Consensus guidelines for the use and interpretation of angiogenesis assays

Consensus guidelines for the use and interpretation of assays for the assessment and analysis of angiogenesis

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Abstract

Angiogenesis is a complex process playing an important role in growth and development, as well as in a large array of different pathologies. Differences exist in the regulation of angiogenesis in different tissues and in different distinct diseases. For the study of dissected aspects of angiogenesis, a plethora of specific angiogenesis bioassays have been developed and are readily available. The study of a separated aspect of angiogenesis in a specific bioassay has many advantages but it should be realized that such approach can also have certain limitations. The current paper aims to present the various in vivo, ex vivo and in vitro bioassays for the assessment of angiogenesis and the most optimal way they should be executed. As such, this collaborative work is the first edition of a set of consensus guidelines on diverse angiogenesis bioassays, to serve for current and future reference.

Introduction

The process of angiogenesis – the formation of new blood vessels from pre-existing ones – is a hallmark of tissue expansion and remodeling in both physiological processes, such as wound healing, inflammation, ovulation and embryo development, and in various pathologies including cancer, atherosclerosis and arthritis [1-4]. Many of these conditions share similar characteristics, for example the occurrence of hypoxia, angiogenic growth factor production, basement membrane degradation, endothelial cell (EC) migration, proliferation and differentiation, and modulation of vascular support cells. However, dependent on the tissue or

disease under investigation, important details may differ considerably. Moreover, ECs in different vascular beds exhibit heterogeneity associated with the differentiated specialized functions of the tissue. For this reason it is often not possible to directly visualize the process and its molecular players in vivo. Therefore, different in vivo, ex vivo and in vitro bioassays have been developed to investigate the specific stages of the angiogenic process. However, making use of specific bioassays to study a part of the process, in order to extrapolate and understand the full process of angiogenesis inherently means doing concessions. It is therefore of extreme importance to understand the full potential of these bioassays. These assays have been instrumental in the study of vascular biology in growth and development [5-7] but also play a key role in the design and development of drugs that modulate for the treatment of many diseases [8-10]. Major examples of where the use of such bioassays are imperative are (i) the development of angiostatic drugs for the treatment of cancer and other angiogenic diseases [11,12], (ii) screening of natural compounds [13], (iii) the efforts to design combination therapies including angiogenesis inhibitors [14-19], (iv) the identification of the crucial role of lymphangiogenesis [20,21], (v) the interrelationship of angiogenesis and immunity [22-24], (vi) the development of imaging as diagnostic strategies [25], (vii) the study of resistance induction [26-28], (viii) development of compounds and strategies for the re-vascularization of ischemic injuries , and (ix) to improve the vascular fitness in aging vessels. The current paper deals with the most important angiogenesis assays and aims at explaining advantages and limitations of these assays. The final goal of this paper not only confines the assessment of angiogenesis through such bioassays but also includes strategies to study angiogenesis in tissues, through means of assessing and quantifying microvessel density, vessel co-option, pericyte coverage and tip cell behavior.

1. Endothelial cell migration assays

EC migration is one of the hallmarks of angiogenesis and identifies an early step in the angiogenic cascade. Directed migration and invasion are key regulatory processes in sprouting angiogenesis. This process is characterized by cell-autonomous motility property but in some cases it acquires the features of collective migration , in which a group of cells coordinate their movements toward a chemotactic gradient and by establishing a precise hierarchy with leader and follower cells. Therefore, dissection of the molecular mechanisms of EC migration is key to the understanding of angiogenesis and to therapeutically manipulate this process, be it with the objective to inhibit sprouting angiogenesis, e.g. in tumors, or to stimulate angiogenesis (e.g. in tissue regeneration or wound healing). A number of 2-dimensional (2D) and 3D cellular migration assays has been established as relatively simple *in vitro* read-outs of the migratory/angiogenic activity of EC in response to exogenous stimuli. Depending on the specific scientific question, different assays are available to quantitatively and qualitatively assess EC migration. The most widely employed cellular assays include different variations of the wound closure and the Boyden chamber assays.

1.1. Types of assays

Cell culture wound closure assay. Lateral migration assays are performed to investigate the pro- or anti-migratory effect of compounds. Applying such assays, it is possible to determine chemokinesis (undirected migration) in response to certain compounds within the cell culture medium. However, it is not possible to determine the directed migration rate towards or away from a compound. This process is called chemotaxis and can be determined in assays supplying a gradient of the migration-inducing compound.

The cell culture wound closure assay is one of the simplest read-outs for the migratory activity of cells. It is a measure for the lateral 2D migration of EC in cell culture to test pro-migratory or anti-migratory compounds. Depending on the migratory effect of the tested substances, the assay is performed over 2 to 4 days. EC are grown to confluency in a cell culture dish and then scraped with a razor blade/pipette tip [29], allowing the EC at the wound edge to migrate into the scraped area. To really examine the motility contribution to the healing

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and to exclude the component related to cell proliferation, ECs are incubated with the anti-mitotic agent mitomycin . One downside of this method is that by scratching the cell layer the width of the scratch is difficult to control and therefore cannot be easily standardized. Moreover, wounding of the monolayer with a sharp object may scratch the surface of the cell culture dish and additionally damage the EC at the migration front. To obtain more reliable and reproducible results, fencing techniques have been developed that allow the controlled release of a confluent monolayer into lateral migration without wounding of the cells or the underlying matrix [30]. Cells are grown as a monolayer in a culture dish containing a silicon template of defined size prior to seeding the cells. The silicon template is removed once the cells reach confluency allowing them to migrate laterally into the area previously occupied by the silicon template. This allows a simple and precise microscopic analysis of lateral migration over 2 to 4 days.

Wound healing assay connected with video-lapse microscopy allows studying in 2D dimension the role of collective migration in angiogenesis and vascular development. The use of aortic rings (see below) and that of specific microfluidic devices represent a further tool to describe this process in a 3D architecture. For instance wound healing assay exploited by single cell analysis and by using chimeric EC sheets obtained by infecting cells with different fluorescent proteins was instrumental to describe the following steps of EC collective migration: (i) in resting state ECs undergo random cell motility in the monolayer with a regulated dynamics of homotypic cell junctions; ii) the presence of cell-free space (i.e. the wound) and a chemotactic gradient result in the appearance at the sheet margin of leader cells, which are characterized by an aggressive phenotype with prominent stress fibers, ruffling lamellipodia and enlarged focal adhesions, formation of peripheral actin cables and discontinuous adherens junctions, which indicate mechanical coupling between leader and follower cells in the migrating cluster ; iii) as leaders start to migrate in the free space, a follower phenotype appears within cells of the monolayer.

Transwell cell migration assay – Boyden chamber assay. The Boyden chamber assay is a useful tool to study directed cell migration (chemotaxis) and cell invasion. It was originally introduced by Steven Boyden in the 1960's for the analysis of leukocyte chemotaxis [31]. Today, a large number of different Boyden chamber devices (depending on individual needs) are commercially available. In this assay, one can distinguish positive chemotaxis (migration towards the attractant) and negative chemotaxis (migration away from a repellent). The assay is based on a chamber of two medium-filled compartments separated by a microporous membrane of defined pore size. In order to study cell migration, EC are placed in the upper compartment and are allowed to migrate through the pores of the membrane into the lower compartment. The chemotactic agent of interest or cells secreting chemotactic agents are present in the lower compartment. The membrane between the fluid-filled compartments is harvested, fixed and stained after a defined incubation time and the number of migrated cells on the bottom side of the membrane is determined by staining and subsequent microscopic analysis. A chemical gradient cannot be maintained for a long time. Boyden chamber assays are therefore usually limited to 2 to 6 hours. Boyden chamber assays are used to measure different types of chemotaxis, including haptotaxis, transmigration and cell invasion. Angiogenesis and transendothelial migration are special forms of haptotaxis, since the trigger for migration is not a chemokine but the presence of cell surface molecules. In this case, the insert of the Boyden chamber is coated with extracellular matrix proteins (collagens, fibronectin) on the bottom. The cells migrate along cellular adhesion sites. Transmigration describes the migration of cells, such as leukocytes or tumor cells through the vascular endothelium towards a chemoattractant. Therefore, this assay is not a measure of EC migration but for the transmigration of cells through the EC layer. Angiogenesis requires the invasion of EC through the basement membrane to form sprouting capillaries. The invasion process involves the secretion of matrix metalloproteases to degrade the basement membrane, the activation of EC and the migration across the basement membrane. This process can also be modeled in a Boyden chamber assay.

The bioactive molecules in Boyden chamber assays can be provided as recombinant molecules or by cells in the bottom chamber secreting specific factors. Manipulation of test cells (gain-of-function, loss-of-function) can

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be introduced into the assay. Migration assays are often performed in combination with tumor cells, pericytes or fibroblasts

The real-time random migration assay

The use of video-lapse microscopy allows measuring migration not only as an end-point result, but gives information on single cell parameters , on morphological changes and on the influence exerted by specific substratum . Subconfluent ECs are plated on plastic surface coated with specific extracellular matrix proteins (i.e. collagens, fibronectin, vitronectin) allowed to adhere, and then observed with an inverted microscope equipped with thermostatic and CO2 controlled chamber (e.g. Leica, DMi8 platform; Nikon, TE micorsope) Images of motile ECs are captured with a 5 min time interval over 4 h. Images were then processed with DIAS software (Solltech). A recent review on tracking algorithms offers a wide and comprehensive selection of the available tools to analyze cell motility. Generally data are displayed as a centroid plot showing the location of the geometrical centre of the cell as a function of time. Directional persistence was calculated by determining the ratio between the net path length and the total path length. Furthermore other parameters such as the total and net distance, the speed, the feature of turning angle can be calculated. Single cell trajectories were plotted using Matlab software and displayed in windrose graphs.

1.2 Limitations and challenges.

Standardization of techniques is one of the most critical issues to ensure the reproducibility of experimental results and one has to be aware that cellular *in vitro* systems represent only a surrogate of the *in vivo* situation. However, compared to *in vivo* experiments, *in vitro* assays are relatively simple to perform and they offer the possibility to pursue large scale screens of compounds affecting EC migration, e.g. supernatants of tumor cells. However, one has to consider the limitations of cellular assays. All the cell culture conditions have to be taken into consideration, as well as possible pitfalls of the assay itself. Pure populations of EC are required for migration assays. Human umbilical vein EC (HUVEC) are widely used for this analysis. However, they derive from a large vessel, whereas angiogenesis is driven by microvessels. HUVEC are primary cells and are only viable for a limited time. Moreover, one has to consider that cells in a culture dish change their expression profile and therefore their phenotype and behavior over time and passage. Moreover, reproducibility of scratch assays relies strongly on the initial degree of confluency [32]. Another important consideration is that EC *in vivo* are exposed to flow and sheer stress, which is missing in static cell culture models. The scratch assay is a straightforward cell culture assay to analyze EC chemokinesis. It does not have a high degree of sensitivity, but it is a useful tool to perform large scale screening experiments. As mentioned above, a potential drawback is the difficulty to standardize the wound areas. This could be solved as described above using silicon templates [30]. Likewise, several commercial suppliers have developed robust assays that also circumvent this problem. For example, IncuCyte ZOOM™ assays use a mechanical tool, called a WoundMaker™, to create 96 identically-sized scratches in each well. The WoundMaker™ is a 96-pin mechanical device designed to create homogeneous, 700-800 µm wide scratch wounds in cell monolayers on 96-well microplates. The device creates wounds without damaging the cells or the underlying plastic or biomatrix. Every scratch has the same

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dimension and is created with the same amount of pressure (www.essenbioscience.com/en/products/incucyte). The cells are traced fully automated. Another example of such device is provided by Peira Scientific Instruments (Beerse, Belgium) together with analysis software and automated hardware [33].

The Boyden chamber assay is somewhat more delicate and requires more experience in handling. The most critical issue is the possible trapping of air bubbles in the lower and upper chambers during assembly. Air bubbles, which are trapped beneath a cell, will cause empty spaces on the filter since the cell is hindered to migrate by the bubble. For manual analysis, this may not of major importance, but it becomes relevant if an automated analysis is performed since a trapped cell is not distinguishable from a non-migrated one. It is definitely worth to invest some substantial time into the setup and troubleshooting of the assay in order to yield robust and reliable results. Therefore, it is recommended to include a checkerboard analysis in order to solidly distinguish between chemotaxis and chemokinesis effects. To this end, different dilutions of the substance are titrated in the upper and lower chamber. Equal concentrations in the upper and lower chamber should lead to the same migration behavior as in the control for a compound that strictly responds to a gradient, i.e. chemotaxis).

1.3. Concluding remarks. In summary, the lateral scratch wound assays [34] and the Boyden chamber assay are both robust and reliable systems to study EC migration. They are suitable for scale-up purposes in order to perform manual or automated large-scale compound screens. Multiple vendors provide multiple scratch and transwell assay systems. These systems offer good reproducibility and adequate throughput capacity.

2. Endothelial cell proliferation assays

Since the proliferation of ECs is a hallmark of angiogenesis, the measurement of this parameter has been broadly applied. Many regulators of angiogenesis have been identified, validated and developed based on screening for an effect on endothelial cell proliferation. ECs are among the most quiescent cells in the body, with proliferation rates approaching 0 under steady-state conditions. Only after activation, usually as a consequence of injury, inflammation or pathological processes such as malignant growth, they start to proliferate [35,36]. The ideal assay to measure EC proliferation would be rapid, reproducible, reliable and translatable and where possible should exclude inter-operator variability, e.g. through quantitative computational readout rather than qualitative researcher-dependent observations [36]. This section presents different methods and will elaborate on problems and pitfalls.

2.1. Types of proliferation assays. A number of different approaches to address cell proliferation have been developed in the last decades. In general, these include the monitoring of cell number, the detection of DNA synthesis by incorporation of labeled nucleotide analogs, measurement of DNA content, detection of proliferation markers and metabolic assays (**Fig. 1**). Depending on the broadness of the definition of cell proliferation, which can range from the narrow description “the fraction of cells dividing over time” to the more general “the doubling time of a population over time”, different assays may be pursued. Next to that, means and equipment available will also dictate the choice for a particular method. As all methods focus on a particular aspect of the process, it is highly recommended to verify results with a complementary assay.

Cell counting. Cell counting can be considered the gold standard for proliferation. Moreover, at least in theory, it is one of the most straightforward procedures of measuring proliferation of a cell population. It can be done using automated cell counters (e.g. Coulter) or by using a hemocytometer after removal of the cells from the culture vessel [36,37].

More recently, different automated platforms have entered the market that allow analysis of cells while present in microplates, such as plate cytometers, automated microscope or high-content screening platforms, that are compatible with cell counting-like procedures. With these, cells can be monitored over time but frequently require staining for detection and (computation-assisted) quantification, by e.g. staining of nuclei. More recently, real-time cell analysis (referred to as RTCA) platforms have emerged, that allow label-free, automated, real-time monitoring of cellular properties during incubation based on electrical resistance measurements. As such equipment requires considerable investment, it will be beyond reach for many laboratories.

DNA labeling. During S-phase of the cell cycle, DNA is synthesized and subsequently divided between the daughter cells (2N→4N→2N). Addition of modified nucleotides to the culture medium will result in incorporation of these into the newly synthesized DNA. Adhering to the narrow definition of proliferation as stated above, this type of assay most closely reflects a means of measuring the fraction of actively dividing cells. It should be realized that this technique does not directly measure cell division or population doublings, but exclusively only incorporation into DNA.

The use of ^{3}H-thymidine, has a long track record [35,37,38,18]. Briefly, cells are pulsed with ^{3}H-thymidine for several hours and radioactivity is measured by liquid scintillation counting. It provides a very accurate representation of actively proliferating cells and is highly sensitive since the amount of incorporated ^{3}H-thymidine is directly proportional to the rate of DNA synthesis [36,37]. Constraints on using radioactive compounds and the rise of alternative methods have limited its use somewhat nowadays. In a similar approach, the incorporation of 5-bromo-2'-deoxyuridine (BrdU) or EdU (5-ethynyl-2'deoxyuridine) can be measured. BrdU or EdU can be (in)directly detected and subsequently be (semi-)quantified using ELISA, flow cytometry or immunohistochemistry [36,37,39], the latter two quantification techniques allowing to determine the fraction of dividing cells. These uridine analogs can be combined with DNA dyes (see below) to gain additional cell cycle information [2].

A more static approach is the measurement of cellular DNA content using intercalating dyes such as PI (propidium iodide) or DAPI (4',6-diamidino-2-phenylindole). Using flow- or plate cytometry, a profile of the distribution of cells over the different phases of the cell cycle can be visualized, represented by DNA contents of 1N (G1/0), 2N (G2/M), or mixed (S). In addition, this method allows for the detection of apoptotic cells that would exhibit a subG1/0 (<1N) DNA content.

An alternative method to study EC cycle is based on the use Fucci (fluorescent, ubiquitination-based cell cyle indicator) technology (Termofisher). It consists of a fluorescent protein-based system that employs both a red and a green fluorescent protein respectively fused to cdt1 and geminin, which are two regulators of cell cycle. These two proteins are ubiquitinated by specific ubiquitin E3 ligases in a specific temporal sequence. In the G1 phase, geminin is degraded; therefore, only cdt1 is present and appears as red fluorescence within the nuclei. In the S, G2, and M phases, cdt1 is degraded and only geminin remains, resulting in cells with green fluorescent nuclei. During the G1/S transition, when cdt1 levels are decreasing and geminin levels increasing, both proteins are present, giving a nuclear yellow fluorescence. More recently, Fucci probe was re-engineered to generate a triple color-distinct separation of G1,S and G2 phases extending the use of this technology to quantitative analyze the interphase of cell cycle.

Proliferation markers. Cell division is a highly coordinated process where specific proteins show a concerted action to allow mitosis to complete. Detection of these proteins, usually through immunochemical procedures, allows the estimation of the fraction of dividing cells. This approach can be used in *in vitro* end-point assays, but can additionally be used to evaluate active EC proliferation in tissue sections (see separate section).

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Metabolic assays. Gradually, the use of cell viability assays has taken a rather dominant position in addressing cell proliferation. While not reflecting this property in its narrowest sense, if properly conducted they accurately represent the number of live cells in an assay system. They are readily available and require minimal handling and infrastructure. The most well-known is the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), in which this yellow salt is taken up by metabolically active cells and converted by mitochondrial dehydrogenase to insoluble purple formazan crystals that cannot leave the cell. As the amount of the converting enzyme is highly stable in a given cell population, the formation of formazan (and hence color intensity) is proportional to the number of viable cells. This is subsequently quantified by solubilization of the crystal containing cells and spectrophotometry. Variations to this method, e.g. involving less toxic reagents, simplified reaction steps or alternative readouts such as cellular ATP levels, have also been widely used [37,33,40].

2.2. Limitations and challenges.

Endothelial cell culture considerations. Studying EC proliferation *in vitro* requires a purified population of ECs compatible with the assay setup. HUVEC are a widely available source, but are of macrovascular origin, whereas angiogenesis *in vivo* takes place at the microvascular level. Therefore, other sources of ECs are necessary for confirmation of results. Foreskin-derived human dermal microvascular EC (HDMEC) are therefore a good alternative.

Like with all primary isolates, cells have a limited life span *in vitro*. Moreover, their mere propagation in culture induces phenotypic changes [36,37]. As such, it is recommended to only use populations of cells that underwent limited population doublings. Though immortalized EC can pose a helpful alternative, it should be recognized that the immortalization itself may or will likely alter growth control and survival mechanisms in these cells [41]. As such, care must be taken to address the generalizability of assay outcome.

In all cases, cell density needs to be carefully controlled. Assay linearity can be compromised when cells are plated too dense (e.g. 50,000 cells/cm^2) or too sparse (e.g. 5,000 cells/cm^2). Loss of cell-cell contact is a potent stimulus for EC to proliferate, whereas EC enter a quiescent state upon confluency, known as contact-inhibition [36,37,42]. Optimization of the dynamic performance can involve synchronization of cells by exposure to low serum conditions (when studying pro-angiogenic molecules), or by stimulation prior to the addition of anti-angiogenic drugs [6].

Assay choice considerations. Each type of EC proliferation assay described here has its own limitations. Though cell counting is the most straightforward method, it can be prone to sampling error when cell detachment is required. Furthermore, it can be labor intensive and requires relatively large samples [36,37]. However, it generally does not require the handling of toxic, mutagenic or radioactive compounds like with several metabolic, DNA labeling and DNA incorporation based assays [36,37,42].

From a methodological point of view, each assay has its strengths and weaknesses. For example, the indirect detection of antigens (e.g. PCNA or BrdU) requires careful procedural optimization. For the latter, the alternative 'click' chemistry by which the analogous EdU can be detected directly, this issue is circumvented [37,36,39]. In addition, assay readout and interpretation are important to consider. When measuring incorporation of nucleotide analogs, one should realize that DNA synthesis is not in all situations confined to chromosomal duplication during S-phase [37,36]. For example, during DNA repair nucleotides are excised and replaced, which is especially relevant when addressing the action of compounds with a potential DNA damaging effect. With the DNA intercalating dyes, care must be taken that duplets are excluded in the gating procedure or with readout in plate-based systems. By nature, this type of assay is mostly suited for truly diploid cells, and not for cells that may display alternative karyotypes. Although the latter is not a common trait of EC, a few reports have addressed this matter in tumor-derived EC [43], and personal observations also indicate

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this may be the case with EC lines. Finally, test reagents may interfere with readout chemistry, e.g. compounds that affect mitochondrial function are less compatible with metabolic assays.

2.3. Concluding remarks. The choice for a particular EC proliferation assay is determined by a number of considerations. Endpoints, test compounds, laboratory infrastructure, scale, required throughput, convenience and cost all influence the applicability of an assay system. The growth of a cell population is influenced by both division and death, which is difficult to simultaneously monitor. Most important is the researchers' awareness that all assays have their strengths and weaknesses and that interpretation of data is done with care, and if possible, validated with an alternative method.

3. 3D models of vascular morphogenesis

To generate blood vessels and vascular networks, ECs must undergo vascular morphogenesis, which can include either vasculogenic and/or angiogenic processes [44]. In vasculogenesis, differentiating ECs migrate, proliferate, aggregate and rearrange to form cords that then undergo lumen formation to generate three-dimensional (3D), tubular blood vessels. In angiogenesis, stimulatory vascular endothelial growth factor (VEGF) promotes EC sprouting from existing vessel walls, the formation of new tubes, and anastomosis (joining) with other vessels to create an interconnected network of vessels. ECs recruit perivascular stromal cells (pericytes) to stabilize this newly-formed network and minimize leak upon blood perfusion. Importantly, not all sprouts become functional vessels. Pruning serves to selectively remove redundant or non-functional vessels to optimize fluid flow through the network [45].

In vitro assays have played a valuable role in our understanding of vascular morphogenesis. These assays provide a simpler platform than animal models for dissecting individual steps within the process while also incorporating 3D matrix to mimic native *in vivo* tissues. Here, we present several of the most reliable and informative assays developed to date and highlight the strengths and limitations of each (**Table 1**). While many types of ECs can be used in these assays, the most commonly used are HUVECs and human endothelial colony-forming cell-derived EC (ECFC-ECs), which generally have a higher proliferative potential. Mouse ECs are not generally used in these assays as they are notoriously hard to maintain in culture. While we use "ECs" to reference both cell sources here, assays using a specific EC source are annotated accordingly.

3.1 Types of assays.

Fibrin Bead Assay. Traditional Matrigel cord-forming or collagen I angiogenic invasion assays are insufficient to model the complexity of angiogenesis, as these assays are two-dimensional and ECs in these assays often form incomplete lumens. Moreover, lumen formation in Matrigel is not unique to ECs as several non-EC cell types (e.g. human prostate carcinoma and glioblastoma cells) also form lumens, complicating the interpretation of results from these assays [36]. In contrast, the fibrin bead assay provides a platform for testing EC sprouting and lumen formation over an extended period (2-3 weeks), incorporates a 3D, extracellular matrix (ECM), and multiple cell types (i.e. stromal pericytes) to model native angiogenesis. ECs (HUVECs) are first allowed to adhere to collagen I-coated Cytodex beads to generate an EC monolayer that mimics the vessel wall of native vessels. These EC-coated beads are then embedded into a fibrin gel with human stromal cells either embedded within the gel or plated in monolayer on top. Tip cells are observed 2-3 days post-plating and elongating sprouts appear 2-4 days after this (**Fig. 2a-b**). When maintained in pro-angiogenic EGM-2 medium (Lonza), lumens form within a week and the assay remains viable up to three weeks, at which point anastomosis between sprouts is often apparent [46]. A detailed, video protocol of this assay is available [47]. Angiogenic sprouting from individual beads is evaluated by phase-contrast microscopy allowing for quantification of sprout number, length of sprouts, percentage of sprout lumenization, and the number of anastomoses. Genetic approaches (siRNA, lentiviral transduction) [48] can modify gene expression in

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individual cell types to dissect cell-autonomous components of the angiogenesis process. Protein expression and localization is measured by fixing bead assays and using modified immunofluorescent staining techniques. More detailed gene expression analyses are made possible by harvesting individual cell types to track RNA expression changes over time through various stages of sprouting angiogenesis.

The use of stromal cells (i.e. lung fibroblasts) is critical to the success of this assay, as these cells secrete angiogenic factors necessary for EC sprouting and lumen formation, including growth factors (e.g. HGF, TGF-α, and Ang-1), as well as ECM molecules, matrix-modifying proteins and matricellular proteins (e.g. collagen I, procollagen C endopeptidase enhancer 1, secreted protein acidic and rich in cysteine [SPARC], trans-forming growth factor-β–induced protein ig-h3 [βIgH3], and insulin growth factor–binding protein 7 [IGFBP7]). These factors act to locally stiffen the matrix, which supports sprouting and lumen formation [49]. This assay represents a significant improvement over conventional, single cell-type angiogenic assays, as the inclusion of multiple cell types more closely mimics the physiological environment. Nevertheless, as this assay uses primary cultures of cells, rather than cell lines, it is important to remember that batch-to-batch variations in stromal cells (and HUVECs) can significantly affect assay results. To partially overcome this issues, it should be appropriate to use ECs pooled from 5-10 umbilical cords. Generally, for this assay to be reproducible, it is crucial to identify stromal cell-HUVEC pairs that yield optimal angiogenic sprouts for this assay to be reproducible.

Collagen Lumen Assay. To understand EC lumen formation mechanics, early assays seeded ECs in monolayer on plastic dishes coated with ECM proteins (i.e. collagen I, collagen III, fibrin, or Matrigel). While these 2D assays are sufficient to induce EC cord formation [50-52], they cannot reproduce the necessary cues for true lumen formation found in native, 3D tissues. Collagen sandwich assays surround ECs within a 3D matrix by seeding the cells in monolayer on collagen I matrix, then covering them with a second layer of collagen [53]. Nevertheless, tube formation fails to occur in a random, 3D growth pattern, forming only in the X-Y plane of the initial gel layer and not in the z-axis. As this does not adequately recapitulate normal vessel growth in a true 3D environment, George Davis and others further optimized these assays, opting instead to embed single ECs (HUVECs) randomly throughout a collagen I matrix. In the simplest version of these assays, HUVECs are seeded at low density ($7x10^5$ cells/mL) under serum-free growth conditions and with the addition of minimal growth factors (phorbol ester, VEGF, and fibroblast growth factor-2 [FGF-2]). After 48 hours, the embedded ECs form intact tubes throughout the gel, with clearly demarcated lumens (**Fig. 2c-d**). Several variations on this assay have since enhanced and optimized lumen formation. First, the addition of several other growth factors, including stem cell factor (SCF), JL-3, stromal-derived factor-1α (SDF-1α), and FGF-2, further promote lumen formation while maintaining serum-free growth conditions. Second, when simultaneously seeded within the same matrix, stromal pericytes are recruited by ECs, recapitulating a key step in vascular morphogenesis. Lastly, to understand the process of EC sprouting and angiogenesis, ECs can be seeded on top of a 3D collagen gel containing the same growth factors and invasion of the underlying gel layer can be quantified. A detailed protocol of the collagen lumen assay and its variations is available for further reading [54].

Real-time imaging of tube formation can be achieved using fluorescent protein-transduced ECs. Alternatively, fixed vessels can be stained with 0.1% toluidine blue and imaged using brightfield microscopy (**Fig. 2d**). More in-depth analyses can be carried out on these fixed vessels using immunofluorescence staining of relevant protein markers or transmission electron microscopy to resolve structural details of formed lumen and remodeled ECM.

Regular users of collagen gels will note that the viscosity, pH, and contraction of these gels can hinder successful execution of assays in the hands of new users. As a result, special care should be taken when pipetting (such as when mixing cells and growth factors) and plating gels to ensure even gel coating of the

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bottom of the well plate. Perhaps most significantly, early gel contraction can limit the useful length of these assays. Users will note that plating gels only in wells within the center of the 96 half-area wells and adding medium or water to the outer wells of the plate will minimize gel contraction, by maintaining local humidity levels. Additionally, seeding fewer ECs within the collagen ($1.5x10^3$ cells/ml) can minimize gel contraction and prolong the assay.

