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## Integrins as “Functional Hubs” in the Regulation of Pathological Angiogenesis

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### Abstract

It is well accepted that complex biological processes such as angiogenesis are not controlled by a single family of molecules or individually isolated signaling pathways. In this regard, new insight into the interconnected mechanisms that regulate angiogenesis might be gained by examining this process from a more global network perspective. The coordination of signaling cues from both outside and inside many different cell types is required for the successful completion of angiogenesis. Evidence is accumulating that the multifunctional integrin family of cell adhesion receptors represent an important group of molecules that play active roles in sensing, integrating, and distributing a diverse set of signals that regulate many cellular events required for angiogenesis. Given the ability of integrins to bind numerous extracellular ligands and transmit signals in a bi-directional fashion, we will discuss the multiple ways by which integrins may serve as a functional hub during pathological angiogenesis. In addition, we will highlight potential imaging and therapeutic strategies based on the expanding new insight into integrin function.

### Keywords

Integrins; Angiogenesis; Functional Hubs; Extracellular Matrix

### Introduction

Angiogenesis can be characterized as an integrated set of cellular, biochemical and molecular processes by which new blood vessels are formed from pre-existing vessels [1]. Central to this process is the concept that networks of “soluble” and “solid state” information must flow in a bidirectional manner between the non-cellular microenvironment and different cell types involved in new vessel development [Figure-1]. The diverse cell types involved in angiogenesis must have the capacity to sense, assimilate, interpret, distribute and respond to complex streams of information in a meaningful way in order for blood vessels to form. A crucial feature in the integration and subsequent distribution of diverse networks such as the World Wide Web, electrical power grids, and cells signaling networks, is the concept of “functional hubs” [2,3]. Functional hubs can act to gather, interpret, integrate and distribute large amounts of complex information to control a given response to changes in bi-directional communication links. Due

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to the integrated nature of cellular signaling networks disrupting a “function hub”, as opposed to any one individual pathway within an integrated system may have a more pronounced effect [4]. Thus, developing novel approaches to selectively disrupt functional hubs, may lead to more effective strategies for controlling complex biological processes such as angiogenesis.

Given the number of networks that need to be organized and integrated for new vessels to form, functional hubs may be operationally important during angiogenesis. A major group of cell surface molecules that may serve as functional hubs during angiogenesis is the integrin family and these molecules will be the primary focus of our discussion [5]. Integrins are multifunctional heterodimeric adhesion receptors composed of  $\alpha$  and  $\beta$  chains derived from separate gene products [6]. These transmembrane receptors are broadly expressed in all cell types that play roles in angiogenesis. Given their transmembrane structure, ability to form associations with adaptor molecules, and finally, their ability to bind many extracellular ligands, these receptors are well positioned to serve as functional hubs [5,6].

Blood vessel formation regulates physiological processes such as embryonic development, wound healing, and reproduction [7–9]. While angiogenesis plays important roles in normal physiology, it also contributes to pathological processes such as diabetic retinopathy, rheumatoid arthritis and tumor growth [7–11]. Given the broad implications of angiogenesis in these processes, the possibility of modulating angiogenesis for therapy has driven the need for a more complete understanding of this complex cascade. Great strides have been made in understanding the molecular events that control angiogenesis and surprising new mechanistic insight is beginning to emerge. Several new studies have uncovered evidence that while certain angiogenesis inhibitors such as those targeting VEGF signaling may inhibit angiogenesis and slow primary tumor growth, they may also be associated with enhanced tumor cell invasion and metastasis [12,13]. While enthusiasm continues for targeting angiogenesis as a therapeutic modality, these and other opposing experimental results from genetic models and pharmacological inhibition studies illustrates the need for a more detailed understanding of this important process.

## Angiogenesis

While several mechanisms can lead to the formation of blood vessels including embryonic vasculogenesis and arteriogenesis, we will focus our discussion on angiogenesis and in particular, the roles of integrins as functional hubs in pathological neovascularization. Angiogenesis can be categorized into at least two types including sprouting and intussusceptive angiogenesis [14,15]. Sprouting angiogenesis involves growth factor stimulation leading to altered cell-cell and cell-extracellular matrix (ECM) interactions, which can enhance proliferation and invasion. These invading cells organize into cords that ultimately form lumens by a process of lumenogenesis [16]. The newly forming blood vessels undergo maturation and stabilization as they recruit supporting cells such as pericytes and smooth muscle cells. In contrast to sprouting angiogenesis, which is associated with extensive proliferation, intussusceptive angiogenesis involves the formation of transluminal tissue bridges that split vessels into higher order branches [4].

In simplistic terms, angiogenesis can be organized into three interconnected stages including an initiation phase, an invasive phase and a maturation phase. During the initiation phase, pro-angiogenic factors can stimulate unique patterns of gene expression leading to altered cell adhesion and proliferation. The invasive phase of angiogenesis can be characterized by enhanced ECM remodeling and invasion. In the maturation phase, endothelial cords undergo lumenogenesis, and recruit cells such as pericytes, fibroblasts, and bone marrow derived cells.

## Pathological Angiogenesis

While some of the basic regulatory molecules and cell types that control vessel development may be shared between normal and pathological angiogenesis, distinct differences also exist. These differences may contribute in part to the conflicting results sometimes observed between genetic models focused on normal embryonic vascular development and pharmacological inhibition studies of pathological angiogenesis. Studies have identified a variety of differences between normal and tumor vessels including alterations in the levels and composition of cell surface molecules. Using Serial Analysis of Gene Expression (SAGE), investigators have identified tumor endothelial cell markers that exhibited differential expression [17]. Endothelial cells isolated from certain tumors exhibited enhanced proliferative capacity, reduced ability to undergo senescence and enhanced resistance to serum deprivation as compared to quiescent endothelial cells [18]. Glioblastoma derived tumor endothelial cells were shown to exhibit enhanced resistance to chemotherapeutic drugs as compared to normal brain endothelial cells [19]. This chemoresistance was thought to be at least partly due to elevated levels of survivin [19]. It is interesting to point out that integrin  $\beta$ 1-mediated interactions with ECM proteins such as fibronectin have been suggested to regulate survivin [20]. In other studies, enhanced expression of ER chaperone protein GRP78 in brain tumor endothelial cells may contribute to their chemoresistance [21]. De-regulation of growth factor signaling in tumor endothelial cells is also thought to play an important role in chemoresistance [22]. Moreover, some endothelial cells isolated from tumors exhibited aneuploidy [23]. Intriguing new studies also demonstrate differences between tumor and normal endothelial cells in terms of how they respond to mechanical forces [24]. It is interesting to note again, that integrins are a major family molecules known to regulate a cell's response to growth factors and mechanical forces. Finally, a variety of differences also exist in the physical structures of normal and tumor vessels. The composition and integrity of the ECM including the basement membrane and interstitial matrix can be significantly altered between normal and tumor vessels.

Differences have been documented between the basement membrane components of embryonic vasculature and adult vasculature. During embryonic development the major type of laminin within basement membranes is largely laminin-8 which contains the  $\alpha$ 4 laminin chain [25]. In contrast, in postnatal mice, increased levels of Laminin-10 are deposited in basement membranes [25]. Interestingly, the  $\alpha$ 5 chain of laminin-10 has been suggested to be an isotype of laminin that has a freely exposed and integrin accessible RGD site capable of being recognized by  $\alpha$ v $\beta$ 3 integrin [26]. If  $\alpha$ v $\beta$ 3 recognition of this laminin RGD site plays a role in the ability of  $\alpha$ v $\beta$ 3 to regulate new vessel development, it would not be surprising that mice lacking  $\alpha$ v $\beta$ 3 exhibited minimal effects on embryonic vascular development since this potentially important ligand for  $\alpha$ v $\beta$ 3 is not highly expressed in this context. However, during adult pathological angiogenesis when Laminin-10 is strongly expressed along with other RGD containing provisional matrix proteins such as fibrinogen and vitronectin, antagonists of  $\alpha$ v $\beta$ 3 inhibit pathological angiogenesis [27]. Thus, when examining integrin function, the expression of their ligands should also be considered.

