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Alexander Birbrair *Editor*

Pericyte Biology - Novel Concepts

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Preface

This book's initial title was *Pericyte Biology: Development, Homeostasis and Disease*. However, due to the current great interest in this topic, we were able to assemble more chapters than would fit in one book, covering pericyte biology under distinct circumstances. Therefore, the book was subdivided into three volumes entitled *Pericyte Biology - Novel Concepts*, *Pericyte Biology in Different Organs*, and *Pericyte Biology in Disease*.

This book, *Pericyte Biology - Novel Concepts*, presents contributions by expert researchers and clinicians in the multidisciplinary areas of medical and biological research. The chapters provide timely, detailed overviews of recent advances in the field. This book describes the major contributions of pericytes to different organs' biology in physiological and pathological conditions. Further insights into the biology of pericytes will have important implications for our understanding of organ development, homeostasis, and disease. The authors focus on the modern methodologies and the leading-edge concepts in the field of cell biology. In recent years, remarkable progress has been made in the identification and characterization of pericytes in several tissues using state-of-the-art techniques. These advantages facilitated the identification of pericyte subpopulations and definition of the molecular basis of the role of pericytes within different organs. Thus, the present book is an attempt to describe the most recent developments in the area of pericyte behavior, which is one of the emergent hot topics in the field of molecular and cellular biology today. Here, we present a selected collection of detailed chapters on what we know so far about the pericytes in various tissues and under distinct pathophysiological conditions. Ten chapters written by experts in the field summarize the present knowledge about the physiological and pathophysiological roles of pericytes.

William B. Stallcup from Sanford Burnham Prebys Medical Discovery Institute discusses the role of NG2 proteoglycan in pericyte biology. Bruno Péault and colleagues from the University of California describe the osteogenic potential of pericytes and their use for therapeutic bone repair. Nabila Bahrami and Sarah J. Childs from the University of Calgary compile our understanding of pericyte biology in zebrafish. Paula Dore-Duffy and Nilufer Esen from Wayne State University update us with the latest approaches for pericyte isolation, characterization, and cultivation.

Cristina L. Esteves and F. Xavier Donadeu from the University of Edinburgh summarize the current knowledge on the properties of pericytes from domestic animals, and outline their potential use in clinical veterinary medicine. Kiminori Sato from Kurume University School of Medicine addresses the importance of pericytes in the human vocal fold mucosa. Theodor Burdyga and Lyudmyla Borysova from the University of Liverpool focus on Ca^{2+} signaling in pericytes. Sharon Gerecht and colleagues from Johns Hopkins University introduce our current knowledge about pericytes derived from human pluripotent stem cells. Betül Çelebi-Saltik from Hacettepe University discusses the possible use of pericytes in tissue engineering. Finally, Abderahim Gaceb and Gesine Paul from Lund University give an overview of pericyte secretome.

It is hoped that the articles published in this book will become a source of reference and inspiration for future research ideas. I would like to express my deep gratitude to my wife, Veranika Ushakova, and to Mr. Murugesan Tamilsevan from Springer, who helped at every step of the execution of this project. This book is dedicated to the memory of my grandfather, Pavel Sobolevsky z"l, PhD, a renowned mathematician, who passed away during the creation of this piece.

My grandfather, Pavel Sobolevsky z"l, PhD (March 26, 1930–August 16, 2018)

Belo Horizonte, Brazil Alexander Birbrair

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Chapter 1
Pericyte Biology: Development, Homeostasis, and Disease

Alexander Birbrair

In the nineteenth century, a French researcher, Charles-Marie Benjamin Rouget, revealed a population of contractile cells associated with small blood vessels, which were initially named after him as the Rouget cells [14]. In the twentieth century, a German scientist, Karl Wilhelm Zimmermann, called these cells "pericytes" due to their anatomical position located in a perivascular position [21]. The word *pericyte* was derived from "peri" meaning "around" and "cyte" from the word "kytos" (cell), illustrating a cell encircling a blood vessel [16]. Until now, pericytes are still identified partially based on their specific anatomical location and morphology. Pericytes are present in all vascularized tissues, surrounding blood vessel walls [12]. They encircle endothelial cells and communicate with them along the length of the blood vessels by paracrine signaling and physical contacts [11]. Previously, the accurate distinction of pericytes from other perivascular cells was difficult, as electron and light microscopy were the sole available techniques capable to image these cells, limiting the information acquired from those works. This resulted in the misleading assumption that pericytes are merely inert supporting cells, limited exclusively to the physiological function of vascular stability. In the last 10 years, the combination of fluorescent and confocal microscopy with genetic state-of-art techniques, such as fate lineage tracing, enabled remarkable progress in the discovery of multiple novel essential functions for pericytes in health and disease, before unexpected [7]. Recently, the rapidly expanding understanding of the pathophysiological roles of pericytes drew the attention of several research groups. Now, we know, for instance, that pericytes may play immune functions [2]: attract innate leukocytes to exit via sprouting blood vessels [19], regulate lymphocyte activation [15, 20], and contribute to the clearance of toxic by-products, having direct phagocytic activity [9]. Pericytes also may behave as stem cells [6], forming other cell populations, as well

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as regulate the behavior of other stem cells in their niches [1, 4, 5, 8, 13]. Very little is known about the exact identity of pericyte ancestors within developing tissues, and there is evidence for multiple distinct developmental sources [3]. Pericytes differ in their embryonic origins between tissues and also within the same organ [3, 17, 18]. Importantly, pericytes from distinct tissues may differ in their distribution, morphology, expression of molecular markers, plasticity, and functions [10]; and, even within the same organ, there are various pericyte subpopulations. This book describes the major contributions of pericytes to different organ biology in physiological and pathological conditions. Further insights into the biology of pericytes will have important implications for our understanding of organ development, homeostasis, and disease.

This book's initial title was "Pericyte Biology: Development, Homeostasis, and Disease." However, due to the current great interest in this topic, we were able to assemble more chapters than would fit in one book, covering pericyte biology under distinct circumstances. Therefore, the book was subdivided into three volumes entitled: "Pericyte Biology: Novel Concepts," "Pericyte Biology in Different Organs," and "Pericyte Biology in Disease."

Here, we present a selected collection of detailed chapters on what we know so far about pericytes. More than 30 chapters written by experts in the field summarize our present knowledge on pericyte biology.

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Chapter 2
The NG2 Proteoglycan in Pericyte Biology

William B. Stallcup

Abstract Studies of pericytes have been retarded by the lack of appropriate markers for identification of these perivascular mural cells. Use of antibodies against the NG2 proteoglycan as a pericyte marker has greatly facilitated recent studies of pericytes, emphasizing the intimate spatial relationship between pericytes and endothelial cells, allowing more accurate quantification of pericyte/endothelial cell ratios in different vascular beds, and revealing the participation of pericytes throughout all stages of blood vessel formation. The functional importance of NG2 in pericyte biology has been established via NG2 knockdown (in vitro) and knockout (in vivo) strategies that reveal significant deficits in blood vessel formation when NG2 is absent from pericytes. NG2 influences pericyte proliferation and motility by acting as an auxiliary receptor that enhances signaling through integrins and receptor tyrosine kinase growth factor receptors. By acting in a *trans* orientation, NG2 also activates integrin signaling in closely apposed endothelial cells, leading to enhanced maturation and formation of endothelial cell junctions. NG2 null mice exhibit reduced growth of both mammary and brain tumors that can be traced to deficits in tumor vascularization. Use of Cre-Lox technology to produce pericyte-specific NG2 null mice has revealed specific deficits in tumor vessels that include decreased pericyte ensheathment of endothelial cells, diminished assembly of the vascular basement membrane, reduced vessel patency, and increased vessel leakiness. Interestingly, myeloid-specific NG2 null mice exhibit even larger deficits in tumor vascularization, leading to correspondingly slower tumor growth. Myeloid-specific NG2 null mice are deficient in their ability to recruit macrophages to tumors and other sites of inflammation. This absence of macrophages deprives pericytes of a signal that is crucial for their ability to interact with endothelial cells. The interplay between pericytes, endothelial cells, and macrophages promises to be an extremely fertile area of future study.

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Keywords NG2 proteoglycan · Blood vessel development · Pericytes · Endothelial cells · Vascular basement membrane · Cell proliferation and motility · Integrin signaling · Growth factor receptor signaling · NG2 knockdown · NG2 ablation · Cre-Lox technology · Tumor growth · Tumor vascularization · Macrophage recruitment

Introduction

The vascular biology literature is overwhelmingly dominated by research on endothelial cells. This is somewhat understandable in light of the critical roles of endothelial cells in forming the vascular lumen, controlling vascular permeability, and sensing and responding to cells and molecules in the circulation [1–5]. By comparison, the relative paucity of research on pericytes greatly undervalues the importance of these mural cells in microvessel biology. Cooperative interactions between pericytes and endothelial cells are essential for most aspects of blood vessel development and function, even at very early stages of vascularization [6, 7]. These pericyte-endothelial cell interactions promote the maturation of both vascular cell types and the maturation of overall vessel structure and function. This maturation includes assembly of the vascular basement membrane, a critical yet also frequently neglected third component of blood vessels in which both endothelial cells and pericytes are embedded [8–12].

Since a number of excellent reviews have summarized the general literature on pericytes [13–17], this chapter will not attempt to cover the same ground. Instead, we will deal more specifically with the importance of the NG2 proteoglycan, also known as chondroitin sulfate proteoglycan-4 (CSPG-4), as a pericyte marker and as a functional player in pericyte biology. Similarly, since NG2 is expressed in other cell types besides pericytes, the chapter will not try to cover the available information about NG2 in the context of all cells. Other reviews will provide useful background in this respect [18–21], and we will select from these reports only key insights into NG2 functions that apply to pericyte biology.

NG2 as a Pericyte Marker

One important factor underlying the relative lack of attention paid to pericytes has been the difficulty in identifying these mural cells. Pericytes are best defined by their intimate, abluminal spatial relationship with vascular endothelial cells. However, the spatial intimacy of this relationship makes it very difficult to distinguish pericytes from endothelial cells in the absence of markers for both cell types, leading to potentially erroneous conclusions regarding vascular cell identities [22, 23]. Since pericytes are the microvessel counterparts of smooth muscle cells, alpha-smooth muscle actin (α-SMA) has often been used for pericyte identification. This reliance on α-SMA as a pericyte marker is at least partly responsible for the failure, until

relatively recently, to recognize the very early participation of pericytes in neovascularization. This is especially true in the case of rodent pericytes, which express α-SMA only with maturation, but not at early stages of development. This inability to recognize immature pericytes led to the concept of pericytes as relatively late participants in vascularization, serving mostly to stabilize maturing blood vessels [24–27]. However, the use of other markers such as 3G5 ganglioside [28] and aminopeptidase A [29] hinted at very early participation of immature pericytes in developing blood vessels. More recently, PDGF receptor-β (PDGFRβ) [30–32] and the NG2 proteoglycan [33, 34] have emerged as convenient and reliable pericyte markers that confirm the presence of these immature mural cells during the earliest stages of microvessel formation [35–37]. Functionally, PDGFRβ is responsible for pericyte recruitment in response to PDGF-B produced by endothelial cells [30, 31, 38]. The functional importance of NG2 in pericyte development and interaction with endothelial cells will be discussed in later paragraphs. The utility of these two pericyte markers holds in both normal and pathological microvessels, and in vessels formed via either vasculogenic or angiogenic mechanisms [33, 34], serving in all cases to distinguish pericytes from endothelial cells (Fig. 2.1a–f). Increasing pericyte maturation in these various vessel types can be monitored by quantifying the percentage of NG2-positive or PDGFRβ-positive pericytes that express α-SMA [32, 39–41].

It is important to note that neither PDGFRβ nor NG2 are expressed exclusively by vascular mural cells. NG2 for example is also expressed by oligodendrocyte progenitor cells (OPCs) in the central nervous system, by activated macrophages in inflammatory pathologies, by chondroblasts and osteoblasts in developing cartilage and bone, by keratinocytes and dermal progenitors in the skin and hair follicles, and by some types of tumor cells (such as gliomas and melanomas) [21, 36, 42–47]. It is therefore not possible to conclude that an NG2-expressing cell is a pericyte without obtaining confirmatory information, such as the expression of other pericyte markers such as PDGFRβ (Fig. 2.1g–i). Even this is often not sufficient proof of identity, since pericytes are closely related to mesenchymal stem cells (MSCs) [48–51] and are seen to express many of the same markers [52, 53]. Because MSCs are not always associated with blood vessels, double labeling for NG2 and an endothelial cell marker such as CD31 is extremely useful in establishing whether an NG2-positive cell is truly perivascular in nature (Fig. 2.1a–f).

In the context of the vasculature, NG2 is expressed not only by pericytes in microvessels but also by smooth muscle cells in developing macrovessels and by cardiomyocytes in the developing heart [33]. NG2 is thus a general marker for vascular mural cells that distinguishes these perivascular elements from their endothelial counterparts. There is nevertheless some heterogeneity of NG2 expression among mural cells. For example, in the developing heart, NG2 expression is strong in ventricular cardiomyocytes (and also in aortic smooth muscle cells) but much weaker in atrial cardiomyocytes [33]. This outflow tract versus inflow tract dichotomy is also observed in microvessels, where NG2 is preferentially expressed by pericytes in arterioles compared to pericytes in venules [54]. NG2 expression can nevertheless be induced in venule pericytes during vascular remodeling [55]. In fact, a general observation regarding vascular NG2 expression is that levels of the proteoglycan

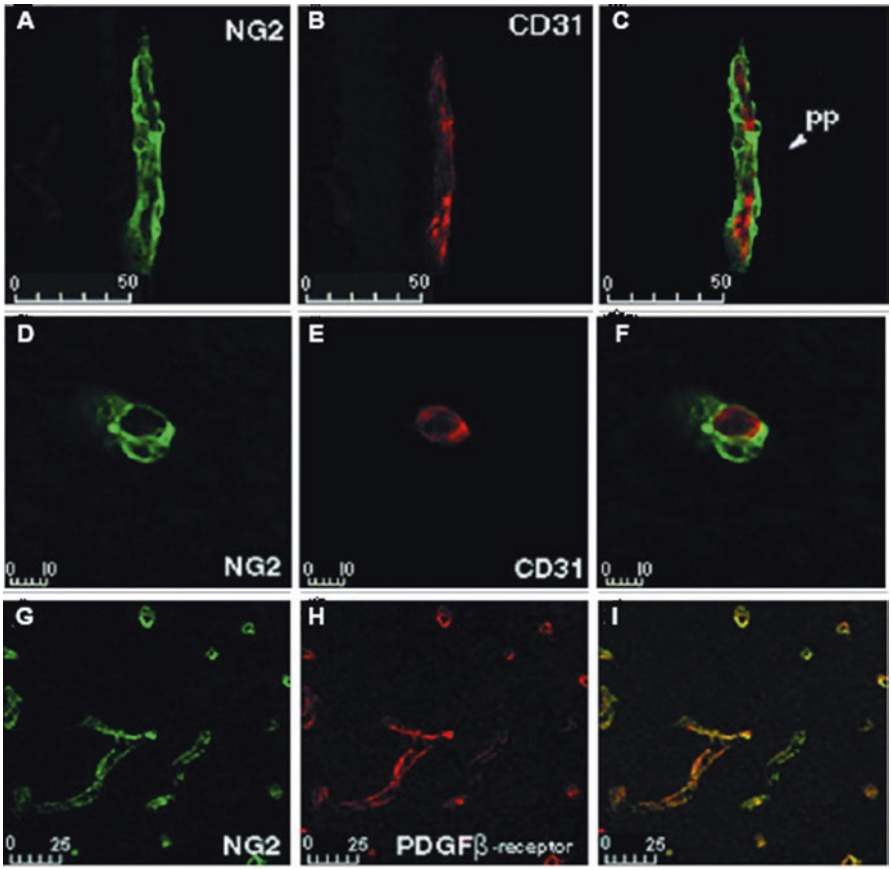

Fig. 2.1 NG2 as a pericyte marker. (**a**–**c**) Double staining for NG2 (**a**; green) and CD31 (**b**; red) in a section of postnatal day 11 mouse retina. The merged image (**c**) reveals the abluminal relationship of pericytes to endothelial cells in this longitudinal view of a developing capillary. pp = primary vascular plexus. (**d**–**f**) Double staining for NG2 (**d**; green) and CD31 (**e**; red) in a section of embryonic day 12 mouse forebrain. The merged image (**f**) clearly shows the abluminal relationship of pericytes to endothelial cells in this capillary cross section. (**g**–**h**) Double staining for NG2 (**g**; green) and PDGFRβ (**h**; red) in a section of embryonic day 12 mouse forebrain. The merged image (**i**) demonstrates the co-localization of both markers on pericytes in the capillary network. Scale bars = 50 μm in (**a**–**c**); 10 μm in (**d**–**f**); 25 μm in (**g**–**i**). (Reproduced with permission from Ozerdem et al. [33])

are downregulated in mature, quiescent vasculature but are dramatically upregulated during vascular remodeling or induced neovascularization. This accounts for the high levels of NG2 seen on pericytes in many types of healing wounds and in tumors, even in adult animals. This phenomenon is consistent with the overall pattern of NG2 expression in many cell types. As a general rule, NG2 is expressed during stages when immature cells are motile and mitotically active but then is downregulated when cells become mature and quiescent [20, 21]. As we will see below, NG2 contributes functionally to the motile, mitotic phenotype of immature cells.

Mechanisms of NG2 Action

As discussed above, NG2 expression is not really specific to a single cell type. Instead, NG2 is expressed by several types of developing cells that exhibit a phenotype characterized by increased mitotic activity and enhanced motility. This type of activated phenotype is critical for the ability of NG2-expressing progenitor cell and tumor cell populations to expand and migrate to new sites. Importantly, a number of studies implicate NG2 as a functional player in the proliferation and motility of these populations. Even though NG2 is a membrane-spanning protein capable of interacting with the cytoskeleton [56–59], it does not appear to possess robust signaling activity of its own. Instead, NG2 promotes proliferation and motility as an auxiliary receptor that enhances signaling through integrins and receptor tyrosine kinase growth factor receptors. In this sense, John Couchman's characterization of membrane proteoglycans as regulators of cell surface domains seems entirely appropriate for NG2 [60].

In the case of growth factors, NG2 has been shown to bind directly to FGF2 and PDGF-A [61, 62] and can therefore act to sequester these factors for optimal receptor activation. This mode of action is similar to that of heparan sulfate proteoglycans, except that with heparan sulfate proteoglycans the growth factors bind to the glycosaminoglycan chains [63], whereas with NG2 they bind to the core protein. As a result of this sequestering activity, NG2-positive cells exhibit more robust mitotic responses to PDGF-A and FGF2 than NG2-negative cells [22, 61, 64, 65].

In the case of integrin signaling, NG2 interacts physically with β1 integrins [66–68], promoting an active integrin conformation and also localizing the integrins to key membrane microdomains. This localization of NG2/β1 integrin complexes is controlled by phosphorylation of the NG2 cytoplasmic domain [69, 70]. Phosphorylation of NG2 at Thr-2256 by protein kinase-C favors localization of the NG2/β1 integrin complex to leading edge lamellipodia, where enhanced integrin signaling promotes cell motility. Phosphorylation of NG2 at Thr-2314 by ERK favors localization of the NG2/β1 integrin complex to apical microprotrusions, where enhanced integrin signaling promotes cell proliferation. We hypothesize that the phosphorylation status of NG2 influences its binding to cytoplasmic scaffolding components such as ERM proteins or PDZ proteins like MUPP1, GRIP1, and syntenin [71–73] that may serve to anchor the proteoglycan in different membrane microdomains.

NG2-Dependent Aspects of Pericyte Function

The first indication of a functional role for NG2 in pericyte biology came from studies of corneal and retinal neovascularization in germline NG2 null mice [64]. Using a technique that mimics retinopathy of prematurity, postnatal day 7 mice were exposed to 75% oxygen for 5 days before returning them to normal oxygen for an additional 5 days. The resulting protrusion of pathological retinal vessels into the

vitreous was much reduced in NG2 null mice compared to wild-type mice, at least partly due to a twofold decrease in the pericyte mitotic index in the NG2 null mice and an accompanying reduction in pericyte number. This pericyte deficit was responsible for a fourfold reduction in pericyte ensheathment of endothelial cells, as reflected by a decrease in the pericyte/endothelial cell ratio from almost 1:1 in wild-type mice to only 1:4 in NG2 null mice [74]. In parallel, using FGF2-containing pellets inserted into the cornea, a fourfold decrease in growth of vessels into the cornea was observed in NG2 null mice, compared to wild-type mice. This difference in corneal neovascularization between wild-type and NG2 null mice was not found with VEGF-containing pellets [61]. A similar discrepancy between FGF2 and VEGF induction of pericyte recruitment into subcutaneous Matrigel plugs [36] suggests that there might be a special relationship between NG2 and FGF2. More detailed studies have subsequently demonstrated that the presence of NG2 on smooth muscle cells is critical for the ability of these cells to proliferate in response to FGF2. NG2 is found to be capable of binding to both FGF2 and the FGF receptor (either FGFR1 or FGFR3) to generate a trimolecular complex that improves interaction of the growth factor with the signaling receptor [61].

Additional information regarding the importance of NG2 in pericyte behavior has come from the use of siRNA to knock down NG2 expression in human pericytes in vitro [41]. NG2-deficient pericytes exhibit a 70% reduction in proliferation and a threefold decrease in PDGF-induced migration compared to control pericytes, in keeping with general expectations for the effects of NG2 on these processes and confirming the results obtained with the in vivo corneal and retinal studies. In pericyte-endothelial cell co-cultures in Matrigel, NG2 knockdown pericytes also exhibit impaired ability to stimulate the formation of endothelial tubes. A role for β1 integrin activation in these deficits (Fig. 2.2a–d) is suggested by finding that NG2 knockdown in pericytes results in a twofold decrease in binding of the HUTS-21 antibody [75] that specifically recognizes the activated conformation of the β1 subunit of the integrin heterodimer [41].

In addition to activating integrin signaling in this *cis* orientation (i.e., when NG2 and β1 integrins are expressed by the same cell), NG2 on the pericyte surface is also capable of *trans* activation of β1 integrin signaling in endothelial cells [68]. This was initially predicted as a possibility based on the ability of soluble recombinant NG2 [76] to stimulate β1 integrin signaling in both human and mouse endothelial cells [67]. Activation of β1 integrin by soluble NG2 (detected via use of the conformationally sensitive antibodies HUTS-21 and 9EG7 [75, 77]) results in increased endothelial cell spreading, migration, and tube formation. Subsequently, pericytes and endothelial cells co-cultured on opposite sides of Transwell membranes were used to demonstrate the ability of cell surface NG2 (i.e., on human pericytes) to activate β1 integrin activation in human endothelial cells [41]. siRNA-mediated knockdown of NG2 in pericytes in these co-cultures results in decreased binding of the activation-specific HUTS-21 antibody to endothelial cells. This decrease in integrin signaling is accompanied by decreased formation of cell-cell junctions in the endothelial monolayer (detected via use of antibody against ZO-1) and by increased

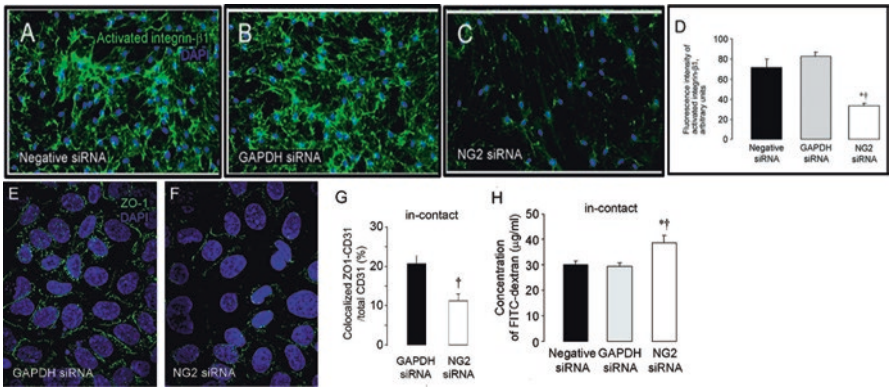

Fig. 2.2 NG2 effects on pericytes and endothelial cells. (**a**–**d**) Pericyte monolayers were immunolabeled with antibody HUTS-21 (**a**–**c**; green) to detect levels of activated β1 integrin (blue = DAPI). Knockdown with NG2 siRNA (**c**) leads to greatly reduced levels of β1 integrin activation (**d**), compared to monolayers treated with (**a**) scrambled (negative) siRNA or (**b**) GAPDH siRNA (**d**). (**e**–**h**) Pericyte and endothelial cell monolayers were cultured on opposite sides of Transwell membranes with 0.4 μm pores. Immunolabeling for ZO-1 (**e**, **f**; green) allows visualization of junctions between endothelial cells (blue = DAPI). Treatment of pericytes with control GAPDH siRNA does not interfere with extensive formation of endothelial junctions (**e**). Treatment of pericytes with NG2 siRNA greatly decreases endothelial junction formation (**f**, **g**). The loss of endothelial junctions in NG2 siRNA-treated co-cultures leads to increased permeability of the endothelial monolayer to FITC-dextran, compared to treatment with scrambled (negative) siRNA or GAPDH siRNA (**h**). $*p < 0.05$ versus negative siRNA; $*\dagger p < 0.05$ versus GAPDH siRNA. (Reproduced by permission from You et al. [41])

leakage of FITC-dextran across the endothelial monolayer (i.e., impaired barrier function) (Fig. 2.2e–h). These results emphasize not only the need for pericyte-endothelial cell interaction during blood vessel formation and function but also the important role played by NG2 in this interaction [41].

In vivo studies of tumor progression using germline NG2 null mice have further extended our understanding of NG2-dependent pericyte function in the context of tumor vascularization. This work has utilized the MMTV-PyMT mouse mammary tumor model [78, 79], as well as a model in which B16F10 melanoma cells [80] are engrafted in the brain. In these models, neither the mammary tumor cells [39] nor the B16F10 melanoma cells [40] express NG2. This allows a specific focus on the role of NG2 in the tumor stroma, of which the vasculature is a major component. Triple labeling for CD31, NG2, and PDGFRβ was used to confirm that pericytes in the vasculature of tumors grown in wild-type mice are strongly positive for NG2. In contrast, pericytes in the vasculature of tumors grown in NG2 null mice are NG2-negative.

In both tumor models, tumor growth is slowed roughly threefold in NG2 null mice compared to that seen in wild-type mice [39, 40]. A major change in the vasculature of both mammary tumors and intracranial melanomas in NG2 null mice is the diminished ensheathment of endothelial cells by pericytes. Pericyte coverage of

endothelial cells is reduced by about 50% compared to that seen in tumors in wild-type mice, reflecting the importance of NG2 in mediating the pericyte-endothelial cell interaction and confirming our in vitro studies with pericytes and endothelial cell co-cultures. This loss of pericyte-endothelial cell interaction is accompanied by large reductions in pericyte maturation (as reflected by expression of αSMA) and by decreased assembly of the vascular basement membrane (as measured by deposition of the basal lamina component collagen IV). These changes in vessel structure result in abnormalities in vessel function. The number of patent tumor vessels in NG2 null mice is only half of that seen in wild-type mice, while vessel leakiness is increased fourfold. Due to these deficits in blood supply to tumors in NG2 null mice, tumor hypoxia is increased severalfold in both mammary tumors [39] and B16F10 tumors [40]. Thus, while the properties of tumor cells are obviously important for tumor growth, these studies underline the importance of effective tumor vascularization in supporting tumor progression. This point has been made in numerous publications [81–83], but our work emphasizes the role of NG2-dependent pericyte function during tumor vascularization, a theme that is also attracting notice by other laboratories [84–86].

In the mammary tumor study, it was noted that many tumor macrophages are NG2-positive and that some changes in macrophage recruitment and phenotype occur in tumors in NG2 null hosts [39]. While this is extremely interesting in light of the importance of tumor macrophages as microenvironmental components that strongly influence tumor progression [87–90], it also indicates that the results from studies with germline NG2 null mice cannot be interpreted solely in terms of NG2 loss by pericytes. Cre-Lox technology was therefore used to produce mice in which NG2 is specifically ablated in either pericytes or in myeloid cells. NG2 floxed mice [91] were crossed with pdgfrb-Cre mice [92] or with LysM-Cre mice [93] to produce pericyte-specific NG2 null (PC-NG2ko) mice [41] or myeloid-specific NG2 null (My-NG2ko) mice [46].

When the studies with B16F10 intracranial tumors were repeated in PC-NG2ko mice, deficits in tumor vascularization and tumor growth were once again detected, even with NG2 still being expressed by tumor macrophages [41]. Reduced brain tumor growth in PC-NG2ko mice (Fig. 2.3h, i) is accompanied by a 30% decrease in pericyte ensheathment of endothelial cells (Fig. 2.3a–c), leading to diminished formation of ZO-1-positive endothelial junctions and reduced assembly of the collagen IV-containing vascular basement membrane (Fig. 2.3d). These structural alterations are accompanied by deficits in vessel function, including diminished vessel patency (Fig. 2.3e), increased vessel leakiness (Fig. 2.3f), and increased tumor hypoxia (Fig. 2.3g) [41]. These results further confirm the importance of NG2-dependent pericyte interaction with endothelial cells during tumor vessel formation and maturation.

In addition to NG2 and β1 integrins, there are of course other molecular interactions that mediate pericyte-endothelial cell communication [6, 94–96]. This is emphasized by our own results with My-NG2ko mice. Specific ablation of NG2 in myeloid cells results in greatly diminished recruitment of macrophages to sites of

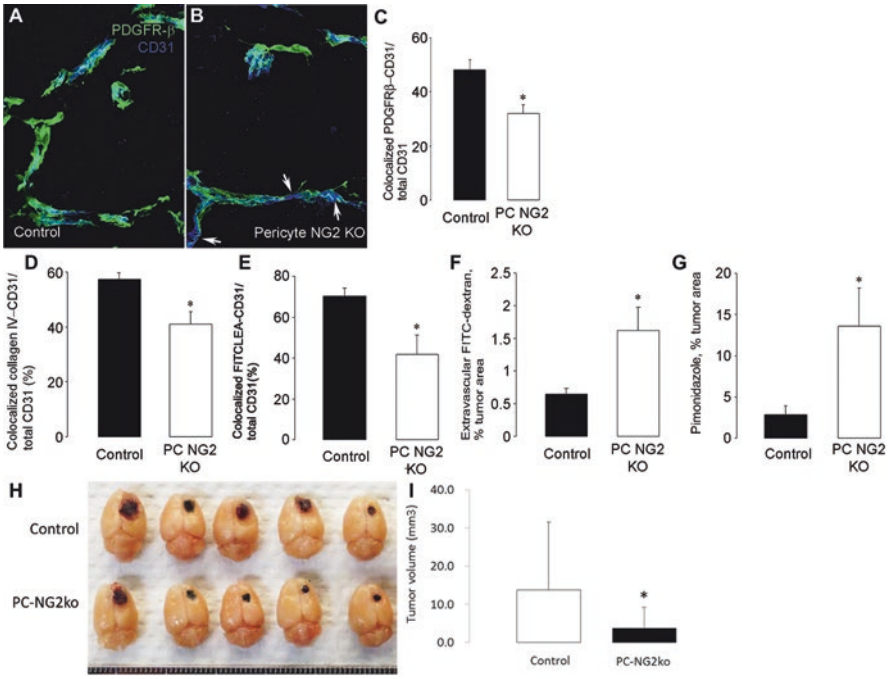

Fig. 2.3 Effects of NG2 ablation on tumor vascularization and growth. (**a**–**c**) Sections from 10-day B16F10 brain tumors in control (**a**) and PC-NG2ko (**b**) mice were double stained for PDGFRβ (green) and CD31 (blue). Z-stacks of confocal images were analyzed for overlap between pericytes and endothelial cells (**c**), revealing decreased pericyte ensheathment of endothelial cells in tumor vessels in PC-NG2ko mice. Arrows indicate vessel segments in which endothelial cells lack pericyte coverage. (**d**) Decreased basement membrane assembly in tumor vessels in PC-NG2ko mice is detected by double staining for CD31 and collagen IV. (**e**) Perfusion with FITC-labeled tomato lectin reveals a decrease in patent tumor vessels in PC-NG2ko mice. (**f**) Leakage of perfused FITC-dextran from tumor vessels is increased in PC-NG2ko mice. (**g**) As a result of vessel deficiencies, tumor hypoxia is increased in tumors in PC-NG2ko mice, as quantified by binding of pimonidazole hypoxia probe. *$p < 0.05$. (Reproduced by permission from You et al. [41]). (**h**, **i**) At 10 days after engraftment, B16F10 tumors are reduced in size in PC-NG2ko mice, compared to controls. *$p < 0.01$. (Reproduced by permission from Yotsumoto et al. [46])

inflammation, including intracranial B16F10 tumors [46] and demyelinated spinal cord lesions [44, 47]. The tumor macrophage deficit in B16F10 tumors in My-NG2ko mice leads to greatly diminished pericyte-endothelial cell interaction, even though the pericytes in these mice retain NG2 expression. As a result, vascular abnormalities in these tumors are even more pronounced than in PC-NG2ko mice. We propose that tumor macrophages are responsible for generating a signal that induces pericyte-endothelial cell interaction that is independent of NG2 [46]. The nature of this signal remains to be identified, as does the mechanism by which NG2 governs macrophage recruitment.

Future Prospects

In addition to its utility for distinguishing pericytes from endothelial cells, the NG2/CD31 combination is also very useful for quantifying the extent of pericyte ensheathment of endothelial cells as one means of characterizing the properties of pathological tumor blood vessels [39–41, 46, 74]. These same publications also discuss a variety of additional ways of characterizing both the structure and function of tumor blood vessels, including the effects of pericyte-endothelial cell interactions on basement membrane assembly, endothelial junction formation, vessel patency, and vessel leakiness. These strategies are likely to be much more informative for understanding tumor pathology than typical determination of vessel density. Although widely used to characterize changes in tumor vascularization, vessel density does not reveal anything about vessel functionality and thus is difficult to relate in a meaningful way to tumor growth.

The discovery that pericytes are very early participants, along with endothelial cells, in vessel morphogenesis is not only enlightening from the standpoint of basic vascular biology but may also have therapeutic implications. For example, the reality of anti-angiogenic therapy for solid tumors has never quite lived up to the initial promise offered by this strategy. One reason for this may be that most anti-angiogenic approaches have targeted only the vascular endothelium. Based on the idea that a multi-targeted approach may offer improved results, some attempts have been made to target both pericytes and endothelial cells in the tumor vasculature via use of different kinase inhibitors [97–99]. For dual targeting strategies, NG2 might provide an additional means of targeting pericytes. In this respect, NG2 antibodies have been used with some success in preclinical studies as a means of inhibiting neovascularization [74, 84–86]. Peptides that bind to NG2 offer another potential means of blocking NG2 function and/or delivering payloads that interfere with other aspects of pericyte function [100, 101]. Conversely, NG2-binding peptides could be used to deliver payloads that stimulate blood vessel development in pathologies such as stroke or myocardial infarction in which neovascularization needs to be accelerated.

Other areas of both intellectual and therapeutic importance will include achieving improved understanding of the relationship between pericytes and MSCs and between pericytes and myeloid cells. As we have seen, pericytes and MSCs both express NG2 and are thought by some workers to be somewhat interchangeable in terms of identity and function [49, 51, 53, 102, 103]. Future goals will include defining the putative interconversion between these two cell types. This scenario could offer opportunities to use pericytes as a source of progenitors for repair of mesenchymal tissues or conversely to harvest MSCs to use in stimulating neovascularization. On the other hand, while myeloid cells may be less likely to assume pericyte-like functions, macrophages can nevertheless be surprisingly perivascular in their localization [36, 88, 104, 105]. Moreover, they exert a powerful influence on tumor vascularization [87, 90, 106], including the ability to control pericyte interaction with endothelial cells [46]. In this respect, macrophages may almost be regarded, along with pericytes and endothelial cells, as a third cellular component of tumor blood vessels, a relationship that certainly merits additional attention from both mechanistic and therapeutic standpoints.

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Chapter 3
Pericytes for Therapeutic Bone Repair

Carolyn A. Meyers, Joan Casamitjana, Leslie Chang, Lei Zhang, Aaron W. James, and Bruno Péault

Abstract Besides seminal functions in angiogenesis and blood pressure regulation, microvascular pericytes possess a latent tissue regenerative potential that can be revealed in culture following transition into mesenchymal stem cells. Endowed with robust osteogenic potential, pericytes and other related perivascular cells extracted from adipose tissue represent a potent and abundant cell source for refined bone tissue engineering and improved cell therapies of fractures and other bone defects. The use of diverse bone formation assays in vivo, which include mouse muscle pocket osteogenesis and calvaria replenishment, rat and dog spine fusion, and rat non-union fracture healing, has confirmed the superiority of purified perivascular cells for skeletal (re)generation. As a surprising observation though, despite strong endogenous bone-forming potential, perivascular cells drive bone regeneration essentially indirectly, via recruitment by secreted factors of local osteo-progenitors.

