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Angiogenesis, signaling pathways, and animal models

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Abstract

The vasculature plays a critical role in homeostasis and health as well as in the development and progression of a wide range of diseases, including cancer, cardiovascular diseases, metabolic diseases (and their complications), chronic inflammatory diseases, ophthalmic diseases, and neurodegenerative diseases. As such, the growth of the vasculature mediates normal development and physiology, as well as disease, when pathologically induced vessels are morphologically and functionally altered owing to an imbalance of angiogenesis-stimulating and angiogenesis-inhibiting factors. This review offers an overview of the angiogenic process and discusses recent findings that provide additional interesting nuances to this process, including the roles of intussusception and angiovasculogenesis, which may hold promise for future therapeutic interventions. In addition, we review the methodology, including those of *in vitro* and *in vivo* assays, which has helped build the vast amount of knowledge on angiogenesis available today and identify important remaining knowledge gaps that should be bridged through future research.

Keywords: Vasculature; Vasculogenesis; Angiogenesis; Intussusception

Introduction

Vascular formation and expansion are divided into three separate biological processes: vasculogenesis, angiogenesis, and intussusception. Vasculogenesis is the process by which the first vessels are formed *de novo* during early embryogenesis.[1] This process begins with the migration of hemangioblasts from the lateral plate mesoderm to the midline, where they aggregate to form a single vascular chord. Hemangioblasts also aggregate within the blood islands of the yolk sac.[2] The cells in the chord and blood islands then differentiate into endothelial cells, polarize, and liquid accumulates, possibly from vesicle fusion both within single endothelial cells (cell hollowing) and between neighboring cells (chord hollowing), to form a vascular lumen, cell-cell junctions, and a basement membrane.[3] The aorta and middle cerebral arteries are the major vessels formed by vasculogenesis.[4] Coinciding with chord hollowing processes, a group of endothelial cells in the ventral floor of the aorta and within blood islands constitute the first hematopoietic region. The erythroblasts formed in this area are seeded into the lumen, giving rise to the blood of the organism.[5]

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Following the formation of major arteries, angiogenesis (sprouting angiogenesis) is responsible for further vascular development. It is a multistep process that begins with the degradation of the plasma membrane and the loss of perivascular cell coverage to allow endothelial cells in the patent vessel to sprout a new daughter vessel.[6] Endothelial cell migration and proliferation are the two main processes involved in the growth of new vessels. Migration is primarily controlled by the leading cell, known as the tip cell, while stalk cells connect the tip cell to a patent vessel.[7] The tip cell surveys the surroundings and, during angiogenic vascular expansion in early embryogenesis, ensures that a new vessel is developed according to a predetermined fate,[8] giving rise to an archetypical form and ensuring regular interspacing of the vasculature, as can be observed among the intersegmental vessels during early embryonic development. Behind the tip cell, the lumen of the new vessel is formed by the cell and chord hollowing.[3] The lumenized vessel begins to lay down a new basement membrane and recruits new pericytes to ensure

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its stability.[9] Angiogenesis is completed when two new vessel sprouts join via anastomosis, which is supported by macrophages acting as "chaperones" that bridge and facilitate the joining of two sprouts.[10] This facilitates the establishment of circulation, which is critical for vascular survival and differentiation into arteries and veins.[11]

Intussusception is the process in which vessels split along their lengths to form two new vessels.[12] This is commonly observed after an initial angiogenic expansion in certain highly vascularized tissues, such as the liver and lungs. During intussusception, endothelial cells sprout into the lumen to form what are known as endothelial luminal pillars that either extend across the vessel lumen or fuse with another pillar from the other side of the vessel to form a transluminal pillar.[12] These pillars are fortified by the deposition of collagen fibrils and recruitment of a mural cell to occupy its core.[13] Several pillars are commonly in close proximity to each other and form a line, which can grow in length and eventually fuse, resulting in longitudinal splitting of the vessel.

