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Mouse models to study angiogenesis in the context of cardiovascular diseases

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1. ABSTRACT

Pathological angiogenesis is a hallmark of various ischemic diseases (insufficient vessel growth) but also of cancer and metastasis, inflammatory diseases, blindness, psoriasis or arthritis (excessive angiogenesis). In response to ischemia (reduced blood flow and oxygen supply), new blood vessels form in order to compensate for the lack of perfusion. This natural process could protect them from the consequences of atherosclerotic diseases (myocardial angina, infarction, hindlimb arteriopathy or stroke). However, neovessel formation is altered in many patients. A better understanding of the mechanisms of functional vessel formation is a pre-requisite to improving the treatment of ischemic pathologies. To this end, it is essential to create easily accessible animal models in which vessel formation can be both manipulated and studied. In this review, we will describe different angiogenic mouse models in the context of cardiovascular diseases, either in an ischemic context (hindlimb ischemia, heart ischemia,

skin model) or in a non-ischemic context (plug and eye assay, wound healing, ovarian model). We will also discuss quantitative techniques for assessing angiogenesis in these assays.

2. INTRODUCTION AND LIMITS

Excessive vessel growth contributes to the development of cancer, metastasis, psoriasis, arthritis, blindness linked to retinopathy and probably other diseases such as obesity, atherosclerosis and infectious diseases. Insufficient vessel growth or lack of vessel stability are responsible for heart, brain and peripheral muscle ischemia but have also been involved in hypertension, pre-eclampsia, neurodegeneration and osteoporosis. Promoting the formation of new collateral vessels in ischemic tissues (therapeutic angiogenesis) is a promising approach for the treating cardiovascular diseases. Conversely, inhibition of the action of key regulators of angiogenesis is a newly explored alternative for the treatment of solid tumors and metastasis. In this review, we will focus on techniques designed to study vessel growth in the context of tissue ischemia.

2.1. Background: angiogenesis, arteriogenesis or vasculogenesis

As currently understood, the formation and growth of new blood vessels involve vasculogenesis (i.e. the *in situ* differentiation of endothelial cells –ECs-, endothelial progenitor cells –EPCs- or vascular progenitor cells), angiogenesis (i.e. the sprouting of new capillaries from existing vessels, and/or postcapillary venules), and arteriogenesis (i.e. the association of newly formed capillaries with mural cells such as smooth muscle cells – SMC- and pericytes). During adulthood, arteriogenesis also signs the formation of collateral arteries that can arise either de novo or from pre-existing, rudimentary vessels (1). For details on the mechanisms of blood vessel growth see the following reviews and articles (2-10). Both angiogenesis and arteriogenesis are involved in new blood vessel formation during adulthood, while the extent of vasculogenesis contributing to neovascularization in adult tissues has not been ascertained (11-12).

2.2. VESSEL GROWTH IN CARDIOVASCULAR DISEASES

Normal tissue functioning depends on adequate supply of oxygen through blood vessels. Atherosclerosis (i.e. lesion of large vessels or macroangiopathy) or diabetes, and hypertension (i.e. lesions of small vessels or microangiopathy) reduce vessel lumen diameter and blood perfusion, leading to tissue ischemia. Atherosclerosis is a major cause of mortality by myocardial angina, infarction, or stroke as well as morbidity by hindlimb arteriopathy. In response to ischemia (reduced blood flow and oxygen supply), new blood vessels form and a collateral circulation is established in order to compensate for the lack of perfusion. This natural process could be very effective in patients and protect them from the consequences of atherosclerotic diseases (13). However, this bypass process shows considerable variation among patients and the reasons for these differences are poorly understood (14). A better understanding of the mechanisms supporting the formation and the maintenance of functional vessels in post-ischemic tissues is a pre-requisite to improving the treatment of ischemic pathologies and to offering therapeutic options by stimulating muscle angiogenesis (3).

Most of our understanding of the angiogenic factors and signaling mechanisms that initiate and control blood vessel formation comes from studies of embryonic vascular development (15-16). Significantly less is known about the regulation of neovascularization in adult tissues, which has long been thought to recapitulate embryonic patterns of vascular gene expression. Hypoxia and blood flow are not the only regulators in adults during ischemic conditions (2). Pioneer works done by oncologists to explain how tumors need to form new vessels to grow, have opened a large field to cardiovascular research (16).

3. ASSESSMENT OF ANGIOGENESIS, ARTERIOGENESIS OR VASCULOGENESIS

There are currently considerable efforts invested into the development of drugs aiming at controlling blood vessel growth in conditions such as ischemia and cancer, characterized by deficient or excessive vessel growth, respectively (4). To this end, it is essential to create easily accessible animal models in which vessel formation can be both manipulated and studied. Vessel formation is a tightly controlled process where EC proliferation and migration are regulated by secreted factors as well as by surrounding cells and matrix. Due to the complex cellular and molecular activities of angiogenic reactions, *in vivo* studies are more informative than *in vitro* studies. In this review, we will focus on adult mouse models that have shown their utility for studying regulation and signaling mechanisms in vessel growth process as well as their feasibility for modulating vessel growth in ischemic or non-ischemic settings. Embryonic vascular development, spheroids of differentiating embryonic stem cells, and tumor models will not be discussed.

3.1. Is there an ideal assay to assess neovascularization?

It should be noted that some neovascularization models will favor angiogenesis, arteriogenesis or vasculogenesis processes, depending on the location and the duration of neovascularization, the context (ischemia, inflammation, metabolism impairment), and the mouse strain (17-19). Hence, in the case of tissue injury, angiogenesis is likely to be the predominant process, with little arteriogenesis occurring; however, in the case of a common femoral artery ligation, arteriogenesis will predominate at the site of ligation, whereas angiogenesis will predominate in the ischemic distal bed. In the case of a lethally irradiated mouse receiving a bone marrow transplant, vasculogenesis will predominate at the sites of injury (20). Unsurprisingly, a single assay that would be optimal for all situations has yet to be described. The ideal *in vivo* assay should fulfill some of the following requirements summarized by Jain *et al* (21):

a) The release rate and the spatial and temporal concentration distribution of angiogenic factor (s) should be known for generating the dose response curves.

b) The assay should provide quantitative measures of 1) the structure of the new vasculature (e.g., vascular length, surface area, volume, number of vessels in the network, fractal dimensions of the network, and extent of basement membrane); 2) the functional characteristics of the new vasculature (e.g., EC migration, proliferation rate, canalization rate, blood flow rate, and vascular permeability).

c) There should be a clear distribution between newly formed and pre-existing host vessels.

d) The assay should permit long-term and, if possible, noninvasive monitoring.

e) It should be cost-effective, rapid, easy to use (routine), reproducible and reliable.

