

INVITED REVIEW

## Cell–matrix adhesion in vascular development

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**Summary.** Vascular development requires correct interactions among endothelial cells, pericytes and surrounding cells. These interactions involve many cell adhesion interactions, including cell–matrix interactions both with basement membranes and with surrounding extracellular matrices. Investigations of the contributions of these various interactions in vascular development and angiogenesis have been rather uneven and incomplete over the past 10–15 years. There has been considerable concentration on a few receptors, matrix proteins and proteolytic fragments with the goal of finding means to control angiogenesis. Many other potential contributors have received much less attention. Even for those molecules that have been subject to intensive investigation, our knowledge is incomplete. This review will survey the spectrum of extracellular matrix (ECM) proteins and cell–matrix adhesion receptors (particularly integrins) that are likely to contribute to angiogenesis and discuss what is known and not known about the roles of each of them.

**Keywords:** angiogenesis, extracellular matrix, integrins.

### Introduction

Vascular development is a complex multi-step process, involving multiple cell types that must interact with one another and with the surrounding cells and extracellular matrix. In addition to the need for endothelial cells to associate with each other and form tubular structures, processes involving cell migration and both cell–cell and cell–matrix adhesion, they must also attract pericytes to surround the endothelial tube and form a joint basement membrane between and around them. Furthermore, correct vascular organization requires interactions of the basic vascular unit (endothelium–pericytes–basement membrane) with the surrounding cells. One example of the importance of this outer layer of cellular interactions for vascular integrity comes from study of the defects in cerebral vasculature in mice

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lacking  $\alpha_v$  integrins [1–3]. Although initial results suggested that this defect might arise as a consequence of loss of  $\alpha_v$  integrins from endothelial cells and/or from pericytes, it turned out on further analysis that the defect lay in the absence of  $\alpha_v\beta_8$  integrin from astrocyte endfeet; this absence interfered with apposition of the glia with the invading vessels and led to vessel dilation and eventual rupture [2,3]. This example illustrates the need for recognition of the complexity of the intercellular interactions necessary for building and maintaining a properly formed vasculature.

In this review we will be concerned with cell–matrix adhesions contributing to these processes. Although a considerable amount of research has been devoted to this topic, we are still far from understanding the functions of the multiple matrix proteins and cell–matrix receptors and, in what follows, I will attempt to point out where further research is needed. Given the availability of genomic sequences, we have a reasonably good ‘parts list’ of the potential matrix proteins and receptors but attention has focused on a few of them at the expense of a systematic analysis. There has been a strong focus on endothelial cells, with less attention paid to pericytes and very little to the parenchymal cells surrounding the vessels. There has also been an emphasis on *in vitro* models of angiogenesis and there is need for a more comprehensive analysis of *in vivo* models using the power of mouse genetics, again with attention to the individual cell types involved, a problem now accessible using cell-type-specific mutation of genes of interest. I concentrate on areas in which we have ourselves expended most effort [Fibronectin (FN) and its receptors and the  $\alpha_v$  integrins] but survey results on other matrix proteins and integrins and note where more research would be valuable.

### Vascular extracellular matrix

Interactions of vascular wall cells (endothelial cells and pericytes) with extracellular matrix involve diverse extracellular matrix molecules, which differ to some degree among vessels and certainly differ depending on the state of the vessel (quiescent, injured or angiogenic) [4]. In resting vessels, endothelial cells are in contact with a basement membrane, which they share with pericytes in the case of small vessels. Vascular basement membranes contain laminins (predominantly laminin-8/ laminin411 and laminin-10/ laminin511), type IV collagens, perlecan, nidogens, collagen XVIII and von

Willebrand factor. The endothelial cells and pericytes in mature, quiescent vessels are non-proliferative and stably attached to the basement membrane. During vascular remodeling and angiogenesis, it is generally believed that the quiescent endothelial layer becomes 'activated' and endothelial cells breach the basement membrane and migrate into surrounding tissue containing different complements of extracellular matrix proteins, which can include collagens and FNs in interstitial extracellular matrix or fibrinogen and FNs in provisional matrices generated after vascular injury and during wound healing. Similarly, the extracellular matrices of tumors also contain fibrinogen and FNs. Other extracellular matrix (ECM) proteins encountered by endothelial cells and pericytes include vitronectin, thrombospondins and tenascins. The effects of ECM on vascular wall cells therefore differ greatly, depending on the state of the vessel and, very probably, to a lesser degree among different vessels. It is evident that the switch from quiescence (adherent to laminins and probably other basement membrane proteins, stably assembled into tubes) to the angiogenic state (migratory, invasive, tube remodeling and formation) involves marked changes in the cell-matrix interactions in which the cells are involved. It is also evident that different sorts of angiogenesis probably involve different forms of ECM and therefore different cell-matrix interactions. This is undoubtedly one reason why there is, as yet, no all-encompassing hypothesis concerning the cell-matrix adhesions that are important for angiogenesis; the likelihood is that there are multiple such interactions that differ in the course of a single angiogenic process and between angiogenesis in different situations (e.g. embryonic, retinal, tumor or wound healing angiogenesis).

