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Comparative vascular biology: A more comprehensive approach to the analysis of circulatory system development

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Abstract

The importance of the vasculature in tumor progression and metastasis is widely accepted. Studies on the regulation of blood vessel formation have potential implications on devising pro- and anti-angiogenic therapies for combating ischemic diseases or cancer, respectively. These studies depend on a detailed understanding of the molecular-genetic and cellular mechanisms of blood vessel formation. However, the limited knowledge of gene expression changes that occur in normal vascular development and in tumor angiogenesis has hindered the development of specific treatments and therapies. As molecules and mechanisms in development are often conserved, studying development of circulatory systems in various models has given insights into mammalian vascular development. The current focus is on comparative studies between vertebrate and invertebrate models to elucidate common themes in the development of circulatory systems.

Keywords: Vascular biology, blood vessel, mouse, embryonic stem cell, *Drosophila.*

1. Conservation of mechanisms in development of circulatory systems

Transport of oxygen, nutrient and waste products is fundamental to all multicellular organisms. The mammalian embryo cannot develop far without a functional cardiovascular system [1]. Indeed, most multicellular animals studied to date have a circulatory system. Hence the fundamental questions of when and how the system arises and functions can be asked in the simplest as well as most complex model organisms. These questions can be addressed by a comparative approach using vertebrate and invertebrate models. The advantages and limitations of vertebrate and invertebrate models allow one to approach the problem from various angles and decipher basic principles of circulatory system development. Several components as well as mechanisms of developing and regulating a circulatory system are often conserved. Well-studied examples are the insect and vertebrate circulatory systems, where several parallels can be drawn during hematopoiesis. Our current understanding of circulatory system development and the utility of the comparative approach to vascular biology are discussed here.

2. Models used for studying the development of circulatory systems

The vertebrate cardiovascular system is the earliest to form and function to carry the vital fluid of life without which the embryo cannot develop. The appearance of precursors of

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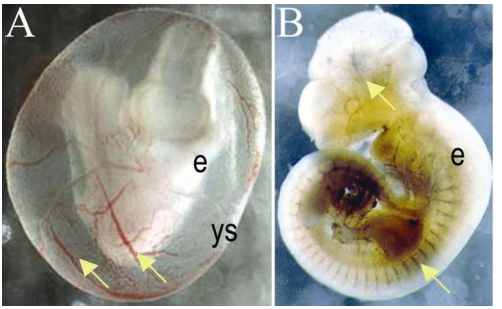

FIG. 1. The mouse embryonic vasculature. (A) Unstained mouse embryo (e) at embryonic day 10.5 encased in its yolk sac (ys) showing an intricate network of blood vessels (arrows). (B) Mouse embryo stained to show expression of PECAM, which marks the blood vessels (arrows).

blood vessels and their support tissues and their subsequent differentiation into a functioning vasculature is poorly understood. While genetically tractable models like the zebra fish are proving to be useful, molecular mechanisms involved in blood vessel formation have been studied mainly in the mouse and chick till recent times. The mouse embryo remains the most accessible model for studying the mammalian vasculature.

2.1. *Mouse embryo model*

2.1.1. *Development of the mammalian vasculature*

The extraembryonic yolk sac mesoderm (Fig. 1A) is the primary site of blood vessel formation by a process called *vasculogenesis*. A putative precursor cell called the hemangioblast differentiates to form an angioblast and a stem cell for hematopoietic lineages giving rise to angioblastic cords of mesoderm that develop into blood islands. The angioblast is the precursor of *endothelial cells* (ECs), which line the lumen of all blood vessels. They differentiate *in situ*, proliferate, migrate and associate to form tubes with tight cell–cell contacts to contain the blood [2, 3]. The primitive vasculature is expanded and remodeled during development or disease by a process called *angiogenesis*.

Angiogenesis is fundamental to both physiological wound healing and the growth of malignant tumors, as it restores or creates a blood supply to a growing tissue. The vascular endothelium represents a complex network of cells producing a large number of active substrates [4]. The physiology of the endothelium affects metabolic and immunological properties of the particular organ or tissue and the whole organism as well. Endothelium-derived factors such as vasoactive agents, peptide growth factors, and cytokines, etc. are involved in the development and progression of many cancers [5].

