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Review Article

# Integrins and angiogenesis: A sticky business

Guido Serini*, Donatella Valdembri, Federico Bussolino*

*Department of Oncological Sciences and Division of Molecular Angiogenesis, IRCC, Institute for Cancer Research and Treatment, University of Torino School of Medicine, Strada Provinciale 142 Km 3.95, 10060 Candiolo (TO), Italy*

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**Abstract**

From an evolutionary point of view, the development of a cardiovascular system allowed vertebrates to nourish the several organs that compose their wider multicellular organism and to survive. Acquisition of new genes encoding for extracellular matrix (ECM) proteins and their cognate integrin receptors as well as secreted pro- and anti-angiogenic factors proved to be essential for the development of vascular networks in the vertebrate embryo. Postnatal tissue neo-vascularization plays a key role during wound healing and pathological angiogenesis as well. There is now clear evidence that building blood vessels in the embryo and in the adult organism relies upon different endothelial integrins and ECM ligands. A successful vascular development depends on fibronectin and its major receptor $\alpha5\beta1$ integrin, but not on $\alpha v\beta3$, $\alpha v\beta5$, and $\alpha6\beta4$ integrins that are instead central regulators of postnatal tumor angiogenesis. Here, endothelial $\alpha v\beta3$ elicits anti- or pro-angiogenic signals depending respectively on whether it is occupied by a soluble (e.g. type IV collagen derived tumstatin) or an insoluble (vitronectin) ECM ligand. The laminin-5 receptor $\alpha6\beta4$ integrin, expressed only by endothelial cells of mature blood vessels, controls the invasive phase of tumor angiogenesis in the adult organism. Finally, regulation of vascular morphogenesis relies upon the fine modulation of integrin activation by chemoattractant and chemorepulsive cues, such as angiogenic growth factors and semaphorins.


*Keywords:* Integrins; Angiogenesis; Growth factors; Semaphorins

**Contents**



## Introduction

Among metazoans, only vertebrates evolved a cardiovascular system capable of transporting over long distances oxygen and nutrients to the many different tissues that compose their multicellular organism. Two distinct morphogenetic processes contribute to the development of the embryonic vasculature: vasculogenesis and angiogenesis [1]. During vasculogenesis, mesodermal cells differentiate into endothelial cell (EC) precursors (angioblasts), which proliferate and coalesce into a primitive network of homogeneously sized vessels known as primary capillary plexus. This initial capillary meshwork is then remodeled by angiogenesis into a mature and functional vascular bed comprised of arteries, capillaries, and veins [2]. Angiogenic remodeling coordinates with the establishment of blood flow

* Corresponding authors. Fax: +39 0119933524.
*E-mail addresses:* guido.serini@ircc.it (G. Serini), federico.bussolino@ircc.it (F. Bussolino).

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and can occur through sprouting, intussusception, i.e. by internal division of the vessel lumen or vascular fusion [2]. Angiogenesis also includes penetration by sprouting of vessels into avascular regions of the embryo and recruitment of mural cells. In multicellular organisms, the formation of organs depends on cell-to-cell and cell-to-extracellular matrix (ECM) adhesion, so does the generation of the cardiovascular system. Notably, compared to lower metazoans, vertebrates built on an entirely new set of adhesion-related genes involved in the development, maintenance, function, and regeneration of the vasculature, i.e. ECM proteins (for instance fibronectin, vitronectin, and fibrinogen) and their heterodimeric integrin adhesive receptors [3]. Up to now, 9 of out of 24 vertebrate integrin heterodimers have been implicated in blood vessel formation, namely α1β1 [4–6], α2β1 [4–6], α4β1 [7], α5β1 [8–12], αvβ1 [13], αvβ3 [14–19], αvβ5 [14,15,17], αvβ8 [15,20–22], and α6β4 [23,24]. Because of the general significance of blood vessel formation to common inherited and acquired human diseases, such as cardiovascular malformations and cancer, there has been much interest in understanding its molecular mechanisms. In particular, over the last 6 years, new light has been shed on the shared and diverse molecular mechanisms through which integrins and their ECM ligands regulate blood vessel formation in the embryo and the adult organism.

