Chapter 3
Endothelial Cell–Matrix Interactions in Angiogenesis and Vessel Homeostasis: A Focus on Laminins and Their Integrin Receptors

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Abstract Integrin-mediated interaction of cells with proteins present in the extracellular matrix and basement membranes direct many cellular processes required for both normal and pathological processes. These interactions guide angiogenesis and vessel homeostasis, both in the embryo and the adult. Mouse genetic studies have explored the contribution of individual integrin heterodimers; laminins and their integrin receptors have emerged as important regulators of these processes. This chapter discusses findings from both in vivo studies and organotypic cell culture models that provide insight into the molecular mechanisms by which the laminin-binding integrins $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, and their ligands, laminin-411 and laminin-511, regulate endothelial cell signaling, cell–cell interactions, and gene expression that contribute to the regulation of endothelial cell function in angiogenesis and vessel homeostasis.

3.1 Introduction

Angiogenesis is a process that is critical to tissue repair, cancer progression, as well as inflammation, and involves the sprouting of endothelial cells from the preexisting vasculature (Potente et al. 2011). Angiogenic signals, such as vascular endothelial growth factor (VEGF), activate endothelial cells leading to the proteolytic breakdown of the endothelial basement membrane, the loosing of endothelial cell–cell junctions, the dissociation of mural cells, and the sprouting of endothelial cells into surrounding extracellular matrix. Sprouting is guided by endothelial tip cells with
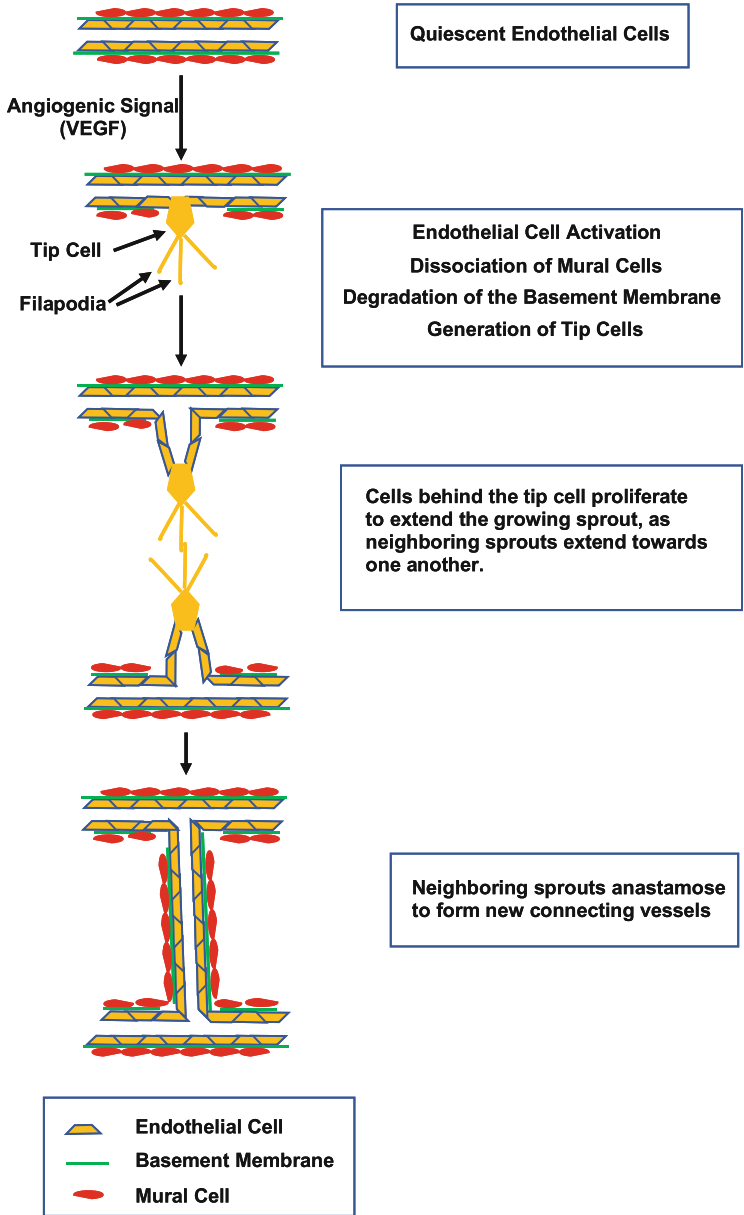
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Fig. 3.1 Angiogenesis. Shown is a schematic representation of the steps involved in angiogenesis

proliferating stalk cells in tow (Potente et al. 2011). Tip cells from neighboring sprouts anastomose to create a network of new vessels that form lumens, assemble new basement membranes, and recruit mural cells (Potente et al. 2011) (Fig. 3.1).

Sprouting endothelial cells can interact with the plasma proteins, fibronectin, vitronectin, or fibrinogen present in the provisional matrix or with collagens present in the interstitial matrix (Senger and Davis 2011; Eming et al. 2007). Endothelial cells can also interact with endothelial-secreted extracellular matrix (ECM) proteins, including fibronectin, and laminins (Hallmann et al. 2005; Avraamides et al. 2008; Turner et al. 2017). Members of the integrin family of adhesion receptors (Hynes 2002a) mediate these interactions that can contribute to the formation and stabilization of endothelial tubes (Hallmann et al. 2005; Turner et al. 2017; Senger and Davis 2011; Xu et al. 2020; Song et al. 2017; Avraamides et al. 2008).

3.2 Integrins in Angiogenesis

Integrins are heterodimeric transmembrane proteins that contain one α and one β subunit. Mammals have 18 α subunits and 8 β subunits that combine to form 24 α/β heterodimers (Hynes 2002a). Their extracellular domains engage ECM and basement membrane components, including fibronectin, collagens, and laminins. Integrin intracellular domains interact with the cell's cytoskeleton and signaling networks to regulate many aspects of cell behavior including cell adhesion, migration, proliferation, survival, and invasion, as well as gene expression, which together contribute to complex biological processes, such as tissue morphogenesis and development (Danen and Sonnenberg 2003; Streuli and Akhtar 2009).

Several endothelial integrin heterodimers, including $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$ $\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$, are known to regulate angiogenesis, and their individual roles can be context-dependent (Avraamides et al. 2008; Senger and Davis 2011; Van Der Flier et al. 2010; Murphy et al. 2015). Much of what is known about the contribution of integrins has been gleaned from mouse genetic studies. The $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ integrins all bind to the RGD motif in fibronectin (Hynes 2002a). Both the global deletion of fibronectin (*Fn1*-null) in mice and the global deletion of integrin α_5 subunit (*Itga5*-null) in mice are embryonic lethal, with both mutants exhibiting defects in embryonic vascular development (Avraamides et al. 2008). The specific deletion of fibronectin alleles in endothelial cells demonstrated that the endothelial expression of fibronectin is required for retinal developmental angiogenesis (Turner et al. 2017). However, the endothelial-specific deletion of the integrin α_5 subunit alleles was not sufficient to alter developmental angiogenesis; however, endothelial-specific deletion of both integrin α_5 (*Itga5*) and α_v (*Itgav*) subunit alleles inhibited embryonic vascular remodeling, suggesting that $\alpha_5\beta_1$ and α_v integrins can compensate for one another's endothelial function during the development of the embryonic vasculature (Van Der Flier et al. 2010). Significant effort both at the bench and in the clinic has been concerned with the RGD-binding integrins, $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$; however, drugs targeting these integrins to suppress tumor angiogenesis have met with discouraging results (Hynes 2002b; Desgrosellier and Cheresh 2010; Paolillo et al. 2016). Additionally, the endothelial-specific deletion of the prominent RGD-binding integrins, as well as fibronectin, failed to inhibit tumor angiogenesis

(Murphy et al. 2015). Thus, it seems that other integrins and matrix components contribute to the regulation of angiogenesis.