Knockout mice lacking many of the basement membrane proteins listed above have been generated. By and large, they have not lent much support to hypotheses implicating those proteins in vascular development [4–6], although in many cases, only developmental angiogenesis has been assessed and further research could, yet, reveal more subtle defects. Examples of knockouts showing no obvious defects in angiogenesis include nidogens, perlecan, vitronectin, and von Willebrand factor. The absence of any obvious angiogenic defects could, of course, arise from the existence of overlapping functions among related (or even unrelated) proteins or from compensation in response to the ablation of a given gene. These two different phenomena (overlapping function and compensation) are frequently lumped together under the rubric of 'redundancy' but this usage is not helpful and it is instructive to keep the two concepts distinct; one is a consequence of a natural overlap in the functions of two genes in a given process, the other is a response to perturbation in response to a mutation – it may or may not be informative of a natural compensatory effect. In either event, the failure to observe an angiogenic defect does not rule out a role for the gene in question; it does, however, show clearly that the gene is not *essential*. In contrast, a defect in vascular development as a consequence of deletion of a given gene provides strong justification for inferring a role, as is the case for FNs and thrombospondins. Mutation of FNs or their

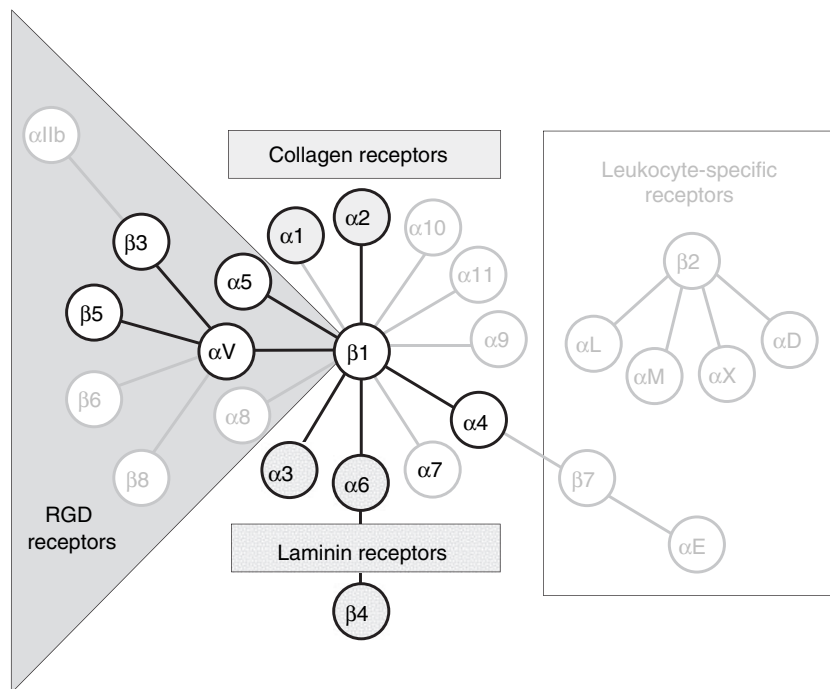
receptors leads to clear vascular and angiogenic defects during embryonic development [1,7–14]. In contrast, deletion of thrombospondins produces little in the way of defects in vascular development but does implicate these proteins as endogenous inhibitors of angiogenesis [15–22]. We will return to discussions of FNs and thrombospondins in later sections.

### Extracellular matrix receptors

Each of the many ECM proteins of vascular basement membranes or in the ECM during angiogenic sprouting has cell surface receptors, predominantly of the integrin family, although other ECM receptors (e.g. dystroglycan, GPIb, GPVI, DDR collagen receptors) are also known. In this brief review, I concentrate on integrins for lack of space and because they have been the most intensively investigated but these other possible matrix receptors should not be ignored in future research; a thorough inventory of the cell-matrix adhesion receptors on endothelial cells and pericytes of different types and in different states would be very useful.