## αv integrins and the fibronectin receptor α5β1 regulate vascular development and tumor angiogenesis

Historically, the largest amount of data pointed to αvβ3 integrin, a receptor for both fibronectin (FN) and vitronectin (VN), and αvβ5 integrin, a VN receptor, as major players in blood vessel formation. Indeed, blockade of αvβ3 [25,26] or αvβ5 [27] integrins with antagonists disrupts tumor and experimental angiogenesis, and the β3 integrin antagonist Vitaxin is currently in clinical trials [28]. In contrast, genetic experiments showed that successful vasculogenesis and angiogenesis depend on FN [29,30] and its main receptor, α5β1 integrin [10], but not on αv integrins [15]. As an outcome of the separation of the endodermal and mesodermal layers of the yolk sacs, E8.5–9.5 vitelline membranes of α5 null mice have abnormally swollen endothelium-lined blood vessels [10]. At the same time, α5 mutant embryos show a decreased complexity of the head primary perineural plexus [10]. In addition, a lower amount of fibrils is deposed within the ECM surrounding blood vessels both in the α5 null yolk sacs and embryos [10]. The fact that FN-null embryos show similar or more severe phenotype [29,30] further corroborates the concept that α5–FN interactions are crucial for embryonic vascular development (Fig. 1, left panel). α5β1 integrin and its ligand FN have been found to be overexpressed in blood vessels of human and mouse tumors [11]. Antagonists of integrin α5β1 integrin blocked tumor angiogenesis [11] and growth and have entered clinical trials as well.

αv gene ablation [15] causes 100% lethality, but developmental vasculogenesis and angiogenesis proceed normally until E9.5. Between E10.5 and E11.5, about 70% of the αv-null embryos die, possibly because of placental defects. By E12.5, the surviving mutants develop intracerebral hemorrhage and die soon after birth [15,20]. While deletion of both β3 and β5 integrin subunits has no effect on vascular development [17], deletion of β8 integrin gene [21] phenocopies the cerebral hemorrhage phenotype of αv-null mice [15,20]. Thus, lack of αvβ8 would be responsible for the selective cerebral blood vessel defects observed in the αv-null mice. Interestingly, these vascular lesions are very similar to those found in cerebral cavernous malformation 1-linked families harboring mutations in the gene encoding for the Krev Interaction Trapped-1 (KRIT1) protein [31], a binding partner for both the integrin-activating GTPase Krev1/Rap1A [32] and the integrin cytoplasmic domain-associated protein 1 (ICAP1) [33]. Recent cell-type-specific genetic ablation [22] and immunohistochemical [34] studies indicate that αvβ8 is expressed by a subset of nestin$^+$/GFAP$^+$ radial glial-like cell processes surrounding blood vessels and is required for the establishment of a proper cerebral blood vessel morphology [22]. In vitro, astrocytic integrin αvβ8 binds and activates latent transforming growth factor-β (TGF-β), which is localized at the basement membrane of brain blood vessels [34]. Activated TGF-β then up-regulates anti-angiogenic genes, such as plasminogen activator inhibitor-1 and thrombospondin-1, which inhibit EC proteolytic activity, migration, and proliferation [34]. The homeostatic interaction between αvβ8 and TGF-β could therefore represent the mechanisms by which astrocytes stabilize the cerebral vasculature (Fig. 1, middle panel).