The $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins bind to both interstitial collagens, as well as collagens present in basement membranes (Hynes 2002a). An endothelial-specific deletion of α_1 subunit gene (*Itga1*) has not been generated. However, mice with null mutations in the α_1 integrin subunit gene, *Itga1-null* animals, are viable and fertile (Gardner et al. 1996), indicating that $\alpha_1\beta_1$ is not required for developmental angiogenesis. However, $\alpha_1\beta_1$ can regulate angiogenesis in the adult. Tumor angiogenesis was suppressed in *Itga1*-null mice, and this was correlated with the increased plasma levels of the metalloproteinases MMP-7 and MMP-9 and defects in endothelial proliferation (Pozzi et al. 2000; Pozzi et al. 2002). However, it is unclear whether endothelial cells are responsible for increased levels of MMPs, as $\alpha_1\beta_1$ is expressed in multiple cell types in addition to microvascular endothelial cells (Belkin et al. 1990; Glukhova et al. 1993; Gullberg et al. 1992; Defilippi et al. 1991; Glukhova et al. 1995; Loeser et al. 1995; Sobel et al. 1998; Senger et al. 1997).

The $\alpha_2\beta_1$ integrin is expressed on microvascular endothelial cells and has been observed on sprouting tips of angiogenic vessels, suggesting a role for $\alpha_2\beta_1$ in angiogenesis (Senger et al. 1997; Enenstein and Kramer 1994). An endothelial-specific deletion of the α_2 subunit gene (*Itga2*) has not been generated. Mice lacking both α_2 alleles (*Itga2*-null mice) have been made. These mice develop normally and are fertile (Chen et al. 2002; Holtkotter et al. 2002); thus, similar to $\alpha_1\beta_1$, $\alpha_2\beta_1$ is not required for developmental angiogenesis. However, adult *Itga2*-null mice exhibited enhanced wound and tumor angiogenesis, suggesting anti-angiogenic role for $\alpha_2\beta_1$ (Zweers et al. 2007; Grenache et al. 2007; Zhang et al. 2008). Surprisingly, the angiogenic response in *Itga2*-null mice was found to be tumor-dependent. An enhanced angiogenic response was triggered by B16F10 melanoma cells, but not by Lewis lung carcinoma cells (Zhang et al. 2008). VEGFR1 is the receptor for the VEGF family member, placental growth factor (PlGF) and is upregulated in endothelial cells from *Itga2*-null mice. B16F10 melanoma cells secrete much higher levels of PlGF compared to Lewis lung carcinoma cells, which explains the differences in the angiogenic response and underlines the importance of crosstalk between tumor and endothelial cells, as well as the tumor microenvironment in the angiogenic response (Zhang et al. 2008). Interestingly, increased expression of metalloproteinases, including MMP-9, was observed in dermal wound tissue from *Itga2*-null mice compared to control animals. In vitro studies suggested the enhanced protease expression was derived from *Itga2*-null keratinocytes, pointing to this as a potential mechanism for enhanced wound angiogenesis (Grenache et al. 2007). Others have shown the importance of integrin-regulated secretion of paracrine factors from keratinocytes in the crosstalk between keratinocytes and endothelial cells in the regulation of angiogenesis during dermal wound healing (Mitchell et al. 2009). Nonetheless, it is unclear how increases in MMP expression inhibit angiogenesis in the case of *Itga1-null* mice and enhance angiogenesis in the case of *Itga2*-null mice. Possibly the cellular source and localization of increased MMP expression determine the effect on angiogenesis. It is notable that these two collagen receptors have opposing roles in the regulation of angiogenesis, raising the question of

whether they balance one another's function, or whether $\alpha_1\beta_1$ or $\alpha_2\beta_1$ plays a dominant role. Analysis of animals lacking alleles for both the integrin α_1 and integrin α_2 subunits demonstrated that both wound and tumor angiogenesis were inhibited, indicating that the *Itga1-null* phenotype is dominant (Ghatak et al. 2016). This was further supported by explant aortic ring angiogenesis assays, which showed that sprouting was inhibited in explants from doubly null mice (Ghatak et al. 2016). Although mostly discussed in terms of their ability to bind to collagen, $\alpha_1\beta_1$ or $\alpha_2\beta_1$ can also bind to a subset of laminin isoforms, which will be discussed in more detail below.

The remainder of this chapter focuses on the contribution of endothelial laminins and their integrin receptors in the regulation of normal and pathological angiogenesis, as well as in vessel homeostasis. We discuss results from both mouse genetic models as well as in vitro angiogenesis assays.

3.3 Endothelial Laminins

Laminins are heterotrimeric proteins, each containing an α, β, and γ chain, are assembled intracellularly and are secreted as heterotrimers (Yurchenco et al. 1997). Laminins are important contributors to the formation and structure of basement membranes, that are present in epithelial, endothelial, muscle, and neural tissues (Colognato and Yurchenco 2000; Yurchenco 2011). In mammals, there are five distinct α chains, four β chains, and three γ chains, that can associate to form at least 15 laminin isoforms, with distinct tissue distribution (Yurchenco 2011). Endothelial cells express two laminin isoforms: laminin-411, which contains the $\alpha4$, $\beta1$, and $\gamma1$ chains, and laminin-511, which contains the $\alpha5$, $\beta1$, and $\gamma1$ chains. It is important to note that laminin-411 and laminin-511 were previously named laminin-8 and laminin-10 respectively (Aumailley et al. 2005).