Keywords Pericyte · Blood vessel · Osteogenesis · Mesenchymal stem cell · Bone · Spinal fusion · Non-union · Tunica adventitia · Perivascular cell · Stem cell

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Introduction

Pericytes are stellate cells in close contact with endothelial cells and embedded within a basal lamina, which form a discontinuous layer in capillaries (<10 μm diameter), and continuous one around microvessels (diameter 10–100 μm) [1]. First described in 1873 by C. Rouget as, visionarily, contractile cells regulating blood flow, it is Zimmermann who coined the term *pericyte* in 1923 to describe cells, also known as *mural cells*, structurally supporting the vasculature [2]. Promotion of angiogenesis, blood vessel diameter regulation [3], and maintenance of vascular integrity and permeability [4] are the main functions attributed to pericytes, through direct cell contact and communication.

As early as the 1970s were pericytes suggested to be also involved in tissue regeneration [5]. It was, however, not before the first decade of this century that definitive experimental evidence was gained that pericytes are native ancestors of mesenchymal stem cells (MSC), the existence of which had been previously documented exclusively in long-term cultures of vascularized organs [6]. Pericytes can differentiate into chondrocytes, adipocytes and osteocytes, regardless of their tissue of origin [6–8], as well as skeletal and cardiac muscle [6, 9], and myofibroblasts at the origin of pathologic fibrosis [10, 11]. Pericytes also support hematopoiesis [12–16] and can modulate immune-inflammatory reactions [17].

Among the potential uses of pericytes/progenitor cells for tissue engineering, the application to bone tissue is most commonly studied [18]. The bone is a richly vascularized organ, and the reaction to bone injury includes the processes of osteogenesis and vasculogenesis that go hand in hand. The theory that mural cells participate in endogenous bone tissue repair has long been posited. Before the advent of cell lineage tracing, the use of intravascular dyes that label mural cells suggested that pericytes participate in osteochondral repair [1, 19]. Later studies using smooth muscle actin (SMA) reporter animals also suggested that endogenous mural cells give rise to bone cells after fracture [20]. SMA is a non-specific marker which labels some pericytes, smooth muscle, and fibroblasts/myofibroblasts. Therefore, to our knowledge, the direct participation of bone-associated pericytes in repair has never been definitely shown. Nevertheless, these observations of the reparative potential of endogenous SMA+ cells, combined with the known mesenchymal progenitor cell properties of human pericytes [6], gave impetus for the use of exogenous pericytes for bone tissue repair. The osteogenic potential of human pericytes and other perivascular cells has been examined in both ectopic and orthotopic models. These findings are briefly reviewed below.

Identification and Purification of Perivascular Cells for Bone Repair

The possibility of using human pericytes/perivascular progenitor cells to speed bone repair was made possible by prior studies that used the cell surface marker CD146, also known as Mel-CAM (melanoma cell adhesion molecule), for the identification and purification of pericytes ([21, 22]; see also [23] for a review). Of note, CD146 expression is by no means specific to pericytes, and as a heterophilic cell-cell adhesion molecule, it is often upregulated when diverse cell types adopt a location on the outside aspect of the endothelial cell [24]. CD146 is also expressed by endothelial cells [25], vascular smooth muscle cells (VSMC) [26], fractions of lymphocytes [27], and tumor cells [28]. Therefore fluorescence-activated cell sorting for a combination of cell surface markers (CD146+CD34-CD31-CD45-) is most commonly used by our group to identify a pericyte among uncultured stromal populations [6, 29, 30]. The potential non-specific identity of CD146+ progenitors has led some investigators to favor the term tissue-specific progenitor cells over pericytes. A presumably analogous CD146+ progenitor cell can also be identified among culture-derived cell populations [22]. CD146+ pericytes/progenitors have been examined for their bone-forming potential alone [31] or in combination with other perivascular mesenchymal progenitor cells derived from the tunica adventitia and typified by CD34 expression and absence of other endothelial cell and pericyte markers (termed adventitial cells or adventitial progenitor cells) [29, 32]. When CD146+ pericytes and CD34+ adventitial progenitor cells are used in combination, they are most commonly referred to as perivascular stem cells or perivascular stromal cells (PSCs) [29], referring to their shared perivascular location. At least in the context of bone tissue engineering, PSCs are most commonly derived from subcutaneous adipose tissue [33]. The rationale for adipose derivation is based principally on the easy access and dispensability of this tissue depot. Once in culture, PSCs are able to undergo differentiation toward multiple mesenchymal lineages under appropriate culture conditions (Fig. 3.1), including osteoblastogenic (Fig. 3.1a–c) and adipocytic cell fates (Fig. 3.1d). Importantly the umbrella term of PSC is used, despite the clear understanding that these perivascular progenitor cells differ in their location and cellular morphology within the vascular wall, markers for in situ detection, frequency within different tissues, and gene network profiles [34]. The functional relevance of cellular differences between CD146+ pericytes and CD34+ adventitial progenitor cells for bone repair outcomes is as yet not known.

We have described methods general to all human organs: fresh tissues undergo mechanical and enzymatic digestion prior to cell isolation and immunolabeling for FACS, with at least one pericyte, endothelial, and hematopoietic cell marker. Sorted populations are seeded in endothelial growth medium 2 (EGM-2) in gelatin-coated plates and passaged using 20% FCS-supplemented Dulbecco's modified Eagle's medium [6]. The immunophenotype CD146+CD34-CD31-CD45-CD56- successfully isolates pericytes in multiple tissues including the skeletal muscle, bone marrow, white adipose tissue, placenta, pancreas, umbilical cord, heart, kidneys,

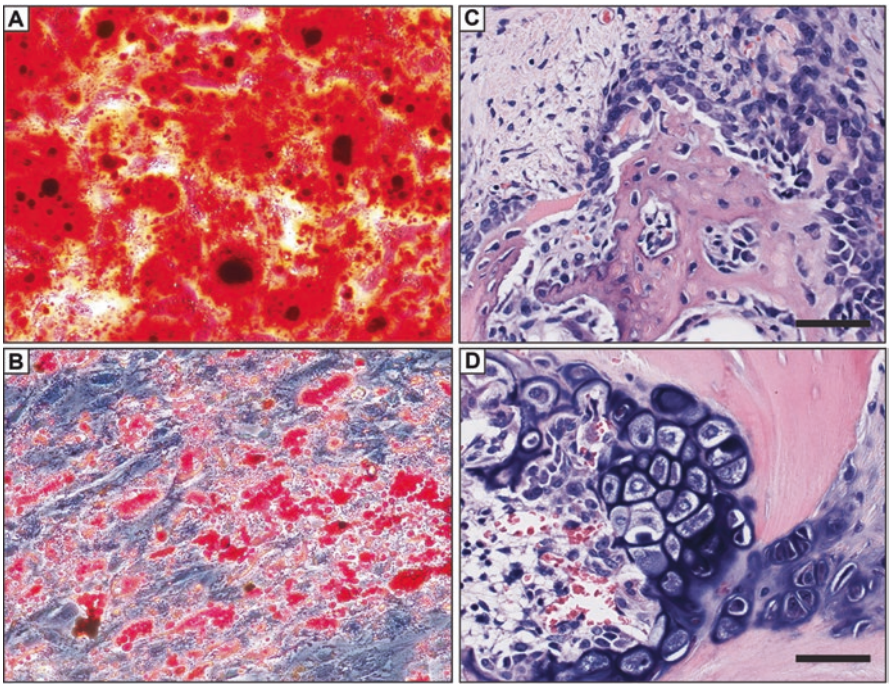

Fig. 3.1 Differentiation of human perivascular stem cells in vitro and stimulation of an osteochondrogenic program in vivo. (**a**, **b**) PSCs are a multipotent progenitor cell type in vitro. (**a**) Human PSCs were cultured in the presence of osteogenic differentiation medium. Frank confluent mineralization was observed among PSC under inductive culture conditions (Alizarin red staining shown). (**b**) Conversely, intracellular lipid accumulation can be visualized within PSC under appropriate adipogenic conditions (Oil red O staining shown). (**c**, **d**) PSC implantation in a rat spinal fusion model induces a combination of intramembranous and endochondral bone formation. (**c**) Woven bone formation, and prominent bone lining osteoblasts in areas of intramembranous bone formation by PSC. (**d**) Chondrocyte hypertrophy and mineralization in areas of endochondral bone formation induced by PSC. Scale bar: 25 um

infrapatellar fat pad, and liver [6, 9, 14, 18, 29, 30, 35–38, 39–41]. Of note, the same immunophenotype has been used to isolate pericytes from other mammalian species, including dog [42], sheep [43], and horse [44, 45], offering large animal models of perivascular cell-mediated tissue regeneration.

Pericytes and Ectopic Bone Formation

Animal models of ectopic bone formation have been used to confirm the capacity for in vivo osteogenic differentiation of implanted human pericytes. Human adipose tissue (AT)-derived CD146+ pericytes have been observed to directly ossify

when implanted in a *SCID (severe combined immunodeficiency)* mouse muscle pouch [23]. Inconspicuous bone is produced when AT pericytes are implanted on a collagen sponge carrier, which represents a relatively inert substance with little osteoinductive properties [29]. In contrast, when AT pericytes are implanted intramuscularly using an osteoinductive demineralized bone matrix (DBM) carrier, robust bone formation is observed [29]. In somewhat similar observations, other groups have shown that CD146+ AT-derived progenitor cells do not form significant bone when implanted in a subcutaneous ossicle model [22]. In contrast, bone-associated (bone marrow or periosteum) CD146+ progenitor cells drive robust bone formation in the same subcutaneous ossicle model [22]. Head-to-head comparisons of AT-derived pericytes and adventitial cells from the same patient sample have been performed [23]. Both perivascular cell types induce vascularized ectopic bone and without substantive differences in the degree of bone formation [23]. This pilot study demonstrated that pericytes and adventitial cells have a similar bone-forming potential and laid the framework for later studies in which these two cell populations were combined. Next, experiments have been performed in which uncultured AT-derived PSCs (combined pericytes + adventitial cells) were implanted intramuscularly and compared with an unsorted/uncultured stromal population from the same patient's adipose sample (termed stromal vascular fraction, SVF) [29]. Here, a DBM scaffold was again used. Results showed that independent of cell number, AT-derived PSC led to more robust intramuscular ossification in comparison to SVF from the same patient sample using quantitative metrics of bone formation by micro-computed tomography, histomorphometry, and select immunohistochemical markers of the bone [29]. Increased bone formation among AT-PSC implants was accompanied by a significant increase in vascularity of the implant site, accompanied by increased elaboration of VEGF (vascular endothelial growth factor) [38]. Ectopic bone formation induced by AT-PSC was also associated with an altered inflammatory milieu within the early wound environment [17]. Overall, these studies showed that AT-derived pericytes or AT-derived adventitial cells either alone or combined result in significant ectopic bone formation. Moreover, and for the first time, it was observed that these FACS-purified cell populations outcompete unpurified stromal cell populations from the same patient sample in terms of bone-forming efficacy.

Pericytes in Calvarial Defect Regeneration

The extent to which AT-derived PSC can induce bone repair was first examined in a mouse calvarial defect model [33]. Here, equal numbers of unpurified SVF or PSC from the same patient's adipose tissue were implanted in a non-healing, circular, full-thickness calvarial defect of the parietal bone. Cells were implanted on a hydroxyapatite-coated polymeric scaffold for an additional osteoinductive effect. Similar to intramuscular implants, radiographic and histologic analysis showed

AT-derived PSC led to a significant increase in bone regenerate at the defect site over an 8-week time course. In comparison, unpurified SVF from the same patient had no statistically appreciable benefit in comparison to a scaffold without cells. In this xenograft model, sparse but present human-specific antigens were detectable within the healing bone defect. Again, and in similarity to intramuscular studies, bone defect vascularity was significantly increased with PSC treatment. Thus, across both ectopic and bone repair models, AT-derived human PSCs have conserved features upon transplantation, including pro-osteogenic/pro-vasculogenic effects of a greater magnitude than unpurified stromal cell fractions. Whether these findings correlated with the enrichment of osteoinductive PSC, or conversely the elimination of an inhibitory cell type within the heterogeneous stroma of SVF, is still a matter of conjecture.

Pericytes in Spinal Fusion

Spinal fusion represents a more functionally demanding environment for a bone graft substitute and represents an assay for the production of contiguous and biomechanically sound bone tissue. The use of AT-derived human PSC as a cellular therapy for bone grafting has been validated in a rat posterolateral lumbar spinal fusion model. In these studies, human AT-PSC implantation was performed across three cell densities in rats, using a DBM scaffold as a moldable carrier. PSC demonstrated a dose-dependent increase in ossification, increase in bone deposition, increase in measurements of bone strength, and complete fusion between lumbar bone segments in all rats [46]. In this model, both intramembranous (Fig. 3.1c) and endochondral bone formation (Fig. 3.1d) was spurred on by PSC implantation. Like in other studies, new bone regenerate was observed to be a product of both direct osteodifferentiation and host osteoblastogenesis. Like the calvarial defect model, paracrine-mediated bone formation of rat origin predominated [46]. In follow-up studies, Lee et al. extended these observations to rats rendered osteoporotic by ovariectomy. Here, increased numbers of implanted human AT-PSC were required to surmount the hormonal changes of estrogen withdrawal [47].

Pericytes for Non-union Fracture Healing

Atrophic non-union is associated with biological failure of fracture healing. Animal studies have shown the vascular ingrowth within atrophic non-union is much reduced at early timepoints [48]. In combination with the observation, the mesenchymal progenitor cell content within fibrous non-unions is reduced, and the proliferative and osteogenic capabilities of these non-union derived cells are likewise reduced [49]. CD146+ AT pericytes were examined in a well-established model of

rat tibial atrophic non-union [48, 50]. Human AT pericytes were percutaneously injected 3 weeks after the establishment of fibrous non-union. Results showed that pericyte injection increased fracture callus size and increased mineralization, eventually resulting in increased bone union [50]. Like in other models, the efficacy of pericyte treatment was primarily a paracrine phenomenon, and in fact species-specific immunohistochemistry failed to later identity residual human cells. These data suggest that at least in the inhospitable microenvironment of atrophic non-union, the benefit of pericytes primarily resides in their trophic abilities.

Discussion

Pericytes have crossed the limits of vascular biology and entered the field of regenerative medicine via their mesenchymal stem cell-cultured progeny. Advantages of using conventional MSCs include the simplicity of the derivation method and possibility to obtain large numbers of cells. On the negative side, MSCs are the cultured product of a heterogeneous mixture of unseparated cells, and in vitro growth involves cell exposure to animal proteins, hence chances of xenogeneic immunization, and entails risks of bacterial contamination and genetic instability. There have been occasional reports of MSC malignant transformation [51]; principally, it is increasingly accepted that MSC recruitment to the tumor stroma can favor cancer development [52]. For all these reasons, it might be beneficial to use purified, non-cultured perivascular cells in place of culture-derived MSCs for cell therapies. Bone repair has been the first envisioned therapeutic use of pericytes and adventitial perivascular cells. Bone structure is relatively simple, and targeted interventions, such as non-union fracture reduction or spine fusion, are usually not life-threatening, providing convenient models in which to gain a proof-of-concept demonstration of the therapeutic usability of perivascular presumptive MSCs. Importantly, PSCs also appear to represent a reliable source of autologous therapeutic cells, regardless of age, gender, and body mass index [30]. Experimentally, as described in this article, pericytes and adventitial cells purified from human or canine adipose tissue exhibited dramatic bone-forming potential in all autologous and xenogeneic in vivo assays performed, including calvarial regeneration and muscle pouch osteogenesis in mice, spine fusion in rats and dogs, and non-union fracture repair in rats. In these tests, PSCs performed at least as well as conventional MSCs are significantly better than the plain stromal vascular fraction. The bone produced following PSC transplantation was histologically normal and mechanically competent. These data illustrate the propensity of perivascular cells to differentiate along the bone cell lineage: culturing human adipose tissue-derived pericytes on a hard hydrogel substrate was sufficient to induce osteogenesis [53], and transcriptome analysis in single adventitial cells revealed expression of genes associated with osteogenic commitment and differentiation [34], which may have an important significance in cardiovascular pathology since adventitial progenitor cells have been shown in

Table 3.1 Summary of in vivo orthopedic models of PSC application

Model	Species/strain	Human cell used
Intramuscular implant	*SCID* mouse	AT pericyte, AT-adventitial cell, AT-PSC
Calvarial bone defect	*SCID* mouse	AT-PSC
Spinal fusion	Athymic rat	AT-PSC
Non-union	SD rat	AT pericyte

the mouse to drive blood vessel calcification, also known as arteriosclerosis [54]. However, even though PSCs are clearly endowed with strong osteogenic potential, a paradoxical yet recurrent observation is that over time little chimerism can be detected in newly developed bone following xenogeneic PSC transplantation, suggesting these perivascular progenitors merely mediate bone formation by recruiting local osteogenic cells and reinforcing the growing belief that MSCs and related tissue regenerative cells function largely via trophic/chemotactic factor secretion [55]. Do pericytes and adventitial cells, which all contribute to MSC cultures and are arranged along blood vessels as a hierarchy of regenerative cells [34], play distinct roles as either osteoblastic progenitors or trophic secretory cells during osteogenesis? This important question is currently under investigation in experiments where either perivascular cell subset or the combination of the two is administered in the same injury setting.

Although recognized in all tissues with canonical markers and characteristic perivascular distribution, pericytes and adventitial cells represent heterogeneous cell populations which also exhibit organ-restricted anatomic, phenotypic, and functional specializations, the complexity of which is being gradually uncovered [11]. Regarding bone formation, we have recently identified novel surface markers which typify PSC subsets endowed with higher osteogenic potential (Ding, Meyers et al., unpublished results), as was already recently done for pro-fibrotic ability [10] and chondrogenic capacity [56]. Ongoing studies will converge to explain the bone healing effect of pericytes and other regenerative perivascular cells, both natively in situ and following purification and transplantation, and contribute to the development of a refined therapeutic product (Table 3.1).

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Chapter 4
Pericyte Biology in Zebrafish

Nabila Bahrami and Sarah J. Childs

Abstract The zebrafish is an outstanding model for studying vascular biology in vivo. Pericytes and vascular smooth muscle cells can be imaged as they associate with vessels and provide stability and integrity to the vasculature. In zebrafish, pericytes associate with the cerebral and trunk vasculature on the second day of development, as assayed by *pdgfrβ* and *notch3* markers. In the head, cerebral pericytes are neural crest derived, except for the pericytes of the hindbrain vasculature, which are mesoderm derived. Similar to the hindbrain, pericytes on the trunk vasculature are also mesoderm derived. Regardless of their location, pericyte development depends on a complex interaction between blood flow and signalling pathways, such as Notch, SONIC HEDGEHOG and BMP signalling, all of which positively regulate pericyte numbers.

Pericyte numbers rapidly increase as development proceeds in order to stabilize both the blood-brain barrier and the vasculature and hence, prevent haemorrhage. Consequently, compromised pericyte development results in compromised vascular integrity, which then evolves into detrimental pathologies. Some of these pathologies have been modelled in zebrafish by inducing mutations in the *notch3*, *foxc1* and *foxf2* genes. These zebrafish models provide insights into the mechanisms of disease as associated with pericyte biology. Going forward, these models may be key contributors in elucidating the role of vascular mural cells in regulating vessel diameter and hence, blood flow.

Keywords Pericytes · Vascular smooth muscle cells · Zebrafish · *pdgfrb* · *notch3* · *tagln* · *acta2* · Neural crest · Mesoderm · Sclerotome

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Early Development of the Zebrafish Vascular System

Zebrafish as a Model for Studying Blood Vessel Development

The optical clarity, rapid development and genetic accessibility of the zebrafish model have allowed us to make enormous strides in understanding the molecular and morphological processes of angiogenesis during development. These include insights into artery-vein specification, tip cell selection, guidance, patterning and the role of blood flow during vascular development. A shift in focus from understanding endothelial cell biology to the genesis and maturation of vascular mural cells is providing new insights into the process of angiogenesis. Vascular mural cells interact with the abluminal surface of endothelial cells to provide stability and integrity to the vasculature [15]. Vascular mural cells consist of smooth muscle cells and pericytes. Although there are strong similarities between the two cell types in terms of gene expression, there are also key differences [22].Vascular smooth muscle cells (vSMCs) wrap around larger blood vessels to provide contractility and support, while pericytes are spaced sporadically along the smaller vessels such as the arterioles, venules and capillaries. In contrast to vSMCs, pericytes do not wrap around vessels, rather they extend their long processes along the surface of the endothelium.

In zebrafish, vascular endothelial cells arise from angioblasts of the lateral mesoderm and become specified at the end of gastrulation (approximately 10 hours post fertilization (hpf)) [39, 44]. First, angioblasts with an arterial identity migrate medially to initiate the formation of the dorsal aorta in the trunk [13, 24, 28, 38]. This is followed by the medial migration of venous-fated angioblasts to form the posterior cardinal vein [24, 28, 31, 38]. In the process of vasculogenesis, these first vessels lumenize and connect with the heart to start a rudimentary blood flow [54]. Secondary angiogenic sprouts then emerge from these vasculogenic vessels to vascularize the brain [8, 47, 49], heart [19], gut [18, 21, 27] and trunk [24], among other organs.

One of the strengths of the zebrafish model is that any defects in vascular development become readily visible through in vivo microscopic observations. Vascular integrity can be easily assessed in the developing zebrafish embryos by detecting the presence of haemorrhage, typically in the brain, and starting around 48 hours post fertilization (hpf). Numerous vascular integrity mutants were isolated in early genetic screens in zebrafish [45]. While some mutants were later shown to have endothelial survival defects [41], other vascular stability mutants were suggestive of primary defects in mural cell development [7, 30, 34]. On the cerebral vasculature, mural cells appear as early 52 hpf, as detected by transmission electron microscopy. The presence of mural cells early during development indicates that perivascular mural cells develop concomitantly with the endothelium and play a crucial role in vascular development, stabilization and maturation.

Developmental Origin of Pericytes in Zebrafish

Determining the Origin of Cerebral Pericytes Using Mutants and Morphants

In vertebrates such as the chick, vascular mural cells of the face and forebrain are largely derived from the neural crest cells [5, 12]. Recently, multiple approaches have been taken to elucidate the origins of head vascular mural cells in the zebrafish. In general, the markers α-*smooth muscle actin (acta2)* and *transgelin (tagln/sm2)* are used to identify smooth muscle cells. The *platelet-derived growth factor receptor β (pdgfrβ), notch receptor 3 (notch3), neural/glial antigen 2 (ng2)* and *chondroitin sulphate proteoglycan 4 (cspg4)* are used to identify pericytes.

The neural crest has been tested as a potential origin for head vascular mural cells in zebrafish since it gives rise to head mural cells in other vertebrates. In zebrafish, neural crest ablation can be achieved by the double knockdown of both *forkhead box d3* (*foxd3)* and *transcription factor ap2α* (*tfap2)* transcription factors [50]. *foxd3;tfap2* double morpholino knockdown embryos show a decrease in the number of *acta2:EGFP*$^{+ve}$ smooth muscle cells around the ventral aorta and the pharyngeal arch arteries, suggestive of a neural crest origin of these mural/smooth muscle cells. Furthermore, single knockdown of either *foxd3* or *tfap2* led to a reduction, but not a complete loss, of *acta2*-positive smooth muscle cells [52].

In terms of the origin of head pericytes, at 72 hpf, *foxd3* knockdown resulted in a decrease in the number of *pdgfrβ*$^{+ve}$ cells, suggesting that pericytes of the cerebral vasculature are also neural crest derived [51]. Similarly, a second study by Ando et al. found that *foxd3*;*tfap2a* double morphants had a decrease in *pdgfrβ*$^{+ve}$ cell numbers in the forebrain, specifically in the choroidal vascular plexus [2]. However, *foxd3*;*tfap2a* double morphants had mural cells still present in the base of the hindbrain, specifically around the basal communicating artery, posterior communicating segment and basilar artery, suggesting that hindbrain mural cells are not neural crest derived [2]. Subsequently Ando et al. interfered with the formation of the paraxial mesoderm by knocking down *tbx6* using morpholino, there was a decrease in mural cell numbers in the hindbrain, suggesting that hindbrain mural cells have a mesodermal origin [2]. Taken together, these gene knockdown experiments provide strong evidence that the brain pericytes (with the exception of hindbrain) and ventral head smooth muscle cells are neural crest derived, while hindbrain pericytes are mesoderm- derived in zebrafish. However, a caveat of these experiments is that morpholino knockdowns frequently have off-target effects [29].

Lineage Tracing of Cerebral Pericytes Using Transgenic Zebrafish

In order to demonstrate a direct lineage relationship between neural crest and mesoderm with head vascular mural cells, ‘switch’ reporters, activated by the expression of Cre recombinase, under lineage-specific promoters (neural crest or lateral mesoderm) have been used.

Transgenic *SRY-box 10:Cre* (*sox10:Cre*) is expressed in early neural crest cells. When combined with a switch reporter, Tg*(ubi:loxP:eGFP:loxP:mCherry)*, it allows the tracing of migratory routes of neural crest cells in the pharyngeal arches, ventral aorta and bulbus arteriosus of the zebrafish. Using this reporter combination, neural crest cells can be seen moving from the sixth aortic arch towards the ventral aorta and covering the ventral aorta by 80 hpf [9]. By 108 hpf, neural crest-derived cells have completely covered the ventral aorta and now begin to cover the bulbus arteriosus. The fibroblast growth factor (FGF) signalling pathway is critical for the migration of neural crest cells towards the ventral head vessels [9]. Thus, in agreement with the knockdown studies, lineage tracing supports a neural crest origin for vascular smooth muscle cells of the ventral aorta.

Unlike in mammals and in birds, where brain pericytes are neural crest derived, the origin of zebrafish brain pericytes was unknown until recently. Using transgenic zebrafish, Ando et al. observed expression of the *pdgfrβ:GFP* reporter at the eight-somite stage in neural crest [2]. Next, using a 'switch' lineage strategy involving pericytes co-expressing *sox10*:Cre and a *pdgfrβ*-driven reporter, Tg(*UAS:loxP-mCherry-loxP-mVenus*), switched mVenus^{+ve} cells were observed in the pharyngeal arches, anterior middle mesencephalic central artery (MMCtAs), choroidal vascular plexus and forebrain at larval stages [2]. This pattern of expression indicated that *pdgfrβ* is expressed in cells that had *sox10* expression at some point during their development. Both expression of pdgfrβ in neural crest, and these lineage experiments support a neural crest origin for brain pericytes in the forebrain. Alternatively and in agreement with the morpholino knockdown studies, there were no mVenus^{+ve} mural cells in the hindbrain, further supporting a non-neural crest origin for hindbrain pericytes [2]. When a *tbx6:*Cre line was used to activate the *pdgfrβ*-driven switch line, positive cells were seen around the hindbrain vessels, suggesting a mesodermal origin for hindbrain pericytes, and also confirming morpholino knockdown data [2].

Developmental Origins of Trunk Pericytes

Determining the Origin of Trunk Pericytes Using Mutants and Morphants

To identify whether there is a mesodermal origin of trunk mural cells in zebrafish, genetic Hand2, 'hands off', mutants (han^{s6}) with defective lateral mesoderm formation were used. Hand2 mutants have no *sm22α/tagln* expression, suggesting that mural/smooth muscle cells around the dorsal aorta in the zebrafish trunk are derived from the lateral mesoderm [42]. As a control, cloche (clo^{s5}) mutants that lack most of their endothelial cells due to a mutation in the *neuronal PAS domain protein 4-like (npas4l)* gene were used. *cloche* mutants were found to maintain *tagln* expression around the aortic floor. Together, these results suggest that in zebrafish mural cells form independently from endothelial cells, unlike in chick where they arise from the transdifferentiation of endothelial cells [3].

Lineage Tracing of Trunk Pericytes Using Transgenic Zebrafish

To test the origins of trunk pericytes, Ando et al. compared the expression of pericyte markers between the *foxd3/tfap2a* morphants (lacking all neural crest) and *tbx6/hand2* double morphants (severe disruption to paraxial and lateral mesoderm). *pdgfrβ:*GFP-expressing mural cells were observed along the dorsal aorta and the intersegmental vessels in morphants lacking neural crest cells, but not in morphants with mesodermal defects. The use of these double knockdown morphants provides one line of evidence for a paraxial and lateral plate mesodermal origin of trunk mural cells [2].

Genetic lineage tracing using Cre recombinase has also been used to definitively determine the lineage of trunk pericytes. *Pdgfrβ:gal4* driving a Tg(*UAS:loxP-mCherry-loxP-mVenus*) switch cassette was crossed to a Cre driver under *sox10* or *tbx6* promoters to activate Cre in neural crest or mesoderm, respectively. Labelling of trunk pericytes, as observed by switched mVenus expression, only occurred with the mesoderm Tg*(tbx6:Cre;my17:EGFP*) driver, supporting a lateral plate mesoderm lineage for trunk *pdgfrβ*-expressing cells [2]. In a separate study, no overlap was found between a neural crest-expressing transgenic, *sox10:RFP*, and a smooth muscle reporter, Tg(*tagln:EGFP*), in the trunk [46].

There is also evidence to suggest that sclerotome cells, derived from paraxial mesoderm, contribute to the pericyte lineage. The photo-convertible protein Kaede was expressed in the sclerotome, driven by the *twist1* promoter. At 4 dpf, Kaede expressed in sclerotome was photo-converted to red. Co-expression of the photo-converted Kaede and smooth muscle *tagln:EGFP* was observed around the dorsal aorta, supporting a sclerotome origin for some trunk mural cells [46]. Stratman et al. were able to observe a few cells migrating from the sclerotome to the dorsal aorta using time-lapse imaging of the Tg(*tagln:nls-egfp-2a-cfp-f)*^{y450} reporter at 2.5 dpf [46]. Together, these studies provide support for a mesodermal and potentially sclerotomal origin of trunk pericytes and smooth muscle cells.

Spatiotemporal Pericyte Marker Expression in the Developing Zebrafish Brain

Pdgfrβ serves as one of the earliest markers of pericytes in zebrafish. In mammals, Pdgfbb ligand expressed in the endothelium signals via its receptor, Pdgfrβ, in pericytes to promote pericyte recruitment to the blood vessels [23, 33]. In the zebrafish head, in situ hybridization reveals *pdgfrβ* expression in the neural crest at 20 hpf [14]. At 48 hpf, cells expressing the pericyte markers *notch3* and *pdgfrβ* appear around the larger arteries and veins of the brain, including the basilar artery and the primary hindbrain channels [51]. Wang et al. observed the *pdgfrβ*- and *notch3*-expressing cells in the brain to be Y-shaped with processes extending at vessel branch points, features similar to a typical pericyte morphology [51]. This early pericyte population also proliferates. At 48 hpf, there are ~20 *pdgfrβ*$^{+ve}$ cells associating with larger arteries,

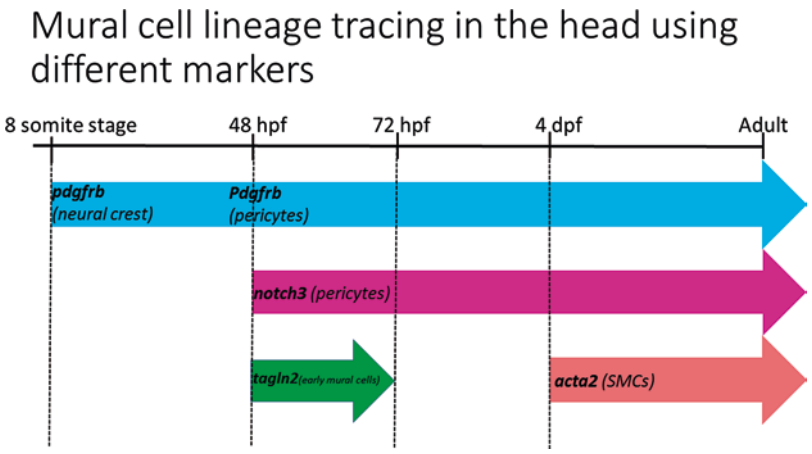

Fig. 4.1 A timeline outlining both the expression of mural cell markers associating with the cerebral vasculature and the cell types they mark at specific stages of development

while at 56 hpf, this number increases to ~50 *pdgfrβ*$^{+ve}$ cells. These pericyte numbers are consistent with increasing pericyte coverage through development.

More recently, transgenic lines have been used to track pericyte appearance on cerebral vessels. Using a BAC transgenic line with GFP knocked into the zebrafish *pdgfrβ* locus, *TgBAC(pdgfrb:EGFP)*, EGFP expression was observed at the eight-somite stage in the neural crest and, at 17 hpf, in the base of the brain [2]. These transgenic lines reveal *pdgfrβ* expression on the cerebral vessels earlier than the signal detected with the use of in situ hybridization (Fig. 4.1). In the hindbrain, transgenic *pdgfrβ*$^{+ve}$ cells are seen at 60 hpf, a time when arteries are being formed at the base of the brain [2]. Pericyte coverage of the hindbrain central arteries is progressive, with few weakly *pdgfrβ*-positive cells sitting ventrally to the forming central arteries and adjacent to the basilar artery in early development. These cells then move along the vessels and proliferate to cover the central arteries by 120 hpf (Fig. 4.2).

In zebrafish, although cerebral smooth muscle cells and pericytes develop in parallel with each other, cerebral smooth muscle cells are first visualized later than cerebral pericytes. In situ hybridization reveals *tagln2* expression in the head at 48 hpf but then becomes difficult to detect at 72 hpf [51]. *tagln* expression is also only weakly visualized in the early brain using TgBAC(*tagln:EGFP*). Thus, in order to examine vSMCs, a smooth muscle actin Tg(*acta2:EGFP*) line was generated [52]. In this transgenic line, *acta2:EGFP* expression does not overlap with the expression of any of the pericyte markers, confirming that *acta2* only marks smooth muscle cells. Tg(*acta2:EGFP*) was used to detect vSMC expression in the zebrafish head. In the dorsal head at 4 dpf, only a few Tg(*acta2:EGFP*) or *acta2*$^{+ve}$ cells can be seen to associate with the cerebral vasculature (Fig. 4.1). By 7 dpf and 11 dpf, *acta2* expression begins to appear around the larger cerebral vessels and continues throughout the zebrafish life.

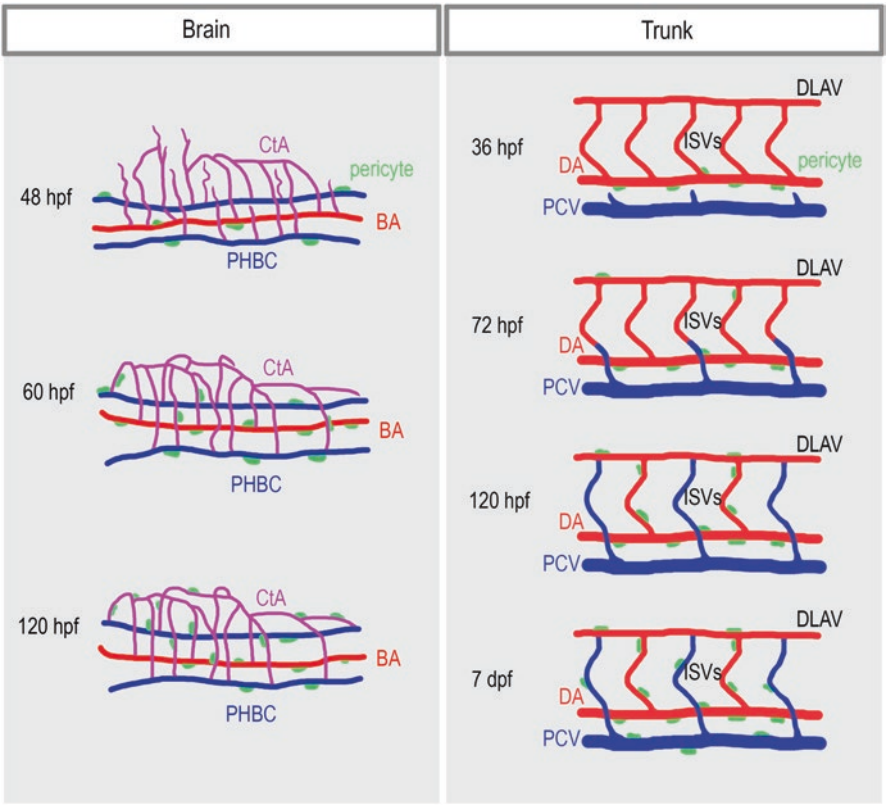

Fig. 4.2 Progressive pericyte coverage of zebrafish vessels. In the brain, *pdgfrb*$^{+}$ cells appear on the basilar artery (BA) and the primordial hindbrain channels (PHBC) at 48 hpf. At 60 hpf, the *pdgfrb*$^{+}$ cells begin to move onto the cerebral central arteries (CtA), and the number of *pdgfrb*$^{+}$ cells increases. At 120 hpf, the *pdgfrb*$^{+}$ cells cover the CtAs. In comparison, in the trunk, *pdgfrb*$^{+}$ cells appear on the ventral surface of the dorsal aorta (DA) at 36 hpf. At 72 hpf, *pdgfrb*$^{+}$ cells can be seen on the DA, intersegmental vessels (ISVs) and dorsal longitudinal anastomotic vessel (DLAV). By 120 hpf, *pdgfrb*$^{+}$ cells have covered ~60% of the ISV, with greater arterial than venous ISV coverage. Finally, at 7 dpf, *pdgfrb*$^{+}$ cells appear on the posterior cardinal vein

Spatiotemporal Pericyte Marker Expression in the Developing Trunk Vasculature

Mural cells of the trunk develop along a similar timeline to those in the head. *pdgfrβ* expression appears in the hypochord of the trunk near the dorsal aorta at 24 hpf and then around the dorsal aorta by 48 hpf, as detected by in situ hybridization [53]. Transgenic zebrafish lines have revealed that *pdgfrβ*$^{+ve}$ cells on the dorsal aorta emerge from a population of cells that first appear on the ventral side of the dorsal aorta and are not derived from the *pdgfrβ*$^{+ve}$ cells of the hypochord [2]. These *pdgfrβ*$^{+ve}$ cells

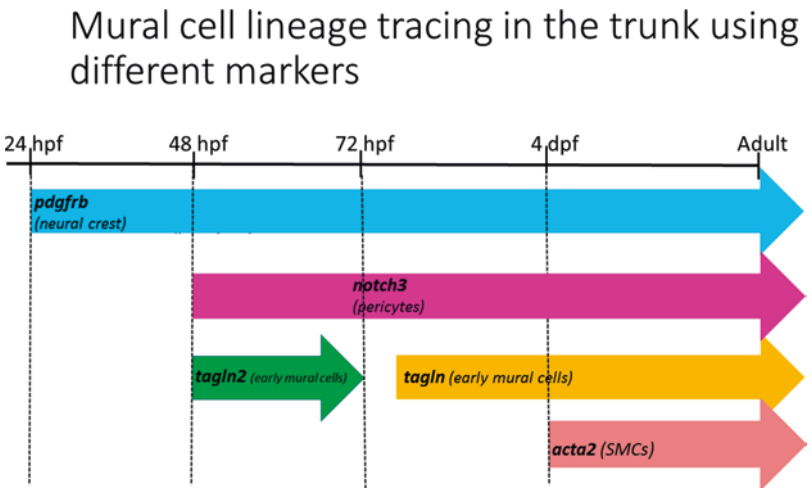

Fig. 4.3 A timeline outlining both the expression of mural cell markers associating with the trunk vasculature and the cell types they mark at specific stages of development

on the ventral side of the dorsal aorta appear at 36 hpf as detected by the time-lapse imaging of Tg*(pdgfrβ:EGFP)* [2]. In contrast to pericytes on the artery, pdgfrβ^{+ve} cells appear much later on the posterior cardinal vein, at 7 dpf [2] (Fig. 4.3).

