Chapter 15

Recent Advances in Angiogenesis Assessment Methods and their Clinical Applications

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Abstract

Angiogenesis, a natural phenomenon of developing new blood vessels, is an integral part of normal developmental processes as well as numerous pathological states in humans. The angiogenic assays are reliable predictors of certain pathologies in particular tumor growth, metastasis, inflammation, wound healing, tissue regeneration, ischemia, cardiovascular, and ocular diseases. The angiogenic inducer and inhibitor studies rely on both in vivo and in vitro angiogenesis methods, and various animal models are also standardized to assess qualitative and quantitative angiogenesis. Analogously, the discovery and development of anti-angiogenic agents are also based on the choice of suitable angiogenic assays and potential drug targeted sites within the angiogenic process. Similarly, the selection of cell types and compatible experimental conditions resembling the angiogenic disease being studied are also potential challenging tasks in recent angiogenesis studies. The imaging analysis systems for data acquisition from in vivo, in vitro, and in ova angiogenesis assay to preclinic, and clinical research also requires novel but easy-to-use tools and well-established protocols. The proposition of this pragmatic book chapter overviews the recent advances in angiogenesis assessment methods and discusses their applications in numerous disease pathogenesis.

Keywords: angiogenesis techniques, *in vitro* angiogenesis, angiogenic mouse models, quantitative angiogenesis, transgenic animal models, angiogenesis in clinical practice, angiogenic inhibitors

1. Introduction

The growth of new microvessels from the parent ones is an integral part of new tissue growth in growing organisms. It plays an essential part in human health while playing key roles in wound healing and tissue development [1]. Similarly, the phenomenon is regularly triggered in certain pathological conditions including rheumatoid arthritis, endometriosis, diabetic retinopathy, macular degeneration, tumor growth, and inflammatory conditions in response to certain antigens and toxins [2]. However, almost every normal tissues lack this phenomenon in adulthood, except cyclical events in the female reproductive organs [3]. Physiological angiogenesis in tissues contains a natural balance between endogenous pro- and anti-angiogenic factors [3]. When this balance gets disturbed and shifts more toward the pro-angiogenic side in certain pathological states (inflammation, ischemia, hypoxia, and cancer), microvascular endothelial cells (ECs) initiate a cascade of angiogenic reactions which may be retracted or progressive and turn microvessels to an angiogenic phenotype [4]. A considerable diversity exists among microvascular endothelial cells in different tissues and organs, and species heterogeneity cannot be ignored in this scenario [4].

Where angiogenesis is useful for tissue growth and development, excessive vessel growth is really problematic and a hallmark to propagate many diseases while contributing to turning tumor cells into cancer, tumor metastasis, psoriasis, arthritis, diabetic retinopathy, and predominantly metabolic disease such as obesity, atherosclerosis, and certain infectious diseases [5]. Conversely, insufficient angiogenesis or neovascularization may cause ischemic tissue states in heart, brain, and peripheral muscles which may lead to high blood pressure, preeclampsia, neurodegeneration, and osteoporosis [5]. In such pathological states, pro-angiogenic therapies which promote compensatory angiogenesis show promise to treat such pathologies [6]. In parallel to that, angiogenic inhibitors found highly effective in clinical trials as successful strategic treatment approaches with or without conventional chemotherapy for the treatment of solid tumors and metastasis [7]. The potential beneficiary of such novel treatment strategies are patients with aberrant ocular angiogenesis and cancer patients, where defective sight and cancer progression are entirely angiogenesis-dependent [7]. Such treatment paradigms are also heralding a new era of the treatment for other commonly occurring angiogenesis-related diseases.

The formation of new vessels involves many different cell types, and an intricate interplay of various endogenous vascular growth factors, receptors, extracellular matrix (ECM) proteins and the humoral factors [8]. To design and develop potentially effective pro- and anti-angiogenic treatments and to understand molecular mechanisms involved in angiogenesis and neovascularization, numerous in vivo and in vitro assays and animal models of angiogenesis have been developed [9]. Similarly, preclinical angiogenesis assays have also used for drug screening, molecular structure activities, and dosage effects of certain approved anti-angiogenic compounds although such assays are not equivalent and relevant to human disease regarding efficacy [10]. The prime objective of this book chapter is to overview current major and newly introduced angiogenic assays with regard to major advantages and limitations from biological, technical, ethical, and economic perspectives. The major assays which we discuss here include

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corneal micropocket assay, CAM (chick chorioallantoic membrane) assay, rodent mesentery, Matrigel plug assays, whole-animal assays (zebrafish), and animal models of angiogenesis in the context of cardiovascular, ocular, and adipose tissue diseases. A precise note on genetically engineered animal models for vascular endogenous genes and their spatial, temporal, and conditional expression is also included [9]. It is beyond the scope of this chapter to cover every angiogenesis assays in details, so we briefly overview quantitative techniques and/or methods to assess/evaluate neovascularization in tissues. We also briefly discuss molecular mechanisms and cell signaling pathways involved in angiogenesis and potential anti-angiogenic therapies, their clinical impact, limitations, and future prospects.

2. Prerequisite for good angiogenesis assays

Before to choose an ideal assay for angiogenesis studies, the investigators and researchers must know the assay kinetics in terms of operating procedures, handling the environment, ethical justification, and assay economy [8]. In vivo angiogenic studies are more informative than in vitro due to complex cellular and molecular activities of angiogenic reactions while providing biology of the assay and showing experimental design are relevant [9]. Similarly, in trauma-based assays (either physical or chemical), where cell damage triggers inflammatory reactions which mimic the release of several pro-angiogenic cytokines, the sensitivity and specificity of the assay are reduced [10]. For such assays, specific precautions must be taken to avoid any inflammatory reaction or to minimize the traumatic tissue state. In parallel to that, the test substance/compound should be designed as being angiogenic in a noninflammatory state. A near to physiological dose of the test compound should be administered for inducing an angiogenic response while to modulate angiogenic assay conditions and dosage response, a dose range of the clinical use must be chosen [10].

Vehicles carrying the test compound in many assays may also affect the pharmacokinetics of the tested drug and alter the dose-response curves among different experimental animals within one group. For such circumstances, the best solution is to compare test animals/samples with vehicle-exposed counterparts [9]. However, for data interpretation, one must be fully acquainted with the fact that how the vehicle-administered tested animals differ from the untreated controls [10]. Spatial and temporal distributions of the tested compounds are also necessary and vital because failure to do so may produce or hinder to generate reliable and rigorous dose-response curves [10]. As in different pathological states newly formed, vessels are delicate in quality and poorly functional, the selection of angiogenic assessment methods (either qualitative or quantitative) also matters to evaluate the morphology and physiology of the neovascularization in diseased tissues [9]. For in vivo angiogenesis assays, histological microscopy provides the detailed information precisely. Mammalian systems adopted for in vivo angiogenesis assays and mouse models for certain cardiovascular, ocular, and cerebral diseases are comparatively more close to relating human pathophysiology than the embryonic CAM assay, embryonic zebrafish (*Xenopus laevis*), and invertebrate (*Hirudo medicinalis*) angiogenic assays [9, 10].