Today, there are no murine assays that respond to all these criteria, however some of the available assays, with the development of new monitoring methods, could provide considerable insights in the mechanisms of new vessel formation.

3.2. Monitoring and assessment of angiogenesis, vasculogenesis and arteriogenesis

The ability to monitor, analyze and quantify blood vessel formation *in vivo* following a particular treatment has been a long-standing challenge. Assessment of angiogenesis can be accomplished either by directly monitoring vessel formation or by indirectly studying the functional effects of this formation. The extent of this ability counts for a large part in the interest of any of the *in vivo* angiogenic models (22).

3.2.1. Imaging vessel morphogenesis

Histological microscopy provides the most detailed information on *in vivo* angiogenesis. Immunohistochemical staining could detect ECs (artery or vein cells), SMCs, pericytes or extra-cellular matrix. Quantification of capillary density, which is recorded as the number of capillaries per mm^2 of tissue section, remains the most commonly assessed histological measure of angiogenesis (23-25). Assessment of capillary density does not preclude assessment in perfusion modification. Changes in muscle fiber types, and evidence of fibrosis, extent of cell death, proliferation or number of muscularized arteries provide interesting additional tissue assessments (26). To study vascular cell proliferation, intra-peritoneal injections of bromodeoxyuridine prior to sacrifice may be helpful. On the other hand, proliferation markers such as antibodies against PCNA, minichromosome maintenance protein 2, or Ki67 could be used directly on tissue sections without any prior injection. Apoptosis process could be investigated through immunochemical techniques using either the TUNEL method or the immunostaining of elements of the apoptosis cascade (e.g. caspases, Bac, Bcl2, Fas). Study of inflammatory cells infiltrating the ischemic area and participating in the neovascularization process could be easily feasible by immunohistochemistry (i.e. using antibodies against myeloperoxydase, CD3, CD4, CD8, CD45, CD60) (27).

Although imaging the permeability of blood vessels is not often a goal in the cardiovascular field, it could be assessed by monitoring the amount of leakage of intravascular tracers (microscopic imaging) (28).

Vascular casts constitute a conventional and potent method to analyze the three-dimensional architecture of micro blood vessels and vascular networks. Specimen are injected with colored gelatin, latex or plastic material followed by tissue digestion and examined by light or electronic microscopy. This approach allows to determine vessel diameter and length, intervascular distances, feature of branching and other aspects not provided by histology on tissue sections (29). Other techniques allow for direct visualization of new vasculature. *Angiographic methods* are excellent for evaluating large arteries and veins. Large collaterals (130 μm diameter) can be observed and perhaps even quantified with this technique; however microvasculature (with the exception of retina) is not accessible to current angiographic methods. Micro angiography offers a higher resolution and some solutions for quantification at the site of angiogenesis in small animals (30). This method is useful in evaluating therapies for hindlimb ischemia but provides only an indirect measure of angiogenesis. However, the multiple potential sources and the complexity of the collaterals, as well as the lack of sufficient radiographic spatial resolution limit this method's ability to accurately measure collateral blood flow. Therefore, angiographic data are valuable mainly as a correlate to other evaluations.

*Micro Computed Tomography (*microCT) triggers great enthusiasm in the cardiovascular field. Micro CT scan is a promising approach in 3D anatomic study of vasculature and namely of neovascularization development after ischemia (31). *Ex vivo* microCT has a high spatial resolution (<20 μm) with an interesting feature of 3D reconstruction of tomographic images. Vessel morphometric parameters such as volume, area, connectivity, thickness, and degree of anisotropy may be applied for quantification of blood vessels growth (32).

Magnetic resonance angiography (MRI) can also permit visualization of collaterals. Contrast-enhanced MRI, with first-pass gadolinium-based contrast agent, can be used to visualize arteries and to obtain regional and muscle-specific perfusion measures (33). Follow-up studies in living animal are possible. Although relatively effective in the limb, the resolution is insufficient to resolve the microvasculature (34).

Finally, *molecular imaging* of angiogenesis has recently received prominent attention, with a number of reports demonstrating the feasibility of the observation of blood vessel growth in tissues by targeting several "angiogenic" EC-specific antigens such as $\alpha v\beta 3$ integrin, VEGF receptors or a NGR receptor (35-36).

3.2.2. Imaging vessel functions

In addition to a direct visualization of the neovessel structures or components, assessment of vessel growth physiological consequences, such as tissue

perfusion, oxygenation, or function is essential (22). The assessment of capillary density does not always preclude assessments in perfusion changes in these models.

Collections of erythrocytes visible in histological sections may not be sufficient evidence of blood flow because they may be static extravascular blood lakes or sites of hemorrhage. Functional blood vessels can be identified by intravital labeling with a tracer that can reach vascular targets only through the circulation. For example, a fluorescent lectin or fluorescent dextran injected intravenously before tissue fixation labels functional vessels but not lumenless endothelial sprouts (37-38).

Blood flow measurement can be achieved with *laser Doppler perfusion imaging*, which uses a laser beam that sequentially scans tissue surface to a depth of a few hundred micrometers. This technique detects the frequency shift of moving blood and then transforms it into a color-coded image representing the microvascular blood flow distribution. Calculated perfusion is expressed as a ratio of ischemic to normal limb (23). Although Doppler flow measurements are useful to identify flow deficits relative to a non-occluded contra-lateral limb, they may be less useful in identifying subtle changes in flow.

Flow probes may be surgically and temporarily placed over a vessel to the ischemic limb, and allow flow changes in response to vasodilator administration (e.g., adenosine or papaverine) to be readily determined.

Another mean of assessing perfusion to the ischemic tissues is to measure the capacity for collateral-dependent blood flow. This could be achieved by measuring blood flow to the distal hindlimb with the use of *microspheres*. Indeed, regional blood flow is proportional to the number of microspheres trapped in the area of interest. However, tissue blood flow has to be determined with the following equation: sample flow (ml/min) / radioactivity (fluorescence) in reference sample = tissue flow / radioactivity (fluorescence) in organ, meaning that microsphere injections has to be monitored (25-39).