Since laminin-411 and laminin-511 contain the same β and γ chains, their endothelial expression in the vasculature can be monitored by immunostaining with antibodies that recognize the laminin $\alpha4$ chain or the laminin $\alpha5$ chain, respectively. In mice, the laminin $\alpha4$ chain is expressed ubiquitously by endothelial cells and is observed in the vasculature beginning at embryonic day 8.8 (Frieser et al. 1997; Iivanainen et al. 1997; Miner et al. 1997). The endothelial expression of the $\alpha5$ chain of laminin is first observed a few weeks after birth and is detected primarily on capillaries and venules (Patton et al. 1997; Sorokin et al. 1997). Because laminin-411 and laminin-511 share the same β and γ chains, the effects of deletion of laminin-411 and laminin-511 on endothelial function can be examined by deletion of the $\alpha4$ (*Lama4*) or $\alpha5$ (*Lama5*) chain alleles, respectively. To date, an endothelial-specific deletion of *Lama4* gene has not been generated. Analysis of *Lama4*-null mice revealed significant hemorrhaging both during embryonic and neonatal development. This was accompanied by defects in the assembly and structure of endothelial basement membranes. Collagen IV and nidogen are also key components of basement membranes; their expression was lacking in endothelial basement

membranes of developing *Lama4*-null mice. Electron microscopy confirmed the presence of defective basement membranes associated with capillary endothelial cells. Together these data suggest that laminin-411 is required for the proper assembly of the endothelial basement membrane that in turn functions to stabilize the embryonic vasculature (Thyboll et al. 2002). Interesting, *Lama4*-null mice recover and develop into adulthood due to the endothelial expression of laminin-511, which becomes more widely expressed by vascular endothelial cells in mutant mice; thus, laminin-511 can compensate for the loss of laminin-411 (Thyboll et al. 2002; Zhou et al. 2004). Mice null for the laminin α5 chain, die during embryogenesis, before laminin-511 is expressed by endothelial cells (Miner et al. 1998). However, an endothelial-specific deletion of the laminin α5 chain alleles (*Lama5*) has been generated. The vasculature in these mice appears to develop normally with no significant defects in vasculature at homeostasis (Song et al. 2013).

3.4 Endothelial Laminin-Binding Integrins

Multiple integrin heterodimers bind laminin isoforms (Hynes 2002a). Endothelial cells express the $\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$ laminin-binding integrins, as do many epithelial cells (Avraamides et al. 2008; Hynes 2002a). The α chains of laminin heterotrimers contain the binding sites for integrins, and thus, the identity of the α chain present in a particular laminin isoform determines its integrin binding partners (Yurchenco 2011). Several approaches have been employed to determine whether $\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$ can bind to laminin-411 and/or laminin-511. Purified laminin-411 or laminin-511 has been used in cell adhesion assays together with antibodies to inhibit the function of specific integrin subunits. Endothelial cell adhesion to laminin-411 was inhibited by function-blocking antibodies to either the α_6 or β_1 integrin subunit, demonstrating that laminin-411 is a ligand for $\alpha_6\beta_1$ (Kortesmaa et al. 2000). K562 cells do not express any laminin receptors. The ability of K562 cells expressing individual recombinant laminin-binding integrins demonstrated that the $\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$ integrins can each function as adhesion receptors for laminin-411 (Kortesmaa et al. 2000; Fujiwara et al. 2001). Endothelial cell adhesion to laminin-511 was inhibited using function-blocking antibodies to integrin $\alpha_3\beta_1$ (Doi et al. 2002). The ability of $\alpha_6\beta_4$ to bind laminin-511 was demonstrated using the murine β_1 *Itgb1*-null GD25 cells that express only one laminin-binding integrin, $\alpha_6\beta_4$ (Kikkawa et al. 2004). The ability of $\alpha_6\beta_1$ to bind laminin-511 was demonstrated using in vitro binding assays (Nishiuchi et al. 2006), as well as in cell adhesion assay with antibodies to block the function of α_6 integrins. Although $\alpha_1\beta_1$ and $\alpha_2\beta_1$ are usually considered collagen-binding integrins, they have also been shown to bind to a subset of laminin isoforms (Humphries et al. 2006, Yurchenco 2011 41). The $\alpha_2\beta_1$ was shown to function in cell adhesion to laminin-411, but not laminin-511 (Stenzel et al. 2011). The ability of $\alpha_1\beta_1$ to bind to these laminin isoforms has not been tested. It is also important to note that the $\alpha_v\beta_3$ integrin was shown to bind a recombinant fragment of the laminin α4 chain (Gonzalez et al. 2002). The significance of the

interaction between $\alpha_v\beta_3$ and the $\alpha4$ chain of laminin-411 to endothelial cell function is not yet fully appreciated. The laminin $\alpha5$ chain contains an RGD sequence and the $\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_1$ and $\alpha_v\beta_5$ were shown to bind recombinant fragments of the $\alpha5$ chain containing the motif (Hallmann et al. 2005; Sasaki and Timpl 2001). Whether these interactions contribute to angiogenesis or vessel homeostasis is yet to be appreciated; however, adhesion to laminin-511 mediated by β_1 and β_3 integrins was implicated in maintaining the surface expression of the adherens junction protein, VE-cadherin (Song et al. 2017), as discussed below.

3.5 Laminins in Pathological Angiogenesis

As mentioned above, embryonic vessels that lack the expression of laminin-411 are leaky, suggesting that laminin-411 functions to promote vessel stability, at least in the embryo (Thyboll et al. 2002). To determine the role of laminin-411 in new vessel formation in adult animals, the effects of inhibiting the expression of laminin-411 were assessed in cornea angiogenesis assays. Compared with wild-type littermates, *Lama4*-null mice exhibited enhanced endothelial sprouting in response to FGF2 (Thyboll et al. 2002), suggesting that laminin-411 may function as a negative regulator of angiogenesis. Interestingly, in wild-type corneas, a well-organized vasculature developed in response to FGF2, but most of these vessels had regressed by 42 days (Zhou et al. 2004). Vessels that formed in *Lama4*-null corneas were disorganized and dilated, but surprisingly at day 42, these vessels had undergone dramatic remodeling into what appeared as a normal vasculature (Zhou et al. 2004). This correlated with the upregulation of laminin-511 expression, suggesting that laminin-511 promotes vessel maturation. To examine the contribution of laminin-411 to tumor angiogenesis, control mice and mice deficient in the $\alpha4$ chain of laminin-411 were challenged with subcutaneously implanted Lewis lung carcinoma cells or B16-F10 melanoma cells (Zhou et al. 2004). Tumor growth and angiogenesis were enhanced in mutant compared to control in response to both tumor types. Additionally, greater B16-F10 lung metastases were observed in *Lama4*-null compared to control mice; however, the mechanisms involved have yet to be identified. Although the tumor vasculature in control mice mostly expressed laminin-411, in *Lama4*-null mice the tumor vasculature exhibited strong expression of laminin-511, which presumably compensated for the loss of laminin-411 (Zhou et al. 2004).

In post-natal retinal developmental angiogenesis, the expression of laminin $\alpha4$ chain RNA localized to extending endothelial sprouts, suggesting a positive role for laminin-411 in regulating endothelial sprouting. *Lama4*-null mice, however, showed enhanced endothelial sprouting, suggesting that the expression of laminin-411 puts the breaks on sprouting (Stenzel et al. 2011). This is consistent with the studies discussed above, which reported enhanced tumor angiogenesis in *Lama4*-null mice.