The observation that antagonists of αvβ3 and αvβ5 integrins inhibit angiogenesis in cancer, arthritis, and ischemic retinopathy, while knockout mice lacking either β3 and/or β5 not only develop normally, but even show enhanced tumor growth and angiogenesis would suggest that these integrins play a negative role on blood vessel formation in the adult life, but not during development. Furthermore, αvβ3 integrin appears to transduce signals that decrease the expression of vascular endothelial growth factor (VEGF)-receptor (R) 2 and VEGF-specific angiogenesis [17,19]; however, it is not clear whether in order to exert its inhibitory function αvβ3 needs to bind soluble or insoluble/polymerized ECM ligands.

Evidences from independent groups indicate that in the absence of ligation [35] or when occupied by a soluble ligand [36–41] αvβ3 can behave as a dependence receptor and mediate pro-apoptotic signals. Indeed, unligated endothelial αvβ3 recruits caspase 8 to the membrane, where it becomes activated in a death-receptor-independent manner and triggers EC apoptosis [35]. Moreover, the soluble C-terminal globular non-collagenous (NC1) domain, which is cleaved by matrix metalloproteinase 9 (MMP9) from the α3 chain of type IV collagen and called tumstatin, acts as an

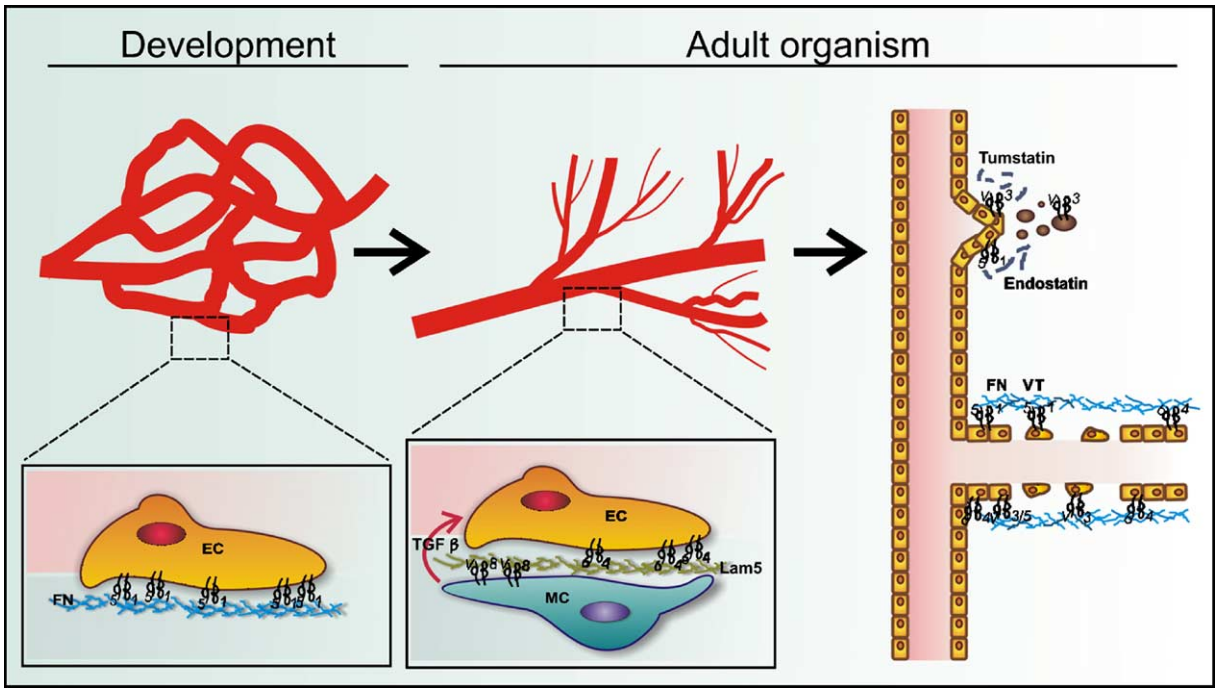


Fig. 1. Role of integrins during blood vessel formation. During embryonic development, the primary vascular plexus (left), originated from the fusion of angioblasts (vasculogenesis), is remodeled in a mature vascular tree (middle) by angiogenesis. In the adult life, angiogenesis occurs in different physiological and pathological settings (right). Successful angiogenesis and vasculogenesis in the embryonic life depend on endothelial cell (EC) interaction with fibronectin (FN) by means of its main receptor α5β1 integrin (left bottom panel). Mature but not developing blood vessels express the laminin-5 (Lam5) receptor α6β4 integrin. In the adult brain, mural cells (MC) surrounding blood vessels express αvβ8 integrins, which are capable of binding, and activate TGF-β that in turn elicits an anti-angiogenic activity on EC, leading to stabilization of the cerebral vasculature (bottom middle panel). The FN and vitronectin (VT) receptors αvβ3 and αvβ5 integrins play a negative role on blood vessels formation in adult life, but not during development. When αvβ3/αvβ5 integrins are unligated or occupied by soluble ligands (tumstatin, endostatin, canstatin, arrestin), they mediate pro-apoptotic signals (upper part left panel). Activation of αvβ3/αvβ5 integrins by insoluble ligands (VT, FN, lactadherin/MFG-E8) is required instead for VEGF-dependent neo-vascularization in the adult organism, but not in the embryo (lower part left panel).