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Retinal Explant Assay. Although *in vitro* assays are high-throughput and can mimic major steps in vascular morphogenesis, these assays do not fully recapitulate the *in vivo*, whole-organ environment [46]. Several *in vivo* animal models, such as mouse retina or zebrafish fins, are valuable tools for studying vascular (re)establishment in a physiologically-relevant context [36,55]. However, the added complexity of these systems makes it more difficult to ascertain the role of individual proteins and growth factors in the vascular morphogenesis process, relying on genetic manipulations or system-wide administration of pharmacologic inhibitors to dissect molecular pathways [36,56]. As such, there is a need to increase assay complexity (and physiological relevance) while developing platforms amenable to *ex vivo* study in the laboratory. Retina explant assays are one such *ex vivo* platform, whereby dissected retinas are maintained and observed for vascular morphogenesis over several weeks in the laboratory. While multiple versions of this assay have been published, a protocol published by Sawamiphak, et al., is most widely used for the study of endothelial sprouting [57]. Briefly, retina cups from embryonic, postnatal, or adult mice are harvested and cut radially to allow flat mounting of the retina interior surface onto a membrane insert. After recovery in media for 2-4 hours, the explants can then be treated with stimulatory or inhibitory agents for up to 4 hours, followed by whole-mount microscopy analysis to evaluate the (anti-) angiogenic effect of these agents on vessel sprouting (**Fig. 2c**). When perfected, each pair of retinas can be harvested and dissected within minutes. Unfortunately, without the support of a 3D matrix, retinal cells cannot survive for long periods, thus making studies for later stages of angiogenesis impossible. To overcome this, Rezzola, et al., have improved the assay by embedding the retinas in different matrices after dissection [58]. In this approach, retinas can be cross-cut into 4 equal pieces and left in serum-free media overnight. The retina fragments are then embedded in Matrigel, collagen I, or fibrin matrix and fed every 2 to 3 days. Depending on the age and the matrix used, sprouts can be observed between day 3 to 6 and anastomosis of neighboring sprouts, similar to what occurs *in vivo* can be observed in 10-14 days [59] (**Fig. 2d**). These explants can be maintained up to 3 weeks before the vessels eventually regress.

Vessel formation can be analyzed in real time using time-lapse imaging or the explants can be fixed and imaged by immunofluorescent microscopy at set experimental time points [59,60]. Gene expression can be manipulated by genetic crossing of the donor mice or, more transiently, by treating retinas with lentivirus or siRNA. Moreover, embedded retinas can be treated with drugs over extended periods to dissect individual signaling pathways.

Several factors are critical to consistently achieve sprouting from dissected retina explants. First, the matrix proteins in which retinas are embedded can greatly influence how vessels sprout. In our experience, Matrigel is far superior to single matrix proteins in inducing sprouting. However, the addition of 10-20% Matrigel in collagen I matrix is sufficient to stimulate sprouting compared to pure collagen I matrix. Second, the use of pro-angiogenic EGM-2 yields more sprouts as compared to basal medium alone. Lastly, as with any tissue explant, the age of the mouse can influence the degree of vessel sprouting. As such, special care should be taken to select mice appropriate for the experimental question at hand. There are many similarities between the mouse retinal explant assay and the traditional mouse/rat aortic ring angiogenesis assay or rat vena cava explant assay [61-63]. However, retinal explant models more closely model true capillary sprouting as the vasculature in these explants is actively developing and remodeling. This makes the retinal explant model uniquely suited to studying microvessel formation and its underlying mechanisms.

Vascularized Micro-Organ (VMO) Platform. To understand all the steps of vascular morphogenesis in a single platform, our group has optimized a vascularized micro-organ (VMO) approach to drive formation of a perfusable vascular network within a 3D hydrogel matrix environment. In contrast to the assays described above, VMO-embedded ECs are exposed to and respond to shear stress, form lumenized vessels, and are perfused with a blood-substitute medium that delivers nutrients to tissues within the matrix, just as in the body. Specifically, this platform utilizes "arteriole" (high pressure) and "venule" (low pressure) microfluidic channels that are joined by a living microvascular network that forms by vasculogenesis in an intervening tissue chamber (**Fig. 2g**). A pressure differential between the two channels is used to drive interstitial flow through the fibrin gel matrix during vessel development, induce vessel formation (through shear-sensing), and drive convective flow through the mature vasculature once formed and anastomosed to the outer channels. This pressure difference is induced by varying the level of medium within fluid reservoirs at either end of the microfluidic channels, thereby creating hydrostatic pressure heads that ensure continuous fluid convection across the cell chamber. To form vessels, human ECFC-ECs and human lung stromal cells are co-loaded within a fibrin matrix into the central cell chamber through an independent loading tunnel. When maintained in pro-angiogenic EGM-2 medium, vessels form within 4-6 days post-loading (**Fig. 2h**). When perfused with 70kDa rhodamine-dextran, a molecule similar in size to albumin, these vessels demonstrate minimal vessel leak – comparable to *in vivo* microvasculature. For readers interested in more information, a detailed protocol for loading and maintaining the VMO platform is available [64].

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The VMO platform is fabricated from polydimethylsiloxane (PDMS), an optically clear, biologically inert polymer widely used in the microfluidics field [65]. The use of this polymer and the dimensions of the platform ensure that live, GFP-transduced EPCs can be imaged and quantified throughout vessel formation. Specific parameters such as vessel network length, branching, and anastomosis can be measured in real-time as can vessel permeability by perfusion with fluorophore-tagged dextran molecules of various molecular weights. Additionally, immunofluorescent staining can be used to quantify expression of specific molecular markers or RNA can be collected to measure changes in gene expression. Lastly, gene expression can be manipulated by treating individual cell types with lentivirus or siRNA prior to loading in the platform.

To ensure robust and reproducible vascular network formation, several steps are critical. First, the fibrin gel matrix must be consistently loaded within the VMO cell chamber. During normal loading, perfusion burst valves at the interface between the tissue chamber and the microfluidic channels ensures a gel/air interface (later a gel/fluid interface) is formed. To simplify loading and minimize specialized training for new users of the platform, current iterations of the VMO platform incorporate a pressure release valve at the loading tunnel that minimizes unintended gel bursting [66]. Second, robust vascular network formation requires that vessels within the chamber anastomose with the outer microfluidic channels. To facilitate the formation of these anastomoses, ECs can be either seeded directly within the microfluidic channels or induced to migrate from the gel by coating the external channels with extracellular matrix [67]. As with the fibrin bead assay, optimal stromal cell-EC pairs should be validated to ensure assay reproducibility.

3.3. Limitations and challenges. With all EC assays, the source of ECs is critical to assay success. Although commercial versions of HUVECs and ECFC-ECs are available, cells from these sources show limited utility in many 3D assays, likely due to a larger than optimal number of cell doublings prior to shipment and use in the laboratory. As a result, the use of primary-isolated ECs will provide the most consistent results and is strongly encouraged. Readers will note that isolation protocols for both cell types are available [68,69]. Additionally, patient-to-patient variation between different EC isolations can lead to inconsistent assay results, an issue that may be avoided by pooling several EC lines prior to use.

Given the many differences between the assays described here, the useful length of these assays varies considerably. Even in well-trained hands, the contraction of gels in the collagen lumen assay effectively limits the useful time frame of assays to 72 hours or less. However, the other assays described here can persist for much longer periods of time, with the fibrin bead assay, retinal explant assay, and VMO platform all suitable for time points up to three weeks under appropriate conditions.

Lastly, the majority of these assays can be run in a relatively high-throughput manner, thereby accelerating the speed with which genetic, molecular, or pharmacologic screens can be conducted to understand vascular morphogenesis. This is especially true with the fibrin bead and collagen lumen assays, which utilize multiple beads or multi-well culture plates to increase assay throughput. Similarly, while initial versions of the VMO platform were cumbersome to load in high-throughput numbers, this platform is now used in an optimized configuration that incorporates up to 16 individual VMO devices within a standard 96-well plate [70]. This design simplifies translation to outside labs and interfacing with existing microscope and plate reader infrastructure. Of all the assays described in this section, retinal explants are most adversely affected by delays between initial dissection, mounting, and plating of tissue samples. This inherently limits the number of animals that can be dissected at once and, for now, limits the number of retinas that can be screened simultaneously.

4. Aortic Ring Assay

Explants of rat aorta have the capacity to sprout and form branching microvessels *ex vivo* when embedded in gels of extracellular matrix. Angiogenesis in this system is driven by endogenous growth factors released by the aorta and its outgrowth in response to the injury of the dissection procedure. This property of the aortic wall first described in the early 1980s [71], led to the development of the aortic ring assay [72] which is now widely used to study basic mechanisms of angiogenesis and test the efficacy of proangiogenic or antiangiogenic compounds [73].

4.1. Benefits and strengths of the Aortic Ring Assay. The aortic ring assay offers many advantages over existing models of angiogenesis. Unlike isolated ECs, the native endothelium of the aortic explants has not been modified by repeated passages in culture and retains its original properties. The angiogenic response can be inhibited or stimulated with angiogenic regulators and analyzed by molecular or immunochemical methods without the confounding effects of serum. Angiogenic sprouting occurs in the presence of pericytes, macrophages and fibroblasts, as seen during wound healing *in vivo* [73]. The different cell types can be identified with specific cell markers by immunostaining whole mount preparations [74] of the aortic cultures. The ultrastructure of neovessels at different stages of development can be evaluated by electron microscopy (**Fig. 3**). Many assays can be prepared from the thoracic aorta of a single animal (approx. 20-25 cultures/rat aorta; approx. 10-15 cultures/mouse aorta). The angiogenic response can be quantitated over time, generating curves of microvascular growth. Aortic cultures can be used to study mechanisms of vascular regression, which typically follows the aortic angiogenic response as seen during reactive angiogenesis in vivo. Aortic rings transduced with viral constructs or obtained from genetically modified mice can be used to study the role of specific gene products in the regulation of the angiogenic response [73].

Recently, rat aortic ring assay was adapted to human arteries by using matrigel as 3D hydrogel. Arteries are maintained in EC basal medium (EBM / MCDB131) supplemented with 5% fetal calf serum and heparin. When growth factors are added (VEGF-A, EGF, FGF-2), capillary outgrowth begins from around day 8 to 12 and continues to develop up day 30. As reported for rat aortic ring, this assay is suitable to gene editing by gain- and loss-of- function approaches and for drug screening.

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4.2. Assay overview. A detailed description of the aortic ring assay protocol is available in previous reports [75,76]. We provide here a summary of key steps for the preparation of the assay. Aortic rings are prepared from the thoracic aorta of 1-2 month old rats or mice. After excision from the animal, the aorta is transferred to

a Felsen dish containing serum-free EBM. Under a dissecting microscope, the aorta is cleaned of blood and fibroadipose tissue using Noyes scissors and microdissection forceps. Care is taken not to stretch, cut or crush the aortic wall during the isolation and dissection procedures. As the dissection progresses, the aorta is rinsed in the four compartments of the Felsen dish. Using a scalpel blade, the aortic tube is then cross-sectioned into 0.5-1 mm long rings. The proximal- and distal-most rings, which may have been damaged during the dissection procedure are discarded. The remaining rings are washed through sequential transfers into eight consecutive baths of serum-free medium, using compartmentalized Felsen dishes. Aortic rings are then embedded individually into thin collagen, fibrin or basement membrane gels as described. Once the gel has set, 500 µl of serum-free EBM is added to each culture. Each experimental group comprises quadruplicate cultures in 4-well NUNC dishes. Aortic ring cultures are incubated in a humidified CO_2 incubator at 37°C for 7-21 days.

4.3. Quantitative Analysis of Angiogenesis in Aortic Cultures. The angiogenic response of the rat aorta can be quantitated by visual counts or by computer-assisted imaging. For visual counts, cultures are examined every 2-3 days and scored for angiogenic sprouting by using an inverted microscope with bright-field optics equipped with 4X to 10X objectives and a 10X eyepiece. Angiogenesis is scored by counting microvessel sprouts, branches and loops according to previously published criteria [72]. Aortic outgrowths can be also quantified by image analysis using low power images of the cultures thresholded to highlight the vascular outgrowths [77-80]. Standard statistical methods are used to analyze data and determine levels of significance between control and treated cultures. An internal control group with untreated aortic rings must be included in each experiment to mitigate the effect of possible interassay variability.

4.4. Critical points. For this assay, we recommend using the thoracic aorta because of its uniform size and intercostal artery branching pattern. The abdominal artery can also be used, but its variable pattern of collaterals and tapering lumen may introduce variability in the angiogenic response. Injury to the aortic endothelium may be an additional cause of uneven sprouting from different rings. Therefore, special care must be taken not to damage the aorta by stretching or letting it dry during the isolation and dissection procedures. Dissection of the aorta and preparation of the aortic ring cultures are best performed in a tissue culture room with HEPA-filtered air to avoid microbial contamination. Best results with this assay are obtained using interstitial collagen or fibrin gels. Collagen can be produced in-house, as described [75,76], or purchased from commercial sources [81]. Fibrinogen and thrombin for the fibrin gel are commercially available. Matrigel, a basement membrane-like matrix of tumor origin, can also be used [82]. Matrigel cultures, however, require growth factor supplements due to the limited ability of the aortic rings to sprout spontaneously in this dense matrix. The growth medium used for the assay should be optimized for the growth of ECs in the absence of serum. Optimal results in collagen and fibrin cultures can be obtained with EBM. When preparing individual collagen gel cultures, given the small volume of gel (20-30 µl), it is important to remove excess growth medium from the aortic rings when they are transferred into the collagen, fibrinogen or Matrigel solution. This is accomplished by gently streaking the aortic ring onto the bottom of the culture dish while holding it from the adventitial side with microdissection forceps. When working with fibrin gels, which set rapidly, no more than four cultures at a time should be prepared, to avoid disrupting the developing gel while positioning each ring. In addition, for fibrin cultures, the culture medium should include a plasmin inhibitor such as epsilon aminocaproic acid (EACA) to inhibit fibrinolysis by the aortic rings, which would rapidly destroy the matrix needed for ECs to sprout.

The rat aortic ring assay is robust and very reproducible when performed by an experienced operator. The mouse aortic ring assay is more variable than the rat aortic ring assay, likely because of the small size of the rings. For this reason, at least twice as many aortic rings should be used for this assay. Miniaturization of the

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assay using IBIDI microchambers and a smaller volume of growth medium (50 µl) is recommended for the mouse aortic ring assay to ensure spontaneous sprouting under serum-free conditions [76]. In all cases, experiments should be repeated 2-3 times to obtain sufficient number of data points for statistical analysis. The growth medium can be replaced on a regular basis (3 times/week) or left unchanged for the duration of the experiment. If the medium is not replaced with fresh medium, the angiogenesis response and the stability of neovessels are enhanced due to accumulation of endogenous growth factors in the system. For immunohistochemical evaluation of the aortic cultures, biomatrix gels should not exceed 20-30 µl and should be well spread as a thin wafer around each ring. Formalin fixation should be limited to 10 min to avoid excessive cross-linking of proteins. In addition, overnight incubation may be needed for optimal penetration of the primary antibody into the gel.

4.5. Limitations and challenges. The main limitation of the aortic ring assay is the lack of blood flow, particularly for angiogenesis-related genes that are regulated by mechanochemical mechanisms. An additional potential limitation is the source of angiogenic ECs, which are arterial and not venous, as neovessels *in vivo* primarily sprout from postcapillary venules. Many studies performed with this assay, however, have shown good correlation of results obtained with the aortic ring assay and in vivo models of angiogenesis. If needed, the aortic ring assay methodology can be applied to veins as reported [63]. Some investigators have described variability of the angiogenic response in different aortic cultures. This is due to the delicate nature of the endothelium which can be damaged because of inadequate handling of the aorta or the aortic rings, drying of the explants, or excessive exposure of these to alkaline pH. Suboptimal preparation of the gels resulting in a defective matrix scaffold can also result in a poor angiogenic response. In addition, the age and genetic background of the animal significantly affect the capacity of the aortic rings to sprout spontaneously or in response to angiogenic factors. Aortic outgrowths in Matrigel are much denser than in collagen and fibrin and more difficult to quantitate by visual counts due to the intricate branching pattern of the endothelial sprouts and the tendency of mesenchymal cells to arrange in confounding networks, which mimic angiogenic sprouts. Immunostaining of the aortic outgrowths with endothelial markers followed by image analysis may overcome this limitation. For quantitative analysis of the angiogenic response, the visual count method (described in detail in reference [76]), becomes challenging when cultures stimulated by growth factors produce 250-300 or more vessels. Since the outgrowths of rings oriented with the luminal axis parallel to the bottom of the culture dish (recommended orientation) are typically symmetrical, angiogenesis in these cases can be quantitated by counting the number of microvessels in half of the cultures and then doubling the score. Alternatively, these cultures can be measured by image analysis [77-80]. Finally, for the whole mount immunohistochemical stain, gel thinness is critical for optimal antibody penetration.

4.6. Concluding Remarks. Many of the molecular mechanisms orchestrating angiogenesis have been discovered, but many others remain to be identified, studied and evaluated as targets for the development of new therapies. The aortic ring assay reproduces *ex vivo* cellular and molecular mechanisms that are essential for the regulation of the angiogenic process. As such, this assay provides invaluable testing ground to test new hypotheses and analyze the efficacy of the next generation of angiogenesis-targeting drugs.

5. Detection methods for tip cells

The tip cell is the first specific angiogenic EC phenotype that becomes activated during angiogenesis, and forms the leading cell on the tip of a vascular sprout. Tip cells are characterized by their position, their long and dynamic filopodia, absence of proliferative activity and their migratory behavior. Stalk cells that follow the tip cells have other properties, they proliferate, produce ECM, and form a vessel lumen. Recently, the tip cell has been studied to decipher the functional relevance of its specific morphology, its coordinated behavior and its

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distinct gene expression profile. Studies in mouse- and zebrafish development, in *in vitro* sprouting assays and in tumor angiogenesis have illustrated that VEGF and Notch signaling are essential for the differentiation of tip and stalk cells. However, a more detailed understanding of the underlying mechanisms of tip cell differentiation is still lacking, especially due to its dynamic phenotype, and the absence of specific and definite *in vivo* markers for tip cells.

5.1. Tip cell detection methods. Tip cells have mostly been studied *in vivo*, i.e. in the developing mouse retina and in zebrafish, and to a lesser extent in vitro in angiogenic sprouting models. The basis for tip cell research was set by Gerhardt *et al.* 2003 [83], who described the tip cell as a specialized EC that could be distinguished from other EC phenotypes (stalk cells, phalanx cells and quiescent ECs) and who provided a comprehensive overview on how to detect these cells by tip cell specific markers using immunostaining or in situ hybridization. In the search for additional tip cell-specific markers whole genome genetic profiling strategies were used [84,85]. In recent years, more and more transgenic models are increasingly being used. **Fig. 4.**

Labeling of tip cells in vivo. The most extensively used model for tip cell research is the developing mouse retina. Here, the first vessels originate from the optic nerve and grow radially to the peripheral retinal margin during the first week after birth. Subsequently, the superficial capillaries start sprouting downwards to form the deep plexus and the intermediate vascular plexus, and in approximately the third postnatal week all vascular layers are completely mature. Sprouting angiogenesis can ideally be studied in the first postnatal week, when tip cells are located in the angiogenic front.

Immunofluorescent staining of retinal vasculature with biotinylated isolectin B4 (IB4) labels sugar (α-d-galactosyl) residues on the endothelium and endothelial tip cell filopodia, but also marks microglia and macrophages. Other useful antibodies against surface bound proteins are anti-VEGFR2, anti-VEGFR3, anti-Pdgfb anti-Dll4 and anti-CD34 antibody [85]. Gene expression of these proteins was found to be enriched in tip cells [83,86] and antibodies preferentially label endothelial tip cell bodies but less intensively their filopodia, endothelial stalk cells and phalanx cells. ESM1 is probably the most specific tip cell marker in mouse retinal tip cells, labeling additionally only some arterial ECs, whereas labeling of CXCR4 is high in tip cells, but also to a lesser extend present in some ECs of the vessel plexus, in arterial ECs and in perivascular cells [87]. The actin cytoskeleton provides a driving force for tip cell movement during angiogenesis, and labeling F-actin with phalloidin [83] is another way of identifying endothelial tip cells, especially by highlighting their filopodia.

As an indirect method to distinguish tip cells from other EC phenotypes, labeling with antibodies against the adherens junction protein VE-cadherin, the adhesion protein PECAM1/CD31 or against proteins in the basal lamina (fibronectin, collagen, laminin) are used in combination with IB4, in which all ECs are labelled, but tip cell filopodia exclusively with IB4 [83,86]. The use of markers of the basal lamina is based on the concept that formation of new sprouts requires degradation of extracellular matrix to allow migration of tip cells, thus showing reduced staining in proximity of tip cells. Indeed, it was recently shown by triple-labeling of tip cells with F-actin, cortactin and collagen type IV that tip cells use so-called podosomes to degrade the extracellular matrix [88]. Labeling with anti-Ki67 or BrdU [83,86] is used as marker of proliferation, a property that is greatly reduced in tip cells.

A useful marker of tip cells employing in situ hybridization (ISH) is the Pdgfb gene [83,86]. Microarray analysis comparing DLL4$^{+/-}$ and wild-type mouse retinas [84] identified additional tip cell enriched genes, including, apelin, angiopoietin-2, chemokine receptor type 4 (CXCR4) and endocan ([84,87,85], that these researchers confirmed to be tip cell specific by ISH and the latter two recently also by immunofluorescent staining [87].

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Transgenic models as tool to identify tip cells. The zebrafish embryo is a popular model for studying tip cells in vascular development due to its small size, transparency and fast development. A combination of the availability of transgenic fish with fluorescent vessels and advanced imaging techniques, including time-lapse videomicroscopy, allows to follow vascular development and angiogenic sprouting in great detail. In situ hybridization of Flt4 is one method to identify tip cells, but transgenic fluorescent lines and cross-breeding of different transgenic lines currently prevail. Fluorescent transgenic fish, such as *Tg(fli1:EGFP)*, driven by the promotor of ETS transcription factor a or *Tg(Fli1ep:Lifeact-EGFP)*, expressing fluorescent F-actin are very helpful to morphologically distinguish vascular sprouts and tip cell filopodia. Identification of tip cells is further helped by automatic tracking of filopodia [89], or by single-cell resolution imaging to track individual nuclei of tip and stalk cells [90].
To study tip cells in the mouse retina, various transgenic mouse models are also available today. Lifeact-EGFP labels actin associated with cell-cell junctions, and apical and basal membranes, and highlights actin-based structures such as filopodia and stress fiber-like cytoplasmic bundles [91]. Mosaic mutant mice were generated by injecting morula's or blastocysts of mice with DsRed or eGFP expressing embryonic stem cells and showed that tip and stalk cells are able to change positions [92]. Recently a Cre transgenic strain was developed (*Esm1-CreERT2*)[87], mimicking tip cell specific expression of ESM1 in the retina [84]. Combining this transgenic strain with the *R26-mTmG*$^{T/+}$ Cre reporter generated a conditional knockout strain that can be cross-bred with global knockout strains for tip cell genes as *Cxcr4*, or stalk cell specific genes as *Notch1*, and *Jag1* to study tip cell fate [87].

Studying tip cells in ex vivo and in vitro models. Ex vivo and in vitro models have the advantage over *in vivo* models that they are suitable for an array of interventions and readouts, and allow a rapid and low cost screening of potential anti-angiogenic molecules. The *ex vivo* aortic ring assays recapitulate many stages of angiogenesis, including migration, sprouting, and proliferation and are suitable to analyze tip-stalk dynamics. Time-lapse videomicroscopy of chimaeric embryonic stem cell spheroids revealed that the sprouts are led by a single tip cell that is followed by proliferating stalk cells, but also showed a dynamic shuffling of tip and stalk cells in this model [92]. Immunohistochemical stainings for cell nuclei (DAPI), vessel walls (Vimentin, CD31), F-actin (Phalloidin), and adherens junctions that connect the ECs in a sprout (β-catenin) allow the discrimination between the leading tip and stalk cells. Tip cells can be indirectly distinguished from the highly proliferative stalk cells using proliferation markers, such as phosphor-histone and Ki-67 [83].

A model of for tip cell studies in vitro based on CD34 labeling. Recently, it has been demonstrated that tip cells can be identified in EC cultures such as primary HUVECs, HMVECs and in immortalized endothelial cultures by staining for CD34, an endothelial and stem cell marker, which stains the luminal side of all ECs in vivo. In contrast, *in vitro* CD34 reliably and specifically stains cells with almost all characteristics of tip cells *in vivo*. This *in vitro* model of tip cells allows studies into the differentiation and regulation of tip cells, as well as the discovery of novel tip cell genes [85].

5.3. Limitations and challenges. Limitations to the use of tip cell markers is that none of the current markers exclusively labels tip cells, as most stain stalk or phalanx cells, and some other ECs of the vascular plexus, arteries or perivascular cells. Therefore, identification of tip cells is relying mainly on their position, morphology, and their filopodial protrusions. To confirm the endothelial identity of tip cells, staining with multiple antibodies is necessary to distinguish between ECs and perivascular cell types. CD34 staining has limited value for in vivo use for this purpose due to its generalized luminal expression on ECs [85]. Alternatively, an indirect method is used, identifying proliferating stalk cells. Furthermore, IHC and ISH require fixation and the accessibility of antibodies in the retina may be dependent on the protein or mRNA targeted. Transgenic models partly

Deleted: is a 3D angiogenesis model that closely mimics in vivo vessel sprouting and contains the key features of angiogenesis: cell proliferation, migration, tube formation, microvessel branching, recruitment of muscle cells and pericytes and remodeling. In another approach, EC-coated beads embedded in fibrin gels and the spheroid model are three-dimensional *in vitro* assays where ECs

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overcome these problems, greatly reducing background in EC-specific reporter lines and show a more equally distributed expression, but are not affordable for every laboratory. *Ex vivo* and *in vitro* assays are faster and cheaper and are more ethically responsible. Disadvantages of these assays are that tip cells in these models are less differentiated from following ECs, tip and stalk cells are able to interchange positions, and cells are of macrovascular origin. Furthermore, sprouts sometimes disintegrate and single migrating tip cells can be identified, leaving the sprouts less suitable for studying sprouting angiogenesis as it occurs *in vivo*.

5.4. Concluding remarks. Tip cells are a subset of ECs with a distinct genetic profile, but their identification remains difficult since a single specific marker for identification of endothelial tip cells *in vivo* has not been identified. Transgenic reporter lines in zebrafish and mice partly overcome this problem, but still rely mainly on identification of tip cells by their localization and filopodial protrusions. For studies of tip cells in vitro, CD34 is a promising marker identifying highly differentiated tip cells in EC cultures

6. Microvessel density and histopathological growth patterns

Microvessel density is often regarded as a surrogate marker of angiogenesis in tumors. Angiogenic tumors contain areas with a high concentration of new but inefficient blood vessels, which have sprouted from existing vessels and are often arranged in a tortuous, glomeruloid tangle. In addition, to these 'hot spots', angiogenic tumors also contain areas of low blood vessel numbers. Weidner developed a method to assess microvessel density in vascular hot spots using pan-endothelial immunohistochemical assays to label CD31, CD34 and von Willebrand's factor and less often αvβ3 integrin, CD105 or type IV collagen [93]. Vascular hot spots are identified by light microscopy at low power magnification by scanning the entire stained tumor section. Individual microvessels are then counted at high power magnification in these regions. These immunohistochemical staining assays are robust, fast and easy to perform and automate. Meta-analyses have confirmed the prognostic value of microvessel density, e.g. in breast cancer [94] and isolated reports support the predictive value of microvessel density for anti-VEGF treatment [95,96]. However, the inter-observer variability of the vessel counting algorithm presents a significant barrier to the use of microvessel density as a biomarker [97].

Although the importance of angiogenesis to tumor biology is well established, multiple observations that primary and metastatic tumors develop and progress in the absence of angiogenesis suggest that other mechanisms are frequently involved in tumor vascularization [98]. This represents a challenge to Folkman's hypothesis that the growth of a tumor is only possible when accompanied by angiogenesis [99]. Moreover, microvessel density cannot distinguish angiogenic and non-angiogenic tumors. In addition to the high numbers of blood vessels in angiogenic tumor hot spots, high microvessel density is indeed also observed in non-angiogenic tumors which have co-opted vessels in organs with extensive microvasculature, such as the liver and the lungs [98]. The differentiation of angiogenic and non-angiogenic tumors is, therefore, not related to the absolute number of vessels per surface area but to the growth pattern of a tumor.

6.1. Histopathological growth patterns. Histopathological growth patterns (HGPs) are defined according to the morphological characteristics of the tumor at the interface with the surrounding normal tissue. HGPs are identified by light microscopy in standard hematoxylin-and-eosin stained tissue sections and distinct HGPs have been described for tumors that grow in the lung, liver, skin, brain and lymph node [98]. Recently, international consensus guidelines for scoring these HGPs have been described [100,101]. One of the important differences in the biology of tumors with specific HGPs is their means of vascularization. Liver metastases may present with one of two common HGPs, replacement or desmoplastic. In the replacement HGP, cancer cells 'replace' the hepatocytes while co-opting the sinusoidal blood vessels at the tumor-liver interface. Patients with colorectal cancer (CRC) liver metastases with a replacement HGP respond poorly to

the anti-VEGFA treatment, bevacizumab, likely because these tumors utilize vessel co-option instead of angiogenesis [101]. By contrast, in desmoplastic liver metastases, the cancer cells are separated from the liver by a rim of desmoplastic tissue in which new blood vessels are formed by sprouting angiogenesis. Desmoplastic CRC liver metastases showed a better response to bevacizumab [101]. Taken together, these observations strongly suggest that HGPs can be used to guide the choice of treatment for individual patients with liver metastases.

Across studies, approximately 50% of patients with CRC liver metastases present with a predominant replacement growth pattern and this proportion extents to 95% when patients with breast cancer liver metastases are considered [100,101]. This clearly demonstrates that non-angiogenic tumor growth is not a rare phenotype. The same applies to primary lung carcinomas and lung metastases for which angiogenic and non-angiogenic HGPs have also been described [102,103]. In the non-angiogenic, alveolar HGP, cancer cells fill the alveolar spaces and incorporate the capillary blood vessels of the alveolar walls. Approximately, 40% of the lung metastases from clear cell renal cell carcinoma present with a non-angiogenic HGP despite the fact that nearly all primary clear cell renal cell carcinoma rely on sprouting angiogenesis, driven by loss of VHL protein function [103].

