also, in the brain, pericytes associated with capillaries are usually α SMA

negative, while those located near arterioles are generally found to express the protein [51]. *In vitro*, the expression of α SMA in freshly isolated capillary pericytes is almost negligible, but with time, and upon the addition of serum-containing medium, nearly 100% of the cells express the protein [52, 53].

Desmin

Desmin is a muscle-specific intermediate filament found in skeletal, cardiac, and smooth muscle cells of vertebrates. It is one of the earliest protein markers for muscle tissue in the embryo, already detected in the somites of myoblasts. The function of desmin has been described by studying knockout mice. These mice develop normally and are fertile, but develop progressive abnormalities and degeneration of cardiac, skeletal, and smooth muscle tissue [54]. Although desmin is sometimes used as a pericyte marker, its expression is unfortunately, like the other markers described, not restricted to pericytes. It is also present in other cells, most prominently in cells of muscle tissue.

Promoter trap transgene X-lacZ4

In the X-lacZ4 reporter mouse, LacZ is expressed in the nuclei of pericytes and vascular smooth muscle cells in a specific fashion [55]. It was created by random insertion of the nslacZ reporter gene behind the 5' flanking region of the adipose specific gene aP2, and the expression pattern was discovered by visualization of β -Galactosidase activity in embryonic and adult tissues. This mouse line has proven to be a valuable tool for the identification of pericytes.

Neuron-glia 2 (NG2)

NG2, also called high-molecular-weight melanoma-associated antigen, HMWMAA, is a cell surface chondroitin sulfate proteoglycan, which is expressed in mural cells during normal vascular development [56] and pathological angiogenesis (eg. tumor growth and wound healing) [57, 58]. NG2 exerts its biological activity by interacting with for instance fibroblast growth factor (bFGF), PDGF-AA, and the kringle domains of plasminogen and angiostatin, and it also appears to be involved in signal transduction pathways that mediate cell spreading and motility [59]. Studies performed on NG2 knockout mice show that the lack of NG2 decreases ischemic neovascularization induced by hypoxia in the retina and by bFGF in the cornea [59]. NG2 has proven to be

a good marker for pericytes in certain situations, but it is of limited value due to a number of shortcomings. First of all, it is expressed in arterioles and capillaries, and some aortic vSMCs, but not in venules [60]. Also, NG2 is not specific to pericytes since it also can be found in nerves (glial progenitor cells, oligodendrocyte progenitor cells, and neurons) [61]. As it is a marker for pericytes in vessels undergoing remodeling, the expression of NG2 in the brain decreases as the vessels mature and become stable and quiescent [62].

Cluster of differentiation 13 (CD13)

CD13, also called pericytic aminopeptidase N (pAPN) [63], is a zinc-dependent ectoenzyme which is involved in the degradation of extracellular matrix (ECM) molecules and non-ECM molecules such as growth factors and neuropeptides [64, 65]. It has been shown to unambiguously identify capillary pericytes and vSMCs in the brain, and it mostly localizes to the plasma membrane of the non-endothelial side of the cell soma (there is less expression in the cytoplasmic processes). Antibodies against CD13 have proven to be extremely useful for the identification of pericytes in the brain, but they have to be used with caution. The antigen is only detectable on microvessels associated with blood-brain barrier (BBB) properties, for instance, and not on microvasculature which is more leaky, such as the area postrema or the choroid plexuses [63].

Other markers

A number of other proposed pericyte markers are listed below, but these will not be discussed in detail. Their usefulness per se is questionable, but in combination with other markers they could be worth considering;

Kir6.1 (ATP-sensitive potassium-channel, Kcnj8) [66]

SUR2B (sulfonylurea receptor 2, Abcc9) [66]

DLK1 (delta homologue 1) [66]

Nestin [67]

Vimentin [52]

VCAM-1 (vascular cell adhesion molecule 1) [68]

OX-42 (integrin α M) [69]

Angiopoietin-1 [70]

3G5-defined ganglioside [71]

ONTOGENY AND PLASTICITY

The origin of pericytes is still an issue under debate, but a general consensus exists around the idea that pericytes can derive from multiple precursors. Some brain pericytes have been shown to stem from the neural crest [72], for example, while it has been indicated that coronary vessel mural cells derive from epicardial cells which, in turn, originate from the mesoderm [73]. Cell marking experiments in chick embryos also suggest that vascular endothelial cells can directly contribute to the developing pericyte population by transdifferentiation [74]. Most pericytes, however, are thought to derive from a mesenchymal cell precursor. TGF- β appears to drive differentiation of PDGFR- β -positive progenitor cells into pericytes that are chemotactically attracted to PDGF-B-secreting endothelial cells in the developing vascular network [42]. Also, *in vitro* studies indicate the existence of a VEGFR-2-positive vascular progenitor cell, derived from embryonic stem cells, which is common for both endothelial cells and pericytes. Under the influence of PDGF-B or VEGF this precursor has the capacity to differentiate into either vSMCs or endothelial cells, respectively [75, 76].

It has been reported that mural cells in the adult may be recruited from the bone marrow [77]. In general, however, it is believed that pericytes arise from local mesenchymal cells within each tissue. Pericytes are considered by many to be multipotent cells that can differentiate into other cell types, such as vSMCs [78-80], fibroblasts [81, 82], osteoblasts [83, 84], chondrocytes [3, 85], and adipocytes [86]. Pericytes have also been suggested to play a role in disease conditions when the surrounding environment is adversely altered. They may then differentiate into fibroblast-like cells that contribute to the collagenous matrix of scars in wound healing, to fibrosis in conjunction with chronic inflammation, and to the formation of fibrous tumor stroma in cancer [87]. Recently, it has been reported that pericytes may serve as multipotent stem cells in the adult brain [51, 67].

FUNCTIONS

Vascular development / angiogenesis

Pericytes play an important role in the formation of blood vessels. In the healthy adult body, blood vessels have already formed and generally remain quiescent, with the exception of transient phases of neovascularization taking place during wound healing and in stages of the female reproductive cycle [88, 89]. This occurs through the process

of angiogenesis, which is the formation of new blood vessels via sprouting and splitting of pre-existing ones. Abnormal or excessive angiogenesis in the adult is often consistent with a developing pathological condition, such as cancer or rheumatoid arthritis [90]. In the embryo, vasculogenesis is initiated very early during development and represents *de novo* formation of blood vessels from differentiating endothelial cell precursors called angioblasts. There is also evidence for the existence of another precursor, the hemangioblast, which can differentiate into both endothelial cells and hematopoietic cells [76]. The precursors differentiate into endothelial cells that migrate to form endothelial tubes and these interconnect with each other in order to build a primitive blood vessel network. This primary capillary plexus is then refined into a functional vessel network by the process of angiogenesis. Finally, the formation of stable and mature blood vessels is achieved via the production of ECM and recruitment of pericytes and vSMCs.