Unlike the expression of pericyte markers on axial vessels early in development, the timing of pericyte marker expression on smaller trunk vessels is unclear and not agreed upon. Using in situ hybridization, *notch3*-expressing perivascular cells can be seen around the dorsal aorta and intersegmental vessels at 48 hpf [2]. However, transmission electron microscopy reveals no mural cell presence around the intersegmental vessels at 72 hpf, a time when mural cells are present around the dorsal aorta [53]. In support of the in situ hybridization results, transgenic *pdgfrβ* reporter lines also reveal the emergence of *pdgfrβ*$^{+ve}$ cells on the intersegmental vessels starting at 48 hpf [2]. Despite the inconsistent timelines surrounding the initial emergence of *pdgfrβ*$^{+ve}$ on the intersegmental vessels, there are consistent reports on the migration of *pdgfrβ*$^{+ve}$ cells to the intersegmental vessels from the *pdgfrβ*$^{+ve}$ cells present on the ventral surface of the dorsal aorta, and from local *pdgfrβ*$^{+ve}$ cell proliferation [51]. By 120 hpf, about 60% of the intersegmental are covered by pericytes, with more arterial than venous coverage [2] (Fig. 4.2). Interestingly, unlike the arterial intersegmental vessels, *pdgfrb*$^{+ve}$ cells of venous intersegmental vessels appear on the dorsal portion of the vessel.

Similar to the inconsistent reports surrounding the timing and location of pericyte markers, there are also conflicting reports surrounding smooth muscle cell appearance on the trunk vasculature. Whole-mount in situ hybridization by Wang et al. showed *tagln2* expression on the intersegmental vessels at 48 hpf, which then decreases by 72 hpf [51], a time when vSMC expression appears on the dorsal aorta [46]. At 80 hpf and at the level of the second somite, *tagln* expression can be seen

around the dorsal aorta and the gut but not around the posterior cardinal vein [42]. At the level of the tenth somite, *transgelin* is localized around the visceral organs but cannot be seen around the aorta, and at the level of the 18th somite, there is no longer any *transgelin* expression [42]. There is therefore an anterior to posterior acquisition of smooth muscle coverage. At 100 hpf, *acta2* appears in the floor plate, aortic arches and on the bulbus arteriosus [52]. Although in situ hybridization data suggests that smooth muscle cells appear on the intersegmental vessels prior to appearing on the dorsal aorta, due to the weak and discontinuous expression of these markers, these results are inconclusive and may reflect expression in either subsets of mural cells or non-mural cells. Using transgenic lines at 4 dpf, only a few vSMCs can be seen on the ventral side of the dorsal aorta, as marked by Tg(*acta2;EGFP*) [52]. The Tg(*tagln:EGFP*) line reveals that not only do $EGFP^{+ve}$ cells increase in number by 5 and 7 dpf but that these cells also begin to wrap around the dorsal aorta [46]. Relative to transgelin, *acta2* expression is more consistent, although it turns on later (Fig. 4.3).

In comparison to embryonic development, there have only been a few studies investigating the development of vascular mural cells in early post-embryonic stages. Of these post-embryonic studies, the majority have focused on the development of vSMCs on the trunk vasculature. At 7 dpf, the vSMCs on the dorsal aorta possess synthetic organelles, and there is no internal elastic lamina (IEL) separating these cells from the endothelial cells [35]. At 14 dpf, using Tg(*tagln:EGFP*) to visualize smooth muscle, continuous layers of vSMCs can be seen around the dorsal aorta [46]. These vSMCs appear as a single continuous layer in the ventral head at 22 dpf, as detected by *acta2* mRNA and *acta2*:EGFP [52]. From these experiments conducted at the larval stages, it appears that the differentiation and expansion of vascular mural cells occur between 1 and 4 weeks of development [35]. At less than 1 month post fertilization (mpf), layers of smooth muscle cells can be seen wrapping around the dorsal aorta [46]. In the adult stage, at 3 months post fertilization (mpf), the IEL finally appears between the differentiated vSMCs and the endothelium [35]. Unlike Stratman et al., Miano et al. found a second layer of vSMCs wraps around the dorsal aorta at this time point [35]. Due to these conflicting reports, further studies are required to confidently report on the presence and differentiation of mural cells during post-embryonic stages. Unlike vSMCs, there is almost no data on pericytes in the adult organism except for descriptions of their presence on brain vessels [10].

Pericyte Recruitment to the Endothelium

Haemodynamic Forces Are Required to Recruit Pericytes to the Endothelium

The initiation of blood flow at approximately 26 hpf in zebrafish induces shear stress on the arterial endothelium. In this low-flow state in zebrafish, endothelial cilia sense and deflect in response to shear stress [11, 17]. The movement of cilia

induces sonic hedgehog signalling, which is important for the early recruitment of vascular mural cells in zebrafish [30]. Blocking endothelial cilia formation (mutants for *ift172, ift81* or *qilin*) leads to intracerebral haemorrhage and a reduction in sonic hedgehog signalling. Whether haemorrhage in this model is due to endothelial defects and/or defective pericyte recruitment, and how sonic hedgehog signalling recruits and promotes pericyte differentiation, remains a subject of study.

The relationship between shear stress and mural cell recruitment to the endothelium is not fully established, and there are conflicting reports. Chen et al. used *gata1* morpholinos to block erythrocyte formation [11]. In these shear stress-compromised zebrafish, vSMCs are poorly recruited to the dorsal aorta. Normally Notch signalling in arteries upregulates *foxc1b* in response to shear stress sensed by cilia. Forced expression of the transcription factor *forkhead box c1b (foxc1b)* is able to rescue vSMC recruitment to the dorsal aorta [11]. Once Foxc1b is induced, mural cells are recruited to the nascent trunk arterial intersegmental vessels, although the mechanism for this recruitment is not known [11]**.** This study contrasts with a second study by Ando et al. that investigated the effects of shear stress on mural cell recruitment. Ando et al. blocked blood flow using a *cardiac troponin T* morpholino (*tnnt2*; blocks cardiac function). In these embryos, mural cell recruitment to the trunk was not impaired [2]. Consequently, Ando et al. concluded that mural cell recruitment to the trunk endothelium is flow-independent [2]. Due to these conflicting reports, the role of shear stress in pericyte recruitment to the trunk vasculature needs further study.

Mural Cell Migration After Recruitment

Once associated with the endothelial cells, pericytes of the cerebral arteries extend their processes along the endothelial cell junctions to cover blood vessels [2]. Normally, it is the tip of pericyte processes and not the entire length of the processes that attach to the inter-EC junctions [2]. To migrate, pericytes use the stiffness of endothelial junctions as scaffolding, with their processes preceding along the junction before relocating their entire cell bodies along the scaffold [2]. In contrast, smooth muscle cells of the ventral head move only minimally once the *acta2* marker turns on [52].

Signalling Pathways Involved in Mural Cell Development, Differentiation and Recruitment

Sonic Hedgehog Signalling Is Required for Pericyte Development

Zebrafish mutants with defects in sonic hedgehog signalling (in mutants for proteins necessary for cilia assembly caused by mutations of *iguana/dZip1*, *ift172, ift81* or *qilin*) exhibit brain haemorrhage. The treatment of embryos with cyclopamine,

a sonic hedgehog inhibitor, also results in brain haemorrhage, similar to that seen with the loss of *notch3* or *pdgfrβ* [26, 30]. Although these studies were performed before pericyte markers were developed in zebrafish, transmission electron microscopy of *iguana* mutants reveals a lack of perivascular mural cells on the brain vessels [30]. The lack of perivascular mural cells in these mutants confirms the crucial role of sonic hedgehog signalling in pericyte development. Among the many genes disrupted in *iguana* mutants and under Sonic Hedgehog control is the pericyte-expressed *foxf2b* gene [4], which is potentially associated with cerebral small vessel disease in humans [10].

TGFβ/BMP Is Involved in Pericyte Development

Bone morphogenetic protein-3 (bmp3) is a ligand of the TGFβ/BMP signalling pathway that is essential for pericyte development in zebrafish [32]. In *bmp3* knockout zebrafish, there are no changes in vessel morphology, but there is a decrease in pericyte number. When *bmp3* function is disrupted by the use of morpholinos, it results in haemorrhage and a leakage in the blood-brain barrier, due to an impairment in pericyte coverage [32]. Thus, *bmp3* signalling plays a crucial role in pericyte formation and the pericyte coverage of blood vessels [32].

Notch Signalling Regulates Mural Cell Development and Recruitment

It is well established that Notch1 is expressed in the endothelium and serves to recruit pericytes to the endothelial lining of blood vessels. Pericytes, on the other hand, express the Notch3 receptor. In the zebrafish brain, *notch3* is expressed in a pattern similar to that of *pdgfrβ* [51]. In the trunk, *notch3* is expressed around the dorsal aorta by 48 hpf and around the intersegmental vessels by 72 hpf. In both the brain and in the trunk, there is co-expression of *pdgfrβ* and *notch3* transcripts in perivascular cells, indicating that *notch3* is expressed in mural cells. To note, since the earliest *notch3*-expressing cells around the dorsal aorta are more likely to be smooth muscle precursors than pericytes, it is likely that *notch3* is expressed in both types of mural cells.

To elucidate the role of *notch3* in pericyte development and proliferation, *notch3* mutants were developed. These mutants show a curved body axis, characteristic of the loss of Notch signalling, but are otherwise morphologically normal. *notch3* mutants have decreased *hey1* and *her9* expression, indicating that these genes are likely targets of Notch3 signalling in pericytes [51]. At 3 dpf, a small percentage of the *notch3* mutants present with haemorrhage in the brain, either with a small focal haemorrhage or with broad haemorrhage in the brain ventricles [51]. To test whether haemorrhage in these mutants is accompanied by defects in the blood-brain barrier,

10 kD and 2000 kD fluorescent dextran dyes and the albumin-binding permeability test compound, Evans blue dye, were injected into the brains of *notch3* mutants [51]. These mutants retained very little of the substances within vessels suggesting that the loss of *notch3* impairs the formation of the blood-brain barrier. *notch3* mutants also had a reduced pericyte population but no defects in the endothelium. At the same time, neural crest precursors of brain pericytes appeared normal. Thus, the effect of Notch3 signalling in pericytes is to positively regulate pericyte numbers and therefore stabilize the blood-brain barrier [51]. Due to the loss of *notch3*, most mutants die by 15 dpf due to cardiovascular defects, and the mutation is not adult homozygous viable [51].

PDGF Signalling Recruits Mural Cells to Vessels

In mammals, platelet-derived growth factors (PDGFs) have multiple ligands (PDGF-AA, PDGF-BB, PDGF-CC and PDGF-DD) and two receptors, PDGFRα and PDGFRβ [37]. Endothelial cells express the ligand (PDGF-BB) in tip cells, while mural cells express the receptor, PDGFRβ. In mice, Pdgfrβ ablation results in mural cell deficiency [15]. In zebrafish, *pdgfrβ* genetic loss of function mutations or inhibition of the Pdgfrβ receptor, using small molecule inhibitors such as AG1296, results in decreased mural cell numbers but not a complete loss [2, 51]. Thus although Pdgfrβ signalling is involved in the proliferation and recruitment of mural cells, it is not essential for their specification.

Pericytes in Zebrafish Organs

Pericytes in the Zebrafish Eye

Pericyte support of the eye vasculature develops relatively late, during late embryo and larval stages as determined via light microscopy [1]. Mural cells first appear near the inner optic circle (IOC), which is associated with the optic vein (OV), and then continue to cover the IOC as the eye develops [42]. At 72 hpf, *pdgfrb*$^{+ve}$ also appears along the hyaloid vessels and the hyaloid artery, indicative of pericytes [51]. These hyaloid vessel mural cells are neural crest derived, as determined by the use of *foxd3*/*tfap2a* and *tbx6*/*hand2* double morphants [2]. In comparison, smooth muscle cells in the eye, as imaged in *acta2* transgenics, in situ hybridization with *acta2* and *tagln,* or *tagln* immunostaining all highlight the presence of vSMCs in the ventral eye, potentially around the ventral fissure near the optic artery [42, 52].

Mural Cells in the Visceral System

Whole-mount in situ hybridization with *acta2* and *acta2:EGFP* transgenics revealed the presence of visceral smooth muscle cells in the gut and in the swim bladder at 72 hpf and at 100 hpf [52]. By 14 dpf, these smooth muscle cells orient radially and circumferentially in the gut and in the swim bladder [52]. The expression of pericyte markers has not been examined in the zebrafish visceral system.

Mural Cell Expression in the Developing Heart

The bulbus arteriosus is a smooth muscle-containing component of the cardiac outflow tract that abundantly expresses *acta2*. Through the use of in situ hybridization and transgenics, *acta2* is also transiently expressed in the myocardium of both the atrium and the ventricle [52]. While *acta2* mRNA is expressed and observed early in the myocardium, persistent expression of acta2:GFP transgene in the myocardium likely does not reflect real gene expression. At 56 hpf, *acta2* mRNA is expressed, while *acta2:EGFP* is not, which could be due to the slower folding of the GPF reporter [52]. However, unlike its early expression, *acta2* mRNA expression ceases in the myocardium by 72 hpf, while *acta2:EGFP* expression remains through life [52]. From the expression of *acta2* mRNA, it appears that smooth muscle cell coverage of the bulbus arteriosus develops between 56 and 96 hpf [42, 52]. *pdgfrβ:GFP*-expressing cells also appear in the heart region at 80 hpf [2]. However, co-localization studies have not been performed to determine if *pdgfrβ, tagln* or *acta2* are co-expressed in the same mural cell population in the heart. Between 100 and 120 hpf, cells expressing *acta2* mRNA and transgene or *tagln* immunostaining can be seen in both the bulbus arteriosus and on the ventral aorta; this coverage continues to increase as development proceeds [42, 52].

Zebrafish Models of Disease Involving Pericytes

Notch3 and CADASIL

In humans, autosomal dominant missense mutations in NOTCH3 lead to cerebral small vessel disease in the CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) syndrome, a hereditary stroke disorder [25, 36]. When Wang et al. modelled *notch3* loss of function in zebrafish, they observed a decreased expression of pericyte markers. Through a number of experimental avenues, Wang et al. hypothesized there is a positive relationship between pericyte numbers and *notch3* expression [51]. While the exact mutations

responsible for CADASIL in humans has not been modelled in zebrafish, the expression of *notch3* in pericytes coupled with the ease of in vivo pericyte marker tracing suggests that the zebrafish can lend insight into CADASIL pathogenesis.

Foxc1 and Foxf2 Are Involved in Mural Cell Development

The FoxC1 and FoxF2 family of forkhead proteins play a role in vascular homoeostasis and differentiation, in both endothelial cells and in mural cells [40, 48]. A conserved region in human Chr6p25 encodes the closely linked FOXQ1, FOXF2 and FOXC1 loci [10]. Both FoxC1 and FoxC2 play a role in the arterial differentiation of trunk endothelial cells and are downstream effectors of Notch signalling in the trunk [20, 43]. However, in the head, *foxc1* is expressed in both the mesenchyme and in vascular mural cells. Furthermore, both the loss and gain of *foxc1* function leads to haemorrhage, reduced *pdgfrβ* expression and reduced numbers of *acta2*-positive mural cells around ventral head vessels [14]. Humans with mutated, deleted, duplicated or single nucleotide polymorphisms (SNPs) within FOXC1 can present with cerebral small vessel disease, a disease attributed to defects in pericytes and in vascular mural cells [14].

More recently, in a large human population-based genome-wide association study (GWAS), novel SNPs associated with an increased risk of stroke through cerebral small vessel disease were identified [10]. These SNPs appear between FOXQ1 and FOXF2. In zebrafish and in mice, *foxf2* serves as a neural crest-expressed transcription factor involved in pericyte development [10, 40]. Zebrafish have *foxf2a* and *foxf2b* genes, which are duplicates of the single mammalian gene. In zebrafish embryos, both *foxf2a* and *foxf2b are* expressed in neural crest and in perivascular mesenchyme, while in the adult fish, they are expressed in brain pericytes [10]. Both *foxf2a* and *foxf2b* have a similar perivascular expression pattern as those of other pericyte markers such as *pdgfrβ* and *notch3*. In zebrafish, *foxf2b* mutants have decreased mural cell coverage and decreased *acta2* expression [10]. Thus, in zebrafish, Foxf2 is identified as a pericyte-expressed gene and a candidate gene contributing to the development of cerebral small vessel disease and stroke since its compromised function alters pericyte differentiation and activity.

The Role of Pericytes in Regulating Vascular Tone

The ability of mural cells to regulate blood flow remains a topic of interest. The optical clarity and the readily availability of zebrafish transgenic lines facilitate imaging of blood vessel diameters, which helps elucidate the mechanisms of mural-cell-induced vascular tone. In zebrafish embryos, the cerebral and trunk vasculature are visible using standard confocal imaging. The dorsal aorta acquires tone between 48 and 80 hpf, as measured by a decrease in diameter. Vessel diameter is inversely proportional to an increase in vascular smooth muscle cell number [46]. Furthermore,

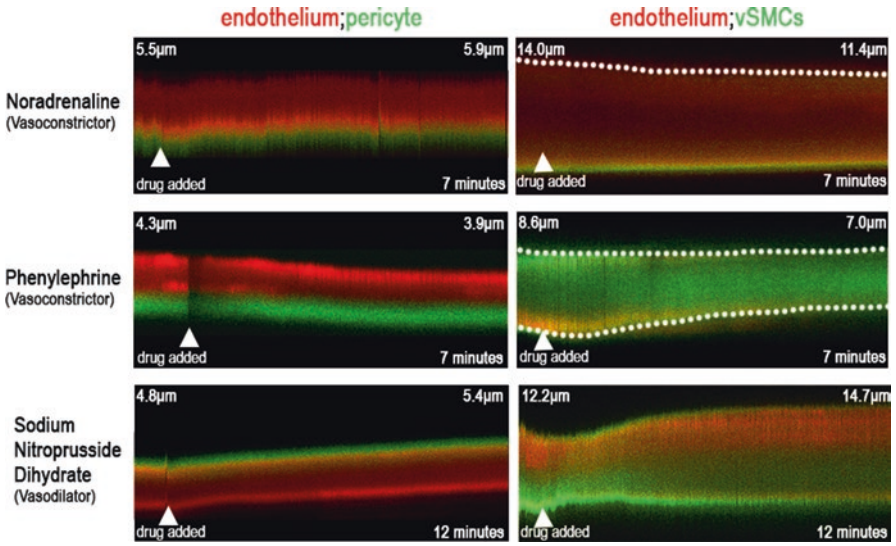

Fig. 4.4 Functional assays of vascular mural cells during zebrafish development. Kymographs of transgenic endothelium (red) and pericytye (green) cells assayed after addition of drugs. Pericyte-covered vessels do not constrict in response to the vasoconstrictors noradrenaline (2 μM) and phenylephrine (10 μM). However, pericytes do relax and cause vessel dilation in response to the dilatory agent, sodium nitroprusside (1 mM). These pericyte-covered vessels dilate by an average of 6%. vSMC-covered vessels constrict in response to both noradrenaline and phenylephrine. However, there is a mixed response from the vSMC-covered vessels in response to sodium nitroprusside; some vessels dilate, while others constrict

pdgfrβ appears to regulate the development of vascular tone. Using a heat-shock-inducible *pdgfrβ*-dominant-negative construct, Stratman et al. observed an increase in aortic diameter by approximately 50% in *pdgfrβ*-inhibited zebrafish [46]. However, since this dominant-negative construct is ubiquitously expressed, an indirect effect on mural cells resulting in the expansion or increased elasticity of the dorsal aorta cannot be ruled out.

Our lab has tested the ability of zebrafish pericytes and vSMCs to provide vascular tone in the brain. Preliminary data using pharmacological vasodilators and vasoconstrictors shows that both pericyte- and vSMC-covered vessels constrict and dilate in response to these agents (Fig. 4.4; Bahrami and Childs, unpublished). Going forwards, the use of zebrafish will provide insights into mural cell function in vivo and in an intact organism.

A Novel Population of Perivascular Mural Cells in the Zebrafish Brain

Recently, a novel perivascular cell population has been observed in the zebrafish brain. Described as mural lymphatic endothelial cells (muLECs), fluorescent granular perithelial cells (FGPs) or Mato cells, these cells lie adjacent to the endothelium,

particularly at vessel branch points, and are similar in morphology to pericytes [6, 16]. Oddly, these cells bud off from the choroidal vascular plexus (i.e. have an endothelial origin) and express lymphatic markers such as *lyve1*, *prox1* and *mrc1*. In terms of both marker expression, muLECs/FGPs/Mato cells are distinct from pericytes and other neuronal lineages that come into contact with the endothelium. However, their perivascular location suggests that Mato cells play a supporting role for the endothelium [6, 16]. There are also suggestions that similar cells exist in mouse and in the human brain, serving as potential scavenger cells. Their exact role and cross-species conservation await further experimentation.

Future Perspectives on Zebrafish Pericyte Research

In the past decade, there has been an increasing focus on vascular mural cell development and activity in both mammals and in fish. Relative to other animal models, the zebrafish offers many advantages for the study of pericytes and vSMCs. These include the development of transgenic reporter lines, molecular markers, lineage tracing and advantages for in vivo observations of mural cell function.

Currently, there remain many unexplored and controversial areas of pericyte biology in mammalian systems. These areas include mural cell plasticity as well as the potential role of pericytes or perivascular cells to act as stem cells for a number of lineages. Given the potential for genetic lineage tracing using fluorescent reporters in vivo, these questions are very accessible in the zebrafish model. Likewise, genetic models to ablate cells in the pericyte lineage could help elucidate the mechanisms of repair following injury, which can be carried out using real-time imaging. For these reasons, the zebrafish model will be an invaluable model to understand both the basic function of pericytes and their role in disease.

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Chapter 5
The Microvascular Pericyte: Approaches to Isolation, Characterization, and Cultivation

Paula Dore-Duffy and Nilufer Esen

Abstract The microvascular pericyte was identified in 1873 by the French scientist Charles Benjamin Rouget and originally called the Rouget cell (Rouget.Sciences 88:916–8, 1879). However, it was not until the early 1900s that Rouget's work was confirmed, and the Rouget cell renamed the pericyte by virtue of its peri-endothelial location (Dore. Brit J Dermatol 35:398–404, 1923; Zimmermann. Z Anat Entwicklungsgesch 68:3–109, 1923). Over the years a large number of publications have emerged, but the pericyte has remained a truly enigmatic cell. This is due, in part, by the paucity of easy and reliable methods to isolate and characterize the cell as well as its heterogeneity and pluripotent characteristics. However, more recent advances in molecular genetics and development of novel cell isolation and imaging techniques have enable scientists to more readily define pericyte function. This chapter will discuss general approaches to the isolation, characterization, and propagation of primary pericytes in the establishment of cell lines. We will attempt to dispel misinterpretations about the pericyte that cloud the literature.

Keywords Human pericytes · Pluripotent cells · Immortalized cell lines · Microvessels · Fat pericytes · Isolation · Cell culture · Magnetic beads · FAC sorting and FACS analysis · Differential adhesion · Migration · PDGFRβ+ · Central nervous system

Introduction

Although a plethora of information exists on the role of the endothelial cell (EC) in vascular hemostasis and tissue homeostasis, less is known of the role played by the microvascular pericyte (PC). The development of techniques for the isolation of defined

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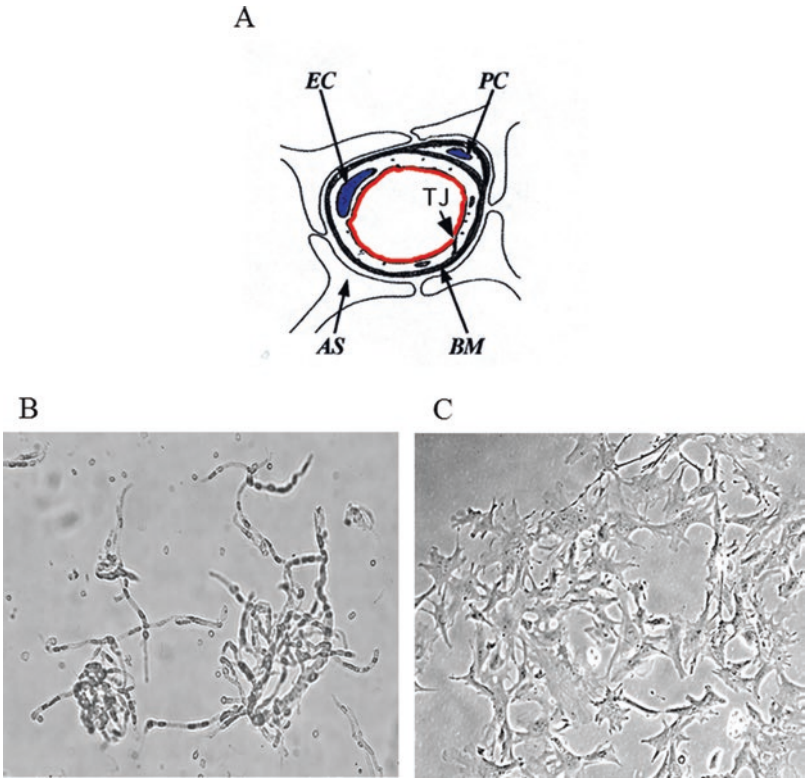

Fig. 5.1 In (**a**) a cartoon rendition of a CNS microvessel in cross section. CNS microvessel fragments (**b**) were isolated according to Dore-Duffy [22]. Pericytes were then subcultured from isolated microvessel fragments after digestion with collagenase. Pericytes (**c**) were separated from endothelial cells by differential adhesion characteristics. Isolated cells are routinely 96–98% PDGFRβ+

populations of microvascular pericytes [1–8] has enabled scientists to examine the function of this unique cell [9] (Fig. 5.1a). A number of approaches have been utilized with varying degrees of success. Even within its microvascular location, pericytes exhibit heterogeneity that can manifest in specific subsets that differ both functionally and phenotypically. Pericyte heterogeneity may even manifest within a specific organ and be in a dynamic flux. Functional changes are complicated by the pluripotent characteristics of the pericyte and its ability to migrate. In this document we will consider basic tenants of cell isolation. We will also discuss how these basic tenants can be applied to pericyte isolation techniques and the preparation of pericyte cell lines.

Cell Isolation and Separation: Basic Considerations

Isolation of any cell type from a heterogeneous population is an integral part of modern biological research. The identification and purification of specific cells are essential for the enumeration of basic cellular properties in pathology. Further,

reliable approaches are needed to enable design and targeting of cell-based regenerative therapies. The main principle governing the isolation and separation of any cell is to utilize the unique properties of the particular cell type. The most widely applied unique cell properties used in cellular isolation and separation techniques are differential adherence, cell morphology, cell size (density/size), and antigenicity (surface markers). High precision single cell isolation methods are usually based on one or more of these properties using either a positive or negative selection approach. Newer techniques such as those incorporating microfluidics make use of additional cellular characteristics (as reviewed, [10]). To isolate pericytes from a heterogeneous population, the unique properties of pericytes need to be exploited.

Preparing for Pericyte Isolation

As with most cell separation procedures, removal of as much extraneous material is important to any primary cell culture preparation. The majority of pericytes are located in microvessels. The ratio of pericytes to endothelial cells in vessels varies from 1:3 to 1:10 depending on the tissues and animal species as well as the technique used to make the determination (publications too numerous to site) [11–16]. Pericytes represent a very small percentage of any given tissue. Removal of nonvascular tissue prior to isolation optimizes chances of purity and cell yield. The approach to be taken may vary depending on the starting material. Choosing an isolation method depends on specific downstream application requirements. For example, enriched pericyte suspensions may be suitable for certain phenotypic analysis, while pure populations are needed for molecular biological analysis and studies on cell to cell to signaling. We have tried virtually all the techniques detailed below for isolation of pericytes from whole tissue and feel that most are not satisfactory without preliminary removal of nonvascular tissue. In general, procedures that result in the best level of pericyte purity are those that do not allow high yields.

Digestion/Homogenization of Solid Tissues for Preparation of Single Cell Homogenates

Whole tissue needs to be first broken down to single cell suspensions or to isolated microvessels that can then be subcultured. Isolation of pericytes from single cell suspensions of whole organ homogenates is difficult and often results in a lot of contaminating cell populations as well as blood cells and plasma components. A number of laboratories have used this approach and have reported varying levels of success [17–21]. My laboratory as well as others has developed techniques to remove nonvascular tissue with subsequent subculture of pericytes from isolated microvessel fragments that are discussed below [22–26]. Single cell suspensions

can be prepared using mechanical disruption with calibrated homogenizers with or without subsequent enzymatic digestion. Most labs have digested the tissue with collagenase or proteinase K for varying periods of time. In general the amount of mechanical or physiological disruption that the pericyte can endure and still remain viable is extraordinary when compared to many other cell types. Isolation of pericytes from fibrous tissue such as lung is difficult and results in low yields (see below). Cell aggregates and debris are removed by using low-speed centrifugation techniques (200–300 × *g*), gravity sedimentation, or sieving techniques.

Isolation of Pericytes from Single Cell Homogenates

In preparations from whole organs, pericytes remain a very small percentage of the total number of cells. Pericyte enrichment requires a positive selection technique because of the large number of heterogeneous cell types present in whole organ cell suspensions. Isolation of the target pericyte can be accomplished using specific antibody. A number of antibodies have been used such as those directed against PDGFRβ, NG2, CD13, and CD146. Positive selection yields preparations with a higher degree of purity. On the other hand, the small percentage of pericytes vis-à-vis other cell types requires that large volumes of cell suspensions be processed in order to be sure of a significant cell yield. To achieve this several cycles of the procedure may be needed. On the downside positively selected cells carry antibodies and other labeling agents that may interfere with downstream culture and assays. If that is a concern, it is preferable to use a negative selection method. The following antibody-mediated techniques have been used to positively select pericytes.

Immuno-panning

One of the earliest developed methods for isolation of specific cells with specific antibody is the panning technique which was derived by Wysocki and Sato [27]. This technique was designed for the fractionation of T and B lymphocytes. Plastic tissue culture dishes are coated with antibodies that selectively bind the cells of interest. In a typical experiment, about 3×10^7 cells can be added to a 100 × 15 mm plastic dish coated with microgram quantities of antibody or secondary antibody if a sandwich technique is desired. The non-adherent cells are removed by washing. In Wysocki's original study, both adherent and non-adherent populations retained immunologic function. The panning technique is sufficiently sensitive to allow detection and separation of cell types comprising as little as 2% of the total population and can be modified to allow the selection of cells by a double-antibody procedure. Panning has been used by Richard Daneman laboratory to isolate optic nerve and CNS pericytes [28, 29]. We have found panning techniques to be more suited to the isolation of pericytes from enriched cell suspensions than preparations with a high degree of heterogeneity.

MACS

The MACS technology was first developed by Miltenyi Biotec™ in the 1990s and since then has become a staple in cell culture labs. It is useful for the rapid and bulk isolation of desired cells. Briefly, the target or nontarget cells depending on a positive or negative cell selection strategy are labeled with 50 nm magnetic microbeads conjugated with antibody and then subjected to a magnetic field. There are three different platforms available for providing the magnetic gradient: (1) magnetic separators, (2) MACS columns, and (3) Dynabeads.

Magnetic Separators – These are powerful magnets into which tubes of suitable sizes can be fitted. This is the simplest MACS procedure wherein following incubation, the tubes are simply placed inside the magnet for the required time. The unbound fraction is drained out and the bound cells remain in the tube. Negative and positive selection can be used.

MACS Columns – These columns consist of a matrix of ferromagnetic spheres which vastly amplify the magnetic field when placed inside a magnetic separator. The cells are injected into the columns and can move freely in the inter-spherical spaces. The labeled cells are retained in the column in suspension (not bound to the magnetic spheres), while the unlabeled cells flow through and can be easily collected. The bound cells can also be eluted out once the column is removed from the separator. Columns provide two advantages compared to simple separators for positive selection – (a) the increase in magnetic gradient increases the sensitivity of detection, especially of minimally labeled cells, and (b) since the labeled cells do not actually bind to the magnetic spheres, there is less stress on the cells.

Dynabeads are hollow, spherical superparamagnetic polymer beads available in sizes ranging from 1 to 3 μm. These beads exhibit magnetic properties only in a magnetic field and have no residual magnetism once the latter is removed. Beads can be coupled with antibodies or other ligands to target pericytes. The basic procedure to use these beads is very simple – pericytes are incubated with antibody-coated beads and placed in a magnetic field to separate the bound and the unbound cells. If targeted cells are in the unbound fraction (negative selection), they are simply aspirated out. In positive selection, the bead-bound target cells are washed and eluted. Dynabead® separation can be performed in conical tubes, micro-tubes, and even multi-well plates, and the latter can be fitted with customized magnets.

The MACS technology is much more useful in purifying enriched populations of cells with limited heterogeneity such as leukocytes. We did not find it user friendly for large quantities of material, and the efficacy varied with the antibodies utilized. It is important to standardize techniques before use.

Fluorescence-Activated Cell Sorting (FACS)

FAC sorting can be used to reliably isolate specific cell types from both whole organ single cell suspensions and enriched cell suspensions containing pericytes (discussed below). However, there are a number of problems. FAC sorting of large

volumes of cells is cost prohibitive, and the cell yields are very low although the final isolates are highly pure. Both positive and negative selection techniques can be utilized. Time must be spent discussing the desired product and outcome with FACS technicians who will be attempting such a run. The criteria for gating cells can make a profound difference in the end results. Technicians are used to sorting leukocytes that have defined staining patterns and are easily gated. Pericyte subsets also vary in size. Familiarity with specific antibodies and their patterns in FACS analysis helps in running an efficient sort. If sterile cells are required for culture, the tubing needs to be cleaned with bleach to sterilize them before sorting. Adequate amounts of sterile saline must be used to wash out the bleach from the tubes or the cells will die during the sort. Even if they do not die, they may have very limited viability. We have found that FAC sorting is useful for preparation of highly pure pericytes from small batches and from enriched populations of cells. FAC sorting is useful for preparation of cell lines. (Remember to save a small sample to conduct a FACS profile to confirm the phenotypic identity of the sorted cell population(s).) FAC sorting techniques for isolation and/or enrichment of pericytes can be found in the following manuscripts [18, 19, 30–36].

The Subculture of Pericytes from Purified Preparations of Cerebral Microvessels

A number of approaches are available to isolate microvessels (Fig. 5.1b) from the brain [8, 22, 24, 36–42], lung [42], kidney [43], skin [44], cardiac tissue [45], and fat [46]. Initially all require either mechanical homogenization or enzymatic digestion of starting material with subsequent enrichment of microvessel fragments. This becomes a problem with the lung tissue due to its fibrous nature. Due to the difficulty of homogenizing the lung tissue, it is recommended to finely mince the tissue before homogenization. Some laboratories have used more rigorous acid digestion, as well as sonication [42]. The overall yield of pericytes from microvessels depends on the starting material and the pericyte to endothelial cell ratio. We have isolated pericytes using most of the available techniques and have found no easy way. Isolation and pericyte enrichment from bulk tissue discussed above is time consuming requiring large amounts of tissue. Thus with the exception of isolating fat pericytes, we routinely subculture them from microvessels. While we published our technique in numerous articles from 1996, the 2003 publication [30] has a detailed technique.