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The prevalence of non-angiogenic tumors and their resistance to anti-VEGF treatment require that a biomarker is identified which accurately reflects this type of tumor growth. The HGPs are a good candidate biomarker. The vascular pattern in a tumor section immunostained with pan-endothelial antibodies or the use of antibodies that mark ECs participating in sprouting angiogenesis are other potential histopathological methods to distinguish non-angiogenic from angiogenic tumors. Indeed, when the number of hot spots is determined by nearest neighbor analysis in digital images of liver metastasis tissue sections stained for CD31, the non-angiogenic, replacement-type metastases clearly resemble normal liver tissue. This contrasts with angiogenic, desmoplastic liver metastases, which show a significantly higher number of vascular hot spots than normal liver tissue and their non-angiogenic counterparts **(Fig. 5).**

Several groups have identified ‘tumor endothelial markers’ or TEMs [104-106]. These are proteins that are selectively upregulated on tumor endothelium compared to normal endothelium. Tumor angiogenesis meta-signatures have been generated using gene expression data of several tumor types by using ‘seeds’ of transcripts that were known to be involved in angiogenesis and expressed by ECs. The resulting set of EC/angiogenesis-related transcripts that correlated with these seeds was then purified by selecting genes that were modulated in response to anti-angiogenic treatment [107]. Interestingly, several of the top ranking genes in this signature have been confirmed as TEM by other independent teams [108]. Although we do not currently know whether TEMs are expressed equally on both angiogenic tumor vessels and co-opted vessels in tumors, it will be intriguing to assess the expression of, e.g. ELTD1, CLEC14a and ROBO4 in the vasculature of replacement versus desmoplastic liver metastases. In primary and secondary non-angiogenic human lung tumors, only pre-existing vessel, with an LH39 positive basal membrane and weak or absent αvβ3 integrin, and arising from the alveolar septa entrapped by the neoplastic cells, were observed [109].

The HGP is not a static phenotype: systemic treatment of patients with CRC liver metastases can alter the HGP [101]. The fact that the HGPs can change in response to treatment reflects the dynamic nature of tumor vascularization driven by either angiogenesis or of vessel co-option. This illustrates the necessity for longitudinal assessment of vascularization mechanisms in tumors during treatment and follow-up of individual patients. In order to achieve this, the histopathological evaluations of the tumor vasculature as described here must be supplemented by medical imaging techniques and/or assays, which utilise small biopsies, circulating tumor cells or circulating tumor derivatives. Moreover, the importance of an accurate biomarker of ongoing sprouting angiogenesis or non-angiogenic vessel co-option is corroborated by the extensively documented and strong link between angiogenesis and immune suppression [110-112] and the surge of clinical studies that combine anti-VEGF and immunomodulatory treatment strategies [113].

6.2. Concluding remarks. Although the assessment of microvessel density (MVD) has a prognostic value in many different tumour types, this parameter cannot distinguish angiogenic and non-angiogenic processes of vascularization. However, the histopathological growth patterns of, for example, lung and liver tumors, accurately reflect the means of vascularization, being sprouting angiogenesis or non-angiogenic co-option of existing blood vessels. We therefore propose to determine both the HGP and MVD when studying the vascularisation of tumors. In addition, non-invasive surrogate markers of the HGP should be developed, e.g. medical imaging parameters, and these markers should be integrated in clinical oncology trials.

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7. Assessment of Intussusceptive Angiogenesis

Angiogenesis is the *de-novo* formation of blood vessels and can follow either a sprouting or a non-sprouting course. One important non-sprouting mechanism is intussusception (growth within itself; known also as vascular splitting). Although both sprouting and intussusception lead to an amplification of the capillary network, the processes involve different cellular mechanisms, which are presumably regulated by different molecules. Sprouting angiogenesis (SA) is localized to the abluminal aspect of vessels and is characterized mainly by local vasodilatation, increased vascular permeability and cell proliferation. It is initiated by proteolytic degradation of the basement membrane, which is followed by migration of proliferating ECs into the extracellular matrix. The sprouts reorganize internally to form a vascular lumen and are finally connected to other capillary segments [114]. Intussusceptive angiogenesis proceeds through transluminal tissue pillar formation and subsequent vascular splitting, and the direction taken by the pillars delineates intussusceptive angiogenesis into its overt variants, namely: (i) intussusceptive microvascular growth (expansion of capillary surface area), (ii) intussusceptive arborization (remodeling of the disorganized vascular meshwork into the typical tree-like arrangement) and (iii) intussusceptive branching remodeling (optimization of local vascular branching geometry), including intussusceptive vascular pruning. It has been shown that intussusceptive angiogenesis takes place not only during physiological processes, but also under pathological conditions including tumor growth, colitis, and in neurotoxic disease, among others [115]. Although the morphological facets of intussusceptive angiogenesis, as well as molecular mechanisms of sprouting are well described, the exact cellular and molecular mechanism of intussusceptive angiogenesis is not yet characterized.

7.1. The concept of intussusception and the methodological challenge. The concept of intussusceptive angiogenesis was first described within the rapidly-expanding pulmonary capillary bed of neonatal rats [116]. Morphological investigations during the last three decades have indicated that the transluminal pillars arise into the vessel lumen according to the four consecutive steps in pillar formation postulated by Burri et al in 1990: 1) creation of a zone of contact between the opposing sides of the capillary wall; 2) reorganization of the junctions between endothelium cells in the contact zone, resulting in central perforation of the bilayer; 3) formation of an interstitial pillar core, which is invaded first by the cytoplasmic processes of myofibroblasts and pericytes, and then by collagen fibrils; 4) the pillars expand in girth (≥2.5μm) and transform into a capillary mesh. The described model with the 4 consecutive steps is based mainly on transmission and scanning electron microscopy methods (scanning ,or transmission electron micrsocope) and these techniques do not allow dynamical observation of the pillar formation, fusion and vascular splitting over time.

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7.2. Limitations and challenges. Intussusceptive angiogenesis is spatially quite a complex process and it is not possible to document it on solitary sections. To overcome this limitation and keep the high resolution, a *3D reconstruction* based on the images taken from the serial ultrathin sections has been reported for the first time in 2000 [117]. This is a very laborious approach feasible only in specialized labs. Even with a profound expertise, only limited number of pillars as a qualitative and descriptive illustration could be visualized. Now

there is a new technique available that makes such task much more feasible - *serial block face 3-dimensional imaging*. With this new technique exact morphological information on relatively big sample volumes (1x1x1mm) within a short time could be acquired: for example the scanning of a block with a size one zebrafish embryo would take around 1 day and would provide the 3D scan of the complete embryo at ultrastructural level. Using the standard approaches it would take weeks if not months. Moreover, the outcome from 3View is of much better quality and without loss of information (slices). This technique enables a quantum leap forward in morphological studies.

Vascular casting has been used repetitively to demonstrate the intussusceptive angiogenesis in 3D. The methodology is based on vascular replica of the microvasculature. As casting media Methylmethacrylate (Mercox, Japan Vilene) or PU 4ii (VasQtec, Switzerland) are used [117]. Few hours after perfusion, the organs have to be excised and transferred to 15% KOH for 2–4 weeks for dissolution of the tissue. After washing, the casts are dehydrated in a graded series of increasing ethanol concentrations and dried in a vacuum desiccator. They are then examined in a scanning electron microscope. A new powerful methodology employing polymer-based contrast agent in combination with micro-computed tomography (micro-CT) has been reported recently [118]. Both casting and micro-CT are very useful for evaluation of large samples, for example entire mouse organs and in a subsequent step for "zoom in" in the area of interest. The vascular casting combined with scanning electron microscope has the potential to demonstrate greatest morphological details (**Fig. 6a**). The micro-CT does not provide such kind of resolution, but is very rapid and the probes are not destroyed and could be preceded for further morphological investigations [118].

The dynamic in vivo observation of the pillar formation and subsequent vascular splitting over time is feasible employing conventional fluorescence microscopy, stereomicroscopy and laser scanning microscopy (**Fig. 6b**). The latter methodologies are widespread, but often cause partially severe, phototoxic artefacts. As a very appropriated model for dynamic observation of the vascular splitting and blood flow (circulating erythrocytes), zebrafish lines with uniform cytosolic expression of the respective fluorescent proteins could be used; for example Tg(Fli1a:eGFP)//Tg(Gata1:dsRed). The embryos should be mounted in low-melting agarose, in the presence of phenylthiourea and could be investigated for 16-20h. To reduce the phototoxicity imaging should be performed with laser scanning microscopes with a long working distance, using heated chamber, fast scanning (less than 1µs pixel time) and lowest possible laser power (in a range of few percentage depending from the used microscope) and time steps of 10-15 minutes. Such dense time pattern is necessary to record the dynamic cellular alterations of pillar formation and splitting. By this setting no signs of photobleaching or phototoxicity (membrane blebbing, cell apoptosis) have been observed.

7.2. Concluding remarks. Intussusceptive angiogenesis can be properly investigated by a combined methodological approach, due to its 3D complexity. Matching the dynamic cellular changes in capillaries from the time lapse *in vivo* observations with morphological data from light microscopy level up to the ultrastructural level, could provide the complex information (**Fig. 6b-d**). The sites of interest should be documented first by intravital microscopy as described above. After the *in vivo* documentation, the area of interest can be harvested, fixed and processed either for paraffin sectioning and light microscopy with immunohistochemistry or alternatively for serial semithin sectioning and 3D analyses using e.g. Imaris Software. To obtain a deeper insight into the morphological substrate and tissue components involved in the process, the same tissue should be proceeded for transmission electron microscope (**Fig. 6**).

8. Alitalo + Fig 7

9. Assay for pericyte recruitment to endothelial cell-lined tubes to promote capillary tube assembly and maturation

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A fundamental question concerns the growth factor and signaling requirements governing how human ECs and pericytes co-assemble to form capillary tubes in a 3D matrix or tissue environment [119-121,54,122]. To this end, bioassays have been developed in 3D collagen or fibrin matrices to address such issues using serum-free defined conditions [123,124] (**Fig. 8**). In either system, pericyte recruitment to EC tubes can be readily observed over a 72-120 h period and a consequence of this recruitment is deposition of the vascular basement membrane between the abluminally recruited pericytes and the ECs that line the branched networks of tubes [120] (Figure 1). Interestingly, EC tube formation results in the creation of networks of extracellular matrix spaces, which are termed vascular guidance tunnels [125], where the EC tubes reside, and is also a physical space into which pericytes are recruited to, along the basal and abluminal EC tube surface [120,125]. Both ECs and pericytes migrate along each other in a polarized manner within these tunnels, resulting in basement membrane deposition underneath the abluminal surface of the EC-lined tubes [119,120,126]. Basement membrane deposition leads to much narrower and elongated EC tubes in EC-pericyte co-cultures compared to EC only tubes which progressively get wider and less elongated with time [120,126].

9.1. Benefits and strengths of the assay. The major benefit and strength of this assay model is its high reproducibility and high replicate number in that it is performed in half-area 96 well plates. The assay typically sets up 60 wells per plate and this can be readily established using one T75 plate of human ECs (e.g. HUVECs) and one T25 plate of human pericytes (brain-derived vascular pericytes) (labeled with GFP). The assay allows us to perform siRNA suppression experiments using either cell type, and we can perform signaling experiments where lysates can be prepared with time and Western blots performed to assess signal transduction cascades. Also, we can examine the recruitment of pericytes with time in the presence or absence of blocking antibodies to various targets (i.e. growth factors, receptors, integrins, extracellular matrix molecules, etc.) or signal transduction inhibitors. We can examine the consequence of pericyte recruitment to EC tubes by examining basement membrane deposition (by immunofluorescence staining or transmission electron microscopy) (Figure 1), or capillary tube widths and lengths. Finally, we can perform real-time video analysis of pericyte and endothelial motility as they co-assemble to form tube networks with abluminally recruited pericytes in 3D-dimensional matrices.

9.2. Assay overview. Human umbilical vein or artery ECs (HUVECs or HUAECs), and human brain derived vascular pericytes or bovine retinal pericytes are trypsinized and seeded within 3D collagen matrices at a density of $2x10^6$ cells/ml for ECs and $4x10^5$ cells/ml for pericytes. Both types of ECs or pericytes work very well in this assay system. FGF-2 and SDF-1α are each added at 200 ng/ml into the collagen gel mix (2.5 mg/ml of rat collagen type I). Twenty-five µl of gel is added into each individual 96 well (A/2 plates from Costar). After 30 minutes of equilibration in a CO_2 incubator, Medium 199 (100 µl per well supplemented with 1:250 dilution of Reduced Serum Supplement II (RS II) [127], 40 ng/ml each of FGF-2, stem cell factor, and IL-3, and 50 µg/ml of ascorbic acid) is added. Cultures are then placed in a CO_2 incubator for 72-120 hr. After this time, cultures are fixed with 3% paraformaldehyde in PBS (120 µl per well). Cultures can then be imaged under fluorescence to quantify EC tube area and pericyte recruitment under the various conditions of treatment. Individual cultures can be immunostained with various antibodies directed to EC or pericyte cell surfaces and/or extracellular matrix antigens (i.e. basement membrane proteins such as fibronectin, laminin and collagen type IV) [120]. Alternatively, cultures could be used to make lysates using SDS sample buffer or used to isolate total RNA to perform gene expression experiments [120]. Real-time movies can be performed to examine pericyte recruitment and measure pericyte motility during capillary assembly and maturation [121]. In the latter case, nuclear GFP-labeled pericytes can be utilized to track pericyte motility [121].

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9.3. Critical steps in the assay. The assay system above is highly dependent on the health of the growing ECs and we make our own growth media and do not purchase commercial endothelial growth media. To each 500 ml bottle of Medium 199, we add 200 mg of lyophilized bovine hypothalamic extract, 50 mg of heparin and 100 ml of fetal calf-serum. Culture flasks are coated with 1 mg/ml of gelatin solution for at least 30 min. prior to seeding trypsinized ECs. The same culture media and gelatin-coated flasks are used to grow pericytes . We prepare our own bovine hypothalamic extracts and also make our own rat tail collagen preps which are suspended at 7.1 mg/ml in 0.1% acetic acid. The assay system that we describe is highly robust and reproducible for our laboratory [120,123,124].

Particularly for longer-term experiments (i.e. multiple days), we make sure that we completely surround each well with 100 µl of water. We fill in the outer wells and each of spaces directly surrounding the wells with that amount of water. This reduces dehydration of media in the microwells and maintains the health of the cultures. For cultures going longer than 72 h, we feed the cultures, by removing 90 µl of media and replacing with 100 µl of media per well. This media is slightly different than that used above to start the assay and is: Medium 199 containing a 1:250 dilution of RS II, ascorbic acid at 50 µg/ml, as well as 80 ng/ml of FGF-2, and 40 ng/ml each of stem cell factor, IL-3 and SDF-1α.

9.4. Limitations and challenges. The major limitations of this *in vitro* assay relate to how reflective the assay model is to the *in vivo* state. Overall, the data and conclusions that we have obtained over the years have been repeatedly demonstrated to be observed using mouse, Zebrafish or quail *in vivo* models [122]. One of the interesting issues is the state of differentiation of the ECs that are used in these *in vitro* assays, and how similar are they to ECs *in vivo* that are undergoing vasculogenesis or angiogenic sprouting. This is a very difficult question to answer, and the answer is currently unknown. Clearly, this is a question that needs to be addressed in future studies. Another potential weakness of our current *in vitro* systems is that we have yet to adapt our model system to flow conditions or transplant the gels into a mouse model to interface the human vessels with an *in vivo* vascular system. Since this has been done previously with other *in vitro* models using HUVECs or other human ECs, there is no reason to think that our specific model system would not perform well using a similar approach.

10. Endothelial cell metabolism

ECs line blood vessels. The majority of ECs remains quiescent for years [128], but upon exposure to pro-angiogenic stimuli (such as VEGF), they can rapidly switch to a proliferative and migratory state in health and disease. At the base of this switch lies a tightly regulated metabolic network, providing the necessary energy and building blocks for ECs to respond accordingly [129,130]. ECs optimized their metabolism to optimally execute these functions, and therefore rewired their metabolism in a highly specific manner [131]. ECs are indeed highly glycolytic, producing large amounts of ATP and lactate even in the presence of ample oxygen [130].

Metabolism is a biochemical reaction network that catabolizes nutrients into metabolic products such as energy and biomass that are needed to survive and adapt environmental challenges. As such, they provide a functional readout of the observed phenotypic changes. Metabolomics is the systematic study of the aforementioned metabolic products and is currently enjoying a revival ("Metabolomics, the apogee of the omics trilogy" [132]). The ultimate goal of metabolomics is to understand: (i) how cells under different circumstances apply and reroute their metabolic pathways, and (ii) what are the required start and end products in relation to the observed phenotype.

In the following section, we will discuss three approaches that have been established to monitor metabolic activities of ECs: (i) tracer metabolomics, (ii) radioactive tracer based metabolic assays, and (iii) measurements of extracellular acidification rate and mitochondrial respiration by the Seahorse XF analyzer.

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These approaches are complementary and it is recommended to perform these assays in parallel for a more complete metabophenotyping.

10.1. Tracer metabolomics. The interpretation of metabolic activities based on the (relative) abundances of metabolites under specific conditions remains a formidable challenge, largely because the network of biochemical reactions is highly interconnected. For instance, an increase of intracellular serine levels can be due to increased uptake but also by de novo synthesis using 3-phosphoglycerate from glycolysis. As such, the majority of metabolites can be linked to multiple sources and by merely measuring the (relative) abundance of metabolites, one cannot readily deduct the responsible pathways and their active interplay. This knowledge is paramount to understanding cellular biology. A specialized field in metabolomics, Tracer Metabolomics [133], tackles this challenge and uses labeled non-radioactive substrates (carrying ^{13}C, ^{15}N, ^{18}O, ^{2}H, etc.) to monitor the distribution of the labeled isotopes throughout the metabolic network and as such, provides the actual contribution of nutrients to specific metabolites and consequently the relative activity of metabolic pathways.

10.2 Experimental tracer metabolomics setup of ECs. A possible cell culture format for ECs is performed using a 6-well, in which at least 150,000 ECs are plated. ECs need to be grown in customized medium of which the substrate of choice is withdrawn and replaced by its non-radioactive isotopically labeled counterpart (tracer). The majority of tracers are nutrients that are taken up by the cell (for instance glucose, glutamine, palmitic acid, etc) of which one of the constituent atom species (^{12}C, ^{14}N, ^{1}H, ^{16}O, etc) is replaced by its non-radioactive isotopologue (respectively ^{13}C, ^{15}N, ^{2}H, ^{18}O, etc). ECs take these labeled nutrients up and process them identically compared to their unlabeled form. Consequently, all of the downstream metabolites and metabolic pathways linked to the specific tracer will incorporate the labeled atoms. It is critical that ECs reach isotopic steady state, a condition that is accomplished when the labeled isotope enrichment in a given metabolite is stable over time relative to the experimental error [133]. Practically, steady state in ECs is reached in 24 to 48 hours using respectively glucose or glutamine tracers [134,135], for palmitic acid up to 72 hours of incubation is required [129]. The difference in labeling time necessary to reach isotopic steady state depends on both the fluxes (i.e. rate of conversion) from the nutrient to the metabolite of interest, and the pool size of that metabolite and all intermediate metabolites [133]. The fractional contribution, this is the fraction of a metabolite's carbon (or other atom) produced from a certain nutrient, in the downstream metabolites as well as their isotopic envelopes (isotopologues) can be quantified using Mass Spectrometry (MS). Prior to MS analysis, ECs are washed using a physiological (0.9%) NaCl solution and metabolites are extracted in an organic (50% methanol-30% acetonitrile-20% H_2O) extraction buffer. It is of utmost importance that the extraction is carried out swiftly because a cell's metabolism changes within the order of seconds to minutes [136]. Extracts are centrifuged to remove the precipitated proteins and insolubilities. The supernatant, containing the polar metabolites, is loaded onto the MS platform.

10.3. Mass spectrometry. The instrument of choice for carrying out (tracer) metabolomics is mass spectrometry (MS), mainly due to its superior sensitivity and speed of analysis in comparison to other technologies such as Nuclear Magnetic Resonance (NMR) Spectroscopy, as well as its flexibility in applying different kinds of chromatography (liquid or gas chromatography, LC or GC) to study different chemical classes of metabolites [137]. We will only focus on the analysis of polar metabolites (amino acids, organic acids, hexose phosphates, etc), apolar species such as lipids are out of the scope for this section. A crucial requirement for the MS in tracer metabolomics relates to its resolution, the ability to accurately quantify the isotopologue profiles of the metabolites of interest. As such, the current benchtop LC-MS OrbiTRAPs are amongst the instruments of choice to conduct tracer metabolomics. Alternatively, GC-linked quadrupole MS can be used as well. For the separation of polar metabolites prior to the MS, two main types of chromatography can be applied: (1)

Hydrophilic Liquid Chromatography (HILIC) [138] and (2) Ion-Pairing Chromatography [139]. The latter has superior binding and separation capacities compared to HILIC, but it restricts any further usage of the chromatograph to Ion-Pairing mode. In this perspective, HILIC provides a better solution, despite the lesser separation capacity compared to the Ion-Pairing setup as it comes without any restrictions to apply different chromatography settings on the same platform.

10.4 Data processing. Data extraction of isotopic profiles occurs via vendor specific software or *in-house* developed software platforms (such as for instance, BIOMEX™) [140]. In general, the workflow starts with *in-house* libraries containing information on the exact mass of the target molecule and its retention time during the chromatographic separation to identify the metabolite of interest. Next, the abundances of the isotopes of different metabolites of interest are quantified. Following a correction for natural abundances [141,133] to facilitate proper interpretation of the labeling data, the fractional contribution is calculated [133].

10.5 Radioactive tracer based assays. As introduced above, unlabeled or labeled metabolites can be quantified by MS or NMR. However, these techniques display some limitations, as they do not quantify the absolute flux, which reflects the rate of turnover of molecules through a metabolic pathway. Radioactive isotope labeling is a widely used experimental approach to analyze metabolic flux. This technique is based on the principle that nutrients labelled with radioactive tracers (e.g. ^{3}H and ^{14}C) at specific positions within the molecule release the radioactive label as $^{3}H_2O$ or $^{14}CO_2$ respectively upon specific enzymatic activities. Consequently, the generated $^{3}H_2O$ and $^{14}CO_2$ diffuse into the medium and can be measured by trapping its release on soaked filter paper and subsequently quantified by scintillation counting [130]. As an example, use of [5-^{3}H]-glucose enables to quantify glycolytic flux [142] because the single tritium at the C5 of glucose is removed by a condensation reaction catalyzed by enolase (**Fig. 9a**), hence releasing radioactive $^{3}H_2O$ in the medium. Similarly, by using [9,10-^{3}H]-palmitic acid, one can measure the radioactive $^{3}H_2O$ formed during fatty acid oxidation [129].
In order to map the different metabolic fates of glucose and other crucial substrates, a set of differently radioactive labeled tracers are needed to identify and quantify the ongoing biochemistry. As an example, in parallel to the glycolytic flux measured by [5-^{3}H]-glucose, the downstream oxidation of glucose can be monitored by quantifying the release of $^{14}CO_2$ by [6-^{14}C] glucose or uniformly labelled [$^{14}C_6$] glucose. Notably, by using [6-^{14}C] glucose, information on the oxidation of glucose in the tricarboxylic acid (TCA) cycle is obtained, whereas the [$^{14}C_6$] glucose tracer generates $^{14}CO_2$ via two different metabolic pathways: the oxidative pentose phosphate pathway (oxPPP) and the TCA. Also, the carbon at position 1 in glucose is liberated in the oxPPP. Hence, using [1-^{14}C] glucose in parallel to [6-^{14}C] glucose, the oxPPP flux can be estimated by subtracting the rate of [6-^{14}C] glucose from the one of [1-^{14}C] glucose. In summary, assessing the choice of the tracer and its specific labeling positions is essential in terms of the biological question that needs to be addressed. Similar approaches are available to investigate other 'non-glucose' related fluxes, e.g. by using [$^{14}C_5$] labelled glutamine to determine glutamine oxidation.

10.6 Measurements of extracellular acidification rate and mitochondrial respiration using a Seahorse XF analyzer. The Seahorse extracellular flux (XF) analyzer enables to assess different aspects of cell metabolism. Apart from providing an indirect readout of anaerobic glycolysis through measurements of the extracellular acidification rate (ECAR) [143], it can also assess oxidative phosphorylation (OXPHOS) (through oxygen consumption rate (OCR)). A commonly used method to determine glycolysis by the Seahorse XF analyzer is the measurement of acidification of the medium (pH changes) in a glycolysis stress test [144]. In this technique, glucose, oligomycin (inhibitor of ATP synthase in the electron transport chain (ETC)), and 2-deoxy-glucose (2-DG; inhibitor of hexokinase) are sequentially administered while measurements of ECAR are being

performed. In the first phase of the experiment, ECs are cultured in medium deprived of glucose or pyruvate (the glycolysis stress medium). Supplementation of glucose feeds glycolysis, which allows estimation of glycolytic rate by calculating the difference between ECAR before and after addition of glucose. ECAR, prior to glucose injection, is referred to as non-glycolytic acidification resulting from other sources of extracellular acidification not attributed to glycolysis (**Fig. 9b**). Next, oligomycin, which inhibits mitochondrial ATP production, lowers the ATP/ADP ratio, and shifts energy production to glycolysis, is supplemented to measure the maximum glycolytic capacity. The difference between ECAR before and after oligomycin supplementation is a measure of the glycolytic reserve capacity. The last injection of 2-deoxy-glucose (2-DG) inhibits glycolysis and the resulting decrease in ECAR confirms that the ECAR produced in the experiment is due to glycolysis. Moreover, inhibition of glycolysis by 2-DG also provides information on non-glycolytic ECAR of cells (**Fig. 9b**). Mitochondrial respiration can be assessed by using a modified Seahorse Cell Mito Stress Test [145]. This test sequentially uses modulators of respiration that target different components of the ETC. Oligomycin, carbonyl cyanide 4-(trifluoromethoxy) phenyl-hydrazone (maximizing OCR by uncoupling the OXPHOS) and antimycin (blocking OXPHOS by inhibiting complex III) are sequentially injected to determine basal mitochondrial OCR (OCR_{BAS}), ATP dependent OCR (OCR_{ATP}), maximal respiration (OCR_{MAX}) and non-mitochondrial respiration ($OCR_{non\text{-}mito}$), proton leak and spare respiration (**Fig. 9c**) [145].

10.7. Limitations and challenges. Tracer Metabolomics provides information on relative pathway activities, qualitative changes in pathway contributions via alternative metabolic routes, and nutrient contribution to the production of different metabolites. In this perspective, a limitation of tracer metabolomics is the lack of quantitatively estimating metabolic fluxes, which provide additional quantitative and complementary information such as pathway directionality. Nonetheless, resolving metabolic fluxes is time and data-intensive [146]. A challenge for tracer analyses is the requirement of expert knowledge at the level of mass spectrometry and biochemistry. This relates to the choice of specific tracers needed to answer biological questions as well as the tools (chromatography, mass spectrometry, software) available to extract and process the required data. The optimal cell culture experiments apply customized medium of which the unlabeled form of the tracer has been withdrawn. This setup maximizes the amount of label entering the cell and increases the likelihood to identify and quantify the labeled 'downstream' targets. A pitfall occurs when the unlabeled form cannot be withdrawn, in this case the labeled compound can be added in equimolar quantities to the medium as the un-labeled compound. This poses several threats: (i) the amount of label incorporated in downstream metabolites is reduced and pathways using low amounts of the tracer might be overlooked; and (ii) doubling the concentration of a specific substrate can induce metabolic changes into the cellular biochemistry, for instance doubling the glucose levels from the physiological 5.5 mM to 11 mM places ECs in a diabetic-like 'high glucose' environment, triggering additional metabolic changes or, in worst case, inducing toxicity.

Not all of the metabolites present in the cell are detectable by MS, the major causes for this relate to the abundance and/or intrinsic technical challenges, such as the ionization potential of the metabolite of interest. To tackle this challenge and still gain information on this missing piece of the puzzle, one needs to look at the fractional contribution and isotopologue profiles of neighboring metabolites that reflect the labeling pattern of the missing component, for instance the lack of oxaloacetate detection can be accounted for by looking at the isotopologue profile of aspartic acid. Consequently, engaging on tracer analyses implies that sufficient metabolites need to be taken into account ensuring full coverage of the pathway(s) of interest.

Seahorse XF analyzer. Although assays performed on a Seahorse XF analyzer requires only small number of cells (30,000 cells/well in 24-well assay plate format), they still present several limitations that one should take into consideration while performing these experiments. Additional metabolic processes, like CO_2 generated TCA activity, can change the pH of the media and interfere with the readout and interpretation of the results. Since ECAR is essentially a measurement of pH, buffering agents (e.g. sodium bicarbonate), are not included

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in the assay medium. Moreover, as bicarbonate and media pH play a role in regulating glycolysis, they could influence and confound measurements of ECAR. In order to increase the accuracy of the performed experiments, application or pretreatment with additional chemical inhibitors (e.g. AR-15585; lactate export blockers) could be of use to determine whether changes in pH come from lactate excretion or other sources of media acidification. Optionally, glutamine can be also withdrawn from the media, in order to assess ECAR coming from glycolytic pathway and not from glutaminolysis [147]. In addition, depending on the used cell type, the concentration of each chemical compound must be carefully determined, in order to obtain the optimal result for each of these measurements. For instance, overdose of oligomycin can lead to maximal inhibition of OCR, which could result in a progressive increase of OCR over time [145].

General limitations of (radioactive) tracer based assays. Cell metabolism is a very dynamic process, and the metabolic needs as well as metabolic activity of ECs has been shown to be correlated with the cell cycle phases [131,148]. Therefore, one should carefully control cell density while performing these experiments. Moreover, the results and interpretation of metabolic studies using radioactive tracers depend on appropriate normalization of the data, as ineffective or poorly chosen normalization methods can lead to erroneous conclusions. Therefore, normalization to total protein concentration, cell number or DNA content are considered as the golden standards in metabolomics.

