Chapter 3
Vasculogenesis and Angiogenesis

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Introduction

The development of the circulatory system is one of the earliest events in organogenesis [1]. This system is comprised of interconnected tubes that form an intricate network for transporting blood and lymph across tissues. The endothelial cells (ECs) line the inner walls of the tubes, and vascular smooth muscle cells surround them on the outside. The circulatory system is comprised of two primary vessel types, namely, the blood vessels and the lymphatic vessels [2]. These vessels differ in the content of their lumen. Blood vessels primarily carry blood, while lymphatic vessels carry lymph fluid. Blood vessels are comprised of arteries and veins. Arteries carry oxygenated blood that flows under high pressure, while the veins carry deoxygenated blood back to the heart under low pressure. The dorsal aorta (DA) and cardinal vein (CV) are the first to arise in a developing embryo. These are formed de novo via the assembly of endothelial precursors into a primitive vascular network (or plexus) through a process called "vasculogenesis" [3]. Once the primitive vascular plexus is established, a complex remodeling process consisting of migration, proliferation, sprouting, and pruning ensues and leads to the development of a functional circulatory system. This remodeling process is referred to as

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"angiogenesis" [4]. Small capillaries and the intersomitic vessels (ISVs; vessels that traverse early body segments or somites) are formed through the angiogenesis mechanism.

Embryonic programs of angiogenesis and vasculogenesis are recapitulated in the adult during repair and healing processes and in mammalian reproduction. Ovulation, menstruation, implantation, and placentation are critically dependent on angiogenesis and neovascularization. The placenta undergoes extensive vascular growth and remodeling throughout pregnancy. Vascularization of the placenta is unique in its requirement for coordinated development of maternal and fetal vessels. This allows formation of large surface areas of exchange between maternal and fetal circulations, necessary for placental function. Specialized placental cells, termed trophoblast cells, are involved in the regulation of this process. Inadequate or abnormal vascularization of the placenta impacts the start of life and has far-reaching consequences on the metabolic and cardiovascular health in adult life [5, 6]. Abnormal angiogenesis and neovascularization are also associated with other pathological conditions such as tumor growth. Tumors secrete EC stimulatory growth factors that induce the host vasculature to sprout and grow in an angiogenic fashion. The new vessels are used by the tumor to traverse to distant sites, thus promoting metastases. In this chapter, we will discuss the basic principles governing vasculogenesis and angiogenesis in the embryo and placenta. In addition, we will discuss the molecules involved in these processes and the role that they play in the development of vascular anomalies.

Origin of Cells of the Vascular Lineage

All tissues of the body emerge from the three germ layers of ectoderm, endoderm, and mesoderm. Post gastrulation, the mesoderm germ layer gives rise to cells of the heart, kidney, muscle, and blood-forming tissues in addition to the cells of the circulatory system, namely, the vascular ECs. The vascular ECs emerge from the lateral most region of the mesoderm termed as the lateral plate mesoderm (LPM). The specification step from the LPM to the precursor cells of the vascular lineage is poorly understood. One putative intermediate step in this specification pathway is the formation of a bipotential precursor cell from the LPM that has capabilities to differentiate into the vascular and hematopoietic lineage. Evidence for the existence of such a bipotential precursor cell is observed during embryonic development, especially in the extraembryonic mouse yolk sac "blood islands"; these are loose aggregates of blood cells surrounded by the endothelium [7]. The outer cells of the blood island clusters appear flattened and subsequently differentiate into ECs, whereas the inner cells become hematopoietic cells [8]. In mouse embryo, this precursor cell is mainly located in the primitive streak (thickening region of the embryo showing noticeable first stages of development) and is only detectable for a very brief period of time [9] and eventually expresses *CD31*, *flk-1*, *flt-1*, and *tie-2* [10] markers, which are characteristic of EC lineage. Similarly, in chick, *flk-1*$^{+}$ cells (Quek1) are abundant in the mesoderm that exits from the posterior primitive streak

during gastrulation [11]. These cells contribute to the formation of both embryonic and extraembryonic mesoderm tissue, which gives rise to the first blood islands [12]. Gene targeting experiments in mouse demonstrate that a functional Flk-1 receptor tyrosine kinase is required for the development of the blood islands, providing further evidence that both hematopoietic and endothelial lineages are derived from a common precursor [13]. This common precursor cell is referred to as hemangioblast, which will be discussed in detail in the next section.

The Case for Hemangioblast

Nearly a century ago, based on observations in chick embryos that both hematopoietic and ECs are closely situated and develop together, a new term called "hemangioblast" was proposed to describe the common precursor cell [14, 15] that gives rise to both lineages. In terms of model systems, zebrafish has contributed immensely to our understanding of hemangioblast because of the identification of the first genetic mutant in vertebrate, "*cloche*," which lacks both ECs and blood cells suggesting that the mutation lies in the critical gene (as yet unidentified) that specifies the critical dual potential hemangioblast cell to vascular and blood lineage [16, 17]. More recently, Vogeli and his group constructed single-cell-resolution fate maps that show individual cells in zebrafish late blastula and gastrula can give rise to both hematopoietic and ECs [18]. Substantial characterization of the hemangioblasts has occurred in vitro through the utilization of the embryonic stem cell (ESC)-derived embryoid body (EB) model system. In the mouse and human ESC-derived EB cultures, single blast colony-forming cells (BL-CFCs) generate colonies that contain both hematopoietic and ECs. These cells emerge in the presence of VEGF-A and bone morphogenetic protein-4 (BMP-4) [10, 19, 20]. Further, they express *flk-1* and the mesodermal marker *brachyury* [21]. Based on the ability of genetically engineered ESCs to differentiate into BL-CFCs in vitro, *flk-1*, *Scl*, *Runx-1*, *Hhex*, *Mixl-1*, *Bmp-4*, *Smad-1*, *Gata-2*, and *Lycat* have all been shown to be essential for the generation of BL-CFCs [22–31]. In addition to these genes, *Lmo*, *fli-1*, and *etsrp* have been recently implicated in the specification of hemangioblast from the posterior LPM in zebrafish [32–37]. Importantly, constitutive activation of *fli1* is shown to be sufficient to induce expression of key hemangioblast genes such as *scl*, *lmo*, *gata2*, *etsrp*, and *flk-1* [38]. At present, our knowledge of hemangioblast specification from LPM is emerging; however, our knowledge downstream of hemangioblast is better known, as will be discussed in the next section.