3. Key components of an ideal angiogenesis assay

It would be interesting to describe that despite the much progress in the field of angiogenesis research, there is no single angiogenic model available which may fully elucidate the entire process and molecular mechanisms of the angiogenic and neovascularization process. Some exogenous and endogenous factors hinder the efforts to develop such an ideal system. Due to cell diversity among different tissues where angiogenesis takes place and intricate interplay among different cell signaling pathways of angiogenic reactions, it is an uphill task to develop and validate a unique assay that is optimal for all situations. However, different modalities and ingenious ways with the passage of time in a particular assay facilitate and provide optimisms for better measurements of angiogenesis than the past. In this context, Vallee et al. [11] conclude that "The design and verification of [new] specific, reliable, repeatable, and precise methodology to measure angiogenesis are considered an imperative of high priority in the field of angiogenesis research." Similarly, Auerbach et al. [10] state "Perhaps the most consistent limitation in all these studies and approaches has been the availability of simple, reliable, reproducible, quantitative assays of the angiogenic response." Moreover, it is challenging although not impossible in several angiogenic assays that the quantification of newly formed vessels regarding numbers and lengths. Similarly, the spatial and temporal distribution of tested compound is also necessary to get strong dose-response curves. Performing an assay in a blinded manner may helpful in this prospect and also to alleviate the influence of any preconceived notions. Analogously, the technical skills to perform any angiogenesis assay are of utmost importance to ensure maximum success.

Despite all these qualms as described above, an ideal angiogenesis assay for quantification of newly formed vessels must feature the following characteristics; first [12], "the release rate [R] and the spatial and temporal concentration distribution [C] of tested compounds should be known to evaluate dose-response curves; second, if tumor cells are used as a source of angiogenic factors, oncogene expression and production of growth factors (either stimulants or inhibitors) must be genetically well defined before the assay proceeding; third, the assay must be designed in a way ensure to provide quantitative measuring parameters of the newly formed vessels (e.g., vascular length [L], surface area [A], volume [V], number of vessels in the network [N], fractal dimensions of the network [Df], and extent of basement membrane [BM]); fourth, the assay should be designed in a way to weigh quantitative measure of morphological characteristics of new vessels (e.g., endothelial cell migration [MR], proliferation rate [PR], canalization rate [CR], blood flow rate [F], and vascular permeability [P]); fifth, a clear demarcation must exist between new and parent vessels; sixth, tissue trauma must be minimized to prevent the formation of new vessels; seventh, in vitro assessment should be verified by in vivo procedures; eighth, angiogenesis assay for long term and with noninvasive monitoring should be preferred; last, the selected assay should be economical, ethically justifiable, robust, and reliable." [12].

4. Process of angiogenesis

Endothelial cell activation, proliferation, and directed migration to form new microvasculature (capillaries) from the parent ones should be a complex process involving many molecular and

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cell signaling pathway events [2]. Some key regulators to switch on or off gene expression are also participating and influence by positive and negative feedbacks of cellular processes. The normal physiological angiogenesis initiates by sprouting of capillaries under the effect of vascular endothelial growth factors (VEGFs) from parent vessels [13]. It continuous during embryonic development and transiently during female reproductive cycle but almost stops in adult tissues except for some wound healing states [13]. Pathologic angiogenesis remains persistent with the continuous proliferation of ECs in different tissue pathologies and particularly in cancer [3]. Many tumor cells are capable of attracting adjacent blood vasculature from nearby tissues [2]. It was evident by the fact that for solid tumors to grow a certain size, neovascularization is necessary otherwise such tumors rarely metastasize as found in thin melanomas which reside on the avascular basement membrane [2, 13]. Also, for tumor growth, the nutrient supply, oxygen, and waste removal are also essential. The new vasculature fulfills this task while providing immune cells, macrophages, and humoral factors to the vicinity of the tumor cells [2].

The parent vessel wall comprises endothelial cell lining, basement membrane, and pericytic cells. Pro-angiogenic growth factors (VEGF, TGF-α, TNF-α) from tumor cells bind to the receptors of ECs and initiate a cascade of cell signaling pathways and angiogenic reactions [2]. Activation and resolution of ECs are two key steps of the angiogenic cascade reactions. When ECs activate and stimulate to grow, the cells secrete proteases, heparanase, and other digestive enzymes that degrade the extracellular matrix (ECM) [13]. ECM degradation allows the secretion of many pro-angiogenic factors from the endothelial cell matrix, and the junctions between ECs become leaky; new microvessel sprouts grow in the direct toward the stimulus [2] (**Figure 1**). For further ECs to grow, proliferate, and migrate, hematopoietic-endothelial progenitor cells (HEPC) also play an essential role [13]. In resolution phase, the new microvasculature tends to mature with the help of pericytic cell adhesion, reconstitution of basement membrane, and

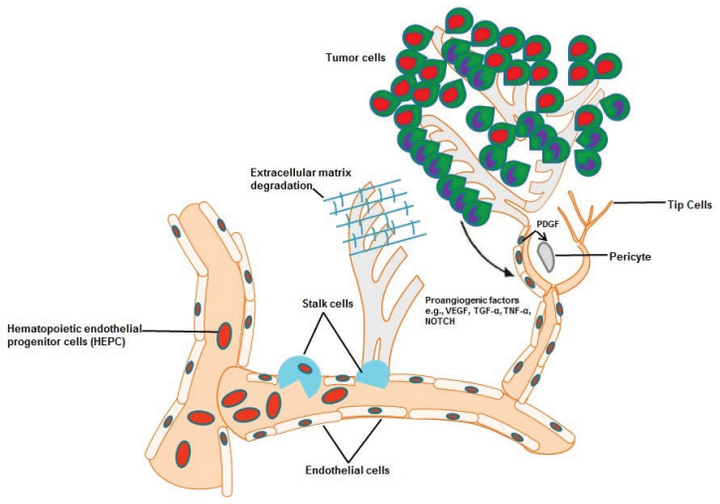

Figure 1. Process of angiogenesis from parent vessels: Angiogenesis sprouting initiates when vascular endothelial growth factors (e.g., VEGF-A) bind to VEGFR-2 receptors located on endothelial cells (ECs). ECs release matrix metalloproteinase (MMP) which degrades extracellular matrix (ECM) from which endothelial tip cells migrate. Vascular endothelial growth factors also regulate Notch cell signaling to inhibit proliferation of endothelial stalk cells. Platelet-derived growth factor (PDGF) released from ECs recruits smooth muscle cells (e.g., pericyte) to stabilize the neovasculature. TGF-α = transforming growth factor, TNF-α = tumor necrosis factor.

formation of cell junctions [14]. Interestingly, the resolution phase in tumor surrounding capillary network remains incomplete which results in irregular and tortuous microvasculature with partial ECs, increased cell permeability, and fragmentary basement membrane [15]. Tumor vasculature is disorganized with poor microcirculation, and vessel diameter changes without any differentiation into arterioles, capillaries, and venules [13]. Similarly, tumor vasculature is sprouting type, so assays which quantify sprouting angiogenesis are very useful to study the kinetics of tumor angiogenesis [15].

