Mechanisms of Vessel Pruning and Regression

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The field of angiogenesis research has primarily focused on the mechanisms of sprouting angiogenesis. Yet vascular networks formed by vessel sprouting subsequently undergo extensive vascular remodeling to form a functional and mature vasculature. This “trimming” includes distinct processes of vascular pruning, the regression of selected vascular branches. In some situations complete vascular networks may undergo physiological regression. Vessel regression is an understudied yet emerging field of research. This review summarizes the state-of-the-art of vessel pruning and regression with a focus on the cellular processes and the molecular regulators of vessel maintenance and regression.

Introduction

A PubMed search for the term “angiogenesis” (June 2015) yields almost 80,000 hits. Conversely, a screen for “VEGF” or “vascular endothelial growth factor” identifies 71,000 publications. This comparison illustrates that the history of the field of angiogenesis research is also the history of VEGF research, with the majority of the angiogenesis literature focusing one way or the other on aspects of VEGF biology. VEGF stands high up in the hierarchy of events that leads to the formation of new blood vessels. It is very much compatible with the tip cell concept of invading angiogenic endothelial cells (ECs) that follow a VEGF gradient to establish novel capillary sprouts (Gerhardt et al., 2003; Potente et al., 2011). The role of VEGF as master switch of angiogenesis induction has made VEGF an attractive target of therapeutic intervention, most notably in the context of oncology as well as in ophthalmology (Carmeliet and Jain 2011).

The focus on VEGF has overshadowed the fact that it takes more than a vascular sprout to grow a hierarchically structured, mature, three-dimensional vascular network. Guided by the need to improve VEGF-targeting therapies, the focus of the field of angiogenesis research has in recent years shifted toward the analysis of network formation mechanisms as well as the study of vessel maturation and remodeling processes. This has led to the discovery of vascular guidance and networking molecules as well as the identification of vessel maturation-regulating molecules such as the angiopoietins (ANG) and the PDGFs (Potente et al., 2011).

When dissecting vessel maturation and remodeling, one can conceptually think of different growth patterns leading to the formation of a vascular network (Figure 1). First, it could be assumed that angiogenesis and vessel maturation are linear processes that start with a sprout and proceed gradually over time (Figure 1A, dotted line). This may be unlikely, considering that nature’s mechanisms usually follow as endpoint equilibration reactions inverse exponential curves, i.e., it is more likely to assume that angiogenesis is initially a rapid process that continues over time with decelerating pace to eventually reach an equilibrium (Figure 1A, red line). Alternatively, triggered by metabolic demands and strong inductive forces, angiogenesis could proceed as an overshooting reaction that leads to the formation of more than necessary blood vessels. Excessive blood vessels would regress over time in a declining rather than an accelerating equilibrium reaction (Figure 1A, black line).

Critically assessing the above-listed three theoretical scenarios, it is all too obvious that angiogenesis in vivo follows the black line of Figure 1A. The intensely studied postnatal mouse retinal vascular network may be the best example for this pattern: vessel density at the sprouting front of a postnatal retina (P2–P8) is substantially higher than in the mature, fully remodeled vascular network of the adult retina (Figure 1A). This trimming of a vascular network is called “vessel pruning” and marks the physiological regression of a subset of microvessels within a growing vasculature.

Beyond vessel pruning, vascular networks may also physiologically undergo complete regression. It occurs, for example, developmentally during the regression of hyaloid vessels (Ito and Yoshioka, 1999) and in the adult during luteolysis (Figure 1B). Luteolysis marks the rapid dissociation of the mature ovarian corpus luteum at the end of the ovarian cycle. Triggered by the downregulation of VEGF and the upregulation of ANG2, the extensive vascular network of the corpus luteum completely dissociates within a few days and gradually resolves into scar tissue over a period of weeks (Goede et al., 1998; Modlich et al., 1996). This prominent example illustrates that nature has secured mechanisms and pathways for the controlled dissociation of established vascular networks. It also highlights that vessel regression may be triggered by the shut-off of ON signals (e.g., VEGF) as well as the active engagement of OFF signals (e.g., ANG2).

Adapting the concepts of vessel pruning and regression to tumors has important conceptual and therapeutic implications. Tumor growth proceeds unidirectionally, i.e., tumors keep growing and the tumor vasculature expands correspondingly following the induction of the angiogenic switch. As such, spontaneous regression does not occur and the vasculature grows...
not occur in human tumors. Instead, anti-angiogenic therapies targeting the VEGF/VEGFR pathway prune the most immature vasculature and leave behind a more normal-appearing network of mature vessels (Carmeliet and Jain, 2011). This process has been called “vascular normalization” and is at the heart of mechanistically explaining the synergistic effects of anti-angiogenic therapy and chemotherapy. Further improvement of the therapeutic efficacy of established anti-angiogenic intervention will require a better understanding of vessel pruning and regression (Figure 1C).

Learning from developmental biology settings, this review is aimed at discussing the state of the art of vessel pruning and regression research. We will review vessel pruning and regression in different physiological processes and discuss the cellular mechanisms and molecular mediators of regressive vessel remodeling and vessel maintenance.

Vessel Pruning and Regression in Different Physiological Processes

Vessel remodeling leading to pruning and regression has been studied in embryonic and postnatal development of mouse (Franco et al., 2015; Korn et al., 2014; Rao et al., 2007; Udan et al., 2013), rat (Hughes and Chang-Ling, 2000), and zebrafish vasculature (Chen et al., 2012; Kochhan et al., 2013; Lenard et al., 2015). Regressive vascular remodeling also occurs during adult reactivation of quiescent vessels such as during corpus luteum regression (Modlich et al., 1996) and upon therapeutic VEGF manipulation (Baffert et al., 2006; Inal et al., 2004). In mouse embryonic development, vascular remodeling is critical for patterning of most vascular beds after primitive vascular plexus formation between E10.5 and E12.5. Mice with genetic inactivation of factors involved in vascular remodeling die during midgestation, and multiple mutations affecting vascular remodeling have been identified so far (Potente et al., 2011).

