# Building blood vessels in development and disease

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**Purpose of review**
This review will examine developmental angiogenesis and tumor-related changes to endothelial cells.

**Recent findings**
Processes that govern developmental angiogenesis become dysfunctional in the tumor environment, leading to abnormal tumor endothelial cells and blood vessels. Recent findings suggest that tumor endothelial cells are permanently modified compared with normal counterparts.

**Summary**
Coordination of numerous intracellular and extracellular programs promotes the formation of new blood vessels that are necessary for both development and certain diseases. Developmental angiogenesis uses canonical signaling modalities to effectively assemble endothelial cells into predictable vessel structures, and disruption of critical signaling factors has dramatic effects on blood vessel development. Solid tumors co-opt developmental cues to promote formation of tumor vessels that sustain their growth, but these angiogenic signals are not well regulated and produce endothelial cell dysfunction. Aberrant growth factor signaling contributes to phenotypic changes and acquired irreversible intracellular signaling, cytoskeletal and genetic modifications in endothelial cells of tumor vessels. Permanently altered tumor endothelial cells may represent a significant population.

**Keywords**
angiogenesis, blood vessel development, cancer, tumor endothelial cells

## INTRODUCTION

Blood vessel formation during embryonic development is exquisitely orchestrated in space and time to produce the right number of vessels of the appropriate size. Remarkably, these vessels function as conduits even as they form and remodel, thus ensuring that the developing organs of the embryo are adequately oxygenized. The scenario in a tumor is radically different: like the embryo, a tumor needs blood vessels to grow (and eventually to metastasize), but tumor vessels are tortuous and leaky, and they do not efficiently alleviate demands for oxygen. Tumors produce angiogenic signals, but not with the same precision and control as in the embryo, and the ongoing hypoxia exacerbates this misregulation. Tumor endothelial cells are clearly phenotypically abnormal, and emerging evidence suggests that they sustain permanent changes that may prevent 'normalization'. Here we examine the major facets of developmental angiogenesis, and contrast the developmental program with recent findings relevant to the misregulated angiogenic program found in tumors.

## WHEN THINGS GO RIGHT: DEVELOPMENTAL ANGIOGENESIS

Blood vessel formation during embryonic development involves differentiation of endothelial cells that organize to form primitive tubes that then expand [1–3]. The first vessel to form is the dorsal aorta, which becomes the main conduit from the heart to the trunk. Mesoderm cells first differentiate into endothelial precursors called angioblasts and then into endothelial cells that coalesce in a process called vasculogenesis; subsequent blood vessel formation most often combines vasculogenic differentiation with angiogenesis. Angiogenesis includes the extension of vessel networks by endothelial cell

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## KEY POINTS

- Developmental angiogenesis relies on precise synchronization of extrinsic inputs and intrinsic signaling cascades to produce functional blood vessels.
- Tumor blood vessels are dysfunctional due to exposure to misregulated and unsynchronized angiogenic signals.
- Tumor-derived endothelial cells remain abnormal outside the tumor, suggesting permanent changes.

proliferation and collective migration called sprouting, subsequent connections to other vessels with lumen formation, and the eventual remodeling of the initial primitive network based on oxygen demand by growing tissues and blood flow. Remodeling is also characterized by stable recruitment to vessels of non-endothelial cells, such as pericytes and smooth muscle cells (Fig. 1, left).