Vasculogenesis Mechanisms in the Developing Embryo

The hemangioblast cell can differentiate down either the vascular lineage or the hematopoietic lineage. If the program for vascular lineage differentiation is initiated, the hemangioblast becomes more differentiated, and this cell type is called

angioblast. The great vessels of the developing embryo such as the aorta and vein are formed directly from this cell population via a process of vasculogenesis [39]. The essential steps in vasculogenesis post angioblast specification are the migration of angioblasts toward the location of future developing vessels, the coalescence of precursor cells to tube formation, and the final maturation of vessels to establish functional circulation [40–42].

Both mice and zebrafish have extensively contributed to our knowledge of vasculogenesis. In mice, the first intraembryonic angioblasts are seen as early as 1 somite (som) stage at the lateral edges of the anterior intestinal portal and ventral to the somites [43]. In zebrafish, angioblasts are observed as early as 1 som in the LPM. An ets-related transcription factor protein (*etsrp or etv2*) was recently shown to be necessary and sufficient for angioblast specification at the LPM [36] and serves as a marker of angioblasts in the developing embryo. Moreover, *etsrp* is induced by *fli1* transcription factor [38], therefore implying that *fli1* is upstream of *etsrp*. Therefore, double positive $fli^{+}/etsrp^{+}$ cells are used as pre-migratory vascular precursor cells (FEVPs) that are ideal for cell tracking during the vasculogenesis processes in zebrafish embryos. The *etsrp* cells in the head, trunk, and tail of a developing zebrafish follow unique patterns of movement, trajectory, and congregation that highlight the complex series of events that occurs when angioblasts are moving from the LPM to the midline. This unprecedented observation illuminates a highly organized series of events that are dictated by spatial and temporal cues [44].

The vasculogenesis process in zebrafish occurs in multiple stages: initially at pre-10 som stage followed by post-10 som stage. A cartoon is depicted in Fig. 3.1 that describes these events. During pre-10 som stage, the FEVPs increase in number at the LPM (at 10 som), and this increase is attributed to a proliferative mechanism that involves the Shh-vascular endothelial growth factor (VEGF)-Notch-Hey2 signaling pathway [44]. It is noteworthy that the number of angioblasts is not critical for their next step in vasculogenesis [45], i.e., migration to midline. At post-10 som stage (10–12 som), angioblast migration starts from the LPM toward the midline, which is in part controlled by signals from the endoderm [45]. At the midline, angioblasts first aggregate to form cord-like structures with no cell-cell junctions established, thereby resembling a poorly organized cluster of cells. However, for angioblast coalescence at the midline to occur effectively, cell-cell junctions need to form first, which occurs at ~17 hpf (16 som) in zebrafish [45]. In addition to cell-cell junctions, adherens junctions are also formed at this stage indicating that angioblasts are in close contact with each other. The next step in vasculogenesis is the coalescence of angioblasts to lumen formation in axial vessels (DA and PCV), which is poorly understood. An EC-derived secreted factor Egfl-7 was identified to regulate vascular tube formation in zebrafish [46], but the mechanisms involved in this process are not clear. A recent study proposes intracellular vacuolization as a proposed model for endothelial tube formation in vivo [47], but this mechanism was shown for smaller vessels and is not clear if it is applicable to larger vessels such as the primary axial vessels (DA and PCV) [45].