During postnatal development, retinal and hyaloid vascular beds undergo extensive hydrostatically coupled vessel regression. In the mouse retina, vessel sprouting starts radially from the center at postnatal (P) day 1, reaching the periphery around P7 (Fruttiger, 2007). In this system, vessel regression starts when sprouting angiogenesis is still ongoing in the outer layers at P5 and continues until P18. Immature vessels in the inner plexus regress, and capillary-free zones around the major vessels develop. Yet regressing vessels are also detected at the advancing vascular front, demonstrating that vascular remodeling is not completely spatially restricted (Franco et al., 2015; Fruttiger, 2007; Korn et al., 2014). Prior to vascularization, the retina is nourished by the pupillary membrane vessels and the hyaloid vasculature. These capillary networks are only transiently present and gradually regress after birth (Ito and Yoshioka, 1999; Poché et al., 2015). Poor retina vascularization resulting in low oxygenation and high VEGF-A levels often cause hyaloid vessel persistence, demonstrating the interdependence of the two vascular networks (Claxton and Fruttiger, 2003; Rao et al., 2007).

During the adult ovarian cycle, growth and regression of the corpus luteum is accompanied by sprouting and regression of blood vessels. Vessel constriction and occlusion are followed by rounding and detachment of single EC and EC sheets from the extracellular matrix and subsequent shedding into the circulation (Goede et al., 1998; Modlich et al., 1996). During

chaotropic with limited remodeling and maturation. Yet the original goal of anti-angiogenic intervention was to starve tumors to death by driving the tumor-associated vasculature into regression (Folkman, 1971). While this may be achieved in some preclinical tumor models, clinical reality has shown that this does
pregnancy, vessel density in the mammary gland and the uterus increases by sprouting angiogenesis and intussusception. Conversely, involution of the breast endothelium after lactation is accompanied by gradual regression of the newly formed capillary plexuses (Andres and Djonov, 2010). Thus, vessel regression is a prevalent process required for vessel patterning in multiple organs at different developmental stages and for maintaining the dynamic nature of some vascular beds throughout life.

**Apoptosis and Migration as Drivers of Vessel Pruning and Regression**

The initial triggers selecting particular branches for vessel regression as well as the fate of regressing ECs are largely unknown. It is an open debate if vessel regression is stimulated by active signaling pathways or if it results from the withdrawal of survival factors or a combination of both. In particular, the contribution of EC apoptosis to regressive vessel remodeling is controversial and likely depends on the context of different vascular beds.

Under some conditions, EC apoptosis may be the primary mechanism triggering vessel regression due to the withdrawal of survival factors such as VEGF (Alon et al., 1995; Baffert et al., 2006; Benjamin et al., 1999; Inai et al., 2004; Meeson et al., 1999). Likewise, in situations of drastically changing metabolic demands, such as during involution of the lactating breast (Andres and Djonov, 2010), therapeutic tumor targeting (Benjamin et al., 1999; Inai et al., 2004), or loss of entire vascular plexuses (Lang et al., 1994; Lobov et al., 2005; Meeson et al., 1996), the vasculature undergoes complete regression involving massive EC apoptosis.

During pupillary membrane vessel regression, macrophages induce a first wave of initiating apoptosis in single EC. Subsequent lumen constriction and flow stasis induce a second phase of synchronous EC apoptosis in which whole vessel segments undergo programmed cell death (Lang et al., 1994; Meeson et al., 1996, 1999). Regression of hyaloid vessels also involves EC apoptosis and subsequent EC clearance by macrophages and/or shedding into the vascular lumen (Mitchell et al., 1998). During mammary gland involution, EC apoptosis partially accounts for vascular regression and peaks together with maximal vessel occlusion (Andres and Djonov, 2010). Moreover, O$_2$-induced vessel regression in retinopathy of prematurity (ROP) models depends on selective EC apoptosis (Alon et al., 1995).

While the contribution of EC apoptosis to vessel regression in the above-listed biological processes is largely undisputed, selective pruning of only a subset of vessels might involve different processes in different situations. In this case, EC apoptosis may not be the primary trigger of regression, but could rather be a secondary effect of impaired blood flow-mediated reduced survival signaling in pruning segments. Disintegration of EC from the vascular network as consequence of EC migration away from the regression fragment and subsequent dissociation from the basal lamina could also lead to EC apoptosis (Dimmeler and Zeiher, 2000; Wietecha et al., 2013). Along these lines, increased EC apoptosis has been correlated with reduced EC numbers and enhanced vessel regression in the postnatal retina upon deletion of the WNT secretion factor Evi/Wls in EC (Korn et al., 2014). Apoptosis-related retinal vessel regression was also observed upon endothelial deletion of the rho guanine exchange factor (GEF) FYVE, RhoGEF, and PH domain containing 5 (Fgdc5) (Cheng et al., 2012; Korn and Augustin, 2012). Thus, if pruning results in a compromised circulation requiring elimination of excessive EC to match tissue needs, EC apoptosis may contribute to regressive vessel remodeling. A study in the zebrafish eye suggests that vessel regression involves all three processes of EC migration, re-deployment, as well as apoptosis. Vessel pruning was triggered in response to low blood flow, and ECs either migrated to neighboring branches in which they were incorporated or died by macrophage-independent apoptosis (Kochhan et al., 2013).