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11. Endothelial cell precursors

Stem- and progenitor cells provide a necessary homeostatic source of tissue specific mature cellular elements that permit appropriate cell functions through replacement of injured, diseased, and senescent cells in many organ systems throughout the lifespan [149]. While the identity, biology, and molecular regulation of hematopoietic-, intestinal-, skin-, and skeletal muscle stem- and progenitor cells is well recognized, little is known of the organization of the reparative cells that comprise the vascular system. In 1997, Asahara and colleagues [150] reported on the identification of circulating progenitor cells for the endothelial lineage. Subsequent studies have clarified that those putative “endothelial progenitor cells” (EPC) did not possess the capacity to undergo a stable lineage switch to the endothelium, but were comprised of numerous hematopoietic cells that can serve paracrine proangiogenic functions to promote vascular repair and replacement but are incapable of integrating as a bona fide EC in the injured vasculature.[3] In fact, these proangiogenic cells could upregulate “endothelial cell” markers and thus give the impression they were becoming ECs at sites of injury, but failed to persist as functional vasculature long term. Only endothelial colony forming cells (ECFC), also called late outgrowth or blood outgrowth ECs (BOEC), are direct EC precursors that form vessels *in vivo* [151]. This segment will briefly discuss available evidence for and remaining controversies regarding the evaluation of endothelial stem- and progenitor cells in mouse and man.

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11.1. Assays to identify endothelial stem and progenitor cells. A simple working definition of a stem cell is a clonal, self-renewing cell, which gives rise to differentiated cell types [149]. A somatic stem cell may be multi-potent (giving rise to multiple types of differentiated cells) or unipotent (differentiating into a single lineage of mature cells). A progenitor cell displays clonal proliferative potential, but progenitor cells lack self-renewal potential. However, progenitor cells can expand into a single lineage of differentiated cells. Multiple assays have been developed to define stem- and progenitor cells that reside in different organs and tissues [149]. Stem cells in any tissue must be interrogated for clonal self-renewal (most stringently proven by long-term *in vivo* persistence with retained capacity for contribution to differentiated progeny [fate mapping approach] and/or use of transplantation assays into primary and secondary recipient hosts). Progenitor cells are often measured with colony forming assays *in vitro* (to prove a precursor product relationship). Stem and progenitor cells may also display distinguishing cell surface markers that can be used to prospectively isolate the cells

through use of monoclonal antibodies and flow cytometry or magnetic bead separation. Most recently, stem- and progenitor cells from some tissues have become definable by single cell gene expression technologies to permit identification by a unique molecular signature [152,153]. Presentation of some selected publications (as examples) identifying putative stem and progenitor cells for the vascular system (using many of the above criteria) follows.

Progenitor cell assays. To identify the progenitor cells for the endothelial lineage, we need to decide what criteria should be used to define cells belonging to the endothelial lineage. Other segments in this paper have made arguments as to the "best" assays used to define EC characteristics and their functions based on published evidence. Obviously there are numerous phenotypic-, morphologic-, physiologic-, genomic-, proteomic- and functional parameters that constitute unique and characteristic behaviors of ECs. The point here is, that one cannot identify cells of the endothelial lineage by expression of a few cell surface markers or of a few RNA transcripts. A useful summary of the gold standard and key "minimal" elements to define an EC are stated in the introduction of this paper. Several selected works that use comprehensive definitions for identifying progenitor cells for the endothelial lineage follow.

Patel and colleagues [154] recently proposed several features that could be used to define resident vascular endothelial progenitor cells for the murine vasculature. Based upon the level of expression of vascular endothelial cadherin (CD144), platelet EC adhesion molecule (CD31), vascular EC growth factor receptor 2 (flk1), and leukocyte common antigen (CD45), ECs present in blood vessels in induced healing wounds or growing in response to implanted tumors were identified as endovascular progenitor cells (EVP), transient amplifying cells, and definitive differentiated cells. Evidence for these different populations displaying different functional states was presented using clonogenic *in vitro* analysis, fate mapping studies *in vivo*, transplantation analysis, differences in gene expression, differences in phenotypic analysis (flow cytometry and immunofluorescence) and the requirement of certain transcription factors for the differentiation of EVP to transient amplifying and definitive differentiated states.

Evidence for the identification of human endothelial progenitor cells (circulating or resident) has been published by several groups. Analysis of the circulating blood of patients following a sex mismatched bone marrow transplant indicated that some of the circulating BOEC were derived from the host, but the most proliferative cells were donor bone marrow derived [155]. Umbilical cord blood was reported to be enriched for clonogenic ECFC that displayed a hierarchy of proliferative potential and *in vivo* vessel formation compared to adult peripheral blood [156]. Furthermore, resident vascular ECs derived from umbilical veins or human abdominal aorta contained clonogenic ECFC with vasculogenic potential. Very recently, human induced pluripotent stem cell (hiPSC) derived ECFC have been reported with properties that are similar to umbilical cord blood ECFC but with some distinct differences in gene expression [157].

Vascular endothelial stem cells. Fang and colleagues [158] reported that stem cells for the endothelial lineage could be isolated from lung blood vasculature. Vascular endothelial stem cells (VESC) expressing the phenotype $CD31^{+}CD105^{+}Sca\text{-}1^{+}CD117^{+}$ (devoid of any mature lineage markers for the hematopoietic system) could be isolated from collagenase digested lung tissue. The VESC displayed clonogenic colony forming activity *in vitro* and when implanted in Matrigel subcutaneously in mice, gave rise to donor derived blood vessels. Indeed, when 15 VESC (GFP-tagged) were implanted with B16 tumor tissue, GFP labeled blood vessels were identified in primary, secondary, tertiary, and quarternary tumor implants. Even a single VESC displayed the capacity to form donor blood vessels *in vivo* in a Matrigel implant. While some flow cytometric estimations of the frequency of the CD117 expressing VESC in different tissues was reported, no detailed visualization of the location of the VESC in arteries, veins, and capillaries in different tissues was presented. In

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addition, no fate mapping studies to identify the contributions of the putative VESC within various vascular beds in homeostatic endothelial turnover were reported.

In the same year, Naito and colleagues [159] used the Hoechst staining method to identify resident vascular ECs in the side-population (SP) that were dormant in steady state, but possessed clonal colony forming activity, produced large numbers of mature endothelial progeny, and when transplanted into ischemic lesions, restored blood flow and reconstituted *de novo* blood vessels at the site of injection. Although the surface markers of the SP cells was similar to primary capillary ECs, the gene expression pattern of the side-population and main population that fail to retain the Hoechst dye, is significantly different. Several unique cell surface markers were identified in the side-population cells that may permit prospective isolation of these putative VESC. Use of the Hoechst method fails to permit identification of the putative VESC in the organ and tissue vasculature during development or after injury and will rely upon further studies using novel cell surface markers.

More recently, Yu and colleagues [160] identified Protein C receptor-expressing ECs as VESC in the mammary fat pad, skin, and retina. These VESC exhibited robust clonal expansion in tissue culture, high vessel reconstituting ability upon multiple rounds of transplantation, and long-term clonal expansion in lineage tracing experiments. Indeed, the VESC were determined to be bipotent, with contributions not only to the endothelium but also to pericytes throughout vessels in multiple tissues. The authors suggested that the VESC identified underwent endothelial to mesenchymal transition to become the pericyte cells in the vascular beds examined [12].

11.2. Limitations and challenges. As alluded to in the introduction, the term EPC has been loosely applied since 1997. Using the definitions of stem and progenitor cells quoted in this segment, most of the >9,564 publications identified by the term EPC in Pubmed (endothelial progenitor cell, Pubmed, July 30, 2017) have failed to provide sufficient evidence that the putative EPC under investigation displays clonal proliferative potential and/or the capacity to directly form ECs that undergo vasculogenesis *in vivo* to form new vessels or integrate long-term into injured vessels *in vivo* as ECs. If the EPC in question could not directly give rise to cells of the endothelial lineage at a clonal level (*in vitro* or *in vivo*) or function as a bona fide EC *in vivo*, then the term EPC should not be applied to those cells [151]. While the work of Patel and colleagues [6] has shown that endothelial progenitors can be identified by applying stringent criteria, the specific sites of EVP, transient amplifying and definitive differentiated cell localization in organs and tissues at homeostasis (artery, vein, or capillary bed), the contributions of EVP to transient amplifying and definitive differentiated cells during homeostasis, differences in the EVP among different organs across the lifespan of the mouse, and determination of whether the EVP represents an endothelial stem cell, remain to be addressed. Human EPCs have been identified [7-9], however, no unique identifying markers have permitted prospective isolation of ECFC from circulating blood or blood vascular endothelium to permit identification of the site of origin of ECFC in human subjects and determination of whether these cells display stem cell activity for the endothelial lineage. Several papers have published evidence for the presence of resident VESC in mice, however, the relationship between the unipotent VESC identified by Fang and colleagues [158] and Naito and colleagues [159], and the bipotent VESC identified by Yu and colleagues [160] remains unclear. Identification of unique and distinguishing characteristics of the VESC that discriminate these stem cells from progenitor and mature endothelial elements awaits further analysis.

11.3. Concluding remarks. Altogether these data suggest that progress in identifying stem- and progenitor cells for the vascular endothelium is possible when using multi-parameter evidence that the putative precursor cells are irreversibly differentiating into cells of the endothelial lineage. Use of multi-parameter analytics will be

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required to permit novel studies on the biology of vascular endothelial stem and progenitor cells and likely may alter our understanding of vascular development and the pathophysiology of vascular diseases.

12. Microfluidic assays

The use of microfluidic cell-culture systems has changed the way in which we study and manipulate living cells, and numerous researchers have leveraged the capabilities of these systems to advance and refine our understanding of angiogenesis and microvascular function. Cutting-edge microfluidic cell culture models for studying angiogenesis have successfully incorporated principles from quantitative analyses of vascular function, *in vitro* flow chambers, microfabrication techniques, and 3D tissue scaffolds. Consequently, microfluidic approaches have enabled unprecedented levels of control of chemical gradients, fluid flow, matrix composition and cell-cell interactions, all of which can be integrated to provide a physiologically relevant context for studying angiogenesis.

12.1. Development and capabilities. Recent advancements in microfabrication and biological integration have helped propel the design and implementation of microfluidic systems, which comprise an emerging class of highly modular *in vitro* culture models of the microcirculation [161,162]. Microfabricated devices can provide cultured cells with a microenvironment similar to that *in vivo*, including the correct extracellular matrix (ECM) composition, chemical species, associations with other cells, and mechanical signals to mimic tissue- and organ-level function [163]. Interestingly, microfluidics allows to generate chemical gradients and enables to study EC chemotaxis in 3D geometry .These systems also contain networks of micron-scale fluid-filled channels that are similar in size and architecture to microvessels *in vivo* [164,165] to faithfully reproduce certain microvascular phenomena *in vitro*. Consequently, the application of microfluidics for studying angiogenesis has emerged as a major research thrust at the interface of microsystems engineering and biomedicine. At present, the majority of microfluidic systems are constructed by soft lithography or replica molding of an elastomeric and biocompatible polymer PDMS)[166]. This process is well-suited to create structures with defined shapes on the micrometer scale that can be used to position cells and tissues, control cell shape and function, and create highly structured 3D culture microenvironments [167-169]. Researchers have used this capability to assess the influence of the host tissue environment and heterotypic cell-cell interactions on angiogenesis [170] by co-culturing vascular ECs organized as an intact vessel structure with other cell types such as cancer cells [171-174], stromal fibroblasts [175,176], vascular smooth muscle cells [177], and bone marrow cells [178]. Applications for these microfluidic co-culture studies include fundamental studies of cell population behaviors, high-throughput drug screening, and tissue engineering [170]. Application of microfluidic systems has also contributed significantly to our understanding of flow-mediated angiogenesis (**Fig. 10**). For instance, multiple studies have shown that interstitial flow potently induces angiogenesis [179-181] and more recently lymphangiogenesis [182,183]. These studies have also shown that the vessel sprouts triggered by interstitial flow preferentially form against the direction of flow [182,179-181]. In other words, vessel sprouts tend to originate from a local pressure minimum and seek the higher pressure vessel or source [180]. Interestingly, this same sprouting behavior was recently observed *in vivo* in avian embryos [184]. In addition to interstitial flow, intravascular shear stress has been shown to control sprouting angiogenesis [185,180] and anastomosis of vessel sprouts [186]. Collectively, these findings suggest that the local fluid mechanical environment can both determine whether sprouting occurs and specify where these sprouts originate from the parent vessel. Moreover, the ability of microfluidic system to apply specific levels of fluid mechanical stimuli in a highly controlled environment will be invaluable towards advancing the mechanobiology of angiogenesis [187].

One limitation to PDMS replica molding is that this process produces microchannels and subsequent endothelial-lined vessel structures with rectangular cross-sections. To address this limitation, alternative

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fabrication techniques have been used to form engineered microvessels with circular cross-sections. The most widely used technique to fabricate this type of microchannel is to cast a 3D scaffold housed within a PDMS chamber around a cylindrical needle or rod of approx. 100 µm in diameter [188]. Once the scaffold has polymerized, the needle or rod cast is removed leaving an open cylindrical microchannel embedded within the 3D scaffold. Scaffold embedded circular microchannels have been used to monitor focal leaks in the endothelial-lined microchannels, by adapting imaging techniques of fluorescent tracer dyes conjugated to macromolecules (e.g. albumin or high molecular weight dextran) that are widely used in intravital microscopy techniques for measuring vascular permeability *in vivo* [189,190]. In addition to quantifying vascular barrier function, circular microchannels have been used to measure vessel sprouting and anastomosis [191].
Since the fabrication of circular microchannels employs a non-lithographic technique, it loses some of the desirable attributes achieved with soft lithography such as rapid prototyping and scalability for high-throughput [164]. In addition, circular microchannels require casting and removal of a cylindrical needle or rod inside a hydrogel, thus they are constrained to simple linear structures [192]. To address the aforementioned limitations of the cylindrically casted microchannels, another circular lumen fabrication technique was recently developed that uses the principle of viscous finger patterning of pre-polymerized hydrogel specified inside PDMS microchannels of different geometries, including branched networks that mimic the topology of bifurcating microvessels [193]. Another hydrogel embedded microfluidic model that is of significance uses micropatterned stencils to produce microvascular network inside natural 3D hydrogels such as collagen and alginate [194,195]. Finally, the recent advent of 3D printed microfluidic vessel networks [196] have enabled the patterning of more complex branching networks that mimic *in vivo* physiology [197].

12.2. Advantages of microfluidics for angiogenesis studies.
The use of chip supported by a microfluidic system for EC culture represents specific advantages. Compared to *in vivo* studies, these systems are relatively straightforward to apply controlled perturbations of extrinsic cues, such as fluid shear stress and biomolecular gradients. Furthermore, in contrast to conventional *in vitro* angiogenesis assays, vessel structures formed in microfluidic systems can be readily perfused to evaluate the effects of fluid flow on vessel maturation. The feature of these systems enables the visualization and the precise quantification of vessel function (e.g. MVD and vascular permeability) in response to various extrinsic cues and allows the construction of distinct tissue compartments (e.g. vascular and perivascular). Moreover, the cells that comprise each tissue compartment can be independently genetically modified to reproduce conditional knockout studies *in vitro*. Interestingly, the material that is typically used for microfluidic devices (PDMS) is fairly inexpensive and enables rapid prototyping and the total volume of biological reagents (e.g. cells, ECM, and culture media) that is required for microfluidic experiments are very low and on the order of microliters. Finally these device can be further implemented with optical sensors for analytical measurements. Altogether these features enable *in vitro* screening platforms for the efficacy of candidate drug compounds in a physiologically relevant setting.

12.3. Limitations and challenges. Microfluidic technologies typically requires specialized fabrication techniques and makes use of challenging complex 3D geometries that require multi-layer construction. These technologies are beyond the research expertise of most biology laboratories. Moreover, despite the beneficial properties of PDMS (e.g. biocompatible, elastomeric, optically transparent, gas and water permeable), there are also several important limitations such as leaching of uncrosslinked oligomers and non-specific absorption of hydrophobic molecules that can critically affect cell-signaling dynamics [198,199].

12.4. Concluding remarks. Existing and emerging technologies for casting, molding or 3D printing are changing the way we study cells in vitro. These methods bridge the gap between tissue culture dishes and animal

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models, allowing more informative and representative coculture systems. Future development may allow recapitulation of additional aspects of vascular biology in vitro such as intussusceptive splitting or network remodeling.

13. Loss-of-function approaches in the developing zebrafish.

In recent years, the zebrafish has become a highly utilized vertebrate model system for studying vascular development and angiogenesis *in vivo*. Vascular development in zebrafish is remarkably similar to that in mice and humans in terms of both anatomy and function. Zebrafish are genetically accessible with externally fertilized, optically clear embryos and larvae that permit high-resolution imaging of developing tissues. Fish also have large clutch sizes for experimentation and their embryos can survive and continue to develop for several days without a functional circulation via passive diffusion of oxygen. Although the beneficial experimental attributes of zebrafish have made this an extremely appealing and approachable model system, like any model organism there are limitations to the studies that can be performed and their interpretation. In this report, we focus on loss-of-function approaches used to study vascular development in the zebrafish, and some of the pitfalls in interpreting data generated using currently available approaches.

13.1. s. Prior to the advent of genome editing technologies in the zebrafsh, other than mutations fortuitously isolated in forward-genetic screens, morpholinos (MOs) were the only available reasonably well-validated loss-of-function method. MOs are specially modified stable antisense oligomers designed to block either gene translation or gene splicing when injected into embryos [200]. In the absence of reverse genetic tools, MO-based experiments rapidly gained popularity in the fish community. Although the advent of this technology was a huge step forward for the zebrafish community, over time many concerns have been raised about the specificity of phenotypes generated from MO injections, particularly the potential for off-target and/or pleiotropic effects from MO injection [201,200,202,203]. These concerns have been greatly heightened by mounting evidence that MOs and genetic mutants for the same genes frequently do not yield comparable phenotypes [201]. In order to promote greater validity of experimental studies using MOs, attempts have been made to develop standards for their use. One recently promulgated set of “community guidelines” suggests use of multiple MO targets, RNA/DNA rescue experiments, dose response curves for titration of the MO, and, importantly, validation with a genetic mutant where possible [200]. It is generally accepted that proper MO use requires rigorous confirmation of their specific blocking ability, either by assessing loss of protein production for translation blocking MOs (if specific antibodies are available) or by assessing the generation of alternate splice variants by RT-PCR when using splice blocking MOs. MO based experiments can also be “rescued” by co-administration of mRNA or transgenes expressing a wild type copy of the targeted gene. However, the best and generally accepted validation for any MO phenotype is confirmation of the same phenotype in a zebrafish genetic mutant. As discussed further below, many mutants are now available from forward-genetic N-ethyl-N-nitrosurea (ENU) mutagenesis screens, “tilling” approaches, or genome editing methods. CRISPR technologies have made it easy and straightforward for any lab to carry out reverse genetic mutation of virtually any gene of interest [203-224]. In short, when possible, all MO experiments should be confirmed and validated with a genetic mutant before they are used extensively for experimental studies.

Common off-target effects noted in MO-injected animals include p53-mediated cell death, defective circulation and “ballooning” (edema), and developmental delay/stunting of embryos and larvae. Altered vascular development and patterning should be interpreted with extreme caution in the presence of any of these phenotypes, as vessel growth and patterning in developing embryos and larvae is highly dependent on the integrity of adjacent tissues and of the animal as a whole (see below for further detailed discussion). The recently published community guidelines noted above include recommendations on how to interpret MO phenotypes, how to validate these phenotypes, and current expectations in the field for MO usage[200].

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Although MO approaches, and especially their use without proper validation, have justifiably come under a great deal of scrutiny (if not outright skepticism) in recent years due to well-documented frequent off-target effects and lack of correlation with mutant phenotypes, it is worth noting a few positive/beneficial features of MOs that make their continued use (with caution) worthwhile. First, translation-blocking MOs can block both the zygotic and maternally supplied activity of target genes, whereas maternal-zygotic mutants can be difficult or impossible to obtain. Second, MOs can be used immediately with any zebrafish line or strain unlike mutants, which need to be crossed into the appropriate genetic backgrounds (when using combinations of mutants and transgenes, this can take many generations). Third, multiple MOs can be injected simultaneously to target two, three, or more members of a gene family with potentially overlapping functions. Fourth, recent work has shown that up-regulation of related compensating gene family members can sometimes occur in genetic mutants (by mechanisms that are not yet clear), while this does not appear to take place in MO-injected animals[202], arguably making MOs a better representation of targeted loss of gene function in these cases. However, if used very extensive additional work is needed to validate all interpretation and distinguish phenotypes from off-target effects.

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13.2 *Genetic mutants*. The ability to carry out large-scale forward-genetic phenotype-based ENU mutagenesis screens for developmental mutants has been one of the major strengths of the zebrafish. Large numbers of mutants have been isolated affecting almost every conceivable developmental process, and these mutants have led to innumerable important new discoveries [225-232]. Forward genetics has been an extremely powerful approach for identifying genes necessary for developmental processes, but until recently reverse-genetic approaches for targeting specific genes of interest were not available in the zebrafish. “Tilling” approaches employing high-throughput next generation sequencing of libraries of mutagenized fish have been used to screen for ENU-induced mutations in specific genes [233] but these approaches are relatively laborious and expensive, and some genes are not as easily mutagenized. More recently, genome editing technologies, and particularly CRISPR/Cas9-based methods, have made reverse-genetic targeting of virtually any gene easy and cost effective for most labs [204-206]. A number of resources now make identifying “ideal” CRISPR cut sites, primer selection, and mutation screening relatively simple [204,234]. Generating specific alleles by homologous integration is still relatively challenging in the zebrafish, but the technology and resources to generate “knock-in” mutants using is also rapidly improving and is likely to soon become accessible to most zebrafish labs [206].

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Although CRISPR mutants offer a simple and effective approach for loss-of-function analysis free of many of the caveats regarding off-target effects associated with MO use, the resulting phenotypes must still be interpreted rigorously. Vascular phenotypes observed in animals that display significant cell death, defective circulation, edema, developmental delay/stunting of embryos and larvae - while they result from defects in the targeted gene - may represent secondary, indirect consequences of the genetic mutation and may not reflect a direct requirement for the targeted gene during angiogenesis. It is also worth noting that transient CRISPR approaches involving, for example, injection of guide RNAs into animals transgenically expressing Cas9 in specific tissues (“CRISPRi”) are subject to the same general concerns regarding off-target effects as MOs, although the spectrum of off-target effects observed with the two approaches will likely be distinct. Phenotypes observed in either MO- or CRISPR-injected animals must be interpreted with caution, and ideally should be verified using a stably transmitted germline genetic mutation.

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The ease and efficiency with which CRISPR mutants can be generated also makes it possible to simultaneously induce and screen for mutations in multiple genes at the same time. This is advantageous for studying the role of gene families in the zebrafish. Gene paralogues are more common in zebrafish than in mammals due to a genome duplication that occurred many millions of years ago during the evolution of teleost fish, and genetic compensation by alternate gene family members can lead to obscured phenotypes[202]. It

has become more common to see reports in which double or even triple mutants have been generated in closely related gene family members[235]. As noted above, compensatory up-regulation of related family members has been shown to occur in at least some genetic mutants[202], and phenotypes comparable to those noted in MO-injected animals have been unmasked by generating double- or triple-mutants.

13.3. Assessing vascular phenotypes in loss-of-function models. Whether MOs or mutants are used, assessment of vascular phenotypes involves a number of specialized considerations, since proper vessel formation can be altered or disrupted by developmental delay or by changes in circulatory flow and local or generalized defects in non-vascular tissues. Below, we discuss some general considerations in assessing vascular phenotypes, and point out some potential pitfalls in utilizing and interpreting zebrafish vascular data.

13.4 Where is the Gene Expressed? One of the first considerations in assessing the vascular function of a particular gene, whether using MOs or mutants, is where is it expressed? This is generally assessed using whole mount ISH of zebrafish embryos and early larvae[236], although in older animals ISH of tissue sections can be used. If a gene shows an exclusively vascular expression pattern (**Fig. 11a-b**), the gene may have a vascular-specific function, and vascular phenotypes observed likely reflect a vascular-autonomous role for the gene. On the other hand, if the gene shows an exclusively non-vascular expression pattern (**Fig. 11c-d**), it is not reasonable to assume that the gene has a vascular-autonomous function, and any vascular phenotypes that are observed are likely indirect. In many cases expression is observed in both vessels and in non-vascular tissues (**Fig. 11e**), and some common–sense judgement must be applied in assessing whether observed loss-of-function phenotypes make sense in terms of the expression pattern of the gene. If significant non-vascular expression is observed, additional experimental approaches will be needed to assess the cell-autonomy of gene function, including transplantation experiments or tissue-specific transgenic expression to “rescue” the phenotype (**Fig. 11f-h**).

13.5 Assessing General Morphology and Development in Morphants or Mutants.
Apparent vascular-specific phenotypes often occur as secondary, non-specific consequences of general developmental delay and other localized or general changes in non-vascular tissues or organs. Staging tables are available for the zebrafish [217] and it is important to determine whether MO injections or genetic mutants result in either overall delays to development or gross embryonic/larval patterning, or localized defects in the development and morphology of specific tissues or organs (**Fig. 12a-b**). For morphants, this should be done by comparing animals injected with specific MO to siblings injected with a control MO and uninjected siblings. For mutants, they should be compared to phenotypically wild type siblings from the same clutch of eggs. In either case, if the overall development of the animal is significantly delayed or readily apparent gross morphological changes are noted compared to the control sibling animals, vascular phenotypes noted in these animals should be interpreted with caution. For example, reduced head size and/or extensive cell death in the central nervous system are common off-target effects noted in both mutant and morphant models, and this secondarily results in vessel defects in the heads of affected animals (**Fig. 12g-h**). The timing and extent of trunk intersegmental vessel growth is frequently used as a convenient and quantitatively robust assay for assessing angiogenesis defects in zebrafish embryos, but delayed development or abnormal formation of the adjacent somites results in defects in vessel sprouting and growth that, again, are not directly linked to vascular-specific functions (**Fig. 12c-f,i-j**).

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13.6. Assessing Circulatory Flow and Cardiac Function. Even in animals with apparently normal overall development, defective blood flow (due for example to heart-specific defects) can affect the timing and extent of vessel growth. Assessing blood flow and cardiac function in the zebrafish is easily accomplished by direct imaging using a dissecting light microscope. For higher resolution, tracers injected into the blood stream such as quantum dots, fluorescent microspheres, lectins, and varying molecular weight fluorescent dextrans can be used to assess blood flow rates, blood flow directionality, vascular leak, vessel drainage sites, and solute uptake [237-240]. Although fish can survive a number of days in the absence of blood flow, animals lacking circulation do eventually become sick, edemic, stunted in growth, and die. If a mutant or MO experiment generates fish with no blood flow or decreased cardiac output, assessment of vascular phenotypes should take place as early as possible during development, during a time frame in which the animal is as healthy as possible. Although embryos and early larvae can develop reasonably normally for one or two days in the absence of circulation (**Fig. 12k-l**), overall vascular development becomes increasingly affected as time goes by (**Fig. 12k-p**). As a general rule, phenotypes in animals deficient in flow should be assessed prior to 6 days post fertilization (dpf), and ideally before 3 dpf, particularly when flow is entirely absent. As noted for animals with general developmental delay or morphological defects, caution should also be taken in interpreting vascular phenotypes observed in animals that lack circulatory flow.

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Quantitative assessment of blood flow and hemodynamics can be accomplished using Particle Velocimetry (PV), in which tracer particles injected into the blood stream are tracked through different vessels [241] and flow speed determined by calculating the displacement of the particles over time [242]. Due to depth and/or the decreased transparency at later stages, PV has mainly been used in embryos or early larvae, although recent advances in confocal microscopy, ultrasound, and tomography tools [243] and methods have facilitated deeper imaging in more opaque tissues.

13.7. Assessing Vascular Patterning. Visualization and assessment of vascular patterning in the zebrafish is easily accomplished using widely available transgenic fluorescent reporter lines labeling vessels. Numerous transgenic zebrafish lines are available with cytoplasmic, nuclear, or membrane-localized fluorescent proteins expressed specifically in cardiovascular cells and tissues [244-246]. These include lines marking the heart endocardium and myocardium, blood and lymphatic vessel endothelium, hematopoietic derived cells, and perivascular cells like pericytes and fluorescent granular perithelial cells. Long-term time-lapse imaging methods have also been developed that permit continuous, real-time imaging of heart and vascular development in these transgenic lines [247]. Together, the availability of fluorescent transgenic reporter lines and methods for high-resolution, dynamic imaging of these lines has revolutionized the study of cardiovascular development.

Alternatively, or in addition, intravascular injection of fluorescent tracers such as quantum dots, fluorescent microspheres, or fluorescent dextrans, or non-fluorescent tracers such as India ink, Berlin blue, or Evans blue dyes can be used to visualize zebrafish blood vessels and their patterning [248,249,237,250,239,251,247,252]. Although the wide availability of vascular-specific transgenic lines has reduced the need for these traditional methods for examining the patterning of developing vessels, microangiography is still the method of choice for assessing vascular integrity, vessel lumenization, and flow (as noted above) in blood vessels, as well as permeability, drainage and solute/fluid uptake in lymphatic vessels. The microangiographic techniques are fast, robust, and cost effective methods for visualizing the vasculature, and can be used to visualize vessels in non-transgenic animals or at more mature developmental stages.