angiogenesis inhibitor by binding to αvβ3 integrin in a non-RGD-dependent manner and eliciting EC apoptosis [36–41]. Col IVα3-null mice display accelerated tumor growth associated with enhanced pathological angiogenesis, while angiogenesis associated with development and tissue repair are unaffected. Interestingly, tumor, but not tissue repair angiogenesis, is associated with robust expression of αvβ3 integrin [41]. Furthermore, the increased rate of tumor growth is rescued in an αvβ3-dependent manner by supplementing Col IVα3-null mice with recombinant tumstatin [41]. These results suggest that physiological levels of circulating tumstatin suppress tumor angiogenesis via the αvβ3 integrin expressed on tumor ECs and provide a clear answer to the unexpected increase of tumor angiogenesis observed in β3 null mice [17]. Thus, MMP-generated fragments of basement membrane collagen (such as tumstatin, endostatin, canstatin, and arresten) could represent endogenous integrin-mediated suppressors of pathological angiogenesis and tumor growth [42] (Fig. 1, right panel).

VEGF-elicited Akt signaling mediated by VEGF-R2 is greatly amplified when ECs adhere on insoluble αvβ3/αvβ5 integrin ligands, such as vitronectin [43,44] or, as lately shown, milk fat globule-EGF factor 8 (MFG-E8)/lactadherin, a secreted glycoprotein that is expressed by vascular ECs [45]. While *Mfge8*-null mice are viable and do not exhibit any detectable vascular defect, the VEGF-induced post-ischemic neo-vascularization and Akt phosphorylation are dramatically blunted in these animals [45]. In addition, administration of exogenous lactadherin in ischemic muscles shows a VEGF-like pro-angiogenic potential [45]. All together, these data identify lactadherin/MFG-E8 as a physiological ligand of αvβ3 and αvβ5 integrins that is required for VEGF-dependent neovascularization in the adult mice, but not in the embryo (Fig. 1, right panel).

## α6β4 signaling controls pathological angiogenesis

A distinguishing feature of α6β4 integrin is the atypical β4 subunit cytoplasmic domain that, compared to the other integrin β subunits, is huge (about 1000 amino acids) and structurally different, being composed by two pairs of fibronectin type III repeats separated by a connecting segment [46]. α6β4 is a versatile laminin-5 receptor involved in at least two aspects of epithelial cytoskeletal organization. On the one hand, linking basement membrane