An additional example that illustrates the differential expression of integrin ligands in the vasculature includes the alternatively spliced forms of fibronectin containing EIIIA and EIIIB domains, which are expressed predominately during embryonic development and pathological angiogenesis, but normally lacking in adult vessels [28]. Collectively, these examples help highlight some of the more obvious differences in blood vessels that may contribute to conflicting results sometimes observed between genetic mouse models of integrin function in embryonic vasculogenesis and pharmacological inhibition during pathological angiogenesis.

Critical to the concept of viewing integrins as functional hubs is the appreciation of the diversity of molecules that integrins bind and regulate. Integrins can associate with a wide array of distinct families of molecules including growth factors and their receptors, cytoplasmic adaptors, signaling molecules, other cell surface adhesion receptors, matrix altering proteases, protease inhibitors, cell surface protease receptors, ECM components and anti-angiogenic ECM fragments [Figure-2]. Given that integration of the numerous signaling networks are required for the formation of new blood vessels, integrins appear well suited to serve as functional hubs.

## Integrin Structure, Ligand Binding, and Bi-directional Signaling

Integrins are receptors with at least 24 distinct members generated from 18  $\alpha$  and 8  $\beta$  subunits [29]. When describing integrin expression as it relates to angiogenesis, we often limit our discussion to endothelial cells and supporting mural cells. Given the evidence implicating many different cell types in angiogenesis, including bone marrow derived cells, fibroblast as well as tumor cells, the regulation of angiogenesis by integrins expressed in these cells may be significantly under estimated. A variety of integrin heterodimers containing  $\beta$ 1,  $\beta$ 3,  $\beta$ 4,  $\beta$ 5 and  $\beta$ 8 subunits have been shown to play roles in angiogenesis and experimental evidence supporting this notion comes from both genetic models and pharmacological inhibition studies [30].

It would be well beyond the scope of this review to discuss in depth integrin structure, ligand binding and signaling. However, in order to fully appreciate the multiple mechanisms by which integrins may regulate angiogenesis, we will briefly touch on some of the more basic features of integrin binding and signaling. Integrins are composed of an extracellular domain with both  $\alpha$  and  $\beta$  chains contributing to a ligand binding surface. The extracellular domains of both the  $\alpha$  and  $\beta$  chains are followed by transmembrane domains and short cytoplasmic tails. Structural studies have provided evidence that integrins exist in at least three different configurations, including a low affinity state characterized by a closed headpiece and a bent conformation. An intermediate affinity state characterized by an extended leg region with a closed headpiece and a fully extend conformation with an open headpiece characterizing the high affinity state have also been described [31].

The ability of integrins to bind and associate with various ECM proteins, transmembrane receptors and soluble ligands largely depends on their structural conformation and these distinct conformations are crucial for regulating both inside out and outside in signaling [32]. Studies demonstrate that the cytosolic proteins such as Talin and Kindlins are important control molecules in integrin function [33,34]. Exposure of a cryptic integrin-binding site within talin is thought to promote interactions with the  $\beta$  integrin tails which may facilitate separation of the  $\alpha$  and  $\beta$  chains [33]. This physical separation may propagate structural changes through the transmembrane region that ultimately allow extension of the integrin leg-like domains and movement of the head-piece away from the membrane allowing enhanced ligand binding [31]. From outside the cell, extracellular ligand binding may facilitate conformational changes and integrin clustering within the plane of the membrane [5]. Integrin clustering may involve lateral associations between the transmembrane domains thereby facilitating cytoplasmic tail interactions with adapter proteins leading to the formation of large multi-protein signaling complexes [5]. Recent estimates have suggested that well over a 100 different types of cytoplasmic molecules may associate with integrins [5]. This large diversity of protein-protein interactions allows integrins to sense changes in the extracellular environment and integrate and distribute this structural and molecular information into diverse signaling networks [33]. In this regard, a recent study has suggested that integrin-mediated interactions with collagen type-I may result in changes in phosphorylation of more than 350 different proteins [36]. Given

this complexity, many questions still remain to be answered as to mechanisms that regulate the molecular switches that define the initiation of a particular integrin-signaling pathway.

Integrin interaction with a variety of ligands has been shown to modulate and integrate signaling networks including MAPK/Erk, PI3k/Akt, Rho/MLCK and the JNk pathways [37, 38]. Integrin engagement is thought to enhance phosphorylation of key residues within their cytoplasmic tails, which can potentiate associations with a variety of adaptor molecules and non-receptor kinases such as Fak, Src and ILK [39]. This capacity of integrins to activate, integrate and distribute information from these signaling networks illustrates the potential of these receptors to serve as functional distribution hubs in bi-directional information transfer.

## Integrins and Angiogenesis

In addition to the differential expression of integrins within angiogenic and quiescent vessels, both genetic models of integrin function and inhibitor studies have provided compelling evidence that integrins regulate angiogenesis. During our discussion, we will highlight the need for careful interpretation of experimental findings in the context of the particular models being examined. To this end, the potential differences between integrin functions during embryonic vasculogenesis and that of pathological angiogenesis will be addressed. It is important to note that a more meaningful interpretation of the biological functions of integrins might be obtained when considering the expression of their ligands in the context of the models being used. The biological functions of a given receptor is linked to the presence or absence of its ligands and this is of particular significance with integrins, given the expanding number of diverse ligand now ascribed to these multifunctional receptors.

### $\beta$ 1 Integrins in Vascular Development and Pathological Angiogenesis

Studies focusing on the functional importance of  $\alpha$ 5 integrin in embryonic development demonstrated that while  $\alpha$ 5 null mice are embryonic lethal, blood vessels were formed prior to their death [40]. However, blood vessel formation appeared disrupted as defects in vessel maturation and stability was observed [40]. These studies are consistent with the possibility that  $\alpha$ 5 integrin may be more important for later steps in vascular maturation and angiogenesis, rather than the early stages of vasculogenesis [40]. The  $\alpha$ 5 integrin subunit can associate with the  $\beta$ 1 chain.  $\beta$ 1-integrin knockout mice are also embryonic lethal, thus making it difficult to establish an in-depth molecular understanding of the roles for  $\beta$ 1 integrins in angiogenesis. In a series of genetic studies where  $\beta$ 1 integrin was specifically knocked out in endothelial and some hematopoietic cells, mutant embryos died before birth, but blood vessels and yolk sac vascular plexus formation did occur, again suggesting limited impact on vasculogenesis (41–43). However distinct defects were observed in sprouting and vascular patterning in these  $\beta$ 1 integrin deficient embryos [41–43].

