Chapter 1

TGF-β Activation and Signaling in Angiogenesis

Paola A. Guerrero and Joseph H. McCarty

Additional information is available at the end of the chapter

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Abstract

The transforming growth factor-β (TGF-β) signaling pathway regulates various cellular processes during tissue and organ development and homeostasis. Deregulation of the expression and/or functions of TGF-β ligands, receptors or their intracellular signaling components leads to multiple diseases including vascular pathologies, autoimmune disorders, fibrosis and cancer. In vascular development, physiology and disease TGF-β signaling can have angiogenic and angiostatic properties, depending on expression levels and the tissue context. The objective of this chapter is to analyze the mechanisms that contribute to the activation and signaling of TGF-β in developmental, physiological and pathological angiogenesis, with a particular emphasis on the importance of TGF-β signaling in the mammalian central nervous system (CNS).

Keywords: TGF-β, vasculogenesis, angiogenesis, VEGF

1. Introduction

Discovery of TGF-βs was the result of independent efforts by several laboratories [1–4] during characterization of a secreted factor from fibroblasts transformed by the Moloney sarcoma virus (MSV). The TGF-β superfamily is now known to be composed of more than 30 chemokines such as TGF-β1-β3, activins, anti-Müllerian hormone (AMH), bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs) and NODAL that can signal via canonical and noncanonical receptors and intracellular effector proteins [5].

The best characterized member of the TGF-β family, TGF-β1, is initially produced from a single gene as a large precursor known as pre- and pro-TGF-βs which undergo two proteolytic cleavage events. The first signal peptide is cleaved in the rough endoplasmic reticulum. Furin, a proprotein convertase, subsequently cleaves the protein into two fragments [6]. The carboxy terminus corresponds to the functionally active cytokine and the large amino

terminus is latency-associated protein (LAP), also referred to as the prodomain. Regardless of this processing by furin, the mature and LAP domains remain associated by noncovalent bonds to form the small latent complex (SLC). This complex subsequently covalently interacts with a second gene product, the latent TGF-β binding protein (LTBP), and is incorporated into a larger latent complex (LLC) that associates with the extracellular matrix (ECM) [6]. Three-dimensional crystal structure of porcine latent TGF-β1 shows a conformation that resembles a ring-like shape [7]. Two domains were defined in the structure: (i) an arm domain that contains an integrin-binding Arg-Gly-Asp (RGD) peptide motif and (ii) a "straitjacket" domain where the mature TGF-β is encased. At the opposite end of the arm domain, LTBP binds the prodomain forming the "ring head" [7] (**Figure 1**).

After secretion, the LLC complex interacts with various ECM proteins, such as fibronectin and fibrillin, and is maintained in an inactivated form [8]. TGF-β is activated by different mechanisms, including interactions with integrins, alterations in pH and extracellular proteases. αv integrin, which forms heterodimers with five different β integrin subunits (β1, β3, β5, β6 and β8), that bind to LAP-TGF-β1 and LAP-TGFβ-3 [9, 10]. However, only αvβ6 and αvβ8 have been shown to activate the latent TGF-β complex [11]. Activation by both αvβ6 and αvβ8 integrins requires the RGD motif in LAP. Activation by αvB6 requires an intact cytoplasmic domain [12, 13] and the presence of other ECM proteins [14]. Activation by αvβ8, however,