A number of studies explain vessel regression independent of EC apoptosis solely as a consequence of EC migration. Live-cell imaging of the vasculature in zebrafish midbrain and cultured mouse embryos suggested that remodeling of redundant loop-forming or parallel vessel segments is independent of apoptosis. In these systems, pruning depends on blood flow-controlled lateral EC migration and subsequent integration into neighboring vessels (Chen et al., 2012; Udan et al., 2013). During vessel remodeling in the mouse embryo, EC migration is followed by fusion of neighboring vessels causing a rapid decrease in vascular complexity and an increase in vessel diameter. The combination of EC migration and fusion is particularly important for shaping the hierarchically ramified arterial tree (Udan et al., 2013).

Recycling of ECs during vessel regression is also observed in the chicken yolk sac, in which vessels disconnecting from the vitelline arteries are re-used for establishing novel vessel connections (je Noble et al., 2004). In the postnatal rat retina, vessel retraction has been shown to involve EC rounding, migration, and subsequent redeployment in new vessels in the immediate vicinity. The localization of regressing branches in the periphery of the retina close to growing vessels supports an EC recycling concept. Moreover, only few apoptotic ECs were detected in the inner plexus of the retina, and numbers did not increase with empty basement membrane sleeves (ebms) accumulating during the second postnatal week. Neither apoptotic ECs nor macrophages were enriched close to regressing vessel segments, favoring a process of apoptosis-independent vessel regression (Hughes and Chang-Ling, 2000). However, the presently used static models do not allow correlation of these ketically different processes, considering that cleaved caspase 3-positive or TUNEL-positive ECs have a short half-life time, whereas ebms may persist for 2–3 weeks during trachea and tumor vessel regression (Baffert et al., 2006; Inai et al., 2004). Likewise, apoptotic ECs would not be expected close to ebms, if EC apoptosis was a consequence of disintegration of the retracting ECs from the regressing vessel. The commonly observed shedding of apoptotic ECs into the vascular lumen and/or their clearance by macrophages further complicates the detection of apoptotic ECs. As the present understanding of vessel remodeling is integrated from studies in wild-type and mutant animals, caution should be taken by comparing physiological and pathological vessel remodeling, which might follow different pathways.

Higher-resolution studies that apply novel technologies allowing dynamic visualization of EC apoptosis and vessel regression are only beginning to emerge. Two recently published studies shed substantially higher-resolution insights into the dynamics
of EC rearrangements during developmental vessel regression, clearly advancing the cellular understanding of the remodeling process. Franco and co-workers have analyzed vascular pruning in the postnatal mouse retinal vasculature and propose a four-step model for vessel regression (Figure 2) consisting of branch selection (Figure 2A), lumen stenosis (Figures 2B and 2C), EC retraction (Figure 2D), and resolution (Figure 2E) (Franco et al., 2015). High-resolution imaging of the retina demonstrated that physiological microvessel regression may occur independent of apoptosis involving flow-induced EC rearrangement and retraction from the regressing branch and EC redeployment in the remaining vasculature (Franco et al., 2015). A second high-resolution and spatiotemporal study of developmental vessel pruning has established the subintestinal vein (SIV) plexus of the zebrafish embryo as novel model to study vessel pruning at the cellular level (Lenard et al., 2015). Developing novel live-cell imaging techniques for analyzing vessel remodeling is essential to improve quantification of vascular regression at the cellular level. Despite the refinement of immunostaining techniques and development of high-resolution imaging in the last years, the present static methods are limited, explaining why vessel remodeling is still an understudied field. Lenard and co-workers describe vessel remodeling as vessel anastomosis in a reversed order independent of EC apoptosis. This process involves cell–cell contact resolution and lumen collapse, as well as EC migration toward opposing sides and subsequent EC fusion with the parental branch. Before complete separation of ECs from the parental vessel, the vascular lumen breaks and reconnects multiple times (Lenard et al., 2015). Taken together, pruning and trimming of vascular beds during developmental regressive vessel remodeling may result from a combination of EC migration, redeployment, and apoptosis, whereas vessel regression during complete vascular plexus resolution is primarily driven by EC apoptosis.

The Contribution of Blood Flow to Vessel Pruning and Regression

Non-perfused vessels are predisposed to vascular pruning, and changes in blood flow act as regulators of vessel regression (Figures 2B and 2C) (Ando and Yamamoto, 2009). It has already been proposed more than a century ago that the vessels with the highest blood flow enlarge and those carrying the least blood flow regress (Thoma, 1893). Several follow-up studies confirmed the importance of hemodynamic forces for vascular remodeling in zebrafish (Chen et al., 2012; Kochhan et al., 2013; Lenard et al., 2015), chicken (le Noble et al., 2004), rabbit (Langille et al., 1989), and mouse (Franco et al., 2015; Garcia and Larina, 2014; Lucitti et al., 2007; Udan et al., 2013). Along these lines, remodeling of the primitive vascular plexus during embryogenesis is initiated after establishment of the first heartbeat and onset of blood flow (Garcia and Larina, 2014; Lucitti et al., 2007). Several mouse mutants with disrupted heart function and impaired blood flow are embryonic lethal due to impaired vascular remodeling of the yolk sac, further stressing the importance of hemodynamic forces for vascular remodeling (Huang et al., 2003).

Pharmacological induction of vasoconstriction or vessel obstruction induced pruning in zebrafish and mouse retina, whereas stimulation of blood flow protected branches from regression (Chen et al., 2012; Kochhan et al., 2013; Lobov et al., 2011). Similarly, pharmacological inhibition of vasoconstriction or induction of vessel dilation reduced blood vessel stability in the mouse retina, indicating that vessel pruning is
dependent on hemodynamic forces (Lobov et al., 2011). Yet it is not clear if mechanical factors like shear stress and circumferential stretch or molecular components such as oxygen or angiogenic factors are the primary mediators of flow-controlled vessel regression (Culver and Dickinson, 2010).

