CHAPTER 6

How Is the Branching of Animal Blood Vessels Implemented?

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The Blood Circulatory System: Tree Analogy versus Network Concept

For centuries, the cardiovascular system of animals has been described as a branching tree with the heart in its very centre.[1] Although this description dates back to Galen[1] (c. 130-200 A.D.), and structural similarities with trees are obvious, it has to be emphasized that, unlike a tree, the animal blood circulation does not contain blind ending branches. The discoveries of Harvey (1578-1657) and Malpighi (1628-1694) demonstrated that the blood circulation, especially when studied at light microscopic dimensions, forms a "closed system" of circuits in which every single blood vessel is continuous with another one. Arterial vessels leave the heart, branch, and connect through a capillary network to corresponding veins that drain the blood back to the heart. It is therefore more appropriate to understand the circulatory system as a network of conductive units of varying sizes.

Given the structural similarities with branching trees, it has been suggested that the growth of the cardiovascular system follows a mode in which tiny tubules in the size range of capillaries "sprout" from existing "mother" vessels. These sprouts, which form new vascular branches, then grow in length until they meet another vessel to which they connect. This process allows for the establishment of flow between both preexisting vessels.[1-7] It is, however, still not clear how the growth of sprouts is directed towards one another so that their fusion is possible. The sprouting mechanism can thus only be of physiological benefit within a network in which the distribution of blood vessels is already quite dense. If the sprout deviates only a few degrees from its closest vessel, the chances for a successful connection are minimal. In addition, since the critical step for the elongation of sprouts is mitosis of endothelial cells, it will take time until a sprout invading an avascular region of tissue meets another vessel to establish the circulation. Only after the onset of the blood perfusion will the nutritional supply of the previously avascular compartment begin. Does an alternative mechanism exist to allow faster and more efficient expansion of the vascular network? To answer this question, we have to review current concepts of vascular morphogenesis in general.

Mechanisms of Blood Vessel Formation, Network Growth, and Remodeling; What Is Their Relationship to the Branching Process?

The cellular mechanisms that are responsible for the establishment, growth, and functional organization (remodeling) of the circulatory system in animals (a) cause the de novo formation of a network pattern and (b) are suited to expand and adapt this network to match the physiological needs of growing tissues and organs. In the embryo, a simple uniform network of tubes of similar sizes is formed initially by the mechanism of vasculogenesis. Endothelial- and blood cells differentiate in situ from mesodermal precursor cells in a pattern of islands (termed "blood

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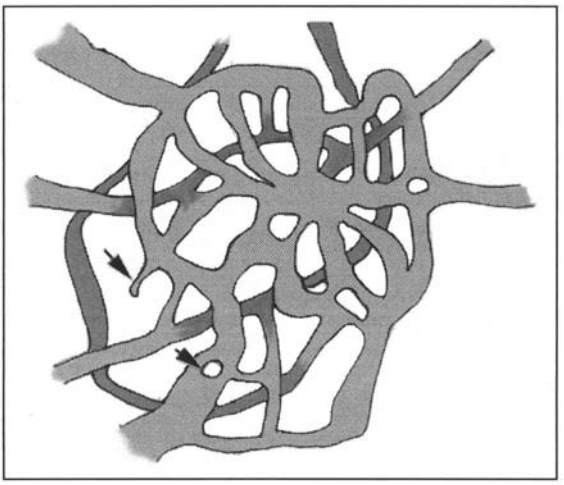

Figure 1. Sprouting- versus intussusceptive concept of vascular morphogenesis. Network expansion by insertion of a new blind-ending tubular branch (sprout, long arrow) inside a branching point (tissue between branches) by out-migration of endothelial cells from the wall of a "mother" vessel. Sprouts thus randomly divide branching points inside the network. Network expansion by insertion of a new pillar (branching point) inside an existing branch, which divides the latter into two segments (branches) according to intussusceptive microvascular growth (short arrow). The pillar has a constant distinct ultra-structure ("pillar core") to guarantee for the stabilization of the network. Branching points within the network and pillars, white areas; blood vessel lumens, grey areas.

islands"). The endothelial cells line these blood islands (the 3-dimensional organization of which is one of connected tubular branches), while the free blood cells fill their lumens. The latter will begin to circulate after the heart has started to function.[8-16]