Importantly, a major ECM ligand for  $\alpha$ 5 $\beta$ 1 integrin is fibronectin, and mice deficient in fibronectin also exhibited vascular defects [44]. Interestingly, mice lacking a functional RGD integrin binding site within fibronectin exhibited an embryonic lethal phenotype and showed significant vascular abnormalities [45]. These studies suggest that integrin recognition of this functional RGD site contributes to new vessel formation. In further studies, isoforms of fibronectin such as alternatively spliced forms containing the EIIIA and EIIIB domains were shown to be restricted in expression to embryonic development and angiogenesis [46]. While little information is available on integrin interactions with these forms of fibronectin, evidence suggests that  $\alpha$ 4 $\beta$ 1 and  $\alpha$ 9 $\beta$ 1 may bind to the EIIIA domain and both these  $\beta$ 1 integrins have been suggested to impact angiogenesis [47]. Interestingly mice lacking either EIIIA or EIIIB exhibited little obvious defects in embryonic vasculogenesis [46]. However, double EIIIA/EIIIB knockouts were embryonic lethal and exhibited cardiovascular defects, vascular hemorrhage, alterations in vascular yolk sac remodeling, mural cell coverage and angiogenesis

[46]. It is interesting to point out that  $\alpha 4\beta 1$  integrin has been suggested to play a role in mural cell recruitment [48–50].

While many studies are consistent with a limited role for  $\alpha 5$  and  $\beta 1$  integrins in the early stages of vasculogenesis, pharmacological inhibition of  $\alpha 5\beta 1$  can disrupt pathological angiogenesis [51,52].  $\alpha 4\beta 1$  may play a functional role in the mobilization and recruitment of monocytes to the vasculature and pharmacological blockade of  $\alpha 4\beta 1$  was shown to reduce the extent of monocyte-stimulated angiogenesis [49]. Blocking  $\alpha 4\beta 1$  interactions with VCAM-1 resulted in reduced pericyte coverage of blood vessels, increased apoptosis and decreased angiogenesis [48]. Moreover, while vasculogenesis did occur in  $\alpha 4$  integrin deficient mice, some vessels exhibited a reduced pericyte and smooth muscle cell coverage [50]. These studies taken together with observation in EIIIA/B fibronectin domain mutant mice provide support for a role for this integrin-ligand pair in angiogenesis.

Collagen and several collagen-binding integrins including  $\alpha 1\beta 1$ ,  $\alpha 2\beta 1$  and  $\alpha 3\beta 1$  have been implicated in regulating blood vessel formation. For example, mice harboring defects in the expression of collagen as well as mice with defects in collagen metabolism have been shown to exhibit vascular abnormalities [53,54]. While, vascular defects were observed in these collagen mutant mice, early embryonic vasculogenesis still occurred (53,54). Previous studies have demonstrated that distinct cryptic collagen epitopes including the HUIV26 and the HU177 sites play roles in postnatal pathological angiogenesis [55–61]. In addition, soluble fragments containing the non-collagenous (NC) domains of collagen were shown to inhibit pathological angiogenesis [62]. Finally, a number of studies have demonstrated that specific antagonists of collagen binding integrins inhibit pathological angiogenesis [63–65].

As mentioned previously, it is important to distinguish between the roles that integrins and their ligands play in normal embryonic vascular development and their roles in pathological angiogenesis, which may differ. For instance, while NC1 domains of collagen-IV chains are known to bind integrins and inhibit pathological angiogenesis, mice deficient in individual collagen chains, and thus their soluble NC domains, exhibited minimal abnormalities in early embryonic vasculogenesis [62,66]. Interestingly, mice lacking the collagen binding  $\alpha 1$  integrin, exhibited few if any defects in embryonic vasculogenesis, yet pharmacological targeting of this integrin inhibited pathological angiogenesis [63,64,67]. In other studies, while mice lacking the  $\alpha 2$  integrin failed to demonstrate an obvious alteration in embryonic vasculogenesis, pharmacological inhibition of  $\alpha 2\beta 1$  inhibited pathological angiogenesis [62, 68]. Finally, other  $\beta 1$  integrins including  $\alpha 6\beta 1$ ,  $\alpha 7\beta 1$  and  $\alpha 9\beta 1$  have recently been reported to regulate blood vessel development and angiogenesis [69–71]. Taken together, these studies and many others, demonstrate the need to distinguish between embryonic vasculogenesis and postnatal pathological angiogenesis in regards to integrin function in new vessel formation.

## **$\alpha v$ Integrins in Vascular Development and Pathological Angiogenesis**

Among the most extensively studied set of integrins in angiogenesis are the  $\alpha v$  containing heterodimers and in particular  $\alpha v\beta 3$ . Numerous studies have provided evidence that pharmacological antagonists of  $\alpha v\beta 3$  inhibit pathological angiogenesis in many in vivo models [27,72–74]. As with the majority of the integrins examined to date, mice lacking  $\alpha v$  exhibited few defects in early vasculogenesis [75]. However, some abnormal leaky vessels were detected in the brain and gut [75]. Given these findings, it would be interesting to examine whether alterations in expression or composition of particular integrin ligands occur within these distinct tissues. In further studies, pathological angiogenesis in these  $\alpha v\beta 3$  deficient mice was shown to be elevated, which may be due in part to increased VEGFR signaling [76]. In contrast, signaling deficient  $\beta 3$  knockin mice harboring mutations in key phosphorylation sites exhibited

few alterations in embryonic vasculogenesis, but cytokine induced and tumor angiogenesis in these mice was inhibited [77].

In more recent studies,  $\beta 3$  signaling deficient mice were shown to exhibit defects in recruitment of bone marrow derived cells to sites of neovascularization [78]. Bone marrow transplants from  $\beta 3$  wild type mice appeared to rescue the defects observed in angiogenesis [78]. Interestingly, in previous studies,  $\alpha v\beta 3$  expressing glioblastoma tumors implanted in  $\beta 3$  wild type mice exhibited enhanced tumor angiogenesis and elevated tumor infiltration of bone marrow derived macrophage as compared to those tumors transplanted in  $\beta 3$  null mice [79]. However, while increased angiogenesis was observed, the primary tumors size was essentially the same [79]. Bone marrow transplantation or macrophage depletion reversed this glioblastoma phenotype, suggesting that host-derived  $\alpha v\beta 3$  expressing cells may differentially regulate angiogenesis and tumor growth. These important studies provide new evidence that  $\beta 3$  integrin expressed in bone marrow derived cells may play a critical role in angiogenesis as well as tumor growth. In further studies, abnormal coronary vessel maturation was detected in male, but not female  $\beta 3$  deficient mice [80]. It would be interesting to speculate that the altered tissue projections within lumens of these coronary vessels may be associated with alterations in intussusceptive angiogenesis given the nature of the morphological changes observed. Finally, while not an  $\alpha v$  containing integrin,  $\alpha 6\beta 4$  has also been implicated in angiogenesis [81]. Deletion of  $\alpha 6$  results in loss of  $\alpha 6\beta 4$  and  $\alpha 6\beta 1$ , which resulted in an embryonic lethal phenotype [82]. However, no obvious defects in embryonic vasculogenesis were reported [82]. In contrast, mice harboring a mutant signaling deficient  $\beta 4$  chain exhibited significant reductions in pathological angiogenesis [81]. Collectively, these examples help illustrate the careful interpretation that is needed in examining the functional impact of integrins in embryonic vasculogenesis and that of pathological angiogenesis. Furthermore, these examples emphasize the need for a more in-depth understanding of the molecular contributions of integrins to angiogenesis from cell types other than endothelial cells.

## Regulation of the Initiation Stage of Angiogenesis by Integrins

Integrins bind and regulate numerous factors known to influence angiogenesis and thereby may act as functional hubs in facilitating the coordination and distribution of information from diverse signaling networks. Integrins may regulate the expression and activity of pro-angiogenic molecules such as growth factors and growth factor receptors as well as anti-angiogenic factors such as ECM fragments [62,82]. Integrin-mediated regulation of growth factor signaling can modulate crucial events during the initiation phase of angiogenesis including proliferation, cell adhesion and expression of matrix altering enzymes. In addition to binding growth factor and growth factor receptors, integrin interaction with ECM components may also impact the expression and activity of receptors that control cell-cell interactions such as cadherins [83].