2.1.2. *Key events in early mammalian hematopoiesis*

Hematopoietic stem cells (HSCs) and ECs arise from common yolk sac mesodermal precursors and develop in close physical association [6, 7]. Primitive hematopoiesis occurs *de novo* in the extraembryonic yolk sac giving rise to blood precursors, which are primarily erythroid in nature [8]. A second round of *de novo* hematopoietic development, termed definitive hematopoiesis, occurs in the mesodermal aorta/gonad/mesonephros (AGM) [9, 10] region of the embryo proper and gives rise to cells that will seed subsequent hematopoietic sites, such as the fetal liver and the bone marrow in mammals [11], as well as to all blood

cell types found in the mature organism. The micro environment of the hematopoietic precursor is also very important for commitment and differentiation to the erythroid lineage. Various growth factors and cytokines signal to activate transcription factors, resulting in altered gene expression profiles. Hematopoiesis is a continuous process throughout adult life whereas the endothelial pool is mostly quiescent. Notable exceptions are the gut, the female reproductive tract and wound healing, where an actively angiogenic endothelium is required. The ability to activate quiescent ECs is also an important requirement for neovascularization in tumors [12].

2.2. *Embryonic stem cell (ESC)-derived model of mouse vascular development*

Analysis of early mammalian vascularization is hindered, as development occurs *in utero.* Hence, molecular markers cannot be used easily to study embryonic cells. This problem is overcome to some extent by using cell lines derived from early embryonic stages [13] and *in vitro* differentiation models [14].

ESCs can proliferate indefinitely and can form derivatives of all three embryonic germ layers [15, 16]. While mouse blastocyst-derived ESCs are well studied, increasing numbers of human ESCs are also becoming available. In addition to their potential role in transplantation therapies, human ESCs will be extremely useful as a basic research tool for understanding the development and function of human tissues. Mouse ESCs are already in use for this purpose and have given important insights into several developmental processes [17].

2.2.1. *Development of the blood vasculature in vitro*

There has been considerable interest in using mouse ESCs as a model to study early development *in vitro.* ESCs are derived from the pluripotent cells of the inner cell mass of blastula-stage embryos, which give rise to the embryo proper and to several extraembryonic tissues [18]. Murine ESCs under appropriate conditions in suspension culture can differentiate to form a structure called the cystic embryoid body (CEB) (Fig. 2). The cystic portion of the CEB is similar to the visceral yolk sac of the mouse embryo. CEBs contain differentiating neuronal structures, clusters of hematopoietic cells that resemble blood islands and a primitive vasculature (Fig. 3) [19–21]. CEBs attached to a substratum also differentiate and form blood islands and provide a more accessible system for experimental manipulation. This model can also be used to study essential developmental genes whose mutants cause embryonic lethality and hence are not amenable to analysis. Pluripotent ES cells and differentiated CEBs make a good model system to study gene expression and regulation in specific lineages during development *in vitro.*

ESCs can also be used to identify and analyze genes involved in blood vessel formation and endothelial function [22]. They can be used to study the molecules and processes by which early stem cells become committed to specific programs of cell differentiation. They tolerate a variety of genetic manipulations *in vitro* and efficiently form chimeras when reinjected into blastocysts [17]. This property has been used extensively to introduce reporter gene constructs into ESCs and analyze their expression in chimeric embryos [23–26]. The technique of gene trapping in ES cells followed by screening for expression patterns after *in vitro* differentiation has been used to identify genes of interest based on their expression