Because primitive vascular networks grow denser and become more extended to reach so-far nonvascularized tissues and organs during subsequent development, new tubular branches have to be added to the system. This process is referred to as angiogenesis. The existence of angiogenesis implies that new branching points between these tubular segments must be established. Angiogenesis by endothelial sprouting involves migration of single endothelial cells to leave the wall of the "mother" vessel, endothelial proliferation to replace the migrating cells, and finally assembly of the latter to form new tubes, which consequently "branch out" of existing ones.[2,17] Branching points thus refer to tissue remnants between the growing and forming sprouts. On the other hand, network expansion and remodeling can also be achieved by the insertion of new branching points within the tubular branches of the network. The analysis and reconstruction of serial sections has revealed that these branching points correspond to tissue columns and possess a specific ultra-structure that is important for the stabilization of the network.[18-24] This process permits growth "within itself", i.e., it follows an intussusceptive* mechanism. One important difference between the sprouting- and the intussusceptive concept of angiogenesis is therefore the formation of branches versus branching points to accomplish vascular network growth and remodeling (Fig. 1).[21-22]

Simultaneously with its growth and expansion, the network has to remodel to achieve an adaptation to the physiological needs of the tissue that it supports. Network remodeling means the differential growth of the uniform branches formed during vasculogenesis to establish a network that contains segments of different sizes (the process is also termed "pruning"[17]) and

* Intussusception refers to "growth by deposition of new particles among the existing particles" (Webster's Encyclopedic Unabridged Dictionary of the English Language, New York, Portland House, 1989).

exhibits a nonuniform distribution of the latter. The addition and deletion of segments (branches) is another important characteristic of the remodeling process and is largely achieved by intussusceptive growth.[21-23] The importance of network remodeling is emphasized by the fact that the main intra-embryonic blood vessels, as the dorsal aortae and the cardinal veins, arise from plexuses established by vasculogenesis.[24] Remodeling based on intussusception occurs while vasculogenesis is still proceeding. This has been confirmed by the discovery of tiny tissue columns, the landmarks of intussusceptive growth, in the yolk sac circulation of the chicken embryo at an extremely early stage, on day E4.0, during vasculogenesis (Patan, unpublished data).

What Are the Cellular Mechanisms That Lead to the Formation of Branching Points According to Intussusceptive Microvascular Growth?

Intussusceptive microvascular growth (IMG) is a mechanism of network formation, growth and remodeling that forms an alternative concept to the sprouting model of angiogenesis. It is based on the idea that the vascular system expands by dividing the blood vessel lumen into subordinate segments (branches) through the insertion of columns of tissue, termed interstitial- or intervascular tissue structures (ITSs, diameter >2.5 μm) and tissue pillars or posts (diameter <2.5 μm).[18-23,25-32] These "tissue columns" correspond to branching points of the network, since they split the blood circulation in divergent directions. Using video microscopy we have demonstrated the existence of tissue pillars, i.e., tiny branching points (diameter often <1 μm), and their formation in vivo.[18-20,30] The analysis and reconstruction of sequential serial sections provide insight about the three dimensional network architecture as well as the pillar morphology and reveal the existence of their precursor stages. The latter technique, together with in vivo video microscopy, permits the determination of the cellular mechanisms that lead to pillar- or branching point formation and has confirmed the wide spread existence of IMG.[18-20,30-33]

New pillars or branching points can form in varying ways. Several different mechanisms have been uniformly detected in the embryo,[23,19-20] as well as in pathological states in the adult organism, as in tissue repair,[32] in cancer,[30-31] during the recanalization of thrombotic lesions,[31-32] and after myocardial infarctions (Patan et al, in prep.). Others, such as segmentation and apposition (see below) are preferentially related to adult angiogenesis and are based on the existence of intra-and extra-vascular fibrin deposits (Patan et al, in prep.).[31-32]

Pillar Formation by Folding of the Blood Vessel Wall

Pillars can separate from the tips of tissue folds that project inside the vessel lumen. Folding is initiated by retraction of the endothelial layer of the vessel wall into the adjacent tissue around the region of the future fold. The fold is lined by endothelial cells of the lateral vessel wall and contains peri-endothelial cell extensions and collagen fibers within its center. A pillar core forms within the intra-luminal tip of the fold during fold elongation. The fold does not contain organized pillar cores at its initial stages of formation. A pillar core is composed of a collagen fiber bundle that can be ensheathed by peri-endothelial cell extensions. The pillar core can also be viewed as the core of the future branching point. In a next step, the intraluminal tip of the fold that contains the pillar core separates to give rise to a free tissue pillar. The latter remains, however, connected to the fold at its bottom and its top. Pillar separation occurs based on thinning of the fold adjacent to the pillar core to form a thin cytoplasmic bridge composed of the extensions of one endothelial cell. Fusion of opposite cell membranes within this extension causes the formation of a transcellular hole that separates the pillar from its fold. This is followed by endothelial cell rearrangement to increase the distance between the pillar and the fold (Fig. 2A).[19,21-23,30-33]