## Integrin-Growth Factor and Growth Factor Receptor Associations

Growth factors such as VEGF, FGF, IGF-1, and EGF can be secreted as well as immobilized within the ECM [84,85]. These growth factors may be recognized in a matrix bound state and stimulate proliferation and motility [84,85]. Evidence suggests that integrins may associate with a number of these immobilized pro-angiogenic molecules. Integrins such as  $\alpha v\beta 3$ ,  $\alpha 3\beta 1$  and  $\alpha 9\beta 1$  may interact with distinct isoforms of VEGF-A and these interactions may enhance endothelial cell adhesion, migration and survival [71,85]. In addition to VEGF, integrins bind FGF1, Angiopoietins (Ang1), Connective Tissue Growth Factor (CTGF) and Cysteine-rich angiogenic protein 61 (Cyr61) [86]. Studies demonstrate that  $\alpha v\beta 3$  may bind immobilized FGF1 and enhanced cell adhesion, migration and proliferation [86]. Moreover,  $\alpha 5\beta 1$  may

associate with Ang -1 and this interaction may promote signaling leading to migration that is independent from the Ang-1 receptor Tie-2 [87].

In addition to interactions with secreted and matrix bound growth factors, integrins also associate with growth factor receptors. VEGF-A- mediated activation of VEGFR2 recruits Src and facilitates phosphorylation of the  $\beta 3$  cytoplasmic tail [88]. The phosphorylated  $\beta 3$  tail may promote interactions between  $\alpha v\beta 3$  and VEGFR2, thereby enhancing endothelial proliferation and survival [88]. Further studies suggest that  $\alpha v\beta 3$  may associate with other growth factor receptors including FGF, IGF-1, PDGF and HGF receptors [89–92]. The molecular mechanisms governing these associations and their functional impact on angiogenesis are not fully understood, but these complexes likely enhance and integrate numerous signaling networks important for new vessel formation. Interactions between  $\beta 1$ -integrins and ECM can also influence growth factor signaling. For example,  $\beta 1$ -integrin collagen interaction was shown to inhibit VEGFR2 signaling by recruitment of the tyrosine phosphatase Shp-2 leading to de-phosphorylating VEGFR2 [93].

In addition to pro-angiogenic factors, integrins also regulate the response of vascular cells to anti-angiogenic factors. The anti-angiogenic chemokine CXCL4 may bind  $\alpha v\beta 3$  in endothelial cells and inhibit adhesion and migration [94]. Moreover, endogenous angiogenesis inhibitors such as such as Canstatin and Tumstatin bind  $\beta 1$  and  $\beta 3$  integrins leading to inhibition of endothelial cell proliferation and survival [95,96]. Finally, the Tissue Inhibitor of Metalloproteinase-2 (TIMP-2) may inhibit angiogenesis by an MMP independent mechanisms involving TIMP-2 binding to  $\alpha 3\beta 1$  integrin [97]. This novel association leads to Shp-1 dependent inactivation of FGF and VEGF receptors, thereby inhibiting endothelial cell proliferation [97].

## Integrin-Mediated Alteration of Cell Adhesion During Angiogenesis

Alterations in cell-cell junctions are thought to play vital roles in the initiation of angiogenesis and embryonic vascular development, as mice deficient in VE-cadherin exhibit vascular abnormalities [98]. Experimental support for integrins in regulating cadherin-dependent endothelial cell behavior comes from studies examining VE-cadherin.  $\alpha v\beta 3$  interactions with fibronectin disrupts VE-cadherin distribution in endothelial cell junctions and this disruption was associated with Src activation [83]. This  $\alpha v\beta 3$ -mediated disruption of VE-cadherin decreased cell-cell interaction and may promote migration. Previous studies have also suggested that FGF signaling may play an important role in regulating cadherin expression in endothelial cells. FGF2 stimulation of endothelial cells in vitro was also shown to decrease the surface expression of several cadherins including E, N, P and VE-cadherins and this inhibition appeared to depend at least in part, on JNK signaling [99]. In this regard, integrin are known to modulate the JNK pathway and integrins such as  $\alpha v\beta 3$  and  $\alpha 5\beta 1$  have been shown to associate with FGF and FGF receptors. Interestingly, Syndecan-1, a cell surface ECM receptor was also shown to bind and subsequently activate  $\alpha v\beta 3$  [100]. This novel syndecan-integrin association appears to play a role in angiogenesis as a specific peptide antagonist (Synstatin) of this interaction inhibited angiogenesis and tumor growth in vivo [100]. Thus, integrin signaling and cross talk with other cell adhesion receptors may play an important role in regulating cell adhesion and integrin activation thereby providing endothelial cells with an enhanced capacity to migrate during the initiation phase of angiogenesis.

## Integrin Mediated Regulation of Protease Expression

The ability of vascular cells to remodel the local extracellular microenvironment is a critical step in angiogenesis and the expression of proteolytic enzymes such as MMPs and serine proteases are thought to regulate angiogenesis in both a positive and negative manner [101].



The coordinated expression of proteases and their respective inhibitors likely facilitate the transition from the initiation phase to the invasive stage of angiogenesis. Endothelial cells, tumor cells, fibroblasts, and bone marrow derived cells all contribute to the enhanced levels of proteases within the angiogenic microenvironment. Thus, it is important to consider integrins expressed in these diverse cell types as to their ability to regulate protease expression. Previous studies demonstrate that  $\alpha 5\beta 1$  binding to the 120kd cell-binding domain of fibronectin enhances expression of MMP-3 and MMP-9 while interaction with the CS-1 domain suppressed MMPs [102,103]. In endothelial cells,  $\alpha 2\beta 1$  interactions with collagen, enhanced expression of MMP-1, while  $\alpha 5\beta 1$  expressed in macrophages increased expression of MMP-9 [104,105]. Over expression of  $\alpha v\beta 6$  in squamous carcinoma cells enhanced expression of MMP-3 and MMP-9 [106,107]. These studies highlight some of the many examples by which integrins may regulate the complex mechanisms that contribute to the early initiation phase of angiogenesis.

## Regulation of the Invasive Stage of Angiogenesis by Integrins

Following initiation, the invasive phase of angiogenesis can be characterized by remodeling of the local microenvironment. While integrins can regulate the expression of proteolytic enzymes, many of these proteases are secreted as zymogens and thus need to be activated and localized to discrete sites to facilitate directed cellular invasion. An expanding body of evidence has demonstrated that integrins play functional roles in the activation and localization of matrix degrading proteases. This enhanced activity leads to structural alterations in the local ECM, providing less restrictive pathways for vascular cell invasion and migration. In addition, structural changes of the ECM can expose Matrix Immobilized Cryptic ECM Epitopes (MICEE) that regulates cell adhesion, proliferation and migration [55–61,108].

## Involvement of Integrins in Protease Activation and Cell Surface Localization

Previous studies have demonstrated that  $\beta 1$  interactions with collagen enhanced the activation of MMPs including MMP-2, MMP-9, and MT1-MMP in a variety of cell types [109–111]. Interestingly, MMP-2 activation following collagen binding appeared to depend on the structural conformation of collagen as the activation of MMP-2 was observed in cell attached to native but not denatured collagen type-I [112]. Moreover, this MMP-2 activation was associated with increased levels of MT1-MMP. Further evidence supporting a role for integrins in regulating protease activity comes from studies examining  $\alpha v\beta 3$  dependent activation of the serine protease tPA in vascular smooth muscle cells [113].