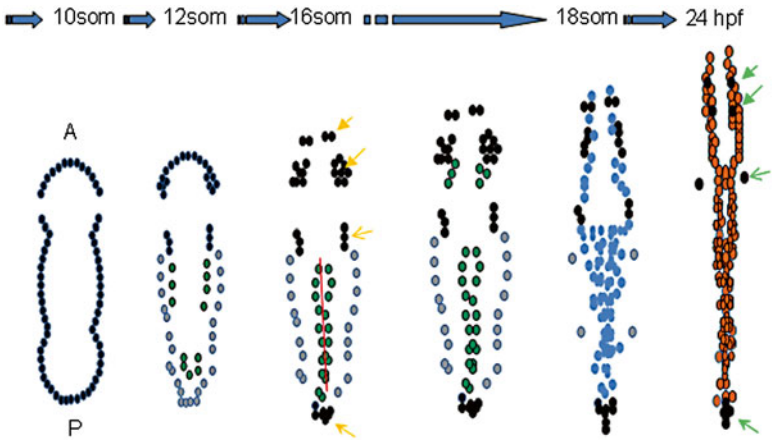

Fig. 3.1 *Etsrp*$^{+}$ angioblast cell development in zebrafish embryo. *Etsrp*$^{+}$ angioblasts appear in head, trunk lateral plate mesoderm (LPM), and tail region at 3 to 10 somite (som) embryos. The head region and the trunk LPM angioblasts contribute to cranial vessels and trunk axial vessels, respectively, during early embryonic vasculogenesis. At 12 to 16 som, head angioblasts show an inward trajectory resulting in four cephalic patches. At the same developmental stages, trunk LPM angioblasts show two distinct populations, anterior and posterior angioblasts. The anterior cells do not undergo substantial change. In contrast, posterior cells (*green circle*, 12 to 16 som) show coordinated movements such as the migration to the midline in an anterior to posterior direction in the trunk. At this time point, four distinct clusters of cells indicated by *yellow arrows* are evident along the axis of the embryo. At 18 som, most of angioblasts (*blue circle*) are closer to the midline in the trunk. At 18 to 20 som, the anterior trunk and the midline angioblasts come together at the site of future lateral dorsal aorta (DA). At 24 hpf, two major axial vessels, dorsal aorta (DA) and posterior cardinal vein (PCV), are observed (*brown circles*), and residual *etsrp*$^{+}$ cell (*green arrows*) remains for the next few hours

Artery vs. Vein (A/V) Specification Mechanisms in Developing Embryo

Once the primary vessels are formed, they take an identity of an artery or vein. Until recently, it was thought that flow dictated artery vs. vein (A/V) formation of primitive vessels [48, 49]. However, recent evidence shows that molecularly, artery and veins are fundamentally distinct in the early embryo thereby challenging the existing paradigm [50]. The embryonic expression pattern of ligand ephrin-B2 (efnb2) (specifically in artery ECs but not in venous ECs) prior to the onset of circulation in mouse embryos and its cognate receptor Ephrin-b4 (Ephb4) (expressed more in vein and less in artery) opened the door for identifying molecular markers that define arterial and venous cell identity [50]. At present, several artery- and vein-specific genes have been identified in vertebrate embryos [51, 52].

During the vasculogenesis process, the specification of angioblasts into artery or venous ECs is a critical step in the formation of arteries and veins, respectively [50]. In zebrafish, the differentiation of angioblasts into arterial and venous ECs first

occurs at 17 hpf (16 som) [45]. The sonic hedgehog (Shh)-VEGF-Notch pathway is involved in A/V specification [52, 53], and a model is proposed to explain how and when A/V specification occurs in vivo. In this model, *shh* expressed in midline notochord cells induces medioventral somites to express VEGF. Because of this VEGF gradient, angioblasts migrate medially toward the midline [53]. VEGF overexpression in *shh*-deficient embryos induces arterial marker *efnb2* but not in *Notch-induced* embryos thereby placing VEGF downstream of Shh but upstream of Notch in arterial differentiation pathway [53]. Similarly in mice, VEGF and its isoforms promote arterial differentiation by inducing *efnb2*-positive vessels [54, 55]. Notch, downstream of VEGF, induces arterial-specific genes such as *efnb2* and represses venous-specific genes such as *flt-4* resulting in A/V specification [56]. Zebrafish embryos with reduced Notch activity have poorly formed DA and PCV often leading to arteriovenous malformation [56]. The hairy-and-enhancer-of-split-related (hey) family of transcription factors is a direct target of the Notch pathway [57]. In zebrafish, the *hey-2* genetic mutant *gridlock (grl)* [58] shows abnormal fusion of the lateral DA leading to aortic coarctation, a cardiovascular defect observed in humans [59]. Although *grl* expression is restricted to DA in zebrafish embryos, it does not appear to play a role in repressing venous markers such as *flt-4*, which might be mediated by another member of the hey family. Interestingly, in the grl^{m145} allele, arterial differentiation is normal, and defects in trunk vessel patterning are apparent suggesting that grl functions in the formation of DA, but its role in differentiation of artery ECs is unclear [52, 58, 60, 61]. Besides hey transcription factors (TF), members of FoxC TF family FoxC1 and FoxC2 are also involved in A/V specification. FoxC1/C2 upregulates the expression of *notch-1*, *notch-4*, and arterial markers *efnb2* and notch target *hey-2* [62] in mouse embryonic ECs suggesting that FoxC TF functions upstream of Notch to promote arterial specification.

In terms of signaling mechanisms, the mitogen-activated protein kinase (MAPK) and phosphoinositide-3-kinase (PI3K) pathways downstream of Flk-1 have been implicated as integral biochemical pathways for A/V specification [63]. Blocking of PI3K signaling pathway and activation of extracellular signal-regulated kinase-1 and kinase-2 (ERK-1/2) MAPK promote arterial fate, and conversely, activation of PI3K and blocking of ERK activation promote venous fate [63]. Compared to arterial specification, less emphasis has been placed on venous specification markers. Of the venous markers, the chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) is the best studied and is a member of the orphan nuclear receptor family that has been shown to actively maintain the venous state by inhibiting Notch activation [64].