In the following section, we present the major and currently used preclinical angiogenesis assays in approximate chronological order of their first publication like chick chorioallantoic membrane (CAM), Matrigel plug, and corneal micropocket assays, while the others described in brief. [10]. CAM, Matrigel plug, and zebrafish assays are very useful for new angiogenic inhibitors screening. For a particular research focus, we provide advantages and disadvantages between different assays in a tabular form feasible for the readers (**Table 1**).

Assay name	Advantages	Disadvantages
***In vivo* angiogenesis assays**		
Corneal micropocket assay	(a) Easy-to-identify newly formed vessels	(a) Atypical assay due to avascular tissue nature
	(b) Easy to perform in animals (e.g., mice, rat, and rabbit)	(b) Induction of nonspecific inflammation to test substance
	(c) Qualitatively permits noninvasive and long-term monitoring	(c) Inaccessible to endogenous blood-borne angiogenic factors
	(d) Immunologically, cross reaction is minimized	(d) Ethical problems as using a major sensory organ for angiogenic assay
	(e) New vessel formation by sprouting	(e) Oxygen exposure may affect angiogenesis
		(f) Not a suitable site for tumor growth
		(g) Tested compounds are few
Chick chorioallantoic membrane assay	(a) Simple to perform and low in cost	(a) Inflammation-mediated angiogenic reactions
	(b) Suitable to study pro- and anti-angiogenic compounds	(b) Very sensitive to change in O_2 tension
	(c) Tumor angiogenesis may assess	(c) Not suitable for metabolically activated compounds
		(d) Embryonic nonmammalian procedure
		(e) Newly formed vessels are difficult to identify
Rodent mesentery assays	(a) Natively sparsely vascularized	(a) Less significant for quantitative angiogenesis in mice than rats
	(b) Lacks physiological angiogenesis	(b) Real-time observation is limited

Assay name	Advantages	Disadvantages
	(c) Angiogenesis induction with little or no trauma	(c) Technically require skills and time consuming
	(d) Suitable for quantitative measurement of microvessel variables (e.g., spatial extension, density, vessel number and length)	
	(e) Suitable to study tumor angiogenesis	
	(f) Sprouting type of angiogenesis	
Sponge/matrix implant assay	(a) Simple and inexpensive to proceed	(a) Nonspecific inflammatory host responses
	(b) Replicate in hypoxic tumor microenvironment so convenient for tumor angiogenesis studies	(b) Implant/sponge composition may vary
	(c) Reproducible and continuous assessment of angiogenesis	(c) s.c is not a reliable route for tumor growth
		(d) Variable drug retention within implant
Disk angiogenesis assay	(a) Inexpensive and easy to perform	(a) Continuous or kinetic observation is limited
	(b) Quantitative assessment of angiogenesis	(b) Encapsulated by granulation tissue
	(c) Wound healing may access	
	(d) Multiple disks can be used at one time	
Matrigel plug assay	(a) Rapid screening of pro- and anti-angiogenic compounds	(a) Matrigel chemical composition is not defined
	(b) An experimental model for tissue regeneration	(b) Three-dimensional plugs are difficult to generate
	(c) Simple to proceed and rapid screening in chambers	(c) Avascular test tissue
Whole-animal models for angiogenic assays		
***Xenopus* laevis (Zebrafish)**	(a) Embryonic and organogenic angiogenesis is assessed	(a) Expensive in breeding condition
	(b) Useful animal model for functional genomic analysis	(b) Non-mammalian and embryonic in nature
	(c) Simple to proceed and relatively fast	
	(d) Easy animal maintenance and significant number of tested animals per statistical analysis	
	(e) Single-drug dosing and small quantities of drugs are required	

Assay name	Advantages	Disadvantages
Mouse models of angiogenesis		
Adipose angiogenesis models		
1. ob/ob mice	(a) Deficient in leptin	(a) Expensive in terms of handling and treatment
	(b) Suitable to study angiogenesis in adipose tissue expansion	(b) Time consuming to assess angiogenesis
	(c) Helpful to test compounds related to metabolic disorders and obesity	
2. Db/db mice	(a) Excellent for role of angiogenesis in insulin resistance and obesity-related diabetes	(a) Difficult to handle and time consuming
Cardiovascular angiogenesis mouse models		
1. Hindlimb ischemic model	(a) Suitable to study arteries growth in tissue hypoxia	(a) Hind limb surgery is complicated
	(b) Can be performed in mice or rat	(b) Skilled and experienced person is required
	(c) Suitable to use for therapeutic agents which augment perfusion to ischemic limb	(c) Degree of tissue hypoxia may vary within experimental animals group
		(d) Residual blood flow may slightly differ in limb after surgery
2. Heart ischemic model	(a) Suitable for pathological and drug evaluation studies	(a) Inflammatory response–mediated angiogenesis
	(b) Efficient neovascularization	
Wound healing assays	(a) Suitable for vascular maturation/ remodelling studies	(a) Inflammatory responsemediated angiogenesis
	(b) Surgery is very simple	(b) limited to skin regeneration
	(c) Pro- or anti-angiogenic compounds can be tested for vessel morphology or regenerative angiogenesis	(c) Regeneration through new tissue formation instead repairing and replacing damaged tissue
	(d) Very easy and robust assay	
Transgenic animal models		
1. Transgenic choroidal neovascularization model	(a) Controlling transgene conditional expression and evaluation of spatial and temporal vascular gene expression	(a) Ethically questionable
2. Transgenic zebrafish model	(b) Knock-down vascular endogenous gene expression	(b) Time consuming
		(c) Differential gene expression may observed within same animal

Table 1. The advantages and disadvantages of major *in vivo, in vitro* and animal models angiogenesis assays.

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5. In vivo angiogenesis models

5.1. The corneal micropocket assay

The firm foundation of systematic angiogenesis research was initiated by Folkman and associates who introduced first time the corneal micropocket assay and chick chorioallantoic membrane (CAM assay) in 1974 [16, 17]. The corneal micropocket assay allows the growth of newly formed blood vessels in vivo, and the techniques were first time applied in rabbits and after that in mice and rat [16]. In this assay, a micropocket is made in the stroma where a pellet containing the growth factors is placed inside the micropocket on the corneal surface of the eye. The growth factors induce a reproducible angiogenic response, and by implanting multiple pellets of different growth factors into parallel micropockets, the various stimuli of angiogenic response may be assessed. The angiogenic response in this assay is entirely due to direct stimulation of blood vessels instead to indirect induction of inflammation reaction. The assay shows minimal inflammatory cellular activity. However, the tested compounds are slowly released from the polymer of the micropocket, and such formulations may cause irritation and ultimately lead to inflammatory reactions which may alter angiogenesis quantification. The micropocket itself is inaccessible to certain blood borne growth factors and blood progenitor cells which may influence angiogenesis. The new vasculature mainly forms through the sprouting from the adjacent limbal area. Being avascular in nature, the corneal assay is useful in visibility and accessibility of new vessel formation and topical application of test drugs and biomicroscopic grading of new vasculature. However, it makes the assay atypical because normal tissues are vascular with few exceptions [16].