Modern Views about Vascular Formation

In addition to the classical view that vascular formation falls into one of three categories–vasculogenesis, angiogenesis, and intussusception, recent findings have revealed an alternative type of vessel formation that lies between or can be considered a combination of these categories. This process, known as angiovasculogenesis, occurs during the formation of the major embryonic vein (cardinal vein) and resembles the development of the lymphatic system. During angiovasculogenesis, endothelial cells sprout and move away from the aorta without retaining a connection to the vessel, followed by the cells reuniting and undergoing a process similar to chord formation and hollowing at their new location ventral to the aorta, thereby forming the cardinal vein.[14]

A nonclassical compound type of vascular formation and expansion has been recently discovered during the development of the extremely dense choroidal vascular plexus. This process was found to occur through the sprouting and migration of single endothelial cells into the eye field, first settling as vascular seeds that are regularly interspaced along the entire eye field and then send out endothelial projections that meet with projections from neighboring vascular seeds to form the first non-lumenized outline of the vasculature. As these primitive vessels become lumenized, the vasculature expands via simultaneous angiogenesis and intussusception, leading to a maximal density of this unique vasculature/vascular membrane of nearly 100%.[15]

A new role of intussusception has recently been described, whereby a vascular sprout that has not met with a second sprout and cannot therefore establish circulation can instead stabilize itself by undergoing intussusception at the tip, leading to the establishment of its own circulation loop to maintain circulation and vessel survival.[16] Although this has only been described under pathological conditions, it suggests that angiogenesis and intussusception could be more interlinked than proposed in the classical view. In line with this hypothesis, we found that the choriocapillaris is activated by hypoxia and under conditions in which the Bruch's membrane is intact; thus, the vessels cannot grow into the retina and undergo intussusception instead. Furthermore, the intussusceptive choriocapillaris becomes leaky,[17] suggesting that intussusception can destabilize vessels under pathological conditions.

Vascular Remodeling

As is commonly observed during development, the initial angiogenic response is often excessive, leading to the formation of an overly dense vasculature in most tissues. These primitive vasculatures are partially regressed and remodeled via two types of vascular pruning: intussusceptive and reverse angiogenic pruning. Intussusceptive pruning involves the formation and fusion of transluminal pillars at the branching points between two vessels,[18] thereby essentially cleaving one vessel from the remaining circulation. During reverse angiogenic pruning, a vessel starts to retract by first blocking the lumen and then forming two tip cells on either side, after which it regresses backward towards the patent vessel.[19] Based on studies of the rapid revascularization response observed after discontinuation of a successful antiangiogenic therapy, the reverse-angiogenesis process has been hypothesized to leave behind empty basement membrane sleeves that could form a low-resistance "highway" for new vessel growth at later stages.[20] However, this idea has been challenged by the finding that upon completion of the reverse-angiogenesis process, the empty basement membrane sleeves appear to be sealed off by basement membrane plugs, which prevents their reuse during the second angiogenic process.[21]

Both the vascular remodeling processes mentioned above mainly refer to the remodeling of the capillary network. Arteriogenesis is a different type of vascular remodeling of great medical interest that refers to the differentiation of a capillary into an artery. This process involves increasing vessel diameter, wall thickness/muscularity, and blood flow-speed.[22] Key insights into the role of blood flow and shear stress in arteriogenesis were from studies on zebrafish, in which high blood pressure was found to activate endothelial cilia, which induce Notch signaling. Notch signaling is important for the recruitment of mural cells to arterial-fated vessels.[23] This pathway ensures the arterial fate of vessels that sustain high blood flow. Whether a similar mechanism is involved in ischemic arteriogenesis warrants further investigation. Another key factor involved in the regulation of vascular diameter is the angiotensin (Ang)-Tie signaling pathway. Ang1-Tie2 signaling is generally believed to cause vessel dilation and arteriogenesis, whereas Ang2-Tie2 signaling is vasoconstrictive and inhibits arteriogenesis.[24–26]

Regulation of Angiogenesis

Under physiological conditions, angiogenesis is both positively and negatively regulated by proangiogenic and antiangiogenic factors, respectively,[27] creating a balance

in which endothelial and vascular mural cells receive signals to maintain their functions[28] and overstimulation, which would lead to angiogenesis, is prevented. However, under pathological conditions, the overexpression of angiogenic factors or inhibition of antiangiogenic factors disrupts this balance, leading to pathological angiogenesis. The most important pathways involved in this process are discussed in the following sections.