The mechanical forces of blood flow are necessary and sufficient for yolk sac remodeling as embryos with a low hematocrit showed impaired capillary plexus remodeling and downregulation of the mechanosensitive factor eNOS, which could be rescued by restoring blood viscosity (Lucitti et al., 2007). Similarly, mice with disturbed hematopoiesis leading to low blood hematocrit were characterized by vascular remodeling defects (He et al., 2008). Hemodynamic forces are particularly important for shaping the arterial tree and during aortic arch remodeling (Buschmann et al., 2010; Yashiro et al., 2007).

Blood flow triggers several downstream effects, which can all contribute to vessel regression, including vessel constriction, EC survival, alignment, and migration (Figure 3) (Chen et al., 2012; Franco et al., 2015; Kochhan et al., 2013; Lenard et al., 2015; Meeson et al., 1996; Udan et al., 2013). Shear stress induces AKT signaling, resulting in Krüppel-like factor 2 (KLF2) activation and subsequent upregulation of nitric oxide (NO)-synthase and superoxide dismutase, thereby promoting EC survival and NO-mediated vessel dilation (Dekker et al., 2005; Dimmeler and Zeiher, 2000). Shear stress-induced survival signaling has also been observed in the pupillary membrane, the corpus luteum, and the trachea as flow stasis resulting from vessel occlusion led to rapid vessel regression (Azmi and O’Shea, 1984; Baluk et al., 2004; Meeson et al., 1996). Shear stress reduction also shown by high-resolution imaging of EC nuclei and Golgi in the postnatal retina (Franco et al., 2015). Differential flow patterns in juxtaposed vessels trigger developmental vessel regression by breaking EC symmetry, resulting in EC retraction from low-flow branches and subsequent incorporation into high-flow segments (Chen et al., 2012; Franco et al., 2015; Kochhan et al., 2013; Lenard et al., 2015). Time-lapse microscopy analysis of cultured mouse embryos similarly revealed directed EC migration from low-flow to high- and pulsatile-flow vessels (Udan et al., 2013). The direction of flow-induced polarized EC migration during vessel regression is determined by the axial polarity between EC Golgi and nucleus (Franco et al., 2015). In the zebrafish midbrain, flow-regulated EC migration is Rac dependent and involves subsequent EC integration in the remaining vasculature (Chen et al., 2012). Similarly, flow is sensed by EC in the zebrafish eye and controls EC rearrangements, migration, and apoptosis, resulting in pruning of low-flow vessels (Kochhan et al., 2013).

The presence or absence of flow has also been shown to determine the mode of pruning and particularly the sequence of lumen collapse and EC rearrangements. Lumen collapse advances EC rearrangements during type I pruning in the absence of flow. These cell rearrangements lead to the formation of a unicellular connection, which subsequently resolves via regression of the last linking cell. In contrast, type II pruning starts in perfused vessels with EC rearrangements, and the lumen is maintained until the last EC forms a transcellular lumen that eventually collapses via self-fusion to form two separate luminal compartments (Lenard et al., 2015). In conclusion, blood flow is...
an essential determinant of EC survival and migration during regressive vessel remodeling.

**Regulation of Vessel Pruning and Regression by Oxygenation and VEGF Signaling**

Similar to sprouting angiogenesis, vessel regression is controlled by tissue oxygenation. Wide capillary-free spaces surround large $\text{O}_2$-rich blood-carrying retinal arteries (Claxton and Fruttiger, 2003). Hypoxia leads to VEGF downregulation, causing massive obliteration of central retinal capillaries (Alon et al., 1995; Claxton and Fruttiger, 2003). Along these lines, astrocyte-specific VEGF deletion resulted in massive vessel regression in the retina in response to hyperoxia (Scott et al., 2010). However, VEGF does not completely rescue hypoxia-induced vessel regression, and other pathways activated by FGF2, ANG2, PDGF, and DLL4 have been described to control VEGF-independent vessel stabilization (Augustin et al., 2009; Hou et al., 2010; Lieu et al., 2011; Lobov et al., 2011). In contrast, hypoxia incubation of pups as well as intravitreal VEGF injection interfered with retinal vessel regression by inducing endothelial phosphoinositide 3-kinase (PI3K)/AKT survival signaling. High $\text{O}_2$ levels following primary plexus formation lead to reduced VEGF levels, thereby inducing EC apoptosis and vessel regression (Figure 3) (Alon et al., 1995; Baffert et al., 2006; Claxton and Fruttiger, 2003).

The involvement of VEGF signaling in vascular regression has also been studied in the trachea angiogenesis model as well as in Lewis lung carcinoma (LLC), rat insulin promoter-SV40 large T-antigen 2 (RIP-Tag2), and xenograft tumor models. Inhibition of VEGF-A signaling in these models or withdrawal of VEGF-A overexpression stimulated vessel regression by promoting cessation of blood flow and EC apoptosis (Baffert et al., 2006; Baluk et al., 2004; Benjamin et al., 1999; Inai et al., 2004). Whereas the surviving vessels were VEGF independent with little branching and uniform caliber, dying VEGF-dependent ECs detached from their basement membrane. The remaining ebms served as scaffolds for vascular regrowth after termination of treatment. In RIP-Tag2 and LLC tumor models, two subsequent courses of VEGF inhibition resulted in two rounds of vascular regression and regrowth along the ebms, demonstrating the plasticity of the regression process (Baffert et al., 2006; Inai et al., 2004; Mancuso et al., 2006).

Repetitive rounds of vessel formation and regression have also been observed in the corpus luteum and during the mammary gland reproductive cycle, which are paralleled by increases and decreases of VEGF production (Modlich et al., 1996). Hyaloid and pupillary membrane vessel (PM) stability also depends on VEGF-mediated survival signaling. Notably, the second phase of synchronous apoptosis during PM vessel regression results from the lack of VEGF in non-perfused and constricted vessels (Meeson et al., 1999; Mitchell et al., 1998). In conclusion, high $\text{O}_2$-tension-controlled VEGF downregulation promotes EC apoptosis, thereby inducing vessel regression and formation of ebms, which may serve as scaffold for vascular regrowth.