Developmental angiogenesis results from a delicate balance of both pro-angiogenic and anti-angiogenic cues in both space and time [4,5]. A primary signal is vascular-endothelial growth factor (VEGF)-A, which interacts with several high affinity receptors found on endothelial cells, Flt-1 (VEGFR-1) and Flk-1 (VEGFR-2, KDR); genetic deletion of any of these components is embryonic lethal [6–9]. Numerous studies show that proper levels and spatial organization of VEGF signaling is crucial to vascular development. For example, VEGF-A RNA is alternatively spliced to give multiple isoforms that differentially interact with the extracellular matrix and may set up a gradient, and disruption of this isoform balance results in abnormal vessel morphology [10,11]. Flt-1 receptor RNA is also alternatively spliced to give both a membrane-localized (mFlt-1) and a soluble (sFlt-1) isoform [12]. Flt-1 negatively modulates VEGF signaling during development by acting as a sink for VEGF-A ligand, and spatial organization of sFlt-1 from endothelial cells is also important for correct vessel morphogenesis [13–16]. In some diseases, VEGF signaling is misregulated both temporally and spatially (see below), leading to abnormal blood vessels.

VEGF-A also regulates sprouting angiogenesis by regulating endothelial cell phenotypes. Gerhardt *et al.* [17] showed that correct vessel sprouting requires that some endothelial cells migrate in response to VEGF-A signaling and form tip cells, whereas other endothelial cells form stalk cells that primarily proliferate and lumenize. Proper vessel network formation requires the correct ratio of tip cells vs. stalk cells, and this is mediated via Notch signaling [18–21]. Basically, membrane-localized Notch ligand Dll4 is upregulated by VEGF-A signaling in tip cells, and this leads to increased Notch

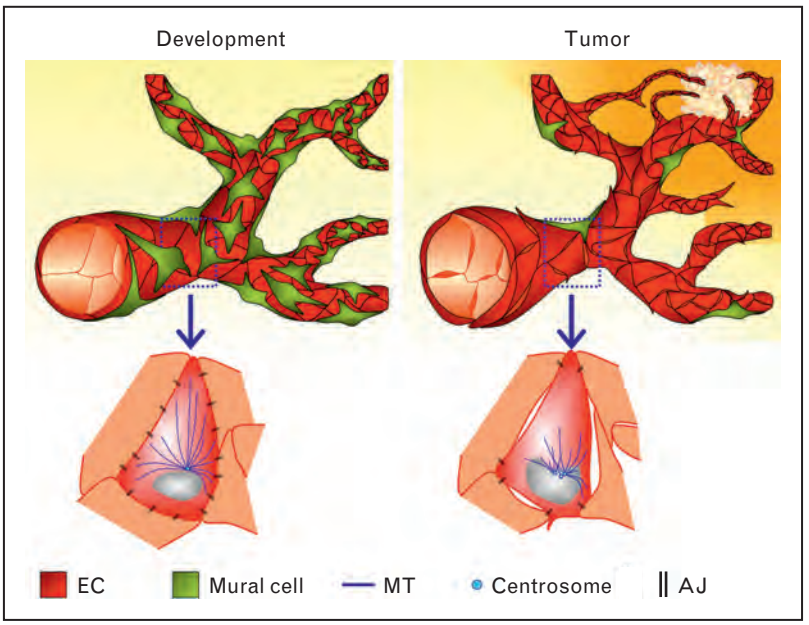


**FIGURE 1.** Diagram of morphological and cellular differences between normal and tumor vasculature. Normal blood vessels (left) display stereotypical patterning, complete mural cell coverage and numerous cell–cell junctions. At the cellular level, normal endothelial cells (ECs) have proper DNA content and cytoskeletal components. In contrast, tumor blood vessels (right) exhibit gross morphological defects marked by atypical vessel patterning, loss of mural cell coverage, and fewer and loosened endothelial junctions. Additionally, tumor endothelial cells have elevated frequencies of aneuploidy and permanent modifications of cellular components such as centrosomes. AJ, adherens junction; MT, microtubule.

signaling in neighboring stalk cells. Notch signaling downregulates Flk-1 and upregulates Flt-1 to presumably alter the overall VEGF-responsiveness of stalk cells. VEGF–Notch interactions are also thought to allow endothelial cells to 'compete' for the tip cell position [22].

