REVIEW ARTICLE

Angiogenesis: a balancing act between integrin activation and inhibition?

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ABSTRACT. Acquisition of new genes encoding for extracellular matrix (ECM) proteins and their cognate integrin adhesive receptors, as well as secreted pro- and anti-angiogenic factors, proved to be essential for the development of functional vascular networks in the vertebrate embryo. There is now clear evidence that post-natal, pathological tissue neo-vascularization is crucial for cancer growth and therapy as well. Integrins are major ECM receptors that can exist in different functional states with respect to their affinity for ECM proteins. Regulation of integrin activation is crucial for their biological functions. In the embryo, the development of a properly patterned network of blood vessels relies upon the fine modulation of integrin activation by chemoattractant and chemorepulsive cues, such as angiogenic growth factors and semaphorins. Such a fine-tuning of endothelial integrin function is likely to be disrupted in cancer. Here, the vasculature is structurally and functionally abnormal and therefore inadequate for an efficient drug and oxygen delivery, which is a mandatory pre-requisite for successful chemotherapy and radiotherapy. It is thus important to identify the molecular mechanisms that regulate integrin function in normal ECs and which are altered in tumor ECs.

Keywords: integrins, semaphorin, neuropilin, plexin, vascular endothelial growth factor

ABERRANT VASCULAR MORPHOGENESIS IN CANCER: AN ADHESION ISSUE?

Solid cancers initially arise as microscopic avascular lesions that must elicit angiogenesis, *i.e*. the formation of new blood vessels from pre-existing ones, to grow beyond a minimal size and metastasize. Notably, the tumor vasculature displays multiple morphological abnormalities that can be due to, what remain, poorly characterized defects in the molecular mechanisms that orchestrate the angiogenic remodeling [1]. As a direct outcome, chemotherapeutic drugs and molecular oxygen, which is mandatory for the formation of reactive oxygen species and hence successful radiotherapy, are inefficiently delivered to cancer cells in many solid tumors [2]. Therefore, as originally proposed by R. Jain [3], normalization of tumor blood vessel architecture could result in a sizeable increase in the effectiveness of standard anti-cancer therapy. Thus, a deeper understanding of the molecular mechanisms that support physiological vascular morphogenesis, and that are likely perturbed in cancer, is required in order to attain the capacity of converting aberrant tumor blood vessel in a quasi-normal vascular network.

The final shape of the cardiovascular system results from the balancing and combinatorial interaction of several factors: *i)* local environmental factors, *e.g*. tissue oxygen and nutrient demand; *ii)* the blood flow; *iii)* genetically programmed extrinsic cues, *e.g*. VEGF-A, Ang-1, ephrins, and semaphorins [4]. During angiogenic remodeling of pre-existing vessels, endothelial cells (ECs) move and change their reciprocal positions and interactions in response to the several guidance cues that control their motility [5]. As a result of a balanced response to fluid shear stress, chemoattractant and chemorepulsive agents, ECs dynamically regulate their adhesiveness both in terms of cell-to-cell and cell-to-extracellular matrix (ECM) contacts. In this regard, EC interactions with the surrounding ECM are of particular relevance since, in multicellular organisms, directed cell motility is a coordinated process largely impinging on the regulation of cell adhesion to ECM [6]. In particular, in vertebrate embryos the ECM protein fibronectin represents the earliest and most abundant vascular basement matrix molecule and is essential for embryonic vascular development [7] and branching morphogenesis [8]. Hence, a minimal hypothesis could be that defective vascular morphogenesis results at least in part from perturbations of vascular EC interaction with the surrounding ECM.