The patterning of vessels in genetically or experimentally manipulated animals can be examined for alterations including reduced/absent vessel growth, excessive vessel growth or branching, altered patterning of vessels, changes in flow patterns/vascular connections, etc. Changes can occur either broadly throughout the animal or may be localized to particular vascular beds. Up to approximately 7 dpf the pattern of blood vessels observed

should be compared to the published staged atlas of vascular anatomy [248]. Although the positioning of major vessels (e.g. dorsal aorta, cardinal vein, and intersegmental vessels in the trunk, or basilar artery in the head) is relatively invariant in normal animals at given stages, the precise location and paths taken by many smaller and/or later forming vessels (e.g. the central arteries of the hindbrain) is less stereotypic, and minor changes in the patterning of these vessels may not be significant. As noted above, it is important to assess vessel defects in genetically or experimentally manipulated animals in the context of the animal as a whole, including whether any developmental delay or gross morphological defects are present. The timing and extent of intersegmental vessel sprouting is frequently used to assess angiogenic phenotypes in developing zebrafish, but their patterning can be strongly affected by developmental delay, non-vascular morphological defects, and reduced or absent circulatory flow (**Fig. 12**).

13.8. Concluding remarks. A variety of approaches for reverse-genetic targeting of specific genes for loss-of-function analysis are now available in the zebrafish, most notably MOs and CRISPR-generated mutants. Although MOs have been somewhat discredited in recent years as a "first line" tool for assessment of loss-of-function phenotypes, they remain valuable when validated by a corresponding mutant with the same phenotype. Furthermore, in some cases mutants are susceptible to genetic compensation by up-regulation of related genes, while morphants are not. Detailed "community guidelines" for use of MOs were recently published and should be referred to [200].

Any researcher generating loss-of-function vascular phenotypes in the zebrafish (or in any other model organism, for that matter) needs to critically assess their data in light of some key questions. Where is the gene expressed? Do mutant or morphant phenotypes make sense when compared to the expression pattern of the gene? The more "vascular specific" expression is, the more likely the associated vascular phenotypes are specific. Conversely, if a gene is ubiquitously expressed throughout the animal, the burden of proof is on the researcher to determine the vascular cell-autonomous function of the gene, using transplantation, tissue specific transgene "rescue" experiments, or other methods. Do mutants or morphants show significant developmental delay or gross morphological phenotypes, and do the vascular phenotypes most likely reflect developmentally "younger" animals or problems in non-vascular tissues? Do the mutants or morphants have absent or strongly reduced circulatory flow? Are vessel phenotypes being assessed in these animals at a time point prior to lack of flow causing significant general defects in development? Taking all of these criteria into consideration when assessing vascular phenotypes resulting from gene knockdown or genetic mutants is important to ensure that reliable, valid conclusions are made when studying regulators of angiogenic development utilizing the zebrafish.

14. Chorioallantoic membrane assays

Although the chorioallantoic membrane (CAM) assay appears to have been first reported by Rous and Murphy in 1911 [253], more than a century ago, to study xenoplastic growth of mammalian tumors, the chick embryo itself has been a target of scientific study beginning with Aristotle. Due to the fact that the fertilized chicken eggs are readily available and the embryos are easily visualized, chicken embryos became a favorite target for experimental biologists and, not surprisingly, the CAM assay became one of the classic procedures for students, being included in one form or another in virtually every college laboratory manual. Due to these advantageous properties the CAM has been widely used not only for direct vascular biology studies, but also in bioengineering, cosmetics testing, transplant biology, drug development, vaso-occlusive therapies or cancer research [254-261]. There are certainly several hundred variations in the experimental protocol, and while all of them involve placing a test material on the CAM or its intravenous injection, the variables – largely unbeknownst to the user – lead to highly significant differences in results and a striking diversity in observations and conclusions. Several aspects of the diversity are dealt with below.

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14.1. In ovo CAM assays. The “standard” (i.e. ”classic”) assay consists of exposing the CAM in the incubated egg by making a window through which the CAM can be accessed and placing a test graft, implant or substance on, in, or through it. After resealing the window, periodical examination of the test area can be performed, ultimately measuring the result, frequently by harvesting the area for chemical or histological analysis.

Incubation temperature. The standard Hamburger and Hamilton stages of chick embryo development for stages 14-35 were based on incubation at 39.4 degrees using White Leghorn chickens [262]. The other stages were based on incubation temperature of 27.5 degrees. Stage 33 (7 days) is when many investigators place a graft or sample on the CAM. This may sound trivial, but at stage 33 the CAM is slowing its growth. If the CAM is accessed on day 7 of an embryo incubated at 37 degrees (common in most laboratories), CAM cells are still rapidly proliferating [35]. Moreover, depending on the source and strain of eggs used there may be a 12-18 hour variation in developmental age.

Making a window. The original method used a hand-held saw blade, which worked well but frequently led to debris falling on the developing CAM. Such debris can provide artifacts difficult to distinguish from results due to planned experimental protocols. Ed Zwilling (in 1952) had a Dremel drill sanding disc in his garage which worked well and is now used nearly universally, but even so, debris, often too fine to see, can materially influence the results [35].

Placement of test material/graft. The CAM itself is, as indicated by its name, multilayered. Placing a filter or graft on the membrane is quite different from inserting it part-way (preferred by many) or penetrating the CAM completely so that it projects through the entire membrane. Results may be completely different depending on the mode of insertion as well as on the physical forces used [263]. A second variable is the choice of location on the membrane. The CAM at stage 30-35 is highly vascularized (hence a good choice for transplants), but there are vessels of all sizes. Choosing a site near a large vessel sets up conditions quite different from choosing a less vascular site. Sprouting will depend on placement as well rate of dispersion of test substances and the elution from discs and filter membranes. The reader is referred to several excellent comprehensive reviews that describe a wide range of modifications and adaptations of the basic CAM assay [254,264-269].

14.2 Ex ovo CAM Assays. Once it was demonstrated that one could carry out CAM assays in shell-less egg cultures [270,271], this modification became a staple variant of the assay. Again, there have been numerous improvements and variants, but all involve transferring the egg content at an early stage (day 2-3) into a container, allowing the CAM to develop and then carrying out procedures as in the standard *in ovo* protocol. The major modification from the original petri dish method is the use of a curved rather than flat-bottomed dish, and modifications have ranged from specially designed watchglass type vessels to plastic wrap loosely fastened over emptied food cans. Major advantages over *in ovo* methods are better visibility, potential to use transmitted light, ready application of several test sites, time lapse monitoring and multiple manipulations. Disadvantages include significant mortality, greater risk of infection, the need for more rigorous control of the incubation environment, and materially greater expense.

14.3. Assessing Vascular Patterning. Visualization and assessment of vascular patterning in the CAM model is one of the most challenging aspects of the assay. Quantitation of angiogenesis by appearance (number of vessels and their hierarchy, degree of branching, etc.), whether by simple inspection or quantitative image analysis, is highly variable. To obtain statistically-significant data requires multiple samples. Monitoring during the course of the experiment is often foiled by the spreading and hence moving of the test site from the observation window. Most importantly, many of the visible measurements cannot distinguish well between neovascularization and vasodilation or between inhibition and vasoconstriction. Several semi-quantitative or

quantitative imaging [272,273] or immunohistochemistry-based [274] methods for vascular and capillary pattering assessment have been developed. The descriptors include e.g. branching points/area, vessel or capillary density, or mean mesh size. Apart from intravenous injection of fluorescent dextrans [275,276] or non-fluorescent tracers such as ink [277,278], MRI and PET imaging, scanning electron microscopy or optical Doppler tomography are now available to facilitate the visualization of the CAM vasculature (see a comprehensive review [254]). For some of these techniques, however, the resolution might be an important limitation and should be considered prior imaging technique selection. Another point to take into account in imaging technique selection is the vascular development stage, vascular permeability and lymphatic uptake. Recently imaging was facilitated by the replacement of the original eggshell by an artificial one [279].

14.4. Limitations and challenges. The CAM assay is a model of developmental angiogenesis. Exponential growth of new blood vessels and capillaries occurs between embryo development day 5 and 9. For testing drugs or transplants it should always be realized that effects are observed in the context of this developmental background. After day 9 the CAM vasculature is fully established and allows investigation under conditions without this background angiogenesis. It should also be realized that the chicken model is genetically further remote from the human system, which in some cases induces difficulties using the CAM. Although the model is very attractive for many researchers, the chicken embryos hatch around development day 21. It is therefore recommended not to exceed day 18 for experimental testing. In some countries regulatory rules apply after development day 14-15, resulting in limitations of experimental testing for only 10-11 days. Nonetheless, there is little doubt that the classic CAM assay has produced a remarkable array of highly valuable information about neovascularization, inhibition of angiogenesis, normal xenograft behavior and tumor growth and development. Its relative simplicity, its avoidance of many of the regulatory rules placed on live animal research, as well as its low cost have made it a reasonable assay for preliminary screening. This limit is further enhanced by the low availability of antibodies that recognize chicken proteins. In the case of more detailed analyses, ISH could be a surrogate tool of immunohistochemistry and a collection of specific probes is available (http://geisha.arizona.edu/geisha/).

14.5. The CAM as a screening platform. The CAM model has been used for many different application in various fields of research. Many excellent papers and reviews summarize these applications, showing that each application demands the use of specific protocols (Table 2**).**

14.6. Concluding remarks. Even in the last three years close to a thousand publications have employed CAM assays, and thousands of CAM assays are used daily in experiments that fail to lead to publications. Whereas classically-trained embryologists are well aware of the complexity of embryogenesis and are almost overly cognizant of the problems encountered when working with a rapidly changing target, researchers less acquainted with the vagaries associated with developing embryos may need to develop a grudging respect for the intricacies underlying the CAM assay. This said, it is deservedly a most important technique, major contributions have resulted from its use, and no doubt it will continue to be an important tool in angiogenesis research. **Add Fig. 13.**

15. *In vivo* angiogenesis plug assay

When identifying angiogenic or anti-angiogenic factors, it is important to have a reliable *in vivo* assay for validation of the activity. A widely used in vivo assay is the in vivo angiogenesis plug assay making use of basement membrane extracts (BME) or Matrigel. This assay is reliable, easy to perform without the need for special equipment, reproducible, quantitative, and quick [280-282]. The assay is performed by injecting e.g. 0.1 ml BME/Matarigel in the subcutaneous space of a test animal. The liquid gellates to form plug, due to the body

warmth of the animal. In time, blood vessels will sprout into the plug. With two plug sites per animal, the setup is quick and allows for multiple test compounds or concentrations to be tested. Thus, drug screening can also be evaluated for effects on the activity of angiogenic or anti-angiogenic factors [283-286]. The drug can either be placed in the plug together with the test factor by mixing with the BME/Matrigel matrix or given to the host animal. Cells or even exosomes can also be tested when mixed into the gel for their production of angiogenic factors. Furthermore, the assay is highly adaptable. For example, the role of certain genes can be evaluated using genetically modified (over-expressing or knock out) mice, or animal models of diseases, such as aged or diabetic animals. Also, the effect of certain drug treatments given to the mice to modulate angiogenesis, can be evaluated. BME/Matrigel is a mixture of basement membrane components derived from an animal tumor [287,288]. It contains predominantly laminin-111, collagen IV, heparan sulfate proteoglycans and various growth factors. BME/Matrigel is a liquid at 4°C and gels at higher temperatures. It is used in various angiogenesis assays, including the tube formation, aortic ring, and plug assays.

15.1. The in vivo plug assay. In this assay, the test compound is mixed with a basement membrane extract at 4°C, known as BME/Matrigel, and then injected, while still cold, subcutaneously into mice where it will gel. After approximately a week, the plug is excised and assayed for angiogenesis by various methods, including histology, the drabkin assay (content of blood), content of fluorescent dextran that was injected intravenously just before plug harvest, etc. [286,285,283,281,280,284]. Complete protocols have been published [286,281].

The assay requires the use of BME/Matrigel without phenol and with low content of growth factors. -reduced. BME/Matrigel is thawed overnight on ice in the refrigerator, because it gels at a temperature above 4°C and is not usable. BME/Matrigel can be supplemented with an angiogenic inducer and it is mandatory to mix it well (do not vortex as bubbles will form) with the BME/Matrigel at 4°C without diluting the matrix as this reduces the gelling properties. The test mixture or the control without test materials should be cold when injected as it will gel quickly in the subcutaneous location due to the warmth of the animal. Injection is done with 1 inch 21-25 g needles, which are changed the after 2 to 4 injections as they may become dull. Mice (n=3) are slowly injected with 0.1 ml into both groin areas (**Fig. 14**) with the tip of the needle as far as possible from the injection site to prevent leakage. A bump should appear at the site where the test material is released from the tip of the needle. It is preferable to hold the syringe in place for about 30 seconds to allow the test material to gel. The syringe is gently rotated to remove the needle to help further seal the injection site hole.
At the end of the experiments, mice are sacrificed. For the harvest, a square segment of skin containing the plug is excised. Alternatively, the skin can be cut around the plug leaving plenty of space so as not to damage the plug. Then the underside is exposed. The plug is small and yellow in color but it may also be pink or red depending on the degree of angiogenesis. The control plug depending on the assay method will be colorless. The plug is safely and gently removed with scissors, embedded in HistoGel, and fixed for histology, followed by sectioning and staining with Masson's Trichrome. Most of the angiogenesis will be from the edges of the plug, and the quantitation can be based on the density of the vessels and/or extent of in growth toward the center of the plug. At least 3 fields per plug located at approximately the same distance from the edge of the plug are analyzed.Alternatively, Drabkin reagent can be used to assess to the amount of blood in the plug or fluorescein isothiocyanate dextran (Mw 150,000-200,000) can be injected into the tail vein of the animals and extracted from the plug for quantitation. However, these approaches can be flawed by either the presence of compressed non-perfused vessels, or by the presence of leaky blood vessel or hemoragic areas. Some researchers isolate RNA from the plugs and use qPCR of EC genes [289] as a quantitation assay.

15.2. Limitations and challenges. As the age and gender of mice can influence the results of this assay it is important to standardize these parameters. Young female C57BL6 mice are recommended as BME/Matrigel

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- The BME/Matrigel matrix should be phenol-free and be growth factor-reduced. ¶
- Thaw the BME/Matrigel in the cold, on ice in the refrigerator overnight is best. The matrix will gel at a temperature above 4°C and not be usable. ¶
- Plan on 100 µl per injection site. ¶
- Plan on at least six injection sites (3 animals) and be sure to include controls with no test materials but containing the vehicle.¶
- When adding the test material be sure to mix it well (do not vortex as bubbles will form) with the BME/Matrigel at 4°C and try not to dilute the matrix as this will reduce the gelling properties.¶
¶
Key steps during the injection¶
- Bring the test mixture and syringes on ice to the animal room.¶
- The test mixture should be cold when injected as it will gel quickly in the subcutaneous location due to the warmth of the animal.¶
- Use 1 inch 21-25 g needles and change the needle after 2 to 4 injections as it may become dull.¶
- Holding the mouse upside down with one hand, inject 0.1 ml into both groin areas (**Fig. 14**) with the tip of the needle as far as possible from the injection site to prevent leakage. A second person can help by holding each leg away from the body during the injection. Inject slowly. A bump should appear at the site where the test material was released from the tip of the needle.¶
- Hold the syringe in place for about 30 seconds to allow the test material to gel.¶
- Gently rotate the syringe as you withdraw the needle to help further seal the injection site hole. Some leaking will occur.¶
¶
Key steps in harvesting and in analyzing the plug¶
- Animals may be sacrificed for the harvest, but care should be taken not to externally damage the plug area.¶
- For the harvest, a square segment of skin containing the plug can be excised. Alternatively, the skin can be cut around the plug leaving plenty of space so as not to damage the plug. Then the underside is exposed. Note the plug is small and yellow in color. It may also be pink or red depending on the degree of angiogenesis. The control plug depending on the assay method will be colorless.¶
- The plug can be safely and gently removed with scissors, embedded in HistoGel, and fixed for histology, followed by sectioning and staining with Masson's Trichrome.¶
- The skin or plug sample can then be analyzed by histology. Most of the angiogenesis will be from the edges of the plug, and the quantitation can be based on the density of the vessels and/or extent of in growth toward the center of the plug. Count at least three fields per plug located at approximately the same distance from the edge of the plug.¶
- Alternatively, Drabkin reagent (D5941, Sigma Aldrich) can be used to assess to the amount of blood in the plug or fluorescein isothiocyanate dextran (Mw 150,000-200,000 Sigma Aldrich) can be injected into the tail vein of the animals and extracted from the plug for quantitation. However, these approaches can be flawed by either the presence of compressed non-perfused vessels, or by the presence of leaky blood vessel or hemoragic areas. Some researchers isolate RNA from ... [11]

originates from this mouse strain. Immunodeficient can also be used but are more costly. Female mice are recommended because they are easier to handle and do not bite as much as the male mice. It is important to note that there can be considerable variability in the plug responses under the same treatment so the use of 3 to 6 mice per data point is recommended, with two injections in each mouse.
The BME/Matrigel should be handled according to the instructions. If the BME/Matrigel has gelled or contains particulate matter after thawing, it should not be used. This may interfere with the even distribution of the test material and can yield artefacts. When mixing the test material with the BME/Matrigel it is not recommended to vortex, as bubbles may be formed that will distort the gel in vivo. Letting the mixture stand on ice for 10 minutes will reduce bubbles. Diluting the BME/Matrigel matrix with too much test substance volume will reduce the gelling capacity and therefore the ingrowth of blood vessels. Purchasing BME/Matrigel that is at least 12-14 mg/ml will reduce this problem. A common problem may be that in certain cases the plug may be hard to see when harvesting. If this happens it may be best to take the larger area of skin around the injection site for histology. It should be noted that fixation can affect some epitopes, therefore frozen sections may also be considered. HistoGel (Thermo Scientific) is recommended for preservation of the fragile plug before fixation. Cracks or bubbles in the matrix may require selection of areas in the plug that are clear of these artifacts.
An adaptation of the assay is an easy to use kit: DIVAA, Directed In Vivo Angiogenesis Assay, Trevigen Inc., that avoids many of the injection and artifact problems [290].

15.3. Concluding remarks. Among the various *in vivo* angiogenesis assays, the plug assys is the most widely used because it is the most versatile, least costly, and easiest to perform. Furthermore, results are available within a week. Care must be taken at all steps in the assay, including in the selection of the recipient mice, handling and injecting the BME/Matrigel, harvesting the plug, and counting the vessels.

16. *In vivo* murine model for vasculogenesis using human endothelial and mural cells.
In this model, human ECs and human mesenchymal cells form perfused vascular networks as rapidly as 4 days after sub-cutaneous injection into immune-deficient mice, providing an in vivo system to study human blood vessel formation and function with the option to manipulate the human cells in vitro prior to implantation. Human ECs are suspended in a liquid ECM or hydrogel with mesenchymal cells that are capable of differentiating into perivascular mural cells and injected into immune-deficient mice (**Fig. 15a**). The selected ECM or hydrogel should form a gel soon after injection to confine the cells. The vascular networks formed within the implant are lined with human ECs and surrounded on the abluminal side by human mural cells [291-294] (**Fig. 15b**). The human ECs can be ECFC isolated from umbilical cord blood, adult peripheral blood or adult white adipose tissue, HUVECs, HDMEC or ECs differentiated from hiPSC [291,292,295,296]. The human mural cell source can be saphenous vein smooth muscle cells [291], mesenchymal progenitor or stem cells (MPCs, MSCs) from bone marrow or cord blood [292,297], or adipose stromal cells[298]. Murine MSC from bone marrow, white adipose tissue, skeletal muscle, and myocardium have also been used [299] as well as the mesenchymal precursor 10T1/2 cell line [300]. A variety of ECMs and hydrogels support formation of human vascular networks including Matrigel [291], type I collagen [293], fibrin [293], PuraMatrix [293] and the photo-crosslinkable gelatin methacrylate [301]. In summary, this two-cell model for building human vascular networks in the mouse is versatile, rapid and relatively simple to perform.

16.1. Background / History. Seminal studies by Schechner and colleagues [302,303] set the stage for the model described above. They suspended HUVECs in collagen/fibronectin gels wherein the HUVECs formed tubular structures in vitro within 20 hours. They then placed gel pieces by incision into the abdominal wall of severe combined immune deficiency mice. Human EC-lined vessels were detected in the implants by 30 days but the investigators found that HUVECs transduced with the anti-apoptotic gene Bcl-2 were better able to

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survive and attract host α-smooth muscle actin (αSMA) positive mural cells. After 60 days, basement membrane deposition, EC-EC junctions and TNF-α induced E-selectin and VCAM-1 were evident [303]. In summary, HUVECs formed well-developed, functional vessels, albeit slowly. The next logical step was to supply mural cells to 1) avoid reliance on host mural cell recruitment and 2) as a source of growth factors for the EC. Including the murine mesenchymal precursor cell line called 10T1/2 significantly increased vessel density beginning at day 14 after implantation [304]. In summary, these early studies represented a "tissue-engineering approach:" pre-assembled human vascular networks transplanted into mice were able to connect with host vessels. George and colleagues showed that the in vitro incubation step significantly accelerated formation of anastomoses with host vessels [305], highly desirable for tissue-engineering applications.

16.2. Assay overview. We adapted these concepts to develop a rapid, simple and non-surgical model for the de novo formation of blood vessels – i.e. vasculogenesis. Our prototypical assay consists of 2-4 x 10^6 human ECFC + human bone marrow MPCs combined at a ratio of 2:3 and suspended in 200-250 microliters of ice-cold Phenol Red-free Matrigel for each implant. The cell/Matrigel suspension is injected sub-cutaneously using a 26 gauge needle; two implants are placed on each dorsal side of 6 week old athymic nu/nu mice. Matrigel forms a gel at 37°C such that cells are confined at the site of injection. Perfused vessels (~100/mm^2) are detected as early as day 4 and are long-lived [292,294]. Inclusion of 1ug/ml FGF-2 in the cell/Matrigel suspension significantly increases the number of perfused vessels detected on day 4 [306]. Negative controls include injecting the ECM or hydrogel without cells or with ECs alone. Detailed descriptions of the methods, including endothelial and mesenchymal cell isolation procedures are provided in these publications [292,69,306].

16.3. Visualizing and quantifying vessels. At desired time points, the cell/Matrigel implants are removed by making a dorsal incision adjacent to the implantation site to expose the subcutaneous space. The implants are 3-5 mm in diameter and easily distinguished from subcutaneous fat by shape, consistency and pink (vascularized) color (**Fig. 15**). Digital photographs of the implants provide initial primary data. The excised specimens are fixed in 10% neutral buffered formalin overnight, followed by paraffin embedding and sectioning (5-7 μm sections). Hematoxilin and eosin (H&E) staining is used identify red blood cell-containing lumens; empty lumens can be counted separately to track non-perfused vascular tubes[294]. This analysis will capture human vessels as well as host vessels that have sprouted into the gel to form connections with the human vessels[307]. Vessels are counted in 10 randomly-selected areas from two H&E-stained sections, ~150μm apart, for a total of 20 areas for each implant. Counters should be blinded to the experimental groups to reduce potential bias. Image analysis software such as ImageJ can also be used. As noted above, vessel density is typically between 50-150 vessels/mm^2.

16.4. Distinguishing human from murine vessels. Three strategies have been used to visualize and quantify human versus murine vessels in the implant. The first is to use human-specific antibodies such as the monoclonal anti-human CD31 antibody (clone JC70A, Dako, Agilent) that has been shown by numerous groups to specifically label human vessels in histological sections. The second approach is to label human ECs prior to implantation with green fluorescent protein or an equivalent fluorescent marker using lentiviral or retroviral constructs. Human mesenchymal cells can be labeled with a different fluorescent marker for dual in vivo tracking[292]. The third approach is to use plant lectins - *Ulex europaeus agglutinin I* (UEA-I) which is specific for human endothelium, and *Griffonia simplifolia* isolectin B_4 (GS-B_4), which has no reactivity with human cells because humans lack the α-linked galactose residue needed for GS-B_4 carbohydrate binding. Tail vein injection of rhodamine-labeled UEA-I and FITC-labeled GS-B_4 ten minutes before removing the implants will label perfused blood vessels and show the connections between human and mouse vessels within the

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implants [307] (**Fig. 15b**). Three distinct patterns can be seen: UEA-I-positive human vessels, GS-IB_4-positive murine vessels, and UEA-I/GS-IB_4-double positive chimeric vessels; each can be quantified and reported as vessels/mm^2 (**Fig. 15c**) [307-309]. Alternatively, biotinylated forms of UEA-I and GS-B_4 can be used to stain histological sections.

16.5. Longitudinal analyses. Vascular volume within the implants can be measured longitudinally by contrast-enhanced ultrasonic imaging using the Vevo 2100 high resolution system (VisualSonics Inc, Toronto, Canada) [294,307]. This requires imaging before and shortly after tail vein injection of an echogenic microbubble contrast agent, preferably with a syringe pump to achieve consistent levels of contrast among the different animals. To assess cell retention over time, luciferase-labeled human ECFCs (Lenti-pUbiquitin-firefly luciferase-GFP) allow one to visualize the location of ECFCs over time in individual mice [292,309,308]. The assay requires an intraperitoneal injection of the substrate luciferin and bioluminescence imaging 30-40 minutes later.

16.6. Advantages. The model is amendable to loss-of-function experiments: specific genes can be tested by shRNA/siRNA knockdown or CRISPR/Cas9 gene editing in ECs or mural cells, carried out prior to implantation. Conversely, specific genes can be expressed using retroviral or lentiviral constructs, providing a gain-of-function test strategy. Alternatively, ECs or mural cells can be treated with drugs prior to implantation, which restricts drug exposure to the cell of interest and avoids confounding systemic effects. These types of experiments have been used to study human vascular tumors and vascular malformations[310-312]. Furthermore, Melero-Martin and colleagues showed that ECFC can be genetically engineered to provide controlled release of a protein of interest from the human vascular networks. In their study, ECFC-lined vascular networks engineered to express erythropoietin increased erythropoiesis in the mouse[313], revealing the engineered network's potential to serve as a drug delivery device. ECs and mural cells, as well as host cells, can be retrieved from the implant and sorted into purified populations for molecular and biochemical analyses, or for transplant into secondary, recipient mice. Finally, ECFC and MPC will form human vascular networks in ischemic myocardium or ischemic hind limbs, and provide physiologic benefit[309,308]; this demonstrates that ECFC + MPCs are vasculogenic in settings beyond the subcutaneous implant site.

16.7. Limitations and challenges. Implanting human vascular cells necessitates use of immune-deficient recipient mice, which precludes the use of many available transgenic murine models. Murine ECFCs or a similarly robust and phenotype-stable murine EC would solve this; murine MSCs from four different tissue sources have already been shown to support ECFC vessel formation in vivo [299]. Therefore, it should be feasible to switch to murine ECs + MSCs for implantation into desired murine strains. Second, the model is not well-suited for high throughput studies because of the relatively large number of human cells required, the expense of immune-deficient mice, and the time-consuming analytical procedures. In vitro 3-dimensional microfluidic models in which perfusable human vascular networks form will provide a valuable intermediary between in vivo and 2-dimensional cell culture models [314].

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16.8. Concluding remarks. This two-cell model is technically simple, reliable and experimentally accessible; it provides a tested for vasculogenic and functional studies in vivo using human endothelial and mural cells. The human cells can be manipulated genetically and/or pharmacologically prior to implantation to address a variety of questions. Human cells (and murine) can be retrieved from the implants at desired time points for cellular and molecular analyses. Human EC from patients or ECs genetically engineered to express a human mutation can be used to model specific vascular pathologies.

17.Corneal Angiogenesis assays

In early 1970' The Folkman's group firstly set up an in vivo angiogenesis by exploiting the anatomical features of rabbit cornea and in particular the absence of blood vessel and the easy access to monitor the neovascularization by a slit lamp after implanting tumor cells or tissues or slow release pellets containing angiogenic inducers. This strategy was further extended to rodents (mouse and rats), which are smaller and less expensive than rabbits and allow studying specific molecular correlations by taking advantages of use genetic engineered mouse models. Corneal assays are instrumental to characterize angiogenic inducers and inhibitors, interactions between different factors and to study cellular and molecular mechanisms of angiogenesis. Interestingly corneal assay can also be used to study other biological processes. For example, studies of lymphangiogenesis were made possible through the implantation of low dose bFGF pellets, which allowed the visualization of lymphatic vessels through specific molecular markers

17.1 Assay overview

The protocol is based on the creation of a small pocket in the cornea to introduce tissue samples, cells or slow-release pellets incorporating growth factors. Surgical procedures are performed on anesthetized animals, in sterile conditions to avoid inflammatory reactions and with the help of a stereotaxic microscope.

Generally the resins used are etylen-vinyl acetate (Elvax 40W, 40% by weight ethylene-vinyl acetate comonomer content with a 'W' amide additive, Dupont) or a mixture of sucralfate and poly(2-hydroxyethyl methacrylate) (Hydron). To produce slow-release Elvax 40 pellets ,1 g of the polymer is washed in 100 ml of absolute alcohol for at least 15 days and then dissolved in 10 ml of methylene-chloride to prepare 10% casting stock solution (60 min in at 37°C). Two hundred microliters of this solution is layered on a teflon film and the methylene chloride is allowed to evaporate under a laminar flow hood. The resulting film of polymer is cut under a stereomicroscope into 1x1x0,5 mm pieces, which are used as implants. Ten implants (see above) are monitored for 14 days after implantation. The casting solution is eligible for the use when the implants performed with a specific casting solution do not induce reactive inflammatory injury at microscope and histological exam of the cornea. To incorporate the test substance, a pre-determined amount of casting solution is mixed with a given amount of the molecule and processed as above described. Alternatively , pellet is incubated with a solution of the testing molecule dissolved in sterile phosphate buffer solution for 40 min at room temperature in a plate rotator and then dryed in a laminar airflow hood under sterile condition .

Elvax-40 containing testing molecules can be stored up to one month at -80°C. Empty pellets of Elvax-40 are used as negative control, while positive control contains VEGF-A (200 ng/pellet).
In the case the experiment protocols require the use of cells or tissues, it is used to implant 5 μl of medium containing 2-5x10^5 cells, or a fragment of 2-3 mg of fresh tissue.