laminins with the intermediate filament cytoskeleton via plakin HD1/plectin, α6β4 mediates the formation on the basal epithelial cell surface of stable adhesive structures known as hemidesmosomes (HDs) that serve an essential mechanical function [46,47]. The N-terminal portion of β4 cytodomain (to amino acid1355) is sufficient for interaction with the plakin HD-1/plectin and association with intermediate filaments [48]. On the other hand, α6β4 also associates with the actin cytoskeleton in dynamic situations, such as epithelial and carcinoma cell migration during respectively wound healing [47] and invasion [46]. Chemotactic growth factors, such as EGF [49], can elicit β4 cytoplasmic tail phosphorylation, the ensuing disassembly of HDs, and the incorporation of α6β4 in F-actin containing lamellipodia [46]. After Src family kinase-mediated phosphorylation of β4 cytodomain, α6β4 signaling goes on through recruitment of Shc and activation of Ras/extracellular-signal regulated kinase (ERK) and phosphoinositide-3 kinase (PI3K)/Akt [49]. Tyrosine phosphorylation sites of β4 involved in the recruitment of Shc and PI3K are located in the C-terminal portion (substrate domain) of the β4 tail, downstream of amino acid 1355 [50]. Upon cessation of signaling, α6β4 mediates the assembly of HDs.

α6β4 integrin is expressed by ECs of mature, but not developing blood vessels (Fig. 1, middle panel). Fittingly, β4 null embryos do not display any defect of either developmental vasculogenesis or angiogenesis [51,52]. However, similarly to αvβ3 and αvβ5, α6β4 integrin even if dispensable for vascular development could be involved in postnatal angiogenesis. Indeed, α6β4 integrin is expressed in medium and small size vessels, but not microvessel in human cancers and tumor xenografts [23,24]. To directly investigate whether α6β4 signaling could be play a role in the invasive phase of angiogenesis in the adult organism, Nikolopoulos and colleagues [24] generated mice carrying a targeted deletion of the substrate domain of β4 tail from amino acid 1356 on (β4 1355 T). These mice, even if α6β4-dependent signaling through ERK and Akt is impaired, do not present any cutaneous or gastrointestinal blistering or any defect of cardiovascular development [24]. However, β4 1355 T mice exhibit an impaired angiogenic response to both basic fibroblast growth factor (bFGF) and VEGF. Interestingly, an accurate analysis of bFGF-containing subcutaneous Matrigel plugs unveiled that, while wild type plugs contained $\beta4^+$/$BrdU^-$ peripheral small-sized vessels from which $\beta4^-$/$BrdU^+$ microvessels sprouted inside the plug, β4 1355 T mutant plugs were almost devoid of those penetrating $\beta4^-$ microvessels without any detectable increase in EC apoptosis [24]. Moreover, nuclear accumulation of phospho-ERK and the p65 subunit of NFκB was impaired in EC of β4 1355 T mutant plugs. This study [24] definitely points to α6β4 integrin as a central regulator of the very onset of the invasive phase of postnatal angiogenesis, when EC proliferation and migration have not been triggered yet (Fig. 1, right panel). The observation that loss of β4 substrate domain significantly inhibited angiogenesis in 80% of tested tumor xenotransplantation models further suggests that α6β4 integrin could be a major player in tumor angiogenesis. Since α6β4 signaling is also crucial for tumor migration and invasion, developing inhibitors of α6β4 signaling could represent the ultimate therapeutic strategy for cancer that holds the promise of attacking at the same time invading neoplastic cells and the surrounding vasculature.