Contrast echocardiography (ultrasound techniques) allows for the noninvasive quantification of blood flow with a continuous intravenous infusion of echogenic gas-filled microbubbles. After destruction of the microbubbles by high-energy ultrasound pulses, the increase of the tissue signal intensity over time reflects the replenishment of the microbubbles in the tissue and is an estimate of blood flow (40-41).

Several approaches are available to measure blood flow and blood volume in a clinical setting but are not applicable to mouse models unless detectors are developed and image resolution is enhanced. In the case of myocardium angiogenesis study, *single-photon emission computed tomography* (SPECT) imaging could be used. However, demonstrating the benefits of angiogenic therapy by SPECT imaging is limited by its spatial resolution and lack of sensitivity (42). *Positron-emission tomography* (PET) and MRI are the main alternatives to SPECT imaging. Although MRI sensitivity is interesting, its use is limited to small animals due to poor spatial resolution. Micro-PET boasts somewhat higher spatial resolution, elimination of attenuation, and quantitative assessment of perfusion. Blood volume within tissue can be determined via labeled carbon monoxide inhalation experiments with ^{11}C or ^{15}O, positron emitters (43). When inhaled, labeled carbon monoxide irreversibly binds to red blood cells and distributes in accordance with vascular volume. The first pass of water labeled with ^{15}O can be used to calculate blood flow within tumors because water is completely diffusible and redistributes quickly (42-44).

Intravital microscopic imaging using fluorescence microscopy or multiphoton laser scanning microscopy could picture blood vessel function. This technique allows to assess blood flow, red blood cell velocity, blood vessel diameter, vascular density, endothelial permeability, leukocyte–endothelial cell interactions, intravascular and interstitial fluid pressures and interstitial diffusion (45). Changes in tissue pH and partial pressure of oxygen (pO_2) and effects of vasoactive agents can also be measured (46).

4. MOUSE MODELS TO INDUCE NEOVESSEL GROWTH

The major mouse *in vivo* assays are presented below, depending on the ischemic context, with a description of the assay, potential applications and limitations.

4.1. Model in an Ischemic context

4.1.1. Hindlimb ischemia

4.1.1.1. Original mouse model of hindlimb ischemia

The model originally developed in mouse was based on a previously developed rabbit model (23). Unilateral hindlimb ischemia was operatively induced in a mouse as follows: vessel exposure was obtained by performing an incision in the skin overlying the middle portion of the left hindlimb. The proximal end of the femoral artery and the distal portion of the saphenous artery were each ligated, and the arteries as well as all side-branches excised. This model, which permitted the use of the contralateral extremity as a control, was developed on C57BL/6 mice that fulfill the criteria of chronic impaired flow after this type of surgery. The outcome of same surgery was different in other mouse strains, e.g.Balb/c, C3H (47).

The original series of investigations characterized and documented the operative reduction and subsequent endogenous restoration of hindlimb blood flow in this animal model (demonstrated with laser-Doppler perfusion imaging). Neovascularization, as evidenced by increased EC proliferation and capillary density, was shown to develop in association with increased expression of VEGF mRNA and protein. This reparative angiogenesis is dependent upon VEGF and FGF up-regulation as confirmed by impaired neovascularization after administration of neutralizing antibodies (23)

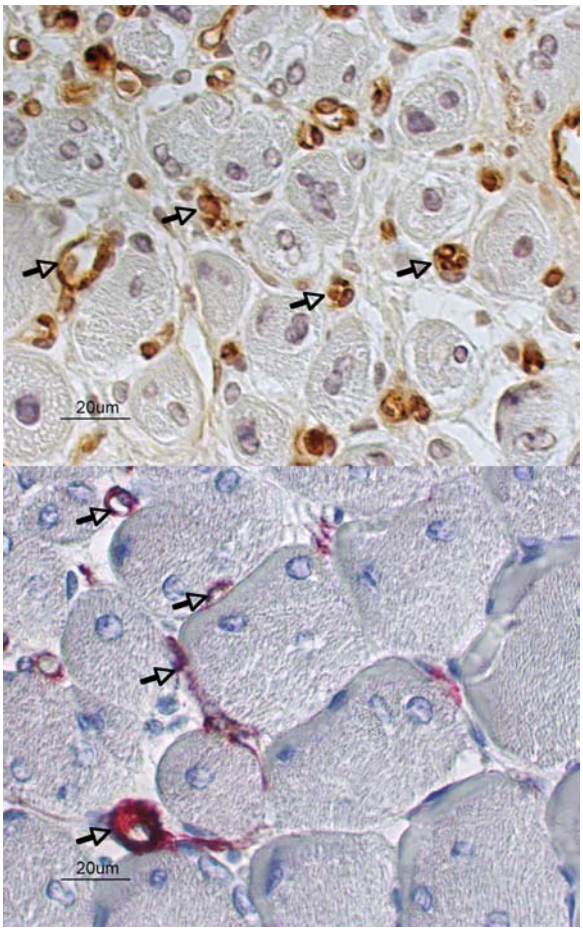

Figure 1. CD-31 and Smooth Muscle α-Actin immunostaining on ischemic muscle section of anterior tibialis at day 15 after the onset of surgery in the mouse hindlimb model. The arrows indicate CD-31-immunoreactive capillaries (stained in brown) and smooth muscle cells (stained in red).

4.1.1.2. Alternative models

The amount of side branches and degree of collateral vessels contribute to the degree of ischemia seen after ligation. Occlusions closer to the aorta, e.g., iliac artery, and occlusions performed with the ligation of potential collateral sources produce more profound effects than simple distal occlusions, e.g., common femoral. Ligations alone, however, often do not result in a significant or sustainable reduction in resting blood flow. Surgical ligation alone produces a light chronic ischemia at rest, but it does appear to produce a reduction in blood flow reserve with stress (24). This model results however in an increase in angiogenic growth factor production and capillary density. It may be more suitable to study the effect (s) of agents preventing angiogenic processes.

4.1.1.3. The mouse model as a preclinical model?

Patients with peripheral arterial occlusive disease present with two distinct clinical manifestations. Patients with intermittent claudication have normal or slightly diminished lower extremity blood flow at rest but exhibit an inability to adequately increase blood flow with exercise. Therefore, the arterial ligation model or the ligation with limited excision model, with an intact capacity to produce an angiogenic and/or arteriogenic response, will exhibit near normal flows at rest but impaired limb blood flow capacity with stress. It is thus well suited for the study of intermittent claudication. The ligation and excision method, especially if performed in the background of an impaired angiogenic and/or arteriogenic response, will result in reductions in blood flow to the ischemic limb, even at rest, and would therefore more closely mimic the clinical situation of patients with critical limb ischemia (48).

