Angiogenesis

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Extracellular matrix (ECM) is essential for all stages of angiogenesis. In the adult, angiogenesis begins with endothelial cell (EC) activation, degradation of vascular basement membrane, and vascular sprouting within interstitial matrix. During this sprouting phase, ECM binding to integrins provides critical signaling support for EC proliferation, survival, and migration. ECM also signals the EC cytoskeleton to initiate blood vessel morphogenesis. Dynamic remodeling of ECM, particularly by membrane-type matrix metalloproteases (MT-MMPs), coordinates formation of vascular tubes with lumens and provides guidance tunnels for pericytes that assist ECs in the assembly of vascular basement membrane. ECM also provides a binding scaffold for a variety of cytokines that exert essential signaling functions during angiogenesis. In the embryo, ECM is equally critical for angiogenesis and vessel stabilization, although there are likely important distinctions from the adult because of differences in composition and abundance of specific ECM components.

The extracellular matrix (ECM) provides a critical framework for angiogenesis through structural support and also by conferring molecular signals essential for all stages of blood vessel formation, including vascular sprouting, lumen formation, vessel maturation, and ultimately vessel stabilization. In addition, ECM provides an immobilizing scaffold for cytokines that are important for angiogenesis. Thus, by providing basic structural support, direct signaling functions, and scaffolding for cytokines, ECM exerts fundamental control over angiogenesis. Moreover, the established functional complexity of ECM together with known mechanisms available for dynamic ECM remodeling

suggest that ECM is capable of exerting precise control over all aspects of angiogenesis and blood vessel maturation.

This article is divided into four parts: (1) Overview of angiogenesis and ECM in the adult; (2) key functions of ECM during angiogenesis; (3) distinctions between the composition of embryonic and adult ECMs, including implications for the involvement of specific integrins in angiogenesis; and (4) ECM remodeling during vascular tube formation and stabilization. The scientific literature on ECM and angiogenesis is vast, and it is therefore impossible to cover this topic completely in a single article. Thus, rather than striving for an exhaustive review of

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the literature, we have sought to provide an overview and also provide specific examples of ECM function in angiogenesis. In particular, we have focused on the importance of ECM in vascular morphogenesis.

OVERVIEW OF ANGIOGENESIS AND ECM IN THE ADULT

Normally, in the adult, quiescent blood vessels are covered on the abluminal (basal) surface with a continuous basement membrane consisting primarily of laminins, collagen type IV, nidogens, and the heparan sulfate proteoglycan, perlecan (Hayashi et al. 1992; Hallmann et al. 2005; Bix and Iozzo 2008). However, during the earliest stages of angiogenesis, such as in response to the angiogenic cytokine VEGF induced by wounding and ischemia, vascular basement membrane is degraded (Sundberg et al. 2001; Rowe and Weiss 2008; Chang et al. 2009). Following disruption of basement membrane, and with the ensuing stage known as vascular sprouting (Nicosia and Madri 1987), vessels become leaky and hyperpermeable to blood plasma proteins (Sundberg et al. 2001). This vascular hyperpermeability causes leakage of the ECM proteins fibrinogen, vitronectin, and fibronectin from the blood (Senger 1996; Sundberg et al. 2001). Fibrinogen is subsequently converted to fibrin through enzymatic coagulation, and together with extravasated vitronectin and fibronectin instantly transform the interstitial collagen matrix to form a new, provisional ECM. Thus, the early stages of sprouting angiogenesis are generally believed to proceed in an environment rich in preexisting interstitial collagens in combination with fibrin, vitronectin, and fibronectin derived from the blood plasma. As vascular morphogenesis proceeds and vascular sprouts acquire lumens and mature, neovessels are again enshrouded in vascular basement membrane with associated pericytes and thereby achieve stability (Grant and Kleinman 1997; Benjamin et al. 1999). Recent studies show that pericyte recruitment to vascular tubes directly controls this basement membrane assembly step in vitro and in vivo (Stratman et al. 2009a, 2010). Thus,

in response to stimulation with angiogenic cytokines, angiogenesis in the adult is generally believed to proceed through the following basic stages: (1) degradation of vascular basement membrane and activation of quiescent endothelial cells (ECs); (2) sprouting and proliferation of ECs within provisional ECM; (3) lumen formation within the vascular sprouts, thereby creating vascular tubes; and (4) coverage of vascular tubes with mature vascular basement membrane in association with supporting pericytes.

It seems logical to expect that vascular basement membrane components associated with normal blood vessel quiescence and stability might directly function in this capacity; and observations in vivo (Risau and Lemmon 1988) together with investigations in vitro, do indeed support a role for basement membrane in conferring vessel stabilization and vascular barrier integrity (Bonanno et al. 2000; Liu and Senger 2004; Stratman et al. 2009a) (see sections Key Functions of ECM during Angiogenesis, and ECM Remodeling during Vascular Tube Formation and Stabilization). Similarly, it seems logical that components of provisional ECM would serve to support the sprouting and lumen-forming stages of angiogenesis, and there is considerable evidence that provisional ECM does indeed serve this role. Although there are distinctly different components of provisional ECM, there is abundant evidence that each of the major components (i.e., interstitial collagens, fibrin, fibronectin, and vitronectin) support EC proliferation and migration (see section Key Functions of ECM during Angiogenesis). Moreover, there is strong evidence that interstitial collagen and fibrin each support key stages of vascular morphogenesis, including cord and lumen formation (see sections Key Functions of ECM during Angiogenesis, and ECM Remodeling during Vascular Tube Formation and Stabilization).

