Mechanisms of Angiogenesis
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New insights into intussusceptive angiogenesis

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Angiogenesis is defined as the growth and development of new capillary blood vessels from pre-existing vasculature [1]. It is requisite in metazoans because the transfer of nutrients and wastes has to be accomplished by diffusion through the tissue and hence, the respiring cells need to be within 100–200 μm of the blood vessels, which is the diffusion limit for oxygen [2]. Angiogenesis is a normal process fundamental in wound healing, reproduction and development. Abnormal angiogenic activity occurs in non-neoplastic diseases such as arthritis, psoriasis, trachoma, and diabetic retinopathy and in the vascularisation of tumours (for details, see [3–8]).

Angiogenesis has two facets: sprouting angiogenesis and intussusceptive angiogenesis. This chapter concentrates on the basic mechanisms, facets and outcomes of intussusceptive angiogenesis.

Concept and definition of intussusceptive angiogenesis

Intussusception is defined in the Merriam-Webster dictionary as INVAGINATION, as may be manifested by the slipping of a length of intestine into an adjacent portion of the same, or the assimilation of new material and its dispersal among pre-existent matter. In 1986, Caduff and co-workers demonstrated tiny holes in the developing lung vasculature and postulated that these were due to intussusceptive (in-itself) microvascular growth a fact proved later by Burri and Tarek [9]. Intussusceptive angiogenesis refers to that process by which new blood vessels grow and develop from pre-existing vasculature through insertion of tissue pillars into the capillary lumina and expansion of the latter to form new capillary networks.

Mechanisms of intussusceptive angiogenesis

The quintessence of non-sprouting angiogenesis by intussusception is formation of a tissue pillar into the vascular lumen of a blood vessel. This phenom-

enon was first observed in the rapidly expanding pulmonary capillary bed of neonatal rats [10]. This was manifested as numerous tiny holes (1–2 μm in diameter) in vascular corrosion casts, which were shown to correspond to slender transcapillary (intraluminal) tissue pillars [9, 10]. Serial sectioning of tissue followed by transmission electron microscopy revealed that the pillars arose by invagination of the capillary wall into the vessel lumen [11].

Stages in pillar formation

Four consecutive steps in pillar formation have been described. During stage I, a zone of contact is established between opposite capillary walls. In stage II, there is reorganization of the inter-endothelial cell junctions and central perforation of the bilayer. In stage III, an interstitial pillar core is formed which is subsequently invaded by pericytes and myofibroblasts that then lay down collagen fibrils. By this stage, transluminal pillars have a diameter of ≈2.5 μm. During the fourth and final stage the pillars increase in girth without undergoing any further change in their basic structure. In addition to developing lung, intussusception has been demonstrated in the chick chorioallantoic membrane (CAM) by Patan et al. [12, 13] and has since been revealed to occur in many organs and species during both normal and pathological microvascular growth. Hence, it appears to be a general phenomenon as seen in the reports of Patan et al. [14–16], Djonov et al. [11, 17–19], Burri and Djonov [20] and Kurz et al. [21]. The concept of intussusception is schematically represented in Figure 1, while Figure 2 illustrates the various stages of intussusceptive microvascular growth (IMG) in the avian kidney.

Nascent reports indicate that non-sprouting angiogenesis is ubiquitous, occurring in tissues such as myocardium [22], skeletal muscle [23] and kidney [19]. The modes of vascular growth described have sometimes been referred to in different names, viz., “longitudinal splitting” [22] and “luminal division” [23], respectively, but both resemble intussusception during the initial stages of inception. In muscular tissue, transluminal pillars appear more elongated than in lung due to the longitudinal and parallel arrangement of the myofibres. The complex spatial structure of transluminal pillars and the inadequacy of older visualization techniques meant that the intussusceptive process eluded many contemporary investigators for a considerable duration of time. Vascular corrosion casting (Fig. 2) and serial sectioning for light or transmission electron microscopy followed by three-dimensional reconstruction (Fig. 3) [9, 11, 17–19], or confocal laser microscopy, are the definite techniques for unequivocal identification of pillars. Cognate methods for three-dimensional imaging, such as nuclear magnetic resonance, micro-computer tomography, angiography and ultrasonics do not have the resolution necessary (at least 1 μm) for the visualization of pillars. This circumstance may explain why intussusception was overlooked in the past.