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REGULATION OF INTEGRIN FUNCTION BY PTB DOMAIN-CONTAINING PROTEINS AND SMALL GTPases

Integrin αβ heterodimers are primary ECM receptors. In mammals, 18 α subunits and 8 β subunits of integrins assemble into 24 distinct receptors, and several integrin heterodimers have been seen to be involved in angiogenesis [5]. Integrins exist in diverse conformational states that are endowed with different binding affinity for ECM proteins *(figure 1)*, and the modulation of integrin shape is central for the fulfillment of their biological functions [9]. In the low affinity state, the large extracellular domain of integrins is bent over the cell surface, whereas α and β transmembrane and cytoplasmic domains tightly interact and are probably stabilized through a juxtamembrane salt bridge involving the arginine of the GFFKR sequence in an α subunit cytodomain and an aspartic residue in the β subunit tail [9]. Interaction of the phosphotyrosine-binding (PTB) domain containing proteins *(figure 1)*, such as talin [10] and kindlin [11-14], with the two NPxY tandem repeats located in the cytoplasmic tail of the integrin β subunit, causes its separation from the α subunit cytodomain, the extension of the extracellular domain, and the uncovering of the ECM binding site of integrins [9]. The talin PTB domain is part of a larger trefoil protein 4.1/ezrin/radixin/moesin (FERM) domain, and can be involved in an intramolecular association with the rod domain that impairs talin binding to the integrin β subunit [10]. Such an inhibition can be relieved upon binding of plasma membrane phosphatidylinositol-4,5 bisphosphate (PIP2) to the talin rod domain *(figure 1)*, thus allowing talin activation, head-to-tail dimerization and integrin binding [10]. In addition, activated talin can also bind the FERM domain of phosphatidylinositol-4-phosphate 5-kinase (PIPKIγ 661), increase PIP2 levels at adhesion sites, and thus trigger a positive feed-back loop *(figure 1)* supporting cell adhesion to the ECM [15].

The small GTPase R-Ras *(figure 1)*, which, *in vivo*, is largely expressed by vascular ECs and smooth muscle cells [16], has been found to promote integrin-mediated cell adhesion to different ECM proteins [17]. However, the molecular details of the effectors that directly mediate R-Ras activity on integrin function are still poorly defined *(figure 1)*. R-Ras (also called R-Ras1), together

