Chapter 2
The NG2 Proteoglycan in Pericyte Biology

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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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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

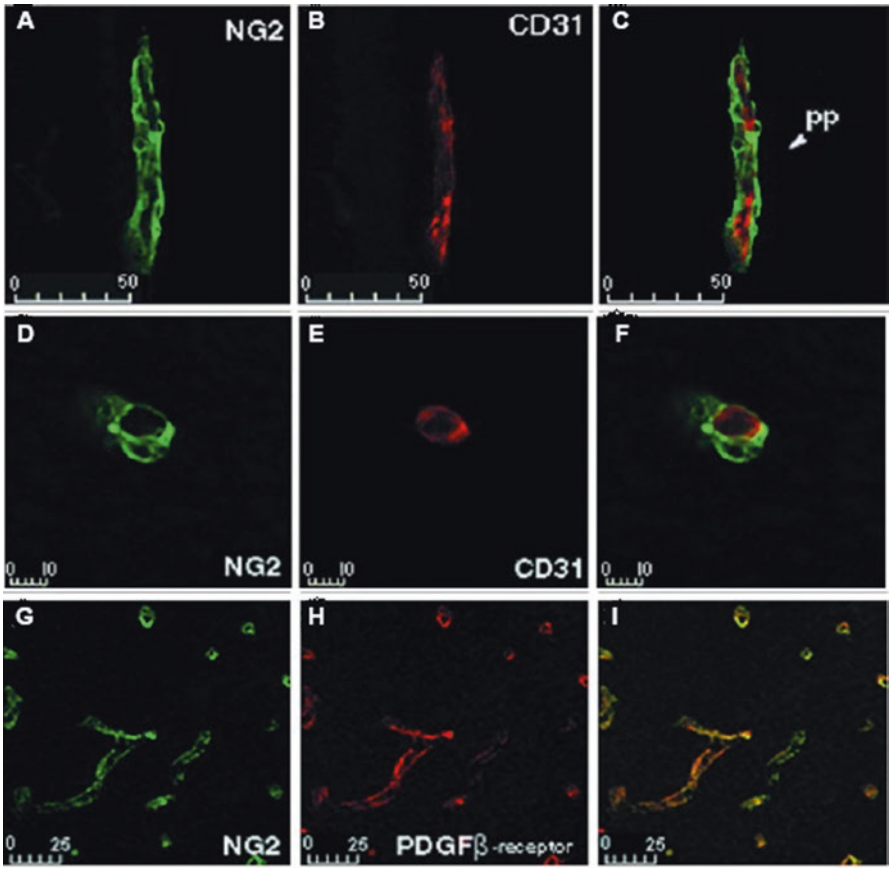

Fig. 2.1 NG2 as a pericyte marker. (**a**–**c**) Double staining for NG2 (**a**; green) and CD31 (**b**; red) in a section of postnatal day 11 mouse retina. The merged image (**c**) reveals the abluminal relationship of pericytes to endothelial cells in this longitudinal view of a developing capillary. 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

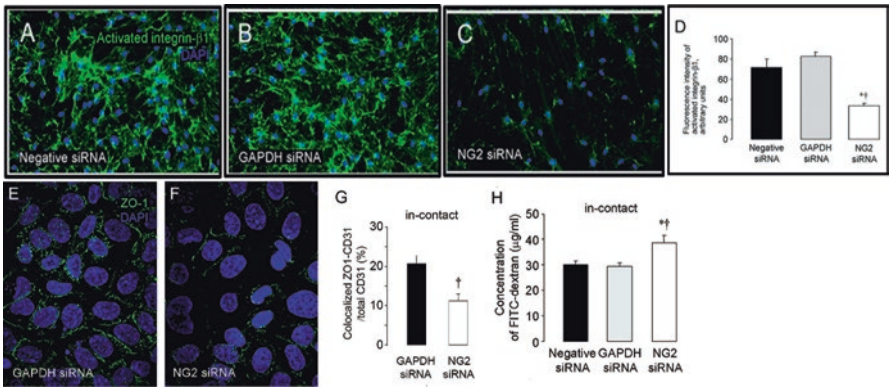

Fig. 2.2 NG2 effects on pericytes and endothelial cells. (**a**–**d**) Pericyte monolayers were immunolabeled with antibody HUTS-21 (**a**–**c**; green) to detect levels of activated β1 integrin (blue = DAPI). Knockdown with NG2 siRNA (**c**) leads to greatly reduced levels of β1 integrin activation (**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

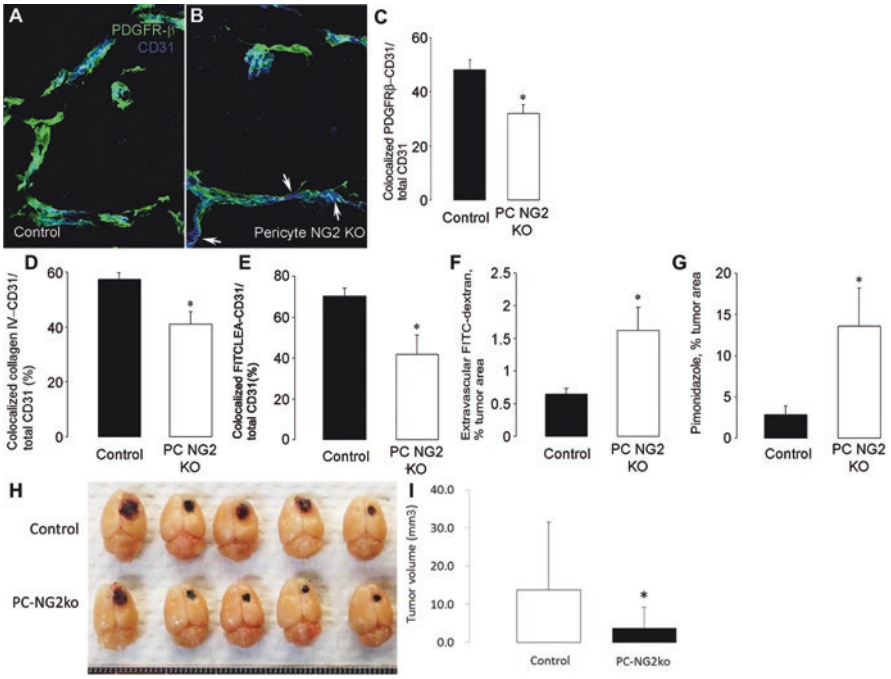

Fig. 2.3 Effects of NG2 ablation on tumor vascularization and growth. (**a**–**c**) Sections from 10-day B16F10 brain tumors in control (**a**) and PC-NG2ko (**b**) mice were double stained for PDGFRβ (green) and CD31 (blue). Z-stacks of confocal images were analyzed for overlap between pericytes and 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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