In addition to playing roles in expression and activation of proteolytic enzymes, evidence now indicates that integrins can bind proteolytic enzymes at the cell surface thereby localizing activity to sites of migration and invasion. In this regard, we previously made the novel observation that  $\alpha v\beta 3$  co-localized with MMP-2 on angiogenic blood vessels and invasive tumor cells and this interaction was associated with enhanced collagen degradation and invasion [114]. Inhibiting  $\alpha v\beta 3$ -MMP-2 interactions reduced angiogenesis and tumor growth, suggesting a functional role for this protease-integrin association [115,116]. Interestingly, since our early observations, many new studies have now documented further examples of integrin-protease associations and their functional implications [Table-I]. Integrin  $\alpha v\beta 3$  has also been shown to associate with the serine protease thrombin, the serine protease receptor uPAR as well as the secreted lysosomal protease procathepsin-X [117–119]. In other studies, MT1-MMP was found associated with  $\alpha v\beta 3$  in endothelial tips cells, while  $\beta 1$  integrin was found associated with MT1-MMP in non-migrating endothelial cells, suggesting that  $\alpha v\beta 3$ - MT1-MMP interactions may promote endothelial cell invasion and migration [120]. In addition to these studies, MMP-9 has also been shown to associate with a number of integrins including  $\alpha M\beta 2$ ,  $\alpha 5\beta 1$  and  $\alpha v\beta 5$  in diverse cell types including tumor cells, epithelial cells and

neutrophils [110,121–123]. Given the numerous studies demonstrating integrin-protease interactions it would not be surprising that many of these associations contribute to the regulation of angiogenesis. Finally, proteolytic exposure of cryptic regulatory elements (MICEE) buried in the three-dimensional structure of ECM proteins has been shown to regulate endothelial and tumor cell adhesion and migration in vitro and angiogenesis and tumor growth in vivo [55–61].

## Integrins in the Regulation of Maturation Phase of Angiogenesis

During the maturation phase of angiogenesis, endothelial cells begin to re-establish new cell-cell and cell-ECM connections. In addition, bone marrow derived progenitor cells may also be recruited to the sites of neovascularization [124]. Following endothelial cord formation, the process of lumenogenesis begins. The molecular mechanisms regulating lumenogenesis is not completely understood, but new insight is beginning to emerge and integrins may play a role [16]. Lumenogenesis is thought to involve several steps and studies have implicated molecules such as CDC42, Rac-1, and MT-1 MMP in this process [16]. Importantly,  $\alpha 2\beta 1$  may also contribute to lumen formation as antibodies directed to  $\alpha 2\beta 1$  inhibit this process [16].

Blood vessels continue to mature by assembling new vascular basement membranes. Recruitment of mural cells such as pericytes and smooth muscle cells contribute to vascular basement membrane assembly. For example, fibroblast, pericytes and vascular smooth muscle cells are all thought to contribute to the formation of basement membranes and their synthesis and proper assembly may be integrin dependent. Genetic evidence supporting this idea comes from studies demonstrating basement membranes abnormalities in  $\alpha 3$  integrin deficient mice [125]. Interestingly, mice deficient in Akt exhibited defects in the assembly of blood vessel associated fibronectin and targeting fibroblast derived from these mice with a stimulating anti- $\beta 1$  antibody reversed this defect [126]. The assembly of other basement membrane components such as laminin and collagen type-IV has also been suggested to depend in part on  $\beta 1$  integrins [127,128]. Blood vessel stability involves pericyte and smooth muscle cell coverage and evidence suggests that recruitment of mural cells to blood vessels may depend on  $\beta 1$  integrins. For instance, vascular maturation was impaired in mice harboring deletions in mural cell  $\beta 1$  integrins and blocking  $\alpha 4\beta 1$  interactions with VCAM-1 resulted in reduced pericyte coverage of blood vessels, leading to increased apoptosis and decreased angiogenesis [48,129].

## Integrins and their Ligands as Selective Targets in Angiogenic Imaging

Concerted efforts are now underway to exploit the new molecular understanding of integrins for imaging and therapeutics. In addition to detecting areas pathological neovascularization, and assessing the efficacy of anti-angiogenic drugs, early detection of tumors that are transitioning from a benign to a malignant phenotype is also of great interest. Detection of the early stages of angiogenesis may provide a means for selective targeting of sites of malignant tumor progression at a time when therapeutic intervention may result in more beneficial effects.

Numerous studies have focused on detecting changes in expression of growth factor receptors and their circulating or matrix bound ligands as potential markers of angiogenesis [130]. In other studies, work has focused on quantifying the relative levels of circulating bone marrow-derived endothelial progenitor cells as surrogate markers of angiogenesis [131]. Importantly, just as investigators have exploited the altered expression of growth factor receptors and their ligands, the differential expression of integrins and their ligands, may also represent clinically useful targets for imaging. One of the better-studied integrins in this context is  $\alpha v\beta 3$ . A variety of studies have documented up-regulation of  $\alpha v\beta 3$  in angiogenic blood vessels as compared to normal vessels, and imaging agents linked to antagonists targeting  $\alpha v\beta 3$  have demonstrated great promise in selectively homing to sites of neovascularization [132]. Given the expanding

body of evidence for the functional contribution of  $\alpha\beta3$  expressing bone marrow derived cells in angiogenesis,  $\alpha\beta3$ -directed imaging may provide a clinically useful approach in the near future.

As we have emphasized throughout our discussion, it is important to consider the corresponding ligands that bind a given receptor, and this concept is also relevant in terms of their potential use in angiogenesis imaging. The alternatively spliced form of fibronectin containing the EIIIA domain, which has been suggested to be a ligand for  $\alpha4\beta1$  and  $\alpha9\beta1$ , is highly expressed in angiogenic blood vessels with little if any expression in normal quiescent adult blood vessels. This differential expression may be useful as a selective target for angiogenesis imaging. Importantly, while ECM proteins are well-known integrin ligands, an early hallmark of angiogenesis involves proteolytic remodeling of the local ECM surround blood vessels. Thus, structurally altered forms of ECM proteins within the vascular basement membrane may also provide a highly selective target to distinguish early angiogenic vessels from quiescent vessels. Studies have suggested that some of the earliest events during angiogenesis are associated with secretion of proteolytic enzymes from resident cells or from cells that have been recruited to the sites of neovascularization [133]. In this respect, we identified the HUIV26 cryptic collagen-IV epitope within the vascular basement membranes of angiogenic tissues. This cryptic epitope was shown to be a ligand for integrin  $\alpha\beta3$  [55]. Importantly, the proteolytic exposure of this cryptic ECM site was shown to be an early event in angiogenesis as exposure of the HUIV26 epitope was observed between 24 and 48 hours following FGF2 stimulated angiogenesis in the chick CAM and could also be detected within 24 hours in the mouse retina following induction of neovascularization [55,134]. Exposure of the HUIV26 cryptic sites was largely dependant on MMP-9, as a specific reduction in the exposure of the HUIV26 cryptic epitope was observed in MMP-9 null mice with only minimal reductions observed in MMP-2 knockout mice [56]. In a separate study, exposure of the HUIV26 site within vascular basement membranes was also detected within 18 to 24 hrs after induction of angiogenesis in the thymus of mice and again this was shown to be dependent in large part on MMP-9 [57].