Pillar Formation by Splitting of Intervascular Walls or Larger ITSs and Pillars

Intervascular walls that separate two adjacent vessels frequently contain pillar cores, which permit their splitting. This is based on thinning and merging of the endothelial layer around this core and cell membrane fusion to form two transcellular holes that separate the pillar at both of its sides. This process causes fusion of neighboring vessels (Fig. 2B, a-b).

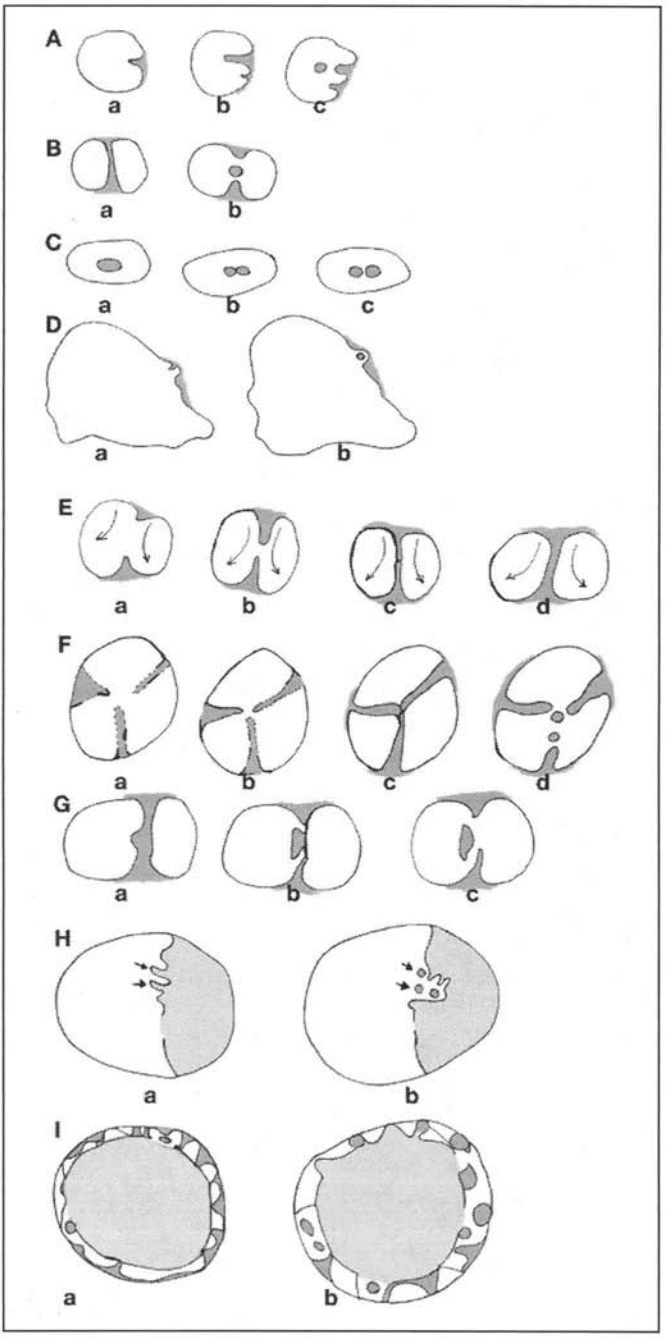

Figure 2. Different cellular mechanisms of intussusceptive microvascular growth. The vessels are depicted in cross-section, pillars (or branching points) appear thus as round spots, i.e., "islands" within the vessel lumens. The figure legend is continued on the next page.