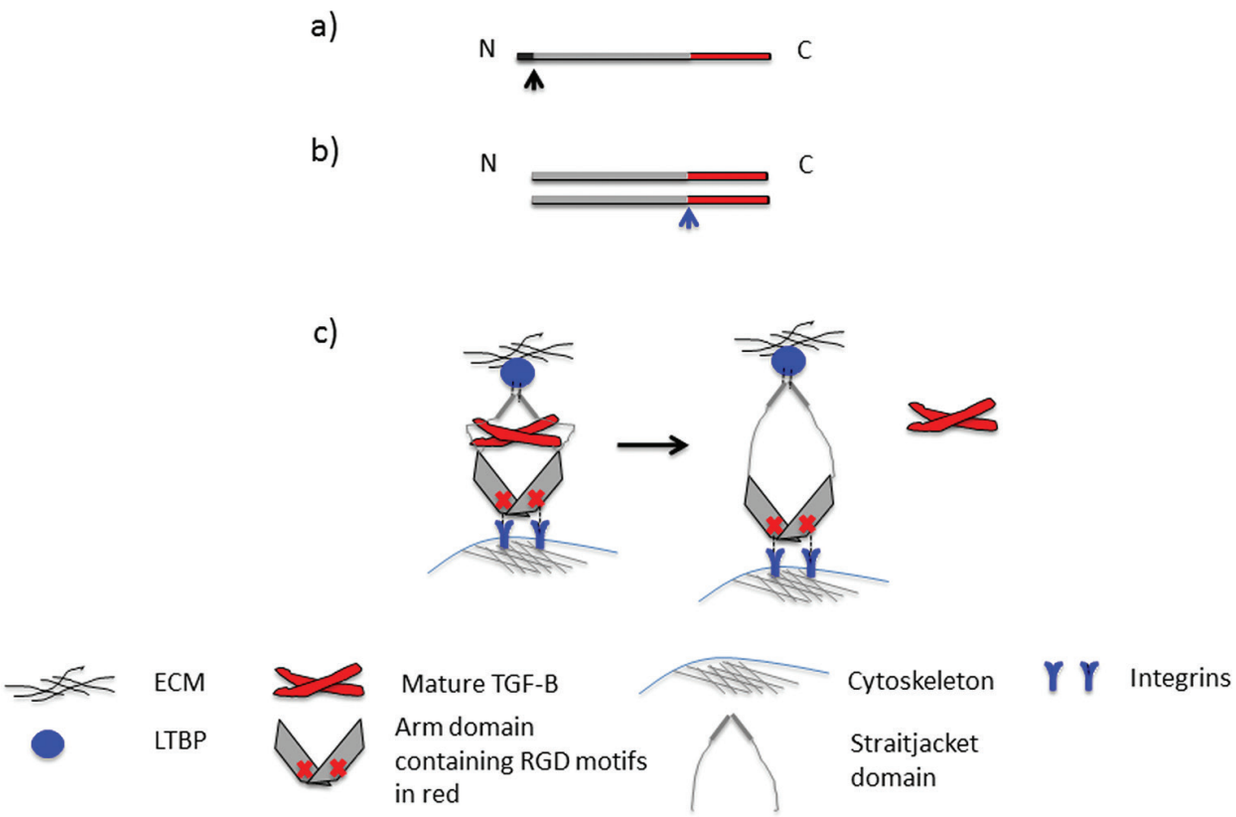

Figure 1. TGF-β processing and activation. **(a)**TGF-β precursor undergoes proteolysis at its N-terminus (black arrow head) which results in the removal of its signal peptide. **(b)**In a second proteolytic cleavage event by furin (blue arrow head), the precursor is separated into a large LAP or prodomain (gray) and the mature TGF-β (red) and **(c)**Schematic view of the closed ring structure (left) and unfastened straitjacket (right) conformation corresponding to the inactive LAP-TGF-β and mature TGF-β, respectively.

does not require the integrin cytoplasmic domain, but it is reported to require the presence of metalloproteinases (MMPs) on the cell surface or in the ECM [9]. Additionally, in T cells, αvβ6 and αvβ8 can activate LAP-TGF-β in cooperation with the glycoprotein-A repetitions predominant protein (GARP) [15, 16].

TGF-β is also activated by proteases. Aspartyl (e.g., cathepsin D) [17], cysteine (e.g., calpain) [18] and serine proteases (e.g., plasmin and kallikreins) and metalloproteases have shown to stimulate the release of chemokine from the latent complex, although most of these studies have been performed *in vitro* [19]. Moreover, TGF-β has been reported to be activated by other nonprotease mechanisms such as neuropilin1 (Nrp1), thrombospondin (TSP-1), F-spondin, pregnancy-specific beta-1-glycoprotein 1 (PSG1) and deglycosylation. Likewise, there are chemical and physical settings that activate TGF-β, for example, heat, ultraviolet radiation, physical shear, detergents and reactive oxygen species [8].

Three-dimensional structural studies of the LLC reveal that the RGD motifs are readily available for integrin engagement. Hydrophobic side chains, which have been identified near the RGD motif, likely enhance integrin binding [7]. In the presence of αvβ6, LLC can bind one or two integrin monomers. However, this binding does not induce required conformational changes to promote the complete activation of TGF-β, which is in agreement with prior mutational studies [12, 13, 20]. Furthermore, in accord with previous studies, the crystal structure of latent TGF-β predicts that pulling forces, emanating from the integrin C-terminal cytoplasmic tail that interacts with the cytoskeleton and binding RGD via the N-terminal extracellular region, are counteracted by associations with the ECM. Therefore, in the latent TGF-β, the straitjacket domain is maintained in a closed conformation until tensile forces are applied from both ends of the structure, resulting in loosening of the straitjacket domain and the release of the mature TGF-β. This study also showed that an additional feature of the prodomain is to prevent access to activating receptors [7].

2. TGF-β signaling pathways

Signaling is regulated by three major receptors: TGF-β receptor type I (TβRI), type II (TβRII) and type III (TβRIII). In general, TGF-β binds TβRIII, which facilitates its delivery to TβRII, a constitutively active kinase, leading to the subsequent phosphorylation and activation of TβRI. In humans, there are seven TβRIs, also known as activin receptor-like kinases (ALK), and five TβRIIs [5]. In most cells, ALK-5 forms a heterodimer with TβRII bound to TGF-β, which activates the ALK-5 kinase domain via phosphorylation of its GS domain. This receptor activation propagates intracellular signaling through 'canonical' effector proteins mothers against decapentaplegic homolog 2/SMAD family member 2 (Smad2) and Smad3, which are transcription factors. Once phosphorylated, these Smad proteins form a complex with Smad4 leading to nuclear translocation and initiation of genes transcription. In most normal cells, TGF-β-mediated activation of Smads leads to inhibition of cell growth. More specifically, the Smad2/3-4 complex partners with foxhead box O (FOXO) factors to activate p21Cip1 (*CDKN1A*), which inhibits cyclin-dependent kinase 1(CDK1), resulting in cell cycle arrest. Similarly, TGF-β can also activate p15Inkab (*CDKN2B*), the CDK4 inhibitor, through the SMAD2-3/4-FOXO1 axis (**Figure 2**) [5].