Among the integrins, nine ( $\alpha 1\beta 1$ ,  $\alpha 2\beta 1$ ,  $\alpha 3\beta 1$ ,  $\alpha 4\beta 1$ ,  $\alpha 5\beta 1$ ,  $\alpha 6\beta 1$ ,  $\alpha 6\beta 4$ ,  $\alpha \nu\beta 3$ ,  $\alpha \nu\beta 5$ ) have been implicated to one degree or another in angiogenesis 23–29 (Fig. 1). These include collagen receptors ( $\alpha 1\beta 1$ ,  $\alpha 2\beta 1$ ), laminin receptors ( $\alpha 3\beta 1$ ,  $\alpha 6\beta 1$ ,  $\alpha 6\beta 4$ ), FN receptors ( $\alpha 4\beta 1$ ,  $\alpha 5\beta 1$ ) and the pair of  $\alpha \nu$  receptors ( $\alpha \nu\beta 3$ ,  $\alpha \nu\beta 5$ ), which have received the most attention (see below). Each of these receptors has been described on endothelial cells (with much less information available about their expression on pericytes), although it must be noted that it should not be assumed that all endothelial cells express the same set of integrins; indeed, it is clear that many are regulated during angiogenesis. It might be expected, based on the discussion above, that the laminin receptors would play their most prominent role in *quiescent* vessels. However, there is good evidence that  $\alpha 6\beta 4$  plays a role in sprouting angiogenesis [30], consistent with evidence that this integrin plays a role in the migration of epithelial cells. Similarly, some results implicate  $\alpha 3\beta 1$ -laminin 411 interactions in angiogenesis [31] and the tetraspanin CD151, a close partner of  $\alpha 3\beta 1$ , has been reported to play a role in angiogenesis [32], supporting the idea that  $\alpha 3\beta 1$  may also. Vascular endothelial growth factor (VEGF) up-regulates  $\alpha 6\beta 1$  and antibodies and siRNA treatments directed against  $\alpha 6\beta 1$  inhibit angiogenesis *in vivo* and endothelial functions *in vitro* [33]. Mice deficient in either  $\alpha 3\beta 1$  or  $\alpha 6\beta 1$  show no obvious deficits in developmental angiogenesis but they have not been extensively tested for other forms of angiogenesis; as we see below, different results can often be observed depending on exactly which angiogenic response is investigated.

Several lines of evidence implicate the collagen receptors,  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$ , in angiogenesis. They are up-regulated by angiogenic growth factors [34,35] and function-blocking antibodies inhibit angiogenesis in several *in vivo* models [36]. Furthermore, mice deficient in  $\alpha 1\beta 1$  show compromised tumor angiogenesis, apparently as a consequence of increased levels/activity of matrix metalloproteinases cleaving plasminogen to



**Fig. 1.** Those integrins reported to be expressed on endothelial cells are marked in bold; other integrins are greyed out. The integrins fall into subfamilies as marked. Integrins are significantly regulated by angiogenic growth factors and not all endothelial cells necessarily express all the integrins shown. Particular attention has been paid to the  $\alpha v$  integrins, which bind to many ligands [including fibronectin (FN)] and to the more specific FN receptors,  $\alpha 5\beta 1$  and  $\alpha 4\beta 1$  (although each of those can also bind other ligands). The collagen ( $\alpha 1\beta 1$ ,  $\alpha 2\beta 1$ ) and laminin ( $\alpha 3\beta 1$ ,  $\alpha 6\beta 1$ ,  $\alpha 6\beta 4$ ) receptor integrins have received less attention, although evidence also implicates them. It is much less clear which integrins are expressed on pericytes and it is clear in a few cases that integrins on cells surrounding the vessels also contribute to vascular structure. Figure adapted from Hynes R.O., *Cell* 110:673–687 (2002).

angiostatin, an inhibitor of angiogenesis [37]. Mice deficient in  $\alpha 2\beta 1$  show no obvious defects in developmental angiogenesis [38]. Clearly,  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$  could have overlapping or compensatory roles in angiogenesis but mice doubly deficient in both these integrins have yet to be studied.

Thus, despite the fact that the laminin and collagen receptor integrins have not been investigated as extensively as have FN receptors and  $\alpha v$  integrins (see below), it seems clear that they do participate and more intensive study of their roles and those of their ligands should prove productive.