Figure 2, continued. A) Pillar (branching point) formation based on folding of the lateral vessel wall. a: Folding is initiated by concentric retraction of the endothelial layer of the vessel wall into the adjacent tissue around the region of the future fold. b: Fold elongation. Folding can simultaneously occur within another region of the vessel wall. c: The intraluminal tip of the fold that contains the pillar core separates to give rise to a free tissue pillar surrounded by the circulation. B) Splitting of a wall that separates two vessels (intervascular wall). a: An intervascular wall can split, if it contains a pillar core. b: This process separates a free pillar and results in fusion of both neighbor vessels. B, b-a: Splitting of an intervascular wall can be reversed based on connection of pillars and folds and their insertion at opposite vessel walls. C) a-c: Following a related mechanism as illustrated in B a-b, a larger pillar or ITS can divide to give rise to two or more smaller pillars. C, c-a: The same mechanism shown in C a-c can be reversed causing the connection of two pillars or ITSs. D) a-b: A similar process as described in A causes the in-situ formation of a small elementary loop within the lumen of a larger vein. Based on this mechanism and remodelling of the elementary loop by division of its central ITS (similar to C, a-c) large systems of compound loops can form. E) Formation of the "flow pillar" a: Within a branching point of blood flow two slender tissue folds project into the lumen from opposite vessel walls, b: elongate and c: attach to each other, if the flow conditions remain un-altered. d: The connection of both opposite folds follows to form a stable tissue pillar. F) a-b: Segmentation involves the deposition of fibrin cords in the vessel lumen. Migration of single endothelial cells along these cords. c. This causes the transformation of the cords to tiny folds and their connection in a spoke-like pattern. d. Further remodelling allows the separation of tiny pillars or ITSs from these folds. G) a: Apposition leads to the formation of a larger ITS via folding of the lateral vessel wall. b: The separating ITS becomes surrounded by a larger part of the vessel lumen termed "outpouch" and spatially related to the wall of an adjacent vessel. c: The process concludes with separation of the central ITS and fusion of the outpouch with the adjacent vessel thus forming a collateral vascular segment that connects both preexisting vessels. H) A large thrombus occludes part of the lumen of a vein. a: Endothelial migration from the lateral venous wall covers the luminal surface of the thrombus and is followed by invasion of fibroblasts. This allows for formation of tiny folds from the endothelial cover layer. b: The folds give rise to pillars and ITSs, which reexpands the venous lumen inside the thrombus. I) A large thrombus occludes the entire lumen of an artery. a-b: Fibroblasts from the media layer of the arterial wall migrate into the intima which subsequently thickens to form two-to three cell layers including thrombus matrix composed of fibrin and coagulated blood. Folding, pillar and ITS-formation and fusion processes transform this circular layer into a ring of communicating vessels. Black lines = endothelial cells, grey areas = extracellular matrix containing also peri-endothelial cells (interstitial tissue), white spaces = vessel lumen.

The critical requirement for the division of ITSs or pillars is the existence of two pillar cores in one ITS or pillar. The endothelial cell extensions will then merge around these cores, followed by thinning and cell membrane fusion to form a transcellular hole that divides the pillar or ITS. This leads simultaneously to the formation of two smaller ITSs or pillars and a new vascular segment between both. The pillars and ITSs remain, however, connected at their bottoms and at their tops (Fig. 2C, a-c).[20,21-23,30-32]

Connection of Adjacent Pillars

The process of pillar or ITS division can be reversed, causing pillar connection with simultaneous deletion of the vascular segment located between them (Fig. 2C, c-a). This means that branching points between vascular segments can fuse to give rise to larger ones. It can also result in the separation of segments to form distinct vessels through the establishment of intervascular walls (Fig. 2B, b-a). The latter mechanism is of special importance in the pathological state, especially in tumors, where it can lead to fragmentation of vessel loops with the subsequent formation of blind ending tubes and vessel regression (S. Patan, in prep.).[30-32,21-22]

"In-Situ Loop Formation"

This mechanism of IMG has been detected in the mouse embryo, but also in the adult organism during tissue repair, in tumors and after myocardial infarctions, especially in humans. Vessel loops are formed in a one step process within the walls of larger veins. The endothelial layer of the vessel wall retreats towards the surrounding tissue to separate a free tissue pillar, which is encircled by a vessel loop (Fig. 2D). In-situ loop formation is thus responsible

for the establishment of a new vessel loop composed of two segments that connect it to the vessel from which it derived. The separation of the tissue pillar or ITS (branching point) forming the center of the loop is a concentric process that occurs simultaneously around the entire circumference of the pillar (and results in fold formation as an initial step, compare to Fig. 2A). This guarantees that the onset of loop perfusion takes place in time to benefit the physiological requirements of the supported tissue. Further remodeling and growth of this "elementary" loop based on splitting of its central ITS (see Fig. 2C) causes the formation of loop systems and ultimately transforms a part of the large vessel into a new vascular network of connected loops.[31-32,21-23]