Thus, a basic model for framing the complex roles of ECM in adult angiogenesis is to envisage the process beginning with ECs residing on a vessel-stabilizing vascular basement membrane ECM, followed by cytokine-initiated exposure of ECs to a provisional ECM that

favors sprouting and lumen formation within that provisional ECM, followed by transitioning of ECs again to interactions with vessel-stabilizing vascular basement membrane. Although undoubtedly oversimplified, the fundamentals of this model are well supported by currently available evidence. Moreover, the basic complexity of ECM (i.e., the large number of distinctly different matrix proteins), combined with additional complexity provided by proteolytic remodeling that generates cell-guidance pathways (see section ECM Remodeling during Vascular Tube Formation and Stabilization) as well as matrix fragments with angiostatic functions (reviewed in Sund et al. 2004; Bix and Iozzo 2005; see also Lu et al. 2011), is well matched to the biological complexity of angiogenesis.

KEY FUNCTIONS OF ECM DURING ANGIOGENESIS

Adhesion to ECM Regulates Endothelial Cell (EC) Proliferation, Survival, and Migration

At the most fundamental level, angiogenesis requires EC activation, proliferation, and survival. Angiogenic cytokines are most generally credited with driving EC proliferation and supporting EC survival; however, EC adhesion to ECM through cell-surface integrins is equally critical (Akiyama et al. 1989; Wary et al. 1996; Meredith and Schwartz 1997; Giancotti and Ruoslahti 1999). In particular, EC adhesion to ECM through integrins is required for efficient cytokine activation of the Erk1/Erk2 MAP kinase signaling pathway (Short et al. 1998; Aplin et al. 1999), and activation of this pathway is necessary for EC proliferation and angiogenesis (Seeger and Krebs 1995; Vinals and Pouyssegur 1999; Roovers and Assoian 2000; Assoian and Schwartz 2001). Adhesion-dependent activation of the Erk1/Erk2 MAP kinase pathway also functions critically in supporting EC survival by suppressing apoptosis (Ilan et al. 1998; Aoudjit and Vuori 2001; Perruzzi et al. 2003). Furthermore, the expression and activities of cyclin-dependent kinases, which are

required for cell cycle progression and therefore for EC proliferation, are also dependent on EC adhesion to ECM (Fang et al. 1996; Zhu et al. 1996; Assoian 1997). Thus, without adhesion to ECM, EC proliferation ceases and apoptosis is induced, underscoring the fundamental importance of ECM for angiogenesis.

Vascular ECs also require adhesion to ECM for migration; and EC migration is essential for sprouting of new blood vessels from the existing vasculature (Ausprunk and Folkman 1977). Angiogenic cytokines stimulate migration, but such motility is strictly dependent on EC adhesion to ECM. Moreover, evidence from in vitro experiments indicates that many of the interstitial and provisional ECM components that are encountered during sprouting angiogenesis, including fibrin and collagen I, are capable of supporting cytokine-stimulated migration (Dejana et al. 1985; Nicosia and Madri 1987; van Hinsbergh et al. 2001; Senger et al. 2002). Additionally, gradients of immobilized ECM components can by themselves drive haptotactic migration in vitro, independently of cytokines (Senger and Perruzzi 1996; Senger et al. 2002). Although the significance of haptotactic, ECM-driven migration in vivo lacks direct confirmation, it seems plausible that the high concentrations of provisional ECM encountered by ECs during the sprouting phase of angiogenesis may drive outward migration through this mechanism. Thus, sprouting ECs may migrate in response to both gradients of angiogenic cytokine (chemotaxis) and ECM (haptotaxis). Regardless, all EC motility, including random migration, is absolutely dependent on EC adhesion to ECM.

Although the general importance of ECM for EC migration, proliferation, and survival is unequivocal, the relative importance of various ECM components in supporting these processes is less clear, often because of functional overlap among different matrix proteins. For example, cytokine activation of the MAP kinase pathway in microvascular ECs and proliferation of microvascular ECs are similarly supported by attachment to either collagen I or vitronectin (Perruzzi et al. 2003). Moreover, a variety of ECM components support EC migration

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(Dejana et al. 1985; Nicosia and Madri 1987; Senger and Perruzzi 1996; van Hinsbergh et al. 2001; Senger et al. 2002), although perhaps not with equal potency (Senger and Perruzzi 1996). There is also evidence that ECM shows maximal activity in promoting EC survival when multiple ECM components are present, indicating that different components of ECM may function cooperatively (Perruzzi et al. 2003).

ECM Function in Formation of Vascular Cords, the Precursors to Vascular Tubes with Lumens

In addition to proliferation and migration, ECs must collectively undergo morphogenesis to form new blood vessels. In the embryo, where this process has been studied extensively, the earliest stages of vascular morphogenesis involve transition of endothelial precursor cells to a spindle-shaped morphology (Drake and Little 1999) together with alignment into solid, multicellular, cord-like structures that form integrated polygonal networks (Vernon and Sage 1995; Drake et al. 1997). These cord-like structures also have been identified during angiogenesis in the adult (Aloisi and Schiaffino 1971). During maturation, the solid vascular cords form hollow lumens, and the ECs are sequestered from the interstitial matrix through establishment of a continuous basal lamina (Aloisi and Schiaffino 1971; Drake et al. 1997), a process that is stimulated by EC-pericyte interactions (Stratman et al. 2009a).