### Inhibitors of angiogenesis

As with any developmental or homeostatic process, angiogenesis must be subject to negative feedback limiting its extent and,

*a priori*, one would expect the presence of endogenous angiogenic inhibitors. There is also considerable interest in discovery and development of inhibitors of angiogenesis for use in therapy of cancer, retinal angiogenesis, etc. [39–42]. Several ECM proteins or fragments thereof have been implicated as negative regulators of angiogenesis [40–42]. Some of these proposed ECM-derived angiogenic inhibitors are better validated than others (Table 1).

The best established are thrombospondins 1 and 2 [15–22]. Both have been shown to act as negative regulators of angiogenesis and tumor growth *in vivo* and of endothelial functions *in vitro*. Knockout and transgenic mice have confirmed their role as endogenous inhibitors *in vivo*. Fragments of TSP-1 containing type 1 TSP repeats induce apoptosis of endothelial cells *in vitro*, acting through the cell surface

**Table 1.** Candidate ECM-derived inhibitors of angiogenesis

Proposed inhibitor	Source	Inhibition of EC functions <i>in vitro</i>	Inhibition of angiogenesis <i>in vivo</i>	Genetic ablation blocks antiangiogenic effects <i>in vivo</i>	Proposed receptor	Dependence on receptor shown <i>in vitro</i>	Dependence on receptor shown <i>in vivo</i>
Thrombospondins		+	+	+	CD36		
TSP-1/TSP-2							
Endostatin	Collagen $\alpha$ (XVIII)	+	+	+	$\alpha 5\beta 1$ integrin?		
Arresten	Collagen $\alpha 1$ (IV)	+	+	+	$\alpha 1\beta 1$ integrin	+	KO
Canstatin	Collagen $\alpha 2$ (IV)	+	+				
Tumstatin	Collagen $\alpha 3$ (IV)	+	+	+	$\alpha v\beta 3$ integrin	+	KO
Endorepellin	Perlecan	+	+				
Anastellin	Fibronectin	+	+		Fibronectin		KO

receptor CD36 [43], although proof that the *in vivo* functions depend on CD36 remains lacking. TSP-1 also inhibits MMP-9 and its release of VEGF from basement membrane; this could provide a second mechanism for inhibition of angiogenesis by TSP-1 [19], although, once again, the formal genetic proof that TSP-1 inhibition depends on MMP-9 is lacking. As MMP-9 has both proangiogenic (release of VEGF) and antiangiogenic (release of tumstatin and maybe other ECM fragments, see below) effects, that proof may be difficult to obtain.

Basement membrane collagens have also been described as sources of angiogenesis inhibitors, the first being collagen XVIII; a proteolytic fragment of the C-terminal domain of this collagen, endostatin, has been studied for a number of years as an inhibitor of angiogenesis [39]. Endostatin has been reported to bind to integrins ( $\alpha 5\beta 1$  and  $\alpha v\beta 3$ ) but it is far from clear whether or not those receptors mediate its inhibitory effects on angiogenesis. For example, no dependence on these receptors for function has been demonstrated and some experiments actually show that  $\alpha v\beta 3$  is *not* required for its effects either *in vitro* or *in vivo* [44,45]. It has also been reported that plasma FN and vitronectin (themselves ligands for the two suggested integrin receptors) are necessary for the *in vivo* effects of endostatin [46]. So the mechanism of action of endostatin remains obscure.

Several fragments of type IV collagen subunits have been described as inhibitors of angiogenesis [40,41]. The most thoroughly investigated is tumstatin, an MMP-generated fragment of the C-terminal domain of  $\alpha 3(\text{IV})$ . Tumstatin binds to integrin  $\alpha v\beta 3$  on endothelial cells *in vitro* and inhibits cap-dependent protein synthesis in a fashion dependent on  $\alpha v\beta 3$  integrin [44]. Furthermore, *in vivo*, levels of tumstatin in the blood are dependent on the presence of collagen  $\alpha 3(\text{IV})$  and MMP-9 and inhibition of tumor angiogenesis and growth are dependent also on the presence of  $\alpha v\beta 3$  integrin [45]. Thus, tumstatin appears to be a well-validated endogenous inhibitor of angiogenesis, dependent on the presence of its precursor (collagen  $\alpha 3(\text{IV})$ ), a cleavage enzyme (MMP-9) and a specific receptor ( $\alpha v\beta 3$  integrin) on endothelial cells [45]. Additional similar proteolytic fragments from other type IV collagen subunits have also been suggested as angiogenesis inhibitors [40,41] (Table 1). Arresten, derived from collagen  $\alpha 1(\text{IV})$ , binds  $\alpha 1\beta 1$  and its antiangiogenic functions are blunted in mice deficient in  $\alpha 1\beta 1$  [47]. However, interpretation of this result is complicated by the fact that  $\alpha 1\beta 1$  suppresses generation of angiostatin by cleavage from plasminogen [37] so that any effects on angiogenesis arising from ablation of  $\alpha 1\beta 1$  can arise from multiple causes. Canstatin, another collagen IV fragment, has also been suggested as an antiangiogenic factor but not extensively analyzed.