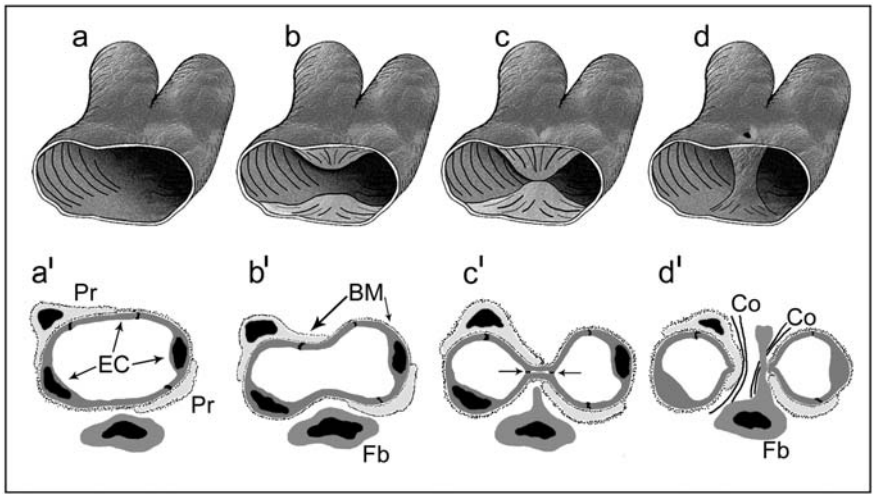

Figure 1. (a–d) Three-dimensional schematic illustrating the steps in the generation of new vascular segments by intussusceptive growth. The process begins with the protrusion of portions of the walls from opposite sides into the vessel lumen (a, b). After contact has been established and fortified (c), the endothelial bilayer becomes perforated centrally and a transluminal pillar is formed (d). (a'–d') Two-dimensional representation of the events depicted in a–d. Endothelial cells (EC) situated on opposite sides of a capillary protrude into its lumen until they contact each other (a'–c'). Once established, this contact is fortified by the formation of interendothelial junctions and then reorganized in such a manner that the endothelial bilayer is perforated centrally. The endothelial cells then retract, and the newly formed pillar increases in girth after being invaded by fibroblasts (Fb) and pericytes (Pr), which lay down collagen fibrils (Co in d'). After [30]. (For colored picture see color plate 2)

Dynamics of pillar formation

A remarkable characteristic of intussusceptive angiogenesis is that it is achieved at a relatively low rate of endothelial cell proliferation. In CAMs, this rate drops dramatically between days 10 and 11 of incubation, coinciding with the peak of intussusceptive pillar formation [11, 17, 24–26]. In the lung vasculature, the capillary volume and surface area were seen to increase 35-fold and 20-fold, respectively [27, 28], in the virtual absence of a change in endothelial cell number [29]. Comparative studies of various organs before and after the onset of intussusception have revealed the total endothelial cell volume to be redistributed during pillar formation by a thinning and spreading of the pre-existing cell population [30]. Endothelial cell attenuation during CAM growth was first documented as a serendipitous finding by Ausprunk et al. [24]. Subsequent morphometric analysis of chick CAMs revealed the thickness of endothelial cells to be reduced by more than 50% between days 10 and 14 of incubation [31].

Direct and definitive evidence for the existence of intussusceptive vascular growth has now been obtained [12] by using chick CAMs [32]. This is an