Physiological and pathophysiological regulation of angiogenesis

Angiogenesis is mainly switched on during development.[29] regeneration[30] (including that of the endometrial mucus membrane during the proliferative phase of the female menstrual cycle), hypoxia,[31] and inflammation.[32]

During development, angiogenesis is required for organ patterning and growth[33] and for sufficient oxygen and nutrient delivery to tissues. Under these conditions, the balance between angiogenic and antiangiogenic factors is maintained, although it is slightly tipped towards angiogenesis[34,35] This allows for the orderly formation of mature and well-structured blood vessels that meet tissue requirements and do not leak. However, in some tissues, the initial angiogenic induction overshoots the metabolic needs and is followed by a vascular remodeling phase that prunes the vasculature and ensures adequate and non-excessive vascularity.[36]

As the vasculature is the primary route for oxygen delivery to tissues, it is not surprising that tissue hypoxia strongly upregulates angiogenic factors to induce the growth of new blood vessels in the hypoxic tissue.[37] Hypoxia also leads to increased blood formation (by upregulating erythropoietin)[38] and vessel diameter (by activating endothelial nitric oxide synthase),[39] thereby coordinating a robust response to re-establish appropriate perfusion and oxygenation of the tissue. Recently, the circadian clock was found to be mechanistically similar to the hypoxia-inducible factor transcriptional machinery and to have an important influence on both hypoxia signaling and angiogenic capacity.[29,40–42] This may explain why angiogenesis, which is important during all growth phases,[43] is mainly active during the night,[29] as the circadian clock induces a pseudo-hypoxic state in tissues, leading to the upregulation of angiogenic factors, specifically at night.[42,44]

Angiogenesis is also induced during inflammation, as the inflammatory response requires the recruitment of immune cells and delivery of circulating inflammatory proteins from the bloodstream to the inflamed tissue.[45,46] By activating angiogenesis, inflammatory factors weaken the vessel wall through basement membrane breakdown and loss of mural cell coverage[47]–the first steps in the angiogenic process (see above)–causing the vessels to leak and facilitating the extravasation of neutrophils, macrophages, and lymphocytes as well as plasma proteins, such as complement factors. Sustained inflammation leads to the expression of angiogenic factors by the recruited inflammatory cells, thereby inducing angiogenesis[48] and increasing tissue perfusion.

Sustained hypoxia and inflammation are present in and drive pathological progression in most common diseases including: (1) rheumatoid or other chronic inflammatory disorders,[48,49] (2) metabolic disorders including obesity and late-stage diabetes,[50,51] (3) cardiovascular disorders including plaque growth and destabilization, ischemic stroke, or myocardial infarction,[52] (4) respiratory disorders such as chronic obstructive lung disease and coronavirus disease 2019,[53,54] (5) eye diseases including age-related macular degeneration, diabetic retinopathy, and retinopathy of pre-maturity,[55] and (6) most types of cancer and premalignant diseases.[56] Under these pathological conditions, angiogenesis is uncontrolled, leading to ectopic vessels with poor stability, maturity, and function being constantly formed and broken down without anastomosing with other vessels. Therefore, these vessels are leaky, leading to the accumulation of interstitial fluid, which compresses the mature vessels and reduces tissue perfusion[57] [Figure 1]. These pathological vasculatures lead to sustained hypoxia and inflammation, creating a vicious cycle that drives disease progression.

Proangiogenic factors for angiogenesis regulation

The most studied angiogenic factor associated with both healthy and pathological angiogenesis is vascular endothelial growth factor (VEGF).[58] VEGF is highly upregulated during hypoxia owing to the presence of four hypoxia-responsive elements (HREs) in the promoter of VEGF-receptor (*VEGFR*) gene.[59] These HREs are bound by hypoxia-inducible factor (HIF)-1, which is stabilized in the absence of oxygen-dependent hydroxylation and ubiquitinylation.[60] VEGF is also produced in high amounts by inflammatory cells, mainly macrophages, in response to stimulation by damage- or pathogen-associated molecular patterns (DAMPs or PAMPs, respectively).[61] During development, VEGF expression is high in tissues before vascularization and is turned off once vascularization is complete.[62] Thus, VEGF plays a central role in the induction of both physiological and pathological angiogenesis. It acts mainly through two receptors, VEGFR1 and VEGFR2.[58] VEGFR1 in endothelial cells mainly sequesters VEGF and thereby inhibits signaling through VEGFR2 but may also be involved in hematopoiesis and homing when expressed on immune cells, survival and proliferation when expressed by cancer cells, and survival and normalization/quiescence in endothelial cells.[63–65] In contrast, VEGFR2 induces cell proliferation, migration, and the expression of matrix metalloproteases that break down the extracellular matrix to allow angiogenic sprouting.[66,67] VEGFR2 is expressed at much lower levels and has weaker affinity for VEGF. However, when VEGF levels become (locally) sufficiently high, VEGFR2 is activated, leading to a switch from vascular quiescence to sprouting.[58] During sprouting, VEGF-induced delta-like canonical Notch ligand 4 (Dll4) expression is important for differentiation of endothelial cells into tip cells, which leads to growth of the sprout.[68] These cells also express platelet-derived growth factor (PDGF)-B, which is required for mural cell recruitment and sprout maturation that lead to vascular stability.[69] Because of these functions, PDGF-B is commonly considered a proangiogenic