Pillar Formation by Connection of Opposing Intraluminal Tissue Folds

This mechanism is directly related to blood flow conditions and has been documented using in vivo microscopy and the analysis of tissue serial sections in the chicken chorio-allantoic membrane and in tumors. In those segments of the vascular network in which flow is diverted into different directions, intraluminal tissue folds derived from opposite vessel walls have a chance to connect and merge to form a new branching point. This is followed by fusion of the adjacent cell membranes of the endothelial cells that line the tips of the folds to form two trans-cellular holes. Matrix elements will invade these holes to stably connect both folds, thus establishing a tiny pillar. This process of fold connection is facilitated by the fact that the opposing folds are located precisely between both "branching" streams of flow and are subsequently pushed into the center of the lumen by the latter. If the flow conditions remain stable and do not change for a longer period of time (2-3 hours), both folds will connect. If, however, the flow directions change and cross over the region where the folds are located, connection cannot occur and the branching point will not be established (Fig. 2E). The latter case has been especially observed in tumors (S. Patan, in prep.).[30,21-22]

Segmentation

In contrast to most other mechanisms of IMG that cause the formation of new branching points, segmentation has so far been detected only during adult neovascularization. It is prominent in tissue repair and in tumors and is responsible for the expansion of a previous network towards regions of new vessel formation. In addition, it is dominant in the remodeling process of large veins and arteries after myocardial infarctions to give rise to multiple smaller segments. A prerequisite for segmentation is the deposition of fibrin in the vessel lumen; the fibrin deposits form a scaffold along which endothelial cells of the vessel wall migrate and which they line. This causes the formation of thin intraluminal folds that connect to each other in the center of the lumen in a spoke like pattern. Gradually the fibrin in the center of these folds is replaced by collagen fibers, which have a stabilization effect and facilitate the further separation of pillars and ITSs (Fig. 2F). In a pathological variation of this mechanism, the folds form valve-like structures corresponding to blind-ending pockets that are not patent and are consequently without perfusion (Patan et al, in prep.).[31-32,34]

Apposition

Apposition is a special mechanism of IMG and has so far been detected only in mouse and human myocardial infarctions. It is a variation of in-situ loop formation. However, in contrast to in-situ loop formation, apposition results in the establishment of a larger segment parallel to the vessel from which it is derived. We term this new segment an "outpouch". Outpouches form by retraction of the endothelial layer around an extra-vascular fibrin deposit which forms the core of the future ITS or branching point. The latter becomes surrounded by the expanding outpouch to form a free tissue column inside the newly established lumen. The fibrin will be replaced by collagen fibers and other matrix elements during this process. Corresponding to the architecture of the smaller loops, the outpouch has a very thin wall composed of a single endothelial layer that facilitates the connection to an adjacent vessel. This occurs by attachment of the

endothelial layer that lines the outpouch to the wall of the adjacent vessel to create a common intervascular wall. Splitting of this wall follows the sequence described above (compare to Fig. 2B). Apposition has thus two important characteristics not involved in in–situ loop formation; the establishment of a new larger vascular segment, the outpouch, and its fusion with a preexisting neighboring vessel (Fig. 2G). We concluded that the outpouch might thus be connected to the "collaterals" that have been observed to connect two preexisting vessels and are specific for newly established vessels after myocardial infarctions. Apposition can occur on the venous, as well as on the arterial side of the circulation (Patan et al, in prep.).