Endorepellin, an 80 kDa C-terminal proteolytic fragment of the ubiquitous basement membrane protein, perlecan, also has antiangiogenic activity [48]. Endorepellin blocks endothelial cell migration and tube formation *in vitro*, and inhibits growth factor-induced angiogenesis in Matrigel plugs and the CAM assay. Endorepellin binds to  $\alpha 2\beta 1$  integrin, which leads to endothelial cell actin cytoskeletal disassembly and focal contact

disruption. Interestingly, endorepellin can also bind to endostatin and counteract its antiangiogenic activity, suggesting that a balance exists between antiangiostatic proteins. Similarly, degradation of FN (a proangiogenic protein, see below) generates an angiogenesis inhibitor called anastellin [49]. Both anastellin and endostatin require the circulating forms of plasma FN and/or vitronectin for their antiangiogenic activities *in vivo* [46], introducing further complexity to angiogenesis regulation.

The model in which vascular basement membrane matrix molecules and, in particular, fragments of them that might be generated during matrix remodeling, act as negative feedback regulators of angiogenesis is appealing, as is the idea that they bind to specific integrins, which have themselves been implicated in angiogenesis. However, as illustrated by this overview and by the summary in Table 1, many of the necessary experiments (especially *in vivo* validation of the endogenous roles of these fragments and their putative receptors) still need to be completed before the generality of this appealing hypothesis can be accepted. It is also important to note that different vascular basement membranes and the ECM around vessels differ at different sites and that not all the proposed inhibitors are necessarily present in any given system. The same goes for the proposed receptors. It will be necessary to demonstrate presence and involvement for each case. Irrespective of their endogenous roles, these ECM fragments may yield valuable pharmacological agents.

### The particular case of $\alpha v$ integrins

The  $\alpha v$  integrin subfamily comprises five members:  $\alpha v\beta 1$ ,  $\alpha v\beta 3$ ,  $\alpha v\beta 5$ ,  $\alpha v\beta 6$ , and  $\alpha v\beta 8$  (24) (Fig. 1) and this subset of integrins has received more attention as potential regulators of angiogenesis than any others [1,6,23,25–27,29,50–52]. Despite this attention, their roles remain controversial, most particularly because of discordance between pharmacological (antibodies, peptides, other small molecules) and genetic (mouse knockouts, human mutations) studies. Part of the reason for the attention is that the closely related integrin,  $\alpha \text{IIb}\beta 3$ , is an excellent target for antithrombotic drugs (abciximab, eptifibatid) and it is an appealing idea that similar strategies targeting  $\alpha v$  integrins might be effective in antiangiogenesis. However, this remains an unfulfilled hope and the exact functions of these integrins in angiogenesis are still unclear; it is not even certain whether they are positive or negative regulators [25–27].

The levels of  $\alpha v\beta 3$  or  $\alpha v\beta 5$  proteins are up-regulated on cultured endothelial cells or on angiogenic blood vessels in response to many different angiogenic growth factors or cytokines, as well as in the vasculature of some but not all tumors. Neovascularization in various of these systems is inhibited upon addition of function-blocking antibodies or peptide-based drugs targeting  $\alpha v$  integrins, particularly  $\alpha v\beta 3$  and/or  $\alpha v\beta 5$ , which are expressed on endothelial cells. In many cases, these agents cause endothelial cell apoptosis and the original idea was that they were acting as antagonists of  $\alpha v$  integrin-mediated adhesion and inducing anoikis.