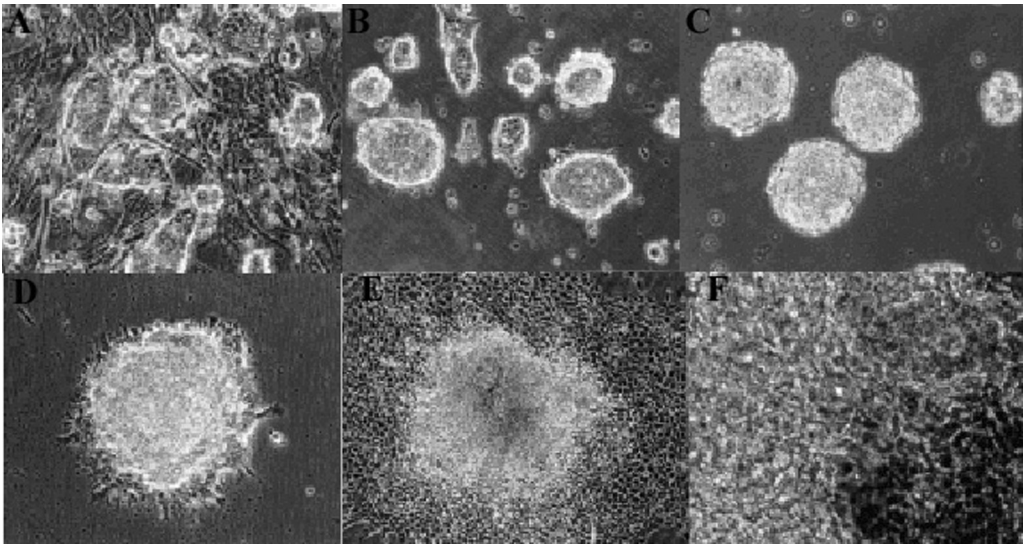

FIG. 2. Differentiation of embryonic stem cells *in vitro*. Undifferentiated ESC cultures grown on fibroblast feeders (A) and off feeders with purified cytokine LIF (B). (C-F) ESC cultures at various days of differentiation to embryoid bodies. (C) day 4 in suspension, (D-F) attached cultures at days 6, 8 and 10, respectively.

pattern. The gene trap approach is based on the assumption that the expression pattern of the reporter gene reflects the expression of the endogenous host gene. Using the gene trap strategy several candidate genes that express in the developing vasculature have been identified [27–30].

2.3. *Drosophila as a model to study vascular development and hematopoiesis*

Vertebrate blood consists of multiple cell types that perform varied and specific functions. Though distinct, all blood cell types are derived from a common, pluripotent precursor or hematopoietic stem cell. *Drosophila* blood consists of only a few terminally differentiated cell types whose functions resemble those of the vertebrate myeloid lineage. Although no hematopoietic stem cells have been identified, *Drosophila* blood cells are thought to be derived from a common set of hematopoietic precursors.

2.3.1. *Drosophila hematopoiesis*

Drosophila hematopoiesis first occurs during embryonic development when hemocytes are derived from the head (procephalic) mesoderm and subsequently migrate throughout the embryo [31, 32]. The hematopoietic repertoire in *Drosophila* is composed of three terminally differentiated cell types–plasmatocyte/macrophage, crystal cells and lamellocytes that develop first in the anterior mesoderm of the embryo and then in the larval lymph gland. No hematopoietic organ has been identified in the adult fly. The embryonic and larval hemocyte repertoires persist in the adult without further development of new cells [33].

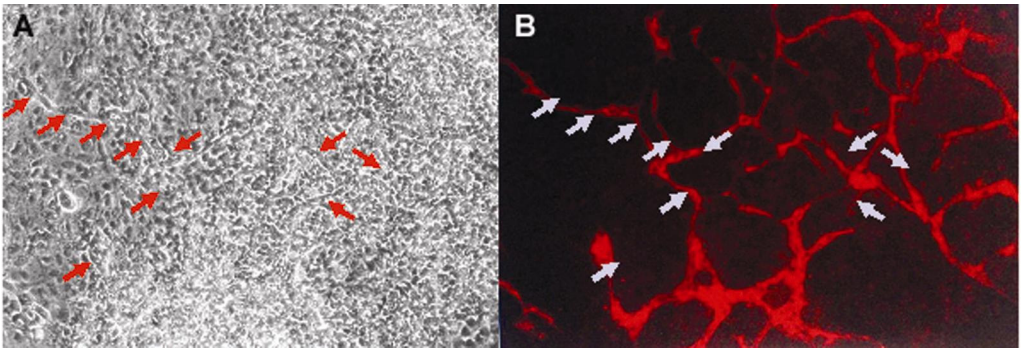

FIG. 3. Formation of blood vessels in vitro. (A) ESC cultures at day 10 of differentiation showing various differentiated lineages including developing blood vessels (arrows). (B) Same culture shown after immunofluorescence staining for the endothelial marker PECAM. Arrows point to red staining showing developing blood vessels.