End-point for assessing changes in perfusion in the mouse hindlimb ischemic model

Calculation of a clinical score based on the number of necrotic toes is useful for the evaluation of treatment effect on recovery from ischemia. Capillary density remains the most commonly used histological measure of angiogenesis (Figure 1). Because muscle atrophy is a common phenomenon in the hindlimb ischemia models, determination of the number of "capillaries per muscle fiber" may be more accurate than the number of "capillaries per millimeter squared" (23-25). Analyses of muscle fiber type, fibrosis, inflammation degree, cell death and proliferation are easy to achieve (26).

pO_2 measurement could be asserted by using a pO_2 probe inserted in the ischemic muscle, by electron paramagnetic resonance (EPR), oximetry and use of fiber-optic probes (27-49)- the surgery typically results in oxygen saturations as low as 64% measured at day 0 post operation - or immunohistochemical techniques (Hydroxyprobe Kit, Chemicon International) to visualize gradients of hypoxia.

Lower extremity blood flow visualization can be achieved with laser Doppler perfusion imaging (Figure 2) or Contrast echocardiography or with the use of microspheres (39). Angiography, Micro CT (Figure 3), and MRI could be used to visualize collateral vessels by employing adapted contrast agents (22).

4.1.1.4. Limitations

Variations in hindlimb injury and blood flow recovery after femoral artery ligation have been documented from species-to-species and in genetic strains within the same species. Vessel location, degree of side branch and collateral vessel occlusion, and species variability all contribute to the degree of ischemia seen after ligation (19-47). Arteriogenesis predominates at the site of ligation, whereas angiogenesis predominates in the ischemic distal bed.

This model can be used to assess the potential utility of therapeutic modalities that might augment perfusion to the ischemic limb. The timing of the perfusion assessments is thus critical. Because all models are associated with some degree of perfusion recovery,

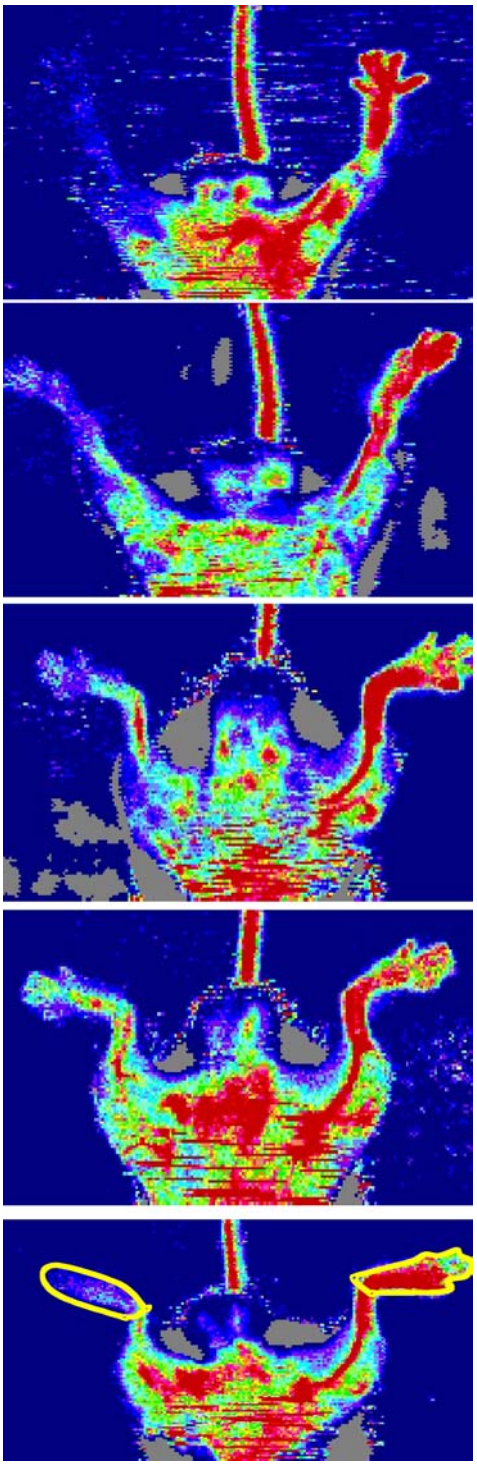

Figure 2. Kinetic of blood flow recovery (Day 0, 12, 18, 28) as analyzed by laser Doppler perfusion imager in the mouse ischemic hindlimb model. The left limb is ischemic and the color-coded image represents the microvascular blood flow distribution. The area where blood perfusion is calculated is delimited in yellow (bottom panel) and the results are expressed as a ratio of ischemic to normal limb blood flow.

interventions or agents tested at the time of surgery, or shortly after, will measure the capacity of an agent to improve the degree of recovery. The downside is that at later time points, perfusion in the ischemic limb will be more like perfusion in the nonischemic limb, and thus the ability to detect the "efficacy" of an intervention or agent may be less important (50).

4.1.2. Heart ischemia

Animal models of myocardial infarction are currently used as angiogenesis models suitable for pathophysiological studies and drug evaluation. The wound healing after myocardial infarction mimics the wound healing processes observed in other tissues, like the skin. Persistent ischemia is characterized by death of cardiomyocytes, followed by an early inflammatory response. Then, the cardiac wound healing process continues with the formation of granulation tissue, which increases the tensile strength of the infarct and prevents cardiac rupture. This granulation tissue is characterized by the presence of many blood vessels (51). Within a few days after infarction new blood vessels start to appear in the wound. This process of neovascularization is very efficient.

4.1.2.1. Permanent coronary occlusion or ischemia-reperfusion model.

Most techniques for producing myocardial infarction in the small rodents are based on coronary artery surgical occlusion. The surgical model of permanent coronary artery occlusion has been later characterized in terms of histological, anatomical and functional variables by various investigators (52-53). With a good consistency in the level of coronary occlusion, infarct size does not vary extensively (54). Surgical occlusion of coronary artery followed by reperfusion has been developed in mice to mimic the clinical setting and also to study angiogenesis in the context of the inflammatory processes associated to ischemia-reperfusion (55). Reperfusion of a previously ischemic myocardium triggers an inflammatory response that reflects cellular response to injury but may also contribute to additional damages and modify angiogenic responses (56).