Recanalization of Thrombotic Lesions

Recanaliztion of thrombotic lesions that are located in large arteries and veins has been detected in a model of tissue repair and in experimentally grown tumors utilizing the same model system.[31-32] In the case of venous recanalization, the thrombus filled the vessel lumen only partially. Therefore the endothelial layer of the lateral vessel wall was able to grow over its free intraluminal surface, followed by in-migration of underlying fibroblasts. This process probably attached the thrombus to the vessel wall and stabilized it by transformation of its fibrin-rich matrix into collagen fibres. In a second step, the endothelial layer at the thrombus surface retreated toward its center to form folds, as well as ITS- and pillar cores composed of fibrin and collagen fibers (Fig. 2H, a). This was followed by separation of ITSs and pillars from these folds and subsequent establishment of vascular segments inside the thrombus matrix (Fig. 2H, b). The latter remodeled to give rise to a new vascular network, which allowed for reperfusion of the region of the former stenosis.[32]

Arterial recanalization followed a different mechanism of IMG, since the thrombus had usually filled the vessel lumen entirely. The endothelial cells of the intima layer of the vessel wall largely retained their original position as a circular lining. At some places, however, the intima ruptured, allowing for blood deposits to undermine it and for subsequent invasion of fibroblasts from the underlying media layer. This caused the intima to thicken and form two- to three cell layers. In places, intimal endothelial cells aligned themselves around the fibrin deposits, coagulated blood and collagen fibers that were ensheathed by fibroblast extensions. This process gave rise to pillar cores and intervascular walls (Fig. 2I, a-b). The latter subsequently formed future branching points between vessel segments and thus transformed the circular layer of cells and matrix into a ring of communicating vessels. Blood flow was subsequently reestablished in the former area of occlusion.[31]

What Are the Advantages of IMG versus Sprouting in the Expansion and Remodeling of the Circulatory Network?

There are several major advantages of adding new segments to the network by dividing vessel lumens based on the insertion of new branching points compared to the addition of new sprouts (branches). First, dividing the lumen based on insertion of a column of tissue can form daughter segments of different sizes, depending on the size and position of the core of the pillar or ITS. In contrast, a newly developing sprout is always very small, about the size of a capillary. Secondly, the tissue pillar or ITS forms while the vessel is still being perfused. The sprout, however, needs to grow, elongate, and fuse with another vessel before its perfusion is established. This implies that inserting tissue columns to modify the network pattern has an immediate physiological benefit. Finally, vessel wall maturation provides the "naked" sprout with a layer of peri-endothelial support cells. Since lumen division based on IMG often occurs in vessels that already possess a fully- matured wall, this step is frequently not necessary.

Pathological Variations of the Cellular Mechanisms of IMG

In addition to the physiological mechanisms of IMG that are listed above, intussusception can follow pathological variations in the adult organism, in tumor angiogenesis and after myocardial infarctions, but apparently not in tissue repair.[31-32] Many of these pathological

variations lead to the formation of blind-ending segments. The latter cause the fragmentation of the network and thus impair the oxygenation of the tissue. This explains the hypoxia that is a characteristic of tumors and the post-infarction myocardium. High and persistent levels of hypoxia contribute to the perpetuation of insufficient angiogenesis in these pathological conditions, which can be deduced from the continuous release of angiogenic growth factors, such as VEGF, FGF-2 and hypoxia inducible factor α (HIF-α).[31-32,35,21-22] Their local concentration inside tumors is assumed to be higher than the one of secreted inhibitors, so that angiogenesis dominates.[36]

Two different pathological features of IMG have been detected in tumors and to some extent also during neovascularization process after myocardial infarctions:

1. Intravital microscopy of the circulation of colon adenocarcinomas transplanted to the skin of nude mice demonstrated that pillars in tumors can form more frequently, but are continuously and rapidly remodeled, subsequently reversing pillar formation through its subsequent deletion. This makes the process of vascularization highly ineffective and probably even increases the oxygen consumption of the local tissue. It also causes the permanent alteration of blood flow on a time-scale of minutes, which is known as "intermittent blood flow" and is a characteristic of the tumor circulation.[30]
2. Pathological variations of the mechanisms of IMG detected in tumors and after myocardial infaction interfere with the formation of regular folds, pillars and ITSs and thus impair the deposition of normal branching points inside the network. A common feature of these pathological variations is the formation of blind-ending tubes. One example of pathological IMG, especially of in-situ loop formation, is the late separation of the central ITS or branching point in the center of the loop when the latter has already reached large dimensions. Another pathological mechanism refers to the secondary disconnection of loop segments, which interferes with the patency of the loop or loop system.[31] Disturbed mechanisms of IMG also include the formation of intervascular walls in tumors that occlude vessel segments, even resulting in vessel regression (Patan et al, in prep.).[30]

What Causes the Existence of Pathological Mechanisms of IMG and Subsequently Disturbed Branching Point Formation?