As mentioned in the Introduction, sprouting is guided by endothelial tip cells, which send out filopodia that direct the growing sprout. VEGF-VEGFR2 signaling in tip cells promotes the secretion of the Notch ligand Dll4 to limit the generation of

additional tip cells nearby (Potente et al. 2011). Dll4-Notch signaling is critical to the regulation of angiogenesis; the loss of Dll4/Notch signaling is known to lead to hyper-sprouting (Eilken and Adams 2010; Phng and Gerhardt 2009). Thus, the loss of laminin-α4 expression phenocopied the defects observed when Dll4/Notch signaling is inhibited. Indeed, Notch signaling was inhibited in *Lama4*-null retinas (Stenzel et al. 2011). Additionally, in vitro adhesion of endothelial cells to laminin-411, but not laminin-511, significantly increased the expression of Dll4 RNA (Stenzel et al. 2011). Antibody inhibition studies and siRNA knockdown experiments indicated that the $\alpha_2\beta_1$ and $\alpha_6\beta_1$ integrins contributed to this regulation (Stenzel et al. 2011). Taken together, these results suggest that $\alpha_2\beta_1$- and $\alpha_6\beta_1$-dependent endothelial cell adhesion to laminin-411 regulates the expression of Dll4 to promote properly regulated endothelial sprouting (Stenzel et al. 2011).

An endothelial-specific deletion of the laminin α5 chain is available (Song et al. 2013). No developmental angiogenesis defects were observed, as expected, since the endothelial expression of laminin α5 chain occurs postnatally. The contribution of laminin-511 to adult or pathological angiogenesis has yet to be explored. However, loss of endothelial laminin-511 decreases endothelial barrier function (Song et al. 2017). Such a phenotype might be permissive to angiogenesis. The contribution of laminin-511 to the regulation of endothelial barrier function is discussed below.

3.6 Laminin-Binding Integrins in Pathological Angiogenesis

Mouse genetic models were established to characterize the function of the laminin-binding integrins $\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$ during development. Mice null for the α_3 (*Itga3*), α_6 (*Itga6*), or β_4 subunit gene (*Itgb4*) died soon after birth with no defects in developmental angiogenesis reported (Kreidberg et al. 1996; Georges-Labouesse et al. 1996; Dowling et al. 1996; da Silva et al. 2010; Germain et al. 2010; Bouvard et al. 2012). To study the role of $\alpha_3\beta_1$ and α_6 integrins during angiogenesis in the adult, several labs used conditional endothelial deletion of either the *Itga3*, *Itga6, or Itgb4* gene (Germain et al. 2010; da Silva et al. 2010; Bouvard et al. 2012; Bouvard et al. 2014; Seano et al. 2014; Welser-Alves et al. 2013). To conditionally delete integrin α_3 subunit alleles in endothelial cells, Cre recombinase was expressed from the Tie-1 promoter (da Silva et al. 2010). This mutant will be referred as ec-*Itga3*-null. The $\alpha_3\beta_1$ integrin was not expressed by angiogenic vessels in mutant mice; however, $\alpha_3\beta_1$ expression was maintained in quiescent endothelial cells in dermal vasculature of ec-*Itga3*-null mice at levels similar to littermate controls (da Silva et al. 2010). Ec-*Itga3*-null mice exhibited increased angiogenesis in three distinct models. Tumor angiogenesis was analyzed in response to the subcutaneous injection of either B16F0 melanoma cells or CMT19T lung carcinoma cells (da Silva et al. 2010). Both tumor cell types showed enhanced angiogenesis in mutant compared to control mice. Hypoxia-induced retinal angiogenesis was also greater in ec-*Iga3*-null

mice compared to control, as was endothelial sprouting in aortic ring explant angiogenesis assays. These results supported a role for $\alpha_3\beta_1$ as a general negative regulator of angiogenesis in the adult. The mechanism reported for the ec-*Itga3*-null phenotype was surprisingly elaborate. The $\alpha_3\beta_1$ integrin was shown to be a positive regulator of the endothelial expression of VEGF, which led to the inhibition of the VEGFR2 RNA expression (da Silva et al. 2010). Thus, endothelial cells from mutant mice express higher levels of VEGFR2, explaining their increased angiogenic activity (da Silva et al. 2010).

There are conflicting reports as to whether and how α_6 integrins ($\alpha_6\beta_1$ and $\alpha_6\beta_4$) regulate adult angiogenesis. The phenotype of targeting the endothelial expression of α_6 integrins is dependent upon the promoter used to express Cre recombinase in endothelial cells (Germain et al. 2010; Bouvard et al. 2014). Like the Tie-1-dependent deletion of the integrin α_3 subunit genes, the Tie1-Cre-dependent deletion of the integrin α_6 subunit alleles (*Itga6*), referred to as ec1-*Itga6*-null, resulted in enhanced tumor angiogenesis (Germain et al. 2010). Subcutaneous injection of either B16F0 melanoma cells or Lewis lung carcinoma cells resulted in larger more vascularized tumors in ec1-*Itga6*-null, compared to those in littermate controls, suggesting that α_6 integrins, like $\alpha_3\beta_1$, are negative regulators of angiogenesis. The authors note that this is consistent with the decreased expression of α_6 integrins in angiogenic vessels associated with invasive ductal carcinoma as compared to normal breast tissue (Germain et al. 2010). The observed enhanced angiogenesis was not due to the upregulation of other integrins reported to promote angiogenesis, such as the $\alpha_1\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$ integrins (Germain et al. 2010). Endothelial cells from ec1-*Itga6*-null mice displayed increased surface expression of VEGFR-2 and downstream signaling, which explain the enhanced angiogenesis observed ec1-*Itgα6*-null mice (Germain et al. 2010).

Tie-2 (Tek) driven Cre recombinase was also used to examine the effect of the endothelial deletion of α_6 integrins (Bouvard et al. 2012, 2014). This mutant is referred to as ec2-*Itgα6*-null. The angiogenic phenotype of this mutant was first examined following ischemic injury to mouse hind limb (Bouvard et al. 2012). A significant reduction in angiogenesis was observed in mutant animals. It is important to note that in addition to endothelial cells, the Tie-2-lineage includes pericytes and subsets of endothelial progenitors and macrophages (Kisanuki et al. 2001). The decrease in angiogenesis observed in ec2-*Itgα6*-null was accompanied by a reduction in the recruitment of bone marrow-derived endothelial progenitor cells and Tie-2 expressing macrophages to the site of injury. Thus, the phenotype of ec2-*Itgα6*-null could be due to the loss of α_6 integrins in multiple cell types (Bouvard et al. 2012). Notably, the loss of expression of α_6 integrins did not affect the surface expression of other integrin heterodimers, or surprisingly, the expression of VEGFR2.

Tumor angiogenesis was also examined in ec2-*Itgα6*-null mice. Angiogenesis was inhibited following the subcutaneous injection of B16F10 melanoma cells (Bouvard et al. 2014). This was associated with a decreased recruitment of Tie-2 expressing macrophages to the site of tumor growth. Macrophages significantly contribute to the tumor microenvironment; thus, their loss could affect the

angiogenic response (Coussens and Werb 2002). Notably, pericyte coverage of tumor vessels was similar in wild-type and mutant mice (Bouvard et al. 2014). Although the reduction in macrophage recruitment to the site of tumor formation in ec2-*Itga6*-null mice, may explain the difference in the angiogenic responses in ec2-*Itga6*-null versus ec1-*Itga6*-null mice, it is difficult to understand how the loss of α_6 integrins affects VEGFR2 expression in one case and not the other.