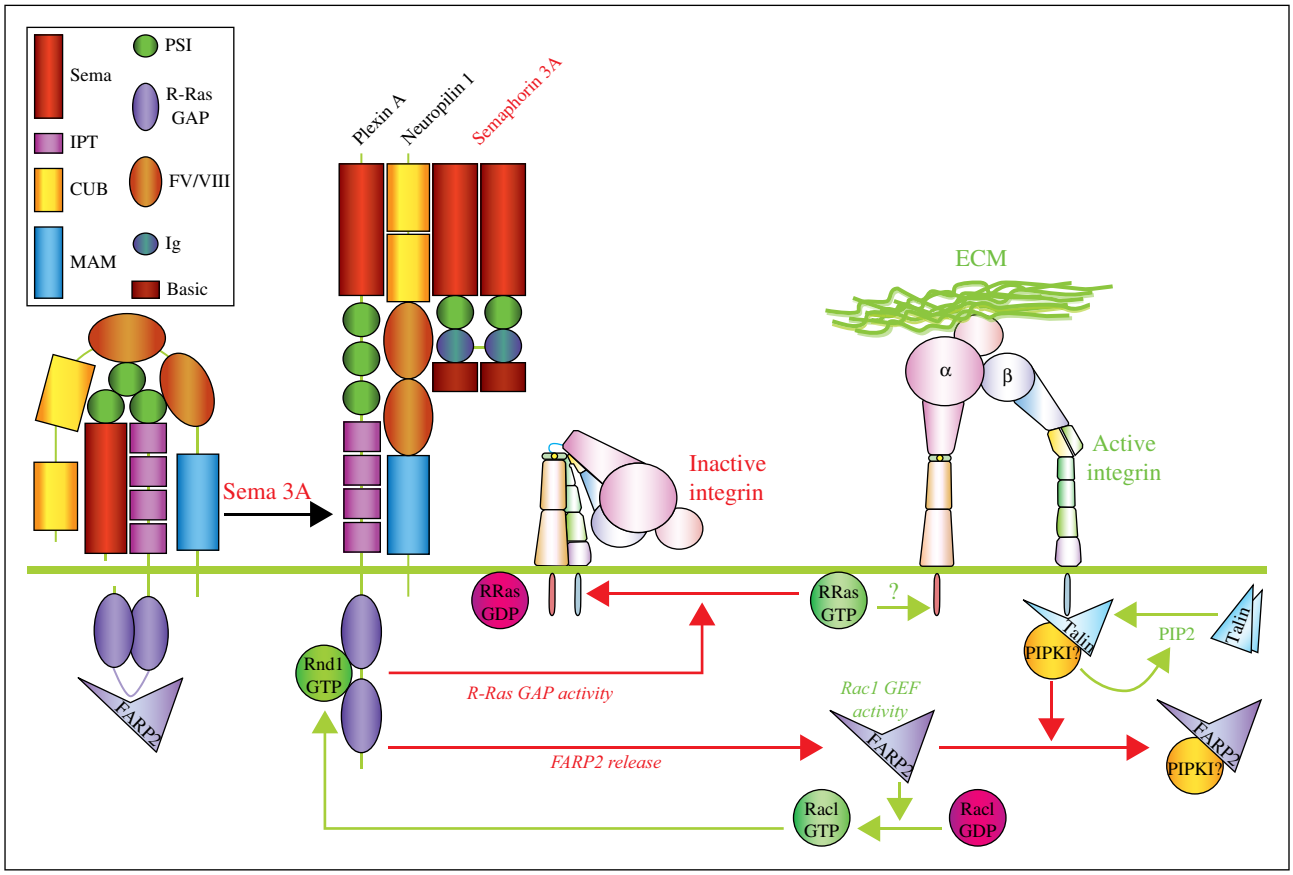

Figure 1

Regulation of integrins by the PTB-domain containing protein talin, R-Ras small GTPase, and the Sema3A/Nrp1/PlexinA system.
αβ integrin heterodimers exist on the cell surface in a bent/low affinity and extended/high affinity conformation, the latter being stabilized by the interaction of the talin FERM domain with the NPXY motif of the β subunit tail. Activated talin binds the PIPKIγ661 enzyme that, producing the talin-activating lipid PIP2, gives rise to a positive feedback loop, which amplifies talin activation and supports cell adhesion to the ECM. The small GTPase R-Ras localizes to adhesive sites and participates in integrin activation through a still poorly characterized mechanism(s). In the absence of Sema3A, PlexinA1 associates with Nrp1 and the FERM domain-containing Rac1 GEF FARP2. Binding of Sema3A to the Nrp1/plexin A1 complex elicits FARP2 release from plexin A1. Free FARP2 then activates Rac1, which somehow favors the association of Rnd1 with the plexin A1 cytodomain and the activation its R-Ras GAP activity that finally promotes the transition of R-Ras from a GTP-bound/active to a GDP-bound/inactive conformation. In addition, once released from plexin A1, FARP2 binds to and inhibits PIPKIγ661 enzymatic activity.