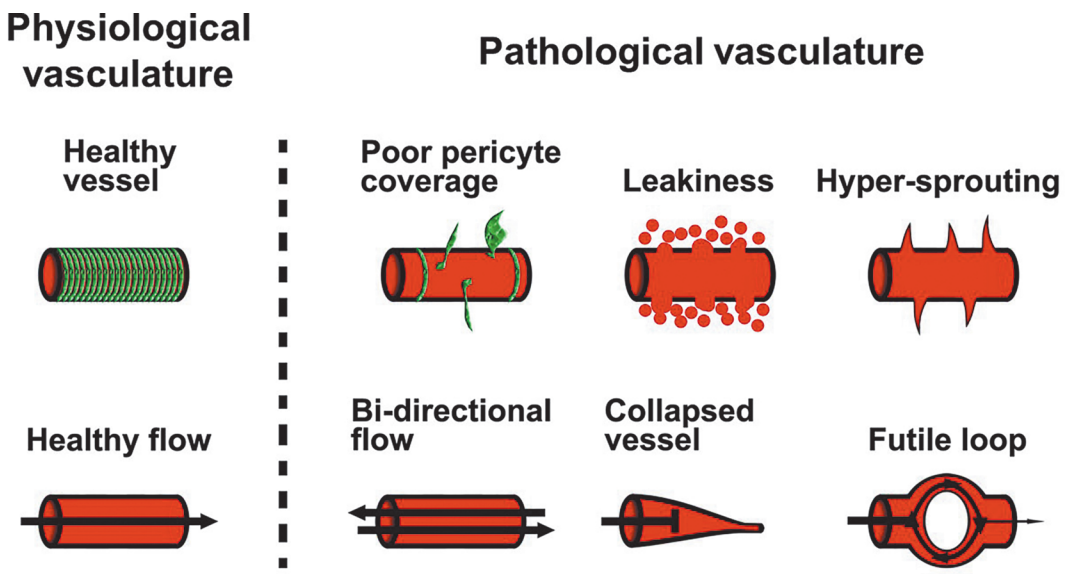

Figure 1: Structural and functional abnormalities of vessels resulting from pathological angiogenesis. Top row: Vessels (shown in red) under physiological conditions have good pericyte coverage (green cells). Under pathological conditions, vessels lose their pericytes, become leaky, and sprout uncontrollably (hyper-sprouting). Bottom row: Vessels under physiological conditions support unidirectional flow. Under pathological conditions, vessels become erroneously wired such that flow can occur in either direction (i.e., the vessel may be both an artery and a vein), become collapsed due to high interstitial fluid pressure, and may form futile loops in which the blood circulates rather than leaves the tissue, thereby reducing perfusion.

factor. Dll4 exerts lateral inhibition by activating Notch in neighboring stalk cells.[70] This is important to prevent excessive tip cell formation and allow only a moderate number of new sprouts to grow. However, this regulation is often disturbed under pathological conditions, wherein notably high VEGF levels or inhibition of Dll4-Notch signaling leads to ectopic tip cell differentiation and sprout formation.[71,72] Thus, Dll4 and Notch can be considered as antiangiogenic factors.

Other potent angiogenic factors are also commonly associated with regeneration or inflammation. For example, fibroblast growth factor 2 (FGF-2), an angiogenic factor that induces both neovascularization and vessel maturation,[73] is released by dead or dying cells to initiate a regenerative process that includes neovascularization.[74] Interleukin (IL)-8, tumor necrosis factor alpha (TNF-α), and transforming growth factor beta (TGF-β) are inflammatory cytokines that have potent proangiogenic activity at low doses,[75–77] although TNF-α and TGF-β are antiangiogenic at higher doses.[76,78] Regeneration and pathological conditions, such as hypoxia or inflammation, induce a plethora of angiogenic factors that act together to drive angiogenesis and either regeneration or disease progression [Table 1].