4.1.2.2. Specific myocardial end-points

To study the progression of cardiac remodeling (cardiac wound healing and angiogenic response) non-invasive and invasive hemodynamic techniques are available today. Echocardiography is an adequate noninvasive tool to follow, over time in the same animal, changes in cavity volume and systolic function after infarct (57-58). Left ventricular catheterization using high fidelity micro tip pressure transducers is the gold standard for the assessment of cardiac function in rodents (54). These methods give an indirect clue of the angiogenic process and the quality of wound healing.

The gold standard for evaluation of cardiac dimensions remains post-mortem morphometric analysis. After measuring the major cavity axis, from the apex to the aortic valve, the left ventricle is cut in slices and the transverse chamber diameters and the free wall and septum thickness are determined with a stereo-microscope (52-53)

Figure 3. Example of high resolution microCT 3D volume rendering of the ischemic hindlimb vasculature obtained 15 days after the onset of surgery. The vasculature has been filled with latex and barium and extracted from the raw data at 36 mm resolution. Vascular tree has been overlapped by the skeleton system, which demonstrates the 3D relationship between these two structures.

Histological evaluation of myocardial composition provides useful insight on the reparative process occurring after myocardial infarction. It is recommendable that microvessel density be assessed in both the remote and the peri-infarct zone.

skin model of graded ischemia

4.1.3. skin model of graded ischemia

To study skin ischemia-induced neovascularization, a recent murine model has been proposed that results in a distinct gradient of soft tissue ischemia. The model consists of lateral skin incisions (2.5 cm in length and 1.25 cm apart) created on the dorsal surface of mice, penetrating the skin, dermis, and underlying adipose tissue. It creates a setting in which the blood supply to the incised tissue is dependent on flow from the 2 sides left intact. Positioning a silicone sheet below the surgically elevated skin prevents capillary growth from the muscle below. As a result, the most central portion of skin undergoes the most severe ischemic insult (59).

Oxygen probe (to measure O_2 tensions) and laser Doppler analysis have confirmed the ischemic gradient leading to the absence of flow and necrosis in the central portion of skin after the surgical onset. Over 14 days, blood flow is restored to the surviving portion of skin. In parallel to laser Doppler, this model offers the possibility of straightforward histological analysis. It has been used to study angiogenesis and mainly vasculogenesis and endothelial progenitor cell recruitment from bone marrow.

4.2. Model in non ischemic context

4.2.1. Plug assay - disc

4.2.1.1. Matrigel plug assay

Matrigel is a solubilized basement membrane preparation extracted from EHS mouse sarcoma, a tumor rich in extra-cellular matrix proteins. Its major components are laminin, followed by collagen IV, heparan sulfate proteoglycans, and entactin 1. Matrigel contains not only basement membrane components but also matrix degrading enzymes, their inhibitors and growth factors (i.e. epidermal growth factor, transforming growth factor beta, platelet derived growth factor, insulin-like growth factor-1, nerve growth factor, and bFGF). Matrigel containing different growth factors are commercially available. At 4°C, it takes the form of a liquid and at room temperature or when injected sub-cutaneously (s.c.) in mouse, Matrigel polymerizes in an active matrix material resembling the mammalian cellular basement membrane. Pure collagen I solubilized basement membrane can be used instead of Matrigel. It is liquid at 4°C and polymerizes when injected (60).

Mice are injected s.c. at the abdominal midline with 0.5 ml of Matrigel supplemented for example with 500 ng of bFGF and/or 10 µg of VEGF165 (61). Host ECs migrate into the plug and form vessel-like structures with fine networks of EC tubes, a process that appears to be specific to these cells and mimics the formation of capillary networks *in vivo* (62). Matrigel plugs are removed between 7 and 21 days postimplantation and prepared for histological examination (Figure 4 and 5). Immunohistochemistry analysis allows detailed angiogenesis characterization of the plug. Injecting mice with FITC-dextran solution could allow quantification of plugs vascularization. In this case, Matrigel plugs are removed, grounded and fluorescence of the supernatant is measured in a fluorometer (63). Quantification can also be achieved by measuring the amount of hemoglobin contained in the plug (17-64). However, the hemoglobin assay may be misleading since blood content is deeply affected by the size of vessels and by the extent of stagnant pools of blood.

In this model, inflammation plays an important role in the neovascularization (17). However the plug contains no other cells, but vascular cells and inflammatory cells, able to influence angiogenic reactions. It has some variability namely in reproducing the volume of the plug. However it is a quick, easy low-cost model to test potential pro- and anti-angiogenic compounds (65).

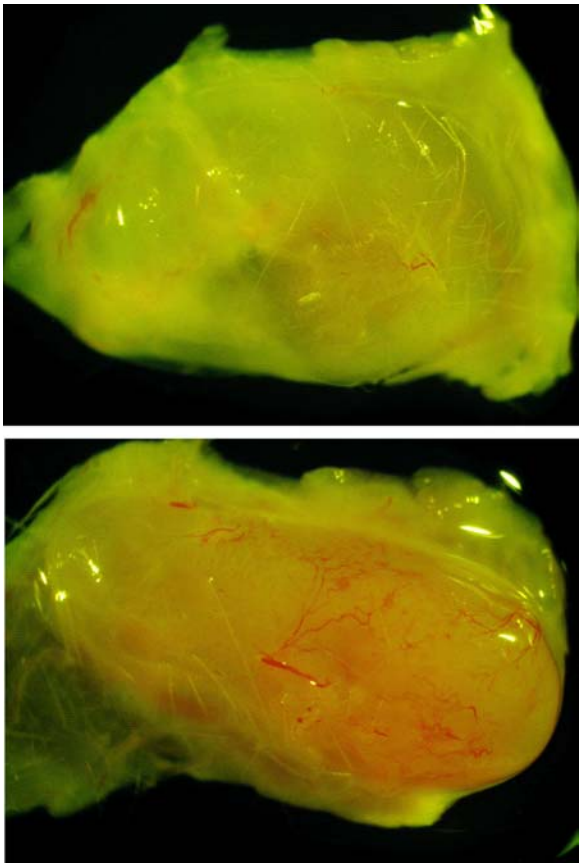

Figure 4. Matrigel plugs removed at 5 days postimplantation. Compared vascular and blood content of plugs without (top panel) and with VEGF (bottom panel).