The ECM is critical for morphogenesis of new blood vessels at several levels. First, ECM serves as a three-dimensional malleable scaffold through which individual ECs and clusters of ECs can transduce tensional forces to other ECs at a considerable distance without direct cell–cell contact. Thus, by generating tension-based guidance pathways within ECM, ECs are able to link up and form multicellular cords (Davis and Camarillo 1995; Vernon and Sage 1995). In addition, ECM can exert important signaling functions directly to regulate EC shape and morphogenesis. For example, three-dimensional interstitial collagen I stimulates

ECs in vitro to assume a spindle-shaped morphology and to align into cords similarly to those observed during angiogenesis in vivo (Delvos et al. 1982; Montesano et al. 1983; Jackson et al. 1994; Richard et al. 1998; Sweeney et al. 1998; Whelan and Senger 2003). Within collagen I gels, these cords mature to form tubes with hollow lumens through formation and coalescence of intracellular vacuoles (Davis and Camarillo 1996) and expansion of the luminal compartment through ECM proteolysis (Chun et al. 2004; Saunders et al. 2006; Stratman et al. 2009b). Importantly, there is considerable evidence that interactions between interstitial collagens and ECs are highly relevant in vivo. As discussed above, sprouting ECs migrate and proliferate within a provisional matrix rich in interstitial collagens (Paku and Paweletz 1991; Sundberg et al. 2001). In addition, ECs isolated from tumors express >10-fold more transcripts encoding interstitial collagens type I and III than ECs isolated from corresponding control tissue (St Croix et al. 2000), thus suggesting that interstitial collagen expression by tumor ECs is conducive for angiogenesis. In further support of this hypothesis, expression of collagen I by isolated EC clones in vitro correlates with spontaneous multicellular organization of these ECs into cords (Fouser et al. 1991; Iruela-Arispe et al. 1991). Finally, proline analogs that interfere with collagen triple helix assembly and β -aminopropionitrile, that inhibits collagen cross-linking, each inhibit neovascularization in animal models (Ingber and Folkman 1988), providing further evidence that collagens play a crucial role in angiogenesis.

The mechanism through which collagen I provokes ECs to form vascular cords requires direct interactions between collagen I and cell-surface integrins. Upon ligation of integrins, collagen I induces actin stress fibers through multiple signaling pathways, including activation of Rho and Src (Liu and Senger 2004) and p38 MAP kinase (Sweeney et al. 2003), suppression of cyclic AMP (Whelan and Senger 2003), and suppression of Rac activity (Liu and Senger 2004). Suppression of cyclic AMP results in reduced activity of cyclic AMP-dependent

protein kinase A (PKA), causing a marked induction of actin polymerization (Whelan and Senger 2003). Coordinate activation of Rho and Src and suppression of Rac activity by collagen I organize polymerized actin into stress fibers, thereby driving EC contractility, transition to spindle-shaped morphology, and ultimately alignment into cords (see Fig. 1) (Liu and Senger 2004). Consistent with the importance of each of these pathways for vascular morphogenesis in vitro, cyclic AMP analogs and inhibitors of Rho, Src, and p38 MAP kinase each block collagen I-induced stress fiber and cord formation (Sweeney et al. 2003; Whelan and Senger 2003; Liu and Senger 2004). Moreover, a dominant-negative RhoA mutant blocks cord formation both in vitro and in vivo (Hoang et al. 2004).

Activation of Src and Rho and suppression of Rac activity by collagen I also disrupts VE-cadherin from intercellular junctions (Fig. 1) (Liu and Senger 2004), and loosening of cell–cell contacts is likely important for cord formation. In marked contrast to interstitial collagen I, basement membrane laminin-111 does not activate Src or Rho or suppress cyclic AMP or protein kinase A activity, nor does it induce actin stress fibers in microvascular ECs (Whelan and Senger 2003; Liu and Senger 2004). Consistent with these distinctions, laminin-111 also fails to induce cord formation or changes in EC shape. Rather, laminin-111 induces persistent activation of the GTPase Rac (Liu and Senger 2004), and such activation is highly consistent with improved endothelial barrier function and vessel maturation (Garcia et al. 2001; Wojciak-Stothard and Ridley 2002; Waschke et al. 2004). However, laminin-111 is not one of the laminin isoforms typically associated with vascular basement membranes (Hallmann et al. 2005), and therefore further investigations with vascular laminin isoforms such as laminin-411 and laminin-421 are required. These findings have suggested to us a model, whereby interstitial collagens of the provisional matrix and laminins of the vascular basement membrane differentially regulate various stages of angiogenesis. As summarized in Figure 1, degradation of the laminin-rich

basal lamina is predicted to reduce Rac activity and thereby diminish integrity of cell–cell junctions during the sprouting phase. Sprouting ECs are exposed to underlying interstitial collagens and begin to invade, resulting in activation of signaling pathways that drive changes to a spindle-shaped morphology and cord formation. Subsequently, as the newly formed capillary sprouts mature into new vessels with mature lumens, the intact laminin-rich basement membrane is reestablished, thereby sequestering ECs from interstitial collagens and reestablishing normal activation levels for signaling pathways that regulate EC stress fiber formation and barrier integrity (Fig. 1).