5.2. Chick chorioallantoic membrane (CAM) assay

The assay was introduced by Folkman and associates, but embryologists used this method to evaluate embryonic tissue grafts for their developmental potential [17]. The assay is useful to study tumor angiogenesis as well as pro- and anti-angiogenesis compound screening [18]. Fertilized hen's egg incubated at 37°C for 3 days is prepared for grafting by removing enough egg albumin to reduce shell membrane adhesion. Carriers containing the tested compound are placed directly onto the CAM by making a rectangular opening in the eggshell. Slow-release polymer pellets, air-dried disks, and gelatin sponges can be used as tested compound carriers; however, Elvax 40 and Hydron which are used to form sponges and membranes remain inert when applied to the CAM [19]. The quantification of angiogenesis can be made 3–4 days after grafting [18–20]. The in ovo CAM assay is relatively simple to perform as described above. However, a complementary in vitro method has also been described during which the chicken embryos grow in Petri dishes after 3 days of incubation. The assay is technically in vitro, but strictly speaking, it presents a whole-animal assay. After three to six days' extra incubation, the CAM develops, and grafts can be assessed for subsequent development. In vitro CAM allows the quantification of blood vessels over a wider area than in ovo CAM assay. Similarly, a large number of samples can be evaluated at one time, and response occurs within a short period of time (i.e., 2–3 days). Furthermore, the test compound can be placed on the underside of the coverslips. Generally, in ovo CAM assay is performed more than in

vitro CAM. The calculated time for CAM angiogenesis response is very critical as between day five and twelve, the experimentally induced acceleration or suppression of embryonic organogenic angiogenesis can be determined. From day 12 onward, endogenous organogenic angiogenesis under the influence of undefined growth factor may initiate, and identification of newly formed vessels under the effect of tested compound becomes vague [18].

5.3. Rodent mesentery assay

The rodent mesentery assay was introduced by K. Norrby and associates in 1986 and refined later on [21, 22]. The peculiarity of the assay is to use the small gut mesentery of small rodents which is considered ideal for the physiological measurement of angiogenesis. It can be exteriorized from the abdominal cavity, and it's "window" like thin membranous parts make it an ideal angiogenic test tissue by using intravital microscopy. Other potential advantage is that the intestinal mesentery of mouse, rat, guinea pig, rabbit, cat, and dog is almost identical. The test tissue is a 5–10 μm thin membrane which is covered by a single layer of mesothelial cells covered on both sides bordering onto a delicate basal membrane. The thin membrane sandwiches a tissue space that contains mast cells, histiocytes, fibroblasts, and some lymphocytes. It is the thinnest tissue found in the body of Sprague-Dawley (SD) rats. Avascular part of the test tissue contains predominantly 52% fibroblasts and 48% of the mesothelial cells in adult male SD rats. The connective tissue elements of varying size including collagen, elastin, and elastic fiber are also a part of mesentery test tissue [21].

A microscopic analysis clearly shows the cellular and vascular components of the mesenteric windows [22]. The microvessel number per mm circumference is increased in the 15-week-old male rat as compared to 5.5-week-old which demonstrates a slow progression of physiological angiogenesis to the peripheral part of the windows. The same phenomenon is noticed in female SD rats with an age increase; however, the increase in microvessel length, density, and vascularization is not seen in untreated male SD rats at the age of 7 weeks. The distal part of the mesentery (i.e., standard test tissue) of these rats shows no significant angiogenesis for 2–3 weeks which is the usual duration of angiogenesis assay. The test compound usually in the form of an intraperitoneal injection (i.p.) reaches all targeted microvessel of the test tissue because the mesothelial cell lining is highly permeable to a wide range of the molecular weight of the test compounds. The test tissue is unaffected by inflammation mediated angiogenesis as it is untouched mechanically, and no surgery is involved. The assay was tested for the first time for mast cell-induced angiogenesis and later on inflammatory cytokines, and humoral growth factors were also tested almost near to physiologic level doses [22].

The quantitative assessment of angiogenesis is performed by immunohistochemically using a specific primary monoclonal antibody against the rat endothelium. The assay allows clear cut identification of even the smallest newly formed vessels in the test tissue. Thus, the quantitative vessel parameters (as discussed on page 4) can be measured easily which are very vital to determine molecular activity, the effect of low molecular weight heparinized preparations, and dose-response curves. Computer imaging and microscopic morphometry may be used to further validate the immunohistochemistry findings in a blinded fashion [22].

5.4. The sponge implant assays

The assay was introduced by Andrade and associates by which tested compound is directly injected into a sponge which is implanted subcutaneously in the rat [23]. The assay is used for continuous assessment of the angiogenesis as sterile polyester sponge implants become vascularized, and the measurement of blood flow in sponge by using Xe^{133} clearance technique produces reproducible and objective angiogenesis. The exudate fluid for biochemical analysis may be extracted after local injection of angiogenic stimulator or inhibitors. The assay is useful to study tumor angiogenesis as the sponge implant may replicate the hypoxic tumor microenvironment although the composition of sponge implant may vary [9]. The potential disadvantage of the assay is a nonspecific inflammatory response to sponge implant which may infiltrate the sponge substance as the subcutaneous implant becomes encapsulate due to granulation tissue. A variable composition of sponge sometimes makes inter-experimental comparison difficult, and use of Xe^{133} becomes complicated [23].

5.5. Disk angiogenesis system (DAS)

The assay was introduced to study wound healing and solid tumor angiogenesis as well as the angiogenic response of soluble substances in mice [24]. A synthetic foam disk composed of polyvinyl alcohol foam and covered on both flat sides by filters is inserted into mice abdomen or thorax which is well tolerated. The disk is easy to assemble, and the tested compound or tumor cells suspension is placed at the center of the disk. The slow release of the tested drug or tumor cell suspension is managed by the use of agarose or ethylene-vinyl acetate copolymer. The disk is removed within a period of 7–21 days, during which microvascular growth occurs centripetally into the disc. Paraffin-prepared sections of the disk are used to microscopically view the vascular growth as well as fibroblasts and connective tissue components. The quantitative vessel parameters can be determined by point counting on histological sections, intravascular volume, and so on. The disadvantage of the assay is inflammation-mediated angiogenesis as the disk is always surrounded by fibroblasts whenever vascular growth occurs. Similarly, the kinetic observation of newly formed vessels is difficult because one disk provides information for only one point in time [9].

5.6. The Matrigel plug assay

The Matrigel plug assay was introduced by Passaniti and coworkers in 1992 [25]. The Matrigel was extracted from Engelberth-Holm-Swarm (EHS) tumor, which is rich in ECM proteins. It is a solubilized basement membrane preparation which liquefies at 4°C but reconstitutes into a gel at 37°C when injected subcutaneously into mice where it is slowly surrounded by granulation tissue. The gel induces highly vascularized response under the influence of angiogenic growth factors in particular bFGF [25]. The assay is noninvasive and easy to administer but time-consuming to handle.