4.2.1.2. Model of disc

The Disc Angiogenesis System (DAS) is a model to study microvascular proliferation which consists of a synthetic foam disc implanted subcutaneously in experimental animals (66). It is covered on both flat sides by filters, leaving only the edge as the area for cell penetration. Microvessels grow centripetally into the disc, together with fibroblasts. A test agent or the suspension of tumor cells to be studied is placed at the center of the disc. The DAS is easy to assemble and implant in abdomen and thorax of the mouse, which tolerates it well. After a period of growth, usually 7 to 21 days, the disc is removed. Planar sections are used to measure and characterize the growth. Multiple discs can be used for each time or dose point, which allows reproducible measurements of vascular growth. Using the DAS, well-established angiogenic agents placed at the center of the disc were found to increase proliferation of ECs and microvessels (67). More generally, the DAS provides a model for wound healing, either uncomplicated or complicated by inflammation, and of angiogenic responses to solid tumors (68).

4.2.2. Eye, retina, cornea

The eye provides a model system with several vascular beds surrounded by avascular tissue, which facilitates identification and quantification of neovascularization (69). The vascular beds can be visualized *in vivo* and the presence of neovascularization can be unequivocally identified and quantified because of the surrounding avascular tissue. In the eye, there are three vascular beds that have been intensively studied, the hyaloidal, retinal, and choroidal circulations.

4.2.2.1. Hyaloid vascular system

During mammalian normal ocular development, a transitory network of vessels, the hyaloid vascular system, is needed for the development of ocular structures especially the lens and the retina (69). Near the end of development, when other circulatory systems in the eye are completed, the hyaloidal vessels regress. This provides an opportunity to study molecular signals involved in vascular regression (70).

4.2.2.2. Retinal vascular model

In rodents, retinal vascular development occurs postnatally, in a stereotypical vascular pattern with a well-defined sequence of events facilitating the study of developmental or physiologic angiogenesis. On the day of birth (postnatal day 0: P0), there are no retinal vessels. In the next few days, until approximately P5, vascular sprouting at the periphery and remodeling at the center occur progressively and simultaneously. At P7, vessels cover the entire surface of the retina and sprouts from superficial vessels begin to grow into the retina to form the intermediate and deep capillary beds. After P10, all three vascular beds of the retina have been formed and remodeled, resulting in an adult retinal circulation (69-71-72).

This model allows the study of different aspects of vessel formation, maturation, and specialization as well as that of specialized endothelial tip cells that lead the outgrowth of blood-vessel sprouts in a single preparation (71-73-74). Retinas are ideally visualized using whole-mount immunostaining and in-situ hybridization techniques, coupled with high-resolution three-dimensional imaging by confocal laser scanning microscopy.

An interesting model to study vessel growth, the role of oxygen and growth factors in the retina is the oxygen induced retinopathy model that mimics the retinopathy of prematurity, a major cause of childhood blindness. Briefly, oxygen-induced retinopathy involves the exposure of neonatal mice to inspired hyperoxia, which induces attenuation of the normal postnatal retinal vascular development, followed by subsequent exposure to the relative hypoxia of room air, which induces retinal vascular proliferation.

4.2.2.3. Cornea model

The corneal model also allows assessing blood vessel growth *in vivo* (75-76). A micropocket assay has been developed where a pellet is placed on the corneal surface inside the pocket. The pellet contains growth factor and induces a reproducible angiogenic response in mice. The visibility, accessibility, and avascularity of the cornea are highly advantageous and facilitate the biomicroscopic grading of the neovascular response and the topical application of test drugs (77-78).

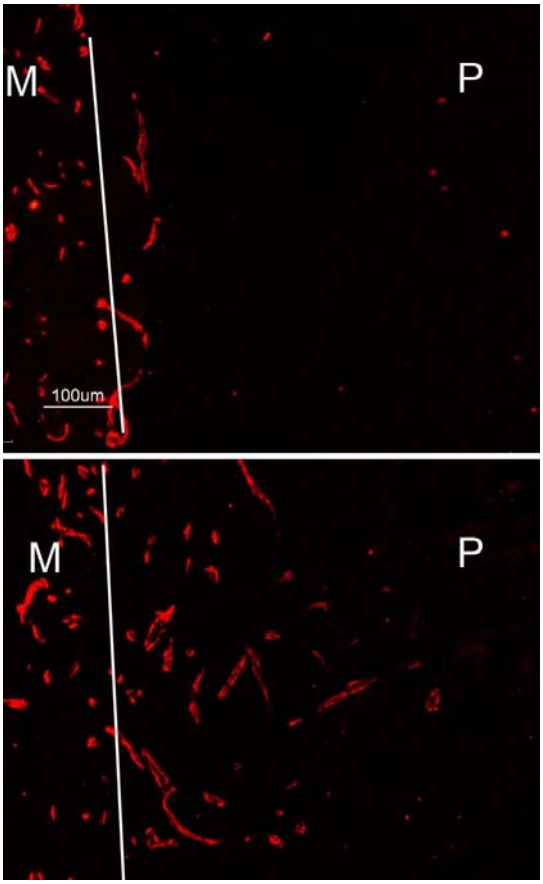

Figure 5. Matrigel plugs stained with anti CD-31 antibody and analyzed by fluorescent microscopy 5 days after implantation. Top panel: matrigel without VEGF. Bottom panel: matrigel supplemented with VEGF showing an increase in capillary density and penetration of the plug.

In this model, neovascular response depends on direct stimulation of blood vessels rather than on indirect stimulation by the induction of inflammation. This model shows the presence of only minimal inflammatory cellular activity (79). However, the corneal pocket itself is inaccessible to the many blood-borne factors and progenitor cells that can influence angiogenesis (17-64).

4.2.3. Skin Wound healing

Angiogenesis is a hallmark of wound healing. In skin wounds, the proliferative phase is characterized by epidermal regeneration, angiogenesis, and fibroblast proliferation as the fibrin clot and debris filling the wound site are replaced by granulation tissue. The granulation tissue is very delicate and bleeds easily because it contains a large number of new vessels but little stabilizing connective tissue. During the development of granulation tissue, capillary sprouts invade the wound clot from adjacent vessels and organize into a microvascular network. At the same time, fibroblasts proliferate within the granulation tissue and begin to lay down collagen and other connective tissue components to reinforce the tensile strength of the repaired tissue. Not all of the initial capillary sprouts ultimately develop into functional vessels. As the granulation tissue matures, the overall cellularity of the tissue and the density of blood vessels decrease (80-82).

