The Dissertation Committee for Steven Alexander Vokes Certifies that this is the approved version of the following dissertation:

The Role of Endoderm in Vascular Patterning

Committee:

Janice A. Fischer, Supervisor

Paul A. Krieg , Co-Supervisor

Alan M. Lloyd

Arlen W. Johnson

S. Martin Shankland

R. Adron Harris

The Role of Endoderm in Vascular Patterning

by

Steven Alexander Vokes, B.A.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

December, 2002

Dedication

This work, symbolic of my higher education, is dedicated to my parents Carol and Emmett Vokes, who played such an integral role in its foundations.

Acknowledgements

I have been extremely fortunate to have excellent mentoring during my time in graduate school. I thank Paul Krieg for his suggestions, enthusiasm, encouragement and friendship during this learning process. He has taught me how to think (and write) critically and insightfully about science. I am also grateful to Amy Cheng Vollmer, my undergraduate mentor. She introduced me to the joys of scientific research, and continues to give me excellent advice whenever I need it most. I thank Peter Vize, whose conversations led to the first experiments described within. In daily interactions, I have benefited from a caste of talented co-workers and advisors. These include Craig Newman, Wendy Gerber, Ondine Cleaver, Tom Carroll, Eric Small, Rob Garriock, Martha Joe, Parker Antin, Ray Runyan, Jean Wilson, Carol Gregorio and all past and present members of the Krieg lab. I am particularly indebted to Tania Yatskievych for her help with the avian experiments. Finally, I thank Janet Heasman, Phil Ingham, David Kimelman and Cliff Tabin for providing reagents or constructs used in these experiments.

The Role of Endoderm in Vascular Patterning

Publication No._____

Steven Alexander Vokes, Ph.D.

The University of Texas at Austin, 2002

Supervisors: Janice A. Fischer and Paul A. Krieg

Angioblasts, the precursor cells that give rise to the endothelial layer of blood vessels, arise from a purely mesodermal population. Individual angioblasts coalesce to form the primary vascular plexus through a process called vasculogenesis. A number of reports in the literature suggest that signals from the adjacent endoderm are necessary to induce angioblast specification within the mesoderm. We present evidence, using both embryological and molecular techniques, indicating that endoderm is not necessary for the induction of angioblasts. While *Xenopus* embryos lacking endoderm contain aggregates of angioblasts, these angioblasts fail to assemble into endothelial tubes. Endothelial tube formation can be rescued however, by implantation of endodermal tissue from sibling embryos. Based on these studies in *Xenopus*, and corroborating experiments using the quail embryo, we conclude that endoderm is not required

for angioblast specification, but does provide an inductive signal for vascular assembly.

In additional experiments using avian embryos, we demonstrate the molecular identity of this inductive signal, showing that endodermally derived Sonic Hedgehog is both necessary and sufficient to form endothelial tubes from angioblasts in avian embryos. This demonstrates a novel role for hedgehog signaling in vascular development and provides a molecular model for vascular assembly.

Table of Contents

List of Figures

Chapter 1: Molecular Vascular Embryololgy[1]

1.1. INTRODUCTION

The establishment of an intact, functional cardiovascular system is a prerequisite for embryonic development in vertebrates. A diagram showing the location of the major blood vessels in the embryonic cardiovascular system is presented in Fig. 1. The importance of this system for delivering oxygen and nutrients to developing tissues is underscored by the early embryonic lethality of embryos deficient in essential cardiovascular genes. Despite a spatiotemporal correlation between the formation of the cardiac and vascular structures, these two systems undergo autonomous developmental programs. In fact, an intact vascular system will form perfectly well in the absence of a beating heart (Knower, 1907; Chapman, 1918). Over the last hundred years, vascular development has been extensively studied by classical embryologists who described the formation of the first blood vessels. However, the absence of early vascular markers, especially markers for vascular endothelial precursor cells (angioblasts), greatly impeded studies aimed at understanding the initial events underlying vascular development. With the advent of molecular biology, it has been possible to study the early events in the formation of the vascular system in greater detail, and to start characterizing the genetic pathways underlying these processes.

---

[1] This chapter, including all figures, is a revised and edited version of a book chapter reproduced with permission from Springer-Verlag. Vokes, S.A. and Krieg, P.A. (2002) Molecular Vascular Embryology. In: Lanzer, P. and Topol, E. (eds) PanVascular Medicine, Integrated Clinical Management. Springer-Verlag, Berlin Heidelberg New York, pp 18-35.

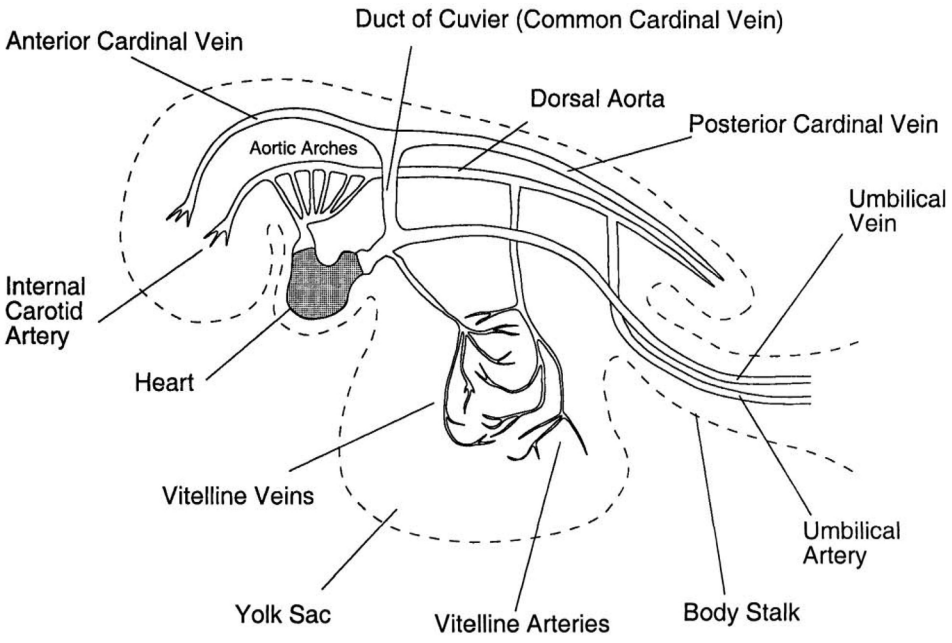

Figure 1. Schematic representation of the vascular system of a mammalian embryo.