The role of pericytes in angiogenesis is not completely understood, but studies suggest that pericytes are involved in three stages of the process; 1. Initiation, 2. Sprout formation and migration, 3. Maturation of new vessels and termination of angiogenesis [51]. When angiogenesis is initiated, pericytes secrete VEGF and induce endothelial proliferation and migration. Subsequently, with the help of proteases (e.g. MMP2) that degrade the basement membrane, pericytes withdraw from the vessels and migrate away, allowing for sprout formation [91, 92]. It has been shown that pericyte-endothelial contacts decrease during angiogenesis, and this coincides with increased sprout formation [93]. There is even evidence pointing towards a role for pericytes in leading newly sprouting vessels into surrounding tissue [94, 95]. The maturation and stabilization of vessels is represented by the termination of angiogenesis and attachment of pericytes to the vessel wall. Several factors have been implicated in this process, including CXCR3-B, heparan sulfate proteoglycans, and angiostatic substances [51]. The recruitment of pericytes, however, seems to be regulated mostly by the paracrine signaling between PDGF-B-secreting endothelial cells and pericytes expressing PDGFR- β . As mentioned earlier, mice deficient for either PDGF-B or PDGFR- β fail to recruit pericytes and die perinatally due to vascular dysfunction. The level of expression and spatial distribution of PDGF-B seems to be important, and also that it derives from the endothelium. Studies show that endothelium-specific ablation of PDGF-B in mice leads to pericyte deficiency, while the deletion of this gene in two

other cell types (neurons and hematopoietic cells) did not affect the vasculature [47, 96, 97].

A number of other signaling pathways involving pericytes have also been implicated in the induction of vascular maturation and stability. Signaling between angiopoietin-1 (Ang-1) on mural cells and its receptor Tie-2 expressed by the endothelium, for example, seems important. Deletion of either Ang-1 or Tie-2 in mice leads to the development of a defective vasculature with poorly organized BM and reduced coverage of pericytes, and the embryos subsequently die before birth [98-100]. Recently it was also shown that Ang-1 was able to induce maturation of pericyte-deficient blood vessels in the retina [101]. The other angiopoietin, Ang-2, seems to have an antagonistic effect and acts merely as a destabilizing factor in the vasculature [99, 102]. Sphingosine-1-phosphate (S1P) signaling via its endothelial GPCR S1P₁ (Edg1) is also involved in vascular stabilization. S1P₁ null mice die before birth due to vascular abnormalities involving defective coverage of mural cells [103], indicating a role in pericyte recruitment. S1P signaling also involves activation of N-cadherin and VE-cadherin [87], which are adhesion molecules involved in the cellular contacts between endothelial cells and pericytes, and between neighboring endothelial cells, respectively. Hence, S1P signaling seems crucial for the interaction and communication between cells in the developing vasculature.

Regulation of blood flow

Vascular smooth muscle cells of larger vessels regulate blood flow via contraction and relaxation. It has long been postulated that pericytes in a similar fashion are contractile cells that contribute to the regulation of blood flow via intricate communication with endothelial cells. Studies show that pericytes indeed do contract in culture and in *ex vivo* brain slices [104-107], but this has been difficult to demonstrate and confirm *in vivo*. Pericytes have been shown to express several contractile proteins, such as actin, tropomyosin, and myosin [108]. Several vasoactive substances that regulate pericyte contractile tone have also been identified. The cells express both cholinergic and adrenergic receptors, for instance, and also binding sites for endothelium-derived prostacyclin and endothelin-1 [109-111]. Pericytes also respond to angiotensin II and have been shown to contract in direct response to this protein *in vitro* [112]. Nitric oxide, which is also produced by endothelial cells, acts in a paracrine manner to

mediate pericyte contraction. It is a potent vasodilator that induces vessel relaxation via cyclic guanosine monophosphate (cGMP), which is expressed by pericytes [113]. These examples demonstrate part of the delicate interplay that takes part between pericytes and endothelial cells and it strengthens the evidence indicating that the two cell types interact in the regulation of blood flow [1].

Immune function

Whether pericytes contribute to immune responses or not is still an issue under debate. More than 10 years ago, several studies, mostly *in vitro*, were conducted to show that brain pericytes might serve as macrophages. The cells were shown to display characteristics reminiscent of macrophages, namely the presence of numerous lysosomes and an efficient uptake capacity via pinocytosis and phagocytosis [114]. Pericytes were also reported to express numerous marker components of macrophages, such as ED2 [69] and the class II major histocompatibility complex (MHC) [115], and to have the capacity to present antigen to T-lymphocytes [116]. Lymphocyte activation as a result of this antigen presentation by pericytes was also shown [117]. Another common macrophage trait attributed to the pericyte is the capability to up-regulate activity in response to certain situations such as tissue injury [118, 119], specific diseases [120, 121], conditions mimicking bacterial infections [122], and the administration of cytokines such as interferon- γ [123, 124]. These results have been and are still being questioned as a completely reliable identification of pericytes is lacking. Some argue that the reported observations might relate to perivascular macrophages instead of pericytes [125]. Hopefully, a more specific pericyte marker will be discovered in the near future so that this issue may be resolved.

Blood hemostasis

A role for brain pericytes in the regulation of blood hemostasis has been suggested, but there is still a limited amount of evidence available. There have been reports of both pro- and anticoagulant activity of pericytes. They have been shown to express functionally active tissue factor, which is essential for the initiation of the extrinsic pathway of blood coagulation [126-128]. Thus, pericytes can coactivate coagulation factors IX and X and also provide a membrane surface for the assembly of the functional prothrombinase complex. Recently it has also been shown that brain pericytes have the ability to negatively regulate endothelial fibrinolysis and amplify the

antifibrinolytic effects of toxins released from bacteria. Also, pericytes were suggested to provide endogenous anticoagulant activity via the expression and secretion of the antithrombin protease nexin-1 [129].

Tissue-specific functions

Pericytes acquire specialized characteristics in different organs, depending on the functions of each organ. Both pericyte density and vessel coverage, for example, vary among tissues. In certain organs, such as the kidney, liver, and brain, pericytes appear to have more specialized roles.

Brain

Of all blood vessels in the body, microvessels of the CNS are those most highly invested by pericytes. It is not completely clear why the brain and retina need higher mural cell coverage than other organs, but several studies indicating that pericytes are critical contributors to the formation and regulation of the blood-brain barrier (BBB) [130, 131] (paper III) are now emerging. The blood-brain barrier is a highly specialized structure consisting of cerebral endothelial cells, astrocyte end-feet, and pericytes [132, 133] (Fig.3). Together they form a selective barrier that restricts the passage of potentially harmful molecules from the vasculature into the brain and regulates the entry of essential nutrients and ions. This is necessary as the CNS is extremely sensitive to a number of substances, and because neuronal function relies on a highly regulated extracellular environment.

BBB endothelial cells are characterized by the presence of an elaborate network of intercellular junction complexes, specific transporters, and the lack of fenestrae. The intercellular junctions are composed of tight junctions (zonula occludens) and adherens junctions (zonula adherens), which essentially connect adjacent endothelial cells in a way that together makes them seem like a continuous cell membrane. The junctions severely restrict the diffusion of polar solutes between the endothelial cells from the blood to the brain extracellular fluid [134, 135] and also confer polarity to the cells by segregating the apical and basal domains of the membrane. A number of molecules are involved in forming these junctions. Tight junctions consist of occludins, claudins (claudin-3, -5, and possibly -12), and junctional adhesion molecules (JAMs) which span the intercellular cleft. These proteins are linked to the cytoskeleton via

cytoplasmic scaffolding and regulatory proteins such as ZO-1, ZO-2, ZO-3, and cingulin [134, 136]. Adherens junctions hold the neighboring endothelial cells together and confer structural support to the tissue. They consist of cadherin proteins that span the intercellular cleft and are linked to the cytoskeleton via the scaffolding proteins α -, β -, and γ -catenin [136]. Most of the proteins mentioned are essential for the formation or maintenance of BBB junction complexes. Studies in mice, for instance, have shown that the loss of either claudin-3 or -5 results in a compromised BBB [137, 138].