ECM Function in Lumen Formation

In addition to inducing formation of vascular cords, three-dimensional (3-D) collagen I induces and supports lumen formation by ECs (Fig. 2) (Davis and Camarillo 1996; Davis et al. 2007; Aplin et al. 2008; Koh et al. 2008; Iruela-Arispe and Davis 2009). Lumen formation also occurs in 3-D fibrin and plasma clots that are also representative of provisional matrix (Nicosia and Madri 1987; Bayless et al. 2000; van Hinsbergh et al. 2001; Sainson et al. 2005; Nakatsu and Hughes 2008) (see section Overview of Angiogenesis and ECM in the Adult). Lumen formation in both collagen I and fibrin is integrin- and Rho GTPase-dependent, involving the formation and coalescence of pinocytic intracellular vacuoles in conjunction with ECM proteolysis (Fig. 2) (Davis and Camarillo 1996; Bayless and Davis 2002; Davis and Bayless 2003; Kamei et al. 2006; Saunders et al. 2006; Stratman et al. 2009b). These intracellular vacuoles coalesce further to form intracellular lumens (Folkman and Haudenschild 1980; Meyer et al. 1997; Yang et al. 1999; Bayless and Davis 2002; Davis and Bayless 2003; Egginton and Gerritsen 2003; Lubarsky and Krasnow 2003) that exocytose by fusion with the plasma membrane allowing for multicellular assembly of tubes by adjacent ECs. Mechanisms that coordinate ECM proteolysis with the EC cytoskeleton to regulate lumen formation are described

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Collagen I versus laminin-1 signaling: A “fire and ice” hypothesis for understanding regulation of vascular morphogenesis by ECM

Figure 1. Signals transduced by interstitial collagen type I and laminin-1 (laminin-111) in dermal microvascular ECs, and consequences for capillary morphogenesis in vitro. In this proposed model, interstitial collagens, but not laminins, activate Src and Rho and suppress Rac and PKA activities through $\beta 1$ integrins. Such signaling results in induction of actin stress fibers, disruption of VE-cadherin, and formation of precapillary cords. In contrast, laminins activate Rac and PKA and suppress Rho activity and therefore do not provoke morphogenesis. Although laminin-1 (revised nomenclature = laminin-111) is not one of the laminin isoforms typically associated with vascular basement membranes (see text) and therefore further testing of this model requires additional investigations with vascular laminin isoforms, these marked distinctions in signaling by collagen type I (“fire”) and laminin-1 (“ice”) suggest a mechanism through which degradation of basement membrane and exposure of activated and proliferating ECs to interstitial collagens within the provisional matrix initiates morphogenesis of new capillary sprouts. (n/c) = no change. (This figure is adapted from Liu and Senger [2004] and Davis and Senger [2005] and is reprinted, with permission, from the Federation of American Societies for Experimental Bio (FASEB) ©2004 and Wolters Kluwer Health ©2005, respectively.)

Figure 2. Fundamental contribution of the ECM-integrin-cytoskeletal signaling axis to the assembly of EC cords and lumens during vasculogenesis and angiogenesis. In this schematic, two models of EC morphogenesis are illustrated. In the *left* panel, confluent human microvascular EC monolayers were overlaid with a type I collagen gel and allowed to undergo cord formation, illustrating how collagenous ECM markedly converts an EC monolayer into an interconnecting network of cords. This process is dependent on the $\alpha 1 \beta 1$ and $\alpha 2 \beta 1$ integrins and the small GTPase, RhoA. In the *right* panels, human ECs are suspended as individual cells in 3-D collagen matrices and are fixed, stained, and photographed at 8 and 48 hr of culture, illustrating intracellular vacuoles and early lumen formation at 8 hr and interconnecting networks of EC-lined tubes at 48 hr. Bar equals 50 μm . The three panels on the far *right* side are cross-sections from plastic-embedded collagen gels revealing EC intracellular vacuoles (*upper two right panels*) or EC lumens (*lower right panel*). Bar equals 25 μm . The vacuole and lumen formation process shown is completely dependent on the $\alpha 2 \beta 1$ integrin and the Cdc42 and Rac1 small GTPases. (This figure is adapted from Davis and Senger [2005] and reprinted, with permission, from Wolters Kluwer Health ©2005.)

further in section ECM Remodeling during Vascular Tube Formation and Stabilization.

ECM as a Scaffold Presenting Cytokines: Implications for Vascular Morphogenesis, Stabilization, and Maturation

ECM also serves as a scaffold for growth factors that exert fundamental control over angiogenesis. Growth-factor-containing ECM allows ECs to respond simultaneously to signals through growth factor receptors and integrins (Hynes 2009; Somanath et al. 2009; Chen et al. 2010). Such cosignaling may provide a mechanism for growth factors to exert distinctly different outcomes, depending on the particular ECM environment (e.g., collagen-rich interstitial matrices versus basement membrane matrices)

and at different stages of vascular morphogenesis and stabilization. Angiogenic cytokines typically have affinity for heparan sulfate and thereby become anchored to heparan sulfate proteoglycans (i.e., syndecans, perlecan, versican, glypicans) either on the EC surface or in the surrounding ECM (Gerhardt et al. 2003; Mitsi et al. 2008). Also, many angiogenic cytokines directly bind to angiogenesis-promoting ECM scaffolds such as collagen type I and fibrin/fibronectin matrices (Kanematsu et al. 2004). For example, VEGF binds to fibronectin (Wijelath et al. 2002; Mitsi et al. 2008), and hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF) each bind directly to collagen type I whereas BMP-4 binds collagen type IV (Ruehl et al. 2002; Rahman et al. 2005; Wang et al. 2008). Interestingly, BMP-1,