with TC21 (also known as R-Ras2) and M-Ras (also named R-Ras3), constitute the R-Ras branch of the wide Ras superfamily of small GTPases [18]. Remarkably, integrin function is activated by R-Ras and TC21/R-Ras2 and inhibited by H-Ras [17]. R-Ras is 55% identical to H-Ras, displays a 26 amino acid N-terminal extension, and its effector region is identical to H-Ras [18]. Intriguingly, H-Ras and R-Ras display a significantly different plasma membrane compartmentalization [19, 20]. Active R-Ras-GTP is targeted to FAs *via* a specific sequence (aa 175-218) within the C-terminal region [19] that is known to be highly variable among different GTPases [18]. Moreover, in R-Ras, a stretch of prolines at the C-terminus bind the SH3 domain of the adaptor protein Nck; changing the prolines at positions 202 and 203 disrupted R-Ras-induced adhesion to the ECM [21]. In addition, while active R-Ras localizes into lipid rafts [20], H-Ras translocates from lipid rafts into non-raft microdomains upon activation [22]. Swapping experiments with H- and R-Ras showed that the R-Ras hypervariable region (aa 193-218) contains a transferable, molecular determinant endowed with the ability of activating integrins and re-localizing the GTPase to lipid rafts [20]. However, changing R-Ras aa 208-218 into H-Ras allows its subcellular compartmentalization in lipid rafts, but does not confer it with the ability of activating integrins [20]. The fact that R-Ras localizes both in FAs and lipid rafts is compatible with the recent observation that integrin signaling regulates lipid raft distribution [23-25]. Together, these data indicate that both targeting to FAs and interaction with specific downstream effectors must be involved in defining the unique capability of R-Ras-GTP to promote integrin function. This is in agreement with the concept that a bipartite recognition process generates specificity within the Ras superfamily. One part of the effector binds to the switch regions and senses the active state of the GTPase, whereas the interaction of another region confers the specificity of the effector [26]. Subcellular localization of active GTPases and specific effectors would further contribute to specificity. The 100% homology of the amino acid sequence of the H- and R-Ras effector loop region is probably why all R-Ras interactors isolated up to now (*e.g*. Raf, RalGDS, Nore1, and PI3K) bind H-Ras as well [17]. These shared interactors cannot provide a reasonable explanation for the opposing behavior that these two GTPases exert on integrin function. Accordingly, the fact that the effector loop mutant D64A of the constitutively active R-Ras V38 still activates integrins, but completely loses any interaction with shared effectors, clearly indicates that these proteins are not needed for integrin activation [27]. Activated R-Ras-GTP is thought to promote cell adhesion by favoring the activation of other small GTPases such as Rap1 [28] and Rac1 [19]. In this respect, it is interesting to note that binding of activated R-Ras to RLIP (Ral interacting protein) 76 leads to Arf (ADP-ribosylation factor) 6 activation, which promotes adhesion-induced GTP loading of Rac1 [29]. However, further work is required to thoroughly dissect the molecular mechanisms by which R-Ras activates integrins.