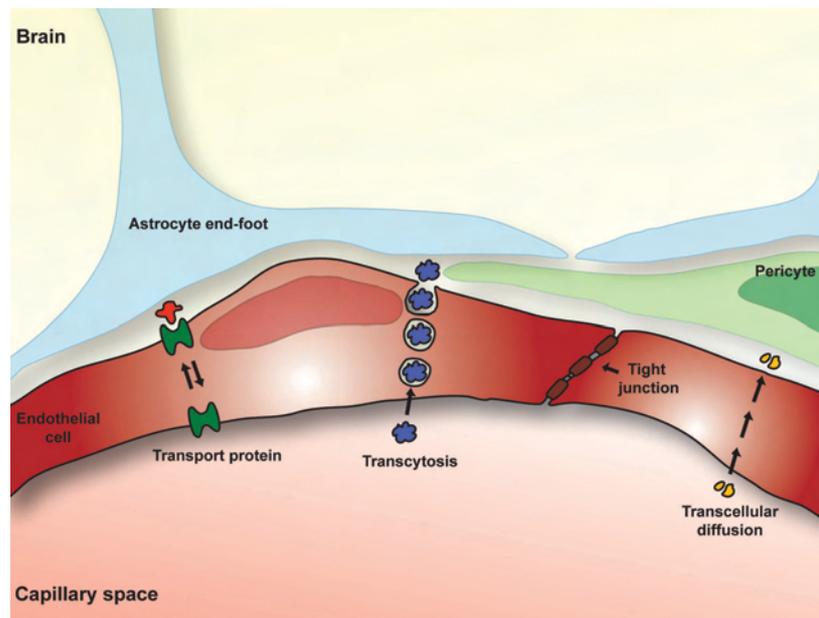


Fig.3. **The neurovascular unit.** Non-fenestrated endothelial cells, astrocyte end-feet, and pericytes form a selective barrier between the blood and the brain. Certain molecules can pass the barrier via active transport, transcytosis, or by transcellular diffusion.

A number of lipid-soluble molecules can diffuse freely through the BBB and enter the brain, but the entry (and exit) of most molecules is restricted in some way (Fig.3). Several transport proteins are involved in mediating the passage of essential nutrients and proteins, and these include carriers for glucose (e.g. GLUT1), amino acids (e.g. LAT1 and CAT1), nucleosides, and other substances [135, 139]. Some of the transporters are energy-dependent and make use of ATP to mainly transport compounds out of the brain. Examples of such transporters are the ATP-binding cassette (ABC) transporters p-glycoprotein (p-gp), multidrug resistance-associated protein (MRP), and breast cancer resistance protein (BRCP) [140]. Macromolecules such as proteins and peptides enter the brain via transcytosis, either receptor-mediated or adsorptive. Insulin and transferrin, for example, cross the BBB via receptor-mediated

transcytosis, while certain plasma proteins can be taken up by adsorptive transcytosis [132].

BBB dysfunction is observed in a number of CNS pathologies. In Alzheimer's disease, for instance, the accumulation of amyloid- β , which might be due to a decrease in pgp transporter expression, is toxic to endothelial cells and astrocytes [141, 142]. There is also an alteration in the expression of other important BBB molecules such as the GLUT1 transporters [143]. In multiple sclerosis there is a breakdown of the BBB due to selective loss of claudin-3 and downregulation of laminin in the basement membrane [137, 144, 145], and in Parkinson's disease pgp efficiency is reduced [146]. Other disease conditions, such as stroke, HIV, epilepsy, brain tumors, and glaucoma are also associated with BBB malfunction [147-153]. In some of the pathologies mentioned, a degeneration of pericytes has been observed [154, 155].

In the BBB, intimate contacts are made between blood vessels and astrocytes. Large nuclei and thick cytoplasmic appendices characterize astrocytes, and it is the endings of the appendices, the so-called end-feet, that tightly attach to blood vessels. As much as 99% of the surface of the brain capillary basement membrane is invested by astrocytic perivascular end-feet [156]. Astrocytes are also in contact with neurons, and thereby represent a line of communication between neurons and blood. Studies show that astrocytes play an important role in BBB formation and function at the physical, transportation, and metabolic levels [132], and they are also involved in regulating cerebral blood flow [157]. The astrocyte end-foot membrane region that is in contact with blood vessels contains a high density of orthogonal arrays of particles (OAPs). These OAPs contain the water channel protein aquaporin-4 (AQP4) and inwardly rectifying Kir4.1, which are both involved in ion and volume regulation. It has been indicated that the OAP-based polarity of astrocytes is important for BBB integrity [158, 159]. The restriction of AQP4 localization to the end-foot membrane is believed to depend on the expression of agrin, an extracellular heparan sulfate proteoglycan in the basal lamina [160-162], which has shown to be important for BBB integrity [163, 164]. Agrin cannot bind to AQP4 directly, but by binding to α -dystroglycan it can couple to AQP4 via α 1-syntrophin. Both α -dystroglycan and α 1-syntrophin are members of the

dystrophin-dystroglycan complex (DCC), which resides in the astrocyte end-foot membrane. α 1-syntrophin also binds to Kir4.1 [132].

Astrocytes are the most abundant cell in the brain, and for a long time, this cell-type has been in focus when it comes to inducing and maintaining the integrity of the BBB. As mentioned earlier, however, increasing evidence points towards a critical role of pericytes as well. It has been shown that BBB tight junctions may form when astrocytes are not present [165], and in mice lacking astrocytic GFAP (glial fibrillary acidic protein), vascular pericyte coverage was increased [52]. This might be a compensatory mechanism by the pericyte to counteract vascular leakage, indicating that astrocytes and pericytes act in unison to regulate barrier function, and that they might even compensate for each other in certain situations. *In vitro* studies also highlight the importance of pericytes for BBB function. The addition of pericytes to co-cultures of endothelial cells and astrocytes seems to stabilize the formation of capillary-like structures [166], and in an endothelial monolayer culture model, pericytes increase endothelial cell barrier function [131, 167]. Pericytes have also been shown to play a protective role in hypoxia-induced BBB disruption [167]. Moreover, pericyte-derived Ang1 can induce the expression of occludin by endothelial cells, thus contributing to the formation of tight junctions [168].

Kidney

In the kidney glomerulus, pericyte-like cells are referred to as mesangial cells. These cells are rounded and compact, and contact a minimal abluminal vessel area. Mesangial cells are important during development of the glomerulus, as they take part in the branching or splitting of the glomerular capillary [169], a process resulting in an increased capillary surface area for blood ultrafiltration. PDGF-B/R β -signaling seems to be instrumental for the development of these cells, as mice deficient for either of these genes lack mesangial cells and form defective kidney glomeruli with a solitary dilated capillary loop [44, 45].

Liver

Itoh cells (also called hepatic stellate cells) are considered as the equivalent of pericytes in the liver [170], and are located between the parenchymal cell plates and the sinusoidal endothelial cells. Itoh cells are involved in vitamin A metabolism [170],

recruitment of inflammatory cells during hepatic tissue repair, and in fibrotic responses to liver diseases [3, 171].