Lymphatic Vessel Specification

Like A/V formation, the origins of lymphatics were controversial, until recent evidence conclusively shows that lymphatic ECs are specified from venous ECs [65, 66]. Similar to arteries and veins emerging from dual potential angioblasts,

lymphatic cells emerge from dual potential venous ECs. Prox-1, a transcription factor, initiates lymphatic specification by expressing in a subset of bipotential venous ECs that differentiate into LECs [67, 68]. Prox-1 is also required for maintenance of lymphatic cell identity [69]. Recent work suggests that Sox-18 transcription factor may in part be responsible for inducing *prox-1* expression [70], thereby placing Sox-18 upstream of Prox-1. The Sox-18-induced *prox-1* expression commits dual potential venous cells to the lymphatic lineage. In addition to Prox-1 and Sox-18, Notch, FoxC2, and Efnb2 molecules that are involved in arterial specification are also involved in lymphatic specification [71]. In humans and mice, *Notch1* and *Notch4* are expressed in the lymphatics [72], and Notch signaling directly regulates VEGFR-3 expression in blood ECs [72]. Of the five isoforms of VEGF, VEGF-C is widely considered to mediate lymphatic sprouting through signals triggered on binding to cognate tyrosine kinase receptor VEGFR-3 in lymphatic ECs. FoxC2, transcription factor (TF) of the forkhead family, is also critical for lymphatic development, is expressed by LECs, and functions in lymphatic differentiation [73]. Loss of FoxC2 in mice results in failure to form lymphatic valves, abnormal accumulation of SMC to the lymphatic vasculature, and lymphatic dysfunction [74]. In terms of ephrins, mice at postnatal day 0 (P0) express both *efnb2* and *Ephb4* in dermal lymphatic vasculature [75]. Efnb2 signals via the intracellular PDZ domain, and deletion of the Efnb2 PDZ domain in mice results in lymphatic defects. These include defective dermal lymphatic remodeling, absence of lymphatic valves, abnormal accumulation of SMC in lymphatic capillaries, and chylothorax [75]. These defects suggest a role for efnb2 signaling in lymphangiogenesis.

Angiogenesis Along the Embryonic Body Axis

In the developing embryo, once the great vessels of the embryo, namely, DA and CV, are formed, secondary vasculature is initiated via an angiogenic mechanism. Zebrafish, chickens, and mice have extensively contributed to our understanding of this process. However, recently, zebrafish studies have provided mechanistic-based evidence of the angiogenesis process in vivo especially in the trunk region [76–78]. In the embryonic zebrafish trunk, vessels that traverse the somite (early muscle) boundaries are formed via the angiogenic process and are referred to as intersomitic vessels (ISVs). The ISVs in zebrafish form by discrete steps, and they first sprout from the established DA in pairs and align along the dorsoventral axis. The dorsalmost cell in the sprouting ISVs is called the “tip cell” or “leading cell,” which progressively navigates through intersomitic boundaries and traverses the length of the chevron-like somites to join with its anterior and posterior neighbors, thus establishing a dorsal longitudinal anastomotic vessel (DLAVs). Interestingly, recent evidence shows the involvement of macrophage cells in linking the adjoining DLAVs in zebrafish and mice [79, 80]. Previously, it was thought that ISVs are comprised of three cells [81], but recent evidence shows that ISVs are comprised of multiple cells that overlap extensively to form a multicellular tube [77]. Recent data from

Blum et al. [77] suggest that ISVs lumen formation is a multicellular process containing an extracellular lumen with adjoining ISVs connected with tight junctions. Also, ISVs show a number of cellular behaviors including cell divisions, cell rearrangements, and dynamic changes in cell-cell contacts [77] that were previously unknown.

The mechanisms utilized for ISV formation are similar to another branching network, the nerves. In vertebrate ontogeny, the neural and vascular networks develop side-by-side [82] and utilize the branching processes of axons and blood vessels, respectively, to penetrate different regions of the body. Axons travel great distances to innervate tissues, while vessels travel shorter distances to perfuse tissues. A growing axon projects a sensory structure called a "growth cone" that integrates directional information provided by attraction and repulsion cues from the surrounding environment. Recent evidence suggests that capillaries sprout similarly from specialized ECs called "tip cells" [83]. Similar to the growth cone, the tip cell is a highly motile structure that constantly extends and retracts filopodia. The original idea came from retinal tip cells [83], which were thought not to proliferate but only provide guidance to the following stalk cell. However, ISVs' tip cells in zebrafish [77] do proliferate suggesting that tip cells from different tissue beds may have different functions. The VEGF and Notch family members participate in the tip vs. stalk cell positioning during angiogenic sprouting [84, 85]. Recent evidence suggests that relative levels of VEGF2 and VEGFR1, cognate receptors for VEGF, dictate which cell becomes a tip cell [86]. Guidance decisions of axons and tip cells are relayed by shared mechanisms at a molecular level [82], and members of the axon guidance molecule family, namely, *ephrins*, *semaphorins*, *netrins*, and *slits* along with their cognate receptors, work cooperatively to guide axonal growth cone pathfinding and vascular development (10) and are reviewed elsewhere [82].

Angiogenesis and Vasculogenesis in Placental Development

In mammals, angiogenic processes are recapitulated in the adult female during reproductive cycle and in pregnancy. The requirement for rapid and extensive neovascularization and remodeling perhaps makes the placenta the most active site of physiological angiogenesis and vasculogenesis. In this section, we discuss the unique aspects of blood vessel formation in the context of hemochorial placentation. This type of placentation is observed in humans, apes, and rodents and is characterized by direct contact of maternal blood with cells of fetal origin. While in all other organs blood flows through tubes of ECs, in the hemochorial placenta, maternal blood flows through channels formed by specialized fetal cells, the trophoblast cells (Fig. 3.2f). Trophoblast cells arise from the extraembryonic epithelium and are a unique cell type of the placenta. They play a critical role in regulating uterine vascular remodeling and fetoplacental vascularization. The next few paragraphs outline the processes involved in the establishment of maternal and fetal circulation in the hemochorial placenta and the regulatory role of trophoblast cells. For ease of