For simplicity only the major vessels are shown. Moreover, only one of the paired dorsal aortae and one of the posterior cardinal veins is illustrated. This simple vascular plexus will undergo extensive remodeling during later development to form the mature vascular system.

Embryonic vascular development can be divided into a number of distinct steps. The first event is the specification of angioblasts from the mesodermal cell layer of the embryo. The second step is the assembly of these free angioblasts into vascular cords followed by formation of the vascular tube. Third is the elaboration of the initial vascular plexus by outgrowth from existing vessels. The final step in the process is the recruitment of vascular smooth muscle cells to the outside of the endothelial tube. It is the properties of these smooth muscle cells that ultimately define the physical and biochemical properties of the resulting artery or vein. As will be described in more detail in the following sections, specific genes can now be associated with the regulation of each of the different steps in vascular development. Although our knowledge of the celluar and molecular mechanisums underlying the functions of these genes is still quite sparse, the importance of these studies is clear, since mutations in these pathways are known to underlie congenital vascular disorders in human patients.

1.2. EMBRYOLOGY OF VASCULAR DEVELOPMENT

1.2.1. General introduction to embryology

As a result of the process of gastrulation, cells within the embryo become divided into three distinct tissue layers, often referred to as the germ layers. These layers are named ectoderm, mesoderm and endoderm, corresponding respectively to the outer, middle and inner layers of the developing embryo. In broad terms, the ectoderm gives rise to the skin and neural tissues, while the mesoderm differentiates into a number of tissues, including the kidneys, heart, blood, muscle and endothelial cells. Endoderm forms the liver, lungs, pancreas

and the lining of the gut. During early development, the primary tissue layers become subdivided into different regions. For example, the outer layer of mesoderm, adjoining the ectoderm is called the somatic mesoderm, while the inner layer adjacent to the endoderm is referred to as the splanchnic mesoderm. These different layers of mesoderm generally develop into distinct tissues. For example, portions of the splanchnic mesoderm develop into cardiac muscle while the somatic mesoderm contributes to the lateral and ventral body wall.

During development, interactions between the different germ layers (usually called inductive interactions) are crucial for establishment of the complex range of organs and tissues that will comprise the mature organism. In molecular terms these interactions often involve the activation of signaling pathways via growth factors or cell surface effectors. Perhaps the most important example of embryonic induction is the formation of neural tissues. In this case, a certain subset of the ectodermal cell layer is induced to form neural tissue through interactions with the mesoderm. The actual processes underlying tissue specification and embryonic induction are complex, but are described in great detail in other sources (Gilbert, 2000; Wolpert *et al*., 1998).

1.2.2. Vasculogenesis

The primary network of blood vessels in the embryo is formed by the process of vasculogenesis, which is defined as the de novo formation of blood vessels by the aggregation of individual angioblasts (Fig. 2). At a fundamental

level, this process involves both the specification of endothelial cell precursors (angioblasts) from mesoderm and then the coalescence of these angioblasts into endothelial tubes. Independent of the events of angioblast specification, some researchers have found it convenient to distinguish two variants of vasculogenesis. In Type I vasculogenesis, blood vessels form from angioblasts that arise in place, i.e. the vessels assemble at the position where the angioblasts are first detected. Type 2 vasculogenesis occurs when individual angioblasts migrate over some significant distance to a new location where they then assemble into a blood vessel (Poole and Coffin, 1991). It should be emphasized that migrating angioblasts that contribute to previously formed blood vessels are not examples of Type 2 vasculogenesis because vascular formation has already occurred. After the delineation of the primitive vascular network via vasculogenesis, subsequent elaboration of the system of blood vessels occurs by angiogenesis. Angiogenesis is the sprouting or splitting of blood vessels from pre-existing blood vessels (see below). In summary therefore, the earliest vascular development is achieved by vasculogenesis, while angiogenesis is the only mechanism of blood vessel formation in later development.

The process of vasculogenesis was originally described by classical embryologists who used histological methods to establish that angioblasts arise from mesoderm and coalesce to form endothelial cords that subsequently become patent blood vessels (His, 1868; Van der Stricht, 1895; His, 1901; Rückert and