Modeling skin wound healing has been approached by creating wound with a punch on the back of mice. It is a full-thickness wound (approximately 8-mm diameter) created by excising the skin and the underlying panniculus carnosus (83). At selected time points after wounding, either diameters of the wound are measured with a caliper or wound closure is recorded with a digital camera. For histological analyses the wound area is excised. Vascularity is analyzed as described in the first section. Wound closure can be compared using different growth factors applied to methylcellulose pellet (84-85).

4.2.4. Ear model

The ear model is a nontraumatic model in the mouse pinnae that allows daily macroscopic examination of vascular tree morphogenesis in a living animal, in response to angiogenic stimuli. A sponge loaded with angiogenic factors is inserted near the margin of the ear, in between and beneath existing vessels. Angiogenesis at the implant site is monitored by photographying the ear under transillumination using a stereomicroscope. Ears are resected for histological examination (86).

4.2.5. Ovarian model

Physiological angiogenesis follows a controlled program that includes a period of vessel maturation and tissue remodeling, then, eventually, a regression of the newly-formed blood vessels during scar formation. The ovary thus represents an exceptional tissue in which one can study the regulation of blood vessel growth and regression (87).

In the ovary, angiogenesis takes place during follicular development and with formation of the corpus luteum (CL). Sprouting ECs invade the growing CL and continue to grow throughout the first third of the ovarian cycle. Thereafter a dense network of vessels characterizes the mature CL. During luteolysis all newly formed vessels regress as most of the developing follicles become atretic (88). These sequences can be carefully monitored in mice with an hyperstimulation of the ovaries induced by injecting 10 UI of pregnant mare's serum gonadotropin, followed 44 hours later by 10 UI human chorionic gonadotropin (60). Ovaries are then collected and studied by histologic methods, *in situ* hybridization or RNA or protein analysis (Figure 6).

5. CARDIOVASCULAR RISK FACTOR ALTERED ANIMALS

Cardiovascular diseases dependent on the atheroclerotic process are the leading cause of death in the modern world. It is a multifactorial and polygenetic disease with two major underlying causes that are disorders of lipid metabolism and metabolic syndrome. Neovessel formation is largely altered in such an environment, representing a limitation to the treatment of patients presenting these disorders (14).

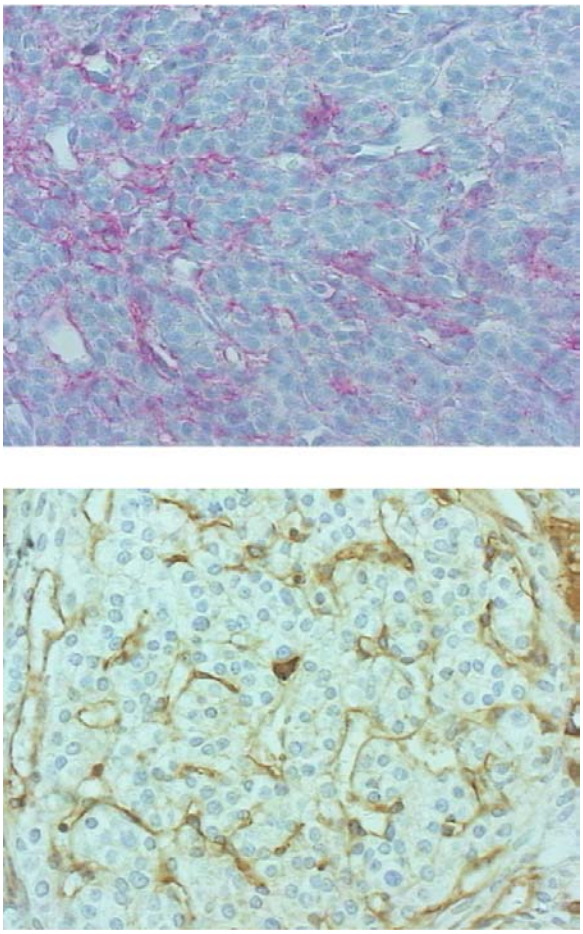

Figure 6. Dense network of vessels characterizes the mature corpus luteum in the ovarian model. In mice, an hyperstimulation of the ovaries is induced by injecting pregnant mare's serum gonadotropine, followed 44 hours later by chorionic gonadotropin. Ovaries are then collected and immunostained with CD-31 to visualize capillaries (brown) and α-actin to visualize smooth muscle cells (red).

Analyzing mechanisms of vessel growth impairment in the presence of metabolic abnormalities prompted the development of animal models that mimic human disease processes. For example, diabetes leads to delayed skin wound healing caused primarily by microangiopathy accompanied by impaired cutaneous blood flow, hypoxia, accelerated inflammation, edema, endothelial-neural dysfunction, and reduction of VEGF-A, Tie2, and the angiopoietin-1 receptor expression and impairment in progenitor cell mobilization and functions (84-89-92). Hypercholesterolemia leads to impaired angiogenesis by affecting growth factor production (27-93).

A number of genetic mouse models based on either spontaneous mutation or genetic manipulation have been developed and used to analyze the influence of metabolic factors on vessel growth and vessel structure (94-95).

5.1. Mouse model of hypercholesterolemia

The most widely used mouse model is the apoE-/- knockout strain. These mice present total plasma cholesterol concentrations of 11 mM, compared to 2 mM for the parent C57BL/6 mouse, and exhibit advanced intimal lesions that are largely confined to the aortic root and aortic arch regions. A high-cholesterol diet results in plasma cholesterol concentrations around 30 mM. Overall, the apoE -/- model can be viewed as an analogue of untreated severely dyslipidemic humans (96).

The LDL Receptor -/- mouse is a model of the human familial hypercholesterolemia due to one of the mutations affecting the LDL Receptor. These mice have only mildly elevated cholesterol levels on a chow diet (5.8 vs. 3.1 mM for homozygotes and wild type, respectively). When fed with a 1.25% cholesterol diet, homozygotes have extremely high plasma cholesterol concentrations in the 41-mM range. These mice exhibit xanthomas and extensive atherosclerotic lesions in the aorta and in other vessels (97).

Mice deficient in the HDL scavenger receptor Class B Type 1 (SR-BI KO mouse) are particularly sensitive to a high-fat/high-cholesterol diet and have total cholesterol concentrations in the range of 40 mM, developing extensive coronary artery disease (98).