PERICYTES IN DISEASE

Tumors

During physiological angiogenesis there is a tightly regulated balance of pro- and anti-angiogenic signals leading to the rapid formation of an organized, mature and stable vasculature. As mentioned earlier, adult blood vessels are generally quiescent, and abnormal angiogenesis is often associated with a pathological situation such as cancer. In tumors, there is an imbalance between the positive and negative angiogenesis signals, leading to an excessive and constant growth of blood vessels that do not mature. The new vessels are irregularly shaped, dilated, tortuous, and leaky, and the vascular architecture is highly chaotic. There is no clear distinction between arterioles, capillaries, and venules, and the vessels are often unable to support efficient blood flow [172, 173].

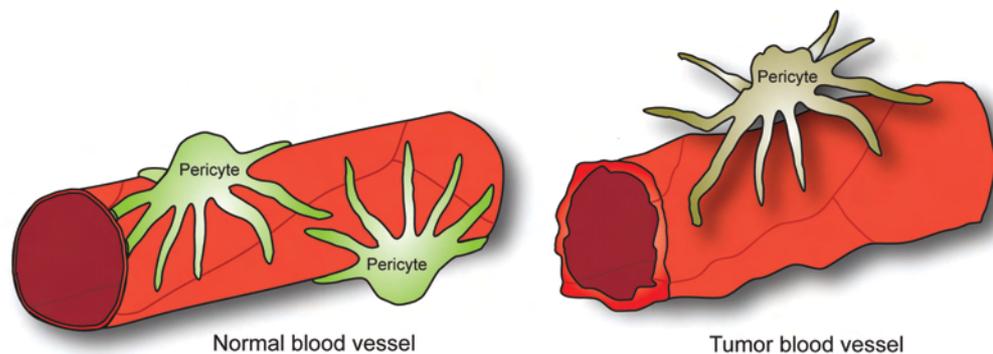


Fig.4. **Tumor pericytes.** Pericytes in healthy tissues are in close contact with the endothelium. In tumors, pericyte numbers are often reduced, and the cells are loosely attached to the abnormal tumor microvessels.

Just like tumor vessels differ from those of the normal vasculature, tumor pericytes are different from normal pericytes (Fig.4). The cells are often loosely attached to the endothelium and extend cytoplasmic processes deep into the tumor tissue. They can also change their expression profile [173]. Pericyte density and vessel coverage is generally reduced in tumors, but it seems to vary and depends on tumor type. Pancreatic islet cell tumors, for example, have a dense pericyte coverage, while in glioblastomas the vessels are invested with relatively few pericytes [174].

The role of pericytes in tumors is not clear, but an involvement of pericytes in angiogenesis has already been implied (see *vascular development/angiogenesis*). Thus, it might not be unreasonable to suggest that the abnormal and reduced numbers of tumor pericytes could at least be partially responsible for the structural and/or functional abnormalities of tumor vessels. It has been shown that a reduction in pericyte density of up to 90% is compatible with life [175], so even a very small number of pericytes, as observed in some tumors, can be important for the microvasculature. It appears that endothelial cells devoid of pericytes are more dependent on VEGF-A signaling for survival and the removal of VEGF-A results in increased pericyte coverage of the tumor vasculature [176, 177]. This suggests that pericytes might protect the endothelium from VEGF-A withdrawal, and together with the proposed stabilizing role of pericytes, it has led to the idea that pericytes could represent potential targets for anti-angiogenic therapy in tumors.

The use of anti-VEGF therapy to treat cancer, in particular in combination with chemo- and radiotherapy, has shown promising results [178, 179]. The success of such combined treatment is based on an initial and transient vascular normalization phase in which intra-tumoral pressure is reduced and drug delivery for chemotherapy is improved [180]. Most tumors, however, become resistant to anti-VEGF-A therapy and the overall survival of patients is not improved. Several mechanisms contributing to this resistance have been proposed, such as the replacement of VEGF-A by other pro-angiogenic molecules such as fibroblast growth factors (FGFs) [181], selective recruitment of immune cells, or the genetic stability of endothelial cells. It is also believed that pericytes are involved in desensitizing the endothelium to VEGF-A depletion, and thus it has been interesting to examine whether treatment efficacy would increase by a simultaneous targeting of pericytes. Pericyte deprivation can be achieved by interfering with PDGF-B/R β signaling, since this pathway is responsible for the recruitment of pericytes to the vasculature (see *Identification of pericytes, platelet-derived growth factor- β*)

A combination of VEGF-A and PDGFR- β signaling inhibitors has been tested in a number of experimental models and increased the antitumor effect when compared to withdrawing VEGF-A alone [182, 183]. The receptor tyrosine kinase (RTK) inhibitors

used to block the PDGF-B/R β pathway, however, are not specific, and so the results of these studies might be difficult to interpret. The inhibitors used could also be targeting other essential pathways involved in tumor or stromal cell (i.e. fibroblasts, inflammatory cells) viability or function. Another concern with combined VEGF/PDGF therapy is the emerging body of evidence showing that pericyte loss in tumor vessels leads to increased metastasis of tumor cells [184, 185]. Also, in a human trial against clear cell renal carcinoma, targeting both VEGF and PDGF did not display an improved therapeutic effect when compared to inhibiting VEGF-A alone, and the treatment was even shown to be toxic [186]. Clearly, the role of pericytes in tumor vasculature needs to be studied further, and the possible risk factors involved with anti-pericyte therapy have to be followed closely.

Diabetic retinopathy

Diabetic retinopathy is a severe condition that people suffering from diabetes face as a complication of their disease. It is the most common diabetic eye disease, and although it is more prevalent in type I than type II diabetes, it may occur in both. The consequences of the disease are quite devastating, and many people lose their vision completely. Diabetic retinopathy is primarily a disease of the retinal capillaries and the general features include vascular occlusions, capillary dilatation, increased permeability leading to edema, and microaneurysms. The disease might also become proliferative and include the formation of new immature vessels that grow into the vitreous and lead to hemorrhage and blindness [187].

The retina has the highest pericyte density in the body, and in diabetic retinopathy, one of the earliest indications of disease is the loss of pericytes from retinal microvessels [188, 189]. It has been suggested that this is one of the defects directly leading to retinopathy, but this has yet to be proven. Another belief is that the absence of pericytes renders the vessels vulnerable and weakened and prone to generate microaneurysms [188]. The mechanism underlying the ‘pericyte drop-out’ is not completely clear, but several studies have shed new light on the issue. Long-lasting hyperglycemia in diabetics has been proposed to have a toxic effect on pericytes. This toxicity manifests itself either directly via the accumulation of harmful metabolites (advanced glycation end products) [190] or indirectly through the upregulation of Ang-2 expression [191].

As mentioned earlier, Ang-2 is an antagonist of Ang-1 and induces vessel destabilization and pericyte loss.

Inflammation

Inflammation is a broad and complex immune reaction initiated by the body in response to injury or infection. In certain diseases, however, such as arthritis, immune responses are triggered inappropriately. The complex process of inflammation involves a response from both the local tissue and the vasculature. The vascular component is characterized by increased vessel permeability to fluid, macromolecules, and immune cells, and there have been speculations around the role of pericytes in these events, mainly through fine-tuning of the vasculature [3, 192]. It has been shown that pericytes have a slight tendency to be located near endothelial cell junctions [4, 193]. Thus, it could be postulated that contraction of the pericyte would lead to an increase in the gap between the endothelial cells and subsequently increase extravasation from the vessel. In invertebrates, it has been shown that endothelial cells can be widely separated and that permeability is primarily controlled by pericytes [194]. Also, as mentioned earlier, a number of vasoactive substances have an effect on pericytes that might lead to vasodilation and subsequently contribute to the inflammatory response.