Our method [22] for isolation of brain capillaries is a modification of methods used by Joo and Karnushina [37], Harik [36], and Bowman and colleagues [39]. Using differential sieving techniques, our microvessels are of uniform diameter, are neuronal- and astrocyte-free, and have less than 3% contamination with precapillary arterioles. The relative purity of the pericyte or endothelial subculture directly reflects the purity of the microvessel starting material. Following digestion with

collagenase treatment, microvessel single cell suspensions can be analyzed by flow cytometry and enriched for pericytes by differential adhesion characteristics [22] (Fig. 5.1c), cell sorting, panning, and/or other techniques for positive or negative removal of one or more populations of contaminating cells. If performed correctly cell suspensions from isolated microvessels will contain an average of 2–3% alpha muscle actin (αSMA)-positive cells, 20–22% pericytes, and 73–75% endothelial cells as assessed by FACS analysis for platelet-derived growth factor receptor beta (PDGFRβ)-positive cells. It is recommended that characterization of pericyte isolations includes dual-label and even triple-label immunocytochemistry with FACS analysis. Pericyte populations even within a microvascular location are phenotypically and functionally heterogeneous. As an example, even minor contamination of microvascular fragments with precapillary arterioles will lead to an enhanced percentage of αSMA+ cells. Hence, pericytes express both αSMA and PDGFRβ, whereas smooth muscle cells will be αSMA+ PDGFRβ-. In our hands expression of NG2 in primary isolates is more variable than PDGFRβ. A minimum of 97% of pericytes express PDGFRβ, while 50–80% express the proteoglycan NG2. All NG2+ pericytes appear to express PDGFRβ.

Generation of Pericyte Lines

Following serial subculture of primary pericytes, cells that continue to grow can potentially become cell lines. Theoretically these are subpopulations of the original primary cell isolate that is capable of continued growth. Pericyte cell lines are very useful because they can be stored in liquid nitrogen and revived for later use obviating the need to conduct a laborious primary isolation procedure. However, preparation of pericyte cell lines is not without difficulty.

Primary pericytes rapidly differentiate in vitro depending on the plating density and environmental cues present in the culture environment. The concept of pericyte differentiation has to be kept in mind when interpreting data. Unfortunately the identification of the state of differentiation or of specific pericyte subsets remains difficult because stage-specific markers have yet to be identified. The generation of pericyte cell lines that exhibit an undifferentiated pericyte phenotype has proven difficult [47, 48]. We have utilized a number of alternative approaches.

We reasoned that for a cell line to become continuous, a so-called “immortalization” event needs to occur. Often continuous culture of a cell results in a natural transformation by somatic mutation. Alternatively this can occur by viral transformation, by an induced mutation, or by hybridization with an immortalized cell line. Thus we first employed the Immortomouse® to prepare CNS immortopericytes (IMPs) [30]. The Immortomouse® harbors a stably incorporated conditionally expressed transgene [49–54], which encodes a variant of the tumor antigen (T-Ag) from the simian virus 40. A missense mutation of T-Ag (TsA) renders this protein functional at 33 °C but inactive at body temperature. In the Immortomouse®, coupling TsA58 to the $H2K^b$ class I promoter ensures expression and confers interferon

gamma (IFNγ)-regulated properties [55]. Thus, the functional expression of T-Ag is conditional on both temperature (33 °C) and the presence of IFNγ in the culture medium.

The Immortomouse® transgenic mouse has been a source for conditionally immortalized cells from the endothelium [53], renal mesangial cells [51], Sertoli cells [54], mesenchymal cells [52], skeletal muscle [56, 57], epithelial cells [58–61], glial cells [62], and osteoclasts [63]. Conditionally immortalized cells continue to replicate at 33 °C in the presence of IFNγ, but do not display a differentiated phenotype [64]. We theorized that primary pericytes cultured from the Immortomouse® would not differentiate at 33 °C, but it was unclear to us whether they would replicate. (In our hands, freshly isolated primary pericytes do not initially replicate in a logarithmic fashion in the presence of serum. There is a lag phase suggesting that only a subset of cells replicate upon initial plating. With prolonged culture we have observed more replication in wild-type pericyte, but we also see evidence of differentiation. The pattern of pericyte proliferation is comparable to that associated with self-renewal.)

Utilizing standard techniques for the preparation of murine CNS primary pericytes, we established a number of mouse IMP lines. Surprisingly the lines exhibited heterogeneous proliferative phenotypes. In our 2011 paper, the IMP line used displayed low levels of cell division at 33 °C until exposed to environmental or exogenous stimuli. It was not until the cells were moved to 37° in standard culture medium that the lines exhibited normal pericyte culture characteristics [30]. One line exhibited no proliferative activity at all but remained quite viable in culture. Since 2011 we have produced additional IMP lines. One line exhibits high replicative activity and we are attempting to clone this line. Yet another may have been transformed. The variability in IMP lines may reflect the heterogeneity of murine CNS pericytes found in capillaries.

We have also prepared pericyte lines from human fat (Fig. 5.2). Using liposuction aspirates (Fig. 5.2a) obtained from a plastic surgeon, we isolated fat pericytes using FAC sorting and/or differential adhesion characteristics of pericytes. Both techniques enrich for PDGFRβ+ cells (Fig. 5.2b). The purity of the cell culture preparations can be enhanced using magnetic bead (Dynabeads), panning or other techniques to enhance the purity of cultures at the first passage. Fat PDGFRβ+ pericytes contain a number of fat progenitor cell subsets. These subsets retain the PDGFRβ+ phenotype but also express fat progenitor markers (Dore-Duffy unpublished observations). The cells exhibit somatic cell memory particularly when cultured at high density and in the presence of growth factors. Preparation of viable lines was easier if cells were plated at low density. At each passage we enriched for PDGFRβ+ cells. Cell lines eventually lost fat progenitor subsets. Pericytes can be isolated from all three layers of liposuction aspirates (soluble layer, fat layer, and the clear layer above the fat layer) (Fig. 5.2). We currently have over 18 human fat lines, and all retain functional characteristics of pericytes, although these lines have not been verified by STR profiling.

We have had less success establishing lines directly from normal murine CNS primary pericytes. While these cells do contain a self-renewing subset of pericytes,

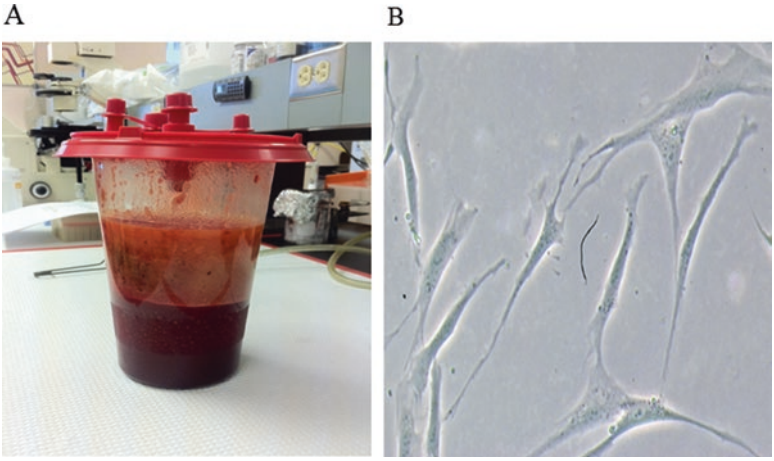

Fig. 5.2 Human liposuction aspirates were obtained during an elective liposuction procedure. The patient, a 40-year-old female, donated her tissue after giving informed consent. Aspirates are kept cold and processed within 24 h. Following gravity sedimentation shown to the left in (**a**), the aspirate separates into a soluble layer at the bottom, fat layer above the soluble layer, and a clear fat layer that lies on the top of the fatty material. Pericytes can be isolated from all layers using somewhat differing techniques. In (**b**) we show 7-day-old PDGFRβ+ pericytes originally isolated from the soluble layer. Human fat pericytes can be passaged and stored and retain phenotype

selecting for this subset has proved difficult. We have tried limiting dilution techniques, sequential exposure to a variety of growth factors over serial passage. Resulting lines are contaminated with varying cells of mesenchymal origin and bear some resemblance to a line which has been described by Greenwood-Goodwin as human pericyte-like progenitor cells [65]. Even at low dilutions, our primary pericytes retain the ability to differentiate along the mesenchymal lineage when cultured in the presence of serum. There are a number of commercially available pericyte cell lines. However, we have had no experience with these cells and they will not be discussed in detail in this chapter. Of note is that human brain microvascular pericytes are available from Neuromics [66]. These cells are also available in a multicellular BBB model system. Pericytes do not appear to differentiate under co-culture conditions making multicellular systems useful experimental tools.

In conclusion, while techniques are available for pericyte isolation and culture, there are no shortcuts. Pericyte populations need to be defined [67]. For there to be real advances in the field, rigorous adherence to protocols and techniques and characterization of starting material is essential. That pericytes within the vascular bed are both phenotypically and functionally heterogeneous, make it imperative to characterize pericyte subsets. That pericytes are pluripotent means that the state of pericyte differentiation must be monitored. With those limitations new advances in cell isolation techniques using laser capture originally used by the Pachter lab [67] show promise. The availability of two-photon imaging modalities [68–71] that now include visualization in live animals [72] has opened a new world to unravel the

mysteries of pericytes. However, caution is needed not to overinterpret data. Many of these techniques visualize very small finite sections of microvessels containing a single pericyte.

Microfluidics. Microfluidic technologies have become more prominent in efficient cell isolation and detection [73–77]. They have been useful in highly rigorous identification of circulating tumor cells [74], stem cell separation, and single cell analysis [77]. The ability to isolate specific types of cells from a large population is important and may have a potential role in pericyte isolation. A number of efficient platforms exist for cell isolation in microfluidic devices including affinity-based approaches for cell detection that involve antibody and aptamers. There are also approaches using physical properties such as hydrophoresis, dielectrophoresis, and acoustophoresis (as reviewed [74]). Wang and colleagues have used microfluidic techniques to develop a 3D in vitro model of the BBB utilizing tissue lines of endothelial cells pericytes and astrocytes [78]. Continued utilization of microfluidic formats should be encouraged for pericyte isolation.

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Chapter 6
Pericytes in Veterinary Species: Prospective Isolation, Characterization and Tissue Regeneration Potential

Cristina L. Esteves and F. Xavier Donadeu

Abstract Although pericytes have long been known for their roles in blood vessel regulation, it was not until a decade ago that their tissue regeneration potential began to be considered, after studies showed that pericytes were the in vivo counterparts of mesenchymal stem/stromal cells (MSCs). The prospective isolation and culture expansion of pericytes brought great excitement as it opened the way to the therapeutic use of well-defined cell populations with known regenerative potential to overcome concerns associated with the use of traditional MSC preparations. Studies first in humans and later in the horse and other domestic species showed that indeed cultured pericytes had key characteristics of MSCs, namely, their immunophenotype and the abilities to grow clonally and to differentiate into mature mesenchymal cells both in vitro and vivo. Several studies with human pericytes, and to a much lesser extent with animal pericytes, have also shown significant promise in tissue repair in different disease models. This review summarizes current knowledge on the tissue regeneration properties of pericytes from domestic animals and outlines future steps necessary for realizing their full potential both in clinical veterinary medicine and in preclinical testing of human therapies using large animal models, including the need for robust approaches for isolation, culture and appropriate in vivo testing of the tissue regenerative properties of pericytes in these species.

Keywords Pericyte · Veterinary · Tissue repair · Mesenchymal stem/stromal cells · Adipose · Regeneration · CD146 · Horse · Dog · Perivascular · CD34 · Adventitial · MSC · Bone marrow · Pig

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Introduction

Pericytes naturally surround the endothelial cell layer of capillaries and microvessels, [4, 67], playing a crucial role in maintaining blood vessel homeostasis, and their dysfunction is associated with disease including diabetes, cancer and fibrosis [67]. Importantly, pericytes can give rise to mature cells from different mesenchymal lineages both in vivo, as demonstrated by cell lineage tracing studies [22, 23, 31, 42, 64], and in vitro [11, 30, 37, 38]. In addition, it has been shown that pericyte and mesenchymal stem/stromal cell (MSCs) markers co-localize in body tissues, and moreover isolated pericytes display typical MSC features in culture. Taken together, these finding indicate that pericytes are in vivo counterparts of MSCs [19, 29, 30, 37, 38, 73] and open the way for the therapeutic use of isolated pericytes as an alternative to the poorly defined MSC preparations currently obtained from cultured-expanded heterogeneous stromal cell populations (Fig. 6.1) [28, 34, 47, 49]. In this regard, the use of pericytes will have positive implications for standardization and consistency in the outcome of treatments. Indeed, the potential of human pericytes for regenerative therapies has already been shown in animal models [14, 27, 36, 41, 48, 70], although appropriate clinical trials are needed to show that pericytes in fact constitute a viable or even superior alternative to MSCs. At present, human MSCs are approved for treatment of paediatric graft versus host disease (in Canada), arthritis (South Korea), Crohn's disease (South Korea) and post-acute myocardial infarction (South Korea and India). In veterinary species, MSCs have

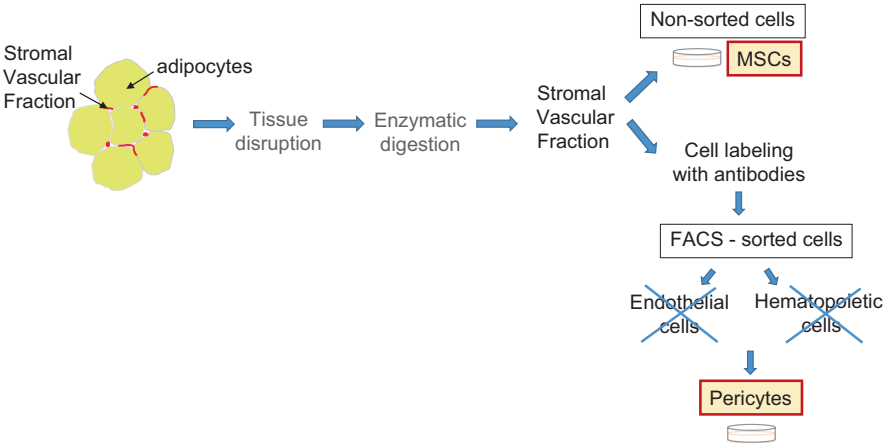

Fig. 6.1 Schematic depiction of the procedures used to derive MSCs and to isolate pericytes from adipose tissue. The tissue first undergoes mechanic disruption (commonly by mincing) followed by enzymatic digestion and centrifugation to obtain the stromal vascular fraction (SVF, containing different cell types including MSC/pericytes, endothelial and haematopoietic cells) free of adipocytes. SVF is either cultured to produce MSCs or stained with specific surface marker antibodies and processed by fluorescence-activated cell sorting (FACS) to remove major contaminant cell populations, endothelia (using CD31 or CD144 antibodies) and haematopoietic (CD45) cells and collect pericytes (stained with CD146) and, if required, other fractions such as adventitial cells (marked by CD34), which are then expanded and characterized in culture

been used for over 15 years, first in racing horses [9, 10, 21, 26, 32, 43, 57, 61, 65] and later also in dogs and cats, having now evolved into a multimillion dollar business worldwide. Although used traditionally for musculoskeletal regeneration in these species, applications outside orthopaedics are now expanding. Similarly to humans, there is a need to improve the quality of MSC preparations used therapeutically in veterinary species, and pericytes offer an attractive alternative.

Here, we provide an overview of the current knowledge on the potential of veterinary pericytes for tissue repair focusing on the methodology for prospective isolation, characterization and potential for tissue regeneration.

Prospective Isolation of Pericytes from Tissues

Pericytes can be visualized by EM and IHC/IF [4, 59] as distinct cells embedded in the basement membrane of capillaries and microvessels and containing fingerlike projections that embrace endothelial cells. Although no surface markers specific to pericytes have been identified to date, a series of antigens including CD146, NG2, PDGFRβ and αSMA have recently been used to identify pericytes in tissues of humans and mice [19, 56, 72] and later in other species including horse [30], sheep [37] and dog [38]. The lack of species-specific antibodies and need for appropriate validation hindered the study and isolation of pericytes in these species.

Pericytes were initially isolated using FACS from the human bone marrow [56] based on the positive selection of CD146 and negative selection of CD45 (an haematopoietic maker). The cells isolated made up only about 0.1% of total nucleated cells in bone marrow and expressed αSMA, NG2, calponin 1 and 3 and PDGFRβ. Concurrently, a seminal study by Péault's group reported on the prospective isolation of human pericytes from several organs including the adult and foetal skeletal muscle, placenta, adipose tissue, skin, myocardium, pancreas and bone marrow [19]. In that study pericytes were isolated using a panel of antibodies (CD146+/CD45-/CD34-/CD56-) to positively select pericytes while excluding haematopoietic (CD45), endothelial (CD34) and myogenic (CD56) cell populations [19]. As expected, cells isolated in this way expressed the typical pericyte genes, NG2, PDGFRβ and αSMA. Later studies also isolated pericytes from other species including the horse, sheep and dog [30, 37, 38]. In some instances single antibodies were used [44, 70]; however, this is not effective to isolate pericytes from crude extracts obtained by enzymatic digestion of tissues [49].

Pericytes in Veterinary Species

The notion that pericyte preparations could be used clinically for regenerative treatments has been recently extended to veterinary species, namely, horse and dog. In these species, clinical MSC preparations are obtained primarily from the bone marrow and adipose tissue, but other tissues have also been used to obtain MSCs [15,

40, 46, 62, 66]. These cell preparations typically contain only a small and variable fraction of true stem/multipotent cells (Fig. 6.1). For this reason, the term "stem/stromal" is preferred to "stem" to define cell preparations currently used for therapy. Moreover, in the absence of species-specific guidelines, MSC preparations from veterinary species are defined following guidelines established for human MSCs by the International Society for Cellular Therapy (ISCT) [8, 25], according to which MSCs should attach to plastic tissue culture vessels, grow clonally, express mesenchymal markers (CD105, CD73, CD90) but not haematopoietic markers (CD45, CD34, CD14 or CD11b, CD79α or CD19) or HLA-DR (MHC class II cell surface receptor) and display multipotency as determined by the ability to differentiate into chondrocytes, osteocytes and adipocytes. The same guidelines are currently used to define prospectively isolated pericytes from all species. Characterization of veterinary MSCs and pericytes has been performed by several studies showing, in general, similar properties to these described for human and rodent cells. However, some differences have also been observed, namely, in the immunophenotype, probably resulting from species-specific differences or the use of antibodies with variable cross-reactivity, and therefore the expression of some MSC markers was not always positively detected in different species. In some instances the MSC marker, such as CD73 in horse, was detected by PCR but not by flow cytometry [52], and due to this inconsistent expression patterns, it was suggested that CD29 and CD44, expressed in MSCs and pericytes, could be used as pan-specific markers [51]. These findings evidenced that although common properties are found for pericytes and MSCs between different species, their characterization needs to take into account the particularities inherent to each species so they can be properly evaluated for future clinical use in regenerative treatments.

The following is a summary of the current knowledge on pericytes and their regenerative potential in different veterinary species.

Horse

Pericytes were successfully isolated using FACS on equine SVF, obtained by depleting adipose tissue of fat cells, followed by enzymatic digestion [30] (Fig. 6.1). SVF was sorted using positive selection for the pericyte marker, CD146, after negatively selecting for endothelial and haematopoietic cells using CD144 and CD45, respectively, and using antibodies previously validated by IHC/IF and flow cytometry [29, 30, 54]. Another cell fraction corresponding to adventitial cells which are associated with the larger blood vessels, and could allegedly act as precursors of pericytes [16], was isolated at the same time as pericytes from equine SVF by positive selection for CD34. This generated two cell fractions with the immunophenotypes, CD146+/CD144-/CD45-/CD34- and CD146-/CD144-/CD45-/CD34+, for pericytes and adventitial cells, respectively. The isolated cells were successfully expanded in DMEM media containing 20%FBS and exhibited typical MSC properties, i.e. generation of colony-forming unit fibroblasts, trilineage differentiation capacity and

expression of MSC markers (CD29, CD44, CD73, CD90 and CD105) as well as, in the case of CD146+ cells, pericyte markers such as NG2 and PDGFRβ. Moreover, compared to MSCs (derived from culture of non-sorted SVF) and CD34-postive cells, the isolated pericytes displayed higher levels of CD146 mRNA but lower levels of transcripts for typical MSC makers. These differences could be attributed to loss by pericytes of MSC surface marker expression during culture or to the presence of cell contaminants in MSCs' preparations such as haematopoietic and/or endothelial cells, which can also express MSC markers [17, 21, 25, 32]. In addition, pericytes organized around endothelial cell networks in culture, resembling their perivascular location in vivo, and compared to MSCs and adventitial cells, they distinctly stimulated angiogenesis in an in vivo chorioallantoic membrane (CAM) assay, in agreement with higher production of the angiogenic factors VEGFA and ANGPT1 compared to the other two cell types. These results indicate that equine isolated pericytes are distinct from standard MSCs and that they maintain fundamental properties of pericytes that naturally contribute to blood vessel homeostasis and are important for the healing process. In this regard, equine pericytes in culture express immunomodulatory molecules including the cytokine IL-6 and the chemoattractants CCL2 and CCL5 known to mediate key trophic effects exerted by these cells in health and disease and that play a fundamental role in the regenerative capacity of MSCs [3, 7, 12, 45, 58] including from horse [5, 20, 50, 53, 68]. Overall, these findings suggest that pericytes may provide distinct benefits over the use of standard, poorly defined MSC preparation for equine regenerative therapies. It will be now essential to assess the potential of equine pericytes in relation to different clinical applications including tendon healing, osteoarthritis, skin lesions, laminitis and endometriosis/endometritis [55], particularly in the context of their allogeneic use where they would offer the most promise.

Dog

Two different studies reported on the isolation and characterization of canine pericytes with the aim of obtaining a cell preparation for clinical use [38, 71]. In the first study, the clinical potential of canine pericytes was tested using an animal model of mannitol-induced transient blood-brain barrier opening. Cells were isolated from adipose tissue using FACS with a single antibody against PDGFRβ. Around 40% of total cells in the initial preparations were sorted, a value much higher than those obtained using a multiple antibody panel in other studies [19, 63], suggesting that PDGFRβ-expressing cells other than pericytes [24] may have been included in the sorted preparations. Moreover, the multipotency and immunophenotype of the cells obtained were not established. PDGFRβ+ cells were transduced with a GFP lentivirus and applied to experimental dogs via intracarotid injection. Higher numbers of cells were detected in the brain when administered after mannitol pretreatment. Moreover, 70% of cells that engrafted did so in a perivascular location, resembling their natural location in tissues. Overall, the results suggested that intra-arterial cell

infusion with autologous PDGFRβ+ cells following hyperosmolar mannitol administration was feasible and a safe therapeutic approach. In the second study [38] cell fractions with immunophenotypes of pericytes (CD45-/CD146+/CD34-) and adventitial cells (CD45-/CD146-/CD34+) were separately isolated from canine adipose tissue in order to investigate their potential for bone tissue engineering. Sorted cell fractions were shown to express the MSC markers, CD44 and CD90; however trilineage differentiation capacity was not assessed. In addition, an antibody to exclude endothelial cells was not included in the sorting panel, further precluding conclusions on the actual identity of the sorted cells. It was found that supplementation with NELL-1 protein increased the ability of these cells to undergo osteogenic differentiation although the authors noted that canine cells had less osteogenic potential than their human counterparts. In contrast to data already available from the horse [28], further studies are required to definitely demonstrate the feasibility of isolating canine pericytes as well as establish their characteristics.

Sheep

Sheep is a preclinical model of choice in orthopaedic medicine. In this context, there is interest in the use of pericytes for testing of novel musculoskeletal regenerative therapies, particularly for joint disease. Pericytes and adventitial cells have been isolated from ovine adipose tissue using the same antibody panels as in a dog study described above [38] and with the aim of evaluating their potential for autologous transplantation in a cartilage defect model [37]. Subsequent characterization steps were performed both individually, or pericyte and adventitial cell fractions were combined as a PSC (perivascular stem cell) preparations. Isolated pericytes remained positive for CD146+ and underwent trilineage differentiation in culture. PSCs labelled with GFP or CMFDA (5-chloromethylfluorescein diacetate) were delivered in either collagen I/III membranes or hydrogels into stifle joints in which a defect had been created. At 4 weeks, the GFP signal was detected in the base of articular defects treated with cell-loaded hydrogel but not in cell-loaded collagen membranes. However, no repair was observed by staining of articular cross sections with Masson's trichrome. Further testing will be needed to more convincingly establish the clinical potential of ovine pericytes in the same and other models of tissue repair This should include optimization of pericyte isolation and characterization, delivery of method to the site of injury and more detailed evaluation of their effects on tissue healing.

Pig

Similarities between human and porcine physiology have led to the pig becoming an important model for understanding human disease as well as for preclinical regenerative therapy testing, notably in relation to the cardiovascular system.

Several attempts were made to isolate porcine pericytes using non-selective methods such as differential cell attachment to tissue culture vessels [6, 33]. A recent study [2] reported the isolation of cells described as "adventitial pericytes" from saphenous vein using immunomagnetic beads to select CD34+ cells after negative selection for CD31+ endothelial cells. At the moment it is unclear what is the relation between "adventitial pericytes", "pericytes" [18, 30, 72] and "adventitial cells" [16, 28]. These porcine cells were multipotent and displayed clonal growth as well as expressed markers associated with pericytes, NG2 and PDGFRβ, but not CD146 [2]. Moreover, transplantation in a swine model of reperfused myocardial infarction led to enhanced tissue angiogenesis and reduced fibrosis, though these effects were not enough to result in functional improvements. Testing of CD146-positive pericyte populations using this model will be of great interest given their distinct angiogenic capacity [30] which could provide an effective tool for cardiac regeneration, as already shown in mice [13].

Conclusions and Future Directions

Following results of a number of studies over the past 10 years showing the regenerative potential of human pericytes, interest in the therapeutic use of pericytes from veterinary species is gaining a real momentum, whether for clinical applications in companion animals (horse and dog) or preclinical testing of prospective human therapies (dog, sheep, pig). The potential to use well-defined cell populations with predicted clinical outcomes makes pericytes very attractive as an alternative to current MSC therapies in animals and humans. Despite clear potential shown in different domestic species, as summarized in this review, data so far obtained is still limited and preliminary. Thus, robust protocols for isolation of pericytes, free of cell contaminants such as endothelia, have yet to be reported from some species, e.g. dog and sheep. This is important towards developing clinical-grade preparations for companion animals. Effects of transplanted cells on healthy and diseased tissue also need to be properly characterized in relation to each disease application of interest, and the most effective routes for administration need to be determined. Again, data already available in humans will be very informative in this regard. Although focus is likely to be placed on specific application in preclinical animal models (e.g. joint disease in sheep and cardiovascular repair in pig), potential clinical applications in companion species (horse and dog) are much wider, including musculoskeletal and many other body systems. A fundamental aspect in companion animals is the need to accurately determine clinical efficacy and safety through appropriately controlled trials, something which has been often overseen in the case of MSC therapies given ethical issues involved in experimental testing in these species. Other novel tissue regeneration applications of MSCs are also being tested, for example, in the combat of bacterial infection in humans [1, 60, 69, 74], horses [17, 35] and dogs [39] expanding the range of pathologies that can be addressed using pericytes.

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Chapter 7
Pericytes in the Human Vocal Fold Mucosa

Kiminori Sato

Abstract

1. The human vocal fold is a vibrating tissue and vascular structures in organs which have the capacity to vibrate require a specific structure suitable for vibration.
2. The structure of the blood vessels is unique at the vocal fold edge as a vibrating tissue, where only small vessels, including arterioles, venules, and capillaries, are present. The capillaries are distributed in the superficial layer of the lamina propria (Reinke's space).
3. The blood vessels enter the vocal fold edge from the anterior or posterior end of the membranous vocal fold and run essentially parallel to the vocal fold edge.
4. Many pericytes can be seen around the capillaries in the human vocal fold mucosa. The cell bodies of the pericytes attach to capillary endothelial cells, and the branching processes encircle the capillaries and attach to the capillary endothelial cells at the tips. The processes of pericytes are in close contact with endothelial cells, sharing a common basement membrane with them. The tips of the processes form intercellular tight junctions with endothelial cells.
5. The pericytes in the vocal fold mucosa appear to provide mechanical support and protection to the capillary walls, particularly during phonation. The pericytes also appear to regulate the diameter of the capillary during and after phonation. Pericytes are also thought to be critical cells in vascular biology and angiogenesis, especially in revascularization following vocal fold tissue injury.
6. At birth, pericytes have already encircled the capillaries in the newborn vocal fold mucosa. The pericytes appear ready to provide support and protection of the blood vessels just after birth.
7. Vascular structures and their permeability are related to the specific structures and specific diseases of the human vocal fold mucosa as a vibrating tissue.

Keywords Pericyte · Capillary · Human vocal fold mucosa · Vibrating tissue

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Introduction

Among mammals, only humans can speak and sing songs. And only the human adult vocal fold has a proper layered structure including a vocal ligament and Reinke's space [4].

The human vocal fold is a vibrating tissue. The fundamental frequency of the tone produced by the bilateral vocal folds reflects their vibrating frequency. During conversational speech, men typically exhibit average fundamental frequencies between 100 and 150 Hz, whereas women typically have average fundamental frequencies between 190 and 250 Hz [1]. Fundamental frequency varies during speech production.

The portion of the vocal fold which vibrates the most during phonation is the vocal fold mucosa, especially the superficial layer of the lamina propria (Reinke's space) and the midpoint of the membranous vocal fold. Vascular structures of organs which have the capacity to vibrate require a specific structure suitable for vibration and such structures minimize hypoxia of the tissue and trauma of the blood vessels.

Histology and histoanatomy reflect the organ's functions very well [13]. From the physiological and pathological point of view, vascular structures and their permeability are related to the specific structures and specific diseases of the human vocal fold mucosa as a vibrating tissue.

Vascular Network of the Human Vocal Fold Mucosa as a Vibrating Tissue

The structure of the blood vessels are unique at the edge of the lamina propria of the human vocal fold mucosa, where only small vessels, including arterioles, venules, and capillaries, are present [4, 8]. The capillaries are distributed in the superficial layer of the lamina propria (Reinke's space) of the human vocal fold mucosa. The arterioles and venules are distributed in the intermediate and deep layer of the lamina propria (vocal ligament) of the human vocal fold mucosa.

The blood vessels enter the human vocal fold edge from the anterior or posterior end of the membranous portion of the vocal fold and run essentially parallel to the vocal fold edge (Fig. 7.1). Away from the edge of the vocal fold, blood vessels increase in number, and large blood vessels run in various directions at the superior and inferior portions of the human vocal fold (Fig. 7.1). The blood vessels in the lamina propria of the mucosa around the human vocal fold edge are clearly separated from and do not network with those in the superior and inferior surfaces of the vocal fold.

In the muscle layer of the vocal fold, the blood vessels enter from the deep portion of the vocal fold. The blood vessels in the lamina propria of the mucosa around the human vocal fold edge are clearly separated from and do not network with those in the vocalis muscle.

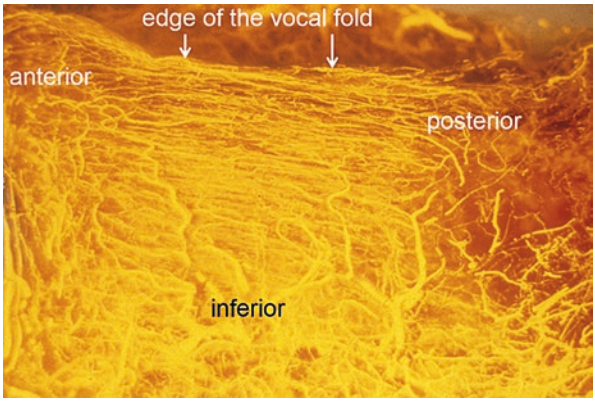

Fig. 7.1 Medial aspect of the human vocal fold. (Silicone rubber compound injection and clearing technique). (Photograph courtesy of Dr. Shigejiro Kurita, from the Department of Otolaryngology-Head and Neck Surgery, Kurume University). Around the vocal fold edge, the blood vessels are small and run roughly parallel to the edge. The vessels around the vocal fold edge come from the anterior and the posterior end of the membranous vocal fold. Large vessels run in various directions at the inferior portion of the vocal fold

At the midpoint of the human membranous vocal fold, especially at the lower surface of the vocal fold, there is a reticulated vascular network [8]. Direct anastomosis between the arterioles and venules is observed sporadically [8].

Microstructure of the Blood Vessels and Pericytes in the Human Vocal Fold Mucosa

There are only small blood vessels, including arterioles (Figs. 7.2 and 7.3), capillaries (Fig. 7.4), and venules (Fig. 7.5) running roughly parallel to the vocal fold edge in the mucosa of the human vocal fold edge. The main blood vessels in the superficial layer of the lamina propria (Reinke's space) of the human vocal fold mucosa are the capillaries.

Arterioles

Generally, arterioles range in diameter from 300 μm down to less than 50 μm [2]. However, the arterioles in the human vocal fold mucosa are relatively thin (Fig. 7.2). Smooth muscle cells completely encircle the blood vessels of arterioles (Fig. 7.2).

The terminal arterioles pass through a short transitional region in which scattered smooth muscle cells persist around the blood vessel (Fig. 7.3). Arterial capillaries,

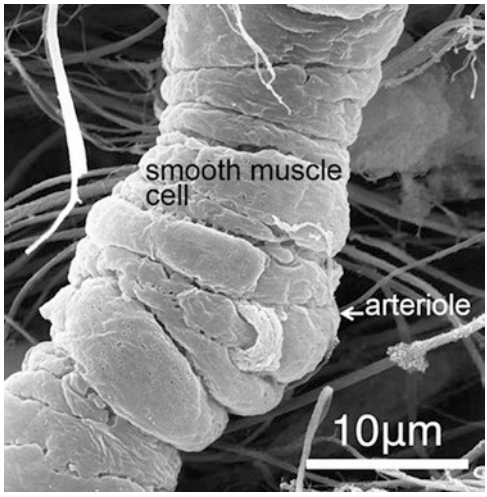

Fig. 7.2 Scanning electron micrograph of arteriole in the vocal fold mucosa. (Modified sodium hydroxide (NaOH) maceration method). Arterioles in the human vocal fold mucosa are relatively thin

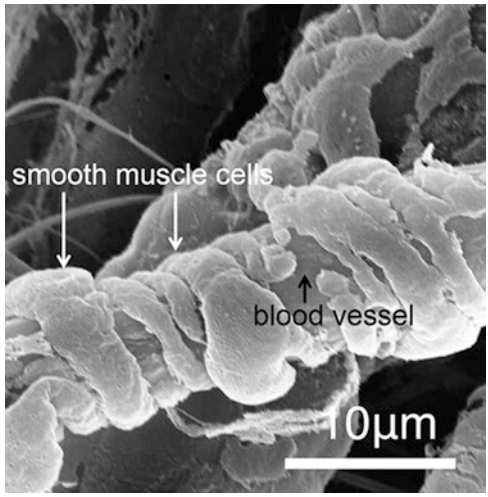

Fig. 7.3 Scanning electron micrograph of the transition area from arteriole to capillary (arterial capillaries). (Modified NaOH maceration method). Arterial capillaries are intermediate in form between smooth muscle cells and pericytes

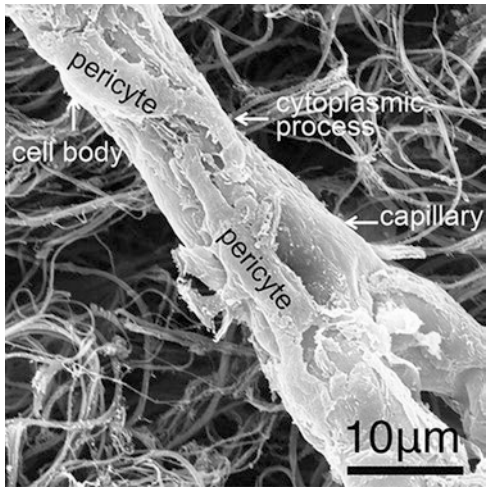

Fig. 7.4 Scanning electron micrograph of capillary and pericytes in the human vocal fold mucosa. (Modified NaOH maceration method)

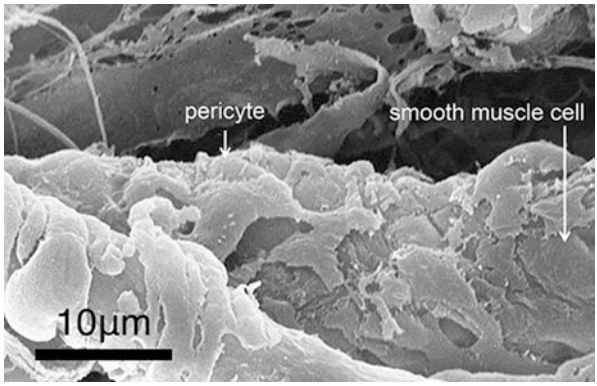

Fig. 7.5 Scanning electron micrograph of the transition area from capillary to venule (venous capillaries). (Modified NaOH maceration method). Venous capillaries are intermediate in form between pericytes and smooth muscle cells

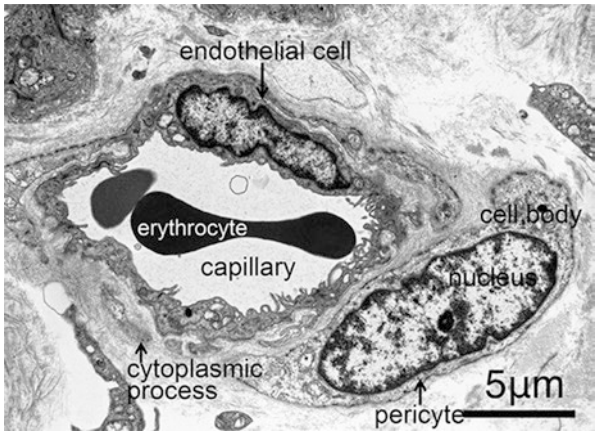

Fig. 7.6 Transmission electron micrographic cross section of a capillary in the superficial layer of the lamina propria (Reinke's space) of the human vocal fold (uranyl acetate and lead citrate stain). A single endothelial cell extends completely around the lumen and the pericyte encircles the capillary

which are the area of transition from an arteriole to a capillary, are intermediate in form between smooth muscle cells and pericytes (Fig. 7.3). The blood vessels then transition to capillaries.