**WNT and Notch Signaling Decide Whether to “Make or Break a Vessel”**

Beyond VEGF, Notch and WNT signaling orchestrate vessel pruning and regression (Figure 3) (Korn et al., 2014; Lobov et al., 2011; Phng et al., 2009). WNT ligands signal via Frizzled (FZD) receptors to regulate EC fate determination, migration, proliferation, and survival by activating canonical and non-canonical downstream cascades, including planar cell polarity (PCP) and WNT/Ca$^{2+}$ signaling (Clevers and Nusse, 2012).

Developmental vascular remodeling critically depends on precisely controlled levels of canonical WNT signaling, as overexpression as well as loss of endothelial ß-catenin cause embryonic lethality around E12.5 (Cattelino et al., 2003; Corada et al., 2010). Deletion of several other WNT signaling components including Wnt2, Wnt4, Fzd4, Fzd5, Rspo3, and Wnt7b similarly resulted in reduced embryonic and extraembryonic vascularization, often correlating with remodeling defects (Ishikawa et al., 2001; Kazanskaya et al., 2008; Monkley et al., 1996; Shu et al., 2002).

WNT/ß-catenin signaling is also critical to stabilize postnatal retinal vessels, as loss of endothelial ß-catenin or Lef1 reduces retinal vessel density, interferes with stalk cell proliferation, and induces premature vessel regression (Phng et al., 2009). Likewise, the EC transcription factor ERG transactivates Fzd4 and Cdh5 expression to induce WNT/ß-catenin signaling, supporting EC proliferation and survival (Birdsey et al., 2015). Canonical WNT signaling-dependent retinal vessel stabilization can also occur independent of WNT ligands via Norrin and TSPAN12-induced Fzd4/LRP5 complex clustering. Along these lines, several WNT signaling mutants including endothelial FZD4-, LRP5-, NDP-, and TSPAN12-deficient mice are characterized by the absence or reduced density of intraretinal and inner ear vessels (Ye et al., 2010).

Besides the canonical WNT signaling pathway, EC-derived non-canonical WNT ligands control vessel pruning and regression by stimulating EC survival and proliferation. Endothelial deletion of the WNT secretion factor Evi/Wls resulted in increased retinal vessel regression. Reduced EC proliferation and increased EC apoptosis correlated with Sta2 and Cdkn1a up- and Tie2 downregulation (Korn et al., 2014). Non-canonical WNT ligand-induced Tie2 expression was also important for survival signaling in cultured ECs and promoted pulmonary vessel stabilization in vivo (Cornett et al., 2013; Masckauchán et al., 2006). The reduced Matrigel plug vascularization in endothelial Evi/Wls-deleted mice was rescued by introducing non-canonical WNT5A, further supporting a role for non-canonical WNT ligands in blood vessel stabilization (Korn et al., 2014).

Shear stress and non-canonical WNT signaling intersect in the regulation of EC survival and cell polarity (Cirone et al., 2008; Lizama and Zovein, 2013). During lymphatic valve remodeling, shear stress enhances nuclear factor of activated T cells (NFAT) signaling in lymphatic ECs, and NFATc3/NFATc4- and calcineurinB-deficient mice are embryonic lethal due to disturbed vessel remodeling (Graef et al., 2001; Sabine et al., 2012). Shear stress also upregulates NFAT target genes in EC and increases endothelial Ca$^{2+}$/CaMKII/eNOS signaling (Nauli et al., 2008; Topper et al., 1996). Moreover, shear stress-sensitive Ca$^{2+}$-channels control downstream ERK/eNOS signaling, and mice lacking these channels are characterized by remodeling defects (Ando and Yamamoto, 2013). Shear stress has also been proposed as upstream regulator of endothelial PCP signaling, controlling microtubule and actin organization to promote EC alignment and division relative to the direction of flow.
vagal signaling, WNT7B stimulates ECs to exit the cell cycle via WNT signaling. In the absence of ANG1-mediated AKT/PI3K survival signaling, WNT7B stimulates macrophage WNT7B production, thereby promoting vessel destabilization and regression. Canonical WNT7B signaling promotes regression of the hyaloid vasculature. In contrast to intraretinal and embryonic vessels, WNT signaling promotes regression of the hyaloid vasculature. ANG2 stimulates macrophage WNT7B production, thereby driving hyaloid ECs into cell cycle via activation of canonical WNT signaling. In the absence of ANG1-mediated AKT/PI3K survival signaling, WNT7B stimulates ECs to exit the cell cycle via apoptosis, resulting in hyaloid vessel regression (Figure 4) (Rao et al., 2007). A tight crosstalk between the ANG/TIE and WNT pathways is also supported by the persistence of hyaloid vessels in various mouse models lacking WNT or ANG/TIE components (Gale et al., 2002; Lobov et al., 2005; Rao et al., 2007).

These apparently opposing effects of WNT signaling on retinal and hyaloid vessels may reflect differences in the microenvironment or in the dynamics of cellular processes. While intraretinal vessels are committed to grow during postnatal development (i.e., vascular pruning), hyaloid vessels are programmed to regress (i.e., bona fide vascular regression). Similarly, radial glia cells impair vessel regression by interfering with endothelial WNT/β-catenin signaling, although radial glia-derived WNT7A and WNT7B support vessel growth early in development (Corada et al., 2010; Ma et al., 2013; Stenman et al., 2008). As such, WNT signaling can intriguingly have distinct and even opposing context-dependent effects at different stages of development and/or in different tissues or vascular beds.