Alzheimer's disease

The most common form of dementia seen in the world is Alzheimer's disease, which causes a progressive loss of neurons in various areas of the brain. The fundamental cause of this debilitating disorder is still unknown, but there is increasing evidence that the deposition and accumulation of amyloid- β protein is a major contributing factor. Pericytes have been implicated in the disease process as it has been shown that these cells are directly responsible for the production of β -amyloid [195, 196]. Pericyte numbers have also been shown to increase in the brain in Alzheimer's [197]. On the other hand, studies show that β -amyloid is toxic to pericytes and causes their degeneration *in vitro* [154]. Evidently, the exact role of pericytes in this disease is not clear and must be investigated further.

Multiple sclerosis

Multiple sclerosis (MS) is a disease of the nervous system that affects the brain and spinal cord. The fatty myelin sheaths that surround and protect nerve axons are

damaged, leading to deceleration of electrical impulses that travel along the nerves and ultimately to nerve degeneration. MS is a debilitating disease as the progressive nerve loss eventually may lead to problems with walking and speaking. Indications suggest that MS is an autoimmune disease, but the true cause of disease is not really known. T cell adhesion to brain microvessels and migration across the BBB into the brain is a major event in MS. The T cells in the brain multiply and release cytokines, leading to inflammation and destruction of the BBB, and thereby allowing more immune cells to invade the nervous system and destroy myelin. VCAM-1, an adhesion molecule which mediates binding of T cells, has been identified on both endothelial cells and pericytes [198]. Also, both cell types have been shown to produce interleukin-1 and -6, which are cytokines involved in the inflammatory response of MS [199]. These data support the theory that implies a role for pericytes in the regulation of T cell infiltration into the CNS in MS.

Other disorders

There are a number of other diseases where pericyte involvement has been implied. As mentioned earlier in this thesis, it has been proposed that pericytes are contractile cells that are involved in the regulation of blood flow, and so it may not seem so far-fetched that the cells play a role in the pathogenesis of hypertension. It has, for instance, been shown that the number of pericytes in brains of spontaneous hypertensive rats increases up to four times compared to controls [200]. Pericytes have also been implicated in the development of atherosclerosis. The calcification of arterial walls appears to occur by a process similar to bone formation, and pericytes have been suggested to be the cellular source of the newly formed bone tissue [201]. This is in line with the proposed ability of pericytes to transform into osteoblasts (see *ontogeny and plasticity*). In glaucoma, the optic nerve is damaged due to a raise in intraocular pressure, and this leads to a progressive, irreversible loss of vision. Pericytes, via their role in the regulation of capillary blood flow, may be involved in the disease process, as blood flow autoregulation seems to be deficient in patients with glaucoma [202].

Adams-Oliver syndrome is a rare inherited disorder which involves a combination of scalp and limb defects. The causes of this disease are not known, but it was recently reported that patients with Adams-Oliver syndrome have a pericyte deficiency [203]. Lymphedema distichiasis is another uncommon genetic disease characterized by the

growth of extra eyelashes and edema of the arms and legs. The disease is caused by mutations in the *foxc2* gene, leading to a lower expression of *foxc2* in the lymphatic endothelium. In patients with lymphedema distichiasis, pericytes are recruited to the lymph vessels, which normally lack associated mural cells [204]. This leads to a failure of lymphatic vessel function as the pericyte-invested lymphatic endothelium attains a more blood vessel-like phenotype.

MOUSE MODELS OF PERICYTE DEFICIENCY

Several mouse mutants with reduced mural cell numbers have been generated, but many of them die before birth. This complicates the study of pericytes, and therefore the need for adult viable mouse models of pericyte-deficiency has been great. A few of these models exist today, and they have proven to be extremely valuable when it comes to addressing the role of pericytes in adult microvasculature. I discuss some of these models below, including the ones used in my studies.

PDGF-B retention motif knockout mice (*pdgf-b*^{ret/ret})

As mentioned earlier, the recruitment of pericytes to vessels depends, in part, on the expression of PDGF-B by the endothelium and its binding to pericytic PDGFR- β . The precise spatial distribution of PDGF-B is important, and this is achieved via the binding to cell surface- and extracellular matrix molecules. Heparan sulfate proteoglycans (HSPGs) are particularly implicated in this process. Positively charged stretches of amino acid residues at the C-terminus of PDGF-B bind to negatively charged sulfate groups on polysaccharide chains of the HSPGs, and in this way the growth factor is retained in the vicinity of the vessels. A specific PDGF-B gradient is created and this is required for proper migration of pericytes to the vessel wall [41].

The HSPG binding motif in PDGF-B does not affect receptor binding or biological activity [203]. Thus, to address the role of PDGF-B retention *in vivo*, the PDGF-B retention motif was deleted in mice by targeted mutagenesis [205]. In PDGF-B retention motif knockout animals, *pdgf-b*^{ret/ret}, PDGF-B is unable to bind HSPGs on the surface of blood vessels and the growth factor gradient is disrupted (Fig.5). This leads to a severe reduction in pericyte density (paper III) and is probably the cause of the observed retinopathy and glomerulosclerosis. The lack of pericytes leads to a severely disorganized retinal vasculature, and in the kidney, the glomerular vascular patterns are

grossly abnormal due to a reduced number of pericyte-like mesangial cells [205]. However, the *pdgf-b^{ret/ret}* mice appear surprisingly healthy and since they survive into adulthood, they serve as a valuable tool for studying the role of pericytes from a loss-of-function perspective.

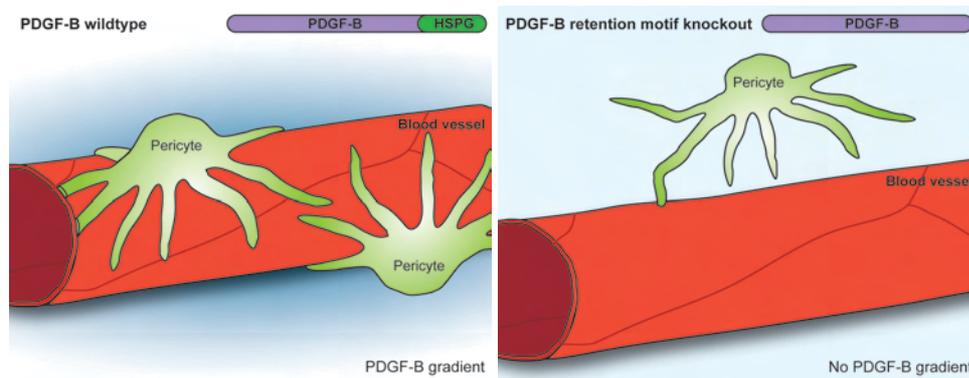


Fig.5. **PDGF-B retention motif knockout.** In wildtype animals, a PDGF-B gradient ensures proper recruitment of pericytes to microvessels. The gradient is disrupted in *pdgf-b^{ret/ret}* mice, and pericytes are unable to attach properly to the endothelium.