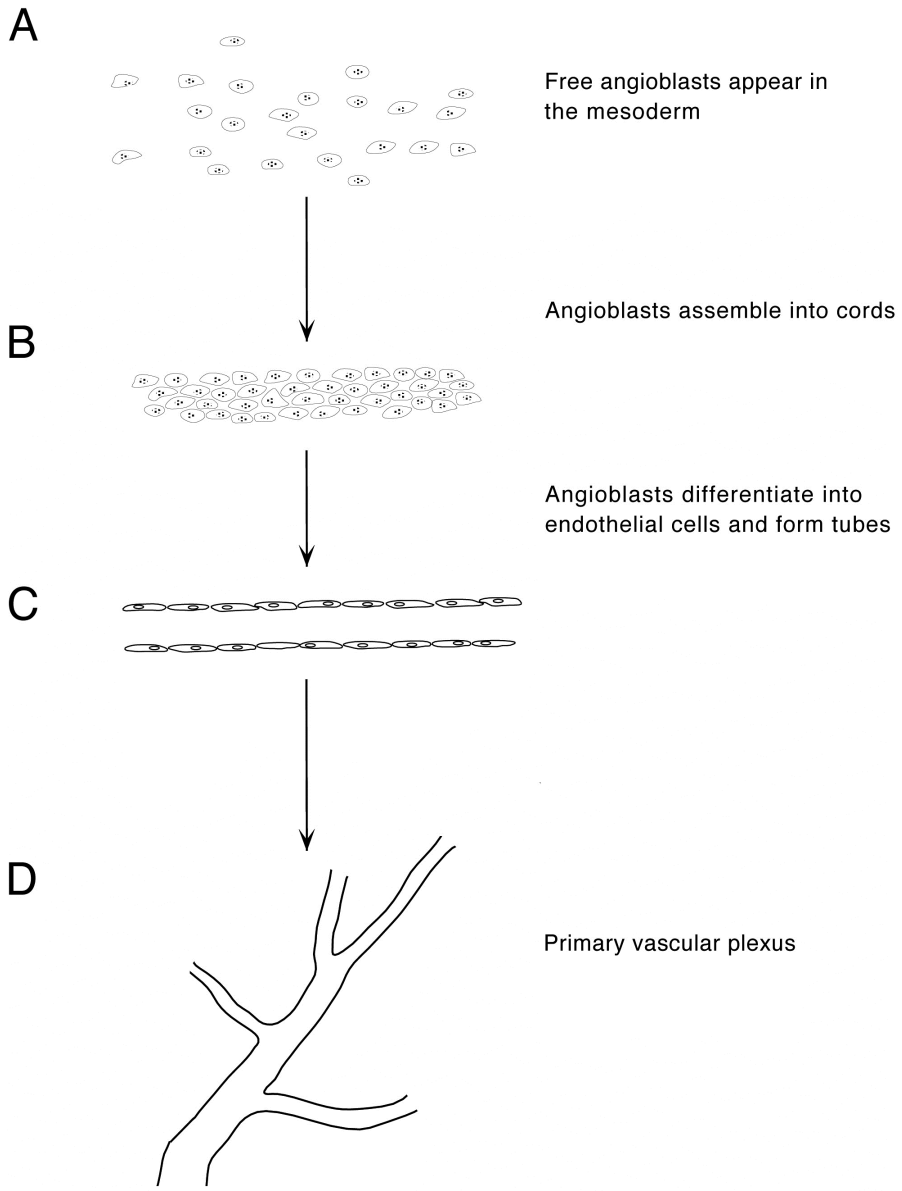

Figure 2. Diagramatic representation of the major events in vasculogenesis.

The basic process involves three steps: (A) specification of vascular precursors (angioblasts) from embryonic mesoderm, (B) aggregation of angioblasts into cords, and (C) lumen formation. The result is the formation of the primary vascular plexus (D).

Mollier, 1906; Dantschakoff, 1908; Stockard, 1915a; Sabin, 1920; Sabin, 1922). These original studies demonstrated that, in amniotes, vasculogenesis occurs both extraembryonically, in the yolk sac surrounding the embryo, as well as intraembryonically. In teleosts (bony fishes) and amphibians, vasculogenesis occurs only intraembryonically (Stockard, 1915b). A major difference between extraembryonic angioblasts and intraembryonic angioblasts lies in their structure. Extraembryonic angioblasts are found in blood islands, containing an outer layer of endothelial cells and an inner layer of red blood cells (See Fig. 3). In contrast to the extraembryonic blood islands, intraembryonic endothelial precursors are almost always first observed as solitary angioblasts (Risau, 1995) and only in rare instances are they closely associated with blood cells. Indeed the independence of these two lineages has been demonstrated in Amphibia, where the entire blood forming region of salamanders can be surgically removed without significantly altering the endothelial network (Goss, 1928). At present it is unclear whether the differences in endothelial cell origins observed between the intra and extraembryonic vasculature reflect fundamental differences in the processes of vascular cell development between these systems, or whether intra and extraembryonic vasculogenesis occur in a molecularly identical fashion.

1.2.3. The avian embryo

The events of embryonic vasculogenesis are better described in the avian embryo than any other organism. This is primarily because the embryonic vascular system is easy to visualize and is readily accessible to experimental

manipulation. Anatomically, the chick embryo is comprised of an outer layer called the area opaca which has yolk directly beneath it, the area pellucida, the inner layer, which does not have yolk directly beneath it and is therefore transparent. Essentially, the area pellucida will form the embryo proper while the area opaca will develop into the extraembryonic yolk sac ectoderm and endoderm (Risau and Flamme, 1995; See Fig. 3). Vascular development in the yolk sac temporally precedes that of intraembryonic vasculogenesis. Formation of the first vessels is initially visible as an aggregation of mesenchymal cells in the splanchnic mesoderm adjoining the extraembryonic endoderm in the middle of the area opaca. This vascularization quickly spreads throughout the entire area opaca with the exception of the area anterior to the head and a small area at the embryonic tail. Together, the regions of the area opaca and area pellucida that form blood vessels are termed the area vasculosa, which corresponds to the entire area that contains mesodermal cells (Risau and Flamme, 1995). After specification, the angioblasts extend cytoplasmic protrusions towards each other and then assemble into cords of angioblasts which subsequently form a continuous strand of endothelial cells. These cells will then form tubular vascular structures. It is important to note that extraembryonic blood vessels contain primitive erythrocytes, while intraembryonic blood vessels are largely devoid of erythrocytes (Sabin, 1920; Houser *et al*., 1961; Gonzalez-Crussi, 1971).

While His postulated that extraembryonic blood vessels might actually be the source of endothelial cells that populated the chick embryo proper (His, 1868;

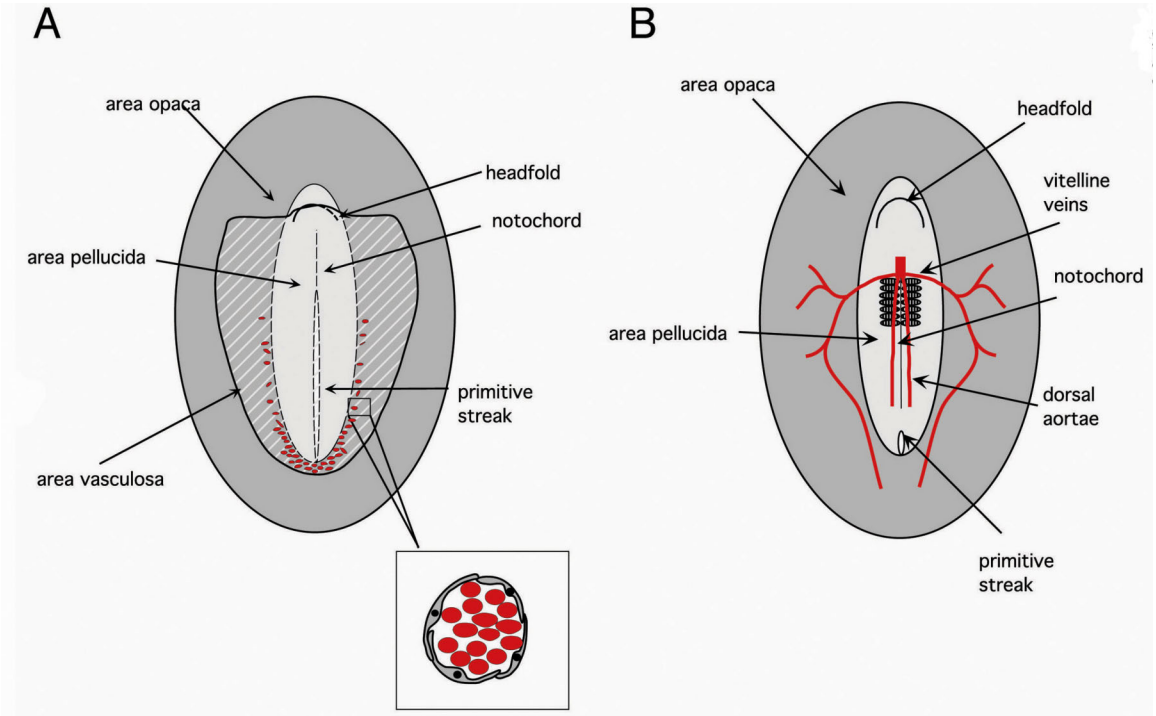

Figure 3. Intra and extraembryonic vascular development in chick embryos.