Recent work has implicated numerous other signaling pathways in developmental blood vessel formation [23–25]. For example, bone morphogenic protein (BMP) regulates sprouting angiogenesis independent of VEGF-A in the zebrafish venous plexus [26▪▪] and has complex interactions with Notch in mammalian models that are antiangiogenic in some cases [27▪,28]. An important antiangiogenic cue is also provided by the semaphorin Sema3E ligand/Plexin D1 receptor interaction [29▪,30]. The G-protein coupled receptor SIPR-1 (EDG-1) stabilizes junctions and is important in limiting vessel sprouting [31▪,32▪,33,34▪]. Numerous studies show that Tie/tek receptors and their ligands the angiopoietins are important for blood vessel formation, and this pathway has a prominent role in vessel remodeling and endothelial–pericyte interactions [35]. Platelet-derived growth factor signaling is also critical to endothelial–pericyte interactions [36].

## THE COMPANY YOU KEEP: TUMOR BLOOD VESSELS ARE ABNORMAL

Tumor angiogenesis relies on the developmental angiogenic tool kit, using the same classic pro-angiogenic and anti-angiogenic cues to recruit blood vessels. An initially undetectable tumor mass sometimes begins to secrete high levels of potent angiogenic factors, such as VEGF and fibroblast growth factor, in a process known as the 'angiogenic switch' [37]. During this phase, transformed cells release pro-angiogenic molecules that not only attract neighboring vessels, but also activate quiescent stromal cells such as fibroblasts and macrophages [37]. The angiogenic switch is effective at recruiting blood vessels, and most solid tumors demonstrate an aberrantly high vessel density compared with non-transformed tissue. However, unlike physiological or developmental angiogenesis, tumor vessels are structurally abnormal and function poorly. Tumor vessels are characterized by reduced blood flow, leakiness and dilation, and a lack of pericyte coverage (Fig. 1, right). In a normal blood vessel network, hierarchy is evident; large veins or arteries bifurcate into successively smaller conduits, connected by a fine capillary network. This beautiful patterning is abolished in tumor vessels. These changes lead to poor oxygen delivery and an accumulation of metabolic wastes [38].

Developmental angiogenic programs underscore the importance of growth factor gradients and regulated signaling in network formation (see above). In contrast, tumor endothelial cells are saturated with pro-angiogenic cytokines that can overwhelm receptor-mediated signaling and sprout guidance mechanisms; for example, sFlt-1 normally negatively modulates VEGF signaling through Flk-1 (VEGFR2), but VEGF signaling is upregulated in many tumors, and in some tumors sFlt-1 is concomitantly downregulated [39]. Downstream effectors such as Akt and Erk are upregulated in tumor endothelial cells and perturb vessel formation [40]. In addition to effects on tumor endothelial cells, vascular-associated stromal cells and pericytes are also altered and potentially contribute to vessel dysmorphogenesis.

Because tumors rely on blood vessel recruitment for growth, anti-angiogenic therapeutic strategies were predicted to be effective. Unfortunately, anti-angiogenic (mostly anti-VEGF) therapies demonstrate mixed results, with some remission but often with recurrence of more aggressive and metastatic tumors over time [41]. There are likely several reasons for this lack of efficacy, including adaptive upregulation of other growth factors, and increased invasiveness of the tumor cells themselves [41,42]. In light of these results, tumor blood vessel normalization for short periods has been proposed. Carmeliet and Jain [41] predicted that regulating antiangiogenesis therapy to equalize the imbalance of pro-angiogenic and anti-angiogenic factors would 'normalize' tumor vessels. Normalization is predicted to increase blood flow and reduce hypoxia and the subsequent release of hypoxia-related growth factors. If elevated pro-angiogenic signaling was the only culprit, this strategy would most likely be more beneficial than has been demonstrated. However, recent evidence suggests that tumor endothelial cells, like transformed tumor cells, accumulate permanent intracellular signaling, cytoskeletal and genetic modifications.