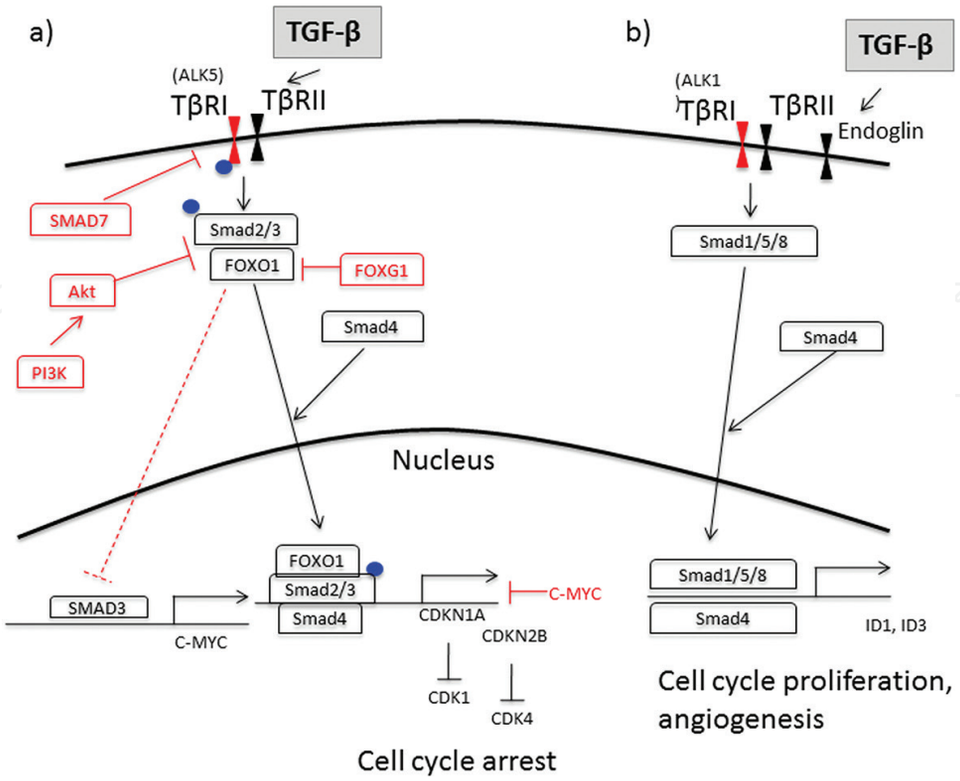

Figure 2. TGF-β canonical pathway. **(a)**In normal cells and early stages of cancer TGF-β promotes cell cycle arrest. Repressors of the pathway are shown in red. Blue dots represent protein phosphorylation and **(b)**in endothelial cells, an alternative pathway promotes cell proliferation.

Likewise, TGF-β acts as a cytostatic factor by decreasing c-Myc expression and downregulating the inhibitor of DNA-binding protein (ID) 1 and ID3 transcription factors. ID1 and ID3 are involved in differentiation, cell cycle progression and self-renewal of stem cells [21, 22]. TGF-β elicits c- Flk-1myc repression by promoting SMAD3 binding to a repressing Smad-binding element (RSBE) at the c-myc promoter [23]. c-Myc can be recruited to the promoters of *CDKN1A* and *CDKN2B* by the Myc-interacting zinc-finger (MIZ-1). This blocks CDK expression and results in apoptosis [24]. Additionally, in endothelial cells (ECs), TGF-β can target a second receptor type 1, ALK-1, which signals through Smad1/5/8 and stimulates angiogenic factors, such as interleukin 1 receptor-like 1 and ID1 (**Figure 2**) [25].

Several proteins are known to antagonize canonical TGF-β signaling. For example, (i) PI3K activates AKT which phosphorylates the SMADs-FOXO complex and inhibits its translocation to the nucleus [21], (ii) foxhead box G1 (FOXG1) inhibits the SMADs-FOXO complex [21], (iii) SMAD7 can trigger TβRI for proteosomal degradation by recruiting SMAD-specific E3 ubiquitin protein ligase (SMURF1) and SMURF2 [26], (iv) SMAD6 blocks SMAD1 through SMAD4 binding, (v) Erk proteins phosphorylate SMADs and inhibit their nuclear translocation, (vi) BAMBI, a pseudoreceptor, dimerizes with TβRI leading to its inactivation, (vii) FKBP12 binds to TβRI and impedes its phosphorylation, activation and signaling [27] and (viii) protein arginine N-methyltransferase 1 (PRMT1) methylates SMAD6 and allows BMP signaling through SMADs1/5 [28–30].