(A) The first extraembryonic blood islands appear at the head process stage, immediately adjacent to extraembryonic endoderm. A stylized blood island (shown in the boxed area) contains future endothelial cells surrounding primitive erythrocytes. The area vasculosa comprises the entire region containing extraembryonic and intraembryonic mesoderm and marks the area that will become vascularized. (B) A 7-somite chick embryo contains fused heart primordia, and the extraembryonic blood vessels connected to the intraembryonic circulatory system through the vitelline veins. The paired dorsal aortae are the most prominent early blood vessels. At this stage many other vessels are present, but for simplicity have been ommited from the diagram.

His, 1901), this was refuted by subsequent experiments (Hahn, 1909; Miller and McWhorter; 1914; Reagan, 1915). The definitive studies were carried out by Reagan, who demonstrated that chick embryos that had their headfolds dissected from the yolk sac, at a period before an extraembryonic invasion of vascular cells could occur, still formed blood vessels.

Unlike extraembryonic vasculogenesis, the formation of intraembryonic angioblasts does not originate in blood islands. In fact, with the exception of aortic endothelial cells in avian embryos, early vasculogenesis does not appear to be connected with hematopoiesis at all (Dieterlen-Lièvre and Martin, 1981; Olah *et al*., 1988; Pardanaud *et al*., 1989). Due to the lack of blood cells in the vessels and the absence of readily recognizable histological features for intraembryonic angioblasts, the early events of vasculogenesis were virtually impossible to describe using classical embryology techniques. By the time blood vessels could be resolved by ink injections (Evans, 1909), they had already undergone a substantial amount of development. The advent of electron microscopy techniques allowed for additional characterization of vascular assembly (Hirakow and Hiruma, 1983; Meier, 1980) but, once again, the absence of molecular markers continued to present difficulties for the identification of angioblasts prior to vascular assembly. Understanding of the early events of vasculogenesis took a great leap forward with the identification of QH-1, a monoclonal antibody which specifically recognizes quail endothelial cells (Pardanaud *et al*., 1987). The enormous advantage of QH-1 was that it allowed accurate identification of free intraembryonic angioblasts prior to vascular assembly and therefore facilitated the first detailed studies of early vasculogenesis. These studies indicated that the first

angioblasts in the embryo proper can be detected at approximately the one somite stage in bilateral sites near the headfolds corresponding to future endocardium, and slightly later in the lateral edges of the anterior intestinal portal and in the area ventral to the somites (Pardanaud *et al.*, 1987; Coffin and Poole, 1988; Sugi and Markwald, 1996). The most prominent concentrations of angioblasts will form the paired dorsal aortae. These angioblasts apparently arose in situ and therefore the formation of the dorsal aortae in chick is an example of type I vasculogenesis (Poole and Coffin, 1991), however, examples presented below will show that the situation is different in some other embryo systems. Additional aggregations of angioblasts mark the future sites of the large vessels, including the vitelline veins, the cardinal veins, the ventral aortae and the aortic arches (Fig. 1) (Pardanaud *et al.*, 1987; Coffin and Poole, 1988). Finally, individual angioblasts are observed throughout the splanchnic mesoderm. These will later assemble into the primary vascular plexus supplementary to the large vessels, and may possibly contribute to large vessel formation through the process of fusion (Drake and Little, 1998). As development proceeds, the primary vessels lumenate (form vascular tubes) and soon after the commencement of heart contractions, blood begins flowing.

As mentioned previously, angioblasts arise exclusively within mesodermal tissue (See Inductive signaling and specification of angioblasts, below.) Vascularization of tissue and organs of non-mesodermal origin (e.g. the brain and the visceral organs) proceeds via invasion of these tissues by blood vessels arising

from the adjacent mesodermal structures by angiogenesis. It is important to note that not all mesodermal tissues are vascularized by the process of vasculogenesis even though almost all mesoderm does have the potential to form angioblasts. Vasculogenesis is predominantly limited to ventrolateral mesoderm and does not occur in dorsoanterior mesoderm, probably due to an inhibitory effect of ectoderm (Augustine, 1981; Pardanaud and Dieterlen-Lièvre, 1999). In the avian embryo, vascular development proceeds in an anterior to posterior wave through the embryo. Therefore, the anterior regions of the paired dorsal aortae may already be clearly defined, lumenated vessels, while the posterior extremities of the same presumptive vessels are merely concentrations of free angioblasts (Drake and Little, 1998).

1.2.4. Other vertebrate embryos

While the embryology of vascular formation is best described for the chick, several other organisms have become increasingly useful for studying vascular development, primarily due to the advent of molecular markers for angioblasts and the increased accessibility of these systems to observation and/or genetic manipulations. Vasculogenesis in mouse embryos has been described using antibody methods (Coffin *et al.*, 1991) and more recently using confocal microscopy techniques by Drake and Fleming (2000). These studies show that extraembryonic vascular cell development is initially detected at E6.5 in the ectoplacental cone of the yolk (extraembryonic mesoderm). Intraembryonic vasculogenesis commences in E7.3 embryos at positions in the mesoderm which

later give rise to the endocardium. By E7.8, endothelial cells have undergone a considerable degree of proliferation and are also present laterally along the flank of the developing embryo in the region where the paired dorsal aortae will form. These dorsal aortae have fused by E8.5 forming a single dorsal aorta located at the midline of the embryo (Drake and Fleming, 2000).

Comparative studies of embryos from different species have revealed some intriguing differences in the temporal sequence of events leading to blood vessel development and have also revealed additional mechanisms involved in determining the architecture of the original vascular plexus. The development of *Xenopus* and zebrafish embryos are especially interesting because they do not generate extraembryonic vessels and therefore only undergo intraembryonic vasculogenesis. In *Xenopus*, angioblasts are first observed in early tailbud embryos, in areas corresponding to the future endocardium and also some head mesenchyme. Slightly later, lines of angioblasts can be observed on each side of the embryo, adjacent and immediately ventral to the somites, where the posterior cardinal veins will later form (Cleaver *et al.*, 1997). A broadly similar pattern of vasculogenesis is observed in zebrafish embryos (Fouquet *et al.*, 1997).