## CHANGES THAT LAST: TUMOR-ASSOCIATED MODIFICATIONS IN ENDOTHELIAL CELL FUNCTION

Tumor cells exhibit dramatic genetic, signaling and cytoskeletal modifications as a consequence of their transformation and atypical growth. Stromal cells resident or co-opted into the tumor environment, such as endothelial cells, seem to reprogram after prolonged exposure to the tumor environment and, consequently, exhibit altered behaviors. Thus, tumor-derived endothelial cells are functionally distinct from endothelial cells derived from normal tissue.

## Migration

At its core, angiogenesis is the collective migration of endothelial cells. Although there are numerous extrinsic modifiers of endothelial cell migration, intracellular cytoskeletal programs are ultimately responsible for cell responses that coordinate cell movements. In an overly simplistic model of endothelial cell migration, pro-angiogenic factors, such as VEGF, bind to receptors on endothelial cells. Ligand-mediated receptor activation leads to upregulation of downstream effectors such as PI3-K or Akt, which in turn activate a variety of signaling proteins (i.e. Rho GTPases) that interact with cytoskeletal structures such as actin microfilaments and tubulin microtubules to initiate sustained, directed cell migration [43,44▪]. Developmental blood vessel morphogenesis leads to network expansion and remodeling. Vessel growth and morphogenesis is reduced postnatally, but it can be activated during physiological angiogenesis and in pathological scenarios such as cancer.

Tumor blood vessels are in a chronic state of migration and remodeling and thus have elevated levels of migratory cytoskeletal proteins, notably proteins of the RhoGTPase superfamily [44▪]. RhoGTPases, which include Rho, Rac1 and Cdc42, are primary drivers of actin polymerization and filopodia formation in endothelial cells [43]. Ghosh *et al.* [45] showed that ex-vivo cultured tumor-derived endothelial cells failed to orient their cytoskeleton when exposed to uniaxial cyclic strain, and had constitutively elevated Rho activity that when blocked restored orientation. Small GTPase-independent changes also perturb tumor endothelial cell migratory programs. It was reported that breast cancer-derived human endothelial cells had elevated expression of TRPV4, a non-voltage-gated $Ca^{2+}$ channel involved in reorientation during shear stress [46]. TRPV4 induced tumor endothelial cell migration and actin remodeling, whereas normal (non-tumor-derived) endothelial cells did not rely on TRPV4 for migratory cues. The root causes for changes in tumor endothelial cells in both investigations are unknown. However, it is notable that modifications originating in the tumor persisted even after endothelial cells were removed and cultured *ex vivo*. This finding indicates that the tumors can trigger permanent alteration(s) in endothelial cell migration, which is not predicted if the tumor microenvironment produces only phenotypic changes. Our preliminary data show that endothelial cells with excess centrosomes, which are microtubule-organizing centers, have altered migratory properties (Kushner *et al.*, in prepration), suggesting that one permanent change that can affect tumor endothelial cell behavior is centrosome over-duplication.

## DNA damage and apoptosis

In primary tumor cells, loss of DNA damage-regulators or cell cycle-regulators is associated with neoplastic transformation and evasion of apoptosis [47▪]. Mounting evidence indicates that the tumor micro-environment alters these pathways in the stromal neighbors of tumor cells. In this regard, it is surprising that few studies have investigated alterations in DNA damage or cell-cycle programs in tumor-derived endothelial cells. Tumor endothelial cells have elevated markers of aneuploidy and chromosomal instability, suggesting that gatekeeper mitogenic and/or apoptotic programs may be dysfunctional [48,49]. An investigation by Dudley *et al.* [50] demonstrated that loss of the tumor suppressor gene p53 in tumor stromal cells reduced responsiveness to p53-activating drugs and the pro-apoptotic drug vincristine. These studies suggest that tumor endothelial cells harbor apoptosis-avoidance mechanisms. Reprogramming the apoptotic circuitry in tumor endothelial cells to resist cell death is another way in which permanent changes distinguish tumor endothelial cells from their normal counterparts.