Capillaries

The capillary wall consists of a layer of endothelial cells, basal laminae, and a sparse network of reticular fibers (Fig. 7.6). The diameter of capillaries in the human vocal fold mucosa averages about 8–12 µm, which permits unimpeded passage of the cellular elements of the blood. The luminal surface of the endothelium is generally smooth, but the thin margins of the adjacent cells overlap slightly, and a thin marginal fold projects a short distance into the lumen. Some endothelial cells of the capillaries are interrupted by circular fenestrations or pores, 60–70 nm in diameter, each closed by a very thin pore diaphragm [5].

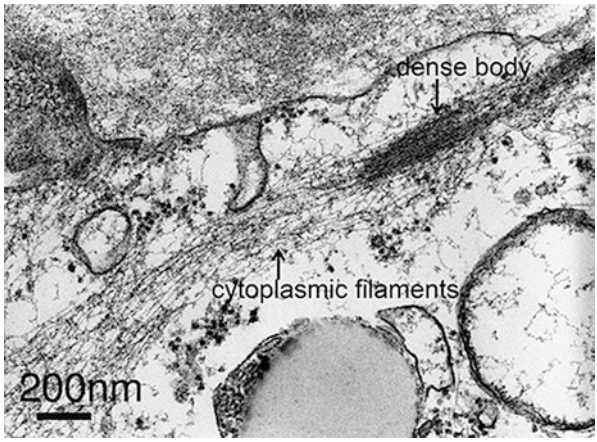

Fig. 7.7 Transmission electron micrograph of the cytoplasm of a pericyte (uranyl acetate and lead citrate stain). Many cytoplasmic filaments come together to form dense bodies

Many pericytes can be seen around the capillaries in the human vocal fold mucosa (Figs. 7.4 and 7.6) [14]. Each pericyte has a cell body and branching cytoplasmic processes (Fig. 7.4). The cell bodies are bulged fusiform or polygonal. Branching cytoplasmic processes consist of short circumferential processes and long and relatively thick longitudinal processes that are parallel to the axis of the blood vessel. The pericytes are 5–10 μm by 15–30 μm and cell bodies are 5–10 μm by 10–15 μm in size. The cell bodies of the pericytes attach to capillary endothelial cells, and the branching processes also attach to the capillary endothelial cells at the tips and encircle the capillaries. The processes of the pericytes are fingerlike or clawlike in appearance and, just like the cell bodies, appear to grasp the blood vessels.

Components in the pericyte cytoplasm including rough endoplasmic reticulum, mitochondria, and free ribosomes are present. Many cytoplasmic filaments can be seen (Fig. 7.7), not only in the cell bodies but in the processes as well. The filaments come together to form dense bodies (Fig. 7.7).

Adjacent to the endothelial cells, a dense meshwork of cytoplasmic filaments in the processes of pericytes is noted (Fig. 7.8). On the outside of the processes, many pinocytotic vesicles are observed in the cytoplasm (Fig. 7.8). Cell bodies and processes of pericytes encircle the capillaries and attach to their capillary walls. The cell bodies of pericytes and endothelial cells remain separated by a gap of 300–500 nm (Figs. 7.6 and 7.8a). The processes of pericytes are in close contact with endothelial cells, sharing a common basement membrane with them (Fig. 7.8b). The tips of the processes form intercellular tight junctions with endothelial cells (Fig. 7.8b).

Venules

Several capillaries unite and form a venule, which is a cylindrical blood vessel 15–20 μm in diameter [2].

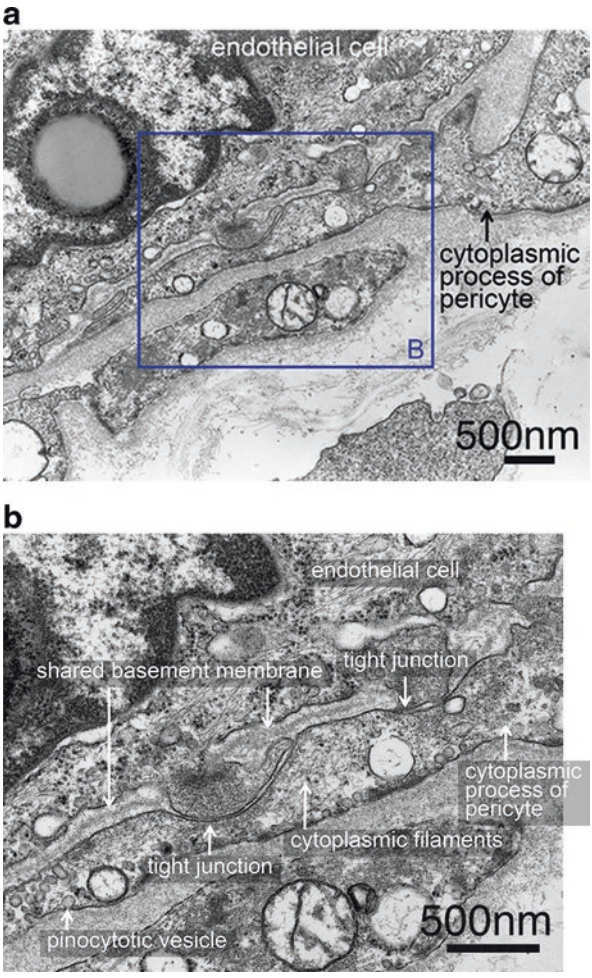

Fig. 7.8 Transmission electron micrograph of an endothelial cell and pericyte. (**b**: region B in **a**) (uranyl acetate and lead citrate stain). The processes of pericytes are in close contact with endothelial cells, sharing a common basement membrane with them and the tips of the processes form intercellular tight junctions with endothelial cells

The terminal capillaries pass through a short transitional region in which scattered smooth muscle cells begin to appear around the blood vessels (Fig. 7.5). Venous capillaries, the area of transition from a capillary to a venule, are intermediate in form between pericytes and smooth muscle cells (Fig. 7.5). The blood vessels then transition to venules.

Development of the Blood Vessels and Pericytes in the Human Vocal Fold Mucosa

The lamina propria of the newborn vocal fold mucosa is a loose structure composed of ground substances and sparse fibers, and no structure corresponding to the vocal ligament can be found. The layered structure in adult vocal folds is not present at birth.

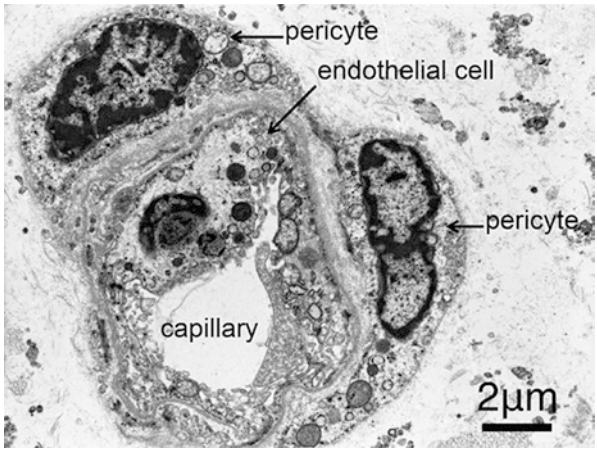

Fig. 7.9 Transmission electron micrographic cross section of a capillary in the superficial layer of the lamina propria of the newborn vocal fold (uranyl acetate and lead citrate stain). A single endothelial cell extends completely around the lumen and the pericytes encircle the capillary at birth

The lamina propria of the newborn vocal fold lacks not only a vocal ligament and layered structure but also the characteristic complex of extracellular matrices seen in adults. The viscoelasticity of the newborn vocal fold mucosa is morphologically not sufficient for phonation. The newborn vocal fold mucosa is not suitable for vibration but is in the process of acquiring the viscoelastic properties of the human vocal fold mucosa as a vibrating tissue.

In the lamina propria of the newborn vocal fold mucosa, capillaries about 10 μm in diameter are present (Fig. 7.9). There are many pericytes around the capillaries. The cell bodies and processes of the pericytes encircle the capillaries in the newborn vocal fold mucosa (Fig. 7.9). At birth, the same microstructure of capillaries in the vocal fold mucosa as in the adult vocal fold mucosa is present.

Physiological Significances of the Vascular Network and Pericytes in the Human Vocal Fold Mucosa

Vascular Network in the Human Vocal Fold Mucosa

Only small blood vessels enter the vocal fold edge from the anterior or posterior end of the membranous vocal fold and run essentially parallel to the vocal fold edge. And these small blood vessels are clearly differentiated from blood vessels in the upper and lower vocal fold mucosa as well as from blood vessels in the vocalis muscle.

Since the portion of the vocal fold which vibrates the most during phonation is the vocal fold mucosa, the structure of the vascular network in the mucosa of the human vocal fold edge is well suited for vibration [4, 8]. The structure of the vascular network in the mucosa of the vocal fold edge is also well suited to prevent circulatory disturbance caused by vocal fold vibration. The vascular structures of the

human vocal fold mucosa that have the capacity to vibrate have a specific structure suitable for vibration and also minimize hypoxia of the vocal fold tissue.

The arterioles form an important segment of the circulation, because they constitute the principle component of the peripheral resistance to flow that regulates blood pressure [2]. The exchange between the blood and the tissue takes place in the capillaries [2]. The venules also have a role in the exchange between the blood and the tissue, and they are particularly important in the changes associated with inflammation [2].

Pericytes in the Human Vocal Fold Mucosa

Zimmermann studied the capillary pericytes using light microscopy with silver staining [20]. Electron microscopic studies have been conducted on capillary pericytes in other organs [7, 9, 11, 17–19]. The number and shape of capillary pericytes differ according to the organs and tissue. The number of capillary pericytes is related to the density of the capillary bed [18]. Their shape and distribution are related to organ function.

The functions of pericytes remain unclear. Synthesis, mechanical support, protection, detection, differentiation, and capillary contraction have been suggested. Cytoplasmic filaments were previously noted in pericytes, and thus they are considered contractile cells that modulate microvascular blood flow [7, 9]. Pericytes are critical cells in vascular biology, especially angiogenesis. They intervene at different levels of blood vessel formation, being involved in endothelial cell stimulation and guidance as well as endothelial stabilization and maturation [12].

Pericytes have been previously noted around capillaries in the vocal fold mucosa [3, 5, 14]. From the morphological point of view, the pericytes in the human vocal fold mucosa are essentially the same as those in other organs [14]. Many pericytes can be seen around capillaries, arterial capillaries, and venous capillaries in the human vocal fold mucosa [14]. The most noteworthy finding concerning the pericytes in the human vocal fold mucosa is the presence of processes thicker than those in other organs [14].

Pericytes in the human vocal fold mucosa encircle the capillary walls. The tips of the processes form tight intercellular junctions with endothelial cells. The cell bodies and processes appear to grasp the vessels and support and protect capillary walls [14]. It is their thickness and firm connection with endothelial cells which render them particularly suitable for such support and protection [14]. Many cytoplasmic filaments can be seen to come together to form dense bodies. The pericytes thus provide great support and protection for the capillary walls [14]. As a result, the vessels in the human vocal fold mucosa, which is the vibrating portion of the vocal folds, do not rupture easily, even during frequent and strong vibrations.

The blood flow of the vocal fold mucosa is reduced during phonation [6] but increases thereafter. The pericytes in the vocal fold mucosa appear to provide mechanical support and protection to the capillary walls, particularly during

phonation [5, 14]. The pericytes also appear to regulate the diameter of the capillary during and after phonation.

Pericytes in the human vocal fold mucosa are also thought to be critical cells in vascular biology and angiogenesis, especially in revascularization following vocal fold tissue injury.

Pericytes have already encircled the capillaries in the newborn vocal fold mucosa. The pericytes appear ready to provide support and protection of the blood vessels just after birth [5, 14].

Deseases Related to the Vascular Network and Pericytes in the Human Vocal Fold Mucosa

Reinke's Edema

Reinke's edema is a common disease of the vocal fold ultimately causing changes in voice quality. The entire length of the membranous vocal fold is edematous and swollen (Fig. 7.10). The primary histopathological feature is edema in Reinke's space. The mechanism for the onset and development of the disease remains unclear. The most frequent etiologic factors of Reinke's edema are considered smoking and aging. Another possible etiologic factor is vocal abuse.

Microscopically, subepithelial vascularization is seen in the vocal fold mucosa with Reinke's edema (Fig. 7.10). The blood vessels are not parallel to the edge of the vocal fold but run in random directions (Fig. 7.10).

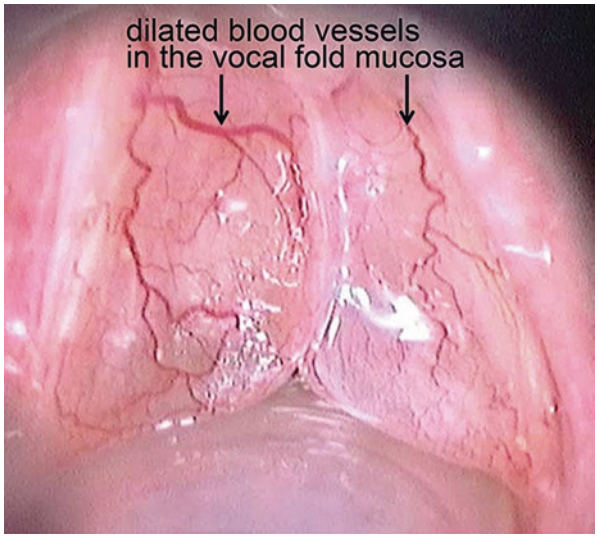

Fig. 7.10 Endolaryngeal microscopic view of Reinke's edema

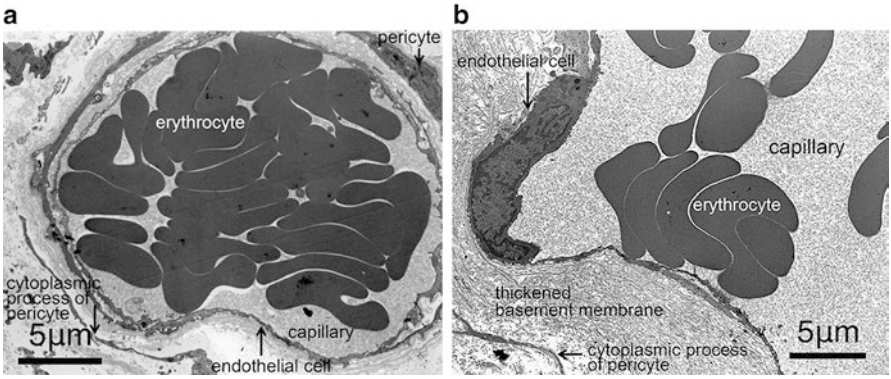

Fig. 7.11 Transmission electron micrograph of Reinke's space of the vocal fold mucosa with Reinke's edema (uranyl acetate and lead citrate stain). The blood vessels are dilated and the blood vessel walls are thin, as are the endothelial cells and pericytes

Edema in Reinke's space appears to be related to blood vessels in this space. Subepithelial vascularization is evident in Reinke's space (Fig. 7.11) [15]. Blood vessels are dilated to 20–30 µm in diameter but capillary diameter varies considerably. The blood vessel walls are thin, as is the cytoplasm of the endothelial cells (Fig. 7.11). The cytoplasm (cell body and branching cytoplasmic processes) of the pericytes is thin (Fig. 7.11). The cell bodies and branching processes are not attached to the endothelial cells of the blood vessels (Fig. 7.11). The number of pericytes has decreased. The pericytes are situated away from the endothelial cells and share the thickened basement membrane with them (Fig. 7.11). The pericytes appear not to adequately support and protect the capillary walls, particularly during phonation. Thus, the blood vessels affected by Reinke's edema are fragile, and this fragility most likely affects the blood circulation in the vocal fold mucosa. Fragile blood vessels not parallel to the edge of the vocal fold, but running in random directions in Reinke's space, are easily injured and collapse during phonation [15]. In addition, the vibrating patterns of the edematous vocal folds with Reinke's edema change bringing an additional adverse effect to the fragile vessels [15].

The exchange between blood and tissue takes place in the capillaries [2]. Morphologically, capillary permeability appears to be higher than normal with Reinke's edema [15].

The etiologic factors bring about hypoxia and ischemia of the vocal fold mucosa. Hypoxia in vitro and ischemia in vivo increase vascular endothelial growth factor (VEGF) mRNA in normal tissues and certain human tumors [16]. These disorders may likely increase VEGF in Reinke's space, with possibly greater subepithelial vascularization and capillary permeability as well [15]. The interstitial cells and/or inflammatory cells in the superficial layer of the lamina propria (Reinke's space) show cytoplasm staining with VEGF, while no staining of this growth factor is found in any component of the lamina propria of normal vocal folds. VEGF is implicated in the control of angiogenesis due to its selective mitogenic stimulation

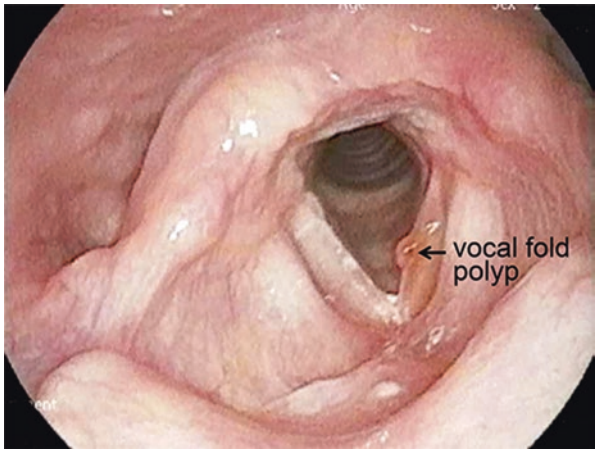

Fig. 7.12 Endoscopic view of a left vocal fold polyp (62-year-old female). The vocal fold polyp is located at the midpoint of the membranous vocal fold. The left vocal fold appears yellow resulting from the presence of hemosiderin which usually accompanies hemorrhage

of vascular endothelial cells and enhancement of vascular permeability. VEGF produced by interstitial cells and/or inflammatory cells in Reinke's space likely promotes vascularization and an increase in blood vessel permeability [15].

Subepithelial vascularization, dilatation and fragility of the blood vessels, and increased permeability of the blood vessels are observed in Reinke's edema [15]. Thus, fragility of and alteration in the permeability of the blood vessels are presumed to cause edema of the superficial layer of the lamina propria (Reike's space), which likely progresses to Reinke's edema [15].

Hemorrhage in Reinke's Space and Vocal Fold Polyps

The frequent violent slapping of the vocal folds against each other caused by overuse or abuse of the voice results in exudation of inflammatory blood products into Reinke's space. Therefore, vocal fold polyps usually occur at the midpoint of the membranous vocal fold, which vibrates the most during phonation (Fig. 7.12).

Trauma of the vessels results in exudation from ruptured blood vessels. The histopathological appearances of these lesions show combinations of the exudation (plasma, erythrocytes, etc.) and interstitial cell and extracellular matrix reactions (Fig. 7.13).

Microvascular Leisions of the Vocal Fold

Microvascular lesions, also called varices or capillary ectasias, are relatively small lesions arising from the microcirculation of the vocal fold [10] (Fig. 7.14). Microvascular lesions are most commonly seen in female professional vocalists.

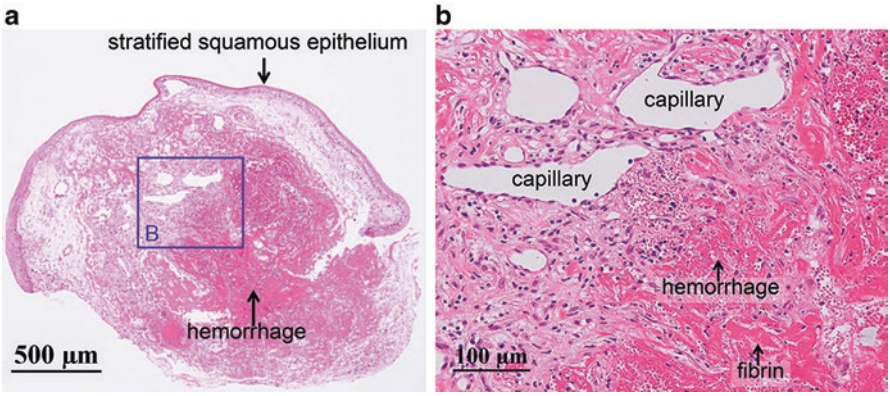

Fig. 7.13 Histopathology of an excised vocal fold polyp. (**a**) Hemorrhage into Reinke's space. (**b**) There is marked fibrin (hyaline pink-stained amorphous material) and erythrocyte exudation (hemorrhage) into Reinke's space. Capillaries are dilated. Connective tissue has been proliferated and inflammatory cells and fibroblasts have infiltrated (region B in **a**)

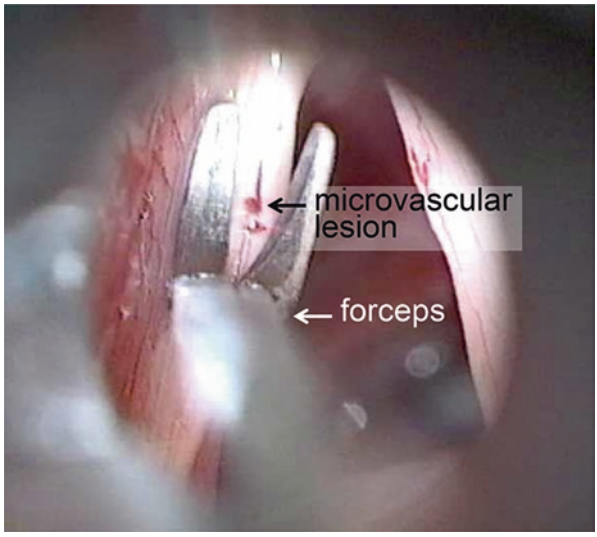

Fig. 7.14 Microscopic view of a microvascular lesion of the left vocal fold during endolaryngeal microsurgery (41-year-old female, soprano singer)

Microvascular lesions are the result of microvascular trauma within the superficial layer of the lamina propria (Reinke's space) of the human vocal fold. Therefore, microvascular lesions usually occur on the surface at the midpoint of the membranous vocal fold, which vibrates the most during phonation (Fig. 7.14). The microvascular lesions are present at the superficial layer of the lamina propria (Reinke's space) of the human vocal fold mucosa just under the basement membrane (Fig. 7.15).

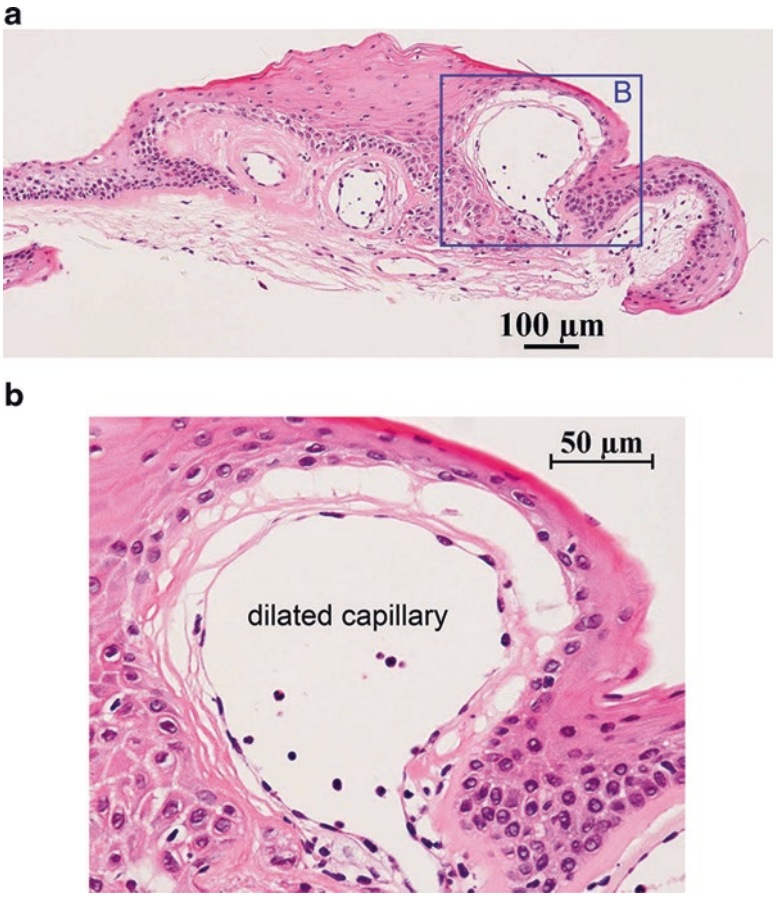

Fig. 7.15 Histopathology of an excised microvascular lesion. Dilated capillaries are observed in the lamina propria of the vocal fold mucosa just beneath the stratified squamous epithelium (**b**: region B in **a**)

Vocal Fold Tissue Injury

Pericytes are critical cells in vascular biology, especially angiogenesis. They intervene at different levels of blood vessel formation, being involved in endothelial cell stimulation and guidance as well as endothelial stabilization and maturation [12]. Pericytes in the human vocal fold mucosa are also thought to be critical cells in vascular biology and angiogenesis, especially in revascularization following vocal fold tissue injury.

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Chapter 8
Ca^{2+} Signalling in Pericytes

Theodor Burdyga and Lyudmyla Borysova

Abstract Microcirculation is the generic name for the finest level of the circulatory system and consists of arteriolar and venular networks located upstream and downstream of capillaries, respectively. Anatomically arterioles are surrounded by a monolayer of spindle-shaped smooth muscle cells (myocytes), while terminal branches of precapillary arterioles, capillaries and all sections of postcapillary venules are surrounded by a monolayer of morphologically different perivascular cells (pericytes). Pericytes are essential components of the microvascular vessel wall. Wrapped around endothelial cells, they occupy a strategic position at the interface between the circulating blood and the interstitial space. There are physiological differences in the responses of pericytes and myocytes to vasoactive molecules, which suggest that these two types of vascular cells could have different functional roles in the regulation of local blood flow within the same microvascular bed. Also, pericytes may play different roles in different microcirculatory beds to meet the characteristics of individual organs. Contractile activity of pericytes and myocytes is controlled by changes of cytosolic free Ca^{2+}concentration. In this chapter, we attempt to summarize the results in the field of Ca^{2+} signalling in pericytes especially in light of their contractile roles in different tissues and organs. We investigate the literature and describe our results regarding sources of Ca^{2+}, relative importance and mechanisms of Ca^{2+} release and Ca^{2+} entry in control of the spatio-temporal characteristics of the Ca^{2+} signals in pericytes, where possible Ca^{2+} signalling and contractile responses in pericytes are compared to those of myocytes.

Keywords Pericytes · Ca^{2+} signalling · Ca^{2+} wave · Ca^{2+} oscillations · Vasoconstriction · Microvascular networks · Precapillary arterioles · Postcapillary venules · Capillaries · Gap junctions · Conducted vasoconstriction · Conducted vasodilation

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Introduction

The most highly investigated and supported functional property of pericytes has been their ability to contract. Ca^{2+} signalling controls the contractile apparatus of pericytes. The advent of Ca^{2+}-sensitive fluorescent dyes, together with video-based imaging and photometric techniques, opened the door for the investigation of pericyte Ca^{2+} signalling. The involvement and relative roles of extracellular and intracellular sources of Ca^{2+} and mechanisms of Ca^{2+} entry and Ca^{2+} release which shape the spatio-temporal profiles of the Ca^{2+} signals induced by putative pericyte vasoconstrictors and vasodilators are under current investigation.Ca^{2+} signalling in pericytes in culture or in isolated microvessels was measured primarily with a photometric ("blind") system of recording of the fluorescent signals using the ratio-metric Ca^{2+}-sensitive indicators, Indo-1 [15, 71] or Fura-2 [40, 41, 51, 84]. Recently the use of confocal Ca^{2+} imaging which brings many advantages to the study of Ca^{2+} signalling in situ has been used. The vessel images permit individual vascular smooth muscle cells (myocytes), pericytes and endothelial cells to be visualized which permits quantitation of the changes of Ca^{2+} signals in these cells and correlate them with the mechanical or electrical events [7, 8, 10, 22–24, 54, 55, 69, 92]. In this chapter, we attempt to summarize our results and those of others in the field of Ca^{2+}signalling of pericytes obtained for the last two decades.

Topology and Morphology of Pericytes

There is a lengthy history of morphological studies of pericytes which led to a firm conclusion that pericytes represent a distinctive cell population of the microvasculature of almost all tissues and organ systems which have ubiquitous distribution and morphology. Traditionally, pericytes are defined as extensively branched cells and based on location and histological characteristics, three different types have been identified: precapillary (arteriolar), capillary and postcapillary (venular) pericytes [93]. These topology and morphology have been confirmed by scanning electron microscopy studies of microvessels in different tissues [19, 29, 31, 36, 47, 60, 76], by live confocal imaging of microvascular network loaded with Ca-sensitive indicators in situ (Fig. 8.1) [8, 23] and recently by live imaging of microvascular networks in vivo using genetically encoded microvascular mural cell labelling [27, 30]. Precapillary pericytes are located in the distal part of the precapillary arterioles. Morphologically, they have thick cell bodies giving several fingerlike processes tightly wrapping around the endothelium (Fig. 8.1a (ii), c(i)). All sections of postcapillary venules are surrounded by fibroblast-like pericytes, with stellate cell bodies giving slender cell projections. These projections appear to be randomly oriented with respect to the vessel axis, and overlap or attach to each other, forming a complicated and dense meshwork (Fig. 8.1a (iv), c(iii–v)).

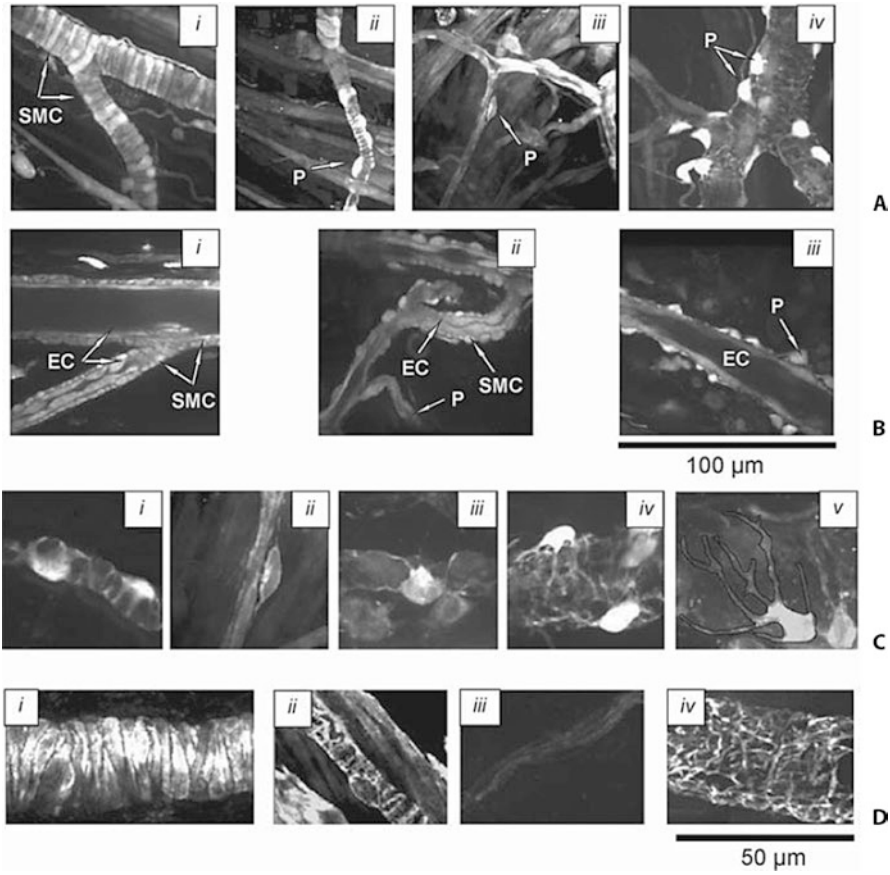

Fig. 8.1 Morphology of the ureteric microvascular network in situ. (**a**) Images of precapillary arterioles (i, ii), capillaries (iii) and postcapillary venules (iv). *P* Pericytes of precapillary arterioles, capillaries and postcapillary venules, respectively; *SMC* smooth muscle cells. (**b**) Radial sections of arcade and transverse arterioles (i), the precapillary arteriole and capillary (ii) and the postcapillary venule (iii), showing a monolayer of endothelial cells (EC) surrounded by a monolayer of myocytes (SMC) and pericytes (P). (**c**) Images showing diversity of shapes of pericytes observed in the precapillary arterioles (i), capillaries (ii) and postcapillary venules (iii–v). (**d**) Images of myocytes (i), precapillary pericytes (ii), capillary pericytes (iii) and postcapillary pericytes (iv) stained with fluorescently labelled phalloidin. (Data were reproduced from [8] with permission)

Mid-capillaries of all tissues are invested with pericytes which have elongated cell bodies with two long slender processes on each side running parallel to the long axis of the capillary tube (Fig. 8.1c). These primary processes taper to smaller processes which partly encircle the capillary wall which gives the appearance that pericytes cradle the capillary tubes [93].

Contractility

The most highly investigated and supported functional property of pericytes has been their ability to contract. There is a general agreement that precapillary pericytes are contractile cells. They exhibit a cytoskeleton similar to vascular smooth muscle cells, with ordered bundles of actin microfilaments [14], and express the tropomyosin [37], the enzyme cGMP-dependent protein kinase [38] and smooth muscle α-actin (α-SMA) [8, 12, 61, 62]. Expression of α-SMA in pericytes of precapillary arterioles has recently been confirmed in α-SMA-GFP transgenic albino mice [12]. Precapillary pericytes stain positive for chondroitin sulphate proteoglycan (NG2), a pericyte marker, and are defined as $NG2^+$ $\alpha\text{-SMA}^+$ phenotype [80]. Contraction of a single pericyte is sufficient to fully occlude precapillary arteriole [8]. Given the unique anatomical location, the pericytes of precapillary arterioles represent prominent controlling elements in the regulation of tissue blood supply and are suggested to act as precapillary sphincters [8, 60, 68, 91]. The term precapillary sphincter has been used to imply the functional significance of the area which controls blood flow to the capillary bed.

Thus the morphological appearances of the precapillary pericytes, their molecular composition and ability to contract are consistent with a contractile phenotype.

The pericytes of postcapillary venules stain positive for α-SMA [8, 12, 23, 56, 61, 62] but negative for NG2 and thus are classified as $NG2^-$ α-SMA phenotype [56, 80]. The information has been accumulated which proves that venular postcapillary pericytes are contractile cells and they can perform several functions in microcirculation which include regulation of blood flow and perfusion [8, 25, 48, 57], aperture of interendothelial junctions [59] and junction protection from overstretching ("umbrella-like" disposition over interendothelial junctions) [77].

There are conflicting data as to whether capillary pericytes are contractile cells. They stain positively for NG2 but in most cases negatively for α-SMA [6, 8, 25, 27, 30, 56, 61, 62]. α-SMA is not expressed in capillaries of transgenic albino mice [12]. The presence of the contractile proteins in capillary pericytes is quite controversial and seems to depend upon the vascular bed from which they originate and their position within the capillary network. For example, smooth muscle myosin is found in capillary pericytes from the heart, diaphragm, pancreas, and intestinal mucosa [37] but not in the brain and retina [30]. The difficulty in distinguishing contractile from non-contractile pericytes on capillaries in vivo has brought the concept of physiological and pathological capillary pericyte contractility in CNS into question [82]. Some authors reported that cerebral ischemia leads to constriction of capillary pericytes that persists after their death and has been proposed to prevent tissue reperfusion, leading to the "no-reflow" phenomenon [26, 64, 86]. Also, recent studies have highlighted the critical roles of capillary pericytes in the regulation of coronary no-reflow after myocardial ischaemia [63]. On the other hand, using a combination of genetically encoded microvascular mural cell labelling, high-resolution imaging of vasomotor activity and functional calcium imaging in vivo, Hill et al. [30] dismiss the idea that capillary pericytes are involved in the regulation

of brain blood flow. Similar results have been reported for the guinea pig inner ear microcirculation [5]. Resolution of these discrepancies is of great importance, and the controversy could be attributed to a drift in pericyte definition [2].