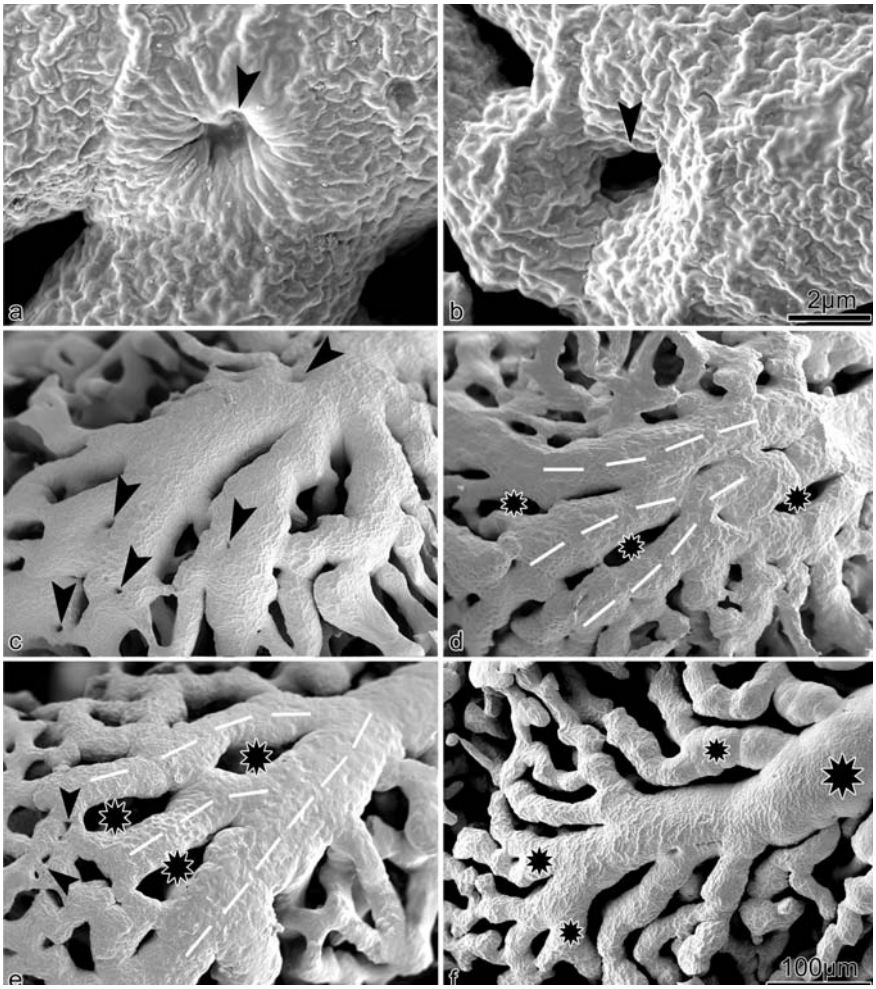

Figure 2. (a–f) Vascular corrosion casts from the avian metanephric kidney at day 15 of incubation illustrating the process of intussusceptive microvascular growth and intussusceptive arborisation. (a) Incipient transcapillary pillars appear as small depressions on the surface of the blood vessel cast (arrowhead) indicating the initial stages of pillar formation (see also Figs 1b, c, b' and c'). (b) As the two opposite components of the pillar approximate, there is fusion and subsequent perforation so that the pillar is now represented by a hole that pierces through the vascular lumen (arrowhead) (see also Figs 1 d, d'). Note that (a) and (b) are at the same magnification. (c–f) Low magnification microvascualar casts showing the various stages of pillar formation and vascular bed expansion. Incipient pillars are represented by depressions or small holes in the cast (arrowheads in c). Notice the irregular nature of the resultant vessels. Subsequently pillars increase in girth and fuse (asterisks in d and e) and in so doing delineate new vascular entities. Further expansion of pillars (asterisk in d and e) separates out the newly formed vessels (interrupted lines in d and e). Note that new pillars (arrowheads in e) now tend to form in the distal part of the vascular tree. Therefore, pillar initiation, augmentation and fusion, results in formation of complex vascular patterns, which include new capillary segments (small asterisks in f) and supplying and feeding vessels (large asterisks in f). Note the virtual absence of pillar holes and the "mature" and hierarchical appearance of the vasculature in (f). (c–f) are at same magnification.

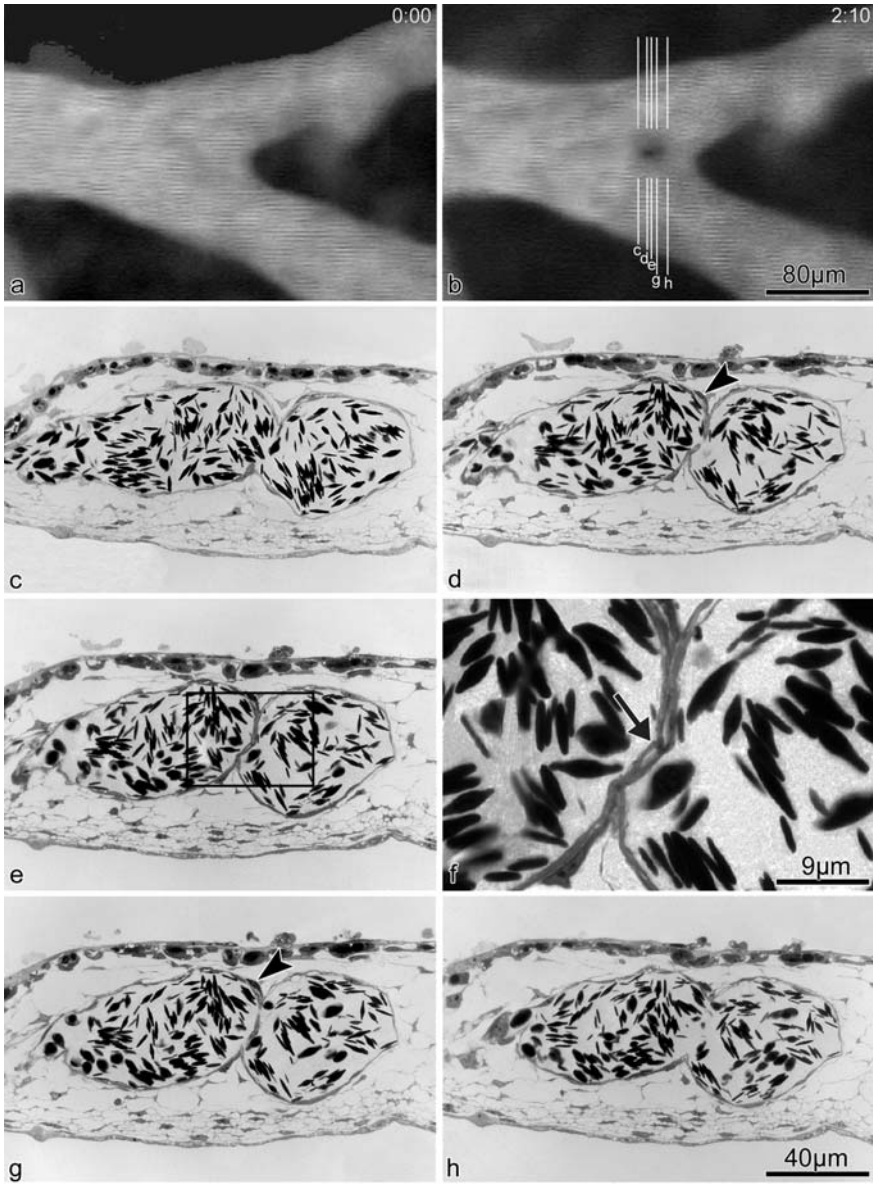

Figure 3. (a, b) *In vivo* video images illustrating pillar formation at a venous bifurcation. After 130 min of surveillance, a dark "spot" became visible, which was present on focusing through the entire breadth of the vessel lumen. (a) and (b) have been procured at the same magnification. (c–h): Semithin serial sections through the dark "spot" revealed the presence of an hour-glass-shaped pillar, created by the simultaneous protrusion of endothelial cells from opposite sides of the vascular wall into the lumen. The intensely stained zone (arrow in f; high-magnification view of the boxed region in e) represents an intercellular junction within the endothelial bilayer. Note mesenchymal cells in a pericytic position near intussusceptive branching remodeling site (arrowheads in d, g). Note that c-e, g, h at same magnification. After [19].