## Regulation of integrin function controls angiogenic remodeling

The final shape of the cardiovascular system results from the complementary and combinatorial interaction of several factors that control EC adhesion and movement: (i) local environmental factors, such as tissue oxygen; (ii) blood flow and fluid shear stress; (iii) genetically programmed extrinsic cues, such as bFGF, VEGF, and angiopoietin-1 [2]. During the angiogenic process, ECs collectively move and change their reciprocal positions and interactions in response to the many stimuli that elicit their motility [53]. To respond to the relative balance of activators impinging on them, ECs need to be capable of dynamically regulating their adhesive behavior both in terms of cell-to-cell and cell-to-ECM contacts. EC interactions with the ECM are of particular relevance since directed cell motility is a highly coordinated process impinging on the regulation of cell adhesion to ECM. Integrins, which are primary ECM receptors, can exist in different functional states with respect to their affinity for ECM proteins, and regulation of integrin activation is crucial for their biological functions [54,55]. In the low affinity state, the extracellular domain of integrins is bent over the cell surface [54,55], whereas the α and β cytoplasmic tails tightly interact through a juxta-membrane salt bridge between the arginine of the GFFKR sequence in the α subunit tail and an aspartic residue in the β subunit tail [56,57]. Interaction of PTB containing proteins, such as talin [58,59], with the NPXY motif conserved in most β integrin subunits leads to an unclasping of α and β subunit tails [57] that associates to an extension of the extracellular domain and results in the transition of integrins to their high affinity state [54,55]. In addition, there are proteins other than talin that activate specific integrins by binding to certain α and β subunit cytoplasmic tails, such as the Rap1 GTPase binding protein RAPL (that interacts with αL tail) [60–62] or the plekstrin homology (PH) domain containing protein cytohesin-1 (that associates with β2 tail) [63–65]. It is likely that other proteins exist that are capable of binding and activating integrins in general or in a subunit-specific way.

The observations that regulated activation of integrins is essential for embryonic morphogenesis in *Drosophila* [66] and that prominent determinants of vascular remodeling (e.g. $O_2$ tension, angiogenic growth factors, and fluid shear

stress) activate integrin-based adhesion could suggest a role for control of integrin function during angiogenesis. Indeed, in several cell types [67–69], low $O_2$ tension promotes cell adhesion to different ECM ligands via hypoxia-inducible factor (HIF), mostly by inducing integrin mRNA and protein expression at the cell surface; however, HIF-1α has been recently shown to induce in ECs a hypoxia-driven VEGF autocrine loop necessary for tumor angiogenesis [70–72]. Furthermore, angiogenic growth factors, including HGF [73–75], bFGF [13], and VEGF [13], act as positive regulators of endothelial integrin function and in ECs migrating towards bFGF high affinity integrins are recruited to the leading edge where they promote new adhesions to support directed cell motility [76]. Moreover, it appears that flow alterations have a major influence in regulating vascular remodeling [77] likely through shear stress that in ECs rapidly stimulates conformational activation of αvβ3 followed by an increase in its binding affinity to ECM proteins [78]. The inside-out signaling through which positive regulators of endothelial integrin function exert their effects is still poorly characterized. HGF [75], VEGF, and bFGF [13] appear to activate integrins through a phosphatidylinositol 3 kinase (PI3K)/Akt-dependent pathway. However, it is not clear whether PI3K is activated directly by docking of its p85 regulatory subunit on VEGF and bFGF tyrosine kinase receptors (TKRs) or via the Ras GTPase subfamily member R-Ras that localizes at ECM adhesive sites [79] and activates integrins [80–82] through PI3K [81,83]. VEGF and bFGF TKRs could activate phospholipase-C-γ leading to diacylglicerol production and activation of R-Ras guanosine exchange factors of the Ras guanine-releasing protein (RasGRP) family (e.g. RasGRP3) [84]. In ECs, VEGF promotes PI3K activity that in turn stimulates integrin linked kinase (ILK) [85], a cytoplasmic PH domain-containing Ser/Thr kinase that recruits several regulators of actin dynamics at ECM adhesive sites [86] where it also binds and activates β1 integrins [87]. Genetic deletion of ILK both in mice and zebrafish is lethal and results in dramatic defects of vascular development that result from a down-regulation of the active conformation of β1 integrins [87].