Antiangiogenic factors for angiogenesis regulation

A balanced angiogenic process, as observed during development and regeneration, requires an appropriate amount of endogenous antiangiogenic factors to counteract the proangiogenic factors and prevent overt ectopic vascularization. Extracellular matrix degradation, which is key for allowing vessel growth through tissues, also provides the best-described endogenous antiangiogenic factors.[79,80] Angiostatin is produced from plasminogen[81] and thrombospondin is liberated upon extracellular matrix degradation to inhibit excessive vascular sprouting.[82] In addition, cytokines that turn off or inhibit tissue-damaging inflammation, such as IL-10 and IL-4, also have antiangiogenic effects.[83,84] However, this may be context- and concentration-dependent, as IL-10 and IL-4, for example, have also been reported to have proangiogenic functions under certain conditions.[85]

Common Models of Angiogenesis

In vitro models

As described in the previous section, circulation is critical for vascular survival. Although some advances have been made in microfluidics that allow the formation of vessel-like structures that can sustain circulation *in vitro*,[86] the majority of *in vitro* models of angiogenesis focus on specific elements of the angiogenic process, such as endothelial cell proliferation and migration, and lumen formation [Table 2]. Endothelial proliferation assays are performed by seeding a relatively small number of endothelial cells in a suitable receptacle, such as a well in a multi-well plate. Researchers may then add different types of pro- or antiangiogenic treatments and examine how they affect the number of endothelial cells over time.[87] Endothelial cells can be counted directly or indirectly. Aside from adding pro- or antiangiogenic molecules to the medium, the endothelial cells can be genetically or epigenetically modified to study specific angiogenic signaling pathways in detail.[88] The wells can also be coated with molecules hypothesized to exert pro- or antiangiogenic effects on endothelial cells, such as basement membrane proteins.[87] As angiogenesis involves both proliferation and migration, several types of endothelial migration assays are commonly used to study this process. Two-dimensional (2D) migration can be studied by scraping off endothelial cells in an area of a confluent monolayer and

Table 1: Pathological condition and effect of proangiogenic and antiangiogenic factors for angiogenesis regulation.

Factors	Pathological condition	Effect on angiogenesis	References
VEGF/VEGFR2	Development, regeneration, hypoxia, and inflammation	Proangiogenic	[66,67]
PDGF-B	Development, regeneration, and hypoxia	Proangiogenic	[69]
Dll4/Notch	Development, regeneration, and reduced in pathological conditions	Antiangiogenic	[70–72]
FGF-2	Development, regeneration, and inflammation	Proangiogenic	[73,74]
IL-8	Inflammation	Proangiogenic	[77]
TNF-α	Inflammation	Proangiogenic	[75]
TGF-β	Inflammation	Proangiogenic	[76]
Endostatin	Development and regeneration	Antiangiogenic	[79]
Canstatin	Development and regeneration	Antiangiogenic	[79]
Tumstatin	Development and regeneration	Antiangiogenic	[80]
Angiostatin	Development and regeneration	Antiangiogenic	[81]
Thrombospondin	Development and regeneration	Antiangiogenic	[82]
IL-10	Inflammation	Context dependent	[83,84]
IL-4	Inflammation	Context dependent	[83,84]

Dll4: Delta-like canonical Notch ligand 4; FGF-2: Fibroblast growth factor 2; IL: Interleukin; PDGF: Platelet-derived growth factor;TNF-α: Tumor necrosis factor alpha; TGF-β: Transforming growth factor beta; VEGF: Vascular endothelial growth factor; VEGFR: VEGF receptor.

then investigating how adjacent cells migrate inward to close the "wound" over time.[89] Alternatively, chemoattraction can be studied using a modified Boyden chamber or transwell assay, in which a membrane with pores of a standardized diameter is placed between an upper and a lower reservoir.[88] Endothelial cells are seeded in the upper reservoir, and chemoattractants are added to either the upper or lower reservoir to assess their ability to stimulate nondirected or directed movement of the cells. The readout requires staining of the cells on the lower side of the membrane (often with Giemsa). Hence, migration is examined at specific time points after cell seeding rather than continuously, as is possible with 2D assays. Following the discovery that endothelial cells spontaneously organize into vessel-like structures within a suitable matrix, studies on vasculogenesis have gained popularity. Matrigel is often selected as the matrix for endothelial cell seeding.[45] Although the process can be monitored over time, most researchers opt to stain endothelial cells at the end of the experiment to gain a clearer view of the vascular network formed. A variation of this assay involves the coating of beads with endothelial cells, followed by their immersion in a fibrin gel.[90] This allows the vessel-like structures to emerge from the surface of the beads, simulating the perpendicular growth of new vessels from patent vessels during angiogenesis. However, a more complex and *in vivo*-like system involves studying the blood vessel growth in organoid systems. In an appropriate matrix, embryonic stem cells can grow into small, disorganized embryo-like structures that may contain blood vessels.[91] In these systems, vessels are commonly studied using standard histopathological techniques with specific antibodies rather than nonspecific stains used in other model systems.