3. Vasculogenesis

During embryogenesis, the development of the vascular system is divided into three stages, vasculogenesis, angiogenesis and arteriogenesis. Vasculogenesis occurs in embryonic organs as well as extraembryonic tissues such as the placenta, yolk sac and allantois [31]. The earliest discernible structures in vasculogenesis, the blood islands, are formed in the mouse yolk sac by embryonic day (E) 6.5–7. This structure contains precursor cells or hemangioblasts, which differentiate to EC and hematopoietic cells [32]. At E8.5, cells located toward the periphery of the blood island, or angioblasts, differentiate into EC, while cells located toward the central region give rise to hematopoietic precursor cells. Next, lumenization takes place; tight junctions and basement membranes develop, and pericytes are recruited to blood vessels and promote maturation [33].

Several growth factors have been identified to regulate vasculogenesis, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), the hedgehog family, neuropilins, integrins, fibronectin and TGF-βs. FGF-2 has been reported to participate in the generation of the angioblast in quail/chick chimeras and in vessel formation [34].

Hedgehog signaling has been shown to be crucial in the initial steps of vasculogenesis. It promotes differentiation of the primitive endoderm into both, endothelial and hematopoietic lineage [35]. For instance, blocking Indian hedgehog (Ihh) causes signaling repression from the visceral ectoderm and consequently abrogation of vasculogenesis and hematopoiesis in anterior epiblast [35]. Deletion of *Ihh* in mouse caused 50% lethality at midgestation with the remaining 50% dying at birth. Defects in blood vessel formation have been proposed as the cause for the lethality, which has been supported by experiments showing: (i) deletion of Sonic hedgehog (*Shh*) in mice resulted in a reduction in vascularization in lung [36], (ii) overexpression of *Shh* resulted in an increase in vascularization in neuroectoderm [37], (iii) depletion of *Shh* in zebrafish caused defective vasculature [38] and (iv) depletion of *Ihh* from stem cell-derived embroid bodies inhibited blood island differentiation [39].

VEGF signaling is crucial in vasculogenesis. Genetic studies have shown that deletion of *Flt-1* (VEGFR1), *Flk-1*/*KDR* (VEGFR2) and one or both alleles of *VEGF* cause embryonic lethality. VEGFR1 mutants exhibit aberrant central localization of the angioblasts in the blood island, instead of their normal localization toward the periphery [40]. These results implied that the growth of ECs was not inhibited in this region and led to the idea that VEGR1 hampers signaling from VEGF by ligand sequestration [33]. In addition, VEGFR2 mutants die around E9. In these embryos, both vasculogenesis and hematopoiesis do not initiate which was explained by faulty blood island in which cell migration was abrogated [41, 42]. Similarly, mutant heterozygous for VEGF die by E11 and showed impaired vasculogenesis and angiogenesis. These embryos showed severe abnormalities, such as underdeveloped brain and heart, decreased number of nucleated red blood cells in blood islands and aberrant vasculature in nervous system and placenta [43].

Neuropilins are co-receptors for VEGF receptors. Nrp1 is found in ECs of arteries, while neuropilin 2 (Nrp2) is found at the endothelium of lymphatic vessels and veins. Deletion of *Nrp1* in mice affects severely the central and peripheral nervous systems, as well as the yolk sac

vasculature [44]. In contrast, depletion of *Nrp2* has no effects in the vasculature of arteries or veins, but it does affect angiogenesis of the lymphatic vasculature [45, 46]. In addition, mice harboring deletions in both neuropilins have shown obstruction in vasculogenesis in the yolk sac and in the formation of the primary vascular plexus [47].

4. Developmental angiogenesis

Angiogenesis is the formation of new blood vessels from existing vasculature. It occurs by mechanisms including sprouting angiogenesis and intussusceptive angiogenesis (**Figure 3**). Sprouting angiogenesis initiates with the selection of endothelial tip cells at the vessel wall. These cells react toward extracellular stimuli and secrete proteolytic enzymes to digest the surrounding ECM. Tip cells are connected to endothelial stalk cells to direct the vascular sprout [33, 48, 49]. Once the new tube is formed and a lumen is established, the vessel is stabilized by the recruitment of pericytes to capillaries or vascular smooth muscle cells (vSMC) to arteries and veins [6, 33], leading to re-establishment of mature blood vessels.

Intussusceptive angiogenesis, also known as splitting angiogenesis, results in the formation of intermediate intracapillary pillars. This mechanism is more efficient than sprouting angiogenesis since it does not require cell proliferation. Instead, it needs the reorganization of existing ECs. In this process, (i) ECs from opposite sides of the blood vessel make contacts, (ii) ECs from both ends reorganize and cause a splitting in the vessel wall, (iii) an interstitial pillar core is generated and (iv) myofibroblasts, pericytes and finally collagen invade the pillar and a basement membrane is formed [33, 49, 50].

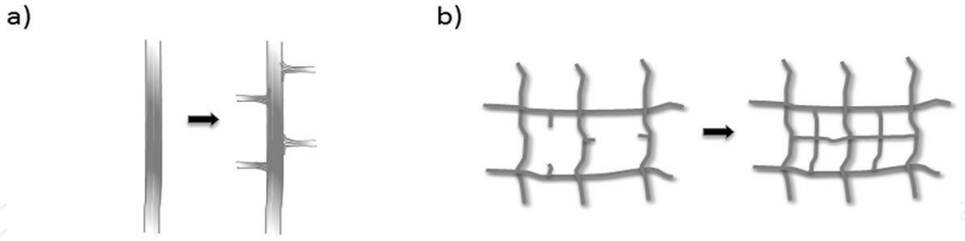

Figure 3. Mechanisms of angiogenesis. **(a)**Sprouting and **(b)**intussusceptive angiogenesis.