Since the pattern of gene expression in tumor endothelial cells is very similar to that in wound healing,[37] it can be assumed that endothelial cells are not responsible for the disturbed mechanisms of IMG. Thus the best available explanation is the existence of nonendothelial, fibroblast-like cells that are incorporated into vessel walls during IMG to replace endothelial cells. Under tissue repair conditions these cells proliferate strongly and express endothelial-specific markers, such as platelet-endothelial adhesion molecule (PECAM/CD31), which indicates that they might differentiate to form endothelial cells.[32] In tumors these fibroblast-like cells are less common.[31] The vessel-wall-invading tumor cells are obviously substituting for these fibroblast-like cells to form "mosaic vessels", in which the endothelial layer is composed of both endothelial-derived and tumor-derived cells.[38-40] Although tumor cells mimic the endothelial cell morphology and thus hide in the vessel wall, they exhibit a pattern of gene expression that is different to the one of endothelial cells that does not include endothelial specific markers (S. Patan, in prep.). This will ultimately interfere with the normal mechanisms of IMG. Therefore it cannot be expected that mosaic vessels give rise to regular tissue folds, pillars, and ITSs.

How Is the Formation of Branching Points at the Molecular Level Regulated?

The currently known pro-angiogenic molecules have been characterized by their effect on endothelial cell proliferation, migration and tube formation. The most prominent ones of these growth factors are fibroblast growth factor 1 and 2 (FGF-1, acidic and FGF-2, basic), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF, scatter factor), vascular endothelial growth factor/vascular permeability factor (VEGF-A, VEGF-B,

VEGF-C), transforming growth factor alpha (TGF-α), and interleukin 8 (IL-8).[41-44] Additionally, an important role in angiogenesis has been established for the Tie/Angiopoietin and the Eph-B/ephrin-B system of tyrosine kinase receptors and their ligands.[23,33,45-50]

It has been shown that the heparin-binding isoform of VEGF-A is especially responsible for vascular branching. Transgenic mice that solely expressed the VEGF-A isoform, which lacks heparin binding (VEGF 120/120), exhibited larger vessels with more endothelial cells but fewer vessel branches as compared to wild-type littermates.[51]

Concerning the formation of pillars at branching points of the circulation (see Fig. 2E), it is known that the alteration of shear stress profiles can cause the expression of angiogenic growth- and transcription factors by endothelial cells; one example is platelet-derived growth factor B (PDGF-B). The receptors for these growth factors are up-regulated in peri-vascular cells of the vessel wall.[52-54] Endothelial cells that are exposed to large shear stress gradients in vitro respond with increased cell division and motility in the vicinity of flow separation.[54] It can be expected that the shear stress profile is high in these areas of flow divergence, while it is low within the branching point itself. Furthermore it has been recently shown that VEGF receptor 2 (VEGFR-2, Flk-1) can be activated by fluid shear stress in a ligand-independent manner, which causes further downstream-activation of endothelial nitric oxide synthase (eNOS).[55] Since eNOS produces nitric oxide (NO) as a response to shear stress [56-57] and NO induces neovascularization,[58-60] a direct link between shear stress and its influence on vascular morphogenesis through a growth factor- receptor independent signaling is established.

Recently, it has been demonstrated that the Angiopoietin-1 (Ang-1)/Tie-2 growth factor receptor system regulates embryonic IMG.[23,33,45-47,50] The ligand, Angiopoietin-1 (Ang-1), is expressed by peri-vascular fibroblasts and mesenchymal cells in the embryo[23] and by pericytes in wound healing.[61] Endothelial cells in the vessel wall express the Tie-2 tyrosine kinase receptor in a paracrine manner.[23] In the embryo, the Ang-1/Tie-2 system is responsible, in cooperation with VEGF, for the recruitment of peri-endothelial cells to the endothelial layer. It also facilitates the interaction between endothelial cells and the extra-cellular matrix to promote cell stretching and motility. The Ang-1/Tie-2 system is critical for both functions, as has been demonstrated in Ang-1- and Tie-2 deficient embryonic mice.[23,33] As is obvious from the analysis and reconstruction of tissue folds, pillars, and ITSs, all cellular mechanisms of IMG depicted in Figure 2 can occur with contribution of peri-endothelial cells to form stable pillar cores. This means that the establishment of regular tissue folds and stable pillars and ITSs is dependent on Ang-1/Tie-2. This fact has been confirmed by the analysis of mice deficient of either the ligand or the receptor. Ang-$1^{-/-}$ or Tie-$2^{-/-}$ mice exhibited defects in vascular network growth and remodeling resulting in an abnormal network pattern and death of the embryo.[23,33]