The Matrigel composition is not fully defined. However, the major components include epidermal, transforming, platelet, nerve, and insulin-like growth factors (e.g., PDGF, TGF, and bFGF) laminin, collagen, heparin sulfate proteoglycans, and entactin [26]. For this reason, care should be taken while using Matrigel assay for the cellular activity studies. It was observed

that when Matrigel with reduced growth factors is implanted, few cells invade the plug or gel. However, with known angiogenic growth factors (e.g., bFGF), mixed with Matrigel and injected subcutaneously, endothelial cells migrate into the gel and constitute vessel-like structures. A fine network of endothelial cell tubes enlarged by micro- and macro-vessel endothelial cells slowly progress to capillary networks in vivo [26].

For the quantitative assessment of angiogenesis, Matrigel and surrounded granulation tissue are removed after 1–3 weeks, and immunohistochemistry and histological sections are measured [27]. However, determining the profiles of capillary-like vessels is difficult. Similarly, the hemoglobin (Hb) test does not differentiate the blood flow in newly formed blood vessels and large parent vessels. Fluorochrome-labeled high molecular weight dextran and quantitative vascular specific indicators are alternative methods to assess neovascularization [27].

The assay is suitable for tissue regeneration experiment model where neovascularization is coupled with organogenesis, fibrosis, and monocytes/macrophages play a pivotal structural role. A possible drawback of the assay is that Matrigel plug contains only capillary network rather than no tissue without any pro- and anti-angiogenic factors to influence angiogenic reactions [28].

A variation of the Matrigel plug assay is the combination of Matrigel and sponge techniques. Five-hundred microliters of Matrigel is injected subcutaneously into mice and solidify for 20–30 min [27]. After that, the mice are anesthetized, skin overlying Matrigel is shaved, and a small nick is made. A similar nick is made to Matrigel plug, and a sterile polyvinyl sponge with the test compound is introduced into the center of the Matrigel plug with the help of tweezers. The same procedure may use for angiogenic growth factors or test tissue to be implanted in the Matrigel plug. By this modification, neovascularization is directional, and assay sensitivity is increased to measure direct angiogenesis as compared to standard Matrigel plug assay. However, the sponge/Matrigel combined assay is time-consuming, and the total number of assayed animals become limited [27].

5.7. Whole-animal angiogenesis model

Zebrafish was introduced in 1999 as a whole small angiogenesis model for the screening of pro-angiogenic compounds which directly influence the newly formed vessels [29]. The choice of the whole animal as a tested tissue was based on the remarkable similarity of zebrafish organs to those of a human at the physiological, anatomical, and molecular levels [30]. Moreover, the short generation time (approx. 3 months) and easy to house in small space and relatively large numbers also facilitate to evaluate many tested animals in one assay [31]. The external development of zebrafish embryos and optical transparency during embryonic stage assists continuous microscopic evaluations of different developmental processes from gastrulation to organogenesis [30]. Furthermore, external mode of fertilization also permits easy access to experiment design and assessment. Small tested compounds dissolve to water diffuse directly to fish embryo and induce distinct and dose-dependent angiogenic effects. Both pro- and anti-angiogenic compounds exhibit similar effects in zebrafish as exerted in mammals [31].

6. Animal models of angiogenesis

In biomedical research, mouse models are of utmost importance for a wide variety of medical tests including gene expression, gene knockout, and medical genetic analysis [32]. For this purpose, SCID, transgenic, and genetically engineered mouse models are of particular interests which allow sophisticated investigations for genetically induced pathological states and molecular pathogenesis of certain genetic disorders. Furthermore, such mouse models are useful to study genes essential for angiogenesis and vascular biology [32]. In parallel to that mice with conditional, global knockouts, over-expressing angiogenic factors are also considerable in this prospect [33]. As remarkable similarity exists between human and murine vasculatures, such tools are valuable to search possible molecular interactions among distinct angiogenic factors in the onset and progression of various human diseases [32, 33]. In the following section, we shed light on some practically used mouse models in the context of pathological angiogenesis which directly plays a part in human diseases.

6.1. Mouse model of angiogenesis in adipose tissue

Genetically engineered mouse model for adipose tissue angiogenesis is highly reproducible and produces robust results because the mice are inbred and share a highly similar genetic background. This approach is irrelevant to humans because high caloric intake and little physical exercise are the predisposing factors for developing obesity instead a little genetics involved. Thus, mice fed on high-fat diet present an ideal animal model to study non-genetically related obesity [34].

6.1.1. Ob/ob mice

The mouse carrying the obese mutation (ob) was first described in 1950, and later on, it was shown that the mutation located in the gene coding for a hormone leptin, which regulates appetite and food intake [35]. The hormone binds to leptin receptor (Ob-R) in the hypothalamus and subsequent cell signaling regulates food uptake, energy expenditure as well as fat and glucose metabolism. Ob/ob mice are deficient in leptin exhibit uncontrolled and continuous food intake which results in a gain of body weight. Consequently, mutated mice weight is three times higher, and body fat content elevates up to fivefold as compared to wild-type species. The mutated mice also show decreased physical activity and energy expenditure, infertility, and immune deficiencies. The mutation is recessive, so the heterozygotes do not display such phenotype [35]. Ob/ob mice can be used as an outstanding model to explore the role of angiogenesis in adipose tissue expansion, and with specific angiogenic inhibitors, obesity may be prevented in such mice [36]. As the leptin kinetics in mice to regulate food intake and obesity are homologous to human, such angiogenic model can be used to search novel therapeutic targets to treat obesity and metabolic disorders [37].

6.1.2. Db/db mice

The mouse strain C57BL/KsJ was first described with an autosomal recessive mutation diabetes (db) in 1966 [38]. Homozygous mice with such mutation are deficient for the leptin

receptor and exhibit a phenotype that resembles human diabetes mellitus. The mice with such mutations are also characterized as an obese phenotype. Furthermore, such mice exhibit infertility and hyperglycemia while heterozygotes are typically lived as wild type. Db/db mice can be used to study molecular mechanisms involved in obesity-related diabetes and insulin insensitivity, and the role of angiogenesis and neovascularization can be elucidated in this regard [38].

6.2. Hindlimb ischemic model of angiogenesis

Most of the angiogenic models described above are very useful to study pathological angiogenesis and search for novel anti-angiogenic treatment in the form of angiogenic inhibitors. However, certain pathological states (e.g., myocardial infarction, stroke, and wound healing/regeneration) in human body require accelerated blood vessel growth to reinstate the proper function of such vital organs [39]. In myocardial infarction, an occluded coronary artery obstructs blood flow to a part of the cardiac muscle tissue which leads to severe tissue hypoxia (ischemia). The cardiac muscle requires a regular supply of oxygen and glucose levels for normal function. To overcome tissue hypoxia, the growth of highly functional arteries is eagerly awaited in such situations. Hind limb ischemia in rat or mice presents an excellent model to study and manipulate newly formed vessels in particular arteries in response to tissue hypoxia [39].