5. TGF-β in vasculogenesis and angiogenesis

5.1. Mutant phenotypes in mice lacking components of the TGF-B pathway

TGF-β mRNA was initially detected by PCR in preimplantation stages and in situ expression was present as early as E7.5, suggesting important roles in early development [51, 52]. In the embryo, proper TGF-β was detected in angioblast progenitors within the primitive heart mesoderm. Likewise, its expression was detected in extraembryonic tissues, including in the allantois mesoderm, and within blood islands of the yolk sac [53].

Deletion of the TGF-β1 gene in mice resulted in 50% lethality in utero, with the remaining 50% of mutant mice surviving up to three weeks postnatally. Histopathology analyses showed multifocal inflammatory cell infiltration and necrosis in several organs, especially the heart and stomach [53, 54]. It was subsequently shown that maternal contributions of *TGFB1* RNA and other genetic and epigenetic factors contributed to 50% postnatal survival [55].

The 50% of TGF-β mutants showed lethality and resorption by E10.5. Analysis of E8.5 embryos did not show significant morphological defects. However, analysis of E9.5 and E10.5 embryos resulted in a range of phenotypic defects within the yolk sac. While in some cases, vasculogenesis was delayed; in other cases, a dramatic reduction in size was observed and it was accompanied by weak and disorganized primary vessels, with some areas displaying complete vessel depletion. Analysis of the yolk sac vasculature indicated that the defects occurred during differentiation of ECs and hematopoietic cells. In contrast, the initial differentiation of mesodermal cells into ECs was not affected [52].

Genetic ablation of TβRII resulted in very similar phenotypes as in TGF-β1 mutants, with alterations in yolk sac vasculature and embryonic lethality by E10.5- E11.5 [56]. Mutants in endoglin also showed defects in vascular vessels within and outside the embryo. Embryo lethality was observed at E11.5, with mutants developing focal hemorrhage [57, 58]. Similarly, engineered mutations in mice that abrogate the expression of *ALK1, ALK5, SMAD1* or *SMAD5* resulted in defects in cardiovascular development [57, 59, 60].

5.2. Roles of TGF-β in angiogenesis

Early work to determine the roles for TGF-βs in ECs was contradictory. TGF-β signaling was initially found to inhibit cell migration and proliferation [61, 62], yet later studies indicated that it promotes cell proliferation [63–66]. The relative levels of expression of TGF-β seem to partially explain these discrepancies, with low doses promoting angiogenesis and higher levels resulting in growth inhibition of ECs and maturation of blood vessels [66, 67]. For instance, during blood vessel coverage by smooth muscle cells, TGF-β paracrine signaling from ECs to mesenchymal cells results in vascular smooth muscle cell and pericyte differentiation [6].

TGF-β also plays a role in the angiogenic process of hypoxic tissue. For instance, during infarction (stroke), neovascularization occurs primarily at the ischemic penumbra (periphery of the infarct), which correlates with high levels of both mRNA and active TGF-β protein [68]. Similarly, during organ transplant VEGF and TGF-β1, levels are increased in devascularized hypoxic tissue. TGF-β3 was also upregulated in hypoxic tissues, but to a lesser degree [69].

TGF-β regulates angiogenesis by different mechanisms; for example, it is involved in vessel proliferation and maturation by alternating two signaling cascades with opposite effects (ALK1 and ALK5). Likewise, TGF-β can promote its own expression, and it upregulates the expression of other angiogenic factors such as, platelet-derived growth factor (PDGF), interleukine-1, basic fibroblast growth factor (bFGF), tumor necrosis factor alpha and transforming growth factor alpha [70]. TGF-β can change the functions of other factors, such as VEGF, from prosurvival to pro-apoptotic [71]. Similarly, *in vitro* work has shown that in ECs TGF-β upregulates the expression of endothelin (*EDN1*), *PDGFA* and *PDGFB*, nitric oxide synthase

3 (*NOS3*), actin, alpha 2, smooth muscle, aorta (*ACTA2*), secreted protein acidic and cysteine rich (*SPARC*), *TSP-1*, fibronectin (*FN1*), collagens (*COL1A1, COL4A1*, and *COL5A1*), plasminogen activator (*PLAU*), serpin family E member 1 (*SERPINE1*) and integrins (*ITGB1, ITGB3, ITGAV, ITGA2* and *ITGA5*). It can also downregulate several genes, such as selectin-E (*SELE*), *KDR*, von Willebrand factor (*VWR*), thrombomodulin (*THBD*), monocyte chemo-attractant protein (*MCP1*), C-X-C motif chemokine ligand 1 (*CXCL1*), integrins (*ITGB1, ITGB3, ITGA5* and *ITGA6*), *TIMP1* and *PLAU* [70].