Postnatal retinal vessel stabilization critically depends on the crosstalk of the WNT/β-catenin pathway with the Delta-like 4 (DLL4)/Notch pathway (Benedito and Hellström, 2013). DLL4/Notch signaling, which controls tip cell selection, also induces Notch-regulated ankyrin repeat protein (Nrarp) expression to promote LEF1-dependent canonical WNT signaling and vessel stabilization. Loss of Nrarp in mice phenocopies the premature regression phenotype of Left1- or endothelial β-catenin-deficient mice, and nrarp knockdown in zebrafish causes aberrant regression of intersegmental vessels. At the same time, NRARP limits Notch signaling and interferes with p21/Rb-dependent cell-cycle arrest. The reduced EC proliferation in an endothelial Nrarp-depleted environment may also trigger vascular remodeling as a more general mechanism to adapt the vascular network to reduced EC numbers (Phng et al., 2009).

DLL4/Notch signaling stimulates vessel pruning and regression by shifting the expression of vasoactive genes toward a vasoconstrictive phenotype, thereby inducing vessel occlusion, cessation of blood flow, and subsequent EC apoptosis (Lobov et al., 2007, 2011). In the retina, Notch signaling is particularly important for vein and perivenous capillary plexus remodeling (Ehling et al., 2013). In conclusion, the balance of WNT, Notch, and ANG/TIE signaling seems to balance vessel formation and regression.

**ANG/TIE Signaling Controls Vessel Pruning and Regression**

The ANG/TIE signaling system is a key player of developmental vessel formation and remodeling and acts as gatekeeper of quiescence in the adult resting endothelium (Figure 3). ANG1 is the constitutive ligand of the TIE2 receptor, and ANG1/TIE2 signaling induces PI3K/AKT-dependent EC survival, mural cell recruitment, and EC quiescence (Augustin et al., 2009). ANG2, which is mainly produced by ECs, has originally been identified as autocrine ANG1/TIE2 antagonist disrupting PI3K/AKT survival signaling, resulting in vessel destabilization and regression (Cao et al., 2007; Gale et al., 2002). In situations of high ANG2 levels,
as for instance in stressed endothelia, the tumor vasculature, and at the tip cell front. ANG2 may act as context-dependent ANG1/TIE2 agonist. Both agonistic and antagonistic functions of ANG2 are important to maintain vessel plasticity (Daly et al., 2013; Hashizume et al., 2010; Kim et al., 2000).

Another modulator of the ANG1/TIE2 signaling system is the orphan receptor TIE1. Tie1-deficient mice have an embryonic-lethal phenotype characterized by vascular defects around E13.5–E14.5 (Puri et al., 1995). TIE1 controls endothelial PI3K/AKT survival signaling in a blood flow-dependent manner and thereby interferes with regression of the stalk cell vasculature (Kontos et al., 2002). TIE1 expression is downregulated by laminar flow and is particularly responsive to acute changes in shear stress, supporting a role of TIE1 in blood flow-controlled vessel remodeling (Porat et al., 2004; Woo et al., 2011).

As regulator of EC survival and migration, ANG/TIE signaling may be critically involved in the control of vascular pruning. This hypothesis is supported by the midgestational lethal phenotype of Ang1- and Tie2-deficient mice showing a persisting capillary plexus that fails to remodel (Dumont et al., 1994; Suri et al., 1996). Conditional deletion of Ang1 in cardiomyocytes phenocopies constitutive Ang1 deletion, suggesting that ANG1-dependent vessel remodeling requires proper heart function and may, thus, be blood flow dependent (Jeansson et al., 2011).

Ang2-deficient mice have a mild vascular phenotype, but global overexpression of Ang2 phenocopies the effects of Ang1/Tie2 deletion favoring an antagonistic role of ANG2 in controlling the constitutive ANG1/TIE2 axis during developmental vessel remodeling (Cao et al., 2007; Gale et al., 2002; Hackett et al., 2002). Despite the absence of an embryonic vascular phenotype, Ang2-deficient mice have a reduced and disorganized postnatal retinal vasculature. The lack of intraretinal capillaries in Ang2-deficient and similarly Akt gain-of-function mice correlated with impaired hyaloid vessel regression. In contrast, AKT inhibition induces EC apoptosis and stimulates hyaloid vessel regression, which can be rescued by ANG1 injection (Gale et al., 2002; Hackett et al., 2002; Rao et al., 2007).

ANG2-blocking antibodies increase remodeling of the central retinal vessels and at the same time reduce sprouting, supporting an agonistic as well as antagonistic role of ANG2 during retinal vessel remodeling (Felcht et al., 2012).

The context-dependent functions of ANG2 can largely be explained by the VEGF dependency of ANG2 function. ANG2 promotes EC death and capillary regression under VEGF-deprived conditions (e.g., in the pupillary membrane, in the hyaloid vasculature, and during corpus luteum regression). In the presence of VEGF, ANG2 supports EC migration, proliferation, and sprouting (Gale et al., 2002; Oshima et al., 2004). ANG2 is primarily detected in tissues undergoing vessel remodeling, including ovary, uterus, and placenta, emphasizing the important role of ANG2 during vessel remodeling (Hackett et al., 2002). In the ovary, ANG2 exhibits a cyclic expression pattern correlating with waves of vessel regression during follicle atresia and corpus luteum regression (Goede et al., 1998). Increased placental vascular remodeling during the first trimester of pregnancy is accompanied by elevated ANG2 levels, and Ang2 overexpression interfered with collateral vessel growth after hindlimb ischemia (Reiss et al., 2007). Moreover, ANG2 is implicated in trophoblast vessel remodeling by interfering with TIE2 activation during chronic airway inflammation (Tabruyn et al., 2010).