5.2. Mouse model of diabetes

Diabetes may be induced by streptozotocin injections that impair pancreatic function and mimic type I diabetes. db/db and ob/ob mice have been studied as a model for metabolic syndrome and type 2 diabetes (94). Mice that are homozygous for the db mutation (mutation of the leptin ObR system) (db/db) displayed obesity, insulin resistance, hyperinsulinemia, and hypertriglyceridemia (99). Numerous studies using the db/db mouse have shown impaired endothelial and vascular function, abnormal cardiac metabolism and function, retinal damage, and glomerular sclerosis consistent with type 2 diabetes (100).

Mice that are ob/ob, i.e. structurally defective leptin that does not bind to the ObR, have no leptin activity and are obese, insulin-resistant, hypertriglyceridemic, but normoglycemic (101). They have cardiac functional defects and vascular dysfunction (94). There are no reports of atherosclerosis or ischemic lesions in these mice.

6. GENETICALLY-ENGINEERED ANIMALS

Gene function can be studied *in vivo* by inducing specific overexpression of a candidate gene, or, on the opposite, by suppressing its expression. This is achieved by genetic manipulations. Over the past two decades, microinjection of fertilized eggs with DNA constructs has become the standard technology for generating transgenic animals. This undoubtedly major technical advance has become widely available and has been used to study gene function and create models for the study of human diseases (102).

6.1. Controlling the spatial and temporal vascular gene

expression

Tissue- or cell-specific promoters directly controlling the expression of target genes in the vascular cells in transgenic animals have been shown to be very useful for investigating developmental and pathophysiological gene function in vasculature. Promoter/enhancer sequences from endothelium-restricted genes have been tested to drive the expression of transgenes via gene transfer vectors. Promoter sequences derived from Flt-1 (vascular endothelial growth factor receptor-1 (VEGFR-1)), the intracellular cell adhesion molecule (ICAM-2), von Willebrand factor (vWF), or endoglin have been reported to be functional in ECs *in vitro* and *in vivo* with various degree of specificity and intensity (103).

The endothelial specificity of many of these promoters has also been confirmed in genetic mouse models. Selective endothelial specific transgene expression is seen in transgenic mice expressing lacZ under the control of minimal promoters derived from Tie 2 (angiopoietin receptor), vWF, Flt-1, thrombomodulin, E-selectin, and ICAM-2 or VE-Cadherin.

Achieving SMC-specific expression is difficult since most SMCs markers are mainly expressed in differentiated states. Because growth and differentiation of SMC are generally exclusive processes, it is difficult to express consistently a transgene in SMCs. However, transgenic mice were obtained using selective promoters of smooth muscle myosin heavy Chain (SM MHC), smooth muscle α-actin, and SM22α.

Transgenic models using vascular cell-specific promoters have provided more detailed information about the function of specific genes in this particular tissue, overcoming limitations of wild type transgene expression (102). However, the specific gene expression is dependent on the properties of the promoters used, i.e. promoter constitutively active, with a variable strength of expression, or with expression starting in embryonic or in the adulthood stage. This could lead to non-viable transgenic animals in case of gene toxicity, limiting studies in adult pathological model, or leading to compensatory responses. More powerful tools are being developed based on conditional transgene expression systems.

6.2. Controlling inhibition of vascular endogenous gene expression

Homologous recombination is an excellent way to suppress the expression of a gene in a whole animal. The method implies that the targeted gene is knocked out at the early stage of embryo development. This does not reflect the subtle regulation to which genes are normally submitted. Conditional recombination may be triggered at a given period of its life and in a given cell type of the animal (104). This implies that two LoxP sequences are initially added to both ends of the targeted gene. LoxP sequences can recombine under the action of the Cre recombinase eliminating the genomic sequence located between the two LoxP, thus knocking out the gene of interest. The presence of Cre recombinase induces the LoxP recombination. Mice harboring a stable transgene containing the Cre recombinase gene under the control of a tissue-specific promoter may be crossed with other transgenic mice in which targeted introduction of LoxP sequences has been performed. The inactivation of the gene induced by LoxP recombination will occur specifically in the tissue in which the promoter is active (105-106).

Another level of control can be added to the system. The Cre recombinase activity could be induced by the administration of a given inducer (tamoxifen or RU 486). These systems offer an additional control for LoxP recombination and gene knock out.

Numerous experiments have shown that in mammal embryos, double strand RNAs strongly induce a specific degradation of the mRNA having the same RNA sequence. This phenomenon is called RNA interference (RNAi). The possibility to use the RNAi phenomenon to knock down genes in a simplified manner is now routinely used. Recent publications indicated that vectors expressing short double strand RNA under the action of vascular-specific promoters can specifically suppress gene expression through an RNAi effect.

6.3. Conditional expression of the transgene

The expression of the transgene can be initiated or terminated rapidly and reversibly by a simple external inducer (107). Together with a specific promoter, the inducible system should provide temporal and spatial control over the expression of the transgene (108). The transgene should have no basal level expression in the "Off" state, whereas high level expression should be achieved when the gene is "On," giving a wide range of induction (109).

This system relies on the use of at least two genes. One, which may be under the control of a tissue-specific promoter, encodes a transcription factor that can specifically activate the gene of interest under the action of a chemical inducer. The Tet-based systems have proved efficient. The original tetracycline-controlled transcriptional activator (tTA), which may be under the control of a tissue-specific promoter, is a regulator with tight control of the gene of interest. The reverse tetracycline-controlled transcriptional activator (rtTA) activates the responsive elements only in the presence of doxycycline, allowing a efficient control over the target transgene (110).

7. CONCLUSIONS

Angiogenic or anti-angiogenic properties of any protein or therapeutic factor are initially evaluated by *in vitro* tests that provide initial information. Then, they have to be confirmed by *in vivo* assays. There are numerous valuable mouse models to evaluate angiogenic properties and mechanisms. Sometimes, *in vivo* assays are both time-consuming and difficult to perform, thereby limiting their development. Quantification is generally more difficult as well. However, *in vivo* assessment is essential because of

the complex nature of angiogenic mechanisms.

It is clear that no single mouse angiogenic model is able to elucidate the entire progress of angiogenesis. Some models would be suitable to study particularly angiogenesis, arteriogenesis or vasculogenesis processes and it could be helpful to combine different models. Moreover, there are differences between mouse strains, specific microenvironments, tissues tested, and the delivery route of tested substance. However, technological progress offers more and more tools every day to thoroughly quantify newly formed microvessels in all the different aspects and patterns using non-invasive techniques. This added to the development of animal models that mimic human disease processes (metabolic abnormalities) and genetically-engineered animals render mouse models more and more attractive.

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