Levels of ALK1 and ALK5 determine TGF-β mitogenic or mitostatic responses in ECs. ALK5-Smad2/3 signaling stimulates transcription of ECM proteins such as fibronectin and plasminogen activator inhibitor type 1, which promote the resolution of angiogenesis by inducing vessel maturation. In contrast, signaling via the ALK1-Smad1/5/8 pathway generates anti-angiogenic responses [25, 51]. This requires a TGF-β accessory receptor, endoglin, which enhances ALK1 signaling and inhibits ALK5 cytostatic phenotype [72]. More recent work has shown that in the mouse eye retina the leucine-rich alpha-2-glycoprotein (Lrg1), which binds endoglin, promotes angiogenesis through Alk1-Smad1/5/8 in the presence of TGF-β [73].

5.2.1. TGF-β signaling in CNS development

In the mammalian CNS, neurons, astrocytes, pericytes and ECs closely interact to form a multicellular neurovascular unit [11]. During embryonic brain development, TGF-β is critical for sprouting angiogenesis of the CNS. In particular, TGF-β has been shown to work in conjunction with αv integrins to regulate paracrine signaling between neuroepithelial cells and ECs within neurovascular units. In mouse, embryos deletion of αv integrin showed vascular defects that were restricted mainly to the brain. This phenotype was recapitulated in β8 integrin mutant embryos. In contrast, deletion of β3 and β5 integrins did not cause brain vasculature abnormalities [74, 75]. Cell type-specific deletion of αv and β8 integrins in nervous system glial cells resulted in developmental intracerebral hemorrhage as well as postnatal motor dysfunction and seizures. Of note, the brain hemorrhage observed in embryos was absence in adult mice, suggesting that a compensatory mechanism that repairs hemorrhage occurs after birth [75, 76]. Interestingly, αv ablation in vascular ECs did not show a phenotype [75]. In later work, in which an outbred background was used to overcome the effects of β8−/− embryonic lethality, it was shown that adult mice lacking β8 integrin displayed neurovascular pathologies [77]. Most notably, adult β8 integrin mutants displayed a reduction in olfactory bulb size and abnormalities at the subventricular zone and rostral migratory stream. Neuroblasts generated in the subventricular zone utilize blood vessels as guides to migrate within the rostral migratory stream and differentiated to neurons within the olfactory bulbs. The size-reduced olfactory bulbs in adult β8−/− mice revealed essential roles for this integrin in promoting neuroblast migration along blood vessels. These defects correlated with a reduction in TGF-β signaling in neurospheres dissected from β8−/− mice [77].

The brain vascular defects observed in *Itgb8* null mutants are also shared by *Tgfb1* and *Tgfb3* loss of function mutants. In addition, mutating the integrin-binding RGD binding site in *Tgfb1* leads to early embryonic lethality [78]. Similarly, mice lacking both β6 and β8 integrins showed similar phenotypes as null mutants for *Tgfb1* and *Tgfb3* [78].

More recently, integrin β8 and Nrp1 have been shown to mediate neuroepithelial-endothelial cell interactions. β8 integrin in the neuroepithelium activates TGF-β signaling in ECs, while Nrp1 suppresses canonical TGF-β signaling, thus controlling normal sprouting angiogenesis [79]. Disruption of TGF-β signaling by targeting β8 integrin or Nrp1 results in excessive vessels sprouting and branching and formation of dysplastic glomeruloid-like vessels that are hemorrhagic [80].

The αvβ8-TGF-β connection in developmental angiogenesis in the brain also regulated neovascularization in the developing retina, where β8 integrin is expressed in astrocytes and Muller glial cells, a neuroepithelial cell type specifically found in the retina [81]. β8−/− retinas display abnormalities in the formation of the secondary vascular plexus, including impaired sprouting and formation of blood vessels with glomeruloid-like tufts. In addition, intraretinal hemorrhage was detected [81]. Furthermore, ablation of αv or β8 integrins but not of *Tgfbr2* in astrocytes resulted in defects in angiogenesis, and blocking TGF-β1 with neutralizing antibodies affected paracrine signaling to ECs [81]. This work was confirmed later in a study showing that *Tgfbr2* deletion in ECs of neonatal mice caused bleeding in the brain and vascular abnormalities, hemorrhage and deficiency in the formation of the deeper vascular network in the retina [82, 83]. Similarly, reduced Smad2 phosphorylation was observed in ECs from retina of *Tgfbr2* knockout mice [82].

6. TGF-β in pathological angiogenesis

Genetic mutations in TGF-β signaling components are associated with various human vascular pathologies. For example, mutations in the TβRIII/endoglin gene are linked to hereditary hemorrhagic telangiectasia (HHT) and Osler-Rendu-Weber syndrome. The disease is characterized, among others, by arteriovenous malformations (AVM) in the liver, brain and lung, telangiectases in skin and mucous membranes and recurrent epistaxis [84]. In trying to understand AVM, it was proposed that TGF-β paracrine signaling from ECs to vascular smooth muscle cells and/or pericytes was reduced. As a consequence, vascular smooth muscle cell differentiation was affected and this resulted in fragile, leaky blood vessels [85]. Alternatively, other suggested mechanisms underlying AVM endothelial cell apoptosis and depletion of smooth muscle cells [6]. In addition, it was discovered that brain AVM present decreased levels of integrin β8 which correlates with decreased TGF-β activation and signaling in ECs [86]. Mutations in human *ITGAV* and *ITGB8* genes also predispose some families to spontaneous brain hemorrhage [87].