Several regulators of vessel remodeling, including shear stress, hypoxia, and VEGF, control ANG2 transcription, demonstrating the extensive crosstalk between the signaling pathways controlling vascular pruning. For instance, the shear stress-regulated transcription factor KLF2 downregulates Ang2 and upregulates Tie2, thereby maintaining the quiescent phenotype after completion of the remodeling process (Augustin et al., 2009). In conclusion, ANG/TIE signaling is a pivotal system for balancing vascular remodeling and angiogenic vessel formation in a context-dependent manner.

Additional Signaling Pathways Involved in Vessel Regression
Beyond the established angiogenesis signaling pathways involving VEGF, ANG/TIE, Notch, and WNT signaling, some additional mechanisms have been reported to control vessel pruning and regression. The GEF FGD5 acts as regulator of vessel regression in the postnatal retina. Endothelial FGD5 induces HEY/p53 signaling and promotes VEGF sequestration by increasing the VEGFR1/VEGFR2 ratio, resulting in EC apoptosis and subsequent vessel regression (Cheng et al., 2012). Moreover, CRIM1, which stimulates autocrine VEGF-A signaling, stabilizes retinal vessels and interferes with regression in the postnatal retina (Fan et al., 2014). Vascular regression during dermal wound healing involves CXCL10 binding to endothelial CXCR3, resulting in disruption of critical integrin contacts, EC detachment, and apoptosis (Bodnar et al., 2009). Remodeling of the extracellular matrix (ECM) is spatiotemporally linked to vessel regression, as cleavage of EC/ECM integrin contacts and release of bioactive ECM fragments critically influence EC survival signaling (Wietecha et al., 2013).

More recently, light has been described as the initial trigger of apoptosis-dependent hyaloid vessel regression and retinal vessel growth inhibition. Light stimulation during late gestation activates intrinsically photosensitive retinal ganglion cells to produce melanopsin, which reduces the number of retinal neurons, thereby limiting tissue hypoxia and VEGF production. Reduced VEGF levels trigger EC apoptosis and suppress retinal vessel growth (Rao et al., 2013). Along these lines, a higher average day length during early gestation was associated with a lower risk for severe ROP (Yang et al., 2013).

The Fate of Pericytes during Vessel Pruning and Regression
The role of pericytes as stabilizers of maturing vessels is well established. Yet their role during vessel regression is rather controversial (Figure 4). It has not been clarified if pericytes stay attached to ebms, undergo apoptosis, or migrate onto surviving vessels. Not only their fate but also pericyte-derived signals controlling vessel remodeling have not been identified. Early studies showed that disruption of endothelial-pericyte associations during hyperoxia treatment of mice resulted in excessive retinal vessel regression and abnormal remodeling (Benjamin et al., 1999). Along these lines, VEGF inhibition-induced vessel regression primarily affects vessels with reduced pericyte coverage, supporting a role of pericytes in stabilizing blood vessels (Benjamin et al., 1999). However, the combination of VEGF
inhibition and PDGFβ receptor targeting did not improve the sensitivity of tumors to anti-VEGF drugs, suggesting that anti-VEGF-induced vessel normalization and regression are independent of pericytes (Nisancioglu et al., 2010). The presence of pericytes was also not sufficient to interfere with VEGF/PDGFR inhibition-induced vessel regression in trachea and in LLC and RIP-Tag2 tumor models. Pericytes temporarily persisted in ebms, but the majority of the pericytes migrated to surviving capillaries and some underwent apoptosis (Baffert et al., 2006; Baluk et al., 2004; Inai et al., 2004).

Pericyte apoptosis in the course of vessel regression has also been observed during regression of the pupillary membrane and hyaloid vasculature (Taniguchi et al., 1999). In contrast, pericyte numbers did not change during several rounds of tumor vessel regression and regrowth. Only the expression of the pericycle marker αSMA was downregulated during regression and returned to baseline upon vessel regrowth (Mancuso et al., 2006). Persistence of pericytes along ebms after Sorafenib-induced tumor vessel regression was also observed in an inducible mouse model of hepatocellular carcinoma (Runge et al., 2014). Although no changes in the pericycle coverage of remaining vessels were observed under conditions of enhanced retinal vessel regression, pericytes have recently been reported to stay attached to ebms of regressed retinal vessels (Franco et al., 2015; Korn et al., 2014; Phng et al., 2009). Further studies are required to investigate the fate of pericytes and their active contribution to vessel regression.

**Role of Inflammatory Cells during Vessel Pruning and Regression**

As during sprouting angiogenesis, macrophages are located close to regressing vessels, where they are believed to play active as well as passive roles (Baluk et al., 2004; Ito and Yoshikawa, 1999). PU.1 mutant mice lacking resident macrophages have persistent hyaloid and pupillary membrane (PM) vessels, supporting a role for macrophages in the regression of these vascular beds (Lobov et al., 2005). Macrophages were initially reported to trigger EC apoptosis in a cell-cell contact-dependent manner and mediate the passive clearance of cell debris (Lang et al., 1994; Meeson et al., 1998; Mitchell et al., 1998). Recently, PM macrophages were described to phagocytose shed EC membrane particles long before PM regression is observed, thereby facilitating the subsequent regression process (Poché et al., 2015). Macrophages provide WNT ligands to induce hyaloid EC apoptosis in an ANG/TIE-dependent manner (Lobov et al., 2005; Rao et al., 2007). They were also detected next to regressing retinal vessels and their numbers increased during ischemic retinopathy (Shen et al., 2007). Anti-VEGF/VEGFR-induced remodeling of the trachea vasculature involves macrophage-mediated passive clearance of apoptotic ECs (Baluk et al., 2004). Similar to macrophages, leukocytes and cytotoxic T cells are capable of inducing EC apoptosis during physiological and hyperoxia-induced retinal vessel pruning (Ishida et al., 2003).