Unlike avian embryos, where the paired dorsal aortae are the first major axial vessels to develop, the first clearly defined axial vessels in *Xenopus* are the paired posterior cardinal veins. The angioblasts that assemble to form these vessels apparently arise in situ (an example of type I vasculogenesis). In

contrast, the dorsal aorta forms from angioblasts that migrate medially, from the pool of precursors in the posterior cardinal vein region, to the midline of the embryo. These cells then assemble into a single dorsal aorta (Cleaver and Krieg, 1998). This assembly, following migration, is an example of type 2 vasculogenesis. At the anatomical level, this migration appears to be mediated by the hypochord, a transient structure in Amphibian and fish embryos, which secretes high levels of the small, diffusible form of VEGF (see below) (Cleaver and Krieg, 1998). A similar situation is thought to take place in zebrafish, where mutants that lack a hypochord fail to form a dorsal aorta. Both the floating head (flh) and no tail (ntl) mutants lack a hypochord as a secondary consequence of defects in notochord formation and also fail to form a dorsal aorta (Fouquet *et al.*, 1997; Sumoy *et al.*, 1997; Weinstein, 1999). It is important to note that no hypochord is present in amniotes such as chicken or mice and therefore other sources of VEGF secretion may pattern dorsal aorta development in these organisms. Indeed, the developing mouse embryo contains high levels of VEGF in the endoderm prior to dorsal aortae formation, suggesting that the endoderm may fulfill the role of the hypochord in mice (Miquerol *et al.*, 1999). Perhaps the major significance of these studies is the observation that angioblasts can migrate large distances within the embryo, in response to growth factor signaling. Although such migrating angioblasts had previously been observed in avian embryos (Noden, 1989; Christ *et al.*, 1990; Wilms *et al.*, 1991; Wilting *et al.*, 1995), their significance for formation of the primary vascular network was unclear. Overall, it appears that angioblast migration in response to growth factor

signaling is playing a major part in determining the location and arrangement of the major vessels in all vertebrate embryos.

1.2.5. Inductive signaling and specification of angioblasts

Both intraembryonic and extraembryonic angioblasts are mesodermal in origin (Van der Stricht, 1895; Rückert and Mollier, 1906; Dantschakoff, 1908). The subsequent cell signaling and tissue patterning events that occur during gastrulation are not required for angioblasts to form (Azar and Eyal-Giladi, 1979; Christ *et al.*, 1991; Krah *et al.*, 1994; von Kirschhofer *et al.*, 1994). By using chick-quail grafting techniques, it has been possible to learn a considerable amount about the angioblastic potential of different types of mesoderm. All embryonic mesoderm with the exception of prechordal mesoderm has at least some capacity to generate angioblasts (Noden, 1989; Wilms *et al.*, 1991; Wilting *et al.*, 1995). Thus the actual development of angioblasts and blood vessels appears to be context dependent. In other words, the tissue environment in and around a specific region of mesoderm is responsible for regulating vascular endothelial cell specification and commitment (Pardanaud *et al.*, 1989; Pardanaud and Dieterlen-Lièvre, 1999; Cox and Poole, 2000).

Despite the critical importance of angioblasts for formation of the embryonic vasculature, the precise origins of these cells remain obscure. Classical embryologists noticed that angioblasts in the extraembryonic blood islands and also in the earliest intraembryonic blood vessels arise in close

proximity to endoderm. This observation raised the possibility that an inductive signaling process between the endoderm and the mesoderm was required for angioblast specification. The hypothesis gained support from a number of different studies carried out using the avian embryo (Wilt, 1965; Miura and Wilt, 1969; Pardanaud *et al*., 1989). For example, in chick tissue culture experiments, when specific portions of the area vasculosa that form the extraembryonic blood islands were separated into the mesectodermal and endodermal components, the mesectodermal component never contained endothelial cells (Wilt, 1965). Endothelial cell differentiation could be restored if the mesectoderm was recombined with endoderm. This suggests that an endodermally derived inductive signal is necessary for endothelial cell formation, at least in the context of blood island formation. In a follow-up study, Miura and Wilt (1969) confirmed the inductive role of endoderm, but found a slight amount of blood formation in isolated mesectoderm, which they suggested could be due to factors in the culture medium. Pardanaud *et al* (1989) proposed that close association with the endoderm is necessary for vasculogenesis based on the close physical proximity of vasculogenic mesoderm to endoderm. It is noteworthy however, that no experiments were carried out to explicitly address this proposition.

While these studies implied that endoderm is required for blood island formation, in the absence of molecular markers it was not possible to identify individual angioblasts prior to blood vessel formation. Even so, the requirement for endoderm during vascular cell development is widely stated in the literature

and has assumed the status of dogma. Recently however, the essential role of endoderm in the specification of angioblasts has been re-evaluated. For example, it has been shown that mouse embryoid bodies lacking the transcription factor, GATA-4, fail to form extraembryonic endoderm. In the absence of endoderm, these embryoid bodies are unable to form blood islands, in agreement with the endoderm induction model. However, use of molecular markers indicated that formation of endothelial cells was not affected in these embryos (Bielinska *et al.*, 1996). This result implies that the primary role of endoderm is in inducing formation of blood island, and not in specifying vascular endothelial cells. Similar results were obtained embryologically by Palis *et al* (1995), who showed that murine yolk sac explants containing extraembryonic mesoderm but not endoderm, still developed endothelial cells, but lacked organized vessels. While these studies hinted that endoderm might not be necessary for angioblast specification, the issue was definitively addressed in a recent study by Vokes and Krieg (2002a). In a series of experiments, we demonstrated that both *Xenopus* and avian embryos contain angioblasts in the complete absence of endoderm. At present therefore, the role of inductive interactions in the specification of angioblasts is uncertain, but it appears that it must be limited to interactions that occur exclusively within the mesoderm.

1.2.6. The Theory of the Hemangioblast

The observation that blood islands are comprised of endothelial cells surrounding primitive erythrocytes led early investigators to postulate that

endothelial cells and blood cells were derived from the same lineage (His, 1868; Sabin, 1920). The putative precursor cell, which would possess properties of both endothelial and hematopoietic lineages, was termed the hemangioblast (Murray, 1932; Wagner, 1980). Despite the fact that this theory is now over a century old, the existence of the hemangioblast, *in vivo*, remains controversial. The proposition however, has gained increased support from certain molecular genetics experiments and it now seems likely that at least some blood and endothelial cells are derived from the same initial population, although the common precursor cell has never been definitively detected within the embryo.