excellent tool for investigating normal vascular growth and remodeling processes, and for monitoring alterations induced by various pro- and anti-angiogenic factors [33]. The use of improved digital techniques in combination with fluorescein-isothiocyanate-dextran injection into the blood stream has enhanced the quality of earlier images. Pillar formation and remodeling have been indubitably observed in capillary plexuses [11, 17], as well as in small arteries and veins [19]. *In vivo* monitoring coupled with histological and ultrastructural analyses of serial tissue sections has demonstrated that pillar formation requires a period of 4–5 h for completion [19] (Fig. 3). This time is decreased to 1 h on doubling the blood flow rate [19], indicating the pivotal role of hemodynamics in control of intussusception. In contrast sprouting angiogenesis is a prolonged process characterized by extensive proliferation of endothelial cells, degradation of extracellular matrix and an increase in vascular permeability. Intussusception occurs in the virtual absence of endothelial cell proliferation, is achieved at low vascular permeability levels, and requires only a short duration for completion. It is a widespread phenomenon that occurs in the vascular systems of all species thus far investigated.

The phases of intussusceptive angiogenesis

The term intussusceptive angiogenesis circumscribes a host of processes that are involved in generation, growth, development and remodeling of vascular entities with diverse morphological and functional outcomes. Though chronologically sequential, the processes overlap both in space and time. Intussusceptive angiogenesis inaugurates with formation of pillars within the capillary bed, which subsequently expand leading to an increase in the complexity of the capillary network (Figs 2, 7), a process referred to as intussusceptive microvascular growth (IMG). In the distal parts of the supplying vessels, pillars may arise in series parallel to the long axis of the vessel and then merge to split the major vessel into small arteries and veins in distal parts of a vascular tree. This process has been referred to as intussusceptive arborization (IAR) and results in the formation of a vascular tree (Figs 4, 7). Thirdly, pillar formation occurring within small arteries and veins can lead to remodeling via an expansion or pruning of vessel branches and an optimization of the branching geometry and of the hemodynamic conditions of the vascular tree, (Figs 5, 7) a process referred to as intussusceptive branching remodeling (IBR).

Intussusceptive microvascular growth (IMG): expansion of capillary plexuses

Continuous pillar formation and growth lead to a rapid expansion of the capillary plexus, thereby affording a large surface area for the exchange of oxygen, carbon dioxide and nutrients. Consequently, new segments of the capil-