However, this simple idea is not well supported by genetic ablation studies in which one or more integrin subunits are removed. Genetic ablation of the  $\beta 3$  or  $\beta 5$  genes, or of both of them, does not lead to overt angiogenic abnormalities [53–55]. Indeed, mice null for both  $\alpha v\beta 3$  and  $\alpha v\beta 5$  are viable and fertile, and blood vessel development proceeds normally in these mutants [55]. Most surprisingly, retinal angiogenesis in response to hypoxic shock as well as s.c. tumor growth and neovascularization are actually enhanced in  $\beta 3$ -null or  $\beta 3/\beta 5$ -null mice [55], although a recent paper reported a deficit in angiogenesis of intracranial gliomas in  $\beta 3$ -null mice [56]. There are no reports of defects in angiogenesis in human Glanzmann thrombasthenia patients and no reported phenotypic differences between GT patients who lack  $\alpha IIb\beta 3$  and those who lack  $\beta 3$  (and, therefore, *both*  $\alpha IIb\beta 3$  and  $\alpha v\beta 3$ ), also implying no major defects in vascular development or angiogenesis in humans lacking  $\alpha v\beta 3$  integrin.

Furthermore, mice lacking the  $\alpha v$  gene, and thus lacking expression of all five  $\alpha v$  integrins, do not develop widespread vascular defects [1–3]. Approximately 70% of  $\alpha v$ -null embryos survive to mid-gestation, having developed a normal vasculature, but die by embryonic day 11 probably because of placental abnormalities. Those  $\alpha v$ -null embryos that do not succumb to the placental defects and survive beyond mid-gestation are carried to term and develop a largely normal vasculature. However, they do develop cerebral hemorrhage, a cleft palate, and die within a day after birth. The hemorrhage is not because of primary endothelial or pericyte defects, but rather involves defective associations between angiogenic cerebral blood vessels and central nervous system glia, where  $\alpha v\beta 8$  integrin is expressed [2,3]. Mice null for the  $\beta 8$  gene develop vascular and other defects very similar to those of the  $\alpha v$ -null mice [57]. Importantly, Cre/lox-mediated ablation of  $\alpha v$  integrins in all endothelial cells fails to produce vascular defects, showing conclusively that those integrins are not necessary in endothelial cells for normal vascular development and angiogenesis [3].

Therefore, the apparent significance of  $\alpha v\beta 3$  and  $\alpha v\beta 5$  in neovascularization based on the antibody and peptide inhibition results vs. those based on genetic ablation data are quite conflicting. It is important to note here that this type of discrepancy between genetic and pharmacological results is the exception rather than the rule, even for integrins – indeed even for  $\alpha v$  integrins. As I will discuss below, the genetic and pharmacological results on  $\alpha 5\beta 1$  integrin and FN are in good agreement; both sets of data implicate this receptor-ligand pair in vascular development. Similarly, genetic and pharmacological data (using many of the same mutants and drugs) are in complete agreement concerning a role for  $\alpha v\beta 3$  in the functions of osteoclasts [58]. Therefore, the discrepancies concerning the roles of  $\alpha v$  integrins in angiogenesis are particular to this system. Interestingly, in  $\beta 3$ - or  $\beta 3/\beta 5$ -null animals there is up-regulation of signaling events mediated by the Flk-1 receptor tyrosine kinase, suggesting that, in the absence of  $\beta 3$  and  $\beta 5$  integrin expression, there is a compensatory response involving enhanced VEGF/Flk-1 signal transduction [55]. Compensation

could be one explanation for the discrepancy between the genetic and the pharmacological results. However, the genetic data do show conclusively that angiogenesis is not dependent on  $\alpha v$  integrins and it should be noted that, notwithstanding many reports of effectiveness of the antibodies and small molecules targeting  $\alpha v\beta 3$  and/or  $\alpha v\beta 5$ , there are also many reports of failures, even given the natural tendency not to publish negative results.