A clear understanding of hematopoiesis in *Drosophila* is not available. The earliest known transcription factor expressed in these hematopoietic precursors is the *Drosophila* GATA factor homolog-Serpent (Srp) (Fig. 4) [34]. Among the Srp-expressing cells, a population starts expressing Glial cell missing (Gcm) and go on to make plasmatocytes. A smaller population of the Srp-expressing cells also expresses the AML-1 homolog Lozenge (Lz). Cells that continue to express Lz form crystal cells, whereas the remaining express Gcm to become plasmatocytes. Lamellocytes differentiate directly from plasmatocytes during encapsulation of foreign invaders and during metamorphosis.

Drosophila hemocytes, have significant roles in development [32, 35–37] and immunity [38, 39]. Developmentally, hemocytes seek out and remove dead cells and debris as well as secrete and remodel extracellular matrix components critical to morphogenesis. An equivalent function is carried out by fetal macrophages during mouse development. Hemocytes also contribute to both humoral and cellular immune responses by secreting antimicrobial peptides and engulfing and encapsulating foreign invaders [40–42].

Many mutations have been discovered in *Drosophila* that cause aberrant proliferation and differentiation phenotypes reminiscent of vertebrate blood disorders (Fig. 4). Additionally, these defects are often associated with the production of cellular masses, called melanotic tumors, which blacken due to melanization.

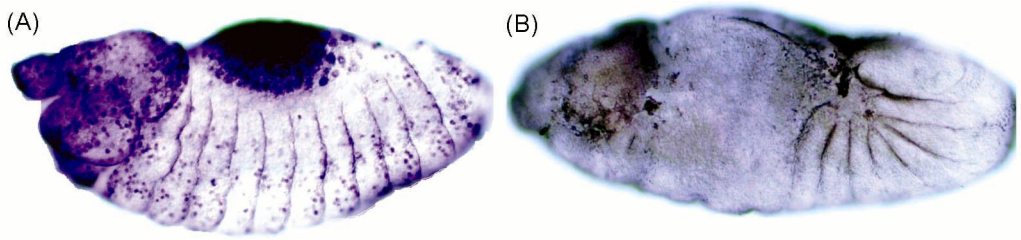

FIG. 4. Embryonic hemocytes in *Drosophila*. Expression of mRNA for a *Drosophila* hemocyte marker (seen as blue staining) in wild type (A) and *serpent* mutant (B) embryos. As *serpent* embryos lack hemocytes, no staining is seen. Anterior is to the left and dorsal is up.

3. Molecular analysis of vertebrate blood vascular development

The vascular system shows several similarities with other organ systems in its developmental program. Like in the developing nervous system and musculature, formation of blood vessels involves the action of specific signals on an undifferentiated sheet of cells (the mesoderm), which results in the selection of primary stem cells (hemangioblast). These stem cells divide to form one or more precursors (angioblast and hematopoietic stem cell), which finally differentiate to generate a specific cell type (endothelial cell or hematopoietic lineages). Hence it is very likely that molecules and signaling pathways involved in determining other organ systems are also used in developing the vasculature.

Although several molecules are known that regulate the commitment to various lineages derived from the HSC, only a few factors that are common to endothelial and hematopoietic lineages are known. Transcription factors play a major role in maintenance of the blood vasculature under normal and pathological conditions [43]. The transcription factor SCL/Tal 1 and the receptor tyrosine kinase Flk1 (VEGFRII) are pivotal in early hematopoiesis and vasculogenesis. In response to signaling through Flk-1 and transcriptional regulation by SCL, large nucleated erythroblasts first arise from the yolk sac blood islands as early as embryonic day (E) 7.5 in the mouse [44]. Their expression patterns first overlap and then complement each other as precursors in the blood island get committed and differentiate. SCL is maintained in primitive erythrocytes and at low levels in ECs, whereas Flk1 is restricted to vascular cells [45–47]. SCL is postulated to act downstream of Flk1, but the mechanism by which these genes regulate hematopoiesis and vasculogenesis is not well understood [48]. Various growth factors and cytokines signal to activate transcription factors, resulting in altered gene expression profiles [49, 50]. For example, the zinc finger transcription factor GATA-1 binds to the regulatory region of many genes expressed in erythroid and megakaryocytic cells [51].