In further studies, we identified a second cryptic collagen epitope termed HU177, and in contrast to the HUIV26 site, the HU177 cryptic collagen site could be selectively exposed within a variety distinct types of collagen [59]. Importantly, the HU177 cryptic sites were shown to be exposed within angiogenic but not quiescent blood vessels in several models [59–61,135–137]. Interestingly, little if any exposure of the HU177 epitope was detected within vessels from the pupillary membranes of mice, however 24 hours after VEGF stimulation the HU177 cryptic epitope could be detected in these tissues [136]. HU177 cryptic epitope was shown to be specifically exposed in a time dependent manner in basement membranes following ischemia induced angiogenesis in the hindlimb model and again its expression correlated with the ischemia induced expression of MMPs. The expression of HU177 epitope was markedly reduced in MMP-9 null mice [137]. Moreover, exciting new studies have recently demonstrated a statistically significant increase in the levels of soluble form of the HU177 cryptic epitope in serum from primary melanoma patients as compared controls [138]. In addition, to these collagen epitopes, we have also identified a novel cryptic epitope within laminin that may also be selectively exposed within the basement membrane of tumor associated angiogenic blood vessels [108]. Collectively, these studies demonstrate that potential of exploiting these novel cryptic ECM sites within well-known integrin ligands as targets for highly selective imaging of angiogenesis and tissue invasion.

## Inhibition of the Integrin Functional Hub During Angiogenesis

As mention above, the notion that integrins serve as functional hubs integrating and distributing networks of angiogenic signaling cues from a variety of cell types, provides a strong rational for targeting integrins to control aberrant neovascularization. Given the known consequences

of disrupting functional hubs associated with other types of network such as transportation systems and electrical power grids, disrupting integrins, might result in important therapeutic benefits alone or in combination with chemotherapy or radiation. Significant efforts have been focused on developing anti-angiogenic agents targeting integrins.  $\alpha v$  containing integrins such as  $\alpha v\beta 3$  and  $\alpha v\beta 5$  have been a major focus in the development of antagonists of angiogenesis and numerous studies have provided convincing data that blocking  $\alpha v$  integrins can inhibit pathological angiogenesis [74]. As discussed previously, blocking  $\alpha v$  integrins may disrupt mechanisms within several phases of angiogenesis including cellular proliferation, migration, survival signaling and proteolytic activity. Several antibodies targeting  $\alpha v$  integrins have been developed, some of which are being currently tested in clinical trials including, Abegrin/Etarcizumab (anti- $\alpha v\beta 3$ ), CNT095 (anti- $\alpha v$ ) and Abciximab (anti- $\alpha IIb\beta 3/\alpha v\beta 3$ ) [139–141]. In addition, peptide and non-peptidic mimetic are also being developed that target  $\alpha v$  integrins such Cilengitide [142]. Interestingly, new strategies aimed at disrupting functional associations of  $\alpha v\beta 3$  with MMP2 (TSRI265) and Syndecan-1 (SSTN) have also been developed and demonstrate anti-angiogenic activity [100,116].

In addition to  $\alpha v$  integrins several antagonists directed to different  $\beta 1$  integrins have also been generated, including Volociximab (anti- $\alpha 5\beta 1$ ), ATN-161 (anti- $\alpha 5\beta 1$ ) and E7820 (anti- $\alpha 2$ ) [51,52,143]. Integrin antagonists such as Efalizumab, a humanized antibody targeting  $\alpha L\beta 2$  and Natalizumab directed to  $\alpha 4$  have been developed to treat inflammatory disease such as psoriasis and Chron's [144,145]. Given the emerging findings that bone marrow derived myeloid cells may contribute to angiogenesis, and  $\beta 2$  integrins can be expressed in a variety of bone marrow derived cells, it would be interesting to determine whether these antagonists may also impact the angiogenic cascade.

### Selective Targeting of Cryptic Integrin Ligands

Given the broad expression of integrins in essentially all tissues as well as their multiple roles in normal as well as pathological conditions, it is critical to carefully determine the most appropriate use of integrin antagonists alone and in combination with existing therapies to fully realize their clinical potential. Complicating the effective use of integrin antagonists in the clinical setting is the emerging realization that certain integrins may regulate angiogenesis and tumor progression in a positive manner while others may negatively impact these processes. Moreover, blocking one specific integrin may alter the functional activity of a second different integrin, a phenomenon known as trans-dominant integrin regulation [146]. Thus, developing novel approaches that selectively inhibit the pro-angiogenic activity without enhancing vessel growth or tumor invasion will be critical to the successful use of integrin inhibitors for the treatment of tumor progression. As mentioned previously, an important aspect in regulating any receptor mediated signaling cascade is to consider both the receptor and its ligands. Just as investigators have targeted the VEGFR and its ligand VEGF, the possibility exists that targeting integrin ligands such as defined ECM proteins may also be of therapeutic utility. However, given the ubiquitous expression of integrin ligands, a highly selective approach is needed.

One potential strategy makes use of the observations that the ECM surrounding angiogenic blood vessels as well as the invasive fronts of tumors are often structurally altered. These structural alterations in existing ECM components may serve as solid-state conformational switches that facilitate an invasive phenotype (Figure 3). A rapidly growing body of evidence is now documenting examples of structurally altered or alternatively spliced forms of ECM components accumulating at sites of angiogenesis and tumor invasion [147–151]. For example, cryptic sites and alternatively spliced forms of many ECM molecules have been detected including cryptic sites in Vitronectin, Fibrinogen, Fibronectin, Laminin and Collagen (Table-II). The localized exposure of these cryptic sites may not simply represent ECM destruction,

but represent solid-state conformational switches, thereby providing temporally controlled and highly selective exposure of novel integrin ligands. The temporally regulated exposure of cryptic integrin ligands may allow the initiation of novel integrin signaling events restricted to sites of tissue invasion.

Cryptic integrin ligands may represent highly selective therapeutic targets that can be exploited to inhibit integrin signaling that is restricted in large part, to sites of tissue invasion. For example, the HUIV26 cryptic site within collagen type-IV was shown to be recognized by integrin  $\alpha v\beta 3$  [55]. Thus blocking  $\alpha v\beta 3$  dependent binding to the HUIV26 cryptic site by targeting the HUIV26 site rather than the integrin receptor, results in highly selective inhibition of  $\alpha v\beta 3$ -mediated signaling at sites of tissue invasion. Several studies have provided evidence that targeting the HUIV26 cryptic epitope inhibits angiogenesis, tumor growth and metastasis in numerous models [55–58]. In similar studies, a second highly conserved cryptic collagen epitope (HU177) that can be exposed in many genetically distinct type of collagen was also identified. Studies have recently shown that specific targeting of this highly restricted cryptic collagen epitope can also potentially inhibit angiogenesis and tumor growth in vivo [59–61]. Importantly, this unique cryptic collagen epitope (HU177) also represents a cryptic integrin ligand (unpublished observations). A murine antibody directed to this site has been humanized and clinical trials are currently underway to examine its effects in patients with malignant tumors [60,61]. Collectively, these studies and others are beginning to provide evidence for the utility of targeting unique cryptic integrin ligands to selectively control pathological angiogenesis.

## Conclusions

With the growing appreciation of the importance of many integrated signaling networks in controlling both physiological and pathological blood vessel formation, additional molecular insight into angiogenesis, and perhaps the development of novel anti-angiogenic approaches, might be achieved by studying this process from a more global rather than a reductionist perspective. In this regard, an interesting concept in systems biology and network theory suggests that targeting certain hubs that integrate circuits within a system may result in a more pronounced disruption than targeting a single pathway. Intriguing evidence suggests that integrins function in sensing changes in both the extracellular and intracellular microenvironments and in turn, integrates and distributes these complex streams of information into diverse signaling networks to ultimately regulate cellular behavior. Thus, in some respects integrins may represent functional hubs that operate during the various stages of angiogenesis. Given the emerging evidence that particular integrins may regulate distinct processes during angiogenesis in either a positive or negative manner, a more global understanding how these receptors coordinate the diversity of pro- and anti-angiogenic stimuli may lead to the identification of highly selective new strategies to regulate pathological angiogenesis.