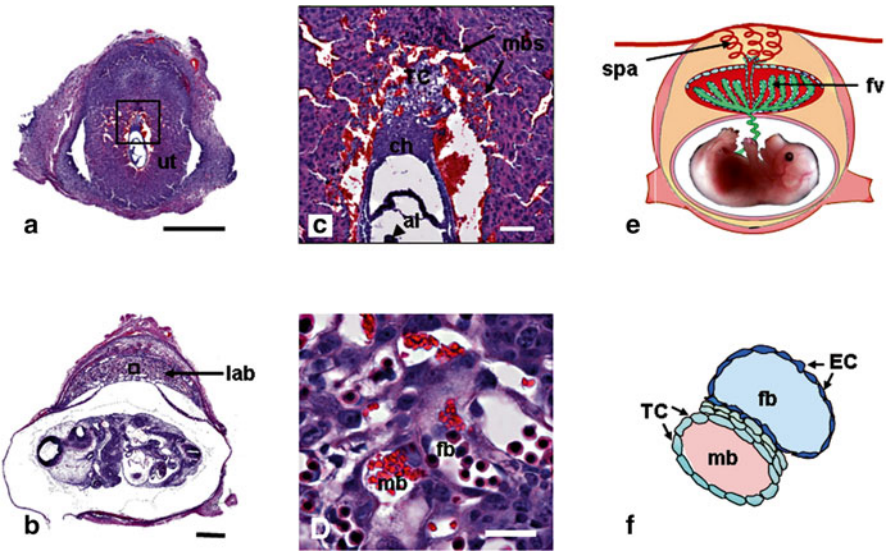

Fig. 3.2 Histological sections and schematic of murine placenta illustrating sites of angiogenesis and vasculogenesis in hemochorial placentation. Hematoxylin-eosin-stained sections of day 7.5 (**a**, **c**) and day 12.5 (**b**, **d**) placenta and schematic drawings of 12.5 placenta (**e**) and an area of placental labyrinth (**f**) are shown. (**c**) and (**d**) are enlarged images of boxed regions in (**a**) and (**b**). Angiogenesis and vascular remodeling in the uterine wall (ut) results in opening of maternal blood sinuses (mbs) to trophoblast cells (TC) surrounding the implanted embryo. The allantois (al) extended by the embryo meets the chorionic plate (ch) of trophoblast stem cells, initiating trophoblast differentiation. Trophoblast lineages exposed to maternal blood adopt EC-like gene expression profile. Spiral arteries (spa) lose their endothelial and smooth muscle layers and convert to low resistance vessels, a process completed by invasion of trophoblast cells into the spiral arteries. Fetal vessels form de novo in the placenta through vasculogenesis in mesodermal cells of fetal villi (fv). Branching morphogenesis and angiogenesis result in the elaboration of fetal vasculature and formation of placental labyrinth (lab). The labyrinth is the site of exchange between fetal blood (fb) and maternal blood (mb). Trophoblast cells (TC) line maternal blood spaces. In contrast, fetal blood vessels are formed from endothelial cells (ECs). The process of fetoplacental vascularization is intimately linked to trophoblast differentiation. Scale bars represent 1 mm (**a**, **b**), 100 μm (**c**), and 25 μm (**d**), respectively. Note that the schematics are not meant to be anatomically accurate

discussion, these processes have been grouped as (1) uterine angiogenesis and vascular remodeling, (2) trophoblast differentiation and pseudovasculogenesis, and (3) fetoplacental vascularization.

The uterus (the organ where the embryo implants) undergoes several changes postconception. These changes, broadly termed as decidualization, include *uterine angiogenesis* and *vascular remodeling*. Uterine angiogenesis is initiated before, and continues after, implantation or the attachment of the blastocyst to the uterus. Trophoblast cells present in the outermost layer of the conceptus mediate this attachment. In rodents, initial uterine angiogenesis involves degeneration of epithelial cells surrounding the implantation chamber; changes in morphology of arterial capillaries in the vicinity, such that their lumens are enlarged and walls become thin

to resemble sinusoids, and hypertrophy of ECs lining these sinusoids [87–89]. As a result of these processes, an anastomosing network of maternal blood sinuses surrounds the implantation chamber and fetal trophoblast cells (Fig. 3.2a, c). In rodents, around 8 days post coitum (dpc), some of the sinusoids open into the implantation chamber and bathe fetal trophoblast cells with maternal blood.

As pregnancy proceeds beyond 9 dpc (corresponding to the end of the first trimester in human pregnancy), the placenta undergoes tremendous growth at a rapid rate. In preparation for this growth, in addition to vascular adaptations at the feto-maternal interface, changes are necessary in larger vessels supplying the uterus. These include significant increases in diameter and length and changes in extracellular matrix composition of arteries that supply the uterus [90]. Extensive remodeling is observed in spiral arteries within the uterus (Fig. 3.2e). The endothelium and smooth muscle layers of uterine spiral arteries are disrupted and replaced by invading trophoblast cells [91]. The net result of these changes is reduced resistance of uterine arteries and an increased and privileged blood supply to the placenta.

Various molecular events involved in uterine angiogenesis and decidualization have been identified [92]. These are regulated by steroid hormones, estrogen and progesterone, and by prostaglandins [93, 94]. Similar to embryonic angiogenesis, VEGF and its receptor VEGFR-2 have also been identified as important mediators of uterine angiogenesis [94, 95]. Angiogenesis can proceed to a large extent with mechanical stimulation of hormonally primed uteri (without blastocyst implantation), suggesting a maternal hormonal control independent of direct regulation by fetal cells. It is, however, clear that fetal trophoblast cells also contribute to uterine angiogenesis. This is exemplified by the observation that abnormal hormonal expression by trophoblast cells in *Gata-2* or *Gata-3* knockout blastocysts implanted in heterozygous mothers (with normal gene expression) results in reduced uterine neovascularization [96]. Similar to uterine angiogenesis, the remodeling of spiral arteries is initiated when trophoblast cells are absent from its vicinity, but requires trophoblast invasion to proceed normally. Among other factors, maternal uterine natural killer cells play an important role in spiral artery remodeling (review [97]).