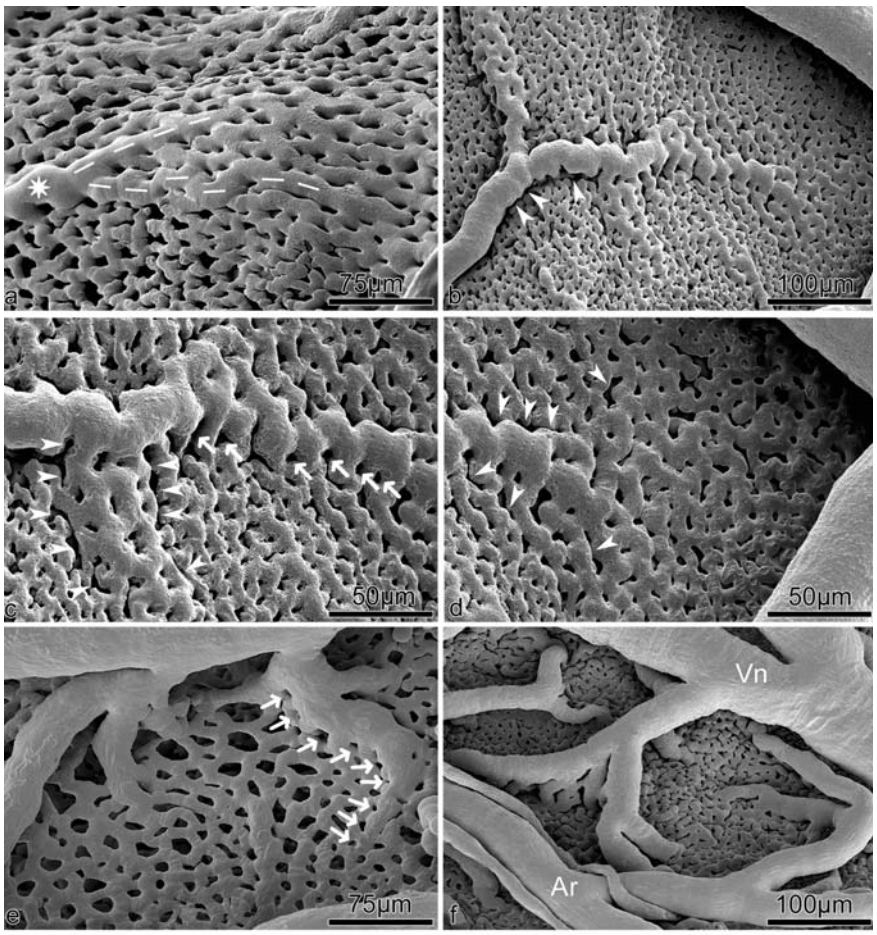

Figure 4. (a–f) Mercox casts of developing CAM vasculature demonstrating the process of IAR. (a) At day 9 of incubation a collecting venule (*asterisk*) with the adjacent triangle-like intensively perfused area is elevated and shifted out from the capillary plane. Rows of pillars demarcate the future blood vessels (hatched lines). (b) By day 10 the proximal part of the collecting vessel is completely separated from the capillary plexus by merging of horizontal tissue pillars (arrowheads). (c–f) IAR is initiated by a change in the pillar axes orientation (*arrows*) from perpendicular in the distal part to horizontal in the proximal part followed by merging into tissue septa. The areas of remodeling expand in lateral (c) and distal (d) directions giving rise to new branching generations by tissue septa formations (*arrowheads*). Note that c and d are taken from b at higher magnification. At day 12 (e), pillar axes (*arrows*) change orientation within a short distance, resulting in a size decrease of the remodelling areas, so that by day 14 (f) the latter disappear and the CAM vasculature consists of two layers: the capillary plexus and the layer of feed vessels, which remain connected by short abrupt vessel bridges. Vn = vein Ar = artery. After [17].

lary network arise with only little changes in the dimensions of its components (Figs 1, 2 and 7), viz., IMG. IMG was first observed in the growing postnatal lung [9, 10] and then in the microvasculature of many other tissues and organs

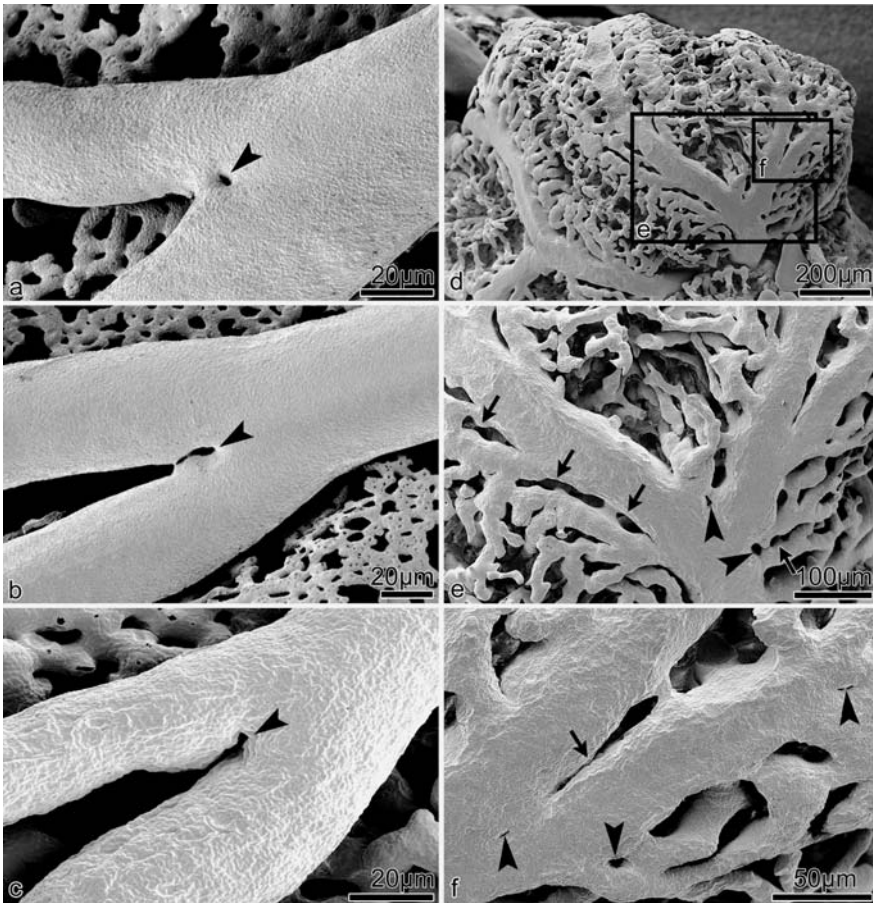

Figure 5. (a–f) Vascular casts illustrating the process of intussusceptive branching remodeling in the chick CAM (a, b,c) and avian metanephric kidney vessels (d, e, f). (a–c) Illustration of the branching angle modification by IBR. The process initiates with a small pillar (small hole indicated by arrowhead in a) which expands (b) until the distal connection between the vessels is severed (c) with a resultant replacement of the bifurcation point to proximal and alteration in the size of branching vessels. (d–f) Vascular casts of a metanephric kidney glomerulus at day 20 of incubation. Series of tissue pillars (arrowheads) and longitudinal folds of the endothelial wall (arrows) are apparent at the bifurcations, indicating ongoing intussusceptive branching remodeling. After [19].

of several species, including the rat [14], the chick CAM [11, 12, 17], retina [17, 19] and kidney [19], in a mouse model of tissue repair [15], in heart development [22], in the human endometrium [34, 35], in cerebral vascularization after stroke [36] and in tumor angiogenesis [15, 18]. It is now evident that IMG represents a general and ubiquitous mechanism of capillary growth. This phenomenon explains the way in which the capillary beds of organs, which arise initially by sprouting and/or vasculogenesis, can undergo rapid expansion

without any compromise in vascular physiology or function, as is reflected by the low vascular permeability conditions and the low rate of endothelial cell proliferation associated with IMG.