Rosa26hPDGF-B mice (R26P⁺⁰)

Hypomorphic PDGF-B mutants were created by ‘rescuing’ PDGF-B knockout mice with the expression of human PDGF-B. This was done by targeting a conditionally silent human PDGF-B (hPDGF-B) transgene to the ubiquitously expressed Rosa 26 locus [206]. Expression of the silent hPDGF-B was activated in endothelial cells of the mice by crossing them with Tie2-Cre mice, which express Cre recombinase under the endothelial specific Tie2 promoter (for detailed information about the Cre-lox system; [207]). The resulting homozygous R26P^{+/+} animals contain two copies of hPDGF-B while the hemizygous R26P⁺⁰ mice contain only one. Both mutants are adult viable and fertile, but display a reduced number of pericytes (paper III). R26P⁺⁰ mice have a greater reduction in pericytes than the R26P^{+/+} animals as they only express half the amount of hPDGF-B. These mice also survive into adulthood and have proven to be extremely useful for the study of pericytes.

Endothelium-specific PDGF-B knockout mice (Tie1Cre⁺, *pdgf-b^{lox/-}*)

The Cre-lox system for conditional gene inactivation was also used to generate an endothelium-restricted knockout of PDGF-B. Mice with a loxP-flanked *pdgf-b* allele were crossed with endothelial-specific Tie1-Cre mice, thereby deleting *pdgf-b* from the

vasculature. The resulting Tie1Cre⁺, pdgf-b^{lox/-} mice develop similar organ defects as the pdgf-b null animals, including cardiac, placental, and renal abnormalities. However, most of the endothelium-specific mutants survive into adulthood with persistent pathological changes [208].

Allelic series of PDGFR- β mutants (F2, F3, F5 & F7)

In order to disrupt individual components of the PDGFR- β downstream signaling pathway, an allelic series of tyrosine-phenylalanine mutations were induced in the PDGFR- β locus, producing F2, F3, F5, & F7 mice. This disrupted the binding sites of signaling components and in some way compromised the activity of the receptor. The number of mutations increases with F-number, i.e. F7 mice had the most severely affected allele. All mutant mice of the series were viable and fertile and lived into adulthood, but displayed vascular defects and pericyte loss in several tissues. There seemed to be a quantitative relationship between the extent to which signals were being transduced and the number of pericytes that formed [209].

PDGFR- $\beta\alpha$ chimeric mice ($\beta\alpha$ /-)

In these mice, the intracellular portions of PDGFR- α and - β have been exchanged. A chimeric receptor encoding the extracellular portion of PDGFR- α was fused to the intracellular portion of the PDGFR- β and targeted into the PDGFR- β genomic locus. The chimeric $\beta\alpha$ allele was then reduced to one by crosses with PDGFR- β heterozygote mice, resulting in $\beta\alpha$ /- mice. Several of these mice died perinatally, but more than half were born and a substantial portion lived into adulthood. The mice were smaller than their littermate controls and displayed kidney defects due to a failure in mesangial cell development. Also, a severe deficit in retinal pericytes resulted in retinopathy [210].

AIMS OF THIS THESIS

Paper I: To analyze the role of the pericyte-specific gene *rgs5* *in vivo*.

Paper II: To investigate whether targeting of pericytes enhances the antitumor effect elicited by anti-VEGF-A therapy.

Paper III: To analyze the role of pericytes at the blood-brain barrier *in vivo*.

Paper IV: To study the cardiovascular abnormalities in PDGF-B/R β null mice and consider the importance the PDGF-B/R β signaling pathway in the recruitment of cells during cardiac development.

PAPER I: GENERATION AND CHARACTERIZATION OF RGS5 MUTANT MICE

RESULTS AND DISCUSSION

As mentioned earlier, RGS5 was identified as a marker for brain pericytes by comparing the vasculature of wild-type and pericyte-deficient PDGF-B null mice. The results from cDNA microarray screens singled out *rgs5* as the most downregulated gene in PDGF-B knockout animals [16]. This was of great interest, since true pericyte-specific genes are hard to come by, and because there is a need for new markers to unambiguously identify pericytes. The function of RGS5 *in vivo* is largely unknown, but its involvement has been implicated in several important processes (see above). Therefore, with the purpose of studying the role of RGS5 *in vivo*, we created a mouse line which lacks the *rgs5* gene and replaced it with a GFP reporter (*rgs5*^{GFP/GFP}).

VelociGene technology [211] was used to generate the *rgs5* mutant mice. We performed RT-PCR and quantitative real-time PCR on cDNA from the animals to confirm the absence of *rgs5*, and immunostaining for GFP gave a strong signal in pericytes of the knockout animals. This signal colocalized with the pericyte marker PDGFR- β , confirming that RGS5 is expressed by pericytes. GFP expression was also detected in perivascular cells in a variety of organs, illustrating the usefulness of the *rgs5*^{GFP/GFP} animals as they allow for easy detection of pericytes and possibly fluorescence-activated cell sorting of the cells from tissue samples.

RGS5 knockout animals are viable and fertile and display no obvious defects. We studied the brain and kidneys further as *rgs5* mRNA expression has been shown to persist in the microvasculature of these organs in adult mice [15]. The architecture of these organs appeared normal and no albuminuria was detected in urine from the mutant animals, implying that the glomerular barrier function is normal. This indicates that RGS5 is not critical for normal renal filtration.

We analyzed retinas of the mutant animals to see whether the lack of RGS5 could compromise the recruitment or distribution of pericytes. In order to compare results

with control animals, which lack GFP expression, we made use of XlacZ4 reporter mice (see above) and immunohistochemical staining for NG2. When counting LacZ-positive pericytes, no difference in numbers was observed between the RGS5 null and control animals. Also, the distribution and blood vessel coverage of pericytes appeared normal.

Quantitative real-time PCR was used to analyze the mRNA expression of known pericyte markers in retinas from the mutant animals. We were curious to see whether the lack of RGS5 had an impact on the recruitment of pericytes in the retina. However, no differences were found in relative mRNA levels of either PDGFR- β or Kir6.1 [42, 66]. We also analyzed the mRNA levels of RGS4 and -16, which are considerably homologous to RGS5, but the results show that neither of these genes are upregulated and are not likely compensating for the loss of RGS5.

In order to study a possible effect of RGS5-deficiency in pathological angiogenesis, we injected tumors in the knockout animals and also made use of the mouse model of oxygen-induced retinopathy (OIR) [212]. T241 fibrosarcoma cells were injected subcutaneously in RGS5 null and control animals and we studied tumor growth and histology of the vasculature. No obvious differences were noted, indicating that the lack of RGS5 does not seem to have a significant influence on tumor growth, tumor neovascularization, or pericyte recruitment to tumor vessels. In the OIR model, mice are subjected to a period of hypoxia, which interferes with vascular development in the retina, followed by 5 days in normoxia. This induces an exaggerated and abnormal vascular growth. The lack of RGS5 did not seem to have a major impact on vessel formation or in pericyte recruitment and distribution in the OIR response.

Considering the putative involvement of RGS5 in hypertension, we studied the effect of RGS5 deficiency on blood pressure (BP) regulation. Results from tail cuff plethysmography experiments indicated that both systolic BP and diastolic BP were decreased in *rgs5*^{GFP/GFP} mice compared to controls. This is the first evidence to date of a functional effect of RGS5-deficiency on BP *in vivo*.

CONCLUSIONS AND FUTURE PERSPECTIVES

In conclusion, mice deficient in RGS5 are viable and fertile and appear normal overall. The vasculature of the animals seems to develop properly with normal pericyte coverage and distribution. The pericyte-specific expression of GFP is convenient and could prove to be a valuable tool for isolation of pericytes. The lack of RGS5 does not appear to have a major influence on pathological angiogenesis, but BP proved to be decreased in the mutant animals. Further studies should be initiated in order to determine the physiological consequence of this decreased BP and to explore further the exact role of RGS5 in the regulation of vascular tone. Also, with recent studies in mind, it would be interesting to see whether these animals could be used to confirm experiments showing that RGS5 has an important role in vascular remodeling and immune infiltration.