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which is a proteinase that activates BMPs as well as procollagens, lysyl oxidase, probiglycan, etc., also recently has been shown to interact with fibronectin (Huang et al. 2009). Furthermore, three hematopoietic stem cell cytokines, stem cell factor (SCF), interleukin-3 (IL-3), and stromal-derived factor-1 α (SDF-1 α) have been shown to control formation of vascular tubes and coassembly of vascular tubes with associated pericytes when incorporated into 3-D collagen matrices (Stratman et al. 2009a). Also, the ECM proteins thrombospondin-1 and thrombospondin-2, which can inhibit angiogenesis (Streit et al. 1999; Armstrong and Bornstein 2003), bind various angiogenic cytokines such as FGF-2, VEGF, and HGF (Margosio et al. 2003) and thereby may prevent their binding to proangiogenic ECM or otherwise interfere with cytokine activity. Angiopoietin-1 has been reported to promote cell adhesion through the integrin $\alpha 5 \beta 1$ and the angiopoietin receptor, Tie-2, has been found to associate with $\alpha 5 \beta 1$, a fibronectin receptor (Carlson et al. 2001; Cascone et al. 2005; Saharinen et al. 2008). One interesting possibility is that angiopoietin-1 becomes anchored to fibronectin matrices to affect vessel formation and/or stabilization through Tie-2 and $\alpha 5 \beta 1$ cosignaling. Of particular interest is the activity of fibrillins, that are major components of the elastin-rich microfibrils required for large-vessel elasticity and compliance, toward binding and regulating the activity of TGF- β isoforms as well as BMPs (Ramirez and Dietz 2007, 2009; Wagenseil and Mecham 2009). Disruption of fibrillin expression increases activation of TGF- β signaling leading to vascular abnormalities such as vascular malformations (Ramirez and Dietz 2007, 2009). Thus, growth factors that control angiogenesis are capable of binding directly to provisional ECM scaffolds implicated in supporting vascular morphogenesis (e.g., collagen or fibrin matrices), ECM that promotes vascular stabilization (e.g., basement membrane matrices), and ECM that regulates vessel elasticity (e.g., elastin, fibrillins). Consequently, cytokine-ECM interactions are likely important in regulating vascular morphogenesis as well as vessel maturation and function.

DISTINCTIONS BETWEEN THE COMPOSITIONS OF EMBRYONIC AND ADULT ECMs: IMPLICATIONS FOR ANGIOGENESIS AND THE INVOLVEMENT OF SPECIFIC INTEGRINS

Embryonic Vasculogenesis Versus Adult Angiogenesis

The preceding sections have focused on ECM as it relates to angiogenesis in the adult. A fundamental and intriguing question that clearly warrants additional investigation involves the composition of ECM associated with vascular morphogenesis in the embryo (vasculogenesis) (Hynes 2007; Davis and Senger 2008). One of the difficulties with investigating ECM function during vasculogenesis is that there are currently no 3-D models that mimic the composition of the embryonic ECM. Thus, the development of such models is an important future goal. Nonetheless, it is clear that there is much less fibrillar collagen during embryonic development in comparison with adult tissues; instead, embryonic ECM is known to be rich in glycosaminoglycans, such as hyaluronic acid, proteoglycans, fibronectin, and tenascins (Hynes 2007; Davis and Senger 2008).

In particular, developing embryos depend on the presence of fibronectin for vasculogenesis; fibronectin knockout mice show severe defects in vascular development together with other abnormalities (Francis et al. 2002; Astrof et al. 2007; Astrof and Hynes 2009). Perhaps the central importance of fibronectin in vasculogenesis involves its mechano-sensitive action in self-assembly during morphogenesis and tissue growth (Sakai et al. 2003; Vogel 2006; Hynes 2007; Smith et al. 2007). Fibronectin contains biologically active cryptic sites that affect this self-assembly process, particularly in its III-I domain (Morla and Ruoslahti 1992; Zhong et al. 1998; Davis et al. 2000; Gao et al. 2003; Smith et al. 2007; Vakonakis et al. 2007; see article by Schwarzbauer and DeSimone 2011). Interestingly, this domain is exposed when fibronectin is absorbed to cell surfaces or ECM (Zhong et al. 1998; Davis et al. 2000; Smith et al. 2007), and it appears to be particularly exposed when cells exert mechanical force

on fibronectin through integrin-based interactions (Zhong et al. 1998; Smith et al. 2007). Of particular interest, EC-pericyte interactions markedly induce perivascular deposition of fibronectin during the formation of vascular tubes (Stratman et al. 2009a). Because pericytes actively migrate along the abluminal surface of tubes during fibronectin deposition, in conjunction with vascular basement membrane matrix assembly (Stratman et al. 2009a), it is likely that tensional forces could be generated by ECs and/or pericytes to facilitate matrix assembly. Disruption of fibronectin assembly also interferes with perivascular collagen type IV assembly; thus, basement membrane assembly is dependent on the deposition of fibronectin that is itself a key component of vascular basement membranes (Stratman et al. 2009a). Thus, fibronectin may play a particularly important role in ECM assembly during processes such as vascular morphogenesis that depend on mechanical forces (Hynes 2007; Zhou et al. 2008; Astrof and Hynes 2009; Stratman et al. 2009a). Also, fibronectin is alternatively spliced (IIIA and IIIB), and these particular isoforms appear to play a particularly critical role in promoting vascular tube assembly and maturation during development (Astrof et al. 2007; Astrof and Hynes 2009).