Trophoblast differentiation and *pseudovasculogenesis* comprise a second group of events intimately coupled with placental vascularization. About 70 % of the mature rodent placenta is made up of zygote-derived trophoblast cells [98]. They regulate many aspects of maternal vascular adaptation to pregnancy and provide the main structural and functional components needed to bring maternal and fetal blood systems in close contact (reviews [99, 100]). The trophoblast cell lineage is specified even before the embryo implants on the uterine wall. In mice, it appears as a sphere of epithelial cells, the trophectoderm, surrounding the inner cell mass and the blastocoel. Trophoblast cells depend on their interactions with embryonic tissues for continued proliferation and regulated differentiation [101]. Cells in direct contact with the inner cell mass maintain the trophoblast stem cell phenotype and form the chorionic plate (extraembryonic ectoderm). These later differentiate and form the bulk of the chorioallantoic placenta in a process initiated by fusion of the allantois (extraembryonic mesoderm extended by the embryo proper) with the chorionic plate (Fig. 3.2c). In mice, chorioallantoic fusion occurs around 8.5 dpc. Almost

immediately, trophoblast differentiation is seen as the expression of *gcm1* in clusters of trophoblast cells in the chorionic plate. The chorion folds into branches initiated at sites of *gcm1* expression [102]. As the fetoplacental vasculature elaborates from the extraembryonic mesoderm filling these branches, trophoblast cells differentiate to form three distinct cell layers surrounding fetal vessels. Gene expression pattern in the early chorion suggests that trophoblast subtypes of these layers may be specified soon after chorioallantoic fusion [103]. Gene knockout studies and tetraploid aggregation assays have led to the identification of a number of genes that regulate trophoblast differentiation [99, 100]. By disrupting specific genes in the trophoblast compartment, their effects on placental development have been evaluated. These studies have underscored a critical role of trophoblast cell differentiation in fetoplacental vascularization.

As alluded to above, distinct subtypes of trophoblast cells constitute the placenta. They have been classified based on their origin, location in the placenta, and gene expression [26, 103–106]. Of these, trophoblast giant cells (TGCs), named after the large size of their polyploid nucleus, are the first to terminally differentiate. Based on their anatomic location, TGCs are thought to play an important role in uterine vascular remodeling. In the murine placenta, for example, at least four subtypes of TGCs occupy the blood-tissue interface [26, 103, 104]. These include spiral artery-associated TGCs (equivalent to endovascular trophoblasts in humans) that invade uterine spiral arteries and replace maternal ECs. In human pregnancies, shallow invasion by trophoblast cells is associated with incomplete remodeling of spiral arteries and obstetric syndromes of preeclampsia and intrauterine growth retardation [107, 108]. It has been further noted that invading trophoblast cells downregulate the expression of adhesion receptors characteristic of epithelial cells and begin to express EC-specific repertoire of adhesion receptors [109, 110]. This process has been termed as endothelial mimicry or *pseudovasculogenesis* and has been found to be incomplete or defective in preeclamptic pregnancies. More recent studies have shown that anticoagulant gene expression, characteristic of ECs, is also widely observed in trophoblast cells [111–113] and that trophoblast differentiation may be programmatically coupled to the acquisition of an EC-like anticoagulant gene expression profile [113]. Thus, the ability to mimic endothelial gene expression is not limited to the subpopulation of trophoblast cells that invade spiral arteries, but rather extends to trophoblast cells resident in the placenta. Trophoblast pseudovasculogenesis is likely to play an important role in the development and maintenance of an effective maternal circulation in the hemochorial placenta.

Trophoblast differentiation is regulated by several growth factors including activin, EGF, TGFβ, IGF-I, IGF-II, and PTHrP [106]. Upon differentiation, trophoblast cells produce a number of angiogenic hormones and their receptors, as well as vasoactive factors and proteases capable of mediating cellular degradation. These include proteins in the prolactin and growth hormone family, the VEGF gene family, adrenomedullin, endothelial nitric oxide synthase, and members of the cathepsin family of proteases [106, 114–116]. The expression of these factors is spatially and temporally restricted and often associated with certain subtypes of trophoblast cells. Subsets of trophoblast cells also express alternately spliced form of VEGFR-1,

a secreted protein also called sFlt-1, which blocks VEGF action. Expression of sFlt-1 by trophoblast cells is thought to be a part of mechanisms by which fetal cells regulate excessive uterine angiogenesis and prevent maternal vessels from growing into the placental junctional zone [117, 118].

Concomitant with trophoblast differentiation, development of fetal vessels in the placenta, termed as *fetoplacental vascularization*, is also initiated with the formation of the chorioallantoic placenta. The fusion of the allantois with the chorion initiates branching morphogenesis at sites of *gcm1* expression. The trophoblast branches get filled with allantoic mesoderm from which the stromal and vascular components of the fetal placenta arise [102, 103, 119]. Fetal vessels in the placenta are formed de novo from hemangiogenic precursors that arise from the mesenchyme of fetal villi (Fig. 3.2e) rather than sprouting of vessels from the embryo into the placenta. Morphologically distinct $CD34^+$ cells can be observed forming primitive cords in human placental villi as early as day 22 of pregnancy [120]. Continuous branching morphogenesis and angiogenic processes produce a complex structure with a large surface area of exchange with maternal blood sinuses (Fig. 3.2d–f).