A second resin is Hydron stabilized by sucralfate. Ten mg of sucralfate are added to solutions of sterile saline (20-50 μl) containing the appropriate amount of testing substances (e.g. 0,05 mg VEGF-A as positive control) that are then speed vacuumed until the mixture is dry (30-60 min). To this suspension 10 μl of 12% Hydron in ethanol are added. This suspension is layered on a sterilized 1,5 x1,5 cm piece of nylon mesh (pore size 0,4x0,4 mm). Both sides of the mesh are covered with a thin layer of Hydron and allowed to dry in sterile conditions. Then the fibers of the mesh are pulled apart under a microscope, and only uniformly sized pellets of 0.4 x 0.4 x 0.2 cm are selected for implantation. All procedures are performed under sterile conditions. Such pellets can be stored at - 80°C for one month without loss of bioactivity.

Pellets are implanted in anaesthetized animals and the eye is further anesthetized with few drops of local anaestetic. A central incision (1,5 x 3 mm in rabbit; 1,5 x 1,5 mm in mouse or rat) is produced into the corneal 3 mm (rabbit) or 1-1,5 mm (mouse, rat) from the limbus and , using a von Graefe knife (2x30 mm), a

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micropocket is dissected to insert the pellet with forceps. This procedure allows the diffusion of test molecule in the tissue, with the formation of a gradient for the ECs of limbal vessels. The distance of the pocket from the limbus can influence the angiogenic response, which depends on the features of the angiogenic inducer

Observations of the implants is made with a slit lamp stereomicroscope over a set period of time on anaesthetized animals. Images are recorded for subsequent analysis. In rabbit an angiogenic response is positive when budding of the vessels from the limbal plexus occurs after 3-5 days and capillaries progress to reach the implant in 7-10 day. Implants with an inflammatory reaction or that are unable to induce angiogenesis within 10 days are discared. The angiogenic activity is evaluated on the basis of the angiogenic score calculated by the formula vessel density/cornea x distance from the limbus The number of positive implants over the total number is also scored.

In the mouse or in the rat the vascular response is measured as the maximal vessel length . By using the slit lamp microscope with an ocular grid, the y-axis of the reticule is located along limbal vessel directly beneath implant. It is possible to refer the eye as a clock with intervals from 1 to 12 and the measurement of the number of clock hours with new vessels is performed during each observation. Two measurements are obtained for each eye: the linear response representing the maximal vessel length (an average of the five longer vessels) extending from the limbus toward the implant and the circumferential response representing the neovascularization zone (measured in clock hours) . The vessel area is calculated according this formula: vessel length x clock hour x 0,2π.

At the end of the experiments cornea are fixed and analyzed for the presence of inflammatory cells and to investigate the vessels features.

17.2. Limitations and challenges

Corneal assays present advantages and disadvantages in different species. The rabbit's size enables an easy manipulation of both whole animal and the eyes and the inflammatory reactions are easily detectable as corneal opacity. However the experimental cost is higher in rabbit than in mouse or rat. Multiple observations are easier in rabbit than in mouse or rat and could be done in not anaesthetized animals. Furthermore the angiogenic response in mouse and rat is highly variable and a large number of animals is required. Thanks to its size the rabbit eye offers a large area for the placement of stimuli in different forms including the presence of two pellets. All species can be also used to test the effect of local or systemic drug treatment on the local angiogenic response. Obviously the systemic drug treatment of rabbit is more expensive than the treatment of mouse or rat.

There are some critical steps in performing a successful corneal assay. The first issue is the preparation of pellets, which need to be uniform, without any inflammatory activity and able to ensure a good distribution of the angiogenic molecule. Surgical procedure is another challenge issue of the assay and it is important to calibrate the appropriate depth of the incision to avoid the eye rupture.

In mouse, this assay permits to induce neo-angiogenesis on different genetic backgrounds, which can be useful to evaluate the effect of a specific gene on angiogenesis.

17.8. Concluding remarks

Corneal angiogenesis assay, which requires high technical competences, represents a powerful tool to monitor and quantify in vivo neo-angiogenesis, to test specific drug treatment and the effect of genetic manipulation. To possibility of a final histological examinations allows an initial description of the cellular mechanisms sustaining the process.

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18. Mouse Oxygen Induced Retinopathy Model.

Pathological angiogenesis plays a pivotal role in the most common causes of blindness including neovascular age-related macular degeneration (AMD) [315], polypoidal choroidal vasculopathy (PCV) [316] or diabetic retinopathy and retinopathy of prematurity (ROP) [317]. Animal angiogenesis models are an essential tool to understand pathways involved in physiologic and pathological angiogenesis for basic comprehension of the biology and to determine the basis for *in vivo* anti-angiogenic drug development in translational cancer and ophthalmic research. Moreover, they are critical in preclinical drug testing.

The oxygen-induced retinopathy (OIR) model was originally developed to reflect the pathological stages of human ROP, vaso-obliteration of retinal vessels with oxygen exposure (phase I), followed by vaso-proliferation (phase II). Oxygen exposure of neonatal dogs, cats, rats and mice creates a relative hypoxic state in the retina with stimulation of growth factors resulting in eventual vaso-proliferation as is seen in preterm infants with ROP [318]. A major distinction of this model is that mice, rats, dogs and cats are born with incompletely vascularized retinas, similar to humans born preterm allowing the study of retinal vascular development. Mice are the least expensive and most genetically manipulative animal model and therefore the most commonly used. Murine retinal angiogenesis occurs post-natally in a tightly regulated, predictable manner similar to humans allowing for manipulations seen post-natally in preterm babies such as oxygen exposure [317].

Since the introduction of the current mouse OIR model in 1994, it has been extensively used in many studies of angiogenesis and retinopathy. A google scholar search with the keywords “oxygen induced retinopathy mice” revealed approx. 45,000 publications with an increase of 9,500 publications in the last year, reflecting the feasibility and practicality of this *in vivo* model in many areas of vascular biology research. The major advantages lie in its reproducibility, affordable costs, short duration (10 days) and accessibility of the retinal vasculature for isolation, imaging and interventions [319,320]. Further the well-established platforms for creating systemic or conditional transgenic strains and access to a wide availability of recombinant proteins and antibodies make it a powerful tool for basic angiogenesis research.

18.1. Assay overview. The current OIR model was developed by Smith et al. [318] and optimized by Stahl et al. in 2009 [321]. In brief neonatal mice with nursing mothers are placed into 75% oxygen from postnatal day (P)7 until P12 and returned to room air (21% oxygen) from P12 to P17 (**Fig. 16a**). Due to the high pO_2 levels during the first hyperoxic phase (P7–P12) retinal vessels stop developing and immature capillaries in the central retina regress resulting in a central zone of vaso-obliteration (**Fig. 16a, b’**) [322]. Interestingly, the oxygen-induced vaso-obliteration (VO) develops very rapidly with peak VO 48 hours after onset of oxygen exposure [318]. The retina starts re-vascularizing slowly under remaining oxygen exposure from P9 to P12 [318,323], likely reflecting increasing oxygen demands of the developing retina. On return to room air at P12, the VO area becomes hypoxic [324] and significant upregulation of HIF-1-dependent, proangiogenic pathways including VEGF ensues resulting in neovascularization (NV) with its peak at p17 [325-327] (**Fig. 16a, b”**). A comprehensive step by step protocol of this mouse model has been published [328][13].

The two main quantifiable components in the OIR mouse model are the NV (maximum at P17) and VO areas. If a study is designed to determine the amount of vasculature lost during the hyperoxic phase the VO area can be measured at P12 or even as early as P8 by manually outlining the VO in an Image software’s such Adobe Photoshop (**Fig. 16c**). In most cases NV and VO are measured at P17 when abnormal vessel growth is at its peak. It has to be kept in mind that the VO area on P17 can differ between experiments with same VO area at p12 depending on the extent of vascular repair, which can be determined by examining the difference between VO at P12 and P17. The current method to quantify NV is the SWIFT_NV method which is a computer aided semi quantitative technique detecting NV tufts and clusters on retinal whole mounts using the free NIH software ImageJ [321]. In brief, using different macros retinal flat mounts are divided into 4 quadrants and background fluorescence is removed allowing NV structures to stand out clearly (**Fig. 16c’, c”**). This enables

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the user to set a fluorescent threshold marking NV, but not normal vessels. Artifacts can be manually excluded during this step. This method greatly improved efficiency, reliability and objectivity compared to the prior methods such as cross-sectional techniques and whole mount grading systems based on predefined characteristics.

18.2. Limitations and challenges. Despite standardization of the OIR mouse model over the last 23 years several essential factors can result in substantial phenotype variability if not controlled. One of these factors is postnatal weight gain (PWG) during the OIR period. A study from Stahl et al. [329] revealed that pups with poor postnatal weight gain (PWG) (defined as pups weighing 5g or less at P17) have a significantly delayed and prolonged vaso-obliteration and NV compared to medium (5-7.5g) and extensive (≥7.5g) PWG. Therefore, the weight of each pup at P7 and again at P17 should be measured. Mice below 6g and over 7.5g at P17 should be excluded from the analysis. To avert poor PWG during OIR several adjustments can be considered. We recommend avoiding large litter sizes over 8 pups by removing some pups (or if pups are too heavy by adding pups to maintain appropriate weight gain) and surrogating C57Bl/6 pups at P2 or P3 with S129 lactating mice due to their lower susceptibility to oxygen toxicity if weight gain is low. It is important to note that in our experience, nursing mothers are no longer fertile after exposure to OIR. Under hyperoxia exposure and especially after transfer to normoxia we found that mothers cannibalize their pups. Therefore, we recommend careful handling after return to normoxia as stress leads to less lactation and increased cannibalization. Another important variable, which needs to be considered, is the phenotype variability between different strains and even within one strain due to vendor-related substrain differences. These differences can be easily addressed by using the same strain from a single vendor. To correct for age and genetic heterogeneity as well as environmental differences the use of littermate controls is strongly recommended. When using transgenic mice, controls with exactly the same background will help to minimize strain dependent phenotype variability. Last, it has to be noted that the OIR mouse model does not cover all aspects of human ROP since mice are not born preterm even though retinal development occurs postnatally as it does with preterm infants. Thus, systemic factors resulting from preterm delivery are not taken into account, such as low IGF-1 seen after preterm birth in humans. Also, mice have no macula (the area of acute central vision) and have fewer cones than humans. However vascular development is very similar.

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18.3. Concluding remarks. The OIR mouse model is a robust, reliable and quantifiable model to investigate developmental and pathological angiogenesis when variables are carefully controlled.

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19. Laser-induced choroidal neovascularization mouse model

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Age-related macular degeneration (AMD) is one of the major causes of vision impairment in the elderly[330]. Although AMD does not lead to complete blindness, loss of central vision makes it difficult for patients to recognize faces, drive, or read. Nonexudative or non-neovascular AMD includes early and intermediate forms of AMD, as well as geographic atrophy, in which progressive loss of retinal cells leads to some loss of visual function. In 10–20% of AMD patients, nonexudative AMD progresses to neovascular AMD, which accounts for ~90% of AMD-associated vision loss with deterioration of central vision[331]. Neovascular AMD is characterized by choroidal neovascularization (CNV), with blood vessels from the choriocapillaris penetrating through Bruch's membrane into the normally avascular subretinal space[332]. However, in some patients, pathological angiogenesis develops from retinal vessels known as retinal angiomatous proliferation (RAP). *In vitro* EC culture models of CNV lack complex *in vivo* cellular interactions of photoreceptors, retinal pigment epithelium, pericytes, inflammatory cells and glial cells[333]. Therefore, reproducible animal models that mimic the pathological angiogenesis in the retina and choroid are needed to study AMD[334]. No models however mimic the aging aspects of the disease, nor do non-primates have a macula.

19.1. Laser CNV model advantages and limitations. A laser-induced *in vivo* model of CNV, first described in 1979, uses photocoagulation to disrupt Bruch's membrane, inducing the growth of new choroidal vessels into the subretinal area[335]. This model is similar to the majority of neovascular AMD in which pathological angiogenesis arises from the choroid. However, it differs from AMD as it is a wounding model unlike neovascular AMD that is initiated with aging changes. The laser-induced CNV model has been used successfully to predict the clinical efficacy of anti-VEGF therapy to suppress neovascular growth in AMD[336]. Laser-induced CNV has been used in mice, rats, rabbits, pigs, and non-human primates. Non-human primates' retinal anatomy (with a macula) is most similar to humans, but primates are costly to maintain, and rarely develop AMD and only do so after decades. Rodent models offer the advantages of lower cost but lack an anatomical macula[337]. Several gene-targeted mouse models, such as the very low-density lipoprotein receptor knockout (*Vldlr*−/−), super oxide dismutase (SOD) knockout (*Sod1*−/−), and apolipoprotein E (APOE) e4 transgenic mice, demonstrate some aspects of CNV such as spontaneous sub-retinal neovascular lesions[333]. However, these models are difficult to further manipulate genetically to determine pathways involved in AMD progression, whereas the laser-induced CNV model can be used in transgenic animals to explore the molecular mechanisms of CNV formation.

C57BL/6J mice are usually recommended for laser-induced CNV experiments, because only pigmented mice absorb laser energy well and respond reliably to laser burns. Previous studies suggest that both gender and age of animals influence the outcome of laser-induced CNV[338]. In one study, mice of both genders more than 12 weeks old developed more severe CNV than mice less than 8 weeks old. Gender difference was only significant in 12-16 weeks old mice, but not in the younger 6-8 weeks old mice[338]. However, Zhu et al. observed larger CNV lesions in female mice at 5–8 weeks old[339]. This discrepancy may be due to differences in analysis time points and fluorescent methods between the studies. Especially noteworthy, the older female mice developed significantly larger CNV lesions than both older male and younger female mice. The larger area of CNV in older female mice is suggested to be related to their high circulating levels of estrogen, which increases pro-angiogenic functions of both ECs and smooth muscle cells *in vivo* and promotes wound healing in both human and animal models Moreover, compared with the younger mice, the lesion area in the older mice had increased variability. These studies suggest that mice of either gender weighing 15-23 g at 6–8 weeks of age are optimal for the laser-induced CNV model for testing efficacy of drugs, although age and gender-matched mice may be essential for specific experiments.

19.2. Assay overview. The general procedure of laser-induced CNV induction involves careful mouse anesthesia, mouse positioning, laser burn, (with optional optical coherence tomography and fundus fluorescein angiography), eye dissection, choroid staining and imaging, and CNV lesion quantification (**Fig. 17a**). Only intact eyes (**Fig. 17b**) without observable structural or morphological abnormalities are used for the laser-induced CNV model. Eyes with anomalous structures (**Fig. 17c**), cataract or visible defects of the cornea or fundus are excluded. Mice are anesthetized with a mixture of xylazine and ketamine , and pupils are dilated with topical drops of Cyclomydril. Two minutes after pupil dilation, lubricating eye drops are applied to the cornea. Four laser burns at equal distance from the optic nerve (which optimally is approximately twice of the diameter of the optic nerve) are induced one by one in each eye by a green Argon laser pulse with a wavelength of 532 nm (**Fig. 17d**). The distance between laser burns must be at least double the diameter of the optic nerve to avoid fusion of lesions. Major retinal and choroidal vessels should be avoided to prevent potential bleeding. The formation of a vaporization bubble immediately after laser photocoagulation indicates the success of a laser burn, which correlates with rupture of Bruch's membrane. Optical coherence tomography immediately after laser photocoagulation may be used to confirm the success of the laser burn with visible rupture of Bruch's membrane (**Fig. 17e**). After laser photocoagulation, the eyes are gently rinsed

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with sterile saline to remove the lubricating eye drops and treated with an antibiotic ointment, erythromycin. Mice are then placed on a pre-warmed warming plate at 35°C after the laser treatment until they fully awakened. Mice with or without treatment can be subjected to fundus fluorescein angiography to evaluate the levels of vascular leakage from CNV lesions 6 days after laser burn (**Fig. 17f-g**). The *in vivo* retinal structure may also be examined by optical coherence tomography, if applicable, to determine the cross-sectional area of CNV lesions 7 days after laser burns. To measure the surface area of CNV lesions, the fluorescence-stained retinal pigment epithelium/choroid/sclera flat-mounts are imaged (**Fig. 17h-i**) and quantified by researchers masked to treatment. The choroidal CNV samples may also be analyzed for RNAs or proteins.

19.3. Limitations and challenges. The laser-induced CNV model in mice has been often characterized as variable and inconsistent. Establishing a set of consistent exclusion criteria is necessary for ensuring reliable data analysis. In a typical study, 10 mice per group with 4 lesions per eye would optimally provide 80 data points for each experimental condition. To account for data or mouse loss, including (1) cataract and corneal epithelial edema before laser photocoagulation, (2) unsuccessful laser burn without Bruch's membrane rupture, (3) odd lesion shape due to mouse movements during laser induction, (4) death of mice post-laser treatment, or (5) damage of the CNV lesions during tissue dissection and processing, more mice may be needed and should be considered in a power analysis to account for an anticipated intervention effect.
To accurately evaluate the laser-induced CNV, some lesions should be excluded. Severe hemorrhages will cause much larger CNV lesions, whereas choroidal damage will yield a CNV lesion much smaller than the fellow CNV lesions in the same eye. First, choroidal hemorrhages encroaching on the lesion should be analyzed and classified carefully: (1) if the diameter of bleeding area is less than that of the lesion, the lesion will be eligible for inclusion of analysis (2) if the diameter of bleeding area is more than that of the lesion but less than 2 times of the lesion diameter, the lesion should be excluded from quantification (3) if the diameter of bleeding area is more than 2 times the lesion diameter, all lesions in the same eye should be excluded from analysis. Second, excessive laser burns that damage not only Bruch's membrane but also the choroid and retinal pigment epithelium should be excluded. These excessive burns can be seen clearly as a solid "hole" in the bright field of choroid imaging. Lesions should also be excluded if (1) the lesion is fused with another lesion, (2) the lesion is either more than 5 times larger than the mean of the lesions under the same experimental conditions, or (3) the lesion is the only one eligible for statistical analysis among all lesions in an eye.
Previous studies provide the optimal settings and conditions to make use of the laser-induced CNV model for the goal of improving the consistency and reproducibility of experimental results for AMD and other pathological angiogenesis research[338].

20. Using transparent window preparations for angiogenesis studies in mice

Our ability to observe living tissues with the microscope improved dramatically in the 1920s when Sandison began implanting transparent windows over wounds in rabbit ears. This allowed him to view the underlying tissue non-invasively and longitudinally [340,341]. In the 1940's, Algire modified Sandison's method to visualize the dorsal skin in mice [342]. With this powerful tool, he was able to perform detailed studies of angiogenesis during wound healing and tumor growth. Intravital imaging techniques have provided unprecedented insight into tumor vessel formation and function [343-345]. By implanting transparent windows in various anatomical locations in the mouse, it is possible to observe angiogenesis associated with tumor development, wound healing and immune/inflammatory responses with high resolution, and in the native microenvironment. To date, transparent window preparations in mice have been developed to observe angiogenesis and lymphangiogenesis in the dorsal skin [346], brain [347,348], cerebellum [349,350], liver [351,352], lung [353], pancreas [354,355], breast [356] and lymph node [357], **Table 3**.

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20.1. Development and capabilities. Advances in intravital microscopy have come with the development of new window models, but also with innovations in microscopy. Multiphoton laser-scanning microscopes provide high resolution, three-dimensional images of vessel anatomy, gene expression and network topology, even in deeper regions of tumors [358]. More recently, optical frequency domain imaging (OFDI) has been applied in window chambers to improve depth penetration and contrast, providing unprecedented images of vasculature non-invasively and longitudinally [356]. Another imaging technology, short wave infrared imaging (SWIR), has been adapted from the defense industry to improve imaging depth penetration and reduce scattering [359].
The development of novel probes for improving contrast has also improved our ability to monitor angiogenesis in vivo [360,361]. Furthermore, advanced imaging technologies such as second harmonic generation imaging allow us to see important components of the angiogenic process such as extracellular matrix fibers [362]. This ability has improved our understanding of the involvement of matrix components in the angiogenic process [363,362,364].
Chronic window preparations allow analysis of vessel function, including hemodynamics [365-367], immune cell trafficking [368], transport of materials in the vascular and interstitial spaces [343,369,358,370-372], the binding kinetics of drugs in vivo [373], and other microenvironmental parameters such as perivascular pH and pO_2 [374-376]. Using intravital reporters, it is possible to study spatiotemporal expression of genes [377], to quantify vessel structure, function and dynamics [356,364], and to probe the abnormal organization, structure and function of angiogenic vasculature (e.g., hyper-permeability, heterogeneous and compromised blood flow) [346,348,378,358,379]. When combined with sophisticated imaging and analysis techniques, we can quantify blood velocity, hematocrit and shear rate *in vivo* [366].
Intravital microscopy through transparent windows has been used to study many aspects of neovascularization and vessel dynamics (**Fig. 18**). In addition to tumor angiogenesis and wound healing, they have been valuable for understanding the integration of engineered tissue grafts into existing vascular beds [304,296]. By following various cell populations as angiogenic host vessels make new connections with those of the graft, it is possible to understand the cellular mechanism of anastomosis [380].
Intravital imaging also allows assessment of therapies that affect the vasculature. For example, it is possible to quantify the abnormalities in structure and function that are hallmarks of tumor angiogenesis, and then assess how these change when the vasculature is normalized with anti-angiogenic or anti-fibrotic therapies [381,382,349,383,384,371]. Intravital optical microscopy [385] has also been useful for calibrating clinical imaging modalities in patients [386].
With information about the structure and dynamics of the angiogenic network, it is possible to analyze performance and efficiency of the vasculature at a deeper level. These analyses allow us to further probe the pathophysiology of tumor angiogenesis and the implications of abnormal vasculature for drug and nutrient delivery [387-390] .
Microfabrication technologies popularized in the past two decades have allowed the design and implementation of more sophisticated chamber designs. By creating custom-designed window chambers from PDMS, a clear, biocompatible polymer, it is possible to control the interactions between host and implanted tissues and to improve imaging by partially constraining the growing tissues [364], (**Fig. 19**).

20.2. Advantages of intravital microscopy / chronic window chambers for angiogenesis studies.
This strategy is characterized by the following features: i) it allows studies in an *in vivo* microenvironment, while *ex vivo* or *in vitro* studies require more extensive validation; ii) it enables visualization and measurement of the dynamics of the same vessels and cells as the new vasculature forms and changes in response to various treatments (**Fig. 20**); iii) it permits a variety of functional studies of angiogenic vessels which is being increasingly recognized in the angiogenesis field; iv) it allows dissection of the role of various mechanical

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(shear stresses, hoop stresses, etc.) and biochemical signals known to govern angiogenesis, v) it allows investigation of the role of different organ microenvironments on vessel formation and function; vi) a wide range or reagents are readily available for the murine system (Abs, genetic manipulation, etc.).

20.3. Limitations and challenges.

Intravital microscopy and window chambers experience some limits: i) they are labor-intensive, making screening studies expensive ; ii) creating the window may cause transient inflammation and Inappropriate implantation of the window can induces tissue damage; the use of compromised windows affects angiogenesis in the windows (wound healing response); iii) for single photon microscopes, depth penetration is limited to a few cell layers due to light scattering. Multiphoton microscopes permit deeper imaging but still limited to a few hundred micrometers. OCT, OFDI and SWIR permit deeper imaging, but are more expensive; iv) commercially available advanced imaging techniques are very expensive and the most advanced imaging techniques are not widely available.

20.4. Concluding remarks. Optical microscopy provides the high resolution imaging needed to distinguish cell dynamics, extracellular matrix components and intracellular features. By placing transparent windows over various organs in animal models, we can take advantage of optical microscopy to follow the formation of new vasculature non-invasively for time periods of weeks or months. This enables studies of neovascularization in normal processes such as wound healing and diseases such as cancer, and allows detailed analyses of responses to drugs that target blood vessels.

21. Mouse hind limb ischemia model

Peripheral arterial disease (PAD) is caused by atherosclerosis and occlusion of peripheral blood vessels, and results in claudication intermittens or in more severe stage in critical limb ischemia. The prevalence of PAD increases with age, affecting 6% of individuals aged 50–60 years, and 10–20% of individuals aged >70 years [391,392], and the upcoming years the number of PAD patients will increase even more due to the aging of the population and the increase in patients with obesity and type 2 diabetes. Unfortunately medical therapies to improve the perfusion of the lower limbs in patients with PAD are hardly effective [393,394]. Therefore the last decade much efforts has been put in defining new strategies to improve the blood flow to the lower extremities and to promote blood vessel growth, strategies that are based on the concept of therapeutic neovascularization, also called therapeutic angiogenesis [393].

Neovascularization consists of three different processes; angiogenesis, arteriogenesis, and vasculogenesis [394]. Angiogenesis describes an expansion of the microvasculature, because of sprouting of ECs from pre-existing capillaries, followed by their proliferation, migration, and capillary formation and is mainly hypoxia driven [395,396]. By contrast, arteriogenesis describes the remodeling of existing arterioles into collateral arteries so that they can deliver more blood flow to the limb [397]. Finally, adult vasculogenesis describes the incorporation of circulating (progenitor) cells into the regenerating microvasculature [393].

For testing these new therapeutic approaches for neovascularization as well as unraveling the complex cellular and molecular mechanisms involved the regulation of the neovascularization process, mouse models mimicking critical limb ischemia have been developed and used. Although these models may vary in crucial aspects, commonly they are referred to as the mouse hind limb ischemia model. In this section we will discuss the critical aspects of the variations in the use of the mouse hind limb ischemia model, their consequences for interpretation of the data obtained and we will discuss the limitations and pitfalls in their use.

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21.1. Mouse model for hind limb ischemia. Couffinal et al. were among the first to describe a mouse model for hind limb ischemia [398]. They induced acute hind limb ischemia by ligating the proximal end of the femoral artery, and the distal portion of the saphenous artery, next the artery and all side-branches were dissected free and subsequently, the femoral artery and attached side-branches were excised completely. The recovery of the blood flow in the ischemic limb due to angiogenesis and arteriogenesis was monitored by laser Doppler Perfusion Imaging (LDPI) [398], **Fig. 21**. In the years to follow many groups used similar approaches and variants of this model [399,400]. Surgical procedures range from a single ligation of the femoral or iliac artery [401-403] to a complete excision of the artery [398] and sometimes even the vein and the nerve are dissected too [404,405]. Several excellent review papers have been written on these variations in the hind limb ischemia model [406-409]. When choosing a particular variant of the mouse hind limb ischemia model for a study, it is important to define the goal of the study. For instance, when testing new pro-arteriogenic approaches there should be an appropriate therapeutic window in which an improvement of blood flow recovery can be monitored. In mice that rapidly form new collaterals, e.g. C57Bl6 mice with a single ligation of the femoral artery, it is difficult to monitor an increase towards an even faster collateral formation [410], and therefore a model with more severe injury is required [411,412]. On the other hand, such a fast model is ideal for mechanistic studies in which the effects of the deficiency or inhibition of crucial factors on blood flow recovery is studied [413,414].

Another crucial factor is which aspect of neovascularisation will be studied. Arteriogenesis is the remodelling of pre-existing arterioles to larger diameter collateral blood vessels required for restoring the blood flow to the distal ischemic parts of the limb, whereas angiogenesis is the sprouting of new (capillary) vessels into the ischemic tissue. One can imagine that the excision model to induce hind limb ischemia results in the disruption of the pre-existing arterioles in the adductor muscle, and thus hampers studies in which the contribution of arteriogenesis only on collateral formation is analysed. The disrupted pre-existing arterioles need to reconnect to the vascular system by sprouting (i.e. angiogenesis). Whereas the effects of pro-angiogenic interventions can better be studied in situations of severe ischemia in the calf muscles, in which case the excision model may be more suitable.

A good comparison of the various models used has also been hampered by the lack of a proper description of the anatomy of the blood vessels in the mouse limb. Hopefully the recent excellent description of the arterial anatomy of the murine hind limb by Kochi et al. [415] will contribute to a better understanding of the outcomes of the different ischemia models.

21.2. Variants of the surgical procedure to induce mouse hind limb ischemia.

General aspects of the surgical procedures. Before surgery, mice were anaesthetised with an intraperitoneal injection of a combination of midazolam (5 mg.kg^{-1}, Roche), medetomidine (0.5 mg.kg^{-1}, Orion) and fentanyl (0.05 mg.kg^{-1}, Janssen). In all models, the femoral vein and nerve were preserved. After surgery, the skin was closed with 6/0 Ethilon sutures (procedures adapted from [407]).

Single electrocoagulation of femoral artery. Directly after incision of the skin in the left inguinal region, the subcutaneous fat pad in the thigh becomes visible and is pulled aside distally. After dissection of the artery from the nerve and vein, ischemia is induced by electrocoagulation of the left femoral artery, proximal to the superficial epigastric artery. Electrocoagulation resulted in complete transaction of the artery. After electrocoagulation, the proximal end of the artery is moving proximally into the surrounding tissue and the distal end is moving distally, so there is a distance of a few millimetres between both ends after the surgical procedure.

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Single electrocoagulation of iliac artery. A bigger skin incision in the inguinal region was made for this procedure. Further, there is no need to cleave the fat pad. For exposure of the iliac artery, a retroperitoneal approach as used. By carefully moving the peritoneum proximally with a cotton swab, a good exposure of the iliac artery was possible. Further, preparation of the artery from the vein was necessary. The internal iliac artery serves as a landmark; direct proximally of the internal iliac artery an electrocoagulation of the common iliac artery was performed.

Double electrocoagulation of both femoral artery and iliac artery. For a double coagulation model, both common iliac artery and femoral artery were electrocoagulated. First, an electrocoagulation of the common iliac artery was performed and subsequently an electrocoagulation of the femoral artery was performed. These coagulations are at the same anatomical levels used in the single electrocoagulation procedures of the femoral artery and the iliac artery. Similar techniques were used as described above.