During development and also in the adult, it is clear that vascular basement membrane matrix assembly represents a fundamental step in the maturation of vessels (Miner and Yurchenco 2004; Davis and Senger 2005; Rhodes and Simons 2007; Eble and Niland 2009; Stratman et al. 2009a; Wiradjaja et al. 2010). This is an example where the functional roles of such matrices in adults and embryos appear very similar. One approach to assess the function of these proteins has been to knock out basement membrane matrix genes in mice (Table 1). It is interesting that the cardiovascular system appears particularly vulnerable to basement membrane gene knockouts because of the mechanical forces and stresses exerted on the heart and vasculature during development and postnatal life. Knockouts of fibronectin, laminin isoforms, collagen IV, and perlecan result in very significant cardiovascular dysfunction

that results in embryonic lethality in most cases (Table 1). Additional defects in the vasculature are likely because of the importance of these basement membrane components for vascular morphogenesis, remodeling, and stabilization.

In adult tissues, collagen matrices appear to present a physical barrier to sprouting angiogenesis and subsequent formation of lumens and tubes, processes that require MMP-dependent cleavage of collagen (Haas et al. 1998; Zhou et al. 2000; Chun et al. 2004; Saunders et al. 2006; Stratman et al. 2009b). In addition, collagen I is highly cross-linked in adult animals through enzymes such as lysyl oxidase that further stabilize this key component of interstitial matrix (Wagenseil and Mecham 2009). An interesting and unanswered question is whether embryonic ECM presents a physical barrier similar to interstitial collagens in the adult and whether different cell surface enzymes are required for modifying embryonic ECM during vasculogenesis.

Integrins in Embryonic Vasculogenesis and Adult Angiogenesis

Integrin binding to ECM plays a critical role in controlling both embryonic vasculogenesis and adult angiogenesis (Davis and Senger 2005; Hynes 2007). Numerous studies have implicated a variety of integrins; and the relative importance of specific integrins likely depends, at least in part, on the composition of the ECM. During embryonic development, fibronectin receptors appear particularly significant with $\alpha 5 \beta 1$ required for formation of vascular tubes (Francis et al. 2002; Hynes 2007; Astrof and Hynes 2009) and $\alpha 4 \beta 1$ and $\alpha 9 \beta 1$ required for development of lymphatic vessels (Avraamides et al. 2008). These observations likely relate directly to the abundance of fibronectin in embryonic ECM (Astrof and Hynes 2009), as discussed above. In the adult, there is evidence that αv integrins (Friedlander et al. 1995; Stupack and Chersesh 2004; Avraamides et al. 2008; Feng et al. 2008), that bind components of provisional ECM derived through leakage of plasma proteins (e.g., fibrin, vitronectin, plasma fibronectin) and $\alpha 1 \beta 1$ and $\alpha 2 \beta 1$

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Table 1. Vascular developmental defects secondary to mouse knockouts of ECM basement membrane matrix genes

Basement membrane component	Mouse knockout phenotype	Specific vascular phenotypic defects/comments	References
Laminin α 4 chain (laminins 411, 421)	Mild, early postnatal lethality, newborns lethargic, pale, and icteric because of anemia and hemorrhage, survivors improve and have normal life span. Corneal angiogenesis assays with FGF-2 reveal early, more intense sprouting and grossly distorted vasculature with apparent hemorrhages and dilated vessels.	Diffuse hemorrhagic phenotype from smaller vessels, particularly associated with parturition. Discontinuous basement membrane in capillaries with reduction in collagen type IV and nidogen staining but normal levels of perlecan. Adult null mice correct these defects and have normal levels of collagen type IV, nidogen, perlecan, and express laminin-511 in capillary basement membranes.	Thyboll et al. 2001
Laminin α 5 chain (laminins 511, 521)	Embryonic lethality between E13.5 and 16.5 with limb, neural tube, and placental defects. Placental labyrinth is malformed which primarily consists of interacting trophoblasts and endothelial cells with intervening basement membranes.	At E13.5-16.5, vessels were present in placental labyrinth, but the branching complexity was markedly reduced and vessel diameters were significantly increased. In normal embryos, trophoblasts and ECs are separated only by a basement membrane while in the mutant, only the ECs remained adherent to the basement membrane creating a cell-free space.	Miner et al. 1998
Fibronectin	Embryonic lethality after E8.5 with deficits in mesodermal development, particularly, notochord, somites, heart, as well as embryonic and extraembryonic vasculature. Combined knockout of alternatively spliced exons EIIIA and EIIIB show embryonic lethality (usually by E10.5) because of cardiovascular defects including failure of vascular remodeling and hemorrhage.	Cardiac and vascular defects vary with the genetic background (more severe defects in 129S4 vs. C57BL/6J background) as well as vessel location. In some cases, defects in EC-mesenchymal interactions (e.g., heart and aorta) may underlie the vascular morphogenic defects observed.	George et al. 1993; George et al. 1997; Astrof et al. 2007