A BALANCING ACT BETWEEN INTEGRIN ACTIVATION AND INHIBITION

High affinity integrins are highly concentrated at adhesion sites [30] and are at the leading edge of migrating ECs [31] where they promote new adhesions to support directed cell motility. Major determinants of vascular remodeling, such as fluid shear stress and angiogenic growth factors, activate integrin adhesive function [5]. Moreover, 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 *(figure 1)* [5, 32]: the inhibitory autocrine loops of Sema3 can hence assure a continuous and subtle modulation of integrin function *versus* an all-or-none activation. Such a fine-tuning of integrin-mediated adhesion to the ECM allows a graded control of EC migration and redirectioning during physiological vascular remodeling. Accordingly, after few minutes of stimulation with either Sema3A or Sema3F, adherent ECs lose their focal adhesions [33] and Sema3A inhibits integrin activation elicited by several pro-angiogenic factors such as VEGF-A165, bFGF, and PlGF2 [32]. Notably, vascular abnormalities usually observed in solid tumors appear to be due to an inbalance between integrin activators and inhibitors in favor of the former *(figure 2)*, *e.g*. VEGF-A [2, 34] and bFGF [35]. Correspondingly, treatment of solid tumors with VEGF-A inhibitors transiently reverts the structural and functional abnormalities of tumor blood vessels *(figure 2)*, resulting in an improved capacity for drug and oxygen delivery [2-4, 34]. Since mutually antagonistic autocrine loops of VEGF-A [36-38] and Sema3 [32, 39-41] are present in ECs, both *in vitro* and during normal angiogenesis *in vivo*, an imbalance in the ratio of autocrine VEGF-A/Sema3 in ECs might happen during tumor progression *(figure 2)* and contribute to the structural and functional defects of the tumor blood vessels [2, 3, 34]. In support of this hypothesis, bone marrow ECs of patients with malignant multiple myeloma lose autocrine loops of Sema3A in favor of endogenous VEGF-A [41]. Therefore, it is tempting to speculate that Sema3A is part of a negative autocrine loop that brings under control VEGF-A signaling in order to self-limit angiogenesis and encourage the development of a functional vasculature *(figure 2)*. Seven, class 3 Sema exists (Sema3A to Sema3G), and it is thus likely that other Sema3 could function as angiogenesis regulators [42]. For example, Sema3F, which signals through Nrp-2, is known to inhibit integrins [32], induce the disassembly of ECM adhesions [33], and inhibit both VEGF-A and bFGF activity on cultured EC [32]. When overexpressed, Sema3F lessens the angiogenic and metastatic phenotype of melanomas and transforms these lesions into benign and encapsulated tumors [43]. Recombinant Sema3A and/or Sema3F could therefore be employed as pharmacological agents in order to restrain excessive VEGF-A- or bFGF-elicited tumor angiogenesis, inhibit tumor progression, and perhaps normalize tumor blood vessels thus favoring the activity of chemotherapeutic drugs and radiotherapy *(figure 2)*. To this end, it is therefore crucial to characterize the signaling pathways that regulate the function of endothelial integrins.