We have previously shown that, during vascular development and experimental angiogenesis, ECs generate autocrine chemorepulsive signals of class 3 semaphorins (SEMA3) that endow the vascular system with the plasticity required for reshaping by inhibiting integrins [88,89]. Inhibitory autocrine loops of endothelial SEMA3 proteins would allow a tunable and fine modulation of integrin function, cell migration, and redirectioning during angiogenic remodeling. The vascular defects we found in *sema3a* null embryos somewhat phenocopy the mutation in *ephrin-B2*, *EphB4*, and *EphB2/EphB3* [53] that also act by modulating integrin function [89]. The receptor complex of five out of six SEMA3, SEMA3E being a notable exception, is constituted by a ligand binding and signal transducing subunit, respectively, represented by neuropilin (Nrp)-1 or 2 and type A (PlexA) or type D (PlexD) plexins [90]. The cytoplasmic domain of plexins is characterized by two interacting Ras GTPase-activating protein (GAP) domains separated by a linker region [91]. Ligand-dependent clustering renders the receptor constitutively active in the presence of Rnd1 [92,93] or Rac [94] that, by binding to the linker region, relieves the interaction between the two RasGAP domains and unleashes plexin GAP activity. Negishi and collaborators found that plexins exert their enzymatic function on the integrin activating R-Ras GTPase [91,93,95] whose inhibition is required both for SEMA3A/PlexinA1- and SEMA4D/PlexinB1-mediated growth cone collapse. All together, our [88,89] and Negishi's data [91,93,95] support the concept that integrin-adhesive receptors are critical effector targets of SEMA/Plexin signaling. In summary, based on the observations that signaling from angiogenic growth factors [13,73–75], ILK [85,87], focal adhesion kinase [96], fluid shear stress [78], the Eph/ephrin system [53,88], and the SEMA/Plexin system [88,89] impinge on integrins, it is tempting to speculate that pro- and anti-angiogenic cues regulate vascular morphogenesis quite generally by modulating integrin activation. Autocrine loops of integrin activators and inhibitors would set basal level of EC adhesion and responsiveness to paracrine integrin activators and inhibitors secreted by the target tissues, which needs to be vascularized, such as developing epithelia in the embryo or carcinomas and healing epithelia in the adult organism (Fig. 2).

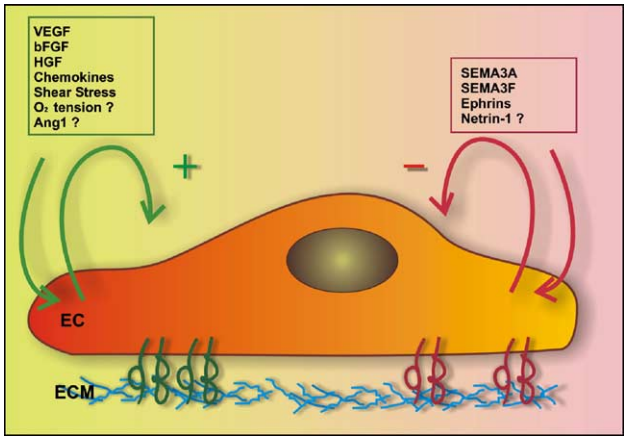


Fig. 2. Angiogenic cues regulate integrin function. Regulation of EC adhesion and motility is crucial for embryonic vascular development. Integrins are primary extracellular matrix (ECM) receptors that exist in different functional states in terms of affinity for ECM proteins (high affinity state, green; low affinity state, red). Autocrine loops of integrin activators and inhibitors set basal level of EC adhesion and responsiveness to paracrine integrin activators and inhibitors secreted by the target tissues. Angiogenic growth factors (e.g. HGF, bFGF, and VEGF), chemokines, and shear stress act as positive regulators of EC integrin function, whereas angiopoietin-1 (Ang1) and low $O_2$ tension promote cell adhesion to different ECM ligands. Autocrine loops of class 3 semaphorins (SEMA3A, SEMA3F) and ephrins negatively regulate integrins and allow EC redirectioning during migration, whereas exogenous netrin-1 induces retraction of EC filopodia.

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