In cancer, angiogenesis is not properly regulated as it occurs in developmental and physiological settings. Hypoxic conditions and proangiogenic factors released by tumor cells promote robust new blood vessel formation. The intratumoral vasculature is often disorganized and leaky with hypertension and acidosis. Similarly, tumor ECs display aneuploidy as well as centrosome and chromosomal amplifications [88]. The TGF-β pathway is affected by either mutations in the main signaling components, in particular SMADs, or by altered expression of repressive factors (e.g., FOXG1, PI3K-AKT and C-MYC) (**Figure 1**). Analysis of copy number alterations using data from The Center Genome Atlas (TCGA) and

analyzed by cBioportal show that Smad2, 3 and 4 are mutated, deleted or amplified in several tumor types, but especially in pancreatic, colorectal and gastric cancers [89, 90].

During tumor growth and progression, TGF-β plays a dual role as both an angiogenic and angiostatic factor [6]. In early-stage tumors, higher TGF-β expression levels are correlative with a better prognosis. TGF-βs exert cell cycle arrest by downregulating c-Myc and Ids 1–3 in late G1 phase and by promoting the expression of cyclin-dependent protein kinase inhibitors [25]. As a proangiogenic factor, TGF-β pathway collaborates with VEGF, PDGF and bFGF in autocrine/paracrine signaling. In highly vascularized tumors, such as GBM and hepatocellular carcinoma (HCC), TGF-β levels are upregulated. In HCC cells, TGF-β induces the secretion of VEGF-A. Accordingly, inhibition of TGF-β by the TβRI/II synthetic kinase inhibitor LY2109761 showed decreased tumor size, vessel density and VEGF expression. This inhibitor also affected paracrine signaling between tumor cells and ECs. These effects on VEGF-A were dependent on SMAD2/3 expression levels [91]. Likewise, TGF-β inhibition in other cancer types such as colorectal cancer and GBM has also led to reduce intratumoral vascularization [59, 92–94]. Of note, the increase in angiogenesis caused by TGF-β in GBM was decreased by inhibition of the JNK pathway [95]. TGF-β family members also promote the secretion of MCP1 and TGF-α [96, 97], which impact inflammatory cells in the tumor microenvironment.

6.1. VEGF regulation by TGF-β

TGF-β promotes the expression of a major regulator of vasculogenesis and angiogenesis, VEGF. The VEGF family is comprised of a large group of secreted glycoproteins that interact with various cell surface receptors. VEGF-A is expressed as four different isoforms (121, 165, 189, 206 a.a.). Isoform 121 has low binding for heparan sulfate and diffuses away from its secreted location creating a chemogradient. Isoform 165 has higher affinity to heparan sulfate compared with 121, and it shows the highest mitogenic capacity among all isoforms. Finally, isoforms 189 and 206 have shown the highest binding capacity to bind heparan sulfates and are known to interact with other components of the ECM [33]. These ligands bind mainly three tyrosine kinase receptors (VEGFR) 1–3. VEGF-A has been a major therapeutic target in cancer due to its upregulation in many cancer types. Unfortunately, anti-angiogenic therapies that target VEGF-A, such as the neutralizing antibody Bevacizumab, have been unsuccessful in clinical studies where patients have shown resistance to anti-VEGF therapy. Similarly, in vitro studies performed in GBM cell lines indicated that irradiation enhances VEGF secretion [98–101].

FGF-2 induces VEGF expression in ECs via paracrine and autocrine signaling mechanisms [71]. TGF-β balances FGF-2 by suppressing the induction of plasminogen activator, a serine protease involved in the migration of cells, which is required in the formation of capillaries during angiogenesis [102]. In contrast, TGF-β induces ECs apoptosis as part of capillary remodeling and at the same time promotes ECs expression of VEGF [103]. VEGF-A is an ECs survival factor and protects ECs from apoptosis [104]. More recent work has shown that VEGF targets p38MAPK resulting in ECs survival. In contrast, in the presence of TGF-β, FGF-2 is activated, promoting VEGF upregulation, p38MAPK activation and apoptosis [71].

6.2. Upregulation of TGF-β in GBM

In GBM, an "angiogenic switch" marks the transition from low to more malignant tumors where ECs proliferate, resulting in a major increase in blood vessels. This excessive growth in vasculature was initially thought to be required for maintaining the aggressive growth rate of GBM. However, recent work suggests that these aberrant blood vessels are also required to maintain glioma stem cells (GSC), which are known to localize in close proximity to ECs in a perivascular niche. These cells secrete VEGF and express VEGFR2, and this complex is stabilized by NRP1. This axis is involved in the self-renewal, survival and tumorigenic capacity of GSCs [105]. Recent work has shown that TGF-β induces differentiation of GSCs into pericytes to support vessel formation and tumor growth [106].

TGF-β cooperates in glioma angiogenesis by enhancing the expression of FGF, VEGF, PDGF-β, and CD44. Increased expression of FGF promotes VEGF expression. High levels of TGF-β in gliomas correlate with poor prognosis, and it is known to work in conjunction with the PDGF-β to increase GSCs proliferation [107]. In vSMC, TGF-β expression increases the levels of PDGF-β, its receptor (βPDGFR) and of EGF receptor (EGFR). βPDGFR promotes VEGF expression and secretion in ECs and signals through PI3K [108].