Total excision of femoral artery. After incision of the skin from the inguinal region till the knee, the subcutaneous fat pad was cleaved for a better exposure. First, preparation of the common femoral artery took place (proximal excision site). Two 8/0 ties were placed around the artery, in the direction of the inguinal ligament as much as possible. Then, dissection of the whole artery from the vein and nerve in the distal direction was performed. All side branches of the artery were carefully dissected free and coagulated. Before excision, preparation of the distal level was performed and again two 8/0 ties were placed around the artery. The distal ligation level was at the popliteal artery level, just distal from the bifurcation of the saphenous artery and the popliteal artery. After cutting the artery between the two ligatures proximal and distal, the whole artery was removed from the surrounding tissue.

21.3. Analysis of blood flow recovery and neovascularization. Traditionally, blood flow recovery is analysed by Laser Doppler Perfusion Imaging (LDPI) where the flow in the footpads of the ligated and contralateral limb is measured and expressed as the ratio between the ligated and unligated footpad. The restoration of the blood flow in time is used as a read-out for neovascularisation in the ischemic limb.

Next to the LDPI based flow analysis immunohistochemical analyses of the neovascularisation is used. For this usually the angiogenic response is studied in the distal part of the limb, the ischemic calf muscle, using EC staining (CD31, von Willebrand Factor) to stain the angiogenic capillaries. In addition the arteriogenic response is commonly studied the adductor muscle. For this the newly formed collateral arteries are stained using antibodies against αSMA to demonstrate the arterial nature of the newly recruited vessels in the adductor muscle.

In addition to these two commonly used methods to monitor the neovascularisation in the ischemic limb of the mouse sometime a functional test is used in which the use of the foot and limb is analysed for instance by assessment of the plantar/dorsi flexion [416].

Moreover, the increased sensitivity of current imaging methods may represent a major step forward. E.g the use of micro-CT analysis which used to be a post-mortem procedure due to the low sensitivity and lack of good contrast agents [407], now has become a method that actually be used as an vivo method for imaging the microvasculature [417], enabling the analysis of the neovascularisation over time. Recently it was demonstrated by Hendrikx et al. [418] that also single photon emission computed tomography perfusion can be used to analyse neovascularisation processes in the mouse hind limb, even with such a resolution that they could demonstrate that LDPI analysis underestimates the revascularisation processes in the model used.

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21.4. Limitations and challenges. One of the major limitations of the currently used hind limb ischemia model is the acute nature of the ischemia, whereas in the patients with peripheral artery disease the ischemia develops gradually. Although an interesting approach for gradual induction of ischemia in mice using amoroid constrictors has been described by Yang et al.[26] some years ago, this model has not received large follow-up.

The capacity for vascular regeneration and neovascularisation differs strongly between commonly used mouse strains [419,420]. For many years it is known that C57Bl6 mice have a stronger neovascularisation capacity than Balb/c mice [421,403] and only recently it has been discovered that this difference in regenerative response not only is linked to a difference in the pre-existing collateral bed in the various strains [419] but that it also can be attributed to a specific gene locus in chromosome 7 of the mouse [422-425]. Because of their slow regenerative response Balb/c mice are frequently used for hind limb ischemia studies, on the assumption that this slow response better mimics the situation in patients with PAD that display a poor regenerative capacity. A recent study by Nossent et al. [426] in which the mRNA expression pattern of angiogenesis and arteriogenesis related genes in the hind limb of C57Bl6 and Balb/c mice were compared, showed that especially in Balb/c mice a stronger upregulation of pro-angiogenic and pro-arteriogenic genes could be observed when compared to C57Bl6 mice, despite the poorer regenerative phenotype in Balb/c. This suggests Balb/c mice lack a thus far unknown factor that is crucial for vascular regenerative response, rather than that this model mimics better the situation in patients with critical limb ischemia.

Since the inflammation and the immune system play an important role in the regulation of the angiogenic and arteriogenic responses in the hind limb ischemia model and several immune cells including monocytes, T-cells and NK cells are crucial in inducing neovascularization [427,414,413,401,403,428-430], it should be realized that working with immune compromised mouse strains for studying for cell therapy approaches with human cells is not ideal and indicates that the results of these studies should be interpreted with caution. This becomes even more clear if the fate of injected cells, and their rapid disappearance even from the same syngenetic mouse is taken into account [430]. A very comprehensive review on the use of various immunecompromised mouse strains in hind limb ischemia models for human cell therapy validations has recently be published be Thomas et al. [416] and provides valuable information on the choice of the proper model.

And last but not least a major limitation for most of the hind limb ischemia mouse studies is that they are performed in healthy young mice whereas the PAD patients usually are older, and have atherosclerosis, type-2 diabetes or other comorbidities. These comorbidities strongly affect the neovascularisation responses in mice [431,432]. Moreover, it should be realised that induction of vascular remodelling also may affect the underlying atherosclerotic disease leading to the arterial occlusion, a phenomenon described as the Janus phenomenon of neovascularisation by Epstein et al. [433] as the interrelated trade-offs inherent in therapies designed to enhance collateral formation and those designed to inhibit atherogenesis.

21.5. Concluding remarks. The mouse hind limb ischemia model is used in many variants and researchers working with this model should be aware of the variations and use that variant of the model that fits best to the goal of their experiments, and should properly describe the used variation.

22. The RIP1-Tag2 transgenic mouse model.

The RIP1-Tag2 mouse model was one of the earliest oncogenic mouse models used in pioneering studies by Hanahan and Folkman to identify and characterize the angiogenic switch and multistep progression to pancreatic neuroendocrine tumors. Due to its high vascularity, and synchronous and rapid tumor development, it became a valuable preclinical tool for developing and evaluating response and relapse from antiangiogenic

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therapy from its earliest stages to the current day. Studies in the RIP1-Tag2 model predicted efficacy and helped to motivate clinical trials that led to the approval of two compounds, everolimus and sunitinib, for human pancreatic neuroendocrine tumors (PNET) in 2011.

22.1. The angiogenic switch. The RIP1-Tag2 mouse was developed by Douglas Hanahan as a result of his interest in using newly developed transgenic technology to use a well characterized rat insulin promoter to express a viral oncogene, SV40 T-antigen, in the beta cells of the pancreatic islets of Langerhans' in order to study oncogenic transformation of normal tissues [434]. The first transgenics died at an early age from hypoglycemia, consequent to the rapid proliferation of beta-islet cells, which produced high insulin levels. Tumors from viable mice were characteristically bright red and highly vascularized, and it was immediately proposed that this was likely the result of what was later called the "angiogenic switch", which Judah Folkman had postulated must occur for tumors to grow beyond a small size [435]. But when and how is this switch activated? Co-culture of the total islet population with ECs in a collagen matrix resulted in proliferation and migration of ECs towards only a small subset of "angiogenic" islets to form capillary tubes, elegantly confirming that the angiogenic switch occurred in hyperplastic islets prior to tumor formation [436]. This also implies that a soluble factor [435], later identified as VEGF through multiple lines of evidence, was critical. In normal adult mammals, the vasculature is quiescent, and new blood vessels are formed through angiogenesis, wherein new capillaries sprout from existing vessels in a process largely driven through VEGF signaling. Hanahan and Folkman proposed that this angiogenic switch is a discrete early step in tumorigenesis, and that the stages of progression that characterize RIP1-Tag2 tumorigenesis, where progressively smaller subsets of quiescent islets sequentially become hyperplastic, angiogenic, and finally progress to invasive carcinomas (**Fig. 22a**), also exist in human tumors [437]. In contrast to the popular hypothesis at that time that the angiogenic switch is induced by upregulation of proangiogenic factors, RT-PCR analysis [438-440] of all VEGF ligands and their receptors, flt-1 and flt-2, as well as FGF-1, indicated that their expression levels were similar in islets before and after the angiogenic switch [441,442]. The fact that normal non-transgenic islets already contained high levels of various proangiogenic factors suggested another mechanism in the activation of angiogenesis. Indeed, the switch from vascular quiescence to an angiogenic state involved the matrix metalloproteinase MMP-9, which was secreted by infiltrating myeloid cells. MMP-9 rendered ECM-sequestered VEGF bioavailable to its receptor, thus triggering an angiogenic switch; this identification was confirmed using co-culture studies of angiogenic islets derived from MMP-9 KO RIP1-Tag2 mice and EC in collagen matrices – in contrast to wild-type islets, the MMP-9 KO islets did not induce EC capillary formation [443]. The result that VEGF-A is a pivotal factor in the angiogenic switch was further confirmed with an islet beta cell-specific knockout of VEGF-A in RIP1-Tag2 mice, which impaired both angiogenic switching and subsequent tumor growth [442].

22.2. Preclinical and clinical studies. These and many other concurrent studies identifying the VEGF/VEGFR2 axis as a powerful therapeutic target led to the development of numerous antiangiogenic compounds. Sarah Parangi and colleagues were the first who used the RIP1-Tag2 model to perform preclinical studies with a combination of three early compounds with antiangiogenic activity - AGM-1470, the antibiotic minocycline, and interferon alpha/beta [444]. The combination could reduce tumor growth, but not prevent it, and was an early preclinical success for antiangiogenic therapy that helped to propel this class of inhibitors into the clinic [445]. The rapid and synchronous multi-step tumor progression that characterized the RIP1-Tag2 model made it then ideal for designing three different trial formats (**Fig. 22a**): early treatment at the hyperplastic stage to block the angiogenic switch commencing prior to tumor formation (prevention trial), treatment of mice bearing small tumors to determine whether tumor growth and progression could be stopped (intervention trial), and treatment of mice with substantial tumor burden and near death to test whether drugs can induce tumor regression and

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promote survival benefits (regression trial). These trials were first tested with AGM-1470, batimastat (BB-94), Fc-endostatin, Fc-angiostatin, or a combination of the latter two compounds and produced distinct efficacy profiles in the various trial formats [444]. These studies suggested that those antiangiogenic compounds needed to be fine-tuned to target specific stages of disease progression and that combinatorial strategies could broaden the effects and enhance survival. Subsequent studies illustrating the importance of targeted therapies that disrupt both tumor ECs and pericytes brought this notion to fruition in combination therapy trials with two different Sugen inhibitors, SU5416 and SU6668, that target VEGFR2 and PDGFR respectively [446]. While the VEGFR inhibitor SU5416 was most potently effective against early-stage disease, SU6668 was shown to block further growth of end-stage tumors suggesting that PDGFR+ pericytes in tumors present a complimentary target to ECs for efficacious antiangiogenic therapy. Combination therapies of these compounds were more efficacious against all stages of islet carcinogenesis than either single agent [446]. Congruently, subsequent studies with sunitinib (SU11248), which targets both VEGFR2 and PDGFR, showed remarkable efficacy in preclinical studies in RIP1-Tag2 mice and produced increased survival relative to monotherapy targeting VEGFR2 [447]. Collectively, these studies were used to incentivize the use of sunitinib in clinical trials for PNET. These clinical trials produced an impressive extension in progression- free survival that lead to the approval of sunitinib in 2011 [448], along with everolimus which primarily targets mTOR signaling. Interestingly, mTOR inhibitors also are very efficacious and produced increased survival in the RIP1-Tag2 model [449]. Thus, the RIP1-Tag2 model predicted the beneficial effects of both sunitinib and everolimus. However, significantly increased overall survival of RIP1-Tag2 mice, but not PNET patients treated with these inhibitors, could be reflective of the consequence of the different lifespans of mouse versus human (approximately 15 weeks versus 70 years), therefore a relatively short life extension could be significant. Also, preclinical studies in the RIP1-Tag2 mice are confounded by the fact that tumors are multifocal and that mice die from hypoglycemia, with relatively low (collective) tumor burdens and little metastasis relative to that characteristic of other mouse tumor models or human cancer. However, it is also notable that evaluation of overall survival in clinical studies is confounded by the fact that placebo treated PNET patients (unlike RIP1-Tag2 mice), elect to “cross-over” to the treatment cohort, likely reducing the calculated overall survival benefit in patients [450].

22.3. RIP1-Tag2 versus PNET tumors. Another major complication in interpreting preclinical studies using the RIP1-Tag2 mouse lies in fundamental biological differences from PNET. Tumors in the RIP1-Tag2 model are rapidly progressive and rarely form metastases, in contrast to the more indolent PNET tumors, although a subset of PNET tumors are more aggressive and metastasize [451]. Further, the tumor gene driving RIP1-Tag2 oncogenesis is a viral protein that interferes with p53 and retinoblastoma tumor suppressor function, while the genes associated with human PNET are predominantly chromatin-remodeling genes, DNA repair genes, mTOR-PI3K pathway genes, and menin mutations [451]. Notwithstanding these differences, expression profiling of human PNET tumors split them into three distinct groups, and remarkably two of them correspond to expression profiles identified in RIP1-Tag2 tumors [452]. Interestingly, human PNET tumors rarely adapt to culture, and while many cell lines from RIP1-Tag2 tumors (βTC cell lines) have been generated, they are surprisingly difficult to culture in comparison to other mouse tumor cell lines in spite of their aggressive growth *in vivo*.

22.4. Adaptation to antiangiogenic therapy. RIP1-Tag2 mice are a powerful model to assess therapeutic response and relapse to antiangiogenics due to their very synchronous tumor progression, and this has made them a potent model to study adaptation to antiangiogenic therapy. The response phase is characterized by tumor stasis and markedly reduced vascularity and vessel normalization blocking hemorrhage formation that produces whitish tumors, while the angiogenic relapse phase is characterized by tumor regrowth and revascularization (**Fig. 22b**). Donald McDonald and colleagues investigated the kinetics and mechanisms

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underlying vessel regrowth upon withdrawal of antiangiogenic therapy, and found that although two different VEGFR inhibitors, AG-013736 or AG-028262, significantly regressed tumor vasculature, they left behind empty sleeves of pericyte-covered basement membrane which functioned as a scaffold for rapid revascularizttion after treatment withdrawal. They concluded that targeting pericytes [453] and the sleeves of basement membrane appeared to be an important strategy to produce an enduring response to anti-VEGF therapy [454]. Casanovas and colleagues performed preclinical studies in the RIP1-Tag2 model, which demonstrated that after a period of response to continuous anti-VEGFR2 monotherapy, tumors rebounded although blockade of VEGFR2 signaling persisted. They found that the mechanism for regrowth was induction of alternative pro-angiogenic factors, including the FGF family and ephrins [455]. In addition, the response phase is characterized by the angiostatic and immunostimulatory polarization of myeloid cells that contributes to the pruning and normalization of the vasculature [456]. Tumors respond to these changes by activating and repolarizing myeloid cells to an angiogenic and immunosuppressive phenotype and by initiating an adaptive immune response by upregulating the negative immune checkpoint regulator, programmed cell death ligand PDL1; both immune adaptations limited the efficacy of VEGF/VEGFR inhibitors [457,458]. Another form of evasion from antiangiogenic therapy that was characterized in the RIP1-Tag2 model (and glioblastoma) is that of increased invasion and metastasis following pharmacological and genetic targeting of the VEGF signaling axis [447], the consequence of increased hypoxia induced by vascular fallout. While increased invasion has not yet been found in PNET treated with antiangiogenic therapy, it appears bevacizumab treatment can cause increased invasion in a subset of patients with glioblastoma multiforme [459], again highlighting the predictive power of this model.

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21.5. Concluding remarks. The RIP1-Tag2 model of beta islet-cell carcinogenesis has been instrumental in studying mechanistic underpinnings of tumor angiogenesis and in revealing adaptations to the environmental stresses elicited by antiangiogenic therapies, leading to valuable insights and predictions regarding clinical successes and failure.

22.6. Limitations and Challenges. In spite of the fact that preclinical studies in the RIP1-Tag2 model predicted the efficacy of two approved therapies, the model is multifocal, rapidly progressive, non-metastatic, and driven by a viral oncogene unlike its human counterpart. Because some PNET patients progress on available targeted therapies, it is desirable to further develop biomarkers of therapeutic response together with Patient Derived Xenograph (PDX) models from resistant tumors in humanized mouse models in order to identify beneficial secondary or concurrent therapies [450].

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23. Detection of 'non-angiogenic' tumors which rely instead on 'vessel co-option'

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While there is an overwhelming body of preclinical/experimental and clinical evidence accumulated over 40 years showing the importance of sprouting neoangiogenesis in tumor growth, progression and metastasis, it is increasingly appreciated that there are many circumstances in which tumors can grow and expand without inducing any new blood vessel capillaries. This realization has major implications for understanding some of the clinical efficacy limitations including failures of antiangiogenic drugs. Thus, new blood vessels can be formed by intussusception (see paragraph 7). Or, certain tumors, especially neural crest derived cancers, may form blood forming channels by "vasculogenic mimicry" whereby tumor cells line the vessel lumen, rather than authentic host ECs. However, the most dominant manifestation of non-angiogenic tumors is likely "vessel co-option" and it is particularly prominent in vascular rich organs that are the most common sites of metastatic disease, namely, lung, liver, lymph nodes and brain [98].

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Broadly speaking, vessel co-option refers to the ability of tumors to parasitize *pre-existing* blood vessels of the tissue, bypassing the requirement of developing new ones. The modern era of vessel co-option may have

begun in 1996-7 with the publication of two pathology reports by Pezzella and colleagues. Therein, a pattern of tumor growth of non-small cell lung cancer and lung metastases was recognized which made use of alveolar air spaces for expansion and alveolar vessels for blood supply *without* an indication of angiogenesis [460,461]. Histopathological descriptions of similar growth pattern in the lungs and analogous patterns in the brain and liver date to the late 1800s and early 1900s. The distinction between so-called 'newly formed' versus 'pre-existing' blood vessels were noted in the past, but the biological significance of each form was generally not appreciated. This has changed in the era of anti-angiogenic therapy – targeting VEGF to impair and cause regression of new vessel sprouts does not deplete pre-existing vessels. Tumor exploitation of vessel co-option has recently been shown to account for instances of intrinsic and acquired resistance to antiangiogenic drugs, both preclinically [103,462,463] and clinically [464,465]. Moreover, growth patterns associated with vessel co-option are emerging as important for patient prognosis [466,467].

There is currently no 'assay' as such to quantitate the extent of vessel co-option in tumors. Conventional measurement of tumor MVD has high potential for error when one considers that normal tissue vessel densities – as well as tumors where cancer cells surround these vessels – can be high [468]. Rather, histopathologic analysis is currently used with certain key features being looked for. Some examples are briefly described below; more detailed descriptions can be found elsewhere [98,468].

23.1. Some 'hallmarks' of detecting vessel co-option in tumors growing within the lungs, liver and brain.

Though the histologic structures of these organs are distinct, tumors within these sites that co-opt pre-existing blood vessels - either as part of its overall mass or throughout - often have some of the following features:

1. *The architecture of the normal host tissue is preserved within the tumor.* This is in contrast with angiogenic tumors, which tend to destroy the surrounding tissue. Tissue architecture preservation has been aided by immunohistochemistry for basement membrane and epithelial markers showing continuity of form across the tissue-tumor interface.
2. *Lack of surrounding capsule or desmoplastic tumor boundary.* Angiogenic tumors often form defined masses or nodules, a symptom of the inflammatory response of the host to the tumor, but this is lacking in 'purely' vessel co-opting tumors.
3. *Invasive, infiltrative and replacing tumor growth.* Minimal compressive tumor growth and compression of normal tissue structures. Rather, tumor cells tend to infiltrate along pre-existing vessels, often in direct contact with epithelial-endothelial basement membranes. Epithelial cells may become destroyed or stripped from their vessels during this process. Tumor borders are irregular.
4. *Incorporation of characteristic cells and structures of the tissue into the tumor.* Parenchymal epithelial cells (e.g. lung pneumocytes, liver hepatocytes, neural glia) and larger intact structures (e.g. lung bronchioles and large vessels in the lungs, portal triads in the liver) are taken up into the expanding infiltrative tumor mass and get locked into the tumor.
5. *Maintained vessel morphology and marker expression.* Co-opted vessels are known to be remodeled by surrounding tumor tissue and therefore may not in all cases appear 'normal'. Newly recruited co-opted vessels in particular may maintain morphologic and molecular characteristics of tissue endothelium (e.g. GLUT-1 and p-glycoprotein endothelial expression in brain tumors).
6. *Low rates of endothelial cell proliferation relative to angiogenic tumors.* Specific molecular markers of co-opted blood vessels are not currently known, thus several criteria are used to distinguish between vessel subtypes and differential growth patterns of tumors. Vessel co-option was first identified in patients, however experimental mouse models have also shown to recapitulate the growth patterns observed in human tumors.

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The major question now for future studies is whether “anti-vascular” therapeutic strategies can be developed that target co-opted vessels, in addition or instead of angiogenic vessels, without impacting the normal vasculature; some possibilities have recently emerged, eg. using antibody-drug conjugates [469].

Shown in **Fig. 23** are preclinical examples of both vessel co-opting and angiogenic tumors. Lung metastases from mice with breast cancer cell (MDA-MB-231/LM2-4) vs. kidney cancer (RENCA). The former develops purely non-angiogenic vessel co-opting lung metastases, whereas the latter forms both angiogenic (shown here) and vessel co-opting lung metastases. Staining for lung-specific epithelial and EC markers show the architecture of blood vessels in lung tissue and metastases. In vessel co-opting lung metastases, alveolar cells surrounding capillaries are taken up by the expanding mass at the tumor border, but alveolar epithelial cells are gradually shed from vessels toward the tumor centre while capillaries remain.

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XX. Murine allantois assay

Allantois is an extra-embryonic structure, which undergoes vasculogenesis, vascular remodelling and angiogenesis and is pivotal in establishing the chorioallantoic placenta and umbilical circulation. Allantois is characterized by a mesenchymal core and by an enveloping mesothelium and appears at E8.0. Vasculogenesis starts in the distal allantois and forms a plexus that connects with the dorsal aorta of the embryo (E8.25) before the chorio-allantoic fusion (E8.5) , which is instrumental in placenta formation (E9.5). This plexus undergoes a deep remodelling resulting in the formation of umbilical artery and vein, which invade the chorion by sprouting angiogenesis. These processes recapitulate the molecular and genetic distinct features of developing vasculature in embryo and in adult life, including the role exerted by signalling and transcriptional pathways triggered by established angiogenic inducers and modulators , such as VEGFs, PDGFs, ANGPTs, ephrins, NOTCH ligands,WNTs.

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Even if less known than other angiogenic assays, murine allantois explant assay represents a powerful tool to investigate general mechanisms of blood vessel formation, including the remodelling of a primitive vascular plexus, the angioblast differentiation, the arterial and venous fate, the sprouting angiogenesis and the maturation of the capillary network by mural cell recruitment. In particular, this assay allowed reaching seminal contributions to vascular biology. For example it was exploited to investigate the role of VE-cadherin, vascular endothelial protein tyrosine phosphatase and sphingosine-1-phosphate in angiogenesis .

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Xx Overview assay

The allantois was dissected from E8.5 mouse embryos using tungsten needles, and was placed individually on collagen or fibronectin-coated coverslips in 8-well culture dishes (BD Biocoat). Explants were cultured in 0.5 ml of culture medium (DMEM 4.5 g/l glucose, 10 mM L-glutamine, Pen-Strep), containing 15% fetal calf serum for 18 hours. Explants then were washed and fixed in 4% paraformaldehyde or Methanol:DMSO (4:1) for 20 min at room temperature and processed for immunohistochemistry or TUNEL assa

Pregnant females are sacrificed on the 8th day of gestation (approximately E7.75) or the following day at the same time (approximately E8.75). It is important to syncronize mice mating to dissect the allantois at the proper stage. Uteri are placed in Dulbecco’s A phosphate-buffered saline for isolation of decidua that is placed in Hepes-buffered DME medium with 7.5% fetal calf serum. With the aid of fine forceps and a scalpel, the embryo is isolated form the uterine tissues under a stereomicroscope. In the embryo, two structures can be recognized: embryonic tissues (clear and cylindrical shaped,) and the ectoplacental cone (redish, cone shaped and located at the end of embryonic tissue in the uterine mesometrial region). Allantoises are mouth-aspirated into a hand-pulled glass microcapillary (60-120 μm diameter).The ectoplacental cone/chorion should cleanly

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break off, leaving an opening to the exocoelomic cavity for the easy aspiration of the allantois. Immediately after inflating the exocoelom, which allows better visualization of the allantois through the yolk sac, aim the tip of the microcapillary toward the distal tip of the allantois and, while gently aspirating, “sheathe” the entire length of the allantois with the tip of the microcapillary. While maintaining minimal aspiration, lift the allantois, still in the exocoelom, toward the meniscus. At the meniscus, gently suction the allantois into the microcapillary, thus leaving behind the embryo, which will drop to the bottom of the dish. The allantois can now be manipulated.

Three different methods can be used to maintain in culture allantois. Allantois can be kept in suspension in rolling cultures allowing the development of a 3D spheroid characterized by a vascualrize core enveloped by a mesothelial layer. Explants are cultured in 0.5 ml of DMEM medium containing 50% rat serum or 5% (“low serum”) or 50% fetal calf serum. For culture longer than 1 day, explants are given completely fresh gas-equilibrated medium at 24-h intervals .

A second system is based on hanging drop system. Allantois is suspended in 0,03 ml of DMEM supplemented with 10% FCS and located onto the underside of a lid of a plastic bacterial dish The hanging drop cultures are placed into a 5% CO2 incubator and after 18 hours the originated 3D spherois can be resuspended in the above mediun containing angiogenenic inducers (e.g. VEGF) and the hanging drop procedure is repeated . Finally, allantois can be easily maintained in adherent conditions (50% rat serum in DMEM mediun) up to 72 hours on glass surface or on plastic surface coated with fibronectin or poly-lysine and differently stimulated Allantois isolated at E8.0 adheres within 12 hours and after 18-20 hours of culture, the explant adopts a circular shape with a vascular plexus, which covers the central area

Limitations and challenges.

The main positive features of this assay is that it can analyze vasculogenesis or angiogenesis dependent on the explant isolation. Actually, when allantois is isolated eraly (E 8.0, headfold stage), explants undergo vasculogenesis, and when isolated later (E 9.5, 22-26 somites), undergo angiogenesis.Second, allantois explants can be imaged with the use of time-lapse microscopy to follow the sequence of events that occur during vessel formation in vitro. Finally, cultured allantois explants can be immunostained for markers of vessel formation and can be sectioned for histologic analysis. It is further evident that allantois isolated from genetically manipulated mouse models can be useful to understand the role of specific genes in vascular development. Finally, because allantois is implicated in placenta vascularization, this model can be specifically exploited to investigate pathogenetic mechanisms of placental diseases . Negative aspects of allantois murine assay is the short time of culture viability (72 hours) and a solid experience in manipulating embryo mice.

Concluding remarks

Allantois angiogenesis assay is not so popular and videly used in the vacular biology community but it useful to discriminate if a vascular phenotype is primitive or secondary to other embryonic genetic defects in particular when the genetic manipulation is early lethal. The robustness of the assay also validated by computer – assisted analysis , could be exploited in drug evaluatin as recently reported Thus, the allantois is an important tool at infancy to adrress relevant issues of vasculogenesis and angiogenesis in development and disease.

23.2. Concluding remarks.

Conclusions

Acknowledgements

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Sci Signal

We apologize for not being able to cite the work of all other studies related to this topic because of space restrictions.

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Abbreviations

AMD, age-related macular degeneration; BME, basement membrane extracts; BOEC, blood outgrowth endothelial cells; BrdU, 5-bromo-2'-deoxyuridine; CAM, chorioallantoic membrane; CD31, platelet endothelial cell adhesion molecule; CD45, leukocyte common antigen; CD144, vascular endothelial cadherin; CNV, choroidal neovasularization; CRC, colorectal carcinoma; CT, computer tomography; D, definitive differentiated cells; DAPI, 4',6-diamidino-2-phenylindole; EACA, epsilon aminocaproic adid; EBM, endothelial basal medium; EC, Endothelial cell(s); ECAR, extracellular acidification rate; ECFC, endothelial colony forming cells; ECM, extracellular matrix; EdU, 5-ethynyl-2'deoxyuridine; EPC, endothelial progenitor cells; EVP, endovascular progenitor cells; GFP, green fluorescence protein; GS-B_4, Griffonia simplifolia isolectin B_4; HDMEC, Foreskin-derived human dermal microvascular EC; H&E, Hematoxilin and eosin; HIF, hypoxia inducible factor; HILIC, hydrophilic liquid chromatography; hiPSC, human induced pluripotent stem cell; HPG, histopathological growth pattern; HUAEC, human umbilical artery EC; HUVEC, human umbilical vein EC; FGF, Fibroblast growth factor; IL-3, interleukin 3; LDPI, laser Doppler perfusion imaging; MMP, matrix metalloproteinase; MO, morpholino; MP, main population; MPC, mesenchymal progenitor cells; MSC, mesenchymal stem cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMR, nuclear magnetic resonance; NV, neovascularization; OCR, oxygen consumption rate; OIR, oxygen-induced retinopathy; PAD, peripheral arterial disease; PCNA, proliferating cell nuclear antigen; PCV, polypoidal choroidal vaculopathy; PDMS, polydimethylsiloxane; PI, propidium iodide; PNET, pancreatic neuroendocrine tumor; PV, particle velocimetry; PWG, postnatal weight gain; RCC, renal cell carcinoma; ROP, retinopathy of prematurity; RS II, reduced serum supplement II; RTCA, real-time cell analysis; SCF, stem cell factor; SCID, severe combined immune deficiency; SDF-1α, stromal-derived factor; SOD, super oxide dismutase; SP, side-population; SPARC, secreted protein acidic and rich in cysteine TA, transient amplifying cells; TCA, tetracarboxylic acid; TEM, tumor endothelial marker; TNF, tumor necrosis factor; UEA, Ulex europaeus agglutinin; VEGF, vascular endothelial cell growth factor; VESC, vascular endothelial stem cells; VMO, vasculatized micro-organ; VO, vaso-obliteration.

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Figure Legends

Fig. 1. (van Beijnum) (**a**) Phase contrast image (left) and binarized image of HUVEC grown in a regular 96-well plate. Simple software solutions can be used to count features in the image. (**b**) Example of MTT assay, with color intensity correlating with cell number. (**c**) DNA staining profile of HUVEC using PI, measured on a plate cytometer. (**d**) Cell viability of HUVEC exposed to sunitinib, measured using a luminescent assay.