## Inflammation

Endothelial cells can differentially express a variety of cell surface antigens to either promote or prevent leukocyte trafficking, which is the extravasation of hematopoietic cells from the circulation into the underlying tissue. Extravasation is promoted when endothelial cells are in an 'activated' state that is a physiologic response to infection and wound healing. Tumor endothelial cells demonstrate chronic activation, with constitutive expression of adhesion molecules that allow pro-inflammatory cells such as neutrophils and macrophages to infiltrate the tumor parenchyma [51]. These activated immune cells secrete pro-angiogenic cytokines that further promote tumor endothelial cell permeability and angiogenesis. Strikingly, ex-vivo cultured tumor endothelial cells retain their activated phenotype [52–54]. Renal tumor-derived endothelial cells exhibit elevated expression of the adhesion molecule NCAM (neural cell adhesion molecule), which is proposed to enhance apoptotic resistance and increase tube formation [54]. How these endothelial cell changes perdure outside the tumor is almost completely unknown.

## Genetics

Preservation of functional defects in tumor-derived endothelial cells may depend, in part, on somatic mutations or epigenetic modifications acquired in the tumor. As previously noted, tumor endothelial

cells exhibit changes in several phenotypic characteristics in the absence of tumor-derived signals, and purified tumor endothelial cells retain aspects of their abnormal phenotype in long-term culture [55]. Although comprehensive genetic analyses of tumor endothelial cells have not been reported, likely due to the difficulty of obtaining a pure population, evidence for chromosomal rearrangements in tumor endothelial cells exists. Tumor endothelial cells in both a xenograft model and in primary human tumors exhibited cytogenetic abnormalities [48,49]. Moreover, excess numbers of centrosomes were observed in tumor endothelial cells compared with normal controls [48]. Cells with excess centrosomes (>2) are prone to chromosomal instability and aneuploidy via promotion of chromosome mis-segregation events during mitosis [47▪]. Our laboratory reported a link between centrosome overduplication and elevated VEGF signaling *in vivo* and in primary endothelial cells, providing the first connection between tumor-associated pro-angiogenic cytokines and centrosome overduplication in endothelial cells [56].

Genetic differences were observed between tumor endothelial cell populations that were harvested from low and high-metastatic tumors. Tumor endothelial cells isolated from high-metastatic tumors had elevated levels of Ch17 translocation, which correlated with elevated stem cell markers and osteogenic differentiation potential [57▪]. The same group reported that tumor endothelial cells proliferated more with paclitaxel (antitumor agent) treatment than normal endothelial cells and had greater MDR1 (multidrug resistance) RNA expression [58▪]. In aggregate, these studies document a spectrum of phenotypic changes in tumor endothelial cells that may be downstream of tumor-induced genetic changes. Beyond genome mutations, epigenetic changes such as DNA methylation and/or histone modifications may lead to tumor endothelial cell phenotypic changes; however, little information is available detailing such modifications. Chung *et al.* [59] showed that epigenetic silencing of CYP24 in tumor endothelial cells correlated with vitamin D insensitivity that was rescued with a methyltransferase inhibitor.

## CONCLUSION

Successful formation of blood vessels during development depends on the coordination of extrinsic and intrinsic angiogenic programs. In this context, a complex circuitry governing timing, spatial regulation of ligand concentrations, and dampening or amplification of signaling events yields functional blood vessels. In contrast, the delicate orchestration of these events is disrupted in the tumor environment, promoting abnormal blood vessel network formation and pathogenesis. Most strikingly, tumor endothelial cells retain changes in behavior and gene expression upon removal from the tumor, suggesting irreversibility of the effects that may contribute to rebound from anti-angiogenesis therapies. Thus understanding the basis of these tumor-induced modifications to endothelial cells will help formulate a clearer picture of how tumor blood vessels differ from normal blood vessels that form during development.

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### Conflicts of interest

*There are no conflicts of interest.*

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- ▪ of special interest
- ▪▪ of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 255).

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