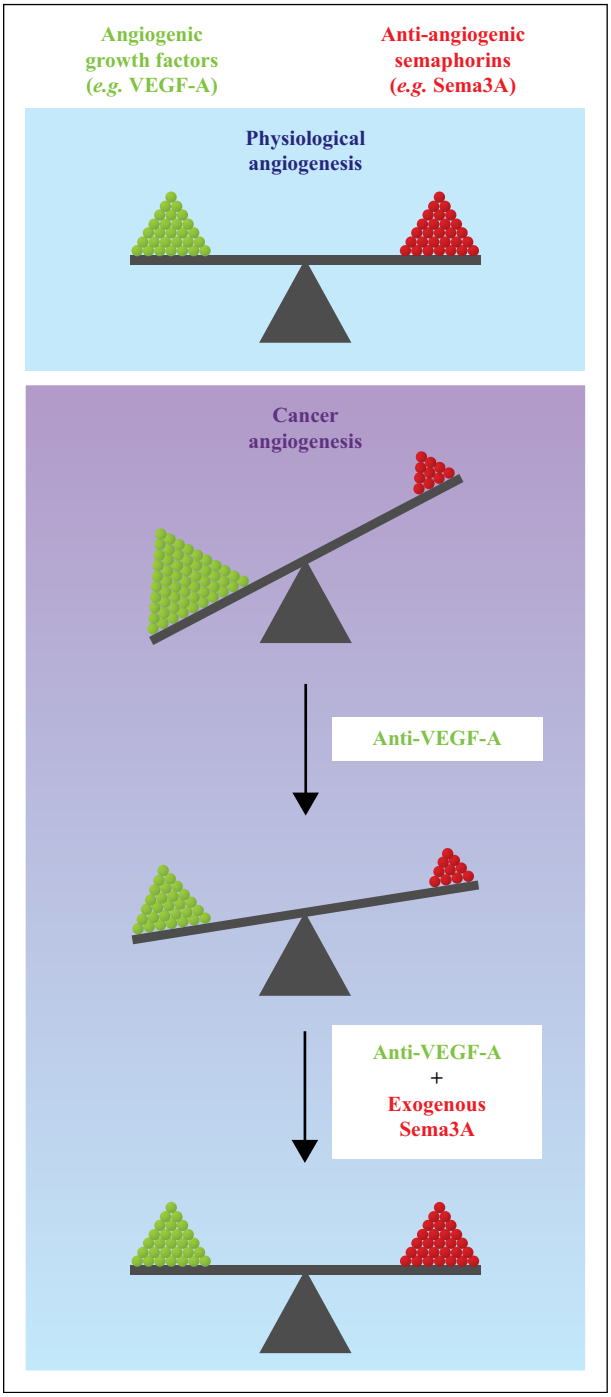

Figure 2
Angiogenesis: a balancing act between integrin activators and inhibitors.
Opposing autocrine loops of VEGF-A and Sema3A are present in angiogenic ECs. An increase in the autocrine VEGF-A/Sema3A ratio in ECs could contribute to the defects in tumor blood vessels and support cancer progression. Treating cancers with VEGF-A inhibitors only transiently reverts the structural and functional vascular abnormalities. The reported resistance to VEGF-A-targeted therapies could be due to the loss of endogenous Sema3A in tumor ECs. Re-establishing Sema3A levels in tumors could synergize with VEGF-A inhibitors and help increase the vascular normalization potential of anti-angiogenic therapies.

In different experimental systems, growth factors [5] and semaphorins [44-46] regulate integrin function by exerting opposite effects on the activation of the small GTPase R-Ras *(figure 1)*. Neuropilins (Nrp) and type A or D plexins are the ligand binding and the signal transducing subunits of SEMA3 receptors respectively. The cytoplasmic domain of plexins is endowed with an R-Ras GTPase-activating protein (GAP) activity *(figure 1)*. Specifically, the juxtamembrane basic sequence of class A plexins directly interacts with FARP2 *(figure 1)*, a Rac guanosine exchange factor (GEF). Sema3A binding to the Nrp-1/plexinA1 complex induces the dissociation of FARP2 from plexinA1 [47]; next, FARP2 GEF activity elicits a rapid increase of active Rac1-GTP that in turn facilitates the binding of the small GTPase Rnd1 to the linker region of plexinA1 cytodomain *(figure 1)* [48]. This event finally activates the R-Ras GAP activity of plexin A1 that that impairs the function of the small GTPase R-Ras and is required for Sema3A inhibition of integrins. In addition, FARP2 holds a FERM domain that mediates its binding to plexin A1 [47]. Upon dissociation from plexinA1, the FERM domain of FARP2 competes with talin for binding to PIPKIγ661 and hence impairs the talin/PIPKIγ661/PIP2/talin positive feedback that supports the formation of ECM adhesion sites *(figure 1)*.

CONCLUSION

It is well accepted that that tissue neo-vascularization is crucial for cancer growth and therapy [49]. However, the architecture and function of tumor vasculature are abnormal, and are ineffective for successful chemotherapy and radiotherapy [2, 3, 34]. The development of a properly patterned vascular tree depends also on the modulation of integrin function by chemoattractant and chemorepulsive molecules, *e.g.* angiogenic growth factors and semaphorins [5]. Such a fine-tuning of endothelial integrin function is likely to be disrupted in solid tumors. Indeed, opposing autocrine loops of VEGF-A [36-38] and Sema3A [32, 39-41] are present in angiogenic ECs, and an imbalance in the ratio of autocrine VEGF-A/Sema3A in ECs could support cancer progression and contribute to the defects in tumor blood vessels *(figure 2)*. Notably, resistance to VEGF-A-targeted therapies, due to loss of responsivity to VEGF-A inhibitors, has been reported [34]. Recently, Vacca and colleagues showed that tumor ECs can lose the autocrine loops of Sema3A in favor of endogenous VEGF-A [41]. Thus, restoring Sema3A in tumors could synergize with VEGF-A blockers and help to improve the efficacy of current anti-angiogenic therapies *(figure 2)*. In addition, it will be important to characterize the molecular mechanisms that regulate integrin function in normal ECs and that could be disrupted in cancer ECs.

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