Fig. 2. (Huges) **3D assays of vascular morphogenesis.** (**a**) A fibrin bead assay uses collagen I- and EC-coated Cytodex beads embedded within a 3D fibrin gel matrix to measure EC sprouting and lumen formation. (**b**) These features are readily resolved using phase-contrast microscopy. (**c**) EC tube formation can be

measured by embedding ECs within a collagen I matrix. (**d**) Once formed, these tubes can be visualized by toluidine blue staining and bright field microscopy. (**e**) Whole-mount, dissected retinas from postnatal mice are mounted within collagen I-Matrigel matrix mix and cultured in pro-angiogenic medium to stimulate EC sprouting. (**f**) Sprout and lumen formation are resolved using phase-contrast microscopy. (**g**) The vascularized micro-organ (VMO) approach utilizes “arteriole” (high pressure) and “venule” (low pressure) microfluidic channels to drive medium diffusion and flow across a cell chamber where microvasculature forms. (**h**) The formed microvasculature (ECs, red) can be measured for leak by perfusion with 70kDa FITC-dextran (green).

Fig. 3. (Nicosia) **Aortic ring assay of angiogenesis**. (**a**) Serum-free collagen gel culture of rat aorta (asterisk) photographed at day 6 (microvessels marked by arrowheads). (**b**) Aortic culture treated with VEGF (5 ng/ml) shows increased number of microvessels (day 6). (**c**) Electron micrograph of aorta-derived microvessel with polarized endothelium (E), patent lumen (L), and surrounding pericytes (P); endothelial tight junctions are marked by arrows. (**d**) Phase contrast micrograph of microvessel composed of an inner core of endothelial cells and surrounding pericytes (white arrowheads). (**e**) Pericytes highlighted with immunoperoxidase stain for NG2. (**f**) Immunofluorescent image of aorta-derived macrophages stained for CD45; an isolectin B4 (IB4)-stained endothelial sprout is visible in the background. (**g**) Confocal image of microvessel double stained for endothelial cells (IB4) and pericytes (αSMA). Magnification bars = 500 μm (A, B), 5 μm (C), 50 μm (D-G).

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Fig. 4. (Ingeborg) **. Identification of tip cells.** The *tip cell* is the leading cell of an angiogenic sprout with long filopodia extensions, followed by *stalk cells* that proliferate and *phalanx cells* that form a matured new capillary. Tip cells in the developing mouse retina can be identified by staining with isolectin B4 (a) a double staining with F-actin and IB4 (b) or a triple staining for F-actin, cortactin and collagen type IV (c). JSH identifies PDGFB (d), apelin (e) and esm1 (f) in the angiogenic front of the mouse retina. Overexpression of Lifeact-EGFP in the mouse retina (g) and in zebrafish (h). Tip cells in endothelial cell cultures are identified by CD34 (i).

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Fig. 5. (Vermeulen) **Microvessel density and histopathological growth patterns**
(**a**) Unsupervised spatial modeling of the blood vessel pattern in normal liver shows a low number of clusters per number of vessel profiles. A selected region of interest (ROI) at the tumor-liver interface of normal liver in CD31-stained tissue is shown (left). The Blood Vessel Analysis algorithm of Definiens™ segments and classifies blood vessel objects (orange) and nuclei (blue) (mid). The Cartesian coordinates (x, y) of the centroids of all vessel objects in one ROI were used in a simplified 'SeedLink' clustering method (23) (right). Centroids with the same color (e.g. red) belong to the same cluster. (**b**) Unsupervised spatial modeling of the blood vessel pattern in a colorectal cancer liver metastasis with a replacement growth pattern shows a low number of clusters per number of vessel profiles. A selected region of interest (ROI) at the tumor-liver interface of replacement growth pattern in CD31-stained tissue is shown (left). The Blood Vessel Analysis algorithm of Definiens™ segments and classifies blood vessel objects (red) and nuclei (blue) (mid). The Cartesian coordinates (x, y) of the centroids of all vessel objects in one ROI were used in a simplified 'SeedLink' clustering method (23) (right). Centroids with the same color (e.g. red) belong to the same cluster. (**c**) Unsupervised spatial modeling of the blood vessel pattern in a colorectal cancer liver metastasis with a desmoplastic growth pattern shows a high number of clusters per number of vessel profiles. A selected region of interest (ROI) at the tumor-liver interface of desmoplastic growth pattern in CD31-stained tissue is shown (left). The Blood Vessel Analysis algorithm of Definiens™ segments and classifies blood vessel objects (red) and nuclei (blue) (mid). The Cartesian coordinates (x, y) of the centroids of all vessel objects in one ROI were used in a simplified 'SeedLink' clustering method (23) (right). Centroids with the same color (e.g. red) belong to the same cluster. (**d**) Tukey boxplots of the normalized number of clusters of blood vessel objects for the desmoplastic growth pattern, the replacement growth pattern and normal liver. There was a statistically

significant difference between the growth patterns as determined by one-way ANOVA ($F(2,22) = 10.8$, $p < 0.001$). A post-hoc Tukey test showed that the number of clusters divided by number of vessel objects was significantly different between the desmoplastic growth pattern and the replacement growth pattern ($p < 0.05$, ‡), but also between the desmoplastic growth pattern and normal liver ($p < 0.001$,‡). However, no difference was found between the replacement growth pattern and normal liver ($p > 0.05$). Outliers are plotted as points (•) and extreme values are plotted as asterisks (*).

Fig. 6. (Djonov) Intussusceptive angiogenesis - the methodological challenge. (**a**) Scanning electron microscopy image of transluminar pillars. Early stage is characterized by tinny pillar (arrow), formed mainly by endothelial protrusions coming from the opposing ECs. At later stages the pillar (arrowhead) is increasing in girth and its core is invaded by perivascular cells, fibroblasts and fibres (visible in the lower disrupted part of the pillar). Lumen of the vessel is marked with asterisks. *Adapted from* [470]. (**b**) Dynamic *in vivo* observation of the regenerating zebrafish fin vasculature demonstrated a newly formed pillar (rectangle). (**c**) 3D-reconstruction based on serial semithin sections from the same area depicted in **b**, (**d**) Transmission electron micrograph demonstrates the transluminal tissue pillars (rectangle in **b** and **c**) at ultrastructural level. Black asterisk indicates the core of the pillar, while arrowhead pointed to cell-cell contacts between the endothelial cells (EC). Er = erythrocyte, Col = collagen fibers. *Adapted from* [471].

Fig. 7 (Alitalo)

Fig. 8. (Davis) **Serum-free defined model of human endothelial cell-pericyte tube co-assembly in 3D collagen matrices.** (**a**) Human EC and GFP-labeled pericytes (Peri) were seeded together at the indicated cell densities and after 120 hr, were fixed and stained with anti-CD31 antibodies. The immunostained cultures were imaged using confocal microscopy. The serum-free defined culture system contains SCF, IL-3, SDF-1α, FGF-2 and insulin (a component of the RS II supplement), which are required to be added in combination. (**b**) Cultures fixed at 120 h were immunostained with antibodies to CD31, laminin (LM), and fibronectin (FN) and were imaged using confocal microscopy or were examined by transmission electron microscopy. Arrows indicate the capillary basement membrane. L indicates lumen while Nuc indicates nuclear labeling. Bar equals 25 μm.

Fig 9. (Carmeliet) **General title** (**a**) Schematic representation of glycolytic flux measurements with [5-^{3}H]-glucose. A single tritium present on 5C glucose is released as water in the ninth step of glycolysis catalyzed by enolase. (**c**) Schematic representation of the modified Glycolysis Stress Test. The first measurements of ECAR are performed while ECs are incubated in glycolysis stress test medium (without glucose and pyruvate). The injection of glucose leads to the saturation of glucose concentration and allows measuring glycolytic rate (blue). Second, injection of oligomycin blocks oxidative ATP production and shifts the energy production to glycolysis, with the subsequent increase in ECAR revealing the cellular maximum glycolytic capacity (green). The difference between glycolytic capacity and glycolysis rate defines glycolytic reserve. The final injection of 2-deoxyl-glucose (2-DG) inhibits glycolysis and the resulting decrease in ECAR confirms that the ECAR produced in the experiment is due to glycolysis. ECAR, prior to glucose injection or after 2-DG injection, is referred to as non-glycolytic acidification (pink). (**c**) Schematic representation of the modified Seahorse Cell Mito Stress Test. First injection of oligomycin blocks ATP synthase and allows the calculation of the ATP coupled oxygen consumption rate (OCR_{ATP}; red). Second, injection of FCCP maximizes the OCR by uncoupling the OXPHOS, enabling to calculate the spare respiration (reserve capacity). Third, antimycin-A treatment blocks complex III of ETC enabling us to calculate the basal mitochondrial respiration (OCR_{BAS};

green), the maximal mitochondrial OCR (OCR_{MAX}; orange), the proton leakage (blue), and the non-mitochondrial OCR (pink).

Fig. 10. (Munn) PDMS microfluidic device for analyzing angiogenesis. Fluid flow can be controlled connecting syringe pumps to the ports, or by imposing hydrostatic gradients. Flow can be directed through the endothelial lumens (green), or across the endothelial junctions, through the central matrix gel. Sprouting occurs through the apertures that flank the central 3D matrix, and is easily visualized and quantified (adapted from [180]).

Fig. 11. (Stratman) **Assessing EC autonomous gene function**. (**a-e**) Whole mount *ISH* of 24-48 hpf zebrafish embryos. (**a-b**) *ve-cadherin* labeling of the vasculature in the trunk (**a**) and head (**b**) of a zebrafish embryo showing vascular specific labeling. (**c-d**) *tagln/sm22* (**c**) and *vegfaa[231]* (**d**) showing labeling of non-vascular (somitic) tissues in the trunk. (**e**) *cds2[232]* labeling of both vascular (arrows) and non-vascular tissues. (**f**) Confocal micrograph of a growing trunk intersegmental vessel in a 32 hpf *Tg(kdrl:mRFP-F)^{y286}* embryo (red vessels), mosaically expressing *Tol2(fli1a:H2B-TagBFP-p2A-egfp-F)* transgene (blue EC nuclei, green EC cytoplasm), showing blue, green and red fluorescent channels and all three merged. (**g-h**) Higher-magnification images of GFP fluorescence (**g**) and merged GFP/BFP/RFP fluorescence (**h**) in a 48 hpf *Tg(kdrl:mRFP-F)^{y286}* transgenic animals mosaically expressing a *Tol2(fli1a:H2B-TagBFP-p2A-egfp-F)*transgene in a single EC in a trunk intersegmental vessel. Scale bars = 20 μm (**f**), 10 μm (**g-h**). Images in panels **f-h** are from reference [472].

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Fig. 12. (Stratman) **Assessing embryonic morphology and effects on vascular patterning**. **(a-f)** Transmitted light (**a,b**), epifluorescence (**c,d**), and confocal (**e,f**) images of 3 dpf developmentally normal (**a,c,e**) or developmentally abnormal (**b,d,f**) embryos from the same *Tg(fli1:egfp)^{y1}* transgenic "wild type" zebrafish population. The normal animal has a normal morphology (**a**) and normal vessel patterning (**c,e**), while the developmentally abnormal animal has a stunted, somewhat malformed trunk (B) and also displays trunk intersegmental vessel patterning defects (**d,f**). (**g,h**) Confocal images of the cranial vasculature in 48 hpf wild type sibling (**g**) and *y284* mutant (**h**) *Tg(kdrl:mRFP-F)^{y286}* animals. The *y284* mutants have small heads heads and eyes, accompanied by reduced and abnormal formation of cranial vessels and aortic arches (brackets). The vascular defects should be interpreted with caution, as they could be primary or solely a consequence of the smaller head (or possibly both). Transplantation and/or mosaic transgenic expression can be used to assess the vascular cell-autonomy of phenotypes. (**i,j**) Confocal (top) and corresponding transmitted light (bottom) images of 48 hpf *Tg(fli1:egfp)^{y1}* wild-type sibling (**i**) and *fused somites (fss^{v66})* mutant (**j**) animals. Improper somite formation in *fss^{v66}* mutants (lack of chevron shaped somite segments, seen in the bright field images) indirectly results in altered intersegmental vessel patterning (**j**, top). (**k-p**) Confocal images of the hindbrain vasculature (**k,l**) or trunk intersegmental vessels (**m-p**) in *Tg(fli1:egfp)^{y1}* control (**k**) or cardiac troponin T type 2a (tnnt2a) (**l-p**) deficient animals at 1.5 (**m**), 2 (**k,l,n**), 2.5 (**o**), or 3.5 (**p**) days post-fertilization (dpf). Control animals have normal blood flow, but tnnt2a-deficient animals have no heart beat and lack all blood flow. Although formation of the vasculature is largely normal for several days in the absence of blood flow (**l,n,o**), abnormalities in vessel growth and patterning begin to appear at later stages, such as enlargement of the dorsal intersegmental vessels at 3.5 dpf (**p**). This illustrates the need to analyze vascular phenotypes as early in development as possible in zebrafish with absent or defective blood flow. All images are lateral views, rostral to the left, except panels **k** and **l**, which show dorsal views, rostral to the left. Images in panels **i** and **j** are from reference [229], images in panels **k** and **l** are from reference [473], and images in panels **m-p** are from reference [474]. Scale bars = 50 μm.

Fig.13 (Nowak) **Chorioallantoic membrane of the chicken embryo (CAM). a)** Bright field image of the CAM vasculature presenting the functional vascular network from capillaries to big vessels imaged at 10 embryo development day **b**) Vascular network of the CAM presented on the fluorescent angiography with FITC-dextran and an intravenous contrast agent. **c**) Fluorescent angiography of the CAM with centrally oriented avascular (black) zone created by vaso-occlusive photodynamic therapy (PDT). Bar corresponds to …. µm (**a**) and 500 µm (**b** and **c**). This figure will be extended.

Fig. 14 (Kleiman) ***In vivo*** **BME/Matrigel plug assay in mice. Injection of BME/Matrigel in both groin areas of a mouse.** Left image is a gel without growth factors, right represents a plug with an angiogenic growth factor.

Fig. 15. (Bischoff) **a**) Schematic of the two cell model. **b**) Tail vein injection of rhodamine-UEA-I and FITC-GS-B_4 labels perfused human (red) and murine (green) vessels on day 7 after cell/Matrigel sub-cutaneous injection. **c**) Quantification of lectin-labeled human and murine vessels shows that perfused human vessels present at day 5 and day 7.

Fig. 16. Add general title (Smith) **a**) The mouse model of Oxygen Induced Retinopathy (OIR). Neonatal mice and their nursing mother are placed into 75% oxygen from P7 to P12, which induces loss of immature retinal vessels, leading to a central zone of vaso-obliteration (VO). After returning to room air at P12, the central avascular retina becomes hypoxic, inducing vascular regrowth with pathologic neovascularization (NV). At P17 the maximum severity of NV is reached. NV starts to regress shortly after P17 and almost no VO or NV remains visible by P25. **b**) Retinal whole mount stained with isolectin-B4-Alexa (red) displaying a normal vascular development at P17 under normoxic condition. B') OIR P12 retinal whole mount showing an extensive VO area without NV. B'') OIR P17 retinal whole mount showing a decreased VO with NV at its maximum. **c**) Quantification of vaso-obliteration (VO) by manually outlining the avascular area with image-processing software (Photoshop, Adobe Systems) **c'**) For computer-aided NV quantification, both the original image and the VO image generated with Adobe Photoshop were imported into NIH's free-access ImageJ software. The SWIFT_NV macro set isolates the red color channel, subtracts background fluorescence and divides the VO image into four quadrants. **c''**) SWIFT_NV then allows the user to outline NV tufts but not normal vessels by setting a fluorescence threshold for each quadrant. The macro set then quantifies all NV pixels from all four quadrants, reports the result as neovascular total area and creates an overlay of NV and original image.

Fig. 17. (Smith) **Experimental Flow Chart of the Image-Guided Laser-Induced CNV Model and Data Collection.** (**a**) Overview of the procedure for CNV induction involving mouse preparation and followed by experimental treatment, sample preparation and analysis. (**b**) Representative image of normal fundus (Green check mark). (**c**) Representative image of anomalous structure (white arrow) in the eye, which is not suitable for laser photocoagulation (Red X). (**d**) Representative image of normal fundus with 4 laser burns shown as bright white spots. (**e**) Representative image of a successful laser burn (white arrow) with 3D optical coherence tomography. (**f, g**) Representative ocular fundus fluorescein angiography images at 5 and 10 minutes after the injection of fluorescent dye at day 6 after laser burn. (**h**) Representative images of flat-mounted choroid with IB4 staining at day 7 after laser photocoagulation. Scale bar: 200 µm. ON, optic nerve. (**i**) Higher magnification of the laser-induced CNV lesion highlighted in panel H. Scale bar: 50 µm. Reproduced with permission from [338].

Fig. 18. (Munn) Tumor angiogenesis in the brain (left) and the dorsal skin (right) visualized using IR frequencies to image deeper into tissue; blood flow creates the contrast, so it is non-invasive (from [356]).

Fig. 19. (Munn) **MMTV tumor vasculature in the cranial window pillar TIC.** (**a**) MPLSM/SHG image of the indicated region in the brightfield image (**b**). Near the edge of the PDMS, the vasculature extends radially into the central chamber. At this time point (day 7 after implantation), the vasculature is mature and has normal morphology in the regions far from the tumor. Four feeding arterioles (red arrowheads) and three venules (blue arrowheads) are indicated. These vessels have significant flow, and have acquired smooth muscle cells in their walls (red, *αSMA*$^{+}$*-DsRed)*. Note that the arterioles generally have more *α*SMA signal than the venules, as expected. (**c**) The vasculature near the growing tumor has dramatically different morphology and flow, as observed in other animal models and human tumors. The tumor was not fluorescently labeled in this group, but is visible as the mass extending from the central tumor (T) in (**b**) (from [364]).

Fig. 20. (Munn) Vascular sprouts entering a cranial window tissue isolation chamber. Time sequence of new vasculature (green) migrating toward the top-left into a cranial window TIC, past the edge of the PDMS disk (dashed line) (imaged using MPLSM and SHG). Alignment of collagen fibers (white) is evident, and SMA+ cells can be seen on the PDMS surface (red). The vasculature (green, FITC-dextran) extends by forming perfused loops and sprouts. As the matrix remodels, the vessels also remodel as they advance. a: D1: day 1, D3: day 3, D6: day 6 post TIC implantation. A pillar structure, which defines the height of the chamber is indicated with * (from [364]).

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Fig. 21. (Quax) Analysis of blood flow recovery in time by Laser Doppler Perfusion Imaging (left panel) and Angiography (right panel).

Fig. 22 (Bergers). **(a).** Multistep Progression to Tumors in RIP-Tag2. Although oncogene expression begins during embryonic development (E8.5), the pancreatic islets initially have a normal anatomical and histological appearance (“normal” stage). Beginning at 4-5 weeks of age, hyperplastic and dysplastic islets begin to appear to comprise about 50% of islets by 10 weeks. Angiogenic islets appear beginning around 6 weeks of age, and represent 10% of all islets at 10.5 weeks. Angiogenic islets are recognized by their dilated blood vessels and microhemorrhagias. Tumors form beginning at 9-10 weeks and represent 2-4% of all the islets by 14 weeks. About half of the tumors at end stage evidence either focal or widespread invasion to the surrounding acinar tissue. RIP-Tag2 mice die at approximately 14 weeks of age primarily due to hyperinsulinemia. **(b).** Antiangiogenic Therapy Response and Relapse. Tumors treated with antiangiogenic therapy using an RTK inhibitor starting at 12-15 weeks ”Response”, or 12-20 weeks ”Relapse”, when vessels have significantly rebounded. Tumors are stained with anti-insulin in blue, vessels are stained with anti-CD31 in red, and surrounding exocrine pancreas is stained with amylase in green.

Fig. 23. (Kerbel) Immunofluorescence and immunohistochemical staining of formalin-fixed mouse lung samples to enable differentiation between vessel co-option and angiogenesis in tumors. **(a,b)** Vessel co-opting tumors are observed in spontaneously formed lung metastases from mice with orthotopically implanted then surgically resected MDA-MB-231/LM2-4 breast tumors. In **(a)**, sections are stained for alveolar cell marker podoplanin, EC marker CD34 and nuclei marker DAPI. Tumor cells can be seen filling alveolar spaces along the border and incorporating alveolar capillaries into the tumor core. In **(b)** is the corresponding section stained for HLA human cell marker and hematoxylin to show the presence of tumor cells with respect to host stroma and lung parenchyma. A bronchiole is also seen to be taken into the tumor and gradually filled with tumor cells. The tumor border is irregular. **(c,d)** Angiogenic growth is observed in spontaneously formed lung metastases from mice bearing intra-renal implanted RENCA tumor cells that later underwent nephrectomy. Sections are

stained for alveolar and bronchial epithelium cell marker cytokeratin 7, EC marker CD34 and nuclei marker DAPI. RENCA tumors grow in 'cannonball' shape, compressing lung tissue and excluding them. The lung-tumor interface of another nodule is shown at high magnification in **(d).** Lung tissue is compressed or "pushed" aside to allow tumor expansion. The tumor border is smooth and microvessels are not associated with alveolar epithelium within the tumor. Scale bar represents 200 μm. Regions in dashed boxes are expanded on the right. "T" = tumor. Arrow = columnar bronchial epithelium.

Table 1. Comparison of 3D Models of Vascular Morphogenesis.

ASSAY	PROCESS					
	Sprouting	Lumen Formation	Anastomosis	Pericyte Recruitment	Perfusion	Pruning
Fibrin Bead Assay	✔	✔	✔	✔		
Collagen Lumen Assay		✔		✔		
Retina Explant Assay	✔	✔	✔			
Vascularized Micro-Organ	✔	✔	✔	✔	✔	✔

Table 2. **Variety of applications with the CAM**

Developmental angiogenesis	**Ref.**
differentiation of vascular endothelium	[270] [475]
membrane proteome associated with the vasculature	[476]
gene transfer / global gene expression	[477-479]
metabolic profiling	[480]
transcriptome analysis in the "wound model"	[481]
vascular and endothelial cell targets from isolated chicken membranes	[482]
Lymphangiogenesis	
Prox-1 in the lymphatic endothelial cells	[274,483]
ingrowth of lymphatics into the tumors	[484]
Embryonic lymphangiogenesis	[485]
Vasomodulating therapies	
radiosensitizing activity	[486,487]
one- / two-photon excitation photodynamic therapy	[488-490] [491]
microbeam radiation therapy	[492]
Tumor angiogenesis	
tumor growth in the CAM	[493,494,266]
experimental metastasis	[495-498]

Interstitial pO_2 gradients in solid tumors	[499,260]
Accessing molecules activity	
growth factor (receptors) inhibitors	[260]
metal-based compounds	[500-504]
inflammatory and tumor cells or purified effector molecules	[505]
prolactin signalling in endothelial cells	[506]
Stem cells	
human Mesenchymal Stem Cells	[507,508]
human skin-derived stem cells	[509]
Drug delivery, Nanoparticles	
drug delivery for cancer treatment	[510]
visible laser irradiation + gold nanoparticles	[511]
screening of nanocarrier vehicles	[512,513]
Engineering	
tissue engineering	[514] [515]

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Table 3. Angiogenesis assays and vascular analysis.

Development of novel imaging techniques	**Ref.**
Direct Measurement of interstitial diffusion and convection of albumin using fluorescence photobleaching	[516]
Tumor induction of VEGF promoter activity in stromal cells	[377]
Interstitial pH and pO2 gradients in solid tumors in vivo	[376]
In vivo measurement of gene expression, angiogenesis and physiological function in tumors	[358]
Two-photon fluorescence correlation microscopy to reveal transport in tumors	[369]
Quantum dots to spectrally distinguish multiple species within the tumor milieu in vivo	[361]
Three-dimensional in vivo microscopy using optical frequency domain imaging	[356]
Simultaneous measurement of RBC velocity, flux, hematocrit and shear rate in vivo.	[366]
In vivo validation of MRI vessel caliber index with intravital optical microscopy in a mouse brain tumor model	[386]
Video-rate resonant scanning multiphoton microscopy	[367]
Next-generation *in vivo* optical imaging with short-wave infrared quantum dots	[517]
Analysis of vessel function	
Regulation of transport pathways in tumor vessels: role of tumor type and microenvironment	[372]
Effect of tumor-host interactions on distal angiogenesis and tumor growth	[518]
Kinetics of vascular normalization in response to VEGFR2 blockade	[381]
pH/pO2	
Calibration and application of fluorescence ratio imaging of pH gradients	[374]
Noninvasive measurement of microvascular and interstitial oxygen profiles	[375]
Simultaneous in vivo high-resolution measurements of interstitial pH and pO2 gradients	[376]
Extracellular Matrix and interstitial transport	

Fluorescence photobleaching with spatial Fourier analysis for measurement of diffusion	[373]
Dynamic imaging of collagen in tumors in vivo using second-harmonic generation	[362]
In vivo imaging of extracellular matrix remodeling by tumor-associated fibroblasts	[363]
Angiotensin inhibition enhances drug delivery by decompressing tumor blood vessels	[371]
Anti-VEGF therapy induces ECM remodeling and mechanical barriers to therapy	[352]

Immune cells	
VEGF and bFGF regulate natural killer cell adhesion to tumor endothelium	[368]
Ly6C^{low} monocytes drive immunosuppression and confer resistance to anti-VEGFR2 therapy	[382]

Analysis of lymphatic vessel function	
Conventional and high-speed intravital multiphoton laser scanning microscopy	[379]
Lymphatic metastasis in the absence of functional intratumor lymphatics	[519]
A genetic Xenopus laevis tadpole model to study lymphangiogenesis	[520]
Investigation of the lack of angiogenesis in the formation of lymph node metastases	[357]

Drug delivery, Nanoparticles	
Vascular normalization improves the delivery of nanomedicines in a size-dependent manner	[370]
Compact high-quality CdSe-CdS core-shell nanocrystals with narrow emission linewidths and suppressed blinking	[360]
Magneto-fluorescent core-shell supernanoparticles	[521]

Engineered vasculature	
Tissue engineering: creation of long-lasting blood vessels	[304]
Paradoxical effects of PDGF-BB overexpression in ECs in vivo	[522]
Engineered blood vessel networks connect to host vasculature via wrapping-and-tapping anastomosis	[380]
Generation of functionally competent durable engineered blood vessels from human pluripotent stem cells	[296]

Mathematical analysis of angiogenesis	
Scale-invariant behavior and vascular network formation in normal and tumor tissue.	[388]
Cancer, angiogenesis and fractals.	[390]
Scaling rules for diffusive drug delivery in tumor and normal tissues.	[389]

Assessing therapies	
Herceptin acts as an anti-angiogenic cocktail	[523]
Targeting placental growth factor/neuropilin 1 pathway inhibits growth and spread of medulloblastoma	[349]
Vascular-endothelial protein tyrosine phosphatase inhibition in tumor vasculature and metastatic progression	[383]
A cerebellar window for intravital imaging of medulloblastoma in mice	[350]

Tissue isolation chambers	
Implantable tissue isolation chambers for analyzing tumor dynamics in vivo	[364]

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Key steps before the injection[U1]

- The BME/Matrigel matrix should be phenol-free and be growth factor-reduced.
- Thaw the BME/Matrigel in the cold, on ice in the refrigerator overnight is best. The matrix will gel at a temperature above 4°C and not be usable.
- Plan on 100 µl per injection site.
- Plan on at least six injection sites (3 animals) and be sure to include controls with no test materials but containing the vehicle.
- When adding the test material be sure to mix it well (do not vortex as bubbles will form) with the BME/Matrigel at 4°C and try not to dilute the matrix as this will reduce the gelling properties.

Key steps during the injection

- Bring the test mixture and syringes on ice to the animal room.
- The test mixture should be cold when injected as it will gel quickly in the subcutaneous location due to the warmth of the animal.
- Use 1 inch 21-25 g needles and change the needle after 2 to 4 injections as it may become dull.
- Holding the mouse upside down with one hand, inject 0.1 ml into both groin areas (**Fig. 14**) with the tip of the needle as far as possible from the injection site to prevent leakage. A second person can help by holding each leg away from the body during the injection. Inject slowly. A bump should appear at the site where the test material was released from the tip of the needle.
- Hold the syringe in place for about 30 seconds to allow the test material to gel.
- Gently rotate the syringe as you withdraw the needle to help further seal the injection site hole. Some leaking will occur.

Key steps in harvesting and in analyzing the plug

- Animals may be sacrificed for the harvest, but care should be taken not to externally damage the plug area.
- For the harvest, a square segment of skin containing the plug can be excised. Alternatively, the skin can be cut around the plug leaving plenty of space so as not to damage the plug. Then the underside is exposed. Note the plug is small and yellow in color. It may also be pink or red depending on the degree of angiogenesis. The control plug depending on the assay method will be colorless.
- The plug can be safely and gently removed with scissors, embedded in HistoGel, and fixed for histology, followed by sectioning and staining with Masson's Trichrome.
- The skin or plug sample can then be analyzed by histology. Most of the angiogenesis will be from the edges of the plug, and the quantitation can be based on the density of the vessels and/or extent of in growth toward the center of the plug. Count at least three fields per plug located at approximately the same distance from the edge of the plug.
- Alternatively, Drabkin reagent (D5941, Sigma Aldrich) can be used to assess to the amount of blood in the plug or fluorescein isothiocyanate dextran (Mw 150,000-200,000 Sigma Aldrich) can be injected into the tail vein of the animals and extracted from the plug for quantitation. However, these approaches can be flawed by either the presence of compressed non-perfused vessels, or by the presence of leaky blood vessel or hemoragic areas. Some researchers isolate RNA from the plugs and use qPCR of EC genes [289] as a quantitation assay.

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