How should we think about this? Clearly there is something special about the functions of  $\alpha v$  integrins in endothelial cells; they are up-regulated in many angiogenic situations but, equally clearly, they are not essential for angiogenesis. What are they doing? Perturbation by antibodies and drugs or by genetic manipulations often yields phenotypic consequences but some of those imply a positive role for  $\alpha v$  integrins in angiogenesis, while others (e.g. the enhanced angiogenesis in mice deficient in  $\alpha v$  integrins) suggest instead a negative regulatory role. I have suggested elsewhere [25] that one way to bring the results into concordance would be to hypothesize that the antibodies and drugs are acting as *agonists of negative signals* rather than as *antagonists of positive signals*. The consequences of tumstatin binding to  $\alpha v\beta 3$  [44,45] are markedly different from those arising from binding of a classic ECM ligand such as vitronectin.  $\alpha v\beta 3$  is a very promiscuous receptor, binding to most proteins containing an RGD sequence as well as to others which do not (e.g. tumstatin) and, as noted, the downstream signaling consequences of engagement of different ligands are not necessarily the same – some may have positive effects and others may have negative effects. Another issue to consider is the possibility of crosstalk among integrins and between integrins and growth factor receptors [59].  $\alpha v\beta 3$  has been implicated in crosstalk with VEGF and PDGF receptors [60,61], with  $\alpha 5\beta 1$  integrin [62] and through it with Tie2 [63]. It could be that  $\alpha v$  integrins are designed to play different roles in different phases or types of angiogenesis, perhaps depending on which ligands are engaged and/or on associations and/or crosstalk with other receptors. Such a situation is clearly the case for receptors controlling attraction and repulsion of neuronal growth cones [64,65]. It seems to me evident that a deeper analysis of the diverse ligands and signal transduction pathways of  $\alpha v$  integrins will be necessary to sort out their true functions in angiogenesis. Such an understanding will also be essential in order to design appropriate drugs to affect angiogenesis via these receptors.

### The particular case of FNs and their receptors

Ablation of the genes for FN [7–9] or for the  $\alpha 5$  subunit of the specific FN receptor,  $\alpha 5\beta 1$  integrin [10,11], causes early embryonic lethality with defects in vascular development (heart and vessels). These early results clearly implicated FN as a key player in vascular development and this ECM protein remains the one most clearly involved.

FN is strongly expressed around developing vasculature [66,67] and, although the levels around quiescent mature vessels are reduced [68], there is marked up-regulation around reactive

angiogenic vessels during wound healing [69,70], around and within tumors [71] and in many pathological states such as atherosclerosis, myocardial infarction, trauma and fibrosis [72]. In FN-null mouse embryos, there are failures in vasculogenesis, vascular remodeling and cardiac development [7–9] and many of these defects are also observed in zebrafish FN mutants [73]. The defects in  $\alpha 5$  integrin-null embryos are very similar, although somewhat less severe [10,11]. This is presumably because there are additional FN receptors,  $\alpha 4\beta 1$  and  $\alpha v$  integrins. To test this possibility, embryos doubly deficient in  $\alpha 4/\alpha 5$  and  $\alpha v/\alpha 5$  were constructed [14]. Although there was no enhancement of phenotype in the  $\alpha 4/\alpha 5$  double nulls, the  $\alpha v/\alpha 5$  double nulls showed more severe defects even than the FN-nulls. This result is consistent with the idea that  $\alpha 5\beta 1$  and  $\alpha v$  integrins serve as somewhat redundant FN receptors in vascular development, but the fact that  $\alpha v$  integrins have so many other ligands complicates the interpretation. Nonetheless, it is clear that FN and its receptors ( $\alpha 5\beta 1$  and possibly others) play important roles in vascular development. This conclusion has been supported by additional studies of angiogenesis in embryoid bodies and teratocarcinomas [12,74], by the up-regulation of both FN and  $\alpha 5\beta 1$  in response to angiogenic growth factors [75] and by antibody and peptide inhibitor studies [75], which confirm a role for the FN- $\alpha 5\beta 1$  ligand-receptor pair in angiogenesis. As mentioned earlier, this clear concordance of genetic and pharmacological data is in marked contrast with the complexity of the results on  $\alpha v$  integrins. An antibody blocking the  $\alpha 5\beta 1$ -FN interaction is now being investigated as a potential antiangiogenic drug [76].

Although the combination of mutations in  $\alpha 5\beta 1$  and  $\alpha 4\beta 1$  showed no enhancement of the early defects shown by the individual mutations, showing that these two receptors do not have overlapping functions in early angiogenesis, there are other data implicating  $\alpha 4\beta 1$  in vascular development.  $\alpha 4\beta 1$ -VCAM-1 interactions play a role in allantois-chorion fusion during formation of the placenta [13]. At a slightly later stage,  $\alpha 4\beta 1$  is expressed on pericytes in cranial mesenchyme surrounding the developing brain and in its absence pericytes fail to spread uniformly along the cranial vessels, leading to vascular defects [77]. This is apparently because of a failure of  $\alpha 4\beta 1$ -FN interactions [77]. In another study of endothelial-mural cell interactions, it was reported that, in proliferating vascular cells, VCAM-1 on the mural cells interacts with  $\alpha 4\beta 1$  on the endothelial cells to mediate apposition of the two cell types and proper vascular development in the chicken CAM [78]. While these two studies appear to address differing roles for  $\alpha 4\beta 1$  integrin in vascular development, each confirms a role for this receptor and its participation, perhaps in several different roles, needs further investigation.