A pro-angiogenic role for $\alpha_6\beta_1$ is supported by the finding that the $\alpha_6\beta_1$ integrin localizes invasive structures referred to as podosomes (Seano et al. 2014) These structures are integrin-extracellular matrix contact sites that aid in targeting proteases to sites of invasion (Linder and Aepfelbacher 2003; Gimona et al. 2008). Antibodies or RNAi approaches to inhibit the activity or expression of α_6 integrins suppressed VEGF-induced formation of podosomes and the recruitment of the metalloproteinase MT1-MMP (Seano et al. 2014). The importance of $\alpha_6\beta_1$ in the formation of these structures was further supported by the finding that podosome formation was impaired in aortic explants from ec2-*Itga6*- null mice, compared to littermate controls (Seano et al. 2014). A tight balance in integrin-laminin interactions appears critical to the formation of these structures in endothelial cells, as more VEGF-induced podosomes were observed in explants from *Lama4*-null mice compared to control (Seano et al. 2014).

As noted above, the integrin α_6 subunit heterodimerizes with either the β_1 or β_4 subunit. Thus, the effects of deletion of the α_6 subunit could be due to the loss of either $\alpha_6\beta_1$ or $\alpha_6\beta_4$. Since the β_1 subunit dimerizes with multiple α subunits, distinct roles for endothelial $\alpha_6\beta_1$ and $\alpha_6\beta_4$ have been inferred from the effects of targeted endothelial deletion of the β_4 integrin subunit gene (*Itgb4*) together with information on the endothelial expression of $\alpha_6\beta_4$ in different vascular beds.

The β_4 integrin subunit has a very large intracellular domain compared with other β subunit intracellular domains. The membrane-proximal region is known to connect the $\alpha6\beta4$ integrin to the intermediate filament cytoskeletal system (te Molder et al. 2021; Mercurio et al. 2001): keratin intermediate filaments in the case of epithelial cells and vimentin intermediate filaments in endothelial cells (te Molder et al. 2021; Homan et al. 1998, 2002). Mice null for the β_4 integrin subunit gene (*Itgb4*) die after birth due to extensive defects in epithelial tissues thought to be mostly due to the loss of a transmembrane connection between the basement membrane and the keratin intermediate filament system (Dowling et al. 1996; Van Der Neut et al. 1996). The membrane distal region is involved in the ability of $\alpha_6\beta_4$ to initiate intracellular signaling events to control cell behavior (te Molder et al. 2021; Mercurio et al. 2001). The global deletion of only the signaling portion of the β_4 subunit cytoplasmic domain was reported to inhibit angiogenesis in several types of assays (Nikolopoulos et al. 2004). The loss of the signaling portion of the β_4 cytoplasmic domain inhibited hypoxia-induced retinal angiogenesis. The subcutaneous injection of a number of different tumor cell lines, including B16F0 melanoma cells and Lewis lung carcinoma cells resulted in significantly decreased tumor angiogenesis in mutant mice compared to wild-type littermate controls (Nikolopoulos et al. 2004). The loss of the signaling portion of the β_4 cytoplasmic domain correlated with reduced nuclear localization of ERK and NFκB, which are known to become

activated downstream of $\alpha_6\beta_4$ and to promote endothelial sprouting and invasion (Nikolopoulos et al. 2004).

As indicated above, a conditional endothelial deletion mutant has been generated using Tie-2-dependent expression of Cre recombinase (Welser-Alves et al. 2013). This mutant is referred to as ec-*Itgb4*-null. No defects in vascular development were observed and analysis of endothelial expression of $\alpha_6\beta_4$ was reported to be restricted to arterioles (Welser-Alves et al. 2013). However, it is important to note that others reported the expression of $\alpha_6\beta_4$ in angiogenic vessels associated with tumors and dermal wound repair, although the $\alpha_6\beta_4$ expression appeared dynamic in angiogenic endothelial cells in some contexts (Nikolopoulos et al. 2004; Desai et al. 2013). Additionally, single-cell RNA sequencing of vascular cells from the murine lung, found β_4 mRNA widely expressed in endothelial cells in veins, venules and with higher expression in capillary endothelial cells (He et al. 2018), consistent with the ability of $\alpha_6\beta_4$ to contribute to angiogenesis. Also of note, the endothelial expression pattern of $\alpha_6\beta_4$ is similar to the endothelial expression of the $\alpha5$ chain of laminin (Patton et al. 1997; Sorokin et al. 1997). The same single-cell RNA sequencing study indicated that arterial endothelial cells of the brain vasculature had relatively higher levels of β_4 mRNA expression (He et al. 2018). The function of $\alpha_6\beta_4$ in brain endothelial cells was analyzed in a chronic hypoxia model (Welser-Alves et al. 2013). Although wild-type and ec-*Itgb4*-null mice did not exhibit a significant difference in the early angiogenic response to hypoxia, the number of arteriolar size vessels was significantly lower in ec-*Itgb4*-null mice, implicating $\alpha_6\beta_4$ in regulating arteriolar remodeling. Previous work indicated a role for TGF-β signaling in this process (Seki et al. 2003). Consistent with this previous work, the expression of the type-I TGF-β receptor, activin-like kinase 1 (ALK1) and the activation of downstream signaling protein was inhibited in ec-*Itgb4*-null mice, providing a mechanism for defective arterial remodeling in mutant mice (Welser-Alves et al. 2013). Nonetheless, with reports of $\alpha_6\beta_4$ expression in venules, capillaries, and angiogenic vessels, it will be interesting to examine the effect of an endothelial-specific deletion of *Itgb4* employing other models of adult angiogenesis.

3.7 Laminin-511 and $\alpha_6\beta_4$ in the Regulation of Endothelial Barrier Function

Previous studies demonstrated that the endothelial expression of $\alpha_6\beta_4$ integrin was upregulated during neuroinflammation (Milner and Campbell 2006). To understand the implication of the upregulation, the effect of the endothelial deletion of β_4 was examined using ec-*Itgb4*-null mice in experimental autoimmune encephalomyelitis (EAE), which is a mouse model for multiple sclerosis (Welser et al. 2017). Although the timing of the onset of disease was similar in mutant and wild-type mice, the clinical outcome was worse in ec-*Itgb4*-null mice, which exhibited increased leuko-cyte infiltration and was accompanied by the loss of the tight junction proteins

claudin-5 and ZO-1 from cell–cell junctions (Welser et al. 2017). These studies implicate the endothelial expression of $\alpha_6\beta_4$ in the protection of the blood–brain barrier by regulating the integrity of cell–cell junctions (Welser et al. 2017).

Leucocyte extravasation from the vasculature occurs predominately at postcapillary venule sites with both laminin-411 and laminin-511 expression (Hallmann et al. 2005). In the EAE model of neuroinflammation, T lymphocytes were found to transmigrate at areas of the endothelial basement membrane that contained laminin-411, but low levels of laminin-511 (Sixt et al. 2001; Wu et al. 2009). In *Lama4*-null mice, the expression of laminin-511 is ubiquitously present in endothelial basement membranes. In the EAE model, *Lama4*-null mice exhibited reduced T-cell extravasation across the blood–brain barrier and decreased disease severity, suggesting that laminin-511 promotes endothelial barrier function (Wu et al. 2009 38). Later studies demonstrated that laminin-511 regulates endothelial barrier function by stabilizing the localization of VE-cadherin at endothelial cell–cell junctions, known as adherens junctions, to limit leukocyte extravasation (Song et al. 2017). Endothelial adhesion to laminin-511 by β_1 and β_3 integrins regulates the localization of VE-cadherin by promoting the activation of the small GTPase protein RhoA (Song et al. 2017). Taken together these results suggest that multiple laminin-511-binding integrins may cooperate to promote the blood–brain barrier in the brain, with β_1 and β_3 integrins functioning to stabilize adherens junctions and $\alpha_6\beta_4$ stabilizing tight junctions. It will be interesting to know whether similar interactions function to limit inflammation in other vascular beds.