Electrical Activity, Ion Currents and Channels

Electrophysiological studies reveal that pericytes are electrically excitable cells [28, 49, 51, 89]. The resting membrane potential of pericytes ranges from −32 to −70 mV [28, 89]. Pericytes of submucosal postcapillary venules generate repetitive action potentials with a slow upstroke and decay associated with vasomotion [25, 57]. In cultured bovine retinal pericytes, repetitive spike-like action potentials (AP) are induced by $BaCl_2$ and norepinephrine. These APs depended on the presence of extracellular Ca^{2+} and are inhibited by the Ca^{2+} antagonist nifedipine [28]. K^+ channel blockers TEA or $BaCl_2$ induce depolarization of pericytes and constriction of vasa recta [89]. The voltage clamp experiments performed on pericytes in culture [51] or isolated microvessels [49, 51, 89] show that pericytes contain L-type voltage-gated Ca^{2+} channels (VGCC). Comparative studies performed on the freshly isolated retinal microvascular complexes [49] and kidney tissue slices [89] show that Ca current in pericytes [49, 89] is 7.5 times smaller than in myocytes. These findings indicate that potential-dependent, dihydropyridine-sensitive VGCC play a more significant role in excitation-contraction coupling in the arteriolar myocytes but may either be sparsely distributed or physiologically silent in the pericytes. In rat retinal pericytes, this difference in VDCC function is, in large part, due to the selective inhibition of VDCCs by endogenous spermine, a polyamine known to inhibit L-type calcium channels [49]. Recently it was reported that T-type VDCC are functionally expressed in pericytes of the myenteric microvasculature of the guinea pig gastric antrum [24].

Pericytes also have a number of other channels such as Ca^{2+}-activated Cl^- (Cl_{Ca}) [23, 25, 52–55, 74] and K^+ (K_{Ca}) channels [46, 89], ATP-sensitive potassium (K_{ATP}) channels [33], inwardly rectifying K^+ channels (K_{IR}) [28, 50, 89], voltage-dependent K^+ channels (K_V) [46, 89], voltage-gated Na^+ channels [87], nonselective cation (NSCC) channels [70], and transient receptor potential channels [44].

Ca^{2+} Signalling and Excitation-Contraction Coupling

The electrical or chemical signals activate the contraction of pericytes via a process known as excitation-contraction coupling (E-C). The second messenger in this transduction system is Ca^{2+}. The generation, modulation and termination of the intracellular Ca^{2+} transient are the essence of E-C coupling in pericytes. To activate the contractile apparatus, Ca^{2+} must increase globally throughout the cytoplasm. Similar to other excitable cells, pericytes have a sophisticated Ca^{2+} control

system that keeps $[Ca^{2+}]_i$ at low levels at rest and ensures that $[Ca^{2+}]_i$ is rapidly and uniformly elevated within each pericyte in response to the electrical or chemical signal. The favourable and large electrochemical gradient provides a strong driving force for the influx of Ca^{2+} via VGCC into the cell during membrane depolarization or Ca^{2+} release from the SR.

VGCC-Dependent Ca^{2+} Entry Induced by Membrane Depolarization

High K^+ is widely used to test the presence of functional VGCC in plasma membrane of excitable cells. High K^+ activates Ca^{2+} entry via L-type VGCC in pericytes of precapillary arterioles, capillaries and postcapillary venules of rat ureteric microvascular bed in situ [8], capillaries of rat retina [49] and kidney vasa recta [89]. High-K^+-induced Ca transient in pericyte is fully blocked by L-type Ca channel blockers. Comparative studies performed on the freshly isolated retinal microvascular complexes [49], kidney tissue slices [89] and ureteric microvascular networks in situ [8] show that L-type VDCC-mediated changes in intracellular Ca^{2+} signals and contractility induced by high-K^+ depolarization are four to six times smaller in pericytes than in the myocytes.

VGCC-Dependent Intercellular Ca^{2+} Signalling

Ca^{2+} influx via L-type VGCC generates global rise of intracellular Ca^{2+} which activates pericyte contraction and produces depolarizing current which can spread from cell to cell via gap junction synchronizing Ca^{2+} signalling and contraction of large group of mural cells producing vasoconstriction [8, 24]. Longitudinal conduction of vasomotor responses provides an essential means of coordinating changes in diameter and blood flow distribution in a complex microvascular network [3, 4].

Elegant electrophysiological experiments performed on isolated retinal microvascular complexes [35, 49, 65, 83, 88] demonstrate that pericytes of capillaries are electrically coupled to myocytes of upstream precapillary (feed, branch) arterioles and constitute a functional unit in which cell-to-cell bidirectional transmission of electrical signals by way of gap junctions is present and highly dynamic. Some agonists and lactate selectively inhibit axial transmission of electrical signals from capillary to arteriolar network [88]. The intercellular transmission of electrical signals between retinal pericytes are also reduced in diabetes due to lower expression of connexion 43 [35]. Overall, the existing data indicate that pericytes and myocytes are electrically coupled in which cell-to-cell transmission of electrical signals by way of gap junctions is present and can be modulated by different vasoactive signals and some pathological conditions.

Synchronized Ca^{2+} Oscillations in Pericytes of Postcapillary Venules

Spontaneous rhythmic contractions controlled by action potentials with a slow upstroke and decay appears to be a common property of postcapillary venules in the wall of several hollow organs distended by their luminal contents [25, 27]. Live confocal imaging revealed that suburothelial venular pericytes exhibit synchronized spontaneous Ca^{2+} transients to develop vasomotion which is abolished by nifedipine, cyclopiazonic acid or Ca^{2+}-activated Cl^- channel inhibitors [54]. Low Cl^- solution disrupts the synchrony of spontaneous Ca^{2+} transients and abolishes vasomotion [54]. Blockade of gap junction with carbenoxolone disrupts the synchrony of Ca^{2+} transients in postcapillary venules [54]. In the presence of L-type Ca^{2+}channel blockers (nifedipine, nicardipine), small fluctuations of the membrane potential and residual asynchronous Ca^{2+} oscillations accompanied by irregular small regional vasoconstrictions are observed [25, 54]. These residual nifedipine-resistant asynchronous Ca^{2+} oscillations are suppressed by inhibitors of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase cyclopiazonic acid (CPA), IP_3 receptors channels blocker 2-aminoethoxydiphenyl borate (2-APB), and phospholipase C inhibitor U-73122 but unaffected by ryanodine [23, 25]. It is suggested that asynchronous Ca oscillations are produced by Ca release from the SR and are mediated by IP_3R channels. Ca^{2+}released from the SR activates Ca^{2+}-activated Cl^- channels which results in depolarization that triggers Ca^{2+} influx through L-type Ca^{2+}channels [23, 25, 52–55]. The depolarization caused by the opening of Ca^{2+}-activated Cl^- channel itself is not sufficiently large enough to spread between postcapillary pericytes, presumably due to a limited spatial spread via gap junctions [25, 54]. Thus, regenerative opening of L-type VGCC is critical for the propagation and synchronization of excitation between pericytes in postcapillary venules of most parts of the GI tract and bladder [23, 25, 52–55]. The Ca^{2+}oscillations in the capillary pericytes of the microvasculature of the myenteric plexus of the guinea pig antrum arise through Ca^{2+}release from SR with synchronization predominantly mediated by T-type VGCC [24]. As a consequence depending on location of the microvascular bed and type of pericyte at least in microvessels of GI tract, L-type or T-type VGCC play a key role in maintaining synchrony amongst pericytes [23–25, 52–55].

Propagating Intercellular Ca^{2+} Waves in Ureteric Microvessels

The myocytes and pericytes of precapillary arterioles and postcapillary venules in rat ureter do not generate spontaneous activity at rest [8]. It has long been established that myocytes of arteries and arterioles are also quiescent at rest but when treated with TEA, they generate spontaneous or evoked spike-like APs [17, 20, 21, 34, 39] sensitive to L-type VGCC blockers [21, 39]. In rat ureteric microvessels pretreated with TEA and BayK, 8644 propagating intercellular Ca waves which appear as Ca spikes accompanied by vasoconstriction of precapillary arterioles and postcapillary venules are generated. These propagating intercellular Ca waves could occur spontaneously or in response to brief (1–2 s) local high-K^+ depolarization or in the presence

of agonists [8]. These Ca waves propagate from the point of stimulation in a regenerative manner at a constant speed and amplitude and synchronize contraction of myocytes and pericytes in precapillary arterioles and postcapillary venules [8]. The speed of propagation of Ca wave between myocytes in arterioles is 1–2 cm/s while between pericytes of precapillary arterioles and postcapillary venules is 1–2 mm/s [8]. These propagating intercellular Ca^{2+} waves and associated vasoconstriction are fully blocked by L-type VGCC blocker nifedipine [8]. Inhibition of gap junctions by 18β-GA reversibly blocks axial spread of the intercellular Ca waves. In the presence of 18β-GA, myocytes and pericyte generate spontaneous asynchronous Ca oscillations associated with random localized vasoconstriction [8]. The intercellular Ca signals between pericytes of isolated vasa recta have also been observed [90].

Collectively the data obtained indicate that myocytes and pericytes are electrically coupled and can generate spreading vasoconstriction. Thus in ureteric microvascular network, transmission of AP-mediated intercellular Ca^{2+}waves between myocytes and pericytes requires participation of depolarization, L-type VGCC and gap junctions [8]. L-type VGCC play a key role in the propagation of intercellular Ca2+ wave between and within arteriolar and venular networks in ureteric microvascular bed in situ [8].

Ca^{2+} Signalling Induced by GPCR Agonists

Pericytes express receptors and respond to a broad range of local and central vasoconstrictors [16]. Microfluorometry has been extensively used to record average changes in the concentration of $[Ca^{2+}]_i$ in the mural cells of isolated retinal microvessels [75], pericytes in culture [11, 15, 51, 71] and isolated microvessels [33, 42, 84, 89]. These data demonstrate that GPCR agonists, e.g. endothelin-1 (ET-1), (Arg^8)-vasopressin (AVP), angiotensin II (Ang II), norepinephrine and ATP, evoke stereotypic Ca^{2+} transients, consisting of an initial spike followed by a sustained plateau component. It was previously thought that in both myocytes and pericytes, contraction is initiated by sarcoplasmic SR Ca^{2+} release and maintained by Ca^{2+} influx [42, 75]. Accumulated data indicate that ET-1, AVP and Ang II, upon binding to their receptors on the plasma membrane of pericytes, activate phosphoinositide phospholipase C-β which then hydrolyses phosphatidylinositol-4,5-bisphosphate to yield IP_3 [71]. In turn, IP_3 initiates Ca^{2+} release from the sarcoplasmic reticulum (SR) through binding and activation of to IP_3Rs channels. The molecular identity of IP3R channels in pericytes has not yet been identified.

IP_3R-Mediated Intracellular Ca^{2+} Waves and Oscillations

Confocal microscopy of intact blood vessels loaded with Ca^{2+}-sensitive dyes has shown that in many blood vessels in vitro or in situ, GPCR agonist-induced contractions are controlled by asynchronous Ca^{2+} oscillations in individual myocytes or

pericytes which appear as propagating intracellular Ca^{2+} waves [7–10, 69, 81]. The asynchronous wavelike Ca^{2+}oscillations are now recognized as a common mode of Ca^{2+} signalling in vascular myocytes of arteries and arterioles [7–10, 32, 69]. In myocytes Ca wave is initiated at one of the ends of myocytes and propagates to the other end in a regenerative manner accompanied by a contraction of an active cell [7–10, 69]. In pericytes agonist-induced Ca^{2+}wave is initiated in one of the processes and propagates to the cell body and other processes [8]. Agonist-induced Ca waves in myocytes and pericytes are observed after long (30–40 min) exposure to Ca^{2+}-free solution and are immune to L-type Ca channel blockers [8]. Ca waves in mural cells are abolished by SERCA pump blocker CPA, IP3R inhibitor 2-APB and PLC inhibitor U73122, but not ryanodine, suggesting a primary role of IP_3-induced Ca^{2+}release from SR in their generation [8]. In the ureter Ca signalling regulating pericyte constriction is different from that regulating arteriole smooth muscle [8]. For example, ET-1 which induces Ca^{2+} oscillations in myocytes of all sections of arteriolar network produces non-oscillatory slowly decaying Ca^{2+}transient associated with strong and long-lasting contraction of pericytes of ureteric precapillary arterioles and postcapillary venules [8]. AVP induces high-frequency Ca oscillations in myocytes but low-frequency Ca oscillations in pericytes [8]. In pericytes containing microvessels, the extent of vasoconstriction depends on the type of agonist and Ca signal produced. Non-oscillatory prolonged Ca spike induced by ET-1 produces much stronger and longer in duration constriction of precapillary arterioles and all sections of postcapillary venules than low-frequency Ca oscillations induced by AVP [8]. Similar to pericytes of the retina [71] and brain capillaries [15], ET-1-induced Ca spike activates strong and prolonged vasoconstriction which lasts up to 10–30 min [8, 78]. In this respect, the responses to ET-1 in pericytes are dramatically different from those seen in myocytes, where the dynamics of the Ca^{2+} signalling and contraction relaxation cycle are very fast [8]. The long-lasting contraction of pericytes induced by ET-1 seems to be the common property of all contractile pericytes and is associated with the complete closure of the precapillary arterioles in the ureter [8] and the vasa recta of the kidney [67] as well as with strong vasoconstriction of all sections of the postcapillary venules in the ureter [8]. The data obtained suggest that Ca signalling and vasomotor responses in pericytes are more heterogeneous than in myocytes with respect to vasoactive agonists, spatiotemporal profile of Ca signals and contractile responses. Several factors such as concentration of IP_3, SERCA pump activity, expression and distribution of IP_3Rs and sensitivity of contractile machinery to Ca may account for these differences, but this needs further investigation. Our recent data reveal that endothelial Ca^{2+} signalling inhibits $[Ca^{2+}]_i$ waves and oscillations mediated by the IP_3R channels in myocytes and pericytes of ureteric microvessels via the L-arginine/NO/cGMP/PKG pathway [9]. These findings are in line with previous results showing that arteriolar dilation by the NO/cGMP/cGKI pathway might involve an inhibition of $[Ca^{2+}]_i$ release via IP_3R channels [18, 79]. The molecular mechanism by which NO inhibits IP_3R is under current investigation. Recently, the PKG substrate, IRAG, has been identified in myocytes, and it has been suggested that it blocks IP_3R activation [1, 13]. Though RyRs have been identified in the myocytes of large arteries and arterioles, there is a distinct lack of evidence for the expression of functional RyRs in

pericytes. Both caffeine, an activator of RyRs, and ryanodine, a selective inhibitor of RyRs, have no effect on agonist-induced Ca^{2+} signals in all types of pericytes [8]. Our recent work [8] supports the conclusion that RyRs do not play a major role in mobilizing Ca^{2+} from the SR in pericytes of ureteric microvessels. However, it is not yet known whether the pericytes of other tissues express functional RyRs.

Voltage-Independent Ca^{2+} Entry

Voltage-independent mechanisms of Ca^{2+} entry induced by different agonists have been reported for pericytes of the retina [70], the vasa recta of the kidney [66, 72, 89] and the suburothelial postcapillary venules [25, 52]. It has been suggested that intracellular nonselective cation channels play a role in the control of Ca^{2+}influx from an extracellular space in retinal pericytes [74]. In the pericytes of the vasa recta, Ang II-induced depletion of the SR Ca^{2+} store activates store-operated channels. These findings are based on observations that SKF 96365, a nonselective blocker of store-operated channels, produces an identical concentration-dependent blockade of Ang II- and CPA-induced responses in the pericytes of the vasa recta. In CNS pericytes, voltage-independent Ca^{2+} entry is mediated by receptor- and store-operated channels [40]. The molecular isoforms of these channels in pericytes are still not known. While the intracellular release of Ca^{2+} in response to GPCR activation is well defined, Ca^{2+} influx in response to agonists in pericytes remains an enigma.

Effects of Metabolic Factors

The control of capillary recruitment and the redistribution of blood flow reside in the terminal arteriolar region of the microcirculation [43, 45, 73]. Recent data suggest that pericytes are metabolically sensitive cells and respond, with the elevation of $[Ca^{2+}]_i$, to several metabolites including lactate [85] and H_2O_2 [41]. Pericytes may also generate hyperpolarizing signals via the activation of K^+ channels and thereby participate in conducted vasodilation [58, 85]. Oxidative stress increases $[Ca^{2+}]_i$ predominantly by Ca^{2+} release from $[Ca^{2+}]_i$ stores. This has been shown to be regulated by tyrosine phosphorylation in rat CNS pericytes [41]. In retinal pericytes, lactate induces Ca^{2+}transients associated with vasoconstriction. It has been suggested that the rise in $[Ca^{2+}]_i$ is caused by the reversal of the Na^+-Ca^{2+} exchanger, i.e. it operates in the Ca^{2+} entry mode. This occurs due to elevation of intracellular Na^+ in the endothelium, in response to the influx of H^+ and lactate via monocarboxylate transporters. When gap junctions are inhibited, lactate induces relaxation of pericytes [85]; however, since Ca^{2+} signals under these conditions have not been monitored in either endothelial cells or pericytes, it is not possible to conclude that this is the only mechanism involved.

Summary and Conclusions

In the diverse areas of developmental and vascular biology and pathology, there is currently an explosion of activity in pericyte research. Collectively, recent work in this area has given solid support for a Ca^{2+} signalling in pericytes and has begun to provide insights into the mechanisms of electro- and pharmacomechanical coupling. The data obtained clearly show that pericytes exhibit a distinct system of Ca^{2+} signalling and contractility. This is encouraging and would allow to develop agents that act selectively on pericytes to treat pathologies associated with pericyte constriction. Further studies are needed to understand mechanisms controlling Ca signalling in pericytes under pathological conditions, e.g. it is not clear which mechanisms of Ca signalling are involved in capillary pericyte constriction caused by ischemia/reperfusion. The determination of the effects of vasoactive molecules on capillary perfusion awaits the development of a method to internally perfuse the microvascular networks in situ and directly visualize fluorescently labelled blood cells flowing through capillaries ex vivo. New techniques will assist in simultaneous investigation of Ca^{2+} signals and cell fluxes using multispectral imaging approaches and genetically encoded Ca^{2+} sensors, which will contribute to a better understanding of how the microvessels are functionally arranged to meet metabolic needs.

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Chapter 9
Pericytes Derived from Human Pluripotent Stem Cells

John Jamieson, Bria Macklin, and Sharon Gerecht

Abstract Pericytes wrap blood microvessels and are believed to play important roles in vascular morphogenesis, maturation, and stability. In addition, pericytes have emerged as candidates for targeting cancer growth and for wound healing. In order to model these processes and test new therapies, it is desirable to have a reliable, scalable source of pericytes. Human pluripotent stem cells (hPSCs), which possess the ability to differentiate into any cell type in the body, have been used to generate pericytes in vitro quickly, consistently, and with high yields. In this chapter, we consider the differentiation of pericytes from hPSCs. We compare the approaches taken by multiple groups and discuss characterization of hPSC-pericytes. Studying pericyte differentiation in vitro provides the opportunity to identify factors influencing pericyte development and to establish the ontogenic relationships between pericytes and similar cells. The development of highly specific, defined pericyte populations from hPSCs will enable downstream applications requiring large quantities of cells, including tissue engineered models and cell therapies.

Keywords Pericytes · Pluripotent stem cells · Differentiation · In vitro · Development · Vasculature · Embryoid body · Mural cells · Tissue engineering · Specification · Protocol · Biomarkers

Introduction

Human pluripotent stem cells (hPSCs) have vast potential applications for cell therapy, regenerative medicine, and disease modeling which are still being realized. hPSCs can be extensively expanded in culture and are capable of producing any cell type in the body. Conducting differentiation in vitro allows for the study the processes of cell fate commitment and specification in a controlled setting, improving our understanding of normal and abnormal development. Several groups have

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demonstrated differentiations of hPSCs toward vascular cell types, including pericytes. hPSC-derived pericytes have been shown to promote revascularization of ischemic tissue, demonstrating their direct utility for cell therapy and tissue engineering.

In this chapter, we first introduce the main sources of hPSCs, including human embryonic stem cells (hESCs), nuclear transfer hESCs (NT-hESCs), and induced hPSCs (hiPSCs). We provide a brief background on the development of each source and its major advantages and disadvantages. We then describe the differentiation of pericytes from hPSCs, introducing techniques such as embryoid body (EB) formation and directed differentiation of monolayer cultures. We discuss the influence of key soluble factors in promoting pericyte differentiation and compare published protocols with regard to cell source and timing. We then introduce in vitro and in vivo characterizations of hPSC-derived pericytes, including assessment of biomarkers and functional assays, such as the stabilization of vascular networks when co-cultured with endothelial cells and the revascularization of ischemic tissue in vivo. We also comment on the potential for differentiation of lineage-specific or tissue-specific pericytes. Finally, we conclude with a discussion of future trends in this rapidly developing field.

Human Pluripotent Stem Cell (hPSC) Sources

The isolation and in vitro expansion of hESCs were first published by James Thompson's group in 1998 [1]. Since, hESCs have served as a catalyst to the fields of tissue engineering and regenerative medicine, with robust hESC-derived vascular, brain, epithelial, and cardiac cell differentiations possible today. The Thompson group was able to successfully culture hESCs by isolating cells of the inner cell mass of the embryo and plating them on a mouse embryonic fibroblast feeder layer. These isolated hESCs displayed a normal karyotype, exhibited high telomerase activity, expressed cell surface markers that characterize undifferentiated cells, and remained pluripotent for over 5 months in culture [1].

With ethical concerns over hESCs coming to the forefront and potential limitation of donor-cell rejection, researchers began developing novel ways to express pluripotency in adult cell types. In 2006, the Yamanaka group successfully reprogrammed human somatic cells into hiPSCs via transfecting four specific transcription factors: OCT4, SOX2, KLF4, and C-MYC [2]. hiPSCs provided an alternative to hESCs that still possessed the capability to derive cell types from all three germ layers and could be created noninvasively from patient cells, creating the opportunity for autologous therapies. However, hiPSCs reportedly exhibit some differences from hESCs that could impose limitations to their utility. There is evidence that hiPSCs may retain an epigenetic "memory" of the parental cells [3] that could affect their propensity for differentiation into various lineages [4].

Thus, some studies have continued to use NT-hESCs, which are reprogrammed by a different method. NT-hESCs are created by a process called somatic cell

nuclear transfer (SCNT). Reprograming these cells occurs in three steps, briefly: induction of the SCNT metaphase II-arrested oocyte, progression from metaphase II to interphase, followed by an electroporation stimulus of the oocyte to release totipotency factors [5]. In comparison to hiPSC generation, the SCNT process is much more difficult and requires donor oocytes [6]. However, some studies have demonstrated that NT-hESCs have methylation and transcriptional profiles that are much more similar to native hESCs [7]. Other studies have shown that both hiPSCs and NT-hESCs are distinct from hESCs to a comparable extent [8, 9]. Thus, research continues in this area to determine whether the potential fidelity advantages of NT-hESCs justify their more laborious production process.

Pericytes Derived from hPSC

hPSCs constitute an ideal source for obtaining relevant quantities of pericytes to support research studies or cellular therapies. Traditional methods of isolating primary pericytes are tedious and tend to suffer from low yields [10]. Primary pericytes also exhibit limited proliferation, batch-to-batch variability, and are known to exhibit phenotypic changes after multiple passages in culture [11]. In addition, primary pericytes of human origin are limited to select tissues, such as the placenta or foreskin, thus studies of brain or lung pericytes, for example, often rely on animal cells which may present cross-species differences [12]. hPSC, on the other hand, are highly expandable in culture and are capable of modeling disease in various human tissues. The development of hiPSCs has even allowed for the noninvasive generation of diseased hPSC lines from patient skin cells. Diseased hiPSCs can then be differentiated into various relevant cell types, enabling the recapitulation and controlled study of cell-specific defects [13–15]. An improved mechanistic understanding of the progression of various diseases at the cellular and tissue level may provide new therapeutic targets.

However, some challenges remain in generating and characterizing pericytes from hPSCs: (1) the diverse origins of pericytes, which are not completely understood, and their resulting unique developmental pathways [16]; (2) a lack of lineage-specific markers for identifying pericytes and for distinguishing them from closely related cell types such as vascular smooth muscle cells (VSMCs) and mesenchymal stem cells (MSCs) [17]; and (3) interactions between multiple environmental factors that jointly coordinate pericyte differentiation [18].

Nevertheless, several groups have developed protocols for differentiating pericytes from hPSCs (Table 9.1). We have summarized notable studies and extracted key information for comparing different studies, including the cell source used, the differentiation technique, and the timeline of the protocol. We also assessed how the resulting cells were characterized in vitro and/or in vivo and whether primary pericytes were included as a positive control. More recently, groups have sought to understand the developmental relationships between pericytes, MSCs, and VSMCs and to design differentiations which attempt to exert finer control over perivascular

Table 9.1 Summary of pericyte differentiation protocols from hPSCs

Author [Ref.]	hPSC source (line or tissue)	Differentiation method	Biomarker expression	Functional assays	Pericyte control	Differentiation duration
Dar et al. [22]	hiPSC (fetal fibroblasts, keratinocytes) hESC (H9.2, I6)	Embryoid body: isolation of CD105/CD73/CD146 cells after 10–14 days spontaneous differentiation	NG2 PDGFRβ CD29 CD44 CD73 CD90 CD105 CD146 Calponin Nestin αSMA (−)	• Cord formation and assembly with ECs on matrigel • Multiple lineage differentiation potential • VEGF secretion • Anastomosis with murine vasculature in matrigel implants in vivo • Accelerated recovery of murine ischemic limb	Human placental PC (for ICC only)	14 days
Kusuma et al. [23]	hiPSC (bone marrow) hESC (H9)	Directed differentiation: mesoderm induction, vascular specification, PC maturation	NG2 PDGFRβ CD44 CD73	• Assembly of 3D vascular networks in collagen or hyaluronic acid hydrogels • Anastomosis with murine vasculature in matrigel implants in vivo	None	18 days
Wanjare et al. [24]	hiPSC (bone marrow) hESC (H9)	Directed differentiation: mesoderm induction, vascular specification, PC maturation	NG2 PDGFRβ CD44 CD73	• Multiple lineage differentiation potential • Alignment and wrapping murine vasculature in matrigel implants in vivo • In vitro migration, invasion, and contractility (not contractile)	Human placental PC and aortic VSMC (for ICC and RT-qPCR only)	18 days

Author [Ref.]	hPSC source (line or tissue)	Differentiation method	Biomarker expression	Functional assays	Pericyte control	Differentiation duration
Orlova et al. [25]	hiPSC (fibroblasts, blood outgrowth ECs)	Directed differentiation: feeder-free culture on matrigel, mesoderm induction and vascular specification, isolation of CD31(−), expansion, derivation of PCs with TGF-β2 and PDGF-BB	NG2 PDGFRβ CD44 CD73 CD105 CD146	• In vitro vascular plexus formation with hiPSC-ECs on gelatin	None	14 days
Greenwood-Goodwin et al. [26]	hESC (ESI-017)	Embryoid body: EB formation and suspension culture (5 days), expansion in adherent cultures (3 days) to obtain a perivascular progenitor (PC-A), that was further differentiated in either MSC media (PC-O) or EC media (PC-M)	*PC-M:* CD34 (−) CD73 CD105 CD133 (−) CD146 NG2 (decr. in cult.) PDGFRβ (decr. in cult.)	• Cord formation and assembly with HUVECs on matrigel (PC-M) • No adipogenic or osteogenic potential (PC-A, PC-M) • Osteogenic potential but no adipogenic potential (PC-O)	Human placental PC and BM-MSCs	8 days (PC-A) 11 days (PC-O) 11 days (PC-M)

(continued)

Table 9.1 (continued)

Author [Ref.]	hPSC source (line or tissue)	Differentiation method	Biomarker expression	Functional assays	Pericyte control	Differentiation duration
Kumar et al. [27]	hiPSC (fibroblast, blood) hESC (H1, H9-EGFP)	Directed differentiation: mesoderm induction (2 days) and mesenchymoangioblast colony formation (12 days) followed by bFGF and PDGF-BB for 3 days and pericyte medium for 14 days to expand immature pericytes, which were further differentiated for 6 days with either 50 ng/mL PDGF-BB and SB431542 (PC1), or 10 ng/mL PDGF-BB and SB431542, VEGF and EGF (PC2), then each was expanded in pericyte medium for 4 additional days	*PC1 (capillary, proinflammatory):* NG2 VCAM1 CD274 aSMA (low) Desmin (low) Calponin (low) MYH11 (−) *PC2 (arteriolar, contractile):* NG2 DLK1 αSMA Desmin Calponin (low) MYH11 (−)	• Cord formation and assembly with HUVECs on matrigel (PC-1 and PC-2) up to 7 days • Limited contractility (PC-1) and moderate contractility (PC-2) determined by gel contraction assay and carbachol area change • Support vascular formation with HUVECs in matrigel plug implants in vivo	Brain PCs for cord formation assay	41 days
Griffin et al. [28] avail. online	hiPSC (fibroblast, male and female lines) hESC (H9)	Embryoid body: neuroepithelial spheres formed from hPSC clusters in neural media (6–9 days). Neural rosettes attach, neural crest-like cells migrate away, and rosettes are mechanically removed. Neural crest-like cells are then directed toward a cranial pericyte (cPC) phenotype using 20 ng/mL PDGF-BB and 2–5% serum	NG2 PDGFRβ ANGPT1 CD13 CD105	• Contractility induced by K^+ • Tube formation and assembly with ECs in fibrin gel • Enhanced junctional protein expression in ECs and reduced barrier permeability • Capillary localization when injected into *Xenopus* • Increased EC transport of transferrin and Aβ42	Post mortem brain pericytes	24 days

cell development. We will discuss this in more detail further on, while considering the differentiation of lineage-specific or tissue-specific pericytes. It is worth noting that pericyte differentiations have also been conducted from multipotent adult stem or progenitor cells [19–21]; however, the scope of this review is limited to highlighting studies focused on the differentiation of pericytes from hPSCs.

To understand the rationale behind in vitro differentiation schemes, it is helpful to begin with a brief description of pericyte origins during embryonic development. The first symmetry-breaking event in embryogenesis is the formation of the primitive streak, which occurs in humans after 2 weeks [29, 30]. This defines the left-right and cranial-caudal body axes and begins the process of gastrulation, which results in the formation of the three primary germ layers – endoderm, mesoderm, and ectoderm, which collectively generate every tissue in the adult human. This process and many subsequent differentiation events are governed by the spatial distribution of growth factors known as morphogens, such as bone morphogenetic protein (BMP), Wnt, fibroblast growth factor (FGF), and transforming growth factor (TGF) [31]. Cell patterning is greatly influenced by precise spatiotemporal gradients of these factors, with cells exhibiting unique responses to ligand concentrations that fall within certain ranges. Thus many in vitro differentiation protocols are designed to guide cells toward a certain lineage, mimicking the concentrations of growth factors known to induce their development. Most protocols developed to date can be categorized by whether they employ embryoid body formation or directed differentiation of a 2D monolayer.

Embryoid bodies (EBs) are three-dimensional aggregates of differentiating hPSCs. These spheroids undergo spontaneous differentiation into all three primary germ layers in culture, closely mimicking the spatial organization of natural embryonic development. Following EB differentiation, purification is required to isolate cell subsets, using fluorescent-activated cell sorting or magnetic-activated cell sorting [31]. In addition, control over the differentiation is limited, as exogenous growth factors may have limited penetration into the inner layers of EBs, resulting in an uneven presentation of differentiation cues [31]. Both Dar et al. and Greenwood-Goodwin et al. used EB culture to obtain pericyte progenitor populations [22, 26]. While Dar et al. dissociated EBs at day 14, Greenwood-Goodwin et al. dissociated EBs at day 5 and cultured singularized cells in adherent culture for an additional 6 days (3 days on fibronectin-coated flasks and 3 days on uncoated flasks).

Directed monolayer differentiations have been developed to better control cellular fate. While they no longer mimic the 3D geometry of the developing embryo, monolayer differentiations provide some key advantages over EBs, including the uniform, defined presentation of growth factors [32]. This allows the differentiation to be "guided" along a certain developmental lineage and carefully designed to provide key growth factors at the correct concentration and timing. The increased homogeneity of monolayer differentiations enables the generation of individual cell types with high purity. This improves differentiation reproducibility and reduces the need for costly purification steps. Kusuma et al. developed a 12-day differentiation protocol for early vascular cells (EVCs), a progenitor able to mature into either endothelial cells (ECs) or pericytes (Fig. 9.1) [23]. This differentiation consisted of

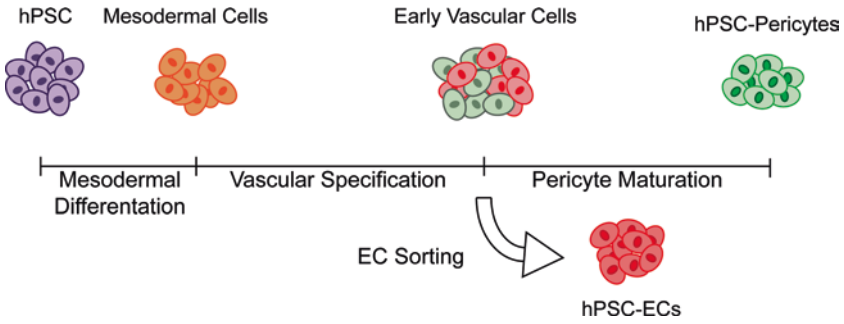

Fig. 9.1 General schematic of monolayer differentiation timeline of hPSC-pericytes. Beginning with hPSC colonies, cells are differentiated to mesodermal cells or mesodermal progenitors. These cells then undergo vascular specification using various growth factors. From this mixed population, pericytes are isolated and further matured

a 6-day mesoderm induction stage followed by 6 days of vascular specification in endothelial growth media supplemented with vascular endothelial growth factor (VEGF) and TGF-β inhibitor. At day 12, EVCs could be sorted for positive expression of VECad and maintained in EC maturation conditions or passaged onto uncoated flasks to expand in conditions favoring pericyte expansion (DMEM with 10% FBS). Similarly, Orlova et al. developed a protocol for the simultaneous differentiation of ECs and pericytes [25]. However, they first induced mesoderm using a defined bovine serum albumin (BSA) polyvinylalchohol essential lipids protocol, with BMP4, Activin A, CHIR99021, and VEGF. After 3 days, these factors were removed, and cells were cultured with VEGF and TGF-β inhibitor for an additional 7 days before sorting on day 10 for positive expression of CD31. Continued culture of CD31- cells in DMEM with 10% FBS similarly leads to pericyte maturation. In each of these studies, ECs and pericytes were differentiated from a common source, with the rationale being that specification toward the vascular lineage would precede endothelial-pericyte bifurcation.

Verification of Phenotype and In Vivo Functionality

Pericyte marker expression has been highly debated among experts; thus characterization of hPSC-pericytes must be robust and thorough. The papers summarized here have investigated the expression of multiple biomarkers as well as in vitro and in vivo functional assays. Common surface markers include platelet-derived growth factor (PDGFR)-β, neural/glial antigen 2 (NG2), and CD13, while the cytoskeletal proteins α-smooth muscle actin (α-SMA), desmin, and calponin are often expressed by contractile pericytes [17, 33, 34]. Dar et al. demonstrated the widest variety of marker verification of hPSC-pericytes utilizing flow cytometry, western blotting, and immunofluorescent staining. As there is no single marker to prove pericyte

phenotype, a rigorous, multi-technique approach is necessary to fully define hPSC-pericyte populations.

Functional assays are also employed to further characterize hPSC-pericytes. Primary pericytes have been shown to differentiate into multiple tissues, including bone, cartilage, and fat, supporting a hypothesis of pericytes as multipotent tissue-resident progenitors, similar to MSCs [35–37]. Thus, a popular assay has been to test the osteogenic, chondrogenic, and adipogenic differentiation potential of hPSC-pericytes [24, 38]. However, a recent in vivo lineage tracing study in mice has convincingly demonstrated that pericytes in vivo do not exhibit this behavior in either aging or pathological settings [39]. Interestingly, these same labeled pericytes, when isolated and cultured in vitro, do behave as MSCs, suggesting multipotency may be induced by in vitro culture. Therefore, this assay may ultimately be characterizing a function that is not native to pericytes in vivo. Other functional assays include in vitro tube formation on 2D matrigel surfaces and within 3D hydrogels [24, 27, 38, 40–42]. Kusuma et al. show pericytes interacting with 3D vascular networks within both collagen and hyaluronic acid-based hydrogels (Fig. 9.2) [40].

hPSC-pericytes have also been tested in in vivo applications. Dar et al. demonstrate the ability for hPSC-pericytes to promote vascular and muscular recovery in hind limb ischemia mouse models. Kumar et al. and Kusuma et al. used matrigel plugs with hPSC-pericytes and human umbilical vein endothelial cells (HUVECs) or early vascular cells, respectively. Kumar et al. showed that when implanted with hPSC-pericytes, HUVEC vessels had a higher vascular lumen diameter, number of vessels per field of view and percent coverage as compared to implantation of HUVECs only [27].