A key control point in blood vessel formation and remodeling is the response of the endothelial cell to developmental or tumor-initiated signals. Several genes are known to have a direct effect on blood vessel formation [52]. However, the pathways that they function in and their role in vascular development are still in the early stages of investigation. Three molecular systems have been implicated in the formation and pathobiology of blood vessels, namely, the vascular endothelial growth factor (VEGF) system, the plasminogen system and the coagulation system [53]. Recent gene-targeting studies have shown that VEGF is a potent modulator of blood vessel formation. The current model for vasculogenesis and angiogenesis involves VEGF and its receptors (the tyrosine kinases Flk-l and Flt-l), and the angiogenic regulators Ang-1, Ang-2 and their receptor Tie-2 [45, 54]. However, the mutant phenotypes of these molecules suggest a far more complex scenario [53, 55]. The initiator of coagulation controls hemostasis as well as maturation of a muscular wall around the endothelium. The plasminogen system has a pleiotropic function in thrombosis, arterial neointima formation after vascular wound healing and allograft transplantation, in atherosclerosis, and in the formation of atherosclerotic aneurysms. Multiple signaling pathways are required to fully execute endothelial differentiation and vascularization. Although elegant descriptions of the events resulting from these interactions exist, only a few of the molecules involved in developmental and pathological blood vessel formation are known.

The rapid cloning of a large number of candidate genes and rigorous screening of these candidate clones should allow for the identification of genes involved in vascularization.

4. Conservation of signaling pathways active in circulatory system development

Several studies underline the existence of parallels in the genetic control of hematopoiesis in *Drosophila* and in mammals. While *Drosophila* has fewer blood cell types and functions than vertebrates, several key components regulating hematopoiesis and vasculogenesis have been conserved through roughly 550 million years of evolution. Hence, the identification of additional players in both vertebrate and invertebrate blood development should allow the rapid elucidation of molecular pathways involved in the development of blood lineages. *Drosophila* is a powerful model system to study basic molecular genetic mechanisms regulating hematopoiesis and to elucidate principles applicable to insect and vertebrate hematopoiesis. Most hematopoietic genes in *Drosophila* and their vertebrate counterparts were studied independently (e.g. Srp/GATA, Ush/FOG, and Lz/AML-1). However, for some molecules like VEGFRII, expression and function were first documented in vertebrates and their fly homolog VEGFR/PDGF was only recently identified by sequence comparison and shown to be required for hemocyte migration [31].

Our laboratory has also identified novel players in mouse vascular development and hematopoiesis and shown that their *Drosophila* homologs are involved in hematopoiesis [56]. We have identified and characterized two novel genes *asrij* and *rudhira* for their developmental expression, subcellular localization, mutant phenotype and regulation. We have shown that these genes are novel markers for ESCs and the developing mammalian cardiovascular system. *Asrij* is expressed from the earliest stages of vasculogenesis and persists in the differentiated endothelium and a subset of the hematopoietic lineage [30]. *Rudhira* is expressed during primitive erythropoiesis and in angiogenic endothelium [57]. As these genes are conserved, we have also extended our analysis to their *Drosophila* counterparts. We have shown that in *Drosophila*, both *asrij* and *rudhira* are associated with the circulatory system. *Asrij* is expressed in hemocytes, the circulating blood cells of *Drosophila* [56]. *Rudhira* is expressed in hemocytes and in pericardial cells that are associated with the heart tube [Inamdar *et al*., unpublished]. Our analysis suggests that *asrij* and *rudhira* have important roles in the developing cardiovascular system.

Signaling pathways and molecules are repeatedly used in different contexts during development resulting in different outcomes. Some of the known and well-characterized signaling pathway molecules are likely to be involved in vascular development. The remarkable conservation of signaling pathways across species ranging from *Drosophila* and *C. elegans* to mouse and human is striking. While this conservation is intensely investigated in the study of developmental systems such as the nervous system and musculature, the role of conserved signaling genes in blood vessel formation has received little attention. Some of the molecules involved in the formation of blood vessels might belong to the well-characterized signaling pathways used in other developmental contexts. Recent reports that some molecules belonging to the Notch [58–60] and Wnt [61–63] signaling pathways are expressed in endothelial cells support this hypothesis. An added advantage to studying known molecules is that the reagents for their analysis and the mutants are already avail-

able. Hence a comparative approach at the molecular and organism level will help rapidly elucidate the mechanisms and principles underlying circulatory system development and function.

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