3.8   Dissecting the Contribution of Laminin-Binding Integrins to Processes Involved in Angiogenesis Using In vitro Angiogenesis Assays

There are several in vitro angiogenesis assays that have been used to identify molecular mechanisms involved in regulating the morphogenesis of endothelial cells into endothelial cords and tubes (Simons et al. 2015). Two organotypic co-culture angiogenesis assays have been particularly useful. These are the "bead sprout" and the "planar co-culture" assays (Nakatsu et al. 2003; Nakatsu and Hughes 2008; Bishop et al. 1999; Donovan et al. 2001; Bajaj et al. 2012; Li et al. 2018). In the bead sprout assay, endothelial cells, usually human umbilical vein endothelial cells, are adhered to gelatin-coated beads and embedded in a fibrin gel (Nakatsu et al. 2003; Nakatsu and Hughes 2008). Endothelial sprouting from beads can easily be observed microscopically by phase contrast or by confocal following immunostaining (Fig. 3.2). Fibrin is present in the provisional matrix of tumors and wounds and provides an adhesive ligand for migration through interactions with RGD-binding integrins (Eming et al. 2007). In the planar co-culture assay, endothelial cells are plated at low density onto dermal fibroblasts, which have grown to confluence (Bishop et al. 1999; Donovan et al. 2001; Bajaj et al. 2012). Endothelial
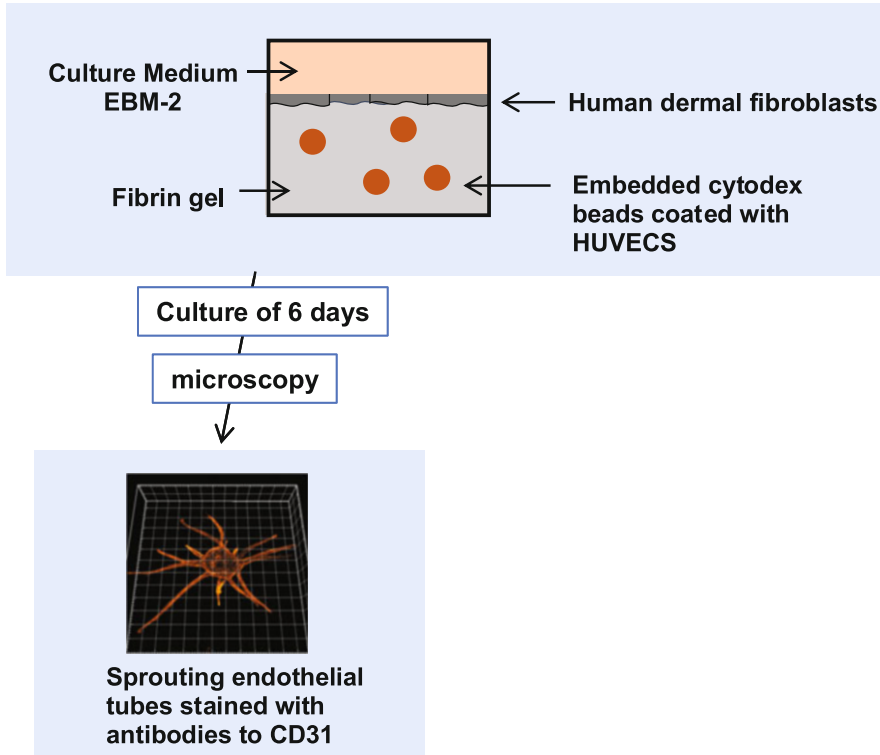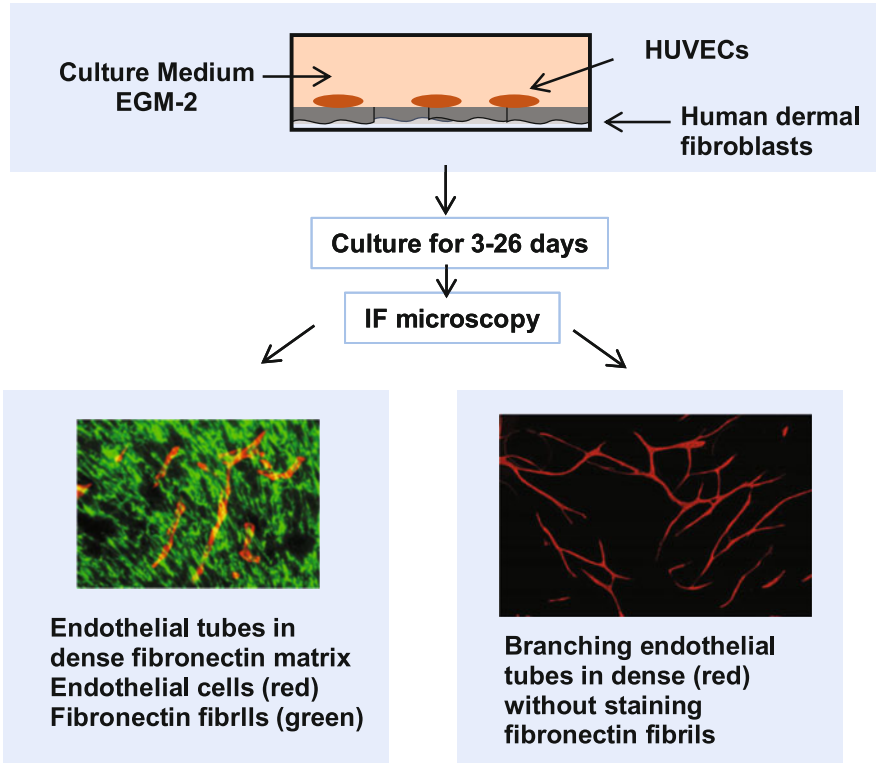
Fig. 3.2 Bead sprout assay. Shown is a schematic of the bead sprout assay

morphogenesis then occurs in the dense fibroblast-secreted ECM, containing fibro-nectin and fibrillar collagen, like what is observed in wound and tumor ECM (Eming et al. 2007) (Fig. 3.3).

Endothelial cells secrete collagen IV (Col IV) and laminin-411 and laminin-511 on their basal surfaces, as they form tubes in both co-culture assays (Fig. 3.4 and (Xu et al. 2020)). Nonetheless, without the demonstration that other components of basement membrane are present and without ultrastructure analysis, it is unclear whether a basement membrane is fully assembled in these assays. However, the expression of these laminins in co-culture allowed for the examination of their contribution to endothelial morphogenesis. RNAi-dependent depletion of either the α4 or α5 chains of laminin-411 or laminin-511 inhibited endothelial sprouting and the formation of endothelial tubes in both the bead-sprout and planar co-culture assays, respectively (Xu et al. 2020).