Perhaps the best evidence for the existence of the hemangioblast comes from studies of avian embryos. During development of the avian embryo, the endothelial cells that comprise the dorsal aorta arise from two distinct populations of mesodermal cells. Angioblasts derived from the splanchnic mesoderm make up the floor of the dorsal aorta, while angioblasts from the somatic mesoderm form the roof and walls of the aorta. The endothelial cells in the floor of the dorsal aorta have been shown, by lineage tracing, to have the potential to give rise to definitive hemopoietic cells (Jaffredo *et al.*, 1998). These results clearly show a close relationship between the endothelial cell and blood lineages, but stop short of definitively proving the existence of the hemangioblast *in vivo*. Additional support for the hemangioblast comes from studies of the VEGF receptor, VEGFR2 (see below). Mice genetically ablated for VEGFR2 fail to form either extraembryonic blood islands or mature endothelial cells (Shalaby *et al.*, 1995)

suggesting that the two lineages are closely related. Intraembryonic development of blood cells could not be assayed because the embyros die before definitive development of the lineage. Despite this phenotype, embryos expressing the sensitive β-galactosidase reporter protein under control of the VEGFR2 promoter, never showed staining in the blood cells in the blood islands, indicating that expression levels are extremely low or non-existent (Shalaby *et al.*, 1995). Independent studies using antibodies have failed to detect VEGFR2 protein in the hematopoietic interior of blood islands or in intraembryonic hematopoietic regions (Drake and Fleming, 2000). Together, these studies suggest that, if VEGFR2 is indeed expressed in hemangioblasts, its expression must very quickly be lost from committed hematopoietic cells. The situation is further complicated by the observation that VEGFR2 $^{-/-}$ embryonic stem cells, in embryoid bodies, have the ability to differentiate into blood cell precursors, demonstrating that VEGFR2 is not strictly necessary for hematopoiesis (Shalaby *et al.*, 1997). The same cells are unable to contribute to vascular structures. One interpretation of these results is that VEGFR2 is necessary for placing these cells in the correct environment to develop into hematopoietic stem cells, but is not strictly required for the hematopoietic pathway.

In addition to VEGFR-2, several other molecular markers are shared between endothelial and hematopoietic lines. These include the transcription factor SCL/Tal (Kallianpur *et al.*, 1994; Drake *et al.*, 1997; Liao *et al.*, 1998; Drake and Fleming, 2000), the cytokine TGF-β1 (Akhurst *et al.*, 1990), the MB1

antigen (Péault *et al*., 1983; Labastie *et al*., 1986) and the von Willebrand factor (Jaffe *et al.*, 1973). Moreover, in avian embryos, there are two lineages that express the antibody QH-1. The first is the splanchnopleural mesoderm, which contributes to both endothelial and hematopoietic cell lineages. The second is the somitic mesoderm which contributes only endothelial cells. This suggests that there is a sub-population of QH-1 positive cells in the lateral plate that have hemangioblastic potential (Pardanaud *et al*., 1996). Likewise, the zebrafish mutation *cloche* (*clo*) has severe defects in both endothelial and hematopoietic cell formation and normal embryonic expression of VEGFR-2 is severely reduced. Therefore *cloche* activity must lie very early in the pathway leading to blood cells and endothelial cells, perhaps within the hemangioblast itself (Stainier *et al*., 1995; Fouquet *et al*., 1997; Liao *et al*., 1997; Liao, *et al*., 1998; Thompson *et al*., 1998; Parker and Stainier, 1999).

Further evidence for the existence of the hemangioblast comes from studies of cells in culture. Using embryoid bodies as a source, a murine cell line (BLast Colony Forming Cells or BL-CFCs) has been derived that expresses markers of both endothelial and hematopoietic lineages (Choi *et al*., 1998; Faloon *et al*., 2000). This cell line can be experimentally manipulated, *in vitro*, to develop along either or both pathways and therefore possesses the properties predicted for the hemangioblast. Nonetheless, such a cell has never been observed *in vivo*, and its elusiveness suggests that hemangioblast cells must be a very transient population.

1.3. MOLECULAR BIOLOGY OF VASCULAR DEVELOPMENT

1.3.1 Differentiation of the Vascular Endothelial Cell Lineage

1.3.1.1. Vascular Endothelial Growth Factor (VEGF) and its Receptors

Vascular Endothelial Growth Factor-A (VEGF-A, hereafter referred to as VEGF) is a secreted dimeric protein that plays a critical role in vascular endothelial cell differentiation and proliferation. Other VEGF family proteins are also expressed in the developing embryo, but, with the possible exception of VEGF-C and its receptor VEGFR3/Flt-4, these do not appear to play a significant role in blood vessel development. For reviews dealing with other VEGFs (VEGF B-E) and the VEGF related molecule Placenta Growth Factor (PlGF), see Eriksson and Alitalo (1999) and Persico *et al*. (1999). The expression of VEGF in mice has recently been examined with great sensitivity by genetically inserting the β-galactosidase reporter module into an untranslated region of VEGF mRNA (Miquerol *et al*., 1999). This study resolved VEGF expression at the cellular level, thereby providing an extremely accurate description of the many domains of VEGF expression in the developing mammalian embryo. VEGF is a powerful mitogen specific for vascular endothelial cells, and also has an important function in mediating the chemotaxis of angioblasts. Postnatally, VEGF plays critical roles in endothelial cell survival (Gerber *et al*., 1999), tumor angiogenesis (Holash *et al*., 1999) and vascular permeability (Keck *et al*., 1989). The VEGF ligand is bound by two high affinity receptors, VEGFR2 (Flk-1/KDR) and VEGFR1 (Flt-

1), both of which belong to the tyrosine kinase receptor family. VEGFR1 and VEGFR2 are expressed exclusively in vascular endothelial cells, and VEGFR2 represents the earliest known specific marker of endothelial cells.