Another role for FN in vascular development is in the development of the heart. In FN-null embryos, organization of the myocardium and endocardium are compromised, the exact degree of defect being a function of the genetic background [7–9]. Using SNP mapping a strain-specific modifier has been mapped to a short region of chromosome 4, in which reside around 20 genes, one of which interacts in some way with the FN

gene during formation of the midline heart [79]. No integrins or other obvious candidate receptors map to this interval.

The final aspect of FN's involvement in vascular development that I wish to address concerns the role of alternative splicing in the functions of FN. FN is alternatively spliced at three regions, which can be either completely (EIIIA and EIIIB, both type III repeats) or partially (the V region), included or excluded, generating up to 12 different variants in rodents and 20 in humans [72]. These splice variants are spatially and temporally differentially expressed in development and disease [66–72]. They are strongly expressed around angiogenic vessels but not around quiescent adult vessels. Most particularly, the EIIIA+ and EIIIB+ isoforms are strongly expressed around developing blood vessels both in embryos and in postnatal angiogenic vessels during wound healing and tumor formation [66–72]. They are also expressed in pathological situations such as myocardial infarctions and atherosclerosis; indeed, in most situations where FN is up-regulated in response to trauma or disease, inclusion of the EIIIA+ and EIIIB+ is also up-regulated. The strong expression of the EIIIA+ and EIIIB+ isoforms in tumor vasculature has led to anti-EIIIB antibody being used as a tumor-targeting and a tumor-imaging reagent [80,81]. This pattern of expression suggested that these isoforms might play some specific role in angiogenesis. However, mice lacking either the EIIIA or the EIIIB segments showed no obvious defects in vascular development [82–84] and specific testing for functions in retinal or tumor vasculature failed to reveal any roles [71]. We have recently generated a mouse strain lacking both these segments in *cis* and the double mutant does show major vascular defects (S. Astrof, R. O. Hynes *et al.*, unpubl. data). It is clear therefore that the strong association of expression of these splice variants of FN with angiogenic vessels does indeed reflect a role for them in angiogenesis. It remains to be seen exactly what are their functions. What do these segments do? There have been reports that the EIIIA segment binds to  $\alpha 4\beta 1$  and  $\alpha 9\beta 1$  integrins [85] and this could converge with the results on involvement of  $\alpha 4\beta 1$ , mentioned above. It is also possible that they bind to other molecules such as adjacent cell surface receptors or growth factors [86] and play some role in the integration of FN-integrin-mediated adhesion with signal transduction.

## Conclusions and prospects

Although it seems as if all the questions concerning cell-matrix adhesion during angiogenesis have been actively addressed in the past decade or more, a closer look at the results available suggests that we have only begun to scratch the surface. Considerable attention has been lavished on the  $\alpha v$  integrins and yet their roles in angiogenesis seem as obscure as ever. There is little doubt that they are playing some important roles and, given the successes with other integrin-directed therapies, they still appear to be attractive targets for drug development. However, it is clear that we need a better understanding of their functions: are they pro- or antiangiogenic or perhaps both at

different times and places? There has been a tendency to extrapolate from limited data sets, leading to overly simplistic interpretations of the results. The discordance between the genetic and pharmacological results on this subfamily of integrins is intriguing, suggesting that we are missing some crucial pieces of the puzzle. In contrast, FN and the  $\alpha 5 \beta 1$  integrin clearly seem to be proangiogenic and offer good prospects for targeted antiangiogenic therapy. While the basic results implicating this ligand-receptor pair have been available for more than a decade, surprisingly little effort has gone into the development of drugs targeting their interaction; one can only hope that this will soon be remedied. Even more neglected have been the collagen and laminin receptors among the integrins and these also seem worthy of more intensive investigation. Interest in proteolytic fragments of extracellular matrix has been significant but it is clear that much further research is necessary to determine which of these are truly useful targets and whether or not the general idea that they constitute a negative feedback loop controlling angiogenesis is valid and to what degree. Angiogenesis is a complex affair and a more systematic approach encompassing multiple angiogenesis models and addressing all the potential players and their roles on the several different cell types involved would seem in order.

### Disclosure of Conflict of Interests

The author states that he has no conflict of interests.

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