The molecular regulators and pathways of fetoplacental vascularization are thought to be similar to those identified for vascularization of the embryo (review [121]). Angiogenesis factors expressed in the allantoic mesoderm include members of VEGF and fibroblast growth factor (FGF) gene families and their receptors [122–125]. Gene knockout studies in mice suggest that these may be involved in vascularization of the yolk sac and, by extension, the allantoic mesoderm [122, 124, 126–128]. Fetoplacental vascularization also involves mechanisms regulated by trophoblast cells. It is clear that trophoblast branching morphogenesis is essential for fetoplacental vascularization. Mice mutants affected in branching morphogenesis are simultaneously affected in fetoplacental vascularization, although the location of primary defect is not very clear [100]. Angiogenic and anti-angiogenic factors secreted by trophoblast cells are candidate mechanisms involved in regulation of fetoplacental vascularization [116, 129, 130].

Abnormal Placental Vascularization and Pregnancy Complications

Optimal vascularization and blood flow are critical for placental function. Defective or inadequate maternal spiral artery remodeling, thrombotic lesions in maternal and fetal vasculature, and benign placental vascular tumors, such as chorioangiomas, are the most common types of pathologies affecting placental blood flow. Depending on the severity of defect, these placental vasculopathies are associated with increased risk of pregnancy complications affecting maternal health and fetal development. While severe defects result in fetal loss, continuation of pregnancy with suboptimal placental function is associated with fetal growth restriction and maternal hypertensive disorders, such as preeclampsia. In rodents, suppression of angiogenesis

reduces fetal and placental size and causes hypertension in the mother [131, 132]. Administration of anti-angiogenic factors such as the soluble form of VEGFR-1 (sVEGFR-1, also called sFlt-1) or the soluble form of TGF-β1 co-receptor, endoglin (sEng), in maternal circulation of pregnant rats leads to severe preeclampsia and fetal growth restriction [133, 134]. An accumulating body of evidence suggests that human pregnancies complicated with preeclampsia are also associated with changes in levels of angiogenic and anti-angiogenic factors in maternal circulation. These include increases in sFlt-1 and sEng levels and lowering of PIGF (placental growth factor), VEGF, and TGF-β1 [133, 135–137]. These findings have opened the possibility of using circulating levels of pro- and anti-angiogenic factors as early biomarkers of preeclampsia and restoring angiogenic balance with therapeutic agents [138]. Consistent with the highly angiogenic nature of the placenta and its ability to suppress inappropriate angiogenesis in adjacent maternal and fetal tissues, placenta has been implicated as the site of origin of infantile hemangiomas, discussed in the next section and in more detail in Chapter 1 [139].

Vascular Anomalies

Vascular anomalies are classified into two distinct groups: vascular tumors and vascular malformations. Infantile hemangioma, classified as a vascular tumor, is the most common tumor of infancy and develops within weeks after birth, rapidly proliferates during the first year of life via endothelial and pericytic hyperplasia, then slowly involutes over a period of years [140, 141]. Infantile hemangiomas share common immunomarkers and a genome-wide transcriptional similarity with placental ECs [139, 142]. This unique similarity, along with perinatal presentation, limited growth and eventual spontaneous involution has led to the hypothesis that infantile hemangiomas arise from embolized placental ECs that become dislodged into fetal circulation [139, 143]. A correlation has been observed between the incidence of infantile hemangiomas and premature birth, low birth weight, and maternal preeclampsia. Examination of a relationship between the presence of placental vascular abnormalities and the incidence of infantile hemangiomas is a matter of active research.

Vascular malformations on the other hand are present at birth, do not proliferate, but grow proportionately with the infant throughout the life of the individual [144]. They are thought to be inborn embryonic errors in vascular development. Vascular malformations are named according to the vessel type they affect. For example, venous malformations are those associated with veins, and lymphatic malformations are those associated with lymphatic vessels. The incidence of venous malformation is 1 in 10,000 [145]. Venous anomalies are subdivided into venous malformations (VM) and glomuvenous malformations (GVM). The majority of cases (95 %) fall into the VM category, which include sporadic VM and cutaneomucosal VM (VMCM), while GVM include 5 % of the cases [144]. The etiology of sporadic

VMs and syndromes such as Maffucci syndrome and Klippel-Trenaunay syndrome that are associated with VMs is unknown.

Besides histological classifications, vascular malformations can be classified according to hemodynamic features during angiography. For example, lymphatic and VMs are "low-flow" lesions, while arterial or arteriovenous malformations (AVMs) are "high-flow" lesions. AVMs have been postulated to result from the defective separation of uncommitted angioblasts as arteries and veins, i.e., impaired arterial specification/differentiation during blood vessel formation. Lymphatic malformations (LMs) may be focal or generalized. LMs usually enlarge slowly over time and often displace adjacent organs. The incidence rate of LMs is 1.2–2.8 per 1000 live births [146]. Despite their "benign" nature, vascular malformations present major medical challenges, both diagnostically and in terms of clinical management. Current therapies are limited in efficacy and have significant complications [147].

Genetic Causes of Vascular Malformations

Vascular malformations are usually focal and sporatic, without familial association. However, inheritance of vascular malformations in families has been observed. Sporadic forms often present with a single lesion, while multiple lesions are often observed in inherited forms. The prevailing hypothesis attributes familial forms of some types of vascular malformations to a "double-hit mechanism" where the primary hit is germline heterozygous resulting in incomplete penetrance, variable expressivity, and multifocality of lesions often observed in inherited VMs [144]. A second somatic hit either in the same gene or in a signaling pathway regulated by the primary hit results in full penetrance of the phenotype including multifocal lesions. Lymphatic malformations on the other hand are usually congenital with no evidence for inheritance, suggesting that somatic mutations in a restricted area of the lymphatic network result in lesions.