In this assay, the arteries supply blood to one back limb of the mice is ligated to stop the blood circulation in the entire limb [40]. The occlusion of arteries leads to tissue ischemia and the initiation of arteriogenesis from collateral arteries. Pro-angiogenic factors and even anti-angiogenic compounds under investigation can be administered to the limb musculature to modulate the arteriogenic response. Doppler angiography is used to evaluate the blood circulation in the hind limb, and the procedure can be repeated in the same animal to know that how the blood flow improves over time. To study newly formed microvessels, the tissue can be excised and stained, and morphology of the blood vessels is elucidated [40].

The assay is the first in class to present therapeutic angiogenesis and widely used in fundamental discoveries to demonstrate that how to generate highly functional and stable arteries therapeutically [39]. On the other side, the potential disadvantage of the assay is very complicated hind limb surgery and requires highly skilled professionals and experienced surgeons. Similarly, the proportion of blood flow in a hind limb may vary after surgery, and it may affect the degrees of tissue hypoxia which ultimately influence on the therapeutic activity of pro- and anti-angiogenic compounds under investigation [39].

6.3. Wound healing assays

The wound healing assay allows to study and evaluate both angiogenesis and vascular maturation/remodeling in injured or damaged tissues [41]. The assay is usually performed on the skin of mice because other accessible tissues (e.g., tail and ears) do not regenerate well. Two circular holes (approx. 5mm in diameter) are punched through the dorsal skin of anesthetized wild-type C57B16 mice. One hole would serve as control while drugs under investigation can be administered on the other. No bandages or sponge is required as no major blood vessels

exist in this region of the skin and wound formation allows very little bleeding in the surrounding area. Wound sealing starts within two weeks, and complete wound healing occurs within a month [41].

Photography and measuring of wound area with calipers provide information about wound size, scar formation, and re-epithelization of the wound [41]. The drugs under investigation (either pro- or anti-angiogenic compounds) in this model may be administered either systemically by oral administration, injection, or topically. The drug effects can be determined by excision of the skin tissue, fixed, and stained with specific dyes. The tested compounds may influence regenerative angiogenesis, vessel morphology, and function. The assay is easy to setup, and surgery is very simple. The wound size remains uniform and homogenous for all animals used in one experiment [41].

The potential disadvantage of the assay is that angiogenesis is inflammation-dependent involving blood clotting phenomenon and other complex biological processes and occurs only in the skin [41]. Similarly, skin tissue regeneration is entirely different as compared to other highly vascular tissues such as the heart and nervous system and does not provide an adequate understanding of the role of angiogenesis in tissue regeneration. Furthermore, tissue regeneration is mainly due to reconstitute new tissue rather than repairing or replacing, which is hard to replace in ischemic insults which produce large patches of dead tissue [41].

6.4. Genetically engineered animal model for angiogenesis

Gene expression analysis and gene function studies are contributing widely to almost every research in life sciences, biomedical research, biotechnology, molecular pathology, and human health [42]. In vivo applied and functional genomic studies are particularly considered by overexpressing a candidate gene or suppressing the gene expression for the purpose of gene knockout [43]. Such approaches are achieved and applicable by genome manipulation of wild-type animals [43]. Similarly, the generation of transgenic animals by injecting desired DNA constructs to fertilized eggs also presents some standard technology in gene expression studies [44]. Transgenic animal models and DNA constructs (e.g., gene expression plasmids and vectors) with desired gene expression are widely used to study gene function and molecular pathogenesis of diseases, and it create models to demonstrate the complex, intricate interplay between gene overexpression or suppression for the molecular epidemiology of human diseases [44]. In the following section, we briefly overview such novel approaches to be involved in the context of vascular angiogenesis.

The control of target gene expression in vascular cells of transgenic animals by cell or tissue expression plasmid with specific promoters is very helpful to study developmental and pathological gene function in the vasculature [42]. It was found that the promoters derived from the sequences of VEGFR-1, ICAM-2, vWF, or endoglin efficiently work in mouse endothelial cells both in vivo and in vitro with specific intensity and specificity [42]. Similarly, lacZ selective transgene expression was seen in ECs cells under the control of promoters derived from Tie 2 (angiopoietin receptor), ICAM-2, or VE-Cadherin [42]. However, the expression of a transgene in smooth muscle cells (SMCs) is difficult to achieve because most SMC markers are expressed differentially, and SMC growth and cell differentiation are an exclusive

process [42]. In contrast, transgenic mice may obtain using selective promoters expressing smooth muscle myosin heavy chain (SM-MHC), smooth muscle α-actin, and SM22α [42]. Such models provide valuable information about the function of a specific gene in a particular tissue, and controlling the expression of such genes may use as a therapeutic approach to certain disease and also for angiogenesis and cancer [42]. However, the transgene expression depends on promoter's characteristics to be used while constructing cell or tissue expression plasmids with the gene of interests including; promoters is constitutively active, capable to express and replicate gene of interest or to express in embryonic or in the adulthood stage [45]. Failure to do so may limit studies in molecular pathogenesis, leads to nonviable transgenic animals or compensatory responses. More powerful tools are being developed based on conditional transgene expression systems [45].

The inhibition of endogenous gene expression is also a potential method to suppress the gene function involved in the molecular pathogenesis of many genetic disorders and infectious diseases [46]. Many studies show that sequence-specific mRNA degradation by double-strand RNA strongly inhibits the function of that gene involved in pathogenesis or propagation of a particular disease [47]. The technique is known as RNA interference (RNAi) and may be used in certain genetic disorders, in cancer, HIV, and other harder to treat infections. Recent publish data indicate that mammalian expression vectors expressing short hairpin RNA (shRNA) under the control of specific vascular promoters inhibit gene expression through an RNAi effect [47].

7. Angiogenesis and cancer

The phenomenon of angiogenesis is fundamental in tumor growth, progression, and metastasis [48]. Angiogenesis itself is the result of a highly orchestrated series of molecular and cellular events including a plethora of genes, signal cascades, and transcription factors which are highly organized and work in a systematic way to generate microvessels in normal physiological angiogenesis [49]. However, the tumor angiogenesis is disorganized, irregular, and not systematic at the level of molecular and cellular events and ultimately propagates many tumors into cancers [48]. The cancer cells contain the ability to stimulate angiogenesis by producing a lot of angiogenic factors including cell growth factors, cytokines, and numerous other molecules [48, 49].

Many pro- and anti-angiogenic molecules involved in the induction of angiogenesis and neovascularization, their receptor ligands, and intracellular signaling pathways have been identified within last 30 years [50]. Much work has been done to develop anti-angiogenic treatment strategies for cancer patients [51]. However, numerous preclinical trials show no promise regarding high efficacy and tolerability with classical anti-angiogenic drugs as monotherapy [51]. It spurred the researchers and investigators to design and develop novel anti-angiogenic compounds to be used in combination with classical cytotoxic agents and radiotherapy [52]. FDA-approved angiogenesis inhibitors in combination with chemotherapy have proven their clinical worth regarding improved patient survival time and patient tolerability in certain cancers [52].