1.3.1.2. Isoforms of the VEGF protein

Depending on the specific organism, as many as five different isoforms of VEGF protein are known to be produced. These different proteins are generated by alternative splicing of the primary transcript from the single VEGF gene (Leung *et al.*, 1989). In humans, the different forms of VEGF protein are called $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$, where the number refers to the length of the protein in amino acids. The precise lengths of the equivalent protein isoforms are slightly different in other organisms, but unless specifically stated otherwise, we will use the human numbering to identify the different isoforms. In all species examined, $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ are the most abundant variants in most tissues. Relative to $VEGF_{121}$, $VEGF_{165}$ has a 44 amino acid domain inserted close to the C-terminus of the protein and $VEGF_{189}$ contains an additional 24 amino acids, also close to the C-terminus. The presence of these additional protein domains has been shown to alter the biochemical properties of the different VEGF isoforms when assayed in cell culture systems. For example, $VEGF_{121}$ is freely diffusible upon cellular secretion, while the medium and large forms have increasing affinities for heparin sulfate, an important component of the extracellular matrix, ECM (Houck *et al.*, 1992). The heparin sulfate proteoglycan, glypican-1, has been shown to bind $VEGF_{165}$ and presumably binds

$VEGF_{189}$ even more efficiently (Gengrinovitch *et al.*, 1999). In transfected cell cultures, both $VEGF_{121}$ and $VEGF_{165}$ are present in conditioned media, while the large form is apparently bound to the cell surface or matrix (Houck *et al.*, 1991). The presence of $VEGF_{165}$ in conditioned media is indicative of its intermediate affinity for ECM components.

In vitro studies suggest that $VEGF_{165}$ is approximately 100-fold more effective at stimulating mitosis than $VEGF_{121}$ (Keyt *et al.*, 1996). Experiments using quail embryos have shown that administration of $VEGF_{121}$ to the chorioallantoic membrane causes a fourfold increase in endothelial cell number relative to controls with no added VEGF, although the degree of proliferation was not directly compared with that obtained by administration of $VEGF_{165}$ (Wilting *et al.*, 1996). For technical reasons, it is difficult to assess the mitogenic potency of $VEGF_{189}$ since it is completely bound to the ECM and therefore is not present in conditioned media. However, within the developing embryo, this property may confer an important regulatory control by limiting the spatial distribution of $VEGF_{189}$. Park *et al* (1993) cultured endothelial cells on ECM that had been conditioned with different VEGF isoforms, and found that, as expected due to its diffusion properties, $VEGF_{121}$ was not present in ECM, whereas $VEGF_{189}$ and $VEGF_{206}$ induced proliferation of endothelial cells at rates that were three to four times higher than controls. ECM conditioned with $VEGF_{165}$ induced proliferation at rates that were approximately twice that of controls. This difference in activity is most likely due to the reduced affinity of ECM for $VEGF_{165}$ compared to the

larger isoforms rather than a reduction in bioactivity. In fact, the amount of $VEGF_{165}$ present in the ECM was undetectable, indicating that very low amounts of $VEGF_{165}$ are sufficient to induce endothelial proliferation. Additional experiments demonstrate that VEGF isoforms that are bound to ECM can be proteolytically cleaved to yield biologically active molecules that are free to move into the conditioned medium (Keyt *et al.*, 1996). Although this effect was demonstrated *in vitro*, it seems likely that such mechanisms will also exist in the developing embryo.

In addition to its role as a mitogen, an increasing body of evidence demonstrates that VEGF acts as a chemoattractant for endothelial cells. This was first demonstrated by showing that endothelial cells in culture migrate towards a source of $VEGF_{165}$ (Waltenberger *et al.*, 1994). This action was posited, although not demonstrated, to occur during vascular development in the mouse embryo by Dumont *et al* (1995). More recent experimental evidence indicates that $VEGF_{165}$, specifically expressed in the lens of transgenic mice, also mediates vascular endothelial cell chemotaxis (Ash and Overbeek, 2000). When tissue ectopically expressing $VEGF_{121}$ is transplanted into the *Xenopus* embryo, it causes *in vivo* endothelial cell migration over distances of hundreds of microns (Cleaver and Krieg, 1998). While the chemoattractant properties of $VEGF_{165}$ and $VEGF_{189}$ have not been directly compared to $VEGF_{121}$ *in vivo*, they would be predicted to be less active than $VEGF_{121}$, due to their limited diffusion properties.

1.3.1.3. Genetic Studies of VEGF

VEGF activity in the early embryo is primarily mediated though the high affinity receptor VEGFR2, which is responsible for transducing the mitogenic and chemoattractant signaling properties of VEGF. In most cases, the embryonic expression domains of VEGFR2 and VEGF are complementary, strongly suggesting a paracrine signaling pathway for VEGF between adjacent tissues (Breier *et al.*, 1992; Yamaguchi *et al.*, 1993; Dumont *et al.*, 1995; Flamme *et al.*, 1995a; Cleaver *et al.*, 1997; Fouquet *et al.*, 1997; Liang *et al.*, 1998).

Gene ablation experiments in mice, and overexpression studies in mouse, frog and avian systems demonstrate the essential function of VEGF and its receptors during early vasculogenesis. Remarkably, the loss of even one copy of the VEGF gene is embryonic lethal in the mouse embryo, demonstrating a striking level of dosage sensitivity (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). Heterozygous (VEGF $^{+/-}$) embryos died at approximately E11 from severe vascular defects, primarily disorganized and leaky blood vessels. For example, the dorsal aorta was poorly developed and much smaller than that observed in wild-type embryos. The defects are much more extreme in embryos totally lacking VEGF function. These embryos die at approximately the same stage as heterozygotes, but contain an extremely low number of vascular endothelial cells and exhibit a complete absence of any organized vascular structures (Carmeliet *et al.*, 1996). As suggested by the lethality of the heterozygous VEGF $^{+/-}$ mouse, expression levels of VEGF are extremely finely regulated by the action of a

number of different cellular mechanisms. In addition to transcriptional regulation, VEGF activity is modulated at the level of translation, via the presence of two distinct internal ribosome entry sites (IRES) (Akiri *et al.*, 1998; Huez *et al.*, 1998; Stein *et al.*, 1998). Although primarily investigated in the context of low oxygen conditions (hypoxia), VEGF is also regulated at the level of mRNA stability (Ikeda *et al.*, 1995; Liu *et al.*, 1995). It is likely that a combination of all these mechanisms acts to regulate the levels of VEGF activity during embryonic vasculogenesis.