PAPER II: THE ABSENCE OF PERICYTES DOES NOT INCREASE THE SENSITIVITY OF TUMOR VASCULATURE TO VEGF-A BLOCKADE

RESULTS AND DISCUSSION

The role of pericytes in conferring resistance to VEGF withdrawal in tumors has been under much debate. It has been postulated that pericytes protect the tumor vasculature and that combined therapy aimed at inhibiting both VEGF and PDGF receptor signaling is more successful when it comes to obstructing tumor growth. A number of experiments have been conducted to test this hypothesis. The RTK inhibitors used to block the PDGF receptor pathway, however, are not specific and could elicit other uncontrolled anti-tumor effects. Also, it is important to note that some of the combined therapies have shown to be toxic and that the lack of pericytes in tumor vessels is associated with an increase in metastasis.

In order to gather more information about the alleged benefits of simultaneously targeting endothelial cells and pericytes in the treatment of experimental tumors, we initiated experiments with the use of two unique tools present in the lab; the pericyte-deficient *pdgfb^{ret/ret}* mouse (see above) and the specific anti-VEGF-A antibody G6-31, which targets both human and murine VEGF-A. We generated three different subcutaneous tumors (T241 fibrosarcoma, B16 melanoma, and Lewis Lung Carcinoma (LLC)) in *pdgfb^{ret/ret}* and control animals and treated them with G6-31 or isotype-matched control antibody. We recorded the growth of these tumors and also studied the vasculature using various immunohistochemical stainings.

With respect to growth curves, our results show that treatment with G6-31 had distinctive anti-tumoral effects on all three tumor models. T241 and B16 tumors grown on control animals displayed a delay in growth upon anti-VEGF-A treatment compared to those receiving control antibody. In the LLC model, the treatment resulted in a negligible response, and this is in accordance with previous results indicating that LLC tumors are refractory to VEGF-A withdrawal [213]. Interestingly, the final tumor volume of all three models grown in *pdgfb^{ret/ret}* mice and exposed to G6-31 did not

differ from the G6-31-treated control tumors. This indicates that the absence of pericytes in *pdgfb^{ret/ret}* tumors did not result in a further decrease in tumor size compared to controls upon anti-VEGF-A treatment.

Pericyte density and attachment to endothelial cells was studied in tumor sections stained immunohistochemically with α SMA, NG2, and CD31 (a blood vessel marker). In the T241 and LLC tumor models, both parameters remained largely unchanged upon treatment with G6-31, while in the B16 tumors, colocalization of pericytes with vessels increased. When comparing tumors grown in *pdgfb^{ret/ret}* mice to those in littermate controls, however, a significant reduction was observed in both pericyte density and endothelial cell coverage. Treatment of these tumors with G6-31 slightly decreased both parameters, again with the exception of the B16 tumors where an increase in pericyte attachment was observed.

We also analyzed the vasculature of the tumors by determining vessel diameter and density. In accordance with reports showing that pericyte-deficient vessels during development are dilated [48], our analysis of T241 and B16 tumors grown in *pdgfb^{ret/ret}* showed a similar tendency. The vessels of pericyte-deficient LLC tumors, however, did not have an increased diameter. Vascular density was reduced in *pdgfb^{ret/ret}* tumors compared to controls, and this is in line with previous studies where PDGFR- β inhibition has resulted in decreased neovascularization [214]. The effect of anti-VEGF-A treatment on vessel diameter in the various tumor models was quite inconsistent, and this is also in agreement with previous studies [183, 215]. A common consequence of anti-VEGF-A treatment is reduced vascular density, and this is what we see in our model. In all three tumor models, grown in either *pdgfb^{ret/ret}* or control animals, there was a reduction in vessel density following treatment with G6-31. This result is contradictory to previous studies showing that pericytes protect tumor vessels upon anti-VEGF-A treatment [177]. If this were the case, we would expect to see a greater reduction in vascular density in tumors grown in the pericyte-deficient model.

CONCLUSIONS AND FUTURE PERSPECTIVES

To summarize, our results indicate that the absence of pericytes does not increase tumor sensitivity to VEGF-A withdrawal. The decrease in tumor size, which was observed as

an effect of anti-VEGF-A treatment, was not greater in the pericyte-deficient model compared to control. Also, vessels devoid of pericytes in tumors grown in the *pdgfb^{ret/ret}* animals were able to withstand withdrawal of VEGF-A and did not regress. The exact role of pericytes in tumor vessels is still far from being understood, but these results shed some new light on the issue. It will be important to keep the new information in mind when initiating further investigations, as the general view so far has been that pericytes protect the vessels and should be targeted in order to improve anti-VEGF-A therapy.

PAPER III: PERICYTES REGULATE THE BLOOD-BRAIN BARRIER

RESULTS AND DISCUSSION

It has become evident over the years that pericytes play an important role at the blood-brain barrier. As mentioned earlier, results from *in vitro* experiments suggest that the cells play a role in endothelial barrier formation [168, 216]. When it comes to their role in BBB-formation *in vivo*, however, evidence is scarce. With the use of in-house pericyte-deficient mouse models (see above), we sought to shed some light on the issue.

Quantification studies performed on tissue sections stained with antibodies for CD13 and PDGFR- β revealed that pericyte coverage in the brains of *pdgfb*^{ret/ret} and R26P^{+/-} animals was 26% and 40% of control levels, respectively. The corresponding figure for homozygous R26^{+/+} mice was 72%. Absolute pericyte numbers were obtained by the use of the before-mentioned XlacZ4 reporter mice, and these were also reduced. Vessel density in the pericyte-deficient mice was reduced and the vessels appeared dilated. Endothelial apical-basal polarity, however, was normal, as depicted by staining for the luminal endothelial marker podocalyxin and the abluminal marker collagen IV.

The brains of *pdgfb*^{ret/ret} and R26P^{+/-} mice appeared enlarged compared to controls, leading to the suspicion of edema and an impaired BBB. We subsequently used a variety of injectable tracers to analyze the integrity of the barrier. One of these was the azo dye Evans Blue (EB), which binds to plasma proteins and can be used to study the extravasation of albumin. EB was retained in the brains of pericyte-deficient mice at a degree which correlated with pericyte density; the fewer pericytes, the more retention. To study whether there was a size-selectivity of the leakage we used a panel of tracers of different molecular weights; 950 Da cadaverine Alexa Fluor-555, fluorescently labeled 66 kDa albumin, 70 kDa dextran, and 200 kDa IgG. All of the markers crossed the BBB in the pericyte-deficient animals. In the R26^{+/+} mice, a small amount of the markers was shown to accumulate in the brain parenchyma, but in the controls, no accumulation was detected. These results clearly show that there is a close association

between pericyte numbers and the leakage of both low and high molecular weight tracers across the BBB.