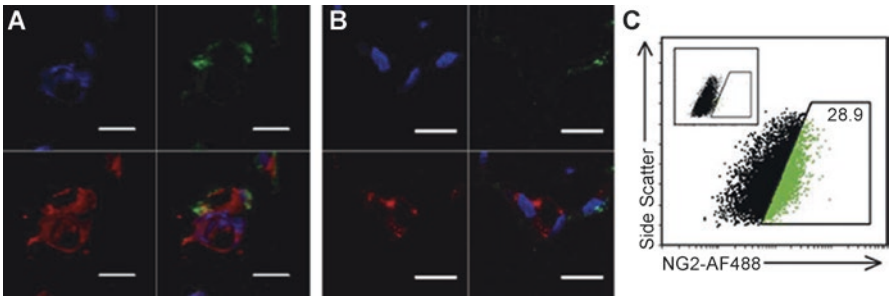

Fig. 9.2 hiPSC-ECs and pericytes interacting in 3D hyaluronic acid hydrogels. (**a**) pericytes (green) integrated onto a hollow EC tubular structure (red) (3D projection) and (**b**) enclosing a luminal structure (confocal z-stack). Scale bar represents 200 μm. (**c**) Flow cytometry analysis ($n = 3$) confirms that a subset of early vascular cells acquires NG2 expression. Inset represents anti-IgG control. (Reproduced from Kusuma et al. [23])

Tissue Specificity and Differentiation of Mural Cell Subtypes

It is well established that pericytes are a heterogeneous group with respect to marker expression and functionality. This is apparent (1) along the arteriovenous axis, where we typically classify mural cells as either vascular smooth muscle cells or pericytes, although some hybrid morphologies exist and definitions are frequently inconsistent [43, 44]; (2) across various organs and tissues, where, for example, pericytes in the liver and kidney (hepatic stellate cells and mesangial cells, respectively) utilize different molecular interactions during recruitment to nascent vasculature [17]; (3) in response to injury, where pericyte subtypes have been established based on differential migration and ECM deposition behaviors post-injury [45, 46]; and (4) in vivo vs in vitro, where it has been demonstrated that pericyte multipotency widely observed in vitro may not occur in vivo [39] and that pronounced changes in pericyte marker expression occur as a result of in vitro culture [26, 47].

In recognition of pericyte heterogeneity, recent studies have sought a deeper understanding pericyte ontogeny and developmental pathways. This will help delineate relationships between pericytes and other closely related cells, such as VSMCs, MSCs, and fibroblasts, and identify whether such relationships are defined by reversible phenotypic modulations, immature vs matured cell populations, or unique terminally differentiated populations. Wanjare et al. demonstrated the differentiation of subsets of VSMCs that exhibited either a synthetic or contractile morphology [48] and demonstrated that pericytes obtained from the same mesoderm also exhibited unique morphology, marker expression, and ECM deposition when compared to VSMCs [24]. In this scheme, media with high (10%) FBS supplemented with PDGF-BB and TGF-beta lead to smooth muscle-like cells (SMLCs), which could continue to be cultured in this media to produce synthetic VSMCs or cultured in low (0.5%) FBS in order to obtain contractile VSMCs. Likewise, Greenwood-Goodwin et al. identified a pericyte progenitor, termed PC-A, which gained osteogenic potential when cultured in MSC media (PC-O) and improved angiogenic support functions when cultured in endothelial growth media (PC-M) [26]. Bajpai et al. developed a VSMC differentiation which proceeds through an MSC intermediate, suggesting that MSCs could be a progenitor for contractile VSMCs and perhaps pericytes [49]. Recently, a thorough investigation was carried out by Kumar et al. on the specification of pericytes and VMSCs from mesangioblasts [27]. In their proposed model, MSCs, immature pericytes, and immature VSMCs are all produced from a common mesenchymal progenitor. They proceeded to mature the VSMCs and differentiate the pericytes into two different subtypes, PC1 and PC2, using PDGF-BB and SB431542 alone (PC1) or PDGF-BB and SB431542 with VEGF and EGF (PC2). Gene expression profiling revealed a proinflammatory, capillary phenotype for PC1 and a contractile, arteriolar phenotype for PC2.

While the above pericyte differentiations have all proceeded through a mesodermal stage, there have been several studies which have demonstrated that VMSCs and pericytes are collectively derived through multiple lineages [50, 51]. In particular, mural cells of the ascending aorta, head, and neck are derived from the neural crest, which is a structure that emerges from the ectoderm. This may help explain some of the diversity observed among mural cells, and it has been suggested that intersections of mural cells of different lineages seem to correlate with areas of vascular disease [18]. This lead to efforts to differentiate lineage-specific VSMCs derived from either the neural crest, the lateral plate mesoderm, or the paraxial mesoderm [52, 53]. Similarly, just recently several groups have developed pericyte differentiations that proceed through a neural crest intermediate. Griffin and Bajpai demonstrate that cranial pericytes (cPC) derived from hiPSC and hESC exhibit a genomic identity that resembles primary brain pericytes more closely than do primary human placental pericytes or primary murine brain pericytes [28]. They also show that cPC exhibit pericyte functionality through incorporation into *Xenopus* vasculature and in vitro assays demonstrating an influence on endothelial cord formation, proliferation, barrier function, and transcytosis. Neural crest-derived pericytes and VSMCs may prove a more suitable alternative to mesoderm-derived mural cells for modeling the vasculature of the CNS and studying neurovascular dysfunction.

Future Directions

Multiple differentiation protocols have been developed to obtain pericytes from hPSCs. These protocols differ in their approaches, and the resulting pericytes therefore present a range of morphologies and gene expression profiles. Clearly, there remains much to be learned about the mechanisms of pericyte development and diversification. Differentiating pericytes from hPSCs in vitro provides an excellent opportunity to identify factors influencing pericyte development and to establish ontogenic relationships between pericytes and similar cells. These studies should be complemented by in vivo lineage tracing studies in order to assess the potential for culture-induced phenotypic changes. hPSC-derived pericytes in multiple studies have demonstrated similar or improved functional capabilities as compared with those of primary pericytes or MSCs. Taken together with their indefinite expandability and potential for autologous transplantations, hPSCs could provide an excellent source for pericytes for research and clinical applications, such as vascularizing organs-on-chips or for direct implantation as a cell therapy.

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Chapter 10
Pericytes in Tissue Engineering

Betül Çelebi-Saltik

Abstract Pericytes have crucial roles in blood-brain barrier function, blood vessel function/stability, angiogenesis, endothelial cell proliferation/differentiation, wound healing, and hematopoietic stem cells maintenance. They can be isolated from fetal and adult tissues and have multipotential differentiation capacity as mesenchymal stem cells (MSCs). All of these properties make pericytes as preferred cells in the field of tissue engineering. Current developments have shown that tissue-engineered three-dimensional (3D) systems including multiple cell layers (or types) and a supporting biological matrix represent the in vivo environment better than those monolayers on plastic dishes. Tissue-engineered models are also more ethical and cheaper systems than animal models. This chapter describes the role of pericytes in tissue engineering for regenerative medicine.

Keywords Pericytes · Tissue engineering · Mesenchymal stem cells · Hematopoietic stem cells · Niche · Scaffold · Bone tissue engineering · Cartilage tissue engineering · Dermal tissue engineering · Vascular tissue engineering · Cardiac tissue engineering · Blood tissue engineering

Introduction

Mesenchymal stem cells (MSCs) have attracted considerable attention as therapeutic cells for regenerative medicine although they are heterogeneous population. It has been described that MSCs originate from two types of perivascular cells, namely, pericytes and adventitial cells, which contain the in situ counterpart of MSC in developing and adult human organs, which can be purified using defined cell

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surface markers [26]. The French physiologist, Charles-Marie Benjamin Rouget identified pericytes as "non-pigmentary adventitial cells" or "intramural pericytes" in 1873, while the German anatomist Karl Wilhelm Zimmermann renamed them as "pericytes" in 1923 [3, 51]. By these expression patterns, pericytes can be separated from other perivascular cells like adventitial cells that are negative for CD146 and positive for CD34 [24]. The majority of pericytes are derived from mesoderm, whereas those found in the retina and brain are derived from the neural crest [48]. These cells are found in capillaries, arterioles, and venules as well as in the subendothelial regions of large-diameter blood vessels (Fig. 10.1) [2]. They engage with endothelial cells through special linkage units and paracrine signals and can increase the proliferation and provide effective endothelialization [35]. They are responsible for the regulation of vascular development, maturation, stabilization, blood flow, and pressure located in the periphery of the vessel wall [29]. Morphologically, pericytes are fibroblast-like cells with prominent nuclei, narrow cytoplasm, and many extensions [48]. Similarities have been described to exist between MSC and pericytes in terms of phenotype and gene expression, suggesting that MSCs indeed represent a progeny of the perivascular cell compartment [25]. Moreover, if one sorts culture expanded human pericytes for the in vivo marker CD146 or smooth muscle actin, the cells obtained have all of the classic markers for human MSCs (CD105, CD90, CD73) [27]. Most pericytes express neural/glial antigen 2 (NG-2) and platelet-derived growth factor receptor beta (PDGFR-β) and lack the expression of hematopoietic and endothelial markers, such as CD45 and CD31 [10, 19]. Although no marker is specific for pericytes, collectively these markers appear to selectively identify an MSC-like pericyte. The purification of pericytes is described as a CD146+CD34-CD45- cell population [42]. Caplan mentioned that these cell sorts clearly documented the equivalency of MSCs with pericytes. These observations lead him to speculate that "all" MSCs are derived from pericytes [11]. Similar to their

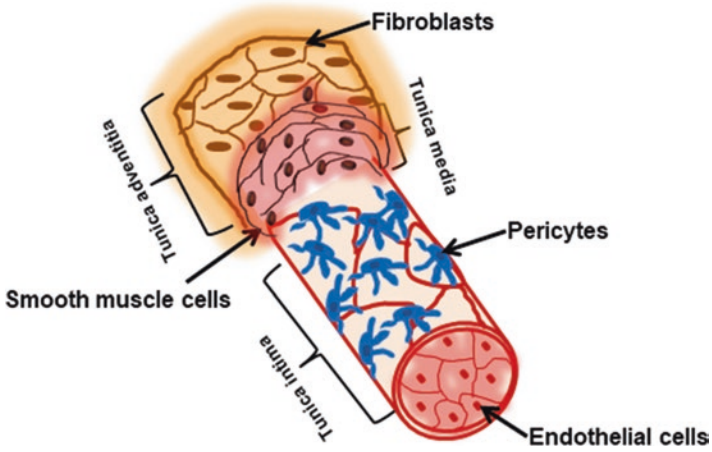

Fig. 10.1 Localization of the pericytes in the vessel

diverse morphology, expression of pericytes phenotypic markers is dynamic and changes at different developmental stages, in addition to being highly variable in different tissues and organs [63].

The production of an engineered tissue in vitro requires the use of cells to populate matrices and produce matrix resembling that of the native tissue [40]. The main successes in this field have come from the use of primary cells taken from the patient and used them with scaffolds to produce tissue for reimplantation. However, this strategy has some limitations, because of the invasive nature of cell collection and the potential for cells to be in a diseased state [40]. The use of embryonic stem cells, MSCs, fetal stem cells (umbilical cord, placenta-derived stem cells), and induced pluripotent stem cells began to become widespread after the development of stem cell field. In addition to these stem cell types, recently, pericytes have become important cell sources for tissue engineering applications [49].

Pericytes in Tissue Engineering

The term "tissue engineering" was officially used at a National Science Foundation workshop in 1988 to mean "the application of principles and methods of engineering and life sciences toward the fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain or improve tissue function" [52]. However, while the field of tissue engineering may be relatively new, the idea of replacing tissue with another goes as far back as the sixteenth century [50]. Tissue engineering applications consist of (1) scaffolds for providing proper three-dimensional (3D) shape of tissue construct and structural support, (2) cells for forming tissues in vitro/within the body, and (3) growth factors for signaling and determining cell fate. Evidence shows that the injected cells do not contribute to the reconstitution of the damaged tissue, highlighting the urgency of new solutions for organ/tissue replacement. Based on these considerations, clinicians and biologists are developing new techniques in the attempt to generate biological tissues "grafts" in vitro, developing the new field of tissue engineering [4]. The reconstruction of tissues can be achieved by the combination of a support material "scaffold" with cells and/or bioactive factors such as growth factors, cytokines, or chemokines (Fig. 10.2) [12, 14, 17]. The scaffold can be of natural or synthetic origin and is meant to provide support to the forming tissue and a matrix for cell retention and controlled bioactive factors release. Natural matrices are made of biologically-derived polymers, such as electrospun collagen, elastin, fibrin, fibronectin, alginates or hydrogels [12–14]. Alternatively, they can consist of entire decellularized tissues, commonly xenografts of porcine or bovine origin [39]. Conversely, synthetic matrices are composed of synthetic polymers like poly-glycolic acid, poly-lactide-co-glycolic acid (PLGA), poly-L-lactic acid, polycaprolactone, and polyurethane [17, 28, 55].

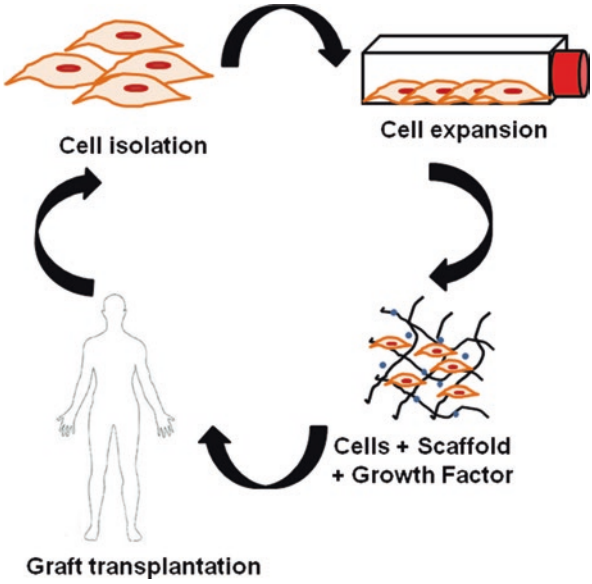

Fig. 10.2 Tissue engineering process

Pericytes in Bone Tissue Engineering

In vitro tri-lineage differentiation of pericytes into mesenchymal cell types has been well documented [32, 34]. The application of pericytes for orthopedic indications is a significant and growing field of study. Among orthopedic indications, the two most promising clinical applications are use in a bone graft substitute for spinal fusion and stimulation of fracture repair. The use of pericytes to stimulate spinal fusion has previously documented preclinical efficacy [22]. Chung et al. confirmed that implanted adipose tissue-derived human pericytes differentiated into osteoblasts and osteocytes; however, the majority of the new bone formation was of host origin. These results suggest that implanted pericytes positively regulate bone formation via direct and paracrine mechanisms [22]. Likewise, pericyte-based stimulation of fracture healing has recently shown proof of principle efficacy in an atrophic nonunion murine model [59]. Animal models of ectopic bone formation have been used to confirm the capacity for in vivo osteogenic differentiation of implanted pericytes by James et al. [42]. Pang et al. investigated mouse incisor tips as a model for the role of dental pulp stem cells in a continuous natural repair/regeneration process. They demonstrated that NG2-expressing perivascular cells (pericytes) differentiated into odontoblast-like cells and facilitated the production of reparative dentine in experimentally damaged mouse incisors [53]. Another research conducted by James et al. revealed that human adipose tissue pericytes seeded onto a PLGA scaffold increased healing of mouse critical-size calvarial defects within 2 weeks of delivery [43]. This is yet another example showing human pericytes potential in skeletal regenerative medicine.

Pericytes in Cartilage Tissue Engineering

Differentiation of stem cells to chondrocytes in vitro usually results in a heterogeneous phenotype. This is evident in the often detected overexpression of collagen type X which, in hyaline cartilage structure, is not characteristic of the mid-zone but of the deep-zone ossifying tissue [1]. In regenerative medicine, methods to better match cartilage developed in vitro to characteristic in vivo features are therefore highly desirable. The number of pericyte-specific studies in the field of cartilage and connective tissue engineering is limited. Zhang et al. co-cultured the articular chondrocytes with pericytes and adventitial cells, respectively, and showed more prominent effects on glycosaminoglycans production and collagen type II synthesis than the adventitial cells [66]. Another research, pericytes from the infrapatellar fat pad (IFP), have been investigated. These cells demonstrated increased chondrogenic potential compared with those from subcutaneous by generating more extracellular matrix (*OL2A1*, *ACAN*, and *SOX9*) than IFP MSCs [38]. The high expression of extracellular matrix by pericytes than culture-derived MSCs makes pericytes as alternative therapeutic agents for cellular therapy and regenerative medicine. It has been discussed by Wu et al. that CD146+ subpopulation represented a chondrolineage-restricted subpopulation of skeletal stem cells and may therefore act as a valuable cell source for cartilage regeneration [62]. Alakpa et al. demonstrated the phenotypic characteristics of human adipose tissue pericytes that cultured on diphenylalanine/serine peptide hydrogels with the more widely used chemical-induced method for chondrogenesis. High levels of collagen type II were noted when pericytes undergo chondrogenesis in the hydrogel without induction media. They suggested also that there was also a balanced expression of collagen relative to aggrecan production, a feature which was biased toward collagen production when cells were cultured with induction media. The study highlighted how material and chemical alterations in the cellular microenvironment have wide-ranging effects on resultant tissue type [1].

Pericytes in Dermal Tissue Engineering

Dermal tissue engineering has revolved around using different cell types for the treatment of cutaneous wounds by direct injection or scaffold-based delivery system. The process of wound healing is a complex and dynamic process involving various players for secretion of soluble mediators and deposition of extracellular matrix along with migration of various cell types, including fibroblasts, keratinocytes, macrophages, leukocytes, endothelial cells, and pericytes [9]. Human umbilical cord pericytes have recently been shown to have great potential for the treatment of skin wounds [65]. The application of human adipose-derived pericytes (alpha-smooth muscle actin (α-SMA)+, PDGFR+, NG2+, and Ang1+) on wounded skin of the rats had beneficial effects due to the increased angiogenesis, extensive collagen

deposition, and reepithelialization [64]. Studies by Rajkumar et al. showed that PDGFR-β inhibition in vivo was accompanied by abnormal microvascular morphogenesis reminiscent of that observed in PDGFR-β−/− mice with significantly reduced immunostaining of the pericyte marker NG2 implying the importance of PDGFR-β signaling during the early phases of wound healing [56]. In systemic sclerosis fibrotic lesions, pericytes showed markers of activation such as PDGFR-β and high-molecular-weight melanoma-associated antigen [41]. Strong evidence showed convergence of microvascular pericytes and resident fibroblasts to a myofibroblast lineage and thereby contributing to systemic sclerosis by synthesizing excessive extracellular matrix components [46]. Laminin alpha (α) 5 (LAMA5), a subunit of the extracellular matrix component laminin-511/laminin-521 (LM-511/LM-521), promoted skin regeneration both in vitro and in vivo [54]. Analysis using immunogold localization revealed that pericytes synthesized and secreted LAMA5 in human skin. Consistent with this observation, co-culture with pericytes enhanced LM-511/LM-521 deposition in the dermal-epidermal junction of organotypic cultures [54].

Pericytes in Vascular Tissue Engineering

Platelets release various factors such as PDGF and transforming growth factor (TGF)-β that promotes pericytes detachment from endothelial cells and migration into the parenchyma. Activated pericytes can express tissue factor to promote activation of the extrinsic coagulation pathway. Platelet activation of pericytes may facilitate or regulate neovascularization. Pericytes can facilitate also angiogenesis through secretion of matrix metalloproteinases (MMPs) (driven in part by the hypoxic environment) to degrade the basement membrane allowing endothelial cells to migrate into the provisional matrix. The dissociation of pericytes from the vasculature allows for destabilization of the endothelial tube, which can promote endothelial migration and proliferation [47]. In particular, pericytes express key adhesion molecules (e.g., intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1), chemokines (e.g., human and murine C-X-C motif chemokine ligand (CXCL)-1, CXCL-8, macrophage inhibitory factor (MIF)), and receptors for proinflammatory molecules (tumor necrosis factor receptor (TNFR)-1, TNFR-II, interleukin-1 receptor (IL-1R), toll-like receptors (TLRs), NOD-like receptors) [16, 58]. In the concept of vascular tissue engineering, the secretory profile of pericytes is important.

Vascular tissue engineering approaches aim to mimic vascular layers using natural or synthetic materials with vascular cell. Current clinically approved polymer-based grafts such as Dacron and polytetrafluoroethylene have shown promising results as large vessel substitutes but perform poorly for small-diameter vessel bypass (≤6 mm) [44]. He et al. cultured human skeletal muscle pericytes with bilayered elastomeric poly(ester-urethane) urea scaffolds. The seeded scaffolds were implanted into rats as aortic interposition grafts for 8 weeks. Results showed

pericytes populated the porous layer of the scaffolds and maintained their original phenotype after the dynamic culture. After implantation, pericyte-seeded vascular grafts showed a significant higher patency rate than the unseeded control [37]. Chong et al. generated biphasic vascular model containing synthetic polymer (polyacrylic acid was grafted onto biaxially-stretched polycaprolactone) seeded with human umbilical cord vein pericytes in the media layer and endothelial cells in the intima layer [21]. They reported that this construct would be suitable not only for vascular applications but for the engineering of layered tissue such as the skin, cornea, or myocardium. Synthetic scaffolds have many disadvantages, so in recent years vascular constructs made from cellularized natural scaffolds were seen to be very promising, but the number of studies comprising this area is very limited. Van der Meer et al. constructed micro-engineered 3D vascular tissue by mixing human umbilical cord vein endothelial cells (HUVECs), pericytes, and the rat tail collagen type I. This is the only study that highlights the interaction of pericytes with collagen type I as a model of vascular graft [60]. We suggested that human umbilical cord vein CD146+ pericytes may be good candidates for generating three-layered small-diameter vascular constructs when combined with human collagen type I, fibrin, elastin, dermatan sulfate, heparin, and fibronectin constituting the human natural vascular components [36]. We recently generated triple-layered vascular construct with natural human extracellular matrix proteins/glycosaminoglycans mixed with smooth muscle cells and fibroblasts differentiated from human umbilical cord vein pericytes and with HUVECs in assistance with cell sheet engineering method. For the treatment of coronary artery diseases, this vascular construct is an important step for generation of fully natural small-diameter ($\leq$5 mm) vascular graft that has the structure closest to the native blood vessel [36].

Pericytes in Cardiac Tissue Engineering

Heart failure, particularly myocardial infarction, is one of the leading causes of morbidity and mortality in the world. Stem cell transplantation therapy has emerged as a popular strategy to treat heart dysfunction. The myogenic capacity and the pro-angiogenic ability of skeletal pericytes were harnessed by organizing them in a poly-ethylene glycol hydrogel-based construct for the repair of ischemic muscle [33]. Wendel et al. produced a cardiac patch by embedded human brain pericytes and human-induced pluripotent stem cell-derived cardiomyocytes into a fibrin gel [61]. Once transplanted onto the infarcted myocardium of a rat, this pericytes/cardiomyocytes patch survived, improved cardiac function, and reduced infarct size [61]. Avolio et al. reported that cardiac pericytes seeded onto clinically approved xenograft scaffolds had penetrated into the graft and colonized after 3 weeks incubation in a bioreactor system and that cells within the graft are viable. Moreover, cells maintain the original antigenic phenotype [5]. Exploiting the paracrine activity of tissue-specific cells rather than using cells isolated from a different tissue becomes attractive for regenerative medicine. In this respect, cardiac stem cells and pericytes

may be uniquely suited to produce paracrine factors instrumental to cardiac and vascular repair and regeneration [31]. Pericyte-like cells isolated and expanded from the adult saphenous vein produce large amounts of angiogenic factors such as vascular endothelial growth factor (VEGF)-A, VEGF-B, angiopoietin (Ang)-1, and miR-132, which are delivered to neighboring endothelial cells through the establishment of integrin-mediated interactions [45]. Secretion of VEGF-A, Ang-1, and miR-132 is further augmented by hypoxia, which mimics in vitro the environment encountered by cells upon transplantation into ischemic tissues [45]. Chen et al. investigated the therapeutic potential of human skeletal muscle pericytes for treating ischemic heart disease and mediating associated repair mechanisms in mice. They found that pericyte transplantation attenuates left ventricular dilatation and myocardial fibrosis and improves cardiac contractility in infarcted mouse hearts. In line with findings in saphenous vein-derived pericytes, hypoxia induced the expression of VEGF-A, PDGF-β, TGF-β1, and corresponding receptors, while expression of basic fibroblast growth factor, hepatocyte growth factor, and Ang-1 was repressed [18].

Pericytes in Blood Tissue Engineering

Hematopoietic stem cells (HSCs) are a rare subpopulation of cells residing in the bone marrow with a well-defined phenotype, lymphoid and myeloid lineage developmental potential, the capacity to reconstitute irradiated host recipients over the long-term in vivo [6]. HSC maintenance, behavior and trafficking are dependent upon information they receive from the niche in which they are localized. The concept of the niche was initially suggested after the work of Schofield and has been defined as a small functional compartment with a specific anatomical position within an organ that homes and regulates stem cell activity, quiescence, self-renewal, and differentiation for healthy tissue maintenance and repair [57]. MSCs have increasingly been implicated in HSC support as major components of the hematopoietic niche [15, 16, 30]. As pericytes were shown to be a reservoir of MSCs in vivo and in addition proximal to HSCs, recent studies have focused on their role in HSC regulation and blood tissue engineering [8]. It has been shown by Chin et al. that a subset of cells differentiated from human pluripotent stem cell defined as $CD146^{hi}CD73^{hi}$ expressed genes associated with the hematopoietic niche and supported the maintenance of functional hematopoietic progenitors ex vivo, while $CD146^{lo}CD73^{lo}$ cells supported differentiation. They discussed that stromal support of hematopoietic progenitors was contact dependent and mediated in part through high JAG1 expression and low WNT signaling. Molecular profiling revealed significant transcriptional similarity between human pluripotent stem cell-derived CD146++ and primary human CD146++ perivascular cells [20]. Primary immunodeficient recipients were engrafted at long-term when injected with CD45+ donor hematopoietic cells from CD146+ co-cultures and could further repopulate secondary recipients. CD146+ cells were able to activate Notch signaling in hematopoietic

progenitors [23], in agreement with previous reports suggesting that Notch signaling regulates the growth and differentiation of hematopoietic progenitors via the microenvironment or niche [7].

Future Perspective

Several preclinical and clinical trials have looked at the therapeutic benefits of systemic infusion of ex vivo isolated and expanded MSCs, but there are problems with the consistency, heterogeneity, and delivery of these cells. Pericytes represent common ancestor cells giving rise to MSCs in the adult. It is clear that pericytes from a range of sources, isolated in numerous ways, and of various phenotypes, show bioengineering potential. However, lack of standardization regarding perivascular marker expression and that of their subtypes renders comparison between studies and overall conclusions difficult. Although recent publications mentioned the proteome and transcriptome profile of pericytes, these cells need to be well defined to be used clinically in the concept of the tissue engineering approach.

Conflict of Interest Statement The author declares that she has no conflicts of interest concerning this work.

Ethical Approval This article does not contain any studies with human participants or animals performed by the author.

Informed Consent This article does not contain any studies with human participants or animals performed by the author.

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Chapter 11
Pericyte Secretome

Abderahim Gaceb and Gesine Paul

Abstract The role of pericytes seems to extend beyond their known function in angiogenesis, fibrosis and wound healing, blood-brain barrier maintenance, and blood flow regulation. More and more data are currently accumulating indicating that pericytes, uniquely positioned at the interface between blood and parenchyma, secrete a large plethora of different molecules in response to microenvironmental changes. Their secretome is tissue-specific and stimulus-specific and includes pro- and anti-inflammatory factors, growth factors, *and* extracellular matrix *as well as* microvesicles suggesting the important role of pericytes in the regulation of immune response and immune evasion of tumors. However, the angiogenic and trophic secretome of pericytes indicates that their secretome plays a role in physiological homeostasis but possibly also in disease progression or could be exploited for regenerative processes in the future. This book chapter summarizes the current data on the secretory properties of pericytes from different tissues in response to certain pathological stimuli such as inflammatory stimuli, hypoxia, high glucose, and others and thereby aims to provide insights into the possible role of pericytes in these conditions.

Keywords Pericytes · Secretome · Angiogenesis · Inflammation · Regeneration · Blood-brain barrier · Cytokines · Chemokines · Growth factor · Microvesicles

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Abbreviations

AD	Alzheimer disease
AGE	Advanced glycation end product
Ang	Angiopoietin
BDNF	Brain-derived neurotrophic factor
CCL	Chemokine (C-C motif)
CD	Cluster of differentiation
CMV	Cytomegalovirus
COX-2	Prostaglandin-endoperoxide synthase 2
CXCL	Chemoattractants
EC	Endothelial cells
EGF	Endothelial growth factor
ENA	Epithelial-derived neutrophil-activating peptide
FGF	Fibroblast growth factor
G-CSF	Granulocyte colony-stimulating factor
GDNF	Glial cell-derived neurotrophic factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GROα/β/γ	Growth-regulated protein alpha/beta/gamma
GUCY1B3	Guanylate cyclase 1 soluble beta3
HB-EGF	Heparin-binding EGF-like growth factor
HGF	Hepatocyte growth factor
HIF-1α	Hypoxia-inducible factor 1-alpha
HIV-1	Human immunodeficiency virus type-1
HLA	Human leukocyte antigen
HO-1	Heme oxygenase-1
ICAM	Intercellular adhesion molecule
IFN	Interferon
IGFPB	Insulin-like growth factor-binding protein
IL	Interleukin
iNOS	Induced nitric oxide synthase
IP-10	Interferon gamma-induced protein 10
ITAC	Interferon-inducible T-cell alpha chemoattractant
JE	Japanese encephalitis
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein-1
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
MMP	Matrix metalloproteinases
MVs	Microvesicles
NGF	Nerve growth factor
NK	Natural killer
NO	Nitric oxide

NOX4	NADPH oxidase 4
NT3	Neurotrophin 3
PDGF	Platelet-derived growth factor
PGES	Prostaglandin E synthase
PLGF	Placental growth factor
RANTES	Regulated on activation normal T-cell expressed and secreted
ROS	Reactive oxygen species
SCF	Stem cell factor
SDF-1	Stromal cell-derived factor 1 alpha
SELE	Selectin E
TARC	Thymus- and activation-regulated chemokine
TGF	Transforming growth factor
TNF	Tumor necrosis factor
VCAM-1	Vascular cell adhesion protein
VEGF	Vascular endothelial growth factor
ZO1	Zona occludens 1

Introduction

Pericytes are lining the endothelial cell layer ubiquitously in all microvessels across the organism [1–3]. They are defined by their position adjacent to endothelial cells (EC) with whom they share a single basement membrane [4–6] which is synthesized by both ECs and pericytes [7–9]. Pericytes are interesting cells due to their versatility and their multiple functions. Well acknowledged is their contribution to vascular homeostasis such as microvessel stabilization, EC survival, and angiogenesis.

Specifically in the brain, where their density exceeds that in most other tissues [1, 10], pericytes contribute to maintenance of the blood-brain barrier, to clearance of metabolites and toxic products from the brain, and to control of endothelial tight junction expression and adherens protein expression and trans-endothelial vesicle trafficking [10–15].

Recently, pericytes are increasingly recognized for their role in tissue repair and regeneration [16–19], and data support their participation in wound healing [20], angiogenesis [21], immunosurveillance, and modulation of neuroinflammation (for review see [22]). The role of pericytes might extend beyond the currently recognized functions due to their secretory properties (for review, see [18] and Fig. 11.2).

Due to their unique localization at the interface between blood and parenchyma in different tissues, these cells are first in line when sensing changes in the environment such as hypoxia, inflammation, pathogens, or changes in glucose concentrations. Pericytes respond to different stimuli by secretion of a variety of growth factors, cytokines and chemokines (see Fig. 11.1), and extracellular matrix that allows them to communicate with neighboring cells and modulate their function via paracrine secretion, but their impact may extend to cell signaling beyond their

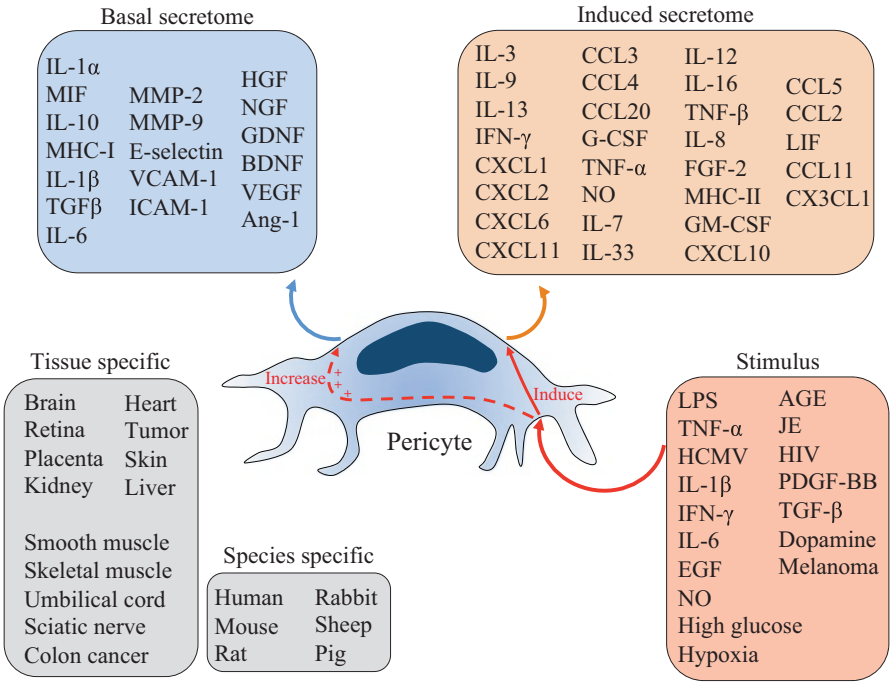

Fig. 11.1 Basal and induced pericyte secretome. Under basal conditions, pericytes release a basal secretome. After exposure to different stimuli, pericytes increase the release of the basal secretome and also release a stimulus-specific-induced secretome. The pericyte secretome is specifically related to the nature of stimulus, the tissue, and species

immediate environment using long-distance communication through secretion of microvesicles and exosomes.

Here we summarize recent evidence on the secretory properties of pericytes in different tissues. Specifically, we group the secretory properties of pericytes in response to different pathological stimuli which may illustrate their reaction under pathological conditions and thus provide insights into their possible role in these conditions.

Most of the data describing the secreted factors of pericytes so far come from in vitro studies using particular stimuli and measuring the secreted molecules. In vitro studies allow controlled conditions, examination of purified and characterized cells or cell lines, and easy ways of measuring changes in gene and protein expression or secreted molecules. It needs to be taken into consideration that cell isolation is based on surface marker expression and cell isolation protocols as well as markers used differ between studies, which may lead to isolation of different pericyte subtypes. The artificial conditions in culture may induce a secretome in pericytes that may not be shared by the corresponding endogenous pericytes in vivo under similar pathological conditions. Secretion in vivo is much more difficult to measure as the readout may not be specific to the cell type examined. Isolation of pericytes from

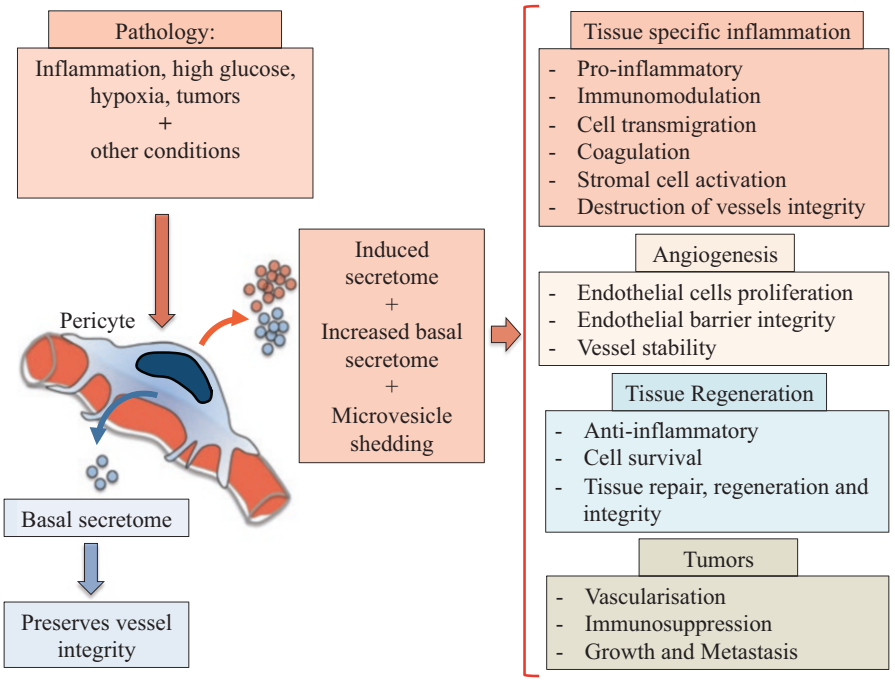

Fig. 11.2 The role of the pericyte secretome under physiological and pathological conditions. The basal secretome of pericytes plays an important role in the maintenance of blood vessel integrity. Exposed to several pathological conditions, the secretome of pericytes has multiple functions in tissue-specific inflammation, immunomodulation, angiogenesis, tissue regeneration, and facilitation of tumor growth and metastasis

tissue exposed to pathological stimuli is a possible way to address the in vivo secretion of pericytes but entails additional procedures such as mechanical and enzymatic manipulation that may impact on the findings.

We summarize examples of data from in vitro studies and, if available, evidence relating to similar conditions in vivo. Although we do not aim for a complete overview, these data will hopefully help to better understand the functions of pericytes in different pathological conditions and further unravel the mystery surrounding this heterogenous cell type that long has been overlooked.