Continued

Table 1. Continued

Basement membrane component	Mouse knockout phenotype	Specific vascular phenotypic defects/comments	References
Collagen type IV, $\alpha 1/2$ chain	Embryonic lethality between E10.5-E11.5; basement membrane laminin and nidogen-1 were detected underlying epithelia and endothelia, although showed weaker and more patchy staining. Areas of basement membrane discontinuity and fragmentation were apparent. Excessive amounts of maternal blood was present in the yolk sac cavity indicative of hemorrhage as was the presence of pericardial bleeding.	Impairment of the placental labyrinth was apparent as its thickness was reduced. At the time of embryonic death, dilated blood vessels were observed. Overall, vascular development appeared relatively normal with reduced capillary density sprouting into the neural layer. However, hemorrhage was observed in the heart and arteries with excessive yolk sac hemorrhage indicative of abnormalities in EC basement membrane contacts or stability.	Poschl et al. 2004
Perlecan	Embryonic lethality between E10-E12 as well as perinatally. The lethality occurs because of hemopericardium and cardiac arrest. There is no evidence for placental or vascular defects.	There are few apparent vascular developmental defects except for some microaneurysms that form in several tissues including lung, skin, and brain.	Costell et al. 1999
Nidogens 1 and 2	Viable mice, some seizure-like symptoms with loss of muscle control in hindlimbs, no discernable vascular phenotype. Overall, normal basement membrane assembly and Immunostaining of major basement membrane components.	Minimal to no vascular abnormalities, some structural abnormalities in basement membrane matrix of brain capillaries. Nidogen 2 appears to be expressed in higher amounts in EC basement membranes compared to other basement membranes. Probable compensation for both nidogens 1 and 2 following knockout.	Murshed et al. 2000; Dong et al. 2002; Miosge et al. 2002
Collagen type XVIII	Viable mice with eye abnormalities. Overall, vascular basement membranes and vascular development (with the exception of the eye) appear normal.	Delayed regression of hyaloid vessels in the vitreous; abnormal outgrowth of retinal vessels which may be secondary to the lack of regression and/or alterations in VEGF expression through changes in retinal hypoxia.	Fukai et al. 2002

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integrins (Senger et al. 1997, 2002), that bind interstitial collagens, are also important for angiogenesis. These observations are consistent with the abundance of provisional ECM in adult tissues during wound repair and other settings in which angiogenesis occurs (see section Overview of Angiogenesis and ECM in the Adult). The role of collagen receptors during embryonic vasculogenesis is less clear. Mouse knockouts have not shown them to be essential (Zweers et al. 2007; Zhang et al. 2008); however, a recent study indicates a role for $\alpha 2\beta 1$ in

zebrafish vascular development (San Antonio et al. 2009). Thus, it seems likely that multiple integrins can regulate neovascularization depending on the composition of ECM. In addition, integrins also play an important role in maturation and stabilization of vascular tubes by binding vascular basement membrane components. In particular, integrins $\alpha 5\beta 1$, $\alpha 3\beta 1$, $\alpha 6\beta 1$, and $\alpha 1\beta 1$ bind basement membrane matrix during vascular tube/pericyte interactions in 3-D collagen matrices (Stratman et al. 2009a).

Figure 3. Vascular guidance tunnels: ECM conduits generated during vessel tubulogenesis that regulate EC-pericyte interactions, ECM remodeling, vascular basement membrane matrix assembly, and vessel stabilization. The process of EC tubular morphogenesis in 3-D collagen matrices leads to the creation of networks of EC-lined tubes, and also networks of vascular guidance tunnels in which ECs reside. The tunnels are formed during lumen and tube formation through EC cell surface proteolysis by MT1-MMP. EC-lined tubes within vascular guidance tunnels recruit pericytes to the tube abluminal surface; subsequently, pericytes migrate along this surface within the tunnels. (A) Time-lapse imaging of EC tubes (RFP-labeled) and pericytes (GFP-labeled) reveals motility of pericytes along the tube abluminal surface over time. Arrows indicate EC tube abluminal surface. Bar equals 25 μm . Active motility of pericytes along tubes leads to EC-pericyte interactions and contributions of basement membrane components, such as collagen type IV, which is deposited continuously along the abluminal surface (right panel; Bar equals 50 μm). (B) Vascular guidance tunnels are formed during EC tubulogenesis through MT1-MMP-mediated proteolysis and can be shown using anticollagen type I antibodies (left panel; Bar equals 50 μm ; the tunnels are the negative stained areas; arrowheads indicate tunnel borders) or by microinjection of tunnels with silicone oil (right panel; Bar equals 20 μm ; arrowheads indicate tunnel borders).