In vivo models

Animal models are required to study the entire angiogenic process *in vivo*. The zebrafish is the most commonly used model for studying developmental angiogenesis, in which the vessels can be monitored in very high spatiotemporal detail using, for example, the transgenic fli1a: EGFP line, which have endothelial cells that express EGFP.[4] This approach allows researchers to identify the angiovasculogenesis process described above, the critical aspects of vascular patterning, and the role of Notch in restricting tip cell formation. Zebrafish are also commonly used to study regenerative angiogenesis during the regrowth of amputated tail fins,[30] pathological angiogenesis relevant to diseases such as diabetic retinopathy,[31,92,93] and tumor metastasis.[94] In these assays, zebrafish are exposed to a pathological stimulus similar to that driving the corresponding human diseases, after which vascular changes are examined over time or at the experimental endpoint. For example, diabetic retinopathy was found to occur after 3 months and progress to neovascular disease involving pathological angiogenesis and vascular leakage by 12 months in *pdx1*-mutant zebrafish exhibiting reduced numbers of insulin-producing beta cells and, therefore, hyperglycemia from approximately 5 days after egg fertilization.[92] Pathological retinal angiogenesis and choroidal angiogenesis, relevant as models of diabetic retinopathy and age-related macular degeneration, respectively, were observed in adult zebrafish exposed to severe hypoxia for 6–10 days.[17,31] Hypoxia was also found to be a potent inducer of tumor angiogenesis and metastasis via the induction of VEGF-VEGFR2 signaling in a zebrafish tumor xenograft model.[94]

Additionally, mice are commonly used as an animal model for *in vivo* angiogenesis assays. A large number of assays have been developed [Table 2] that can be exploited to study angiogenesis in different tissues, such as the eye, ischemic tissue, skin, and tumors, as well as the role of various pathophysiological stimuli, such as tissue damage, inflammation, and hypoxia. Because of its avascular nature, the cornea has frequently been used to evaluate the angiogenic or antiangiogenic properties of natural or synthetic factors and drugs.[73] Such corneal angiogenesis assays often start with the implantation of a pellet containing angiogenic factors, a suture, or the local application

Table 2: Summary of experimental angiogenesis models.