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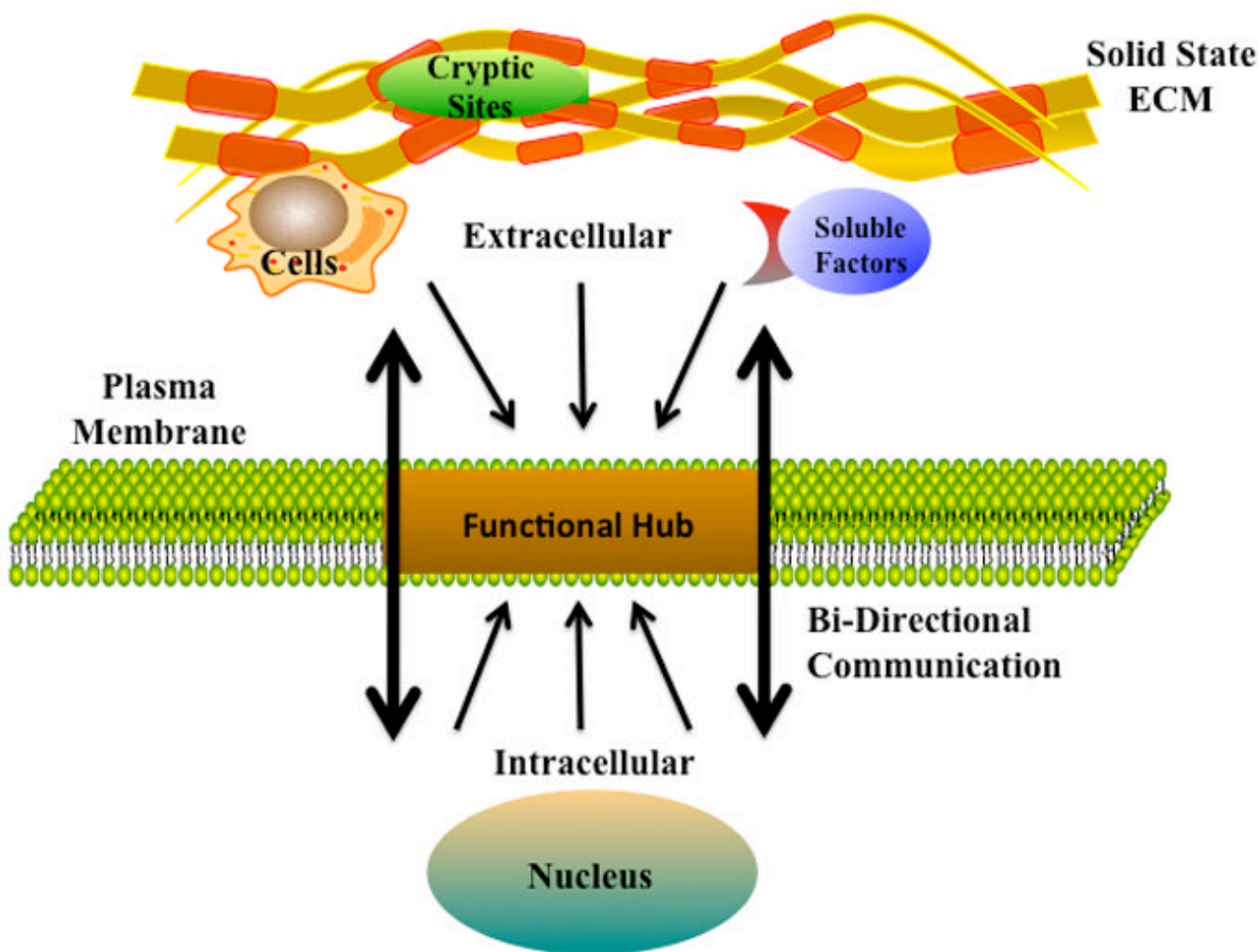
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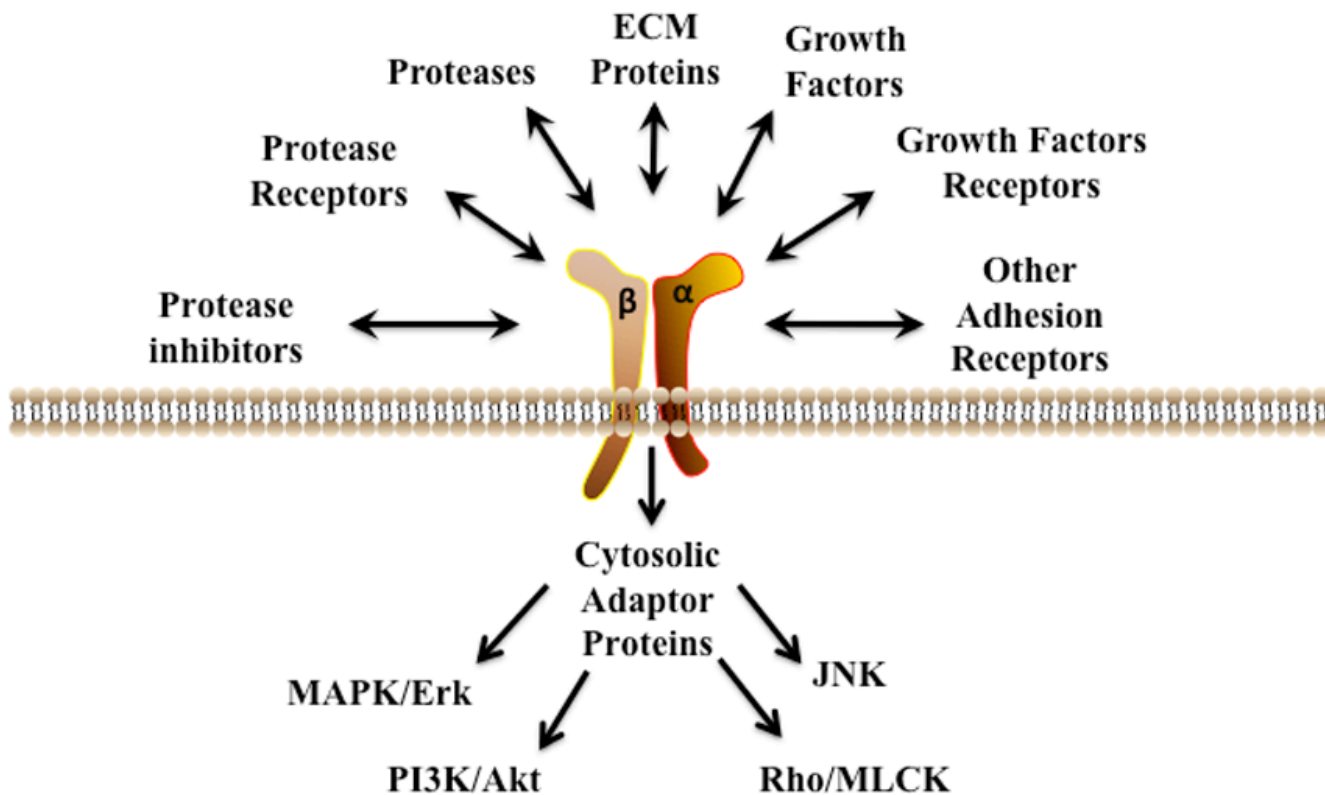
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## Integration of Information Networks within the Extracellular and Intracellular Angiogenic Microenvironment



**Figure 1. Bi-direction Flow of Information During Angiogenesis**  
 Angiogenesis involve bidirectional integration of numerous extracellular and intracellular angiogenic cues. Schematic representation of the bi-directional flow of signaling cues through a functional hub that gathers, interprets and distributes the regulatory stimuli needed for the formation of functional blood vessels.