Intussusceptive arborization (IAR): formation of a feeding vascular tree

The hierarchical organization of the vasculature generally resembles the branching pattern of a tree and hence the name vascular tree [1]. Major supplying vessels give way to feeding vessels, which break into even smaller arterioles and venules. The latter groups give way to numerous capillaries. At each level, the sizes of the vessels decrease but the number of individual vascular entities increases. Notably the original pattern of vasculature formed from vasculogenesis or aniogenesis hardly resembles a tree. The culmination of the latter pattern is thoroughly and adroitly crafted through the process of intussusceptive arborization (IAR). As a capillary plexus grows, the perfusion distance between arteries and veins increases, which necessitates an adaptation in the system of supplying and draining vessels. Intussusceptive pillar formation has been shown to be involved in the differentiation of parts of the capillary plexus into immediate pre- and postcapillary feeding vessels, viz., IAR [11, 17]. IAR furnishes a mechanism whereby preferentially perfused segments of a capillary plexus can be transformed into terminal arterioles and collecting venules by changing their size and position, the number of sprays in a bunch of feeding or collecting vessels being thereby increased. IAR is initiated by the formation of serried "vertical" pillars, which demarcate future feeding vessels. These pillars undergo reshaping into narrow tissue septa that progressively fuse to delineate a new vascular entity. The remaining connecting bridges are "severed" by the formation of "horizontal" folds, the feeding vessels being thereby definitively separated from the capillary plexus. As a result of this process, a complex arterial and venous vascular tree arises to form a second layer of draining and feeding vessels (Fig. 4).

Intussusceptive branching remodeling (IBR): optimization of branching geometry

The concept of symmorphosis postulates a quantitative match of design and function so that an organism does not invest in superfluous structures. This has been demonstrated to be the case for the circulatory system [37]. In development and maturation of vasculature, structural-functional optimization is achieved by the process of intussusceptive branching remodeling (IBR). The branching geometry of supplying vessels is adapted to optimize the pre- and postcapillary flow properties. IBR can also lead to the removal of putative supernumerary branches (vascular pruning), thereby optimizing the efficiency of the blood supply and the hierarchy of the vascular tree.

Implementation of IBR is accomplished via transluminal pillars and folds, which occur close to the bifurcation sites of arteries and veins of up to 120 um in diameter. These structures appear *de novo*, and are capable of rapidly changing the vascular geometry and the hemodynamic properties at the affected branching points [19, 21, 30]. The pillars located close to bifurcation points enlarge (pillar augmentation) until their distal ends approximate, contacts and merge with connective tissue in the branching angle (Figs 3, 5). Pillars located more than 8–10 μm from the bifurcation point tend to elongate into flat longitudinal folds that protrude progressively into the lumen until this is subdivided into two distinct channels (for more details see [19]). Thus, IBR narrows the branching angle by relocating the branching point more proximally. This may represent an important adaptive response to the continually increasing blood flow and blood pressure during embryogenesis and growth. Direct experimental evidence for this hypothesis has been furnished by Frame and Sarelius [38] who reported the bifurcation angle of golden hamster cremaster muscle vessels to be modified in response to blood flow alterations. A 12%–14% reduction in the branching angle of retinal arteries has also been reported to occur in hypertensive human subjects [39]. Secondly, IBR optimizes the hemodynamic conditions at bifurcation sites by remodeling the diameter of one or both branches (mainly by "pillar augmentation"). Consequently, IBR yields a branching pattern that approximates to the ideal predicted by "Murray's Law" of minimal power consumption and constant shear stress [19, 21], a case of symmorphosis [37].

Pruning as a result of IBR

Intussusceptive vascular pruning (IPR) may be considered to be a facet of IBR. Presumably, in normal growing tissue, IPR severs the vascular branches that are no longer required. It is implemented by the successive asymmetric formation of pillars, which occasion the subtotal lumen obstruction of one of the daughter branches. The reduction in blood flow associated with the narrowed bore probably contributes to the regression, retraction, and ultimate atrophy of the affected branch (Figs 6, 7). The pruning phenomenon was first described for retinal vessels by Ashton [40] and is thought to be stimulated by growth factors and by oxygen tension [1, 41]. The thinning, retraction, and atrophy of vessel branches have been well described by Clark and Clark [42]. The latter authors demonstrated the complete separation of a side branch from the main vessel within 3 days and its disappearance by the fourth day. All previous reports dealing with vascular pruning described the phenomen, but did not recognize the crucial role played by eccentric pillar formation and fusion i.e., IPR.