Whereas the gene ablation studies show that a reduction in VEGF levels is embryonic lethal due to failure of vascular formation, overexpression of VEGF in *Xenopus* and avian embryos causes ectopic blood vessel development in normally avascular regions, as well as hypervascularization and fusion of blood vessels (Drake and Little, 1995; Flamme *et al.*, 1995b; Cleaver *et al.*, 1997; Drake and Little, 1999). Additional genetic experiments in mouse have addressed the developmental function of the different VEGF isoforms. Embryos lacking the exons encoding the $VEGF_{165}$ and $VEGF_{189}$ isoforms, and consequently expressing predominantly $VEGF_{121}$, are viable throughout embryonic development, but die postnatally (by approximately P14) due to either internal bleeding or multiple cardiac problems (Carmeliet *et al.*, 1999a). Therefore, despite the apparent differences in mitotic activity and biochemical properties of the different VEGF isoforms revealed in cell culture studies (see above) this experiment demonstrates

that the $VEGF_{121}$ isoform alone is sufficient to regulate the great majority of cellular and morphological events that occur during early vascular development.

1.3.1.4. Genetic Studies of VEGF receptors

The phenotype of embryos lacking function of the high affinity VEGF receptor, VEGFR2, is even more severe than that of the VEGF knockout and indicates an absolute requirement for VEGFR2 in endothelial development. Embryos die between E8.5 and E9.5, lacking yolk sac blood islands and all intraembryonic vessels. The embryos fail to form endothelial cells and show a complete absence of blood cells. This latter defect suggests that VEGFR2 is essential for some early aspect of hematopoietic development (Shalaby *et al.*, 1995). As mentioned above, VEGF-/- embryos die at about E11, approximately two days later than embryos lacking VEGFR2 function. Although both are embryonic lethal, the non-equivalence of the $VEGF^{-/-}$ and $VEGFR2^{-/-}$ phenotypes suggests that a low level of receptor binding, presumably by other members of the VEGF family, is able to partially rescue endothelial cell development in the embryos lacking VEGF-A activity. Additional experiments using chimeric embryos demonstrated that VEGFR2-/- cells are never present in the vascular endothelium, indicating a cell autonomous requirement for VEGFR2 (Shalaby *et al.*, 1997).

Genetic ablation of the other high affinity VEGF receptor, VEGFR1, is also embryonic lethal, in this case due to severely malformed vascular channels

(Fong *et al.*, 1995). In these VEGFR1-/- embryos, an excessive number of endothelial cells were present, leading to the presence of endothelial cells in the interior as well as the periphery of extraembryonic blood islands. Within the embryo itself, endothelial cells are present inside abnormally enlarged vascular structures. More recent work demonstrates that an alteration in cell fate determination in VEGFR1-/- embryos causes an increase in the number of both vascular endothelial cells and blood cells, relative to wildtype (Fong *et al.*, 1999). This increased density of endothelial cells is responsible for the defects in vascular assembly, since VEGFR1-/- endothelial cells are capable of forming normal endothelial channels when they are present in chimeric embryos. As an interesting aside, even though VEGFR1 has a tyrosine kinase domain, ablation of this domain does not effect normal vascular development, providing that the ligand-binding domain is intact (Hiratsuka, *et al* 1998). Based on these observations, it is proposed that the principal role of VEGFR1 in vasculogenesis is to sequester excess VEGF ligand, thereby regulating endothelial proliferation (Fong *et al.*, 1999).

In addition to the two tyrosine kinase receptors, VEGFR2 and VEGFR1, it was recently discovered that neuropilin-1 can also act as a receptor for VEGF, in particular the $VEGF_{165}$ isoform (Soker *et al.*, 1998). This observation was unexpected, since neuropilin-1 had previously been characterized as a receptor that mediates semaphorin signaling during axonal pathfinding (He and Tessier-Lavigne, 1997). When neuropilin-1 and VEGFR2 are co-expressed in the same

cell, addition of $VEGF_{165}$, but not $VEGF_{121}$, causes an approximately 3-fold increase in migration of endothelial cells (Soker *et al.*, 1998). Recently, neuropilin-1 was also shown to bind $VEGF_{165}$ when co-expressed with VEGFR1 (Fuh *et al.*, 2000). A significant role for neuropilin in embryonic vascular development is demonstrated by mouse gene ablation experiments which show that mice lacking neuropilin-1 exhibit both axonal defects and cardiovascular defects, including problems with formation of the dorsal aorta and the extraembryonic blood vessels (Kawasaki *et al.*, 1999). An additional neuropilin receptor, neuropilin-2, also binds $VEGF_{165}$, suggesting that it too will play a role in vasculogenesis (Gluzman-Poltorak *et al.*, 2000).

1.3.2 Vascular tubulogenesis

Vascular tubulogenesis, the process by which angioblasts coalesce into vascular cords and then form a continuous, tubular network is not well understood. Nonetheless, it is clear that this is a highly coordinated process within the developing embryo. In avian embryos, for example, the entire primary vascular network assembles over a period of less than 8 hours, between the 4-somite and 8-somite stages of development. During this process, angioblasts assemble into solid clusters of cells that then form tubes (Hirakow and Hiruma, 1983; Coffin and Poole, 1988). The initial formation of a vascular lumen occurs when a 'slit-like space' opens up between two angioblasts. These spaces enlarge, combining with other such spaces to form a hollow endothelial tube (Houser *et al.*, 1961). At least in some cases, lumen formation precedes the formation of a

continuous endothelial network (Hirakow and Hiruma, 1983; Drake and Jacobson, 1988). In order to form a lumen, angioblasts must presumably acquire polarity, containing apical and basal surfaces. Study of this process has been precluded by the lack of known cell polarity markers in angioblasts. As might be expected, cell adhesion molecules and extracellular matrix components are thought to play pivotal roles in this process.

Amongst the factors known to be essential for vascular assembly is the extracellular matrix (ECM). ECM is a complex mixture of proteins and glycoproteins, and a broad range of different experimental approaches have demonstrated that ECM proteins, including laminin, integrins, collagen and fibronectin serve regulatory functions during blood vessel assembly. In the case of collagen, cell culture studies have shown that endothelial cells which are able to synthesize type 1 collagen will spontaneously form endothelial cords, whereas endothelial cells that do not express type 1 collagen will not form these aggregates (Vernon *et al.*, 1995). It has been proposed that angioblasts associating with ECM fibers are capable of coalescing into aggregates, through forces exerted on the ECM. This establishes the rough boundaries of the vascular network, which subsequently forms a continuous tissue layer, either by protrusive cellular extensions or by recruiting additional angioblasts (Drake and Little, 1998).

One family of ECM proteins implicated in vascular assembly is the integrins, a large group of related glycoproteins that effect cell adhesion by binding to an array of extracellular matrix components (for general reviews see Yamada and