## Integrins as Functional Hubs: Integrating Networks of Angiogenesis Regulatory Molecules

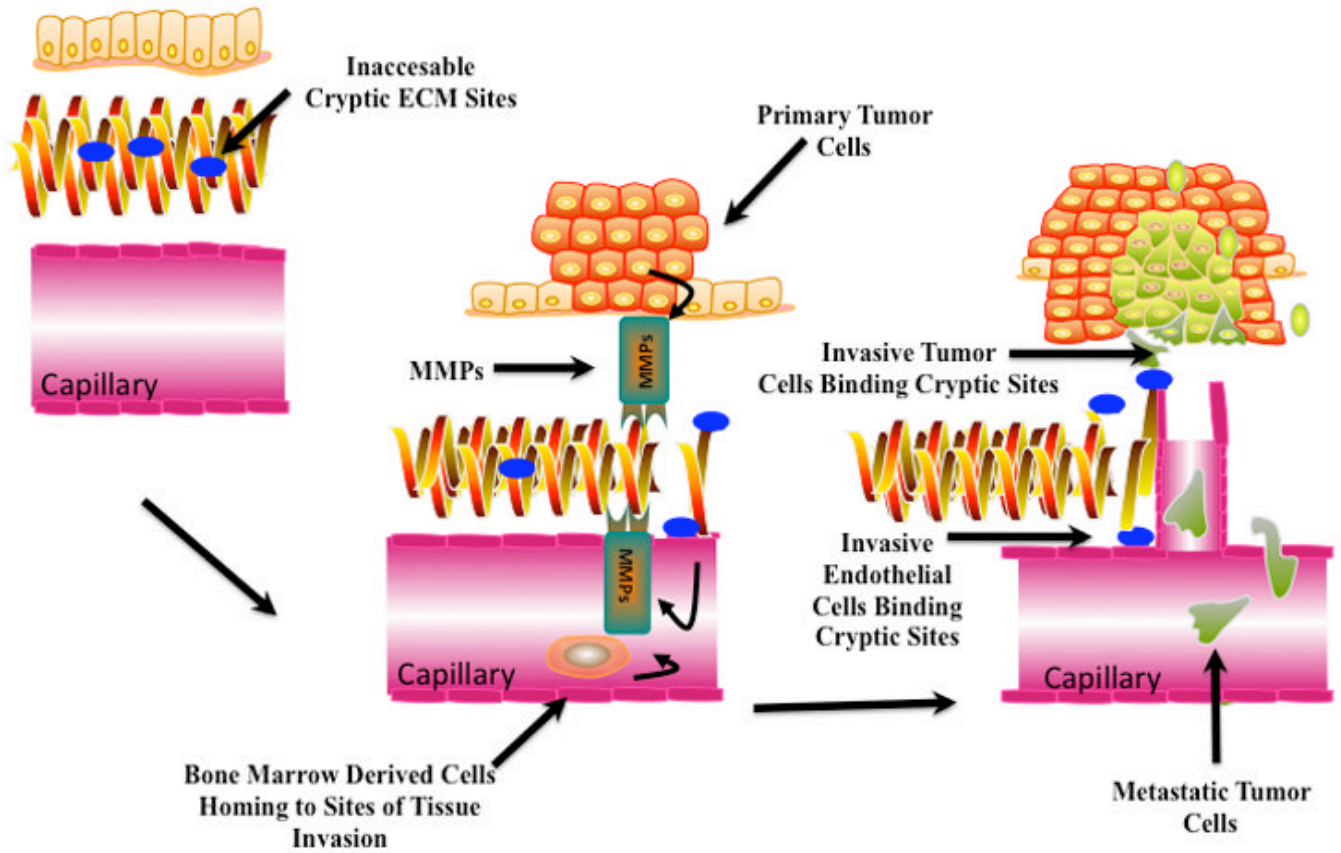


**Figure 2. Integrins as Functional Hubs During Angiogenesis**

Angiogenesis requires the coordinated activity of a wide array of distinct families of molecules and integrin receptors have the ability to bind many different angiogenesis regulatory factors. Schematic representation illustrating the diversity of angiogenic regulatory proteins and signaling networks that integrins may coordinate during new blood vessel formation.



### Solid State Conformational Switches: Exposure of Cryptic Epitopes Within the ECM



**Figure 3. Solid-State Conformational Switches within the ECM**  
 A crucial event during angiogenesis involves structural modification of the local ECM. Schematic representation of proteolytic exposure of solid-state conformational switches (Cryptic Epitopes). Proteolytic activity from both resident cells and bone marrow derived cells homing to sites of angiogenesis may contribute to the localized exposure of these cryptic regulatory switches. Many solid-state cryptic sites represent integrin ligand that regulates invasive cellular behavior restricted to sites of tissue invasion. These cryptic sites may allow highly selective targeting for imaging and therapeutic intervention.

**Table I**

## Examples of Integrin-Protease Associations

<b>Integrin</b>	<b>Proteolytic Enzyme</b>	<b>Reference</b>
$\alpha 2\beta 1$	MMP-1	152
$\alpha 4\beta 1$	ADAM-7	153
$\alpha 4\beta 1$	ADAM-28	153
$\alpha 5\beta 1$	MMP-9	119
$\alpha 5\beta 1$	ADAM-15	154
$\alpha 5\beta 1$	ADAM-17	155
$\alpha 6\beta 1$	ADAM-2	156
$\alpha 6\beta 1$	ADAM-9	157
$\alpha 9\beta 1$	ADAM-9	158
$\alpha 9\beta 1$	ADAM-7	158
$\alpha 9\beta 1$	ADAM-12	159
$\alpha 9\beta 1$	ADAM-28	158
$\alpha 9\beta 1$	ADAM-33	158
$\alpha 4\beta 7$	ADAM-28	160
$\alpha v\beta 3$	MMP-2	114
$\alpha v\beta 3$	MT1-MMP	120
$\alpha v\beta 3$	Thrombin	117
$\alpha v\beta 3$	ProCathepsin-X	119
$\alpha v\beta 3$	ADAM-15	160
$\alpha v\beta 3$	ADAM-23	161
$\alpha v\beta 5$	MMP-9	162
$\alpha v\beta 5$	ADAM-9	163
$\alpha L/\beta 2$	MMP-9	164
$\alpha M/\beta 2$	MMP-9	165
$\alpha M/\beta 2$	Elastase	164
$\alpha M/\beta 2$	Protease-3	167

**Table 2**

Examples of cryptic ECM sites and alternatively spliced forms of ECM

Cryptic ECM Site/Alternatively Spliced	Receptors	References
Cryptic HUIV26 Site in Collagen-IV	$\alpha\beta3$	55
Cryptic HU177 Site in Collagen	$\beta1$	59, Unpublished
Cryptic STQ Site in Laminin	Unknown	108
Cryptic Site in Laminin	Unknown	151
Cryptic Site (RGD) in Vitronectin	$\alpha\beta3$	147
Cryptic Site in Vitronectin-2	Unknown	148
Cryptic site in Fibrinogen	$\alpha\beta6$	149
Cryptic Site in Fibronectin	$\alpha\beta3, \alpha5\beta1$	150
Alternatively Spliced EIIIA Domain	$\alpha4\beta1/\alpha9\beta1$	28
Alternatively Spliced EIIIB Domain	Unknown	28