Angiogenesis in GBM develops by two mechanisms: microvascular cell proliferation and sprouting. The origin of microvascular proliferation seems to arise from hypoxia, where cells migrate away from the hypoxic center as a result of an increase in the levels of migration-related genes. As a consequence, the center becomes necrotic and the cells surrounding it form a palisade, which secretes angiogenic factors such as VEGF. This promotes neighboring angiogenesis, known as glomeruloid-like microvascular proliferation, composed of both endothelial and smooth muscle cells. In contrast, the increase in angiogenic capillaries can be observed by staining with markers such as CD31 and Factor VIII-related antigen (**Figure 4**) [109].

TGF-β1-3 levels and endoglin-ALK1 signaling are elevated under hypoxic conditions. In addition, under hypoxia, *Snail* and *Slug* expression levels are increased. These genes are known to be involved in endothelial to mesenchymal transition and in sprouting angiogenesis and are regulated by TGF-β in ECs [110].

Integrins are important activators of the TGF-β pathway. They are important in regulating tumor angiogenesis by serving as receptors for ECM components, such as laminin, tenascin, fibronectin and collagens [111]. αvβ3 is a receptor for various secreted ECM proteins, such as von Willebrand factor, TSP-1, fibrinogen, proteolyzed collagen, fibronectin and vitronectin, and it is involved in angiogenesis and vascular remodeling. For instance, αvβ3 integrin associates with MMP-2 in blood vessels of melanoma tumors, and this binding facilitates collagen degradation in vitro [112]. In GBM, TGF-β enhances αvβ3 integrin adhesion and expression and promotes integrin-mediated motility [111].

αvβ8 is expressed in GBM cell lines and primary tumors samples. GBM cell lines overexpressing αvβ8 show an increase in proliferation but when injected into the brain generate tumors with a decrease in vascularity. Silencing αv or β8 integrin in transformed astrocytes or in human GBM cell lines leads to decreased in TGF-β signaling, resulting in increased tumor size, intratumoral hemorrhage and decreased tumor cell invasiveness [113, 114].

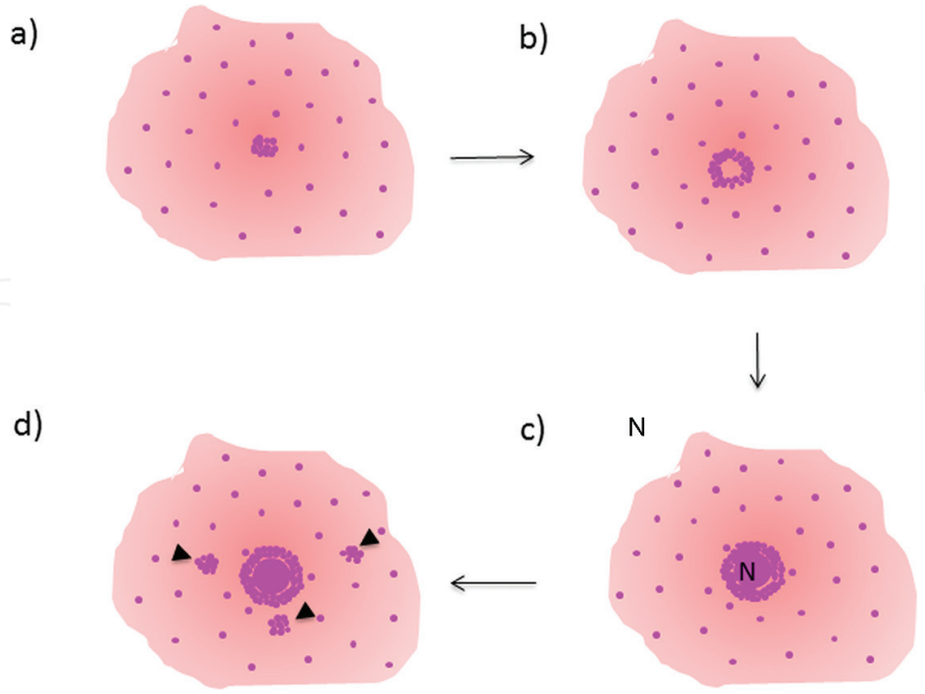

Figure 4. Microvascular proliferation. **(a)**Hypoxia center, **(b)**cell migration, **(c)**necrosis (N) and **(d)**glomeruloid microvascular proliferation (arrow heads).

In summary, TGF-β signaling is a crucial pathway in the angiogenesis of normal and tumor cells facilitating interactions between endothelial and epithelial cells. The vast majority of research has been focused on the activation of TGF-β, but more work is necessary to understand how the pathway is repressed. This could help to move forward current therapeutic attempts to target components of the TGF-β signaling pathway.

Author details

Paola A. Guerrero and Joseph H. McCarty*

*Address all correspondence to: jhmccarty@mdanderson.org

Department of Neurosurgery, University of Texas MD Anderson Cancer Center, Houston, TX, USA

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