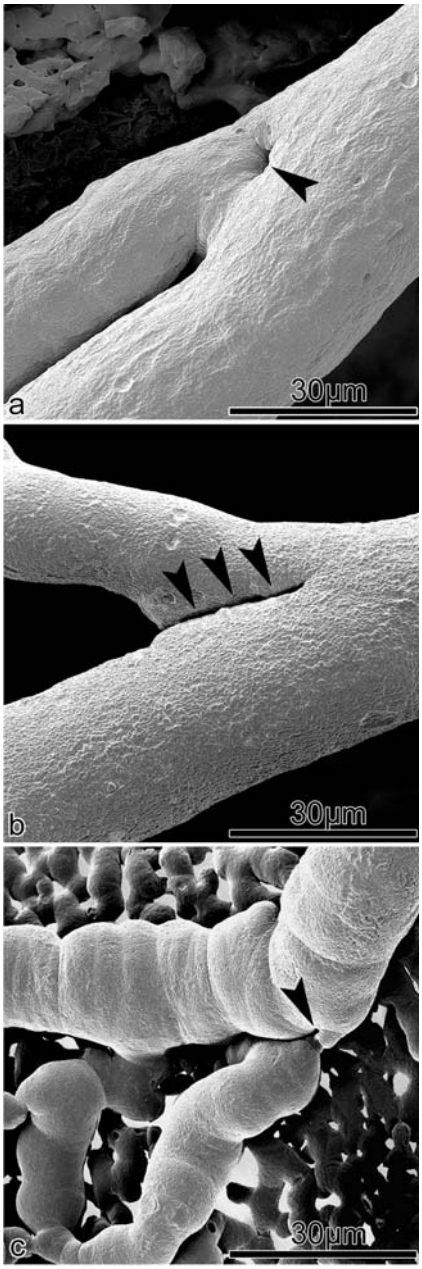

Figure 6. (a–c) Vascular casts of feed vessels in 12- and 13-day-old chorioallantoic membranes illustrating the putative role of intussusceptive branching remodeling in vascular pruning. Initially, a single (arrowhead in a) and later multiple (arrowheads in b) eccentrically located pillars arise. This mode of vessel splitting results in complete luminal obstruction (arrowhead in c) and severance of one of the daughter branches. Adapted from [19].

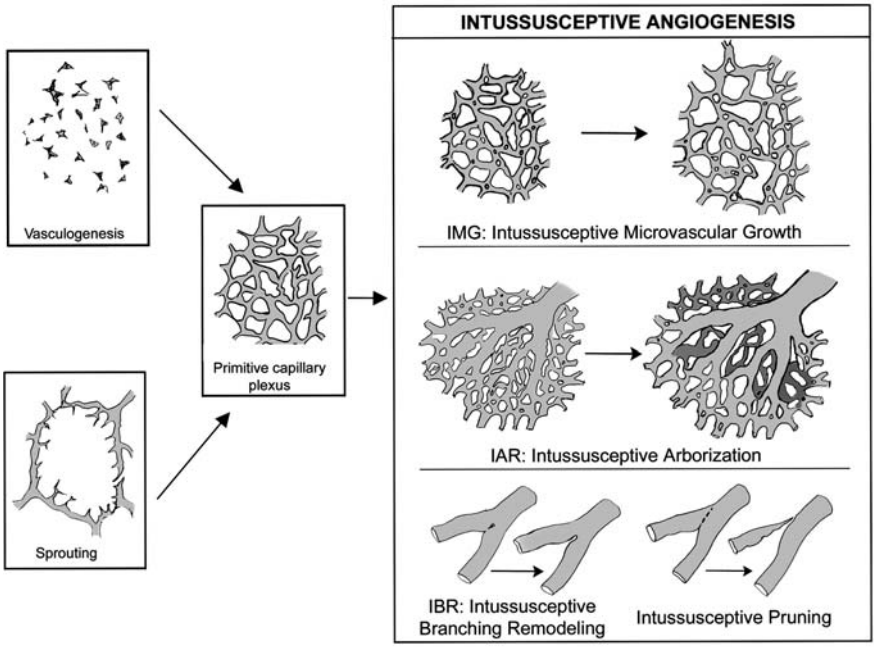

Figure 7. Diagramatic synopsis of intussusceptive angiogenesis (IA). When a primitive capillary plexus is generated by vasculogenesis or sprouting, intussusception is triggered and is responsible for rapid vascular growth and remodeling. IMG is responsible for rapid capillary expansion; IAR for subsequently segregation of feeding vessels from the capillary plexus and IBR optimizes branching geometry and is responsible for vascular pruning. After [30].

Sprouting and intussusception: two complementary angiogenic mechanisms

The various techniques used for *in vitro* and *in vivo* study of angiogenesis, such as three-dimensional collagen gels, corneal implants, tumor implantation, wound healing and embryonic grafting, elicit only capillary sprouting during tissue neovascularization. Blood flow in the aforementioned models is limited and intussusception would not be expected to occur. Consequently, and perhaps also because of the visualization difficulties alluded to above, recognition of intussusception has eluded many investigators of angiogenesis. In the studies conducted this far, it has been shown that the vascular system inaugurates by vasculogenesis [1] followed by an early "sprouting phase" characterized by appearance of multiple capillary sprouts that invade the mesenchyme and, after fusion, form the primary capillary plexus. During the second "intussusceptive phase", capillary sprouting is supervened and perhaps also superseded by transcapillary pillar formation. Further vascular growth and remodeling thus occurs primarily by intussusception. Ultimately, the rapid expansion of the