Variables	Features	Species	Advantages	Disadvantages	References
In vitro models	EC proliferation and apoptosis	Mouse, human	Simple, low cost	*In vitro* culture rejects *in vivo* characteristics, hard to distinguish antiangiogenic or toxic, and rely on cell type and culturing medium	[96]
	EC motility	Mouse, human	Simple, low cost	*In vitro* culture rejects *in vivo* characteristics and rely on cell type and culturing medium	[97,98]
	EC invasion	Mouse, human	Simple, low cost, useful for detecting ECM degradation	*In vitro* culture rejects *in vivo* characteristics and rely on cell type and culturing medium	[99]
	Tube formation	Mouse, human	Simple, low cost, relevant for microvessel formation	–	[100]
	Embryoid body assay	Mouse, rat	Relevant for embryonic vasculogenesis	Requires totipotent cells	[101]
	Arterial ring assay	Mouse, rat, human	Simple, low cost, contains non-EC cell types for better mimicking angiogenesis, relevant for microvessel branching	Arteries do not represent pathological vessels nor microvessels	[102,103]
	Retinal explant	Mouse	Low cost, relevant for microvessel formation	Technically demanding, short time window	[104]
	Organoid	Mouse, human	Self-organizing, 3D, transplanted into host	Technically demanding, high cost	[105]
In vivo models	Chicken embryo chorioallantoic membrane assay	Chicken	Low cost	Technically demanding, difficult to evaluate in real time, difficult to evaluate due to existing vessels	[98]
	Corneal micropocket assay	Mouse, rat, rabbit	Suitable for both angiogenesis and lymphangiogenesis, suitable for angiogenic factor studies, relevant for tumor- or growth factor-induced angiogenesis, easy to visualize angiogenesis	Technically demanding, and vessel growth is limited to 2D	[106,107,108]
	Matrigel plug assay	Mouse	Simple, relevant for growth factor-induced angiogenesis, vessel growth in 3D	Technically demanding, difficult to evaluate in real time	[109]
	Chronic transparent chamber assay	Mouse, rabbit	Easy to visualize angiogenesis	Technically demanding	[110]
	Zebrafish embryo assay	Zebrafish	Simple, low cost, easy to visualize angiogenesis, vessel growth in physiological conditions	Requires transgenic fish	[111]
	Corneal alkali burn-induced corneal angiogenesis	Mouse, rat, rabbit, dog	Simple, low cost, relevant for chemical-induced neovascularization, relevant for limbal stem cell deficiency, relevant for inflammation-induced neovascularization	Burn time must be precise	[112,113]
	Tumor angiogenesis	Mouse, rat	Simple, low cost, clinically relevant	Difficult to evaluate in real time	[114,115]
	Laser-induced choroidal neovascularization	Mouse, rat	Simple, low cost, relevant for neovascular age-related macular degeneration	Laser burn must be precise	[116]
	Wound healing	Mouse, rat	Simple, low cost	Mouse heals differently from human due to contraction	[117]
	Infection-induced angiogenesis	Mouse, rabbit	Simple, relevant for pathogen-induced angiogenesis	Different pathogens produce different phenotypes	[118]
Simulation models	Computer simulation	–	Low cost, suitable for studying angiogenic patterns	Based on existing knowledge	[119]
	3D vascular networks on microfluidic chip	–	Low cost	Not representing pathological aspects such as leakage, involvement of immune cells, etc.	[120]
	3D printing of vascular networks	–	Suitable for studying patterns of vascular networks	Technically demanding	[121]

EC: Endothelial cells.

of hydroxide to produce an alkali burn to induce inflammation in the cornea. Vascular ingrowth into the cornea can be robustly detected after 3 days and progresses for approximately 1–2 weeks or longer depending on the angiogenic stimulus.[106–108] In addition, the postnatal development of the retinal vasculature in mice has proven

to be an ideal system for understanding the molecular regulation of angiogenesis.[95] In this model, whole eyes are recovered from newly born mice (e.g. 5–7 days after birth), the retinae are isolated and stained with an endothelial cell marker and flat-mounted by cutting each 3–4 times, giving the characteristic "flower" appearance. Confocal microscopy can be used to quantify the vasculature, including the number and location of tip cells, the number of filopodia, how far the vessels have grown from the center of the retina, and the vascular density. This model is critical for understanding the molecular underpinnings of tip-cell regulation.[68] The advantages and disadvantages of *in vitro* and *in vivo* models commonly used in angiogenesis studies are listed in Table 2.[96–121]

Conclusion

Since the seminal discovery that angiogenesis is critical for tumor growth and cancer progression more than 50 years ago,[114,115] we have come to understand that angiogenesis is indeed a critical driver of pathological phenotypes in a majority of common and serious public health threats, including cancer, eye diseases, chronic inflammatory diseases, metabolic diseases, and cardiovascular diseases. Thus, targeting angiogenesis holds significant promise for the treatment of these diseases; however, this has been proven to be more difficult than initially anticipated because of the complex molecular regulation of angiogenesis, the toxicity associated with available antiangiogenic drugs, and an incomplete understanding of the differences of pathological angiogenesis from physiological angiogenesis and vascular homeostasis. To date, a large number of assays and tools for angiogenesis research have been developed, allowing the investigation of highly intricate theories in a previously unattainable level of sophistication and detail. As discussed in this review, great progress has been made in elucidating the basic processes and molecular mechanisms that regulate angiogenesis. However, new evidence pointing to molecular differences between endothelial cells depending on their location in the vascular tree,[122] the organ-specific regulation and function of blood vessels,[123] and the importance of the circadian clock in regulating angiogenesis and antiangiogenic therapies,[29,124] has shown that many important aspects related to therapeutic modulation of angiogenesis remain poorly understood and require further investigation. Future studies that consider these aspects will undoubtedly provide important insight that will allow for more effective use of proangiogenic- or anti-angiogenic therapies, leading to better treatment outcomes, reduced suffering, and prolonged survival of millions of patients.

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Conflicts of interest

None.

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