Significant advances have been made in identifying genetic and molecular determinants of a variety of vascular anomalies. Mutations in *TIE2* [145, 148] for multiple cutaneous and mucosal VMs, in *GLOMULIN* [149] for GVMs, and in *VEGFR-3*, *FOXC-2*, *NEMO*, and *SOX-18* for lymphedema or related syndromes have been identified [150]. For a more complete listing, see Chapter 4. The concept of somatic mutations as a possible cause for vascular anomalies although not absolute is definitely gaining strength. We recently identified mutations in two genes, namely, *sucrose non-fermenting-related kinase-1* (*SNRK-1*) and *dual-specific phosphatase-5* (*DUSP-5*) in patients with vascular anomalies [151]. *Snrk-1* and *Dusp-5* both participate together in controlling early endothelial precursor cell population in zebrafish. We have observed *DUSP-5* mutations, but not *SNRK-1* mutations, in infantile hemangioma. Because IH is characterized by initial rapid growth and eventual involution, we hypothesize that loss of *dusp-5* function may promote vascular precursor cell migration or growth during the tumor's proliferative phase. This

may occur via the removal of *dusp-5*'s blockade of *snrk-1* signaling. Alternatively, *DUSP-5* mutations might promote endothelial apoptosis during involution because this has been observed in Dusp-5 loss-of-function differentiated ECs [151]. The coordination of *dusp-5* and *snrk-1* signaling in regulating angioblast numbers and migration in the zebrafish suggests that somatic mutations in either gene might cause or modulate congenital vascular malformations. We found *DUSP-5* mutations in 1 of 3 IH specimens and in 12 of 17 vascular and lymphatic malformations. All *DUSP-5* mutations observed in vascular anomaly specimens were not conservative substitutions, but ones that would alter protein structure, charge, and in turn, function. Thus, we conclude that alterations in Dusp-5 may be associated with a variety of vascular abnormalities.

Interestingly, the *SNRK-1* mutations observed were found exclusively in venous and lymphatic malformations, suggesting that loss of *SNRK-1* expression influences these vascular anomalies. The lymphatic vascular system develops from the venous system [152], and venous and lymphatic malformations are often mixed as was the case in 5 of 8 tissue specimens containing a *SNRK-1* mutation. Our data suggests that somatic mutations in *SNRK-1* and *DUSP-5* may be found in a minority of cells present in affected tissues, although direct evidence will be obtained from sequencing these genes from blood cells of these patients. Based on high expression of *SNRK-1* in infantile hemangioma tissue (Paula North & RR data not published), we hypothesize that the mutation is present in ECs or EC precursor population. However, its presence in other supporting cells such as mesenchyme [153] cannot be excluded. To better evaluate the clinical relevance of *SNRK-1* and *DUSP-5* mutations, future studies will focus on analyzing tissues from more patients with vascular anomalies and correlate our findings with clinical presentations. Nevertheless, our studies suggest that the pathogenesis of vascular malformations may be impacted by aberrant *dusp-5* and *snrk-1* signaling. Recently, reactive oxygen species have been reported to inhibit the catalytic activity of specific tyrosine phosphatases (Dusp-1) in an angiomyolipoma cell model [154] and treatment using nicotinamide adenine dinucleotide phosphate (reduced form) oxidase (Nox) inhibitors abolished tumor growth in vivo in a mouse model of hemangioma via blockage of angiopoietin-2 production [155]. Therefore, vascular anomaly therapy based on blocking aberrant kinases and phosphatases is an avenue worth exploring in the clinic.

Summary

The circulatory system is a critical component for a developing embryo. The cells of the circulatory system primarily originate from mesoderm tissue and more specifically from the LPM. Because blood cells and ECs emerge side-by-side from LPM, a common precursor cell, namely, hemangioblast that gives rise to both lineages, has been speculated. Hemangioblasts committed to a vascular lineage are called angioblasts. Angioblasts proliferate at LPM and migrate to midline where they coalesce to form the primary vessels of the embryo via a vasculogenic process.

During this process of migration to the midline, angioblasts differentiate into arteries and veins, and the Shh-Notch-VEGF-Notch signaling pathway is responsible for A/V specification. Once the primary vessels are formed, secondary vessels emerge, and those that emerge in the trunk region are called ISVs that are formed via an angiogenic process. The ISVs utilize axon guidance mechanisms and are guided by tip cells that sense local milieu, which directs the following stalk cell. Angiogenesis and vasculogenesis are rarely invoked in the adult, except during reproduction, wound healing, and in pathological conditions. The placenta is the site of most rapid and extensive neovascularization and remodeling observed in the adult. At the molecular level, angiogenic and vasculogenic mechanisms involved in placentation appear to recapitulate those observed during embryonic development. Vascularization of the placenta differs from other organs in its requirement for coordinated elaboration of maternal and fetal circulations that occurs in a matrix of trophoblast cells. Dysregulation of vascular growth control can result in formation of vascular anomalies. Several gene products have been identified to be mutated in vascular anomalies which, despite their "benign" nature, present major medical challenges, both diagnostically and in terms of clinical management. Current therapies are limited in efficacy and have significant complications. Identification of specific mutations in the lesions of individual patients will lead to better prediction of biological behaviors and outcomes and to the development of effective, targeted therapies.

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