As discussed above, $\alpha_6\beta_1$ and $\alpha_6\beta_4$ bind to both laminin-411 and laminin-511. The same organotypic angiogenesis assays were used to explore the contribution of α_6 integrins to endothelial morphogenesis. RNAi-dependent depletion of α_6

Fig. 3.3 Planar co-culture assay. Shown is a schematic of the planar co-culture assay

integrins inhibited both endothelial sprouting and tube formation (Xu et al. 2020). The surface expression of the $\alpha_2\beta_1$, $\alpha_5\beta_1$ or $\alpha_v\beta_3$ integrins, previously shown to regulate angiogenesis, was not altered by depletion of α_6 integrins. Notably, the expression of $\alpha_3\beta_1$ was significantly increased (Xu et al. 2020), which is interesting as the endothelial-specific depletion of the α_3 subunit gene promoted angiogenesis (da Silva et al. 2010). However, depletion of $\alpha_3\beta_1$ did not enhance endothelial sprouting in our co-culture assays, as discussed further below (Xu and Laflamme 2022).

The bead-sprout assay allows the easy isolation of endothelial cells for the analysis of gene expression, as the layer of fibroblasts is easily removed (Li et al. 2018). Several genes have been associated with endothelial sprouting and angiogenesis, including *VEGFR2*, *CXCR4*, *ANGPT2*, *DLL4*, *PDGFB*, *NRP1*, *JAG1*, and *MMP14* (MT1-MMP) (de Smet et al. 2009; del Toro et al. 2010; Strasser et al. 2010). To determine whether endothelial α_6 integrins or their laminin substrates regulate the expression of any of these genes in the bead-sprout assay, RNAi technology was employed to deplete endothelial cells of either α_6 integrins, laminin-411 or laminin-511 and changes in gene expression were analyzed by qPCR. Defects in endothelial morphogenesis correlated with significant decreases in the expression of the
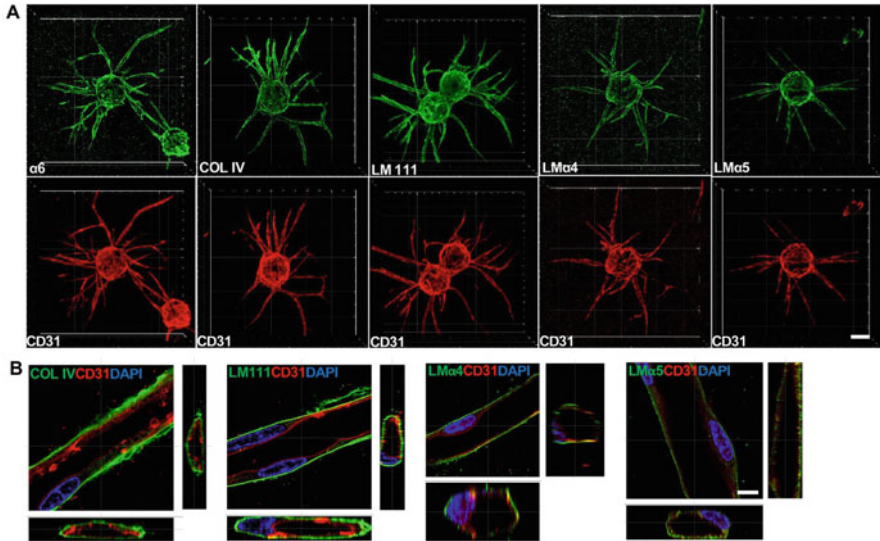
Fig. 3.4 Laminin-411 and laminin-511 are deposited by growing sprouts in organotypic cultures. (**a**) Confocal images of sprouting endothelial cells stained in green for collagen IV (COLIV), laminin β1 and γ1 chains (LM 111), laminin α4 chain (LM-α4) and laminin α5 chain (LM-α5). CD31 staining is shown in red. Scale bar, 100 μm. (**b**) High magnification confocal images of lumenized sprouts and basement membrane components expressed on the endothelial basal surface. Main images show xy sections, with xz and yz shown below. Nuclei are stained with DAPI. Scale bar, 6 μm (Xu et al. 2020)

chemokine receptor (*CXCR4*) and angiopoietin-2 (*ANGPT2*), as well as α5 chain of laminin-511, L*AMA5* (Xu et al. 2020). A similar decrease in the expression of chemokine *CXCR4* receptor and *ANGPT2* was observed when endothelial cells were depleted of the α5 chain of laminin-511, suggesting that the interaction of α_6 integrins with laminin-511 significantly contributes to the regulation of these pro-angiogenic genes (Xu et al. 2020). Importantly, the expression of recombinant *CXCR4* in α_6-depleted endothelial cells partially rescued endothelial tube formation (Xu et al. 2020). It will be interesting to test whether the expression of recombinant *ANGPT2* alone or in combination with recombinant CXCR4 is sufficient to restore endothelial morphogenesis in the absence of α_6 integrins and whether α_6 integrins regulate the expression of these pro-angiogenic genes in vivo. Notably, no overlap was observed in the genes regulated by α_6 integrins and laminin-411. Of note, Dll4 was upregulated in endothelial cells depleted of the α4 laminin chain, but this increase did not reach statistical significance (Xu et al. 2020).

To distinguish the contribution of $\alpha_6\beta_4$ in organotypic co-culture assays, RNAi was employed to inhibit the expression of the β_4 subunit (Xu and Laflamme 2022). The depletion of the $\alpha_6\beta_4$ integrin also inhibited endothelial sprouting. Interestingly, this was accompanied by a decrease in the expression of ANGPT2 mRNA. It is unclear whether $\alpha_6\beta_4$ acts alone or collaborates with $\alpha_6\beta_1$ in this regulation. The

depletion of $\alpha_6\beta_4$, however, did not alter the expression of CXCR4, implicating $\alpha_6\beta_1$ in this regulation (Xu and Laflamme 2022).

As discussed above, in vivo studies implicated the $\alpha_3\beta_1$ integrin as a negative regulator of angiogenesis (da Silva et al. 2010). Also, the expression of the $\alpha_3\beta_1$ integrin was enhanced in endothelial cells depleted of α_6 integrins in bead sprout assays (Xu et al. 2020), suggesting a potential inhibitory role in organotypic assays, as well. Surprisingly, RNAi-dependent depletion of $\alpha_3\beta_1$ also inhibited endothelial sprouting in co-culture angiogenesis assays. However, this inhibition was not accompanied by the downregulation of *CXCR4*, *ANGPT2*, or *LAMA5* expression, suggesting that $\alpha_3\beta_1$ regulates sprouting by distinct mechanisms. This is consistent with the inability of α_6 integrins to compensate for the loss of $\alpha_3\beta_1$ and vice versa. Notably, the expression of neuropilin-1 (NRP1) mRNA was most significantly decreased in α_3-depleted cells (Xu and Laflamme 2022). NRP1 is enriched in endothelial tip cells and functions as a co-receptor for VEGFR2 receptor signaling (Kofler and Simons 2015). Thus, the downregulation of NRP1 expression may be responsible for the inhibition of sprouting observed when endothelial cells are depleted of $\alpha_3\beta_1$. A comparison of gene expression regulated by the $\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$ integrins suggested that these laminin-binding integrins regulate distinct but overlapping sets of genes previously identified to be important in angiogenesis (Xu and Laflamme 2022).