In this respect it is important to note that a recent analysis of embryonic mice deficient of the Tie-1 tyrosine kinase receptor confirmed its role as an inhibitor of IMG. Tie-1 (the ligand for which is still unknown) appears to negatively regulate endothelial cell motility (through interaction with the extracellular matrix) as well as the ability of these cells to form transcellular holes. Consequently, the comparison to wild type embryos demonstrated that Tie-1 deficient mice exhibited increased vessel numbers and "hyperactive" endothelial cells. The latter appeared overstretched, and possessed a large number of filopodia and transcellular holes.[23,50]

FGF-2 facilitates regular neo-vascularization after myocardial infarctions by interfering with pathological mechanisms of IMG. In experimental myocardial infarctions in adult mice (in which the left coronary artery is ligated), the one-time intramyocardial injection of FGF-2 close to the suture increases additionally the number of normal vessels in the transitional zone between healthy myocardium and scar tissue (Patan et al, in prep.).

On contrary the Thromboxane receptor (TP) that has a function in blood coagulation is responsible for a strong inhibition of neo-vascularization after experimental infarctions. As demonstrated by the analysis of TP-deficient transgenic mice, the absence of TP causes an extremely strong and long lasting vascularization response in the scar tissue based on increased pillar formation (Patan et al, in prep.).

Interestingly, the analysis of transgenic mice deficient of another tyrosine kinase receptor family, the Eph/B receptors and their ligands, the ephrins, has demonstrated functions in embryonic angiogenesis that correspond to the one of the Ang-1/Tie-2 system.[48-49] In this case ligand and receptor are attached to the cells and are differentially expressed on arterial (the ligand ephrin-B2) and venous (the receptor Eph-B4) endothelial cells.[48] Ephrin-B2 is interestingly also expressed on peri-vascular cells.[49,62] Ligand and receptor that engage in bi-directional signaling are subsequently also responsible for the coordinated interaction of arterial- and venous endothelial cells.[62-63]

The Notch gene family encodes large transmembrane receptors that are involved in intercellular signaling. Notch1 and Notch4 are expressed in endothelial cells in the embryonic vasculature.[64] Notch1$^{-/-}$ and Notch1/Notch4 double mutant embryos displayed severe defects of embryonic vascular remodeling corresponding to ones detected in Ang-1-, Tie-2-, ephrin-B2 deficient embryos.[65]

The analysis of transgenic mice deficient of connexin 45 (Cx45) demonstrates a similar phenotype concerning embryonic angiogenesis. Not surprisingly, Cx45, a gap junction protein, is involved in the communication between endothelial-, as well as endothelial- and peri-endothelial cells.[66]

RECK, a membrane anchored glycoprotein, was found to be essential for mouse vascular remodeling in the embryo. It inhibits three matrix metalloproteinases (MMPs: MMP-9, MMP-2, and MT1-MMP)[67] and is widely expressed in mesenchymal and vascular smooth muscle cells. RECK deficiency caused increased proteolysis of collagen 1 and defects in the basal lamina with subsequent failure of remodeling of the primitive vascular plexus to form a mature one.[68] Since collagen fibers (collagen type 1) are an indispensable component of every pillar- or ITS core, the important role of RECK for the regulation of the balance between synthesis and proteolysis of collagen can be estimated. This guarantees the sufficient formation of branching points.

In an attempt to investigate the role of the cytoskeleton and Rho GTPases in the process of branching morphogenesis, small GTPase Rac was found to be responsible for matrix induced changes in endothelial cell morphology, while p21-activated kinase, an effector of Rac, was required for cell motility.[69] Correspondingly WAVE2, a protein preferentially expressed in endothelial cells during embryogenesis is responsible for Rac-induced membrane ruffling as it is necessary for changes of cell morphology. Embryos lacking WAVE2 exhibit an increased number of transcellular holes corresponding to Tie-1$^{-/-}$ embryos. In contrast to Tie-1 deficient embryos, WAVE2$^{-/-}$ mice possess fewer endothelial filopodia and reduced vessel numbers compared to wildtype littermates.[70]

Future work should identify the interaction and precise function of these molecular regulators, which are transformed into a sequence of cues to regulate each cellular mechanism of branching morphogenesis.

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