capillary network (IMG) coupled with vascular tree formation (IAR) and the dynamic adaptation of the latter by branching remodeling (IBR) results in functionally efficient vascular networks (Fig. 7). Three factors are thoutght to stimulate the switch from sprouting to intussusceptive angiogenesis. These include the relatively short duration required for intussusceptive angiogenesis, the low cost metabolically and energetically and finally the non interference with the local physiological conditions. This process does not depend upon extensive endothelial cell proliferation, basal membrane degradation or the invasion of surrounding tissue and "physiological" levels of transpermeability that permit vascular growth and remodeling to occur within a functionally uncompromised organ are maintained. In summary, we emphasize the fact that in new embryonic capillary networks intussusceptive angiogenesis (IA) initiates with IMG, which starts with the formation of the first pillars. This is followed by IAR, which establishes a vascular tree. IBR finally remodels the maturing vascular network. However, the three mechanisms may be contemporaneous in the same organ since as some vascular entities are maturing, others are in formative stages.

Control and regulation of intussusceptive angiogenesis

Pillar formation is quintessential in intussusceptive angiogenesis. Through pillar formation new vascular networks are formed, augmented, expanded and remodeled to accommodate changes in functional needs. Based on the method of implementation and morphofunctional outcomes, IA can be considered to comprise three cognate processes, namely IMG, IAR and IBR. Presumably, each process is controlled by a specific program that is initiated and regulated by definite molecules, cells and hemodynamics. Information concerning the identity of these is gradually emerging.

Hemodynamics

Hemodynamic forces are obvious and important determinants of vascular architecture. Djonov et al. [19] demonstrated that clamping of one of the dichotomous branches of an artery in the CAM microvasculature increases blood flow and/or pressure in its counterpart with an almost immediate effect on branching morphology. IBR is initiated within a few minutes, pillars are detected after 15–30 min, and the branching angles are decreased by about 20% after 40 min. Shear stress, which acts tangentially on capillary walls and is known to be modified by experimental increases in blood flow, may be responsible for these changes. Shear stress is related to the diameter of a vessel. Hence, insertion of a pillar into the blood stream near a branching point will reduce this force in post-pillar vessel segments [19, 21].

Molecular control of intussusception

Endothelial cells are known to sense changes in shear stress. Such changes are transduced by molecules such as PECAM/CD31 [43] into the interior of the cell. This mechanotransduction system then leads to changes in the transcription rate of many proteins, such as eNOS, adhesion molecules and angiogenic factors [44, 45]. Physiological or pathophysiological adaptations to changes in shear stress involve interactions between pericytes, macrophages and endothelial cells [46]. It has been demonstrated morphologically that pericytes and/or periendothelial cells are recruited during the initial and final phases of vascular pillar formation in several organs [11, 19] and are thought to contribute either to the synthesis and mechanical stabilization of the transcapillary pillar core or to the maintenance of a low vascular permeability during intussusception.

The putative inducers of sprouting angiogenesis include angiopoetins and their Tie-receptors [47], PDGF-B [48] and ephrins and their Eph-B receptors [49, 50] and probably also influence vascular remodeling. Angiopoietin-2 and PDGF-B are both essential for pericyte recruitment in the retina [51], brain [48] and placenta [52] and therefore may be important in formation and maturation of the tissue pillars during intussusceptive angiogenesis. The injection of PDGF-B into fully developed CAMs, for example, leads to formation of abundant large pre- and postcapillary microvessels but not to the expansion of capillary meshes [53]. On the other hand, knockout mice lacking angiopoietin-1 and Tie-2 show abnormal vascular development in which the vessel growth is arrested at a primitive stage and further remodeling does not occur [54]. Conversely, over-expression of angiopoietin-1 or of angiopoietin-2 simultaneously with VEGF is associated with the formation of "large" vessels, and numerous small holes in the capillary plexus [55], a finding reminiscent of intussusception. Ashton [40] has shown that when VEGF is maintained at moderate but constant levels, the vessels remain at an immature state whereas down-regulation of this factor is associated with pruning. VEGF appears to be an early promoter of angiogenesis while the angiopoietins and their receptor Tie-2 as well as the ephrins and their corresponding Eph receptors appear to act at a somewhat later stage of angiogenesis [3] and are probably associated with regulation of intussusception. VEGF promotes formation of new capillary segments and vascular maturation through pericyte and smooth muscle recruitment [56, 57]. Such periendothelial cells are important for vascular integrity and maturation. Newly formed vessels that are denuded of such cells become VEGF-independent and fail to mature [51]. Treatment of CAM [58] and the retinal vasculatre [59] with VEGF results in both sprouting and intussusceptive angiogenesis. Whether the latter process occurs consequent to sprouting is unclear, but evidently VEGF plays an important role in its initiation. VEGF is a highly potent and universal regulator of vascular responses to tissue oxygenation levels [41, 57], and probably responds to variation in oxygen concentration with the appropriate angiogenic responses [41]. Though many molecules have been implicated in angiogenesis, their precise role in initiation and

progression of intussusceptive angiogenesis remains to be investigated. The interplay between such molecules, local hemodynamic conditions and oxygen tension in regulation and control of intussusceptive angiogenesis are currently fertile areas for investigation.

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