The Tie-1-dependent deletion of α_3 resulted in increased mRNA expression of VEGFR2 in $\alpha_3\beta_1$-deficient endothelial cells (da Silva et al. 2010). However, depletion of $\alpha_3\beta_1$ in bead sprout assays did not result in increased VEGFR2 expression. In fact, RNAi-dependent depletion of the α_3 subunit resulted in a small but significant decrease in VEGFR2 with two of the three siRNA targeting sequences (Xu and Laflamme 2022). The differences in the effects of depleting the endothelial expression of $\alpha_3\beta_1$ in vivo versus in cell culture experiments may be due to the timing when $\alpha_3\beta_1$ expression is lost. As mentioned above, expression of $\alpha_3\beta_1$ in ec-Itga3-null mice appears to occur at some point after the initial response to angiogenic stimuli (da Silva et al. 2010); in contrast, in bead sprout assays, the expression of $\alpha_3\beta_1$ was already inhibited before the onset of the assay. Thus, this in vivo model may not be ideal to examine the role of $\alpha_3\beta_1$ during the initial steps of angiogenesis. Perhaps the use of a promoter that is active in quiescent endothelial cells to drive the inducible expression of Cre recombinase, such as the VE-Cadherin promoter may be better for this purpose.

The stable interaction of endothelial cells with their underlying basement membrane promotes vessel stability. As mentioned above, the endothelial expression of laminin-511 promotes endothelial barrier function (Song et al. 2017). Since α_6 integrins, but not $\alpha_3\beta_1$, were shown to regulate the endothelial expression of laminin-511, the effect of deleting α_6 integrins or $\alpha_3\beta_1$ was examined in the planar co-culture assay ((Xu et al. 2020) and Xu and LaFlamme, unpublished data). The inducible expression of either an α_6, α_3, or non-targeting shRNA was used to inhibit integrin expression after the formation of endothelial tubes and to analyze the effect on the maintenance of endothelial tube stability. Indeed, depletion of α_6 integrins after the establishment of endothelial tubes resulted in the loss of detectable
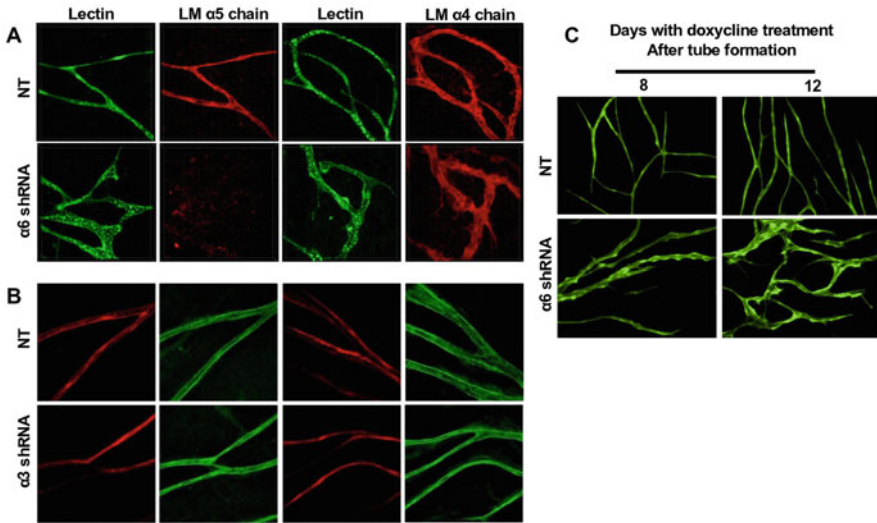
Fig. 3.5 Laminin-511 expression and tube morphology are regulated by α_6 integrins and not by the $\alpha_3\beta_1$ integrin. Planar co-cultures were set up with endothelial cells transduced with lentiviral vectors to direct the doxycycline-inducible expression of α_6 (**a**), α_3 (**b**) or non-targeting (NT) shRNA, together with fluorescent protein as a readout of shRNA expression. (**a**) After the formation of endothelial tubes, cultures were treated with doxycycline for up to 8 days and imaged. Induction of α_6-targeting led to the loss of laminin-511 expression and changes in endothelial tube morphology (Xu et al. 2020). (**b**) This did not occur following the induction of non-targeting or α_3-targeting shRNAs. (**c**) Changes in tube morphology are even more pronounced 12 days after induction of α_6 shRNA (Xu et al. 2020)

laminin-511 expression, as well as normal tube morphology (Fig. 3.5). Notably, the expression of laminin-411 was maintained as expected (Fig. 3.5 and (Xu et al. 2020)). The depletion of $\alpha_3\beta_1$ integrins did not affect laminin-511 expression or tube morphology (Fig. 3.5). This suggests an important role for the association of α_6 integrins and laminin-511 in stabilizing endothelial structures. Unfortunately, the role of $\alpha_6\beta_4$ in the process was not examined in a similar assay, as the endothelial expression of both $\alpha_6\beta_4$ and laminin-511 have been associated with promoting endothelial barrier function in vivo (Welser et al. 2017; Wu et al. 2009).

Laminin-binding integrins likely regulate angiogenesis by multiple mechanisms, as they are known to activate pathways to promote migration and invasion not only in endothelial cells, but in other cellular contexts. Additionally, the laminin-binding integrins, $\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$ form complexes with the tetraspanin CD151 (Sterk et al. 2002). *Cd151*-null mice exhibit decreased angiogenesis in multiple in vivo and ex vivo models, including tumor angiogenesis and aortic ring explant assays (Takeda et al. 2007). Additionally, integrin-laminin-dependent activation of multiple signaling pathways was decreased in *Cd151*-null endothelial cells that adhered to laminin (Takeda et al. 2007). In the case of $\alpha_3\beta_1$, CD151 forms a complex with the membrane-anchored matrix metalloproteinase MT1-MMP (*MMP14*) and $\alpha_3\beta_1$ to

promote appropriate proteolysis, and the loss of CD151 results in a dramatic loss of $\alpha_3\beta_1$/MT1-MMP association (Yanez-MO et al. 2008), suggesting an additional mechanism by which $\alpha_3\beta_1$ can contribute to angiogenesis.

3.9 Concluding Remarks

Mouse genetic models have provided important insights into mechanisms that both positively and negatively regulate angiogenesis and vessel stability. These studies have highlighted the importance of individual laminins and laminin-binding integrins in limiting angiogenesis and inflammation. Organotypic cell culture models have provided additional insight into the underlying molecular mechanisms. Cross-fertilization of ideas regenerated from in vivo and cell culture experimental approaches are likely to facilitate the identification of new therapeutic targets to limit pathological angiogenesis and inflammation.

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