In the coming section, we briefly overview molecular mechanisms of major cell signaling pathways involved in the induction of angiogenesis, and at the end, some brief glimpse about the clinical impacts of newly developed angiogenic inhibitors will be described. The cellular events in the regeneration and propagation of tumor angiogenesis are already explained briefly at page 5 and depicted in **Figure 1**.

7.1. VEGF intracellular signaling

Vascular endothelial-derived growth factor (VEGF) is one of the most important and potent angiogenic molecules which play an integral role in tumor angiogenesis [50]. It presents the first in a class of cytokines which induce vascular leakage and therefore also known as vascular permeability factor. Until now, six members (VEGF-A to VEGF-F) of this unique family of cytokines have been discovered [53]. VEGF-A is mainly involved in angiogenesis and vasculogenesis whereas VEGF-B is a survival factor for ECs, SMCs, and pericytes [54]. VEGF-C and VEGF-D are essential for lymphangiogenesis, and PGF also acts as a survival factor for ECs and modulates VEGF cell signaling [55].

Vascular endothelial growth factors activate ECs by binding to a family of class III transmembrane receptor tyrosine kinases (RTKs) expressing at high levels in endothelial cell lineage [53]. VEGF-R1 and VEGF-R2 are located on ECs and activate during angiogenesis while VEGF-R3 induces intracellular signaling in lymphatic cells. VEGF-R1 acts as a decoy receptor as it is RTK defective and acts as a negative regulator of angiogenesis (**Figure 2**) [54]. The angiogenic multiple cell signaling pathways are initiated as VEGF-A binds to VEGF-R2, and the receptor dimerizes and intracellular receptor domains are phosphorylated in ECs and induce overexpression of growth factors, cell proliferation, mitogenesis, chemotaxis, and prosurvival signaling (**Figure 2**) [55]. VEGF-C binds to VEGFR-3 and initiates mitogenesis in lymphatic cells and stimulates hyperplasia in parent lymphatic vessels [53–55]. The production of VEGF is regulated by several growth factors produced by the tumor cells including, endothelial growth factor (EGF), transforming growth factor (TGF-α & β), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) [54]. Some hormones (e.g., estrogen, thyroid-stimulating hormone (TSH)) and interleukins (e.g., IL-1 & 6) also stimulate VEGF-induced intracellular events in other types of cells [56].

7.2. Notch signaling pathways

The Notch receptors are located on stromal cells and expressed as a heterodimeric complex of two domains, that is, the Notch extracellular domain (NECD) and Notch intracellular domain (NICD) which are associated with each other via noncovalent interactions (**Figure 3a**) [57]. The Notch cell signaling may mimic direct tumor angiogenesis however actively involved to trigger dormant tumors [1]. Notch ligand Delta-like 4 (DLL4) induces cell signaling pathways to improve vascular functions by endocytosis and nonenzymatic dissociation of Notch heterodimer in host stromal cells (**Figure 3a**) [1]. DLL4 inhibition may promote cell proliferation response in ECs which ultimately increase angiogenic sprouting and vessel branching [58]. Despite increased endothelial cell vascularity, the tumor cells perfuse poorly, which reduces cell oxygen concentrations (i.e., increased hypoxia), and consequently, tumor growth is inhibited [59].

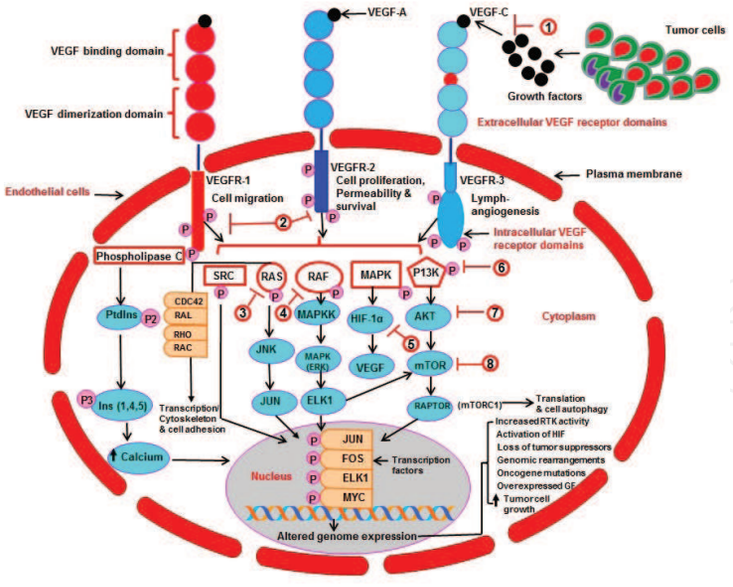

Figure 2. VEGF-induced intracellular signaling in tumor angiogenesis and angiogenic inhibitors with targeted active sites: The binding of vascular endothelial growth factors (VEGF) to respective transmembrane receptors stimulates a plethora of intracellular signaling pathways which regulate nuclear transcription factors for altered gene expressions of normal cell responses including loss of tumor suppression, activation of hypoxia inducible factor (HIF-α), increased receptor tyrosine kinase activity, increased tumor cell growth, and repression of oncogene mutations. Angiogenic inhibitors to their targeted active sites are also shown with numerical circles in the figure. Only anti-angiogenic compounds approved by the US Food and Drug Administration (FDA) for the treatment of numerous solid tumors and carcinomas are depicted where circle 1 represents growth factor inhibitors (bevacizumab, aflibercept); circle 2, growth factor receptor inhibitors (sunitinib, sorafenib); circle 3, RAS inhibitors (tipifarnib, lonafarnib); circle 4, RAF inhibitors (sorafenib); circle 5, HIF-1α inhibitors (geldanamycin, chetomin, echinomycin, 2ME2); circle 6, PI3K inhibitors (wortmannin, LY294002); circle 7, AKT inhibitors (FARA-A); and circle 8, mTOR inhibitors (rapamycin and analogues). JNK = JUN N-terminal kinase; MAPK = mitogen-activated protein serine/threonine kinase, MAPKK = MAPK kinase, PDK1 = phosphoinositide-dependent protein kinase-1; PLC = phospholipase C; PtdInP2 = phosphatidylinositol 4,5-bisphosphate, Ins (1,4,5) = inositol 1,4,5-triphosphates.

In contrast, DLL4 expressed in ECs stimulates Notch 3 receptors located on adjacent cells (e.g., colorectal cancer or T-cell acute lymphoblastic leukemia cells) to activate tumor progression from dormant to active phase [60]. Such findings consider Notch pathways a potential therapeutic target for the design and development of novel anti-angiogenic compounds, although the Notch cell signaling shows a mixed behavior of tumor progression and inhibition in clinical assays [58, 59].

7.3. Transforming growth factor-β (TGF-β)

Transforming growth factor is a ubiquitously expressed paracrine polypeptide of approximately 25 kDa molecular weight [61]. TGF 1 to TGF 3 are three highly homologous isoforms of the polypeptide and discovered in humans and mammals [62]. TGF-β is initially synthesized as a zymogen, and after secretion, an associated peptide is proteolytically sliced to release active form of the growth factor [63].