The Pericyte Secretome Under Basal Conditions

Under control culture conditions (usually using Dulbecco's Modified Eagle medium (DMEM) and 10% fetal bovine serum), *brain pericytes* and *pericytes from several other organs* release a basal secretome including cytokines and express adhesion molecules such as interleukin (IL) IL-1α, macrophage migration inhibitory factor

(MIF), IL-10, major histocompatibility complex (MHC)-I, IL-1β, transforming growth factor (TGF) β, IL-6, E-selectin, vascular cell adhesion molecule (VCAM)-1, and intercellular cell adhesion molecule (ICAM)-1 [23–28].

In order to maintain blood-brain barrier integrity, brain pericytes also produce the angiogenic factors vascular endothelial growth factor (VEGF) and angiopoietin (Ang)-1 as well as matrix metalloproteinases (MMP)-2 and MMP-9 which mediate VEGF activation in vitro [29, 30]. Furthermore, pericytes express mRNA or protein for several neurotrophic factors under basal conditions such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), and hepatocyte growth factor (HGF) [31, 32]. The secretion of these growth factors in pericytes isolated from, e.g., endoneurial tissue (sciatic nerve as an example for peripheral nerve pericytes), might facilitate axonal regeneration in peripheral neuropathy [31].

The organ of origin seems to specifically define the type of molecules released by the pericytes under basal conditions (see Table 11.1). For example, pericytes from the *human placenta* constitutively express the chemoattractants CXCL1, CXCL5, CXCL8, MIF, monocyte chemoattractant protein-1 (MCP-1), CCL3, tumor necrosis factor (TNF) and the granulocyte colony-stimulating factor (G-CSF), and IL-6 [34]. However, pericytes in the human kidney and liver have been shown to express latent forms of TGF-β1 and TGF-β2, which can be increased by stimulation with endothelial growth factor (EGF) [37].

Generally, after exposure to different stimuli, pericytes usually significantly not only increase the basal secretome but release additional molecules here defined as "induced secretome" (see Fig. 11.1). This induced secretome is specifically related to the nature of stimulus but may slightly differ depending on the pericyte origin (tissue specific and species specific) (see Table 11.1).

The Secretome of Pericytes Under Inflammatory Conditions

Most of the data available on the secretome of pericytes come from the stimulation of *brain pericytes* with different inflammatory stimuli in vitro. In response to these different pathogenic stimuli, pericytes have been shown to release a large plethora of pro-/anti-inflammatory molecules, pleiotropic cytokines, and several chemokines. Here we describe the pericyte secretome in response to inflammatory stimuli such as lipopolysaccharide (LPS), TNF-α, Il-1beta, interferon (IFN)-γ, and specific pathogens (see Table 11.1).

LPS

LPS is a stimulator of the innate immune system and one of the most studied pericyte-activating molecules in vitro. LPS resembles a strong inflammatory stimulus able to induce the secretion of pro- and anti-inflammatory molecules [26, 56].

Table 11.1 The stimulus-specific secretome of pericytes

	Stimulus	Secreted factors	Tissue	Species	References
Pericyte secretome	Basal condition	IL-1α	Brain, retina, smooth muscle	Human, mouse	[23, 33]
		MIF	Placenta	Human	[24, 34]
		IL-10	Brain, retina	Mouse	[23, 24]
		MHC-I	Brain, kidney, skin	Mouse, rat	[25, 35]
		IL-1β	Brain, umbilical cord	Human, pig	[26, 36]
		TGF-β	Brain, kidney, liver, heart, sciatic nerve	Human	[27, 37]
		IL-6	Brain, retina, heart, smooth muscle	Human, mouse, pig, rat	[23, 26, 34, 38–40]
		HGF	Brain	Human	[32]
		MMP-2		Pig	[30]
		MMP-9			[30, 41]
		NGF	Brain, sciatic nerve	Human	[31]
		GDNF			
		BDNF			
		Ang-1	Brain, sciatic nerve, colon cancer	Human, rat, sheep	[29, 42–44]
		E-selectin	Brain	Human, mouse	[23, 45]
		VCAM-1	Brain, kidney, skin	Human, mouse, rat	[25, 28, 35, 45]
		ICAM-1	Brain, kidney, skin, placenta		[25, 28, 33–35, 45]
		VEGF	Brain, sciatic nerve, skeletal muscle, retina	Human, rat, pig, sheep	[29, 30, 40, 43, 46, 47]
Immune and inflammatory agents	LPS	IL-3	Brain	Mouse	[23]
		IL-9			
		IL-13			
		IFN-γ			
		CXCL1		Human	[34, 45]
		CXCL2			
		CXCL6			
		CCL3		Human, mouse	[23, 34]
		CCL4		Mouse	[23]
		CCL20			

(continued)

Table 11.1 (continued)

	Stimulus	Secreted factors	Tissue	Species	References
	LPS	IL-1α	Brain, retina, smooth muscle	Human, mouse	[23, 33]
		MIF	Placenta	Human	[24, 34]
		IL-10	Brain, retina	Mouse	[23, 24]
		G-CSF		Human	[34]
		TNF-α		Human, mouse	[23, 38]
		NO	Brain	Human, mouse, pig	[23, 26, 33, 36]
		CXCL10		Human, pig	[26, 33, 36, 48]
		IL-8	Brain, retina	Human, mouse, pig	[23, 24, 26, 33, 36, 38, 49]
		CCL5 (RANTES)		Human, mouse	[23, 24, 38, 49]
		CCL2	Brain, retina, heart	Human, rat, pig	[26, 33, 36, 48, 50]
		IL-6	Brain, retina, heart, smooth muscle	Human, mouse, pig, rat	[23, 26, 34, 38–40]
		E-selectin	Brain	Human, mouse	[23, 45]
		VCAM-1	Brain, kidney, skin	Human, mouse, rat	[25, 28, 35, 45]
		ICAM-1	Brain, kidney, skin, placenta	Human, mouse, rat	[25, 28, 33–35, 45]
	TNF-α	IL-1α	Brain, retina, smooth muscle	Human, mouse	[23, 33]
		MIF	Placenta	Human	[24, 34]
		G-CSF	Brain, retina		[34]
		NO	Brain	Human, mouse, pig	[23, 26, 33, 36]
		IL-7	Retina	Human, mouse, pig	[33, 36]
		IL-12	Retina, brain	Human, pig	[26, 33, 36]
		IL-16			[26, 33, 36, 48]
		TNF-β			
		GM-CSF			
		CXCL10			
		TGF-β	Brain, kidney, liver, heart, sciatic nerve	Human	[27, 37]
		IL-8	Brain, retina	Human, mouse, pig	[23, 24, 26, 33, 36, 38, 49]

(continued)

Table 11.1 (continued)

	Stimulus	Secreted factors	Tissue	Species	References
	TNF-α	CCL5 (RANTES)	Brain, retina	Human, mouse	[23, 24, 38, 49]
		CCL2	Brain, retina, heart	Human, rat, pig	[26, 33, 36, 48, 50]
		IL-6	Brain, retina, heart, smooth muscle	Human, mouse, pig, rat	[23, 26, 34, 38–40]
		MMP-9	Brain	Pig	[30, 41]
		NGF	Brain, sciatic nerve	Pig, human	[31]
		GDNF		Human, rat, sheep	[29, 31, 42–44]
		BDNF			
		Ang-1	Brain, sciatic nerve, colon cancer		
		VCAM-1	Brain, kidney, skin	Human, mouse, rat	[25, 28, 35, 45]
		ICAM-1	Brain, kidney, skin, placenta	Human, mouse, rat	[25, 28, 33–35, 45]
		VEGF	Brain, sciatic nerve, skeletal muscle, retina	Human, rat, pig, sheep	[29, 30, 43, 46, 47, 50]
	IL-1β	NO	Brain	Human, mouse, pig	[23, 26, 33, 36]
		IL-7	Retina		[33, 36]
		IL-12		Human, pig	[26, 33, 36]
		IL-16			
		TNF-β			
		GM-CSF			
		IL-1β	Brain, umbilical cord	Human, pig	[26, 36]
		IL-8	Brain, retina	Human, mouse, pig	[23, 24, 26, 33, 36, 38, 49]
		CCL5 (RANTES)	Brain, retina	Human, mouse	[23, 24, 38, 49]
		CCL2	Brain, retina, heart	Human, rat, pig	[26, 33, 36, 48, 50]
		IL-6	Brain, retina, heart, smooth muscle	Human, mouse, pig, rat	[23, 26, 34, 38–40]
		ICAM-1	Brain, kidney, skin, placenta	Human, mouse, rat	[25, 28, 33–35, 45]
		VEGF	Brain, sciatic nerve, skeletal muscle, retina	Human, rat, pig, sheep	[29, 30, 43, 46, 47, 50]

(continued)

Table 11.1 (continued)

	Stimulus	Secreted factors	Tissue	Species	References
	IFN-γ	CXCL10	Brain	Human, pig	[26, 33, 36, 48]
		MHC-I	Brain, kidney, skin	Mouse, rat	[25, 35]
		MHC-II			
		CCL2	Brain, retina, heart	Human, rat, pig	[26, 33, 36, 48, 50]
		ICAM-1	Brain, kidney, skin, placenta	Human, mouse, rat	[25, 28, 33–35, 45]
	IL-6	MHC-I MHC-II	Brain, kidney, skin	Mouse, rat	[25, 35]
		ICAM-1	Brain, kidney, skin, placenta	Human, mouse, rat	[25, 28, 33–35, 45]
Infections	HCMV	TNF-α	Brain, retina	Human, mouse	[23, 38]
		IL-1β	Brain, umbilical cord	Human, pig	[26, 36]
		IL-8	Brain, retina	Human, mouse, pig	[23, 24, 26, 33, 36, 38, 49]
		CCL5 (RANTES)	Brain, retina	Human, mouse	[23, 24, 38, 49]
		IL-6	Brain, retina, heart, smooth muscle	Human, mouse, pig, rat	[23, 26, 34, 38–40]
		CXCL11	Brain	Human	[38]
	JE, HIV	IL-6	Brain, retina, heart, smooth muscle	Human, mouse, pig, rat	[23, 26, 34, 38–40]
Diabetes	High glucose	IL-10 (decrease)	Brain, retina	Mouse	[23, 24]
		IL-8		Human, mouse, pig	[23, 24, 26, 33, 36, 38, 49]
		CCL5 (RANTES)		Human, mouse	[23, 24, 38, 49]
		CCL11			[24, 49]
		VEGF	Retina	Human	[51]
		TGFβ2			
Tumors environment, stroke	Hypoxia	CCL2	Brain, retina, heart	Human, rat, pig	[26, 33, 36, 48, 50]
		IL-6	Brain, retina, heart, smooth muscle	Human, mouse, pig, rat	[23, 26, 34, 38–40]
		VEGF	Retina	Bovine	[52]
		LIF	Heart	Rat	[50]

(continued)

Table 11.1 (continued)

	Stimulus	Secreted factors	Tissue	Species	References
Growth factors	EGF, AGE	TGF-β	Brain, kidney, liver, heart, sciatic nerve	Human	[27, 37]
	TGF-β	CX3CL1	Brain	Human	[53]
		VEGF	Brain, sciatic nerve, skeletal muscle, retina	Human, rat, pig, sheep	[29, 30, 43, 46, 47, 50]
Signaling molecules	NO	VEGF	Brain, sciatic nerve, skeletal muscle, retina	Human, rat, pig, sheep	[29, 30, 43, 46, 47, 50]
		FGF-2	Retina	Human, rabbit, sheep	[29, 31, 54]
	Dopamine	Ang-1	Brain, sciatic nerve, colon cancer	Human, rat, sheep	[29, 42–44]
	PDGF-BB	IL-33	Tumor	Mouse	[55]
Tumors	Melanoma	VCAM-1	Brain, kidney, skin	Human, mouse, rat	[25, 28, 35, 45]

Primary mouse brain microvascular pericytes, when stimulated with LPS in vitro, secrete 18 cytokines, including IL-12, IL-13, IL-9, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, eotaxin, chemokine (C-C motif) ligand (CCL)-3, and CCL-4 as well as nitric oxide (NO) [23].

The transcriptional profile of LPS-treated *human brain pericytes* revealed that 22 genes were upregulated by more than fivefold. Of these, ten genes encoded chemokines and cytokines (CXCL10, CCL20, IL8, CXCL1, IL6, CCL2, IL1B, CXCL2, IL1A, and CXCL6), and three genes encoded adhesion molecules (ICAM1, VCAM1, and selectin E (SELE)). At the protein level, pericytes secrete several pro-inflammatory cytokines and chemokines (IL-6, IL-8, CXCL1, CXCL2, CXCL3, CX3CL1, CCL5, and CCL2) and upregulate the adhesion molecules ICAM-1 and VCAM-1, which results in an increased adhesion of peripheral blood leukocytes to LPS-treated pericyte monolayers in vitro [45, 53, 57] (Table 11.1).

We have recently shown that LPS treatment of *human brain pericytes* in vitro induced a highly significant release of cytokines and chemokines with mainly pro-inflammatory properties such as growth-regulated protein alpha/beta/gamma (GROα/β/γ), IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-7, regulated on activation normal T-cell expressed and secreted (RANTES), chemokine CCL-17 (TARC), IL-1α, macrophage colony-stimulating factor (MCSF), stromal cell-derived factor 1 alpha (SDF-1α), and IL-2 [58], some of which have been previously described to be secreted by *mouse* [23] *and human* [38] *brain pericytes* after an inflammatory stimulus.

Similar secretive functions of pericytes have also been demonstrated for bacterial infection, where LPS induced the secretion of IL-1 and IL-6 [56].

However, LPS triggers not only *mouse and human brain pericytes* to secrete pro-inflammatory molecules but also interleukins considered to be anti-inflammatory (IL-10, IL-13) [23, 58].

IL1-β, TNF-α

TNF-α and IL-1β are two microglial cytokines known to be upregulated in neurodegenerative and cerebrovascular diseases and are often associated with the disruption of the blood-brain barrier. TNF-α and IL-1β stimulate pericytes to produce IL-1, IL-6, IL-7, IL-8, IL-12, IL-16, TNF-β, RANTES, G-CSF, GM-CSF, MCP-1, interferon gamma-induced protein (IP-10), and NO [26, 33, 36, 59], while the macrophage migration inhibitory factor (MIF) is secreted only after TNF-α stimulation [34]. Neutrophil recruitment from the blood to the tissue is a central component of the acute inflammatory response. Pericytes play an important role in the regulation neutrophil transmigration [34]. Thus, e.g., IL1-β activated *human placental pericytes* to express ICAM-1 and IL-8, the principal adhesion molecule and chemokine, respectively, which regulate human neutrophil recruitment and increase local inflammation [33].

In *human retinal microvascular pericytes*, inflammatory stimuli such as TNF-α and IL-1β induce a fivefold or higher increase in inflammatory mediators such as IL-1β, IL-6, IL-7, IL12 (p40), IL-16, IL-1ra, leptin, TNF-α, TNFRII, TNF-β (all cytokines), epithelial-derived neutrophil-activating peptide 78 (ENA-78), eotaxin, IL-8, MCP-1, MIP-1α, MIP-1β, RANTES (all chemokines), adhesion molecules such as ICAM-1, VCAM-1, as well as coagulation and homeostasis factors such as factor VII and tissue factor [24, 33, 49].

T-cell adhesion to the brain microvascular endothelium and subsequent migration into the brain parenchyma is one of the major events in the development of multiple sclerosis. The transmigration of T cells into the brain is clearly dominated by VLA-4/VCAM-1 interactions. VCAM-1 is highly expressed by *human brain perivascular pericytes* in vivo, and TNF-α stimulates pericyte expression of VCAM-1 in vitro [28] and increases MMP-9 secretion by pericytes [41].

IFN-γ

Primary rat pericytes constitutively express low levels of ICAM-1 and MHC class I molecule, which are upregulated in response to IFNγ. IFNγ also induces the expression of the MHC-II molecule, and pericytes acquire the capacity to present antigens to primed syngeneic rat T-lymphocytes to maintain neuroimmune networks at the blood-brain barrier [25]. The regulatory immunophenotype of

tumor-derived pericytes is characterized by their increased expression of MHC-II, as well as secretion of a range of co-stimulatory and co-inhibitory molecules. Many of these are known to be IFNγ-inducible proteins [35, 60]. IFNγ strongly increases IP-10 and human leukocyte antigen (HLA) expression in human brain pericytes, and this induction is differentially modulated by TGFβ1, but not M-CSF [48]. Similarly, MCP-1 expression in pericytes increases after IFNγ treatment [59]. IP-10 and MCP-1 are chemokines involved in the chemoattraction of monocytes/macrophages, T -cells, natural killer (NK) cells, and dendritic cells and in the promotion of T-cell adhesion to endothelial cells. IP-10 and HLA are widely used indicators of glial activation and neuroinflammation and are upregulated in many brain disorders.

Exposure of Brain Pericytes to Specific Pathogens (CMV, HIV, JE)

Pathogens such as cytomegalovirus (CMV), human immunodeficiency virus type-1 (HIV-1) [39], and Japanese encephalitis (JE) virus [40] have been shown to stimulate the secretome of *brain pericytes* in similar but slightly different ways.

Pro-inflammatory cytokines IL-8, interferon-inducible T-cell alpha chemoattractant (ITAC), and RANTES as well as TNF-α, IL-1β, and IL-6 are upregulated in *primary human brain vascular pericytes* infected with CMV [38].

In vitro, both human HIV-1 and JE virus infections stimulate *brain pericytes* to secrete IL-6 [39, 40], which is reported to be critical for the survival and regeneration of several types of neurons [61]. However, IL-6 secretion after JE virus infection led to attraction of neutrophils contributing to the destruction of the blood-brain barrier through the degradation of the tight junction protein zona occludens 1 (ZO1).

Some of the pericyte-derived cytokines exert clear pro-inflammatory functions. IL-8, RANTES, IL-1β, TNF-α [38], IP-10 [48], and MCP-1 [59] together with adhesion molecules [45] all promote the engagement of T -cells, granulocytes, monocytes/macrophages, and NK -cells, thereby triggering the inflammatory response [33, 34], whereas other secreted molecules are anti-inflammatory, suggesting that pericytes have a more complex role in the regulation of the immune response to inflammation. These immunomodulating properties of pericytes are likely important in their communication with the surrounding environment and provide a mechanism by which pericytes participate in the tissue-specific inflammatory processes (Fig. 11.2).

At least brain pericytes are now accepted as important mediators of neuroinflammation and considered active players in the inflammatory cascade with potential pathophysiological implications (for review see [22]). This emerging role of pericytes in inflammatory processes indicates that therapies addressing the inflammatory pericyte secretome may be a new avenue to modulate inflammatory pathologies.

The Secretome of Pericytes Upon Exposure to High Glucose

Pericytes may also be a key player in inflammatory diseases such as retinal inflammatory diseases including diabetic retinopathy.

Under *normal conditions*, human retinal microvascular pericytes are usually highly immunosuppressive and protect retinal endothelial cells from inflammation-mediated apoptosis and thereby preserve retinal integrity in vitro [49].

Human retinal pericytes profoundly inhibit activated T-cell proliferation and inflammatory cytokine production by secreting IL-10. Neutralization of IL-10 as well as hyperglycemic conditions both impair this T-cell inhibitory activity [49].

In response to 72 h of high glucose treatment in vitro, however, *human retinal microvascular pericytes* acquire an inflammatory phenotype and hypersecrete eotaxin, G-CSF, IL-8, and RANTES [24]. The level and number of secreted inflammatory mediators are even higher in *human retinal microvascular pericytes* when compared to retinal endothelial cells and monocytes [24]. High glucose also significantly increases the levels of TGFβ-2 and VEGF secreted by *human retinal pericytes* [51] (Table 11.1).

This indicates a crucial role for the secretome of *retinal pericytes* in inflammation that is modulated by glucose deregulation (Fig. 11.2).

The Secretome of Pericytes and Control of the Tumor Microenvironment

Immune evasion within the tumor microenvironment supports malignant growth and constitutes a major obstacle for successful immunotherapy. Immature pericytes play a critical role in tumor vascularization and seem to promote local immunosuppression in malignant gliomas and other tumors [32, 62]. *Tumor-derived vascular pericytes* negatively influence CD4(+) T-cell activation and proliferation and promote anergy in recall response to antigen by CD4(+)CD44(+) T -cells via regulator of G -protein signaling IL-5- and IL-6-dependent pathways in melanoma tumors [35]. Cultured immature *human brain perivascular pericytes* suppress mitogen-activated T-cell responses via the expression of prostaglandin E synthase (PGES), inducible nitric oxide synthase [61], HLA-G, HGF, and TGF-β. These factors that are secreted by pericytes in the tumor environment functionally contribute to immunosuppression of T-cell activity thus leading to an environment facilitating tumor growth and invasion [32].

Besides their role in facilitating tumor immune evasion, pericytes secrete factors that promote metastasis such as IL-10 and IL-4, ILs that are reported to help tumor cells evade the immune surveillance, thereby inducing tumor progression [63]. As an example, the interaction between pericytes and tumor-associated macrophages facilitates the production of IL-33 by pericytes, which has been shown to promote metastasis in a xenograft mouse models of cancer [55]. Several studies are

now supporting the role of tumor-associated pericytes and their secretome in the immune evasion paradigm within the tumor microenvironment and suggest that targeting these cell populations might be crucial in the context of successful cancer immunotherapy [64] (Fig. 11.2).

The Secretome of Pericytes in Angiogenesis

Angiogenesis is a mechanism necessary for vascular remodeling in adulthood and serves to reestablish the blood flow under pathological conditions by formation of new blood vessels [65]. Pericytes are a key player in this process. They first detach to allow proliferating endothelial cells to form new angiogenic sprouts [66]. Once the angiogenic sprouts are formed, pericytes are recruited to the newly formed blood vessels to stabilize them [67]. This process requires complex multistep signaling pathways and a high degree of spatial and temporal coordination between EC and pericytes, and is facilitated by bilateral cell signaling.

Pericytes secrete several angiogenic factors, in particular VEGF-A and VEGF-B, TGF-β, Ang-1, and miR-132 (Table 11.1).

Cultured *retinal pericytes* stimulate in vitro angiogenesis of EC and umbilical cord vein EC by secretion of fibroblast growth factor (FGF)-like molecules. The angiogenic, proliferative, and migratory activities of these cells exposed to conditioned medium from pericytes could be inhibited using an antibody to basic (b)FGF [54]. Communication between EC and pericytes in angiogenesis is also controlled, in part, by the Ang/Tie-2 system and VEGF. During angiogenesis, VEGF and FGF2 target EC to initiate angiogenesis and stimulate NO production. Conversely, NO causes an increase in VEGF, FGF2, Ang-2, and guanylate cyclase 1 soluble beta3 (GUCY1B3) expression by microvascular pericytes [29]. It has been shown that Ang-1 is expressed by *human dermal pericytes* in vitro and in vivo, and Ang-1 has a role on in vessel maturation not only in development but also for vessel maturation after angiogenesis in adult tissues [42]. Using in situ hybridization, Wakui and colleagues elegantly showed that the angiogenic phase might be initiated by an increase in Ang-2 and VEGF expressed by rat EC and pericytes, while the microvessel maturation phase might be initiated by a relative increase in Ang-1 expressed by pericytes and a decrease in VEGF [43]. Interestingly, pericytes secrete Ang-1 and Ang-2 at higher concentrations than cardiac stem cells, and a similar trend was observed with bFGF [68] suggesting the important implication that has for the use of pericytes for cell therapy, where the outcome may depend on the balance between cooperative and competitive interactions at the level of the secretome. Angiopoietins are important in the regulation of vascular remodeling, stabilization, and maturation of immature vessels [69, 70].

Pericytes play a role in the regulation of vessel stability; another mechanism for this stabilization may be via pericyte-derived VEGF. VEGF gene expression in the developing retinal vasculature was observed in pericytes contacting newly formed microvessels. Pericytes produce VEGF that may act in a juxtacrine/paracrine

manner as a survival and/or stabilizing factor for ECs in microvessels [46]. Moreover, pericytes have the potential to regulate the endothelial barrier integrity via the production of matrix metalloproteinases MMP-2- and MMP-9-mediated activation of VEGF in vitro [30]. Interestingly, in the human skeletal muscle, VEGF is located in intracellular vesicles in pericytes around microvessels that are located in the subsarcolemmal regions that are between the contractile elements in the muscle, illustrating a possible mechanism in humans by which skeletal muscle fibers can control capillary growth by releasing VEGF from intracellular vesicles during contraction [47]. In addition, bidirectional TGF-β signaling between ECs and pericytes also regulates their maturation and differentiation [9, 71] and thereby contributes to vessel stability and tissue perfusion. TGF-β and its receptors are expressed on both cell types, pericytes and ECs.

Platelet-derived growth factors (PDGFs) are endogenous growth factors that occur in several different isoforms. The PDGF-B gene product forms the biologically active PDGF-BB dimer, which binds to PDGFRα and β, whereby it has the highest affinity for PDGFRβ. Pericytes highly express the receptors for PDGF-BB, and PDGF-BB signaling is important for pericyte recruitment to the blood vessels for vessel stabilization. However, PDGF-BB secreted by ECs not only recruits but also elicits a secretory response in pericytes.

We have demonstrated that PDGF-BB promotes the secretion of several neuroprotective and angiogenic growth factors by *human brain pericytes* via activation of PDGFRβ on pericytes [58]. In response to PDGF-BB stimulation in vitro, human brain pericytes released HGF, a paracrine growth factor with important roles in morphogenesis and organ regeneration. In addition, placental growth factor (PLGF), an angiogenic factor that belongs to the VEGF family, was secreted by human pericytes after PDGF-BB treatment in vitro. In addition, PDGF-BB stimulated release of insulin-like growth factor-binding protein (IGFBP), neurotrophin 3 (NT3), heparin-binding EGF-like growth factor (HB-EGF), brain-derived neurotrophic factor (BDNF), bFGF, βNGF, and VEGF, whereas the release of stem cell factor (SCF) was decreased [58].

Furthermore, PDGF-BB significantly decreased the secretion of TNFα and increased IL-13, IL12p40/70, IL-15, IL-6, IL-8, MCP-1, and IP-10 by *brain pericytes* in vitro. This secretome of pericytes stimulated by PDGF-BB could be blocked using an antibody to PDGFRβ [58]. These data suggest that PDGF-BB elicits more functions in pericytes than simply recruitment to EC but may actually modulate the microenvironment by directing the pericyte secretome from an inflammatory to a trophic secretome.

The Secretome of Pericytes Under Hypoxic Conditions

Upon hypoxia, pericytes have been shown to also increase their production of neurotrophins such as NGF, BDNF, NT4-5 [72], as well as miR-132 in vitro [73].

When exposed to hypoxia, *human pericytes* dramatically increase expression of angiogenic molecules and corresponding receptors, while the expression of bFGF, HGF, EGF, and Ang-1 is repressed. Hypoxic pericyte-conditioned medium significantly increases host angiogenesis as well as supports microvascular structure formation in vivo and stimulated formation of capillary-like networks with/without ECs in three-dimensional co-cultures [50].

In the retina, hypoxia induces an increase of VEGF in *retinal pericytes* [52].

Also, transplanting *skeletal muscle pericytes* into the center of an acute myocardial infarction model (characterized by, e.g., hypoxic conditions) improves cardiac function through the production of IL-6, leukemia inhibitory factor [7], prostaglandin-endoperoxide synthase 2 [36], hypoxia-inducible factor 1-alpha (HIF-1α), and MCP-1, aside from VEGF-A and TGF-β1, and leads to reduction of cardiac fibrosis, protection from hypoxia, and promotion of angiogenesis [50].

Similarly, when pericytes are exposed to oxygen-glucose deprivation in vitro, they change their phenotype, downregulate pericyte markers, and express inflammatory markers such as CD11B, IBA1, galectin-3 (GAL3), TNF-α, and MHC-II [74].

The Secretome of Pericytes After Other Pathological Stimuli

Exposure to NO

Interestingly, blood-brain barrier impairment and disruption by the production of reactive oxygen species (ROS) or cytokines have been observed in several pathologies like neurodegenerative, cerebrovascular, and other neuroinflammatory diseases [75]. NO is a signaling molecule that plays a key role in the pathogenesis of inflammation and is considered a pro-inflammatory mediator. It plays a role in the immune responses of cytokine-activated macrophages, which release NO in high concentrations. In addition, NO is a potent neurotransmitter at the neuron synapses and contributes to the regulation of apoptosis [76]. Treatment of *microvascular pericytes derived from corpus luteum* with varying doses of the NO-donor DETA-NO for 8 h causes a dose-dependent increase of mRNA expression of VEGF, FGF2, Ang-2 and NO receptor, and GUCY1B3, which provides further evidence for the role of pericyte and NO in angiogenic factor expression, and for the potential interactions of pericytes with EC via NO production [29].

Exposure to Advanced Glycation Products

Advanced glycation end products (AGEs) are the late products of nonenzymatic glycation produced under oxidative conditions. They are proteins or lipids that become glycated after exposure to sugars; they are prevalent in the diabetic

vasculature and contribute to the development of atherosclerosis and a variety of other microvascular and macrovascular complications and as such appear to be a key factor in the development of diabetic neuropathy [77].

AGEs are able to induce pericyte production of TGF-β and fibronectin, with consequent hypertrophy and thickening of the basal membrane of the blood-brain barrier with loss of its permeability features [27]. Moreover, pericytes stimulated with AGEs also increase the expression of metalloproteases MMP-9 and MMP-2, contributing to the barrier disruption.

Exposure to TGF-β

The TGF-β protein family is strongly engaged in developmental angiogenesis, but they are also regulators of vascular integrity in the adult [78]. TGF-β mediates an increase in VEGF expression by pericytes in vitro, a mechanism for vessel stabilization via pericyte -EC communication [46]. The effects of TGFβ1 on pericyte secretion in vivo are difficult to infer. However, TGFβ1 seems to affect inflammatory properties of pericytes by increasing the release of IL-6, MMP-2, and NADPH oxidase 4 (NOX4) and reducing pericyte phagocytosis [53]. TGFβ1 attenuates the expression of key chemokines and adhesion molecules involved in CNS leukocyte trafficking and the modulation of microglial function in pericytes. TGFβ1 treatment of *primary human brain pericytes* induces the expression of several inflammatory-related genes (NOX4, COX2, IL6, and MMP-2) and attenuates others (IL8, CX3CL1, MCP1, and VCAM1) through SMAD2/3 transcription factors. A synergistic induction of IL-6 is seen with IL-1β/TGFβ1 treatment, whilst TGFβ1 attenuates the IL-1β-induced expression of CX3CL1, MCP-1 and soluble VCAM-1. Furthermore, TGFβ1 attenuates the phagocytic ability of pericytes, possibly through downregulation of the scavenger receptors CD36, CD47, and CD68 [53].

Microvesicles and Pericytes

Microvesicles (MVs) are small membrane-enclosed vesicles that are shed from the plasma membrane of a variety of cell types. They are present in the blood of healthy individuals, but their levels are often modified under pathological conditions. MVs offer an elegant solution to cells to exchange biomolecules since, in one vesicle, it is possible to find lipids, proteins (receptors and enzymes), second messengers, mRNA, miRNA, and cell organelle fractions or proteins. Despite of these mix of components, MVs possess specialized functions and play a key role in several pathologies by regulating coagulation, angiogenesis, cell survival, modulation of the immune response, and inflammation. The content and the number of MVs depend on the cells they originate from, the stimulus of production, and the mechanism of vesicle generation [79–81].

Our group demonstrated for the first time that *human brain pericytes* release MVs when stimulated in vitro with PDGF-BB or LPS, respectively. Interestingly, the number of MVs released after PDGF-BB stimulation was significantly higher compared to LPS stimulation or control conditions, which did not differ from each other. However, the MVs released by LPS contained high amounts of the inflammatory cytokines IL-6, IL-8, MCP-1, and IP-10, all cytokines that are also released in the medium by pericytes after LPS stimulation (see above). MVs released by pericytes upon PDGF-BB stimulation instead contained a three- to fourfold increased amount of the growth factors BDNF, bFGF, bNGF, VEGF, and PLGF compared to control conditions [58]. These growth factors transported in MVs may provide an effective mechanism for long-range communication of neuroprotective and angiogenic information between pericytes and other cells. Further studies are needed to explore the proteins and miRNA composition of pericyte-derived MVs and their potential function in neuroregeneration and also regeneration of other tissues and organs.

The Role of the Pericyte Secretome -Regenerative Potential

Several of the pericyte-secreted molecules are known to have a function in tissue repair and immunomodulation (for review see [18]). This hypothesis is strengthened by studies reporting an uncontrolled activation of the immune response in several organs or neurodegeneration in pericyte-deficiency models [12, 82].

Besides the modulation of the inflammatory responses, the secretion of anti-inflammatory molecules can be beneficial in the maintenance of tissue integrity and contribute to tissues repair and regeneration. In many neurological disorders, for example, brain inflammation is increased, and blood vessel integrity is strongly affected [83, 84].

Interestingly, some of the cytokines secreted by pericytes have an important role in adult neurogenesis and may affect neuronal survival and regeneration in pathologic conditions. Pericytes also express CX3CL1, also known as fraktaline, which binds its receptor CX3CR1 expressed on microglial cells modulating their activation and reducing, e.g., neuroinflammation in Alzheimer disease (AD) and alpha-synuclein-induced neurodegeneration [22, 85] as well as Aβ clearance and the reduction of p-tau accumulation in AD [86].

LIF, even if scarcely expressed in the central nervous system under physiological conditions, promotes neural stem cell self-renewal [87]. IL-9, shown to be secreted by pericytes in response to LPS [23], has been implicated in the survival of postmitotic neurons [88]. TGF-β is a multifunctional cytokine secreted by pericytes that supports blood-brain barrier integrity and function and by doing that counteracts secondary neurodegeneration due to blood-brain barrier leakage [71]. In AD, TGF-β has been shown to prevent the formation of amyloid plaques and neurofibrillary tangles and to protect the tissue from oxidative stress [89].

Pericytes express IL-33 in response to platelet-derived growth factor-BB (PDGF-BB) [55], which is a controversial cytokine that may exert both pro-inflammatory and protective functions [90]. It has been shown that IL-33-mediated decrease of CNS β-amyloid peptides in vitro [91] is consistent with a decreased IL-33 expression in patients with AD [91]. As mentioned above, both HIV-1 and JE virus infections stimulate brain pericytes in vitro to secrete IL-6 [39, 40], which is reported to be critical for the survival and regeneration of several types of neurons [61]. IL-6 has also been shown to regenerate lesioned peripheral nerves [92, 93] and to support motor neuron survival via upregulation of BDNF in animal models of motor neuron disease [61, 94].

Protective features of the pericytes secretome have been demonstrated not only in the brain: Pericyte secretion of restorative molecules including IL-6, LIF as well as COX-2, heme oxygenase-1 (HO-1) not only increased cardiac function, but reduced cardiac fibrosis and infiltration of inflammatory cells, protected from hypoxia and promoted angiogenesis after transplantation of skeletal muscle pericytes in an acute myocardial infarction model [50]. TGF-β, produced by vascular pericytes from the kidney and the liver, has a potential implication for the pathophysiology of liver regeneration and chronic liver and kidney diseases [37]. Peripheral nerve pericytes modify the blood-nerve barrier functions and promote peripheral nerve regeneration through the secretion of various soluble factors such as NGF, BDNF, and GDNF [27].

Future Trends

The secretome of pericytes has multiple implications of which only few are currently understood (Fig. 11.2). Future research detecting the secretome in vivo or analyzing secreted MVs will further help to elucidate their function. Also selected deletion of certain cytokines or chemokines in pericytes may provide more insights in the contribution of the pericyte secretome and its components to the physiological homeostasis, disease progression, or reparative processes. Conditioned media, selected molecules, or MV engineering may offer new possibilities to impact on disease progression and prevention. In addition, cell therapy grafting and injecting pericytes to harvest their angiogenic or regenerative secretome are already currently explored.

Concluding, it is clearly evident that pericytes are fundamental in the regulation of the immune response in the body and may be an important therapeutic target in several inflammatory conditions by balancing overshooting inflammatory reactions or a target to address immune evasion of tumors. In addition, pericytes provide a vast angiogenic and trophic secretome that should be exploited for regenerative processes.

Therapies modulating pericyte secretion for immunomodulating and regenerative purposes might therefore constitute a new and innovative way for the treatment of inflammatory pathologies or neuroprotective and regenerative therapies.

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Correction to: The Microvascular Pericyte: Approaches to Isolation, Characterization, and Cultivation

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Correction to:
Chapter 5 in: A. Birbrair (ed.), *Pericyte Biology - Novel Concepts*, Advances in Experimental Medicine and Biology 1109, https://doi.org/10.1007/978-3-030-02601-1_5

The author has spotted an error on page 61, in the middle of the second paragraph from bottom. The content has been revised and the corrected version is as follows:

Even at low dilutions, our primary pericytes retain the ability to differentiate along the mesenchymal lineage when cultured in the presence of serum. There are a number of commercially available pericyte cell lines. However, we have had no experience with these cells and they will not be discussed in detail in this chapter. Of note is that human brain microvascular pericytes are available from Neuromics [66]. These cells are also available in a multicellular BBB model system. Pericytes do not appear to differentiate under co-culture conditions making multicellular systems useful experimental tools.

In conclusion, while techniques are available for pericyte isolation and culture, there are no shortcuts. Pericyte populations need to be defined [67]. For there to be real advances in the field, rigorous adherence to protocols and techniques and characterization of starting material is essential. That pericytes within the vascular bed are both phenotypically and functionally heterogeneous, make it imperative to characterize pericyte subsets.

The updated online version of this chapter can be found at https://doi.org/10.1007/978-3-030-02601-1_5

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