To study the route of extravasation, we first analyzed inter-endothelial junctional complexes in pericyte-deficient animals. Using the markers VE-cadherin and ZO-1/claudin-5 we could study adherens and tight junctions, respectively. The level and distribution of these markers were quite similar in mutants and controls, revealing only subtle abnormalities. Next, we followed the pattern of our fluorescent tracers more closely to explore how the extravasation was effectuated. The speckled appearance of high molecular weight tracers which had accumulated in endothelial cells of pericyte-deficient mice prompted us to consider transcytosis. The distribution of horseradish peroxidase (HRP, 44kDa) was studied by transmission electron microscopy, and HRP was found to localize to macro-vesicles in the *pdgfb^{ret/ret}* mice. Uptake of HRP into micro-vesicles, however, was similar in mutant and control animals. Thus, it seemed evident that high molecular weight molecules were crossing the BBB in pericyte-deficient animals via macrovesicular transcytosis.

Recently it was shown that the tyrosine kinase inhibitor imatinib could counteract brain edema in an experimental mouse stroke model [217], so we were tempted to test this treatment in our pericyte-deficient mice. In both the *pdgfb^{ret/ret}* and *R26P⁺⁰* animals, imatinib almost completely reversed tracer extravasation in a dose-dependent manner. Interestingly, imatinib treatment seemed to result in a retained uptake and inhibited release of tracer from endothelial cells, as the post-treatment scenario involved increased accumulation and enhanced punctuate distribution of tracer in the cells. This implies that imatinib inhibits the exocytosis step of the transcytosis process.

Affymetrix gene expression arrays were used to analyze whether the lack of pericytes regulates the expression of endothelial BBB markers. Transcription profiles of brain microvascular fragments isolated from control and pericyte-deficient brains were assessed. The results obtained show that most BBB endothelial markers and other general endothelial cell markers are present in the mutant animals, but some markers display altered expression levels. Transferrin receptor (CD71), for example, was predicted by the gene expression arrays to be slightly downregulated in pericyte-deficient vessels. We confirmed this at the protein level by staining brain tissue from

control and mutant animals with antibodies specific for CD71. The unaltered expression level of Glut1 was validated by the same method. These results show that the lack of pericytes influences the gene and protein expression profile of BBB endothelial cells.

Next, we wanted to take a closer look at the astrocyte component of the BBB and investigate whether pericytes and astrocytes communicate, and also whether the lack of pericytes leads to changes in astrocyte end-foot distribution or polarization. Three different astrocyte markers, which all localize to the surface of the astrocyte end-foot that contacts the endothelium, were used for this purpose: AQP4, α -syntrophin, and laminin α 2 (lama2). The expression of all three markers was weaker in pericyte-deficient mice than in controls. Also, AQP4 and α -syntrophin were associated with detached pericytes and pericytes extending between endothelial cells of two neighboring vessel branches. It might therefore seem as though pericytes express signals that help induce the attachment of astrocyte end-feet to the vasculature. Polarization of the astrocyte end-feet also appears to be deficient in mutant animals, judging by the staining pattern of AQP4, α -syntrophin, and lama2 at the astrocyte-endothelial interface.

CONCLUSIONS AND FUTURE PERSPECTIVES

We show that BBB function is impaired in pericyte-deficient mice, as injected tracers extravasate into the brain parenchyma. The extent of leakage correlates with degree of pericyte loss. Our tracers cross the BBB via macromolecular transcytosis, and the tyrosine kinase inhibitor imatinib inhibits the exocytosis step of this process in a dose-dependent manner. In other words, treatment of pericyte-deficient mice with imatinib reverses the extravasation of injected tracers. The lack of pericytes was shown to regulate the expression of certain BBB endothelial markers, and also that of several astrocyte-specific markers. In addition, astrocyte end-foot polarization, distribution, and association with the vessel wall was affected in the mutant animals.

Taken together, our results provide important new insight into the role of pericytes in regulating the mammalian BBB *in vivo*. The observed leakage across the BBB in our

pericyte-deficient models is compatible with life, and the mice survive into adulthood. Thus, these models may prove to be valuable in the further study of BBB impairment and its consequences on bodily functions and diseases. Also, the fact that immunoglobulins are able to cross the pericyte-deficient BBB is highly interesting from a pharmaceutical point-of-view, and a deeper understanding of the mechanisms regulating the entry of such molecules could prove to be invaluable.

PAPER IV: PDGF-B SIGNALING IS IMPORTANT FOR MURINE CARDIAC DEVELOPMENT: ITS ROLE IN DEVELOPING ATRIOVENTRICULAR VALVES, CORONARIES, AND CARDIAC INNERVATION

RESULTS AND DISCUSSION

Maturation of the mammalian heart depends on recruitment of cells from the epicardium and cardiac neural crest to the primitive heart tube. Cardiac neural crest cells (cNCCs) are involved in the development of the aortic arch, segmentation of the outflow tract, semilunar valve formation, development of the cardiac conduction system and innervation. The epicardium, which covers and protects the heart, derives from the pro-epicardial organ (PEO), which, in turn, develops from the dorsal mesoderm. Via epithelial-to-mesenchymal transformation (EMT), the epicardium gives rise to epicardium-derived cells (EPDCs). These EPDCs migrate into the heart and contribute to the interstitial fibroblasts and the coronary vessel wall, and they are also implicated in proper development of the atrioventricular valves and Purkinje fibers (involved in contraction of the heart) [218].

Mice mutant for PDGF-B or $-R\beta$ have been shown to display dilated hearts with abnormalities such as underdeveloped coronary arteries and ventricular septal defects (VSDs) [42, 44, 208], and the expression pattern of these proteins during avian cardiogenesis indicates that the pathway is involved in coronary maturation [219]. In this study, the cardiovascular aberrations of these knockouts were analyzed further. The role of PDGF-B/ $-R\beta$ signaling in the cardiac contribution of EPDCs and cNCCs was also explored.

Pdgf-b and *pdgfr- β* knockout and control embryos ranging in age from embryonic day (E) 13.5 to E17.5 were collected and thoracic tissue samples were immunohistochemically stained for a panel of different markers (α SMA, periostin, ephrinB2, EphB4, VEGFR-2, Delta-like 1 (Dll-1), phospho-histone H3, and Neural cell adhesion molecule (NCAM)). A number of cardiovascular aberrations were observed in the *pdgf-b*^{-/-} embryos. Perimembranous and muscular VSDs were observed in almost all mutants, and the atrioventricular (AV) valves were underdeveloped. Also,

the compact myocardium was extremely hypoplastic, concomitant with decreased levels of myocardial proliferation and no observed apoptosis, and a number of coronary malformations were observed. Finally, as observed by staining for NCAM, nerves in and surrounding the heart were hypoplastic. Abnormalities in the *pdgfr-b* mutant embryos were similar to the ones seen in *pdgf-b*^{-/-} embryos, but to a slightly different extent.

CONCLUSIONS AND FUTURE PERSPECTIVES

It was hypothesized that PDGF-B/R β signaling is important for the development and recruitment of EPDCs and cNCCs to the primitive heart. Indeed, several of the abnormalities seen in the mutant embryos are comparable to malformations observed as a result of impaired PEO-outgrowth towards the heart or defective EMT. In line with observations showing that PDGF-B can induce epicardial EMT *in vitro*, we now suggest that PDGF-B is involved in epicardial EMT and recruitment of EPDCs *in vivo*. It is also proposed that PDGF-B/R β signaling has a role in cNCC development, as some of the irregularities found in mutant embryos were comparable with neural crest abnormalities and since the cardiac nerves were hypoplastic. Several congenital heart malformations in humans are reminiscent of the defects observed in PDGF-B/R β mutant mouse embryos, and might therefore be a result of impediments in the PDGF-B/R β signaling pathway. A possible future prospect would be to study patients with such heart conditions in search for PDGF-B and -R β related mutations.

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