ECM REMODELING DURING VASCULAR TUBE FORMATION AND STABILIZATION

MT1-MMP and Formation of Vascular Guidance Tunnels within ECM

Studies in vitro indicate that ECM proteolysis by cell surface MT1-MMP plays a critical function in facilitating and expanding EC tubes (Chun et al. 2004; Saunders et al. 2006; Stratman et al. 2009b). Proteolysis also clears ECM to create vascular guidance tunnels (Fig. 3) that facilitate mural cell recruitment, followed by basement membrane assembly, and vessel maturation and stabilization (Chun et al. 2004; Davis et al. 2007; Stratman et al. 2009a,b). As summarized in Figure 4, complex

signaling cascades involving the functional coupling of Cdc42 with MT1-MMP and integrins together with PKC epsilon, Src, Pak-2, Pak-4, B-Raf, C-Raf, Erk 1/2 and the polarity components Par3, Par6b, and atypical PKC isoforms are essential for formation of vascular tubes in 3-D collagen I (Koh et al. 2008, 2009; Sacharidou et al. 2010). Importantly, MT1-MMP is required for the formation of vascular guidance tunnels (Fig. 3), but once vascular guidance tunnels have formed, ECs are able to migrate within these physical spaces independently of MMP activity (Stratman et al. 2009b). Interestingly, previous work indicates that the tumor vasculature appears to regrow (after induction of tumor vessel regression with anti-VEGFR2

Figure 4. Molecular signaling events controlling vascular tube morphogenesis, matrix remodeling, and stabilization in 3-D extracellular matrices. Multicomponent signaling complexes control EC lumen and tube formation in 3-D matrices. A key aspect of this mechanism is the integration of Cdc42-mediated signaling with cell surface proteolysis through MT1-MMP. A downstream kinase signaling cascade is activated leading to EC cytoskeletal changes, survival, and transcriptional control that regulates EC tubular morphogenesis. EC tube formation leads to the generation of networks of vascular guidance tunnel spaces within the ECM that are occupied by ECs, thereby allowing for EC motility and remodeling events (illustrated in Fig. 3). Pericytes are recruited to these EC-lined tunnels and EC-pericyte motility within these tunnels and along the tube abluminal surface leads to ECM remodeling and vascular basement membrane matrix assembly. Both ECs and pericytes have been shown to contribute basement membrane matrix components (illustrated in Fig. 5), as well as TIMP-2 and TIMP-3, which together control the formation and stability of the vascular basement membrane.

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Figure 5. Pericyte recruitment to EC-lined tubes leads to vascular basement membrane matrix assembly in 3-D matrices. Green-fluorescent protein (GFP)-tagged bovine retinal pericytes were mixed with ECs in 3-D collagen matrices over a period of 5 days, during which EC tubes formed and recruited pericytes. Cultures were fixed at 5 days and immunostained for CD31 (panel A) or for collagen type IV (panel B); bar equals 50 μ m. Thin plastic sections were obtained and photographed under transmitted light (panel C); bar equals 25 μ m. (Panel D) RT-PCR and protein analyses of EC-only and EC-pericyte cocultures revealed specific changes in ECM and integrin expression, indicating distinctly different regulation in EC-pericyte cocultures in comparison with ECs alone (Stratman et al. 2009a).

treatments) along previously formed basement membrane sleeves following cessation of the therapy (Mancuso et al. 2006).

Pericyte Recruitment Stimulates ECM Remodeling and Vascular Basement Membrane Matrix Assembly within Vascular Guidance Tunnels

Identification of vascular tunnels suggested the possibility that they serve a more complex function than simply providing an abluminal surface for ECs during formation of vascular tubes. Indeed, in a coculture model involving pericytes and ECs, pericytes were observed selectively along the abluminal surface of tubes and importantly within vascular guidance tunnels (Fig. 3) (Stratman et al. 2009a,b). Thus, ECs

and pericytes coassociate within the tunnels. In addition, time-lapse microscopy showed dynamic motility of pericytes along the EC abluminal surface within the tunnels (Fig. 3) (Stratman et al. 2009a) and showed that both ECs and pericytes were simultaneously motile. Interestingly, pericyte recruitment to EC-lined tubes along the abluminal surface led to continuous deposition of basement membrane matrix (Figs. 3 and 5). In the absence of pericytes, EC-lined tubes are not able to deposit a basement membrane, a finding that was confirmed with electron microscopy (Stratman et al. 2009a); thus, pericyte recruitment to tubes in vitro and in vivo regulates this process (Stratman et al. 2009a, 2010).

Why is pericyte recruitment necessary to stimulate vascular basement membrane matrix

assembly around vascular tubes? Key findings from studies designed to answer this question are summarized in Figure 5. In particular, synthesis of fibronectin as well as specific laminins, nidogens, and perlecan, together with integrins that bind basement membrane ECM, were more abundant in EC/pericyte cocultures in comparison with EC-only cultures (Stratman et al. 2009a). Thus, both ECs and pericytes adapt to their changing environment during vascular basement membrane assembly by increasing expression of integrins that recognize this new matrix.

CONCLUDING REMARKS

By providing mechanical stability and cell adhesion, ECM has been shown to be essential for all stages of angiogenesis. In addition to providing basic support for EC proliferation, survival, and migration, ECM also regulates key stages of blood vessel morphogenesis and maturation. Although angiogenic cytokines such as VEGF are often represented as the key mediators of neovascularization, there is a growing body of evidence that ECM and dynamic changes in the composition of ECM are equally important, particularly in regulating vascular morphogenesis and the stabilization of new blood vessels. Thus, at a minimum, angiogenesis should be viewed as a collaboration between cytokines and ECM, wherein ECM actively controls, rather than merely supports, the formation, architecture, and maturation of new blood vessels.

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