Taken together, immune cells such as macrophages and leukocytes are involved in vessel pruning and regression (Figure 4), but further research is required to establish a more detailed view of their precise contribution to the different forms of vessel regression in various vascular beds.

**Vessel Regression during Tumor Angiogenesis**

The tumor vasculature has an abnormal and chaotic structure characterized by tortuous and leaky vessels that lack hierarchical organization and arterio-venous identity (Carmeliet and Jain, 2011). Therefore, it is questionable if the tumor vasculature undergoes organized morphological processes as they occur during physiological sprouting, maturation, and remodeling. In particular, the process of vessel remodeling involving complex morphological rearrangements and regulation by a plethora of different factors may be abnormal in the tumor vasculature. For instance, blood flow, an important regulator of physiological vessel remodeling, is irregular and compromised in the tumor due to the tortuous and leaky vessel organization. Moreover, signaling mediators involved in the control of vessel formation and remodeling including VEGF and ANG2 are upregulated in the tumor environment and, thus, might rather promote chaotic vessel growth (Carmeliet and Jain, 2011; Gerald et al., 2013).

Anti-angiogenic tumor therapy was originally pioneered with the aim to starve tumors to death by stimulating massive tumor vessel regression (Folkman, 1971). Anti-VEGF/VEGFR treatments target immature vessels for EC apoptosis-dependent regression. At the same time, such treatments induce vessel normalization and stabilize mature VEGF-resistant vessels, thereby improving tumor perfusion and facilitating chemotherapy (Carmeliet and Jain, 2011). As in physiological remodeling processes, anti-angiogenic targeting most likely involves vessel remodeling as consequence of EC apoptosis (regression), but also as result of EC rearrangements and migration (normalization).

Ongoing research is aimed at improving the hitherto limited efficacy of anti-angiogenic intervention by developing therapeutic strategies that precisely balance vessel regression and normalization. On the one hand, tumor starvation needs to be promoted, and on the other hand, drug delivery should be facilitated. Toward this end anti-ANG2 treatments are in advanced clinical development. Such treatments appear to be of limited efficacy as monotherapy, but show improved efficacy in combination with anti-VEGF/VEGFR regimens (Daly et al., 2013; Gerald et al., 2013). Canonical WNT signaling has also been described to induce vessel normalization by inducing pericyte recruitment and may similarly be a promising target for future combination therapies (Reis et al., 2012).

**Role of Deregulated Vessel Pruning and Regression in Disease Development**

A number of diseases are directly or indirectly caused by perturbed microvascular remodeling and/or aberrant vessel pruning and regression. Only some prominent examples can be discussed here. Notably, retinal hypovascularization diseases such as diabetic retinopathy, age-related macular degeneration (AMD), ROP, diabetic retinopathy (DR), Norrie disease (ND), familial exudative vitreoretinopathy (FEVR), and Coats disease are the globally leading causes of blindness. These disorders involve the failure of hyaloid vessel regression or reduced vascularization of the retina, resulting in subsequent neovascularization. Whereas the pathophysiology of these diseases is similar, the underlying causes are different (Jager et al., 2008; Ye et al., 2010). Vessel obliteration in ROP is induced by exposure of
premature newborns to high O₂ concentrations, and AMD develops as consequence of age-related defective retina vascularization (Chen and Smith, 2007; Jager et al., 2008). Destruction of retinal vessels in DR involves hyperglycemia, oxidative stress, advanced glycation endproduct formation, and hemodynamic changes (Tarr et al., 2013). In contrast, ND, FEVR, and Coats disease are hereditary disorders characterized by X-linked NDP mutations and in the case of FEVR also autosomal FZD4, LRP5, and TSPAN12 mutations (Ye et al., 2010). Thus, mutations in WNT signaling mediators are responsible for impaired retinal vascularization, pointing to the importance of WNT signaling for vessel stabilization.

Alzheimer’s disease involves brain hypoperfusion, which is caused by vascular risk factors or cerebrovascular disorders including stroke and ischemia, results in downregulation of VEGF expression. Subsequent vessel regression induces hypoxia and accumulation of neurotoxins including amyloid-β-peptide, which form oligomers that are anti-angiogenic and cause cerebral β-amyloidosis (Sagare et al., 2012). Perfusion deficits also cause other neurodegenerative disorders by decreasing VEGF expression (Quaggebeur et al., 2011). The involvement of vessel pruning and regression in multiple diseases highlights the importance of improving the mechanistic understanding of these processes under physiological as well as pathological conditions.

**Future Perspective**

Although morphological rearrangements during regressive vessel remodeling as well as contributing signaling pathways have been investigated to some extent, further research is required to improve our understanding of physiological as well as pathological vessel pruning and regression. Most notably, the contribution of EC migration and apoptosis to vessel pruning and regression in different vascular beds and under different conditions needs further study. Does the involvement of apoptosis in vessel regression depend on the extent of vascular regression? Is apoptosis more prominent in regression processes resulting in drastic reduction of network complexity? Improved in vivo systems suitable for high-resolution live imaging, taking the dynamics of the pruning process into account, are required to unravel the fate of ECs during vessel pruning and regression. The design of novel techniques should particularly aim at deciphering the contribution of EC proliferation/angiogenesis/maturation, vascular constriction/dilation, and cellular extension/regression to the remodeling process. The role of bystander cells including pericytes, neural cells, and macrophages and signals provided by these cells for controlling vessel remodeling are still terra incognita and require further investigation. Although vessel pruning and regression have been described in a multitude of disease processes, the exact morphological rearrangements and involved signaling pathways are still poorly understood. Do physiological and pathological pruning and regression follow the same mechanistic principles? Understanding the contribution of EC migration, rearrangement, and survival to vessel pruning and regression in the chauropic tumor vasculature is of particular importance to improve the efficacy of anti-angiogenic therapy and other forms of angiomanipulative therapies.

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