Active TGF-β binds to constitutively active serine/threonine kinase TGFBR2 receptors to activate TGFBR1 in a heterodimer complex which controls transcription via activation of canonical signal pathways mediated by a family of SMAD proteins (SMAD1-5) (**Figure 3b**) [64]. The

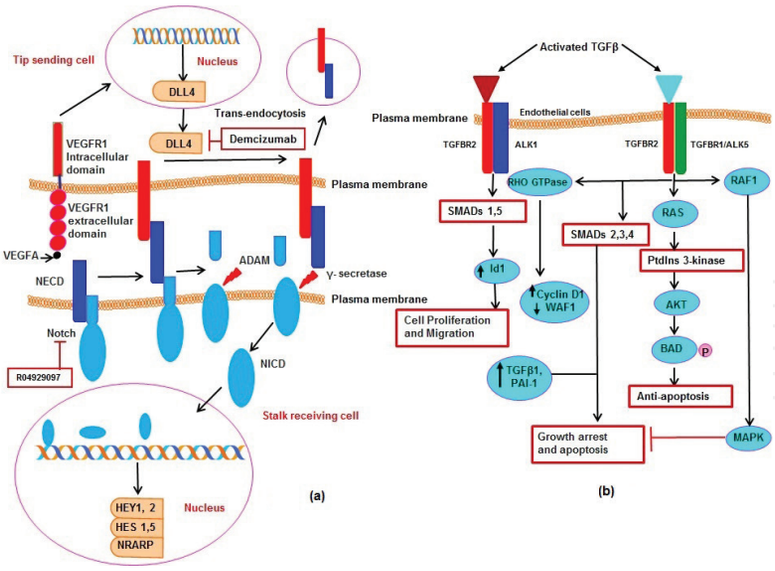

Figure 3. A schematic diagram of Notch and TGF-β induced cell signaling pathways in tumor angiogenesis: (a) Notch cell signaling pathways: The ligand DLL4 dissociates Notch heterodimers by nonenzymatic degradation and cell endocytosis. Notch extracellular domain exposes Notch to ADAM metalloproteases and γ-secretase in sending cells (tip cells) for proteolytic cleavage and the release of Notch intracellular domain which translocates to the nucleus of receiving cells (stalk cells) for the transcriptional activation of Notch target genes (shown in the nucleus of the stalk cells). The DLL4 ligand and Notch inhibitors are also depicted in the red rectangular boxes. (b) Transforming growth factor (TGF-β) induced intracellular signaling pathway: In normal cells, the binding of TGF-β to transmembrane TGFBR2 receptors activates TGFBR1 receptors which upregulate the expression of a series of SMAD proteins (SMAD 2, 3, and 4) and cause cell cycle arrest and apoptosis. However, TGF-β stimulates other molecular pathways in transformed cells to inhibit cell apoptosis and accelerates cell migration and metastasis. In contrast, a second type 1 receptor (ALK1) is expressed in ECs which stimulates cell proliferation and migration via activating SMADs 1 and 5 genes.

activation of SMAD 1 and 5 proteins in transformed cells inhibits apoptosis and mediates cell proliferation and migration via the activation of other cell signaling pathways [65]. However, in normal cells, the stimulation of SMAD 2, 3, and 4 exhibits cell cycle arrests and apoptosis [66]. Similarly, the SMAD 2, 3, and 4 proteins increase the expression of PAI-1which is essential for vessel maturation in angiogenesis (**Figure 3b**) [66].

8. Clinical impact of angiogenic inhibitors

The discovery and development of angiogenic inhibitors have raised the hopes to treat a lot of tumors and carcinomas and ultimately to reduce the morbidity and mortality related to tumors and cancers [67]. Five classes of anti-angiogenic compounds have established and are still under investigation on the basis of potential antitumor drug targeted sites including proteases inhibitors (MMP synthesis inhibitors), ECs proliferation and migration inhibitors, vascular endothelial growth factor inhibitors, cell matrix protein inhibitors, and angiogenic inhibitors with unique mechanisms (**Figure 2**) [68, 69].

Although the anti-angiogenic compounds approved by the FDA show therapeutic efficacy in some categories of cancer as monotherapy, however, sufficient published data recently reveal this fact that angiogenic inhibitors are best therapeutic choices for tumors when used in

combination with traditional chemotherapies [70, 71]. However, one would not expect in the first instance that angiogenic inhibitors might reduce the intratumoral delivery of cytotoxic agents (traditional chemotherapy) by decreasing perfused blood vessels with impaired blood flow and decrease drug transport in treated tumor cells [48, 72]. It would also increase tissue hypoxia and inhibit tumor cell proliferation although proliferating cells are an easy target for chemotherapy [48, 72].

To overcome such hurdles and to enhance synergistic therapeutic potential of chemo and anti-angiogenic drugs when used in combination, Kerbel proposed three mechanistic approaches in this scenario to be adopted; first, normalization of tumor microvessels by anti-angiogenic compounds [73, 74]; second, maximum tolerated dose chemotherapy during the break periods of successive courses [72, 75], and third, use of known chemotherapeutic agents having anti-angiogenic effects [72]. The additional advantages of chemotherapy while improving their anti-angiogenic effects may be grabbed by adopting "metronomic chemotherapy" which states that "the administration of chemotherapeutic agents at relatively low, minimally toxic doses on a frequent schedule of administration at regular close intervals, with no prolonged drug-free breaks [76, 77]." By such approaches, endothelial cells are directly killed, and progenitor ECs are suppressed in circulation. Furthermore, minimal use of toxic doses lowers the frequency of adverse events in treated patients [72, 76, 77]. Such treatment strategies may be adopted for a prolonged period of time with angiogenic inhibitors in the treatment of advanced solid tumors with little side effects as validated by phase II clinical trials; however, phase III clinical studies are extensively demanded in this direction [70, 71].

9. Conclusions

In vivo, in vitro, and in ova assays for angiogenesis assessment are the reliable approaches in basic research and to some extent in real-world clinical practices. However, in vivo systems are difficult to perform and time consumable, and the process of quantification is much complicated than in vitro assays. Conversely, these are relatively better due to complex nature of the vascular response to the test compound. In vitro angiogenesis assays may perform in a short period and provide the accurate and reliable outcome of angiogenic processes. Mouse models based angiogenesis assays have also standardized to an improved understanding of tumor angiogenesis and lymphangiogenesis. Similarly, such models are also used to assess vasculogenesis and arteriogenesis in ischemic heart diseases, blindness, psoriasis, and arthritis. Angiogenesis assessment always plays a focal role to determine the pathogenesis and progression of certain challenging diseases in human populations in particular human cancer. An ample understanding of angiogenesis research in tumor progression, by knowing the molecular mechanisms and cellular pathways, also opens the ways to design and develop effective anti-angiogenic inhibitors. The manipulation of the human genome in a precise and predictable manner due to recently developed molecular techniques has opened new gates for the generation of more reliable models for angiogenesis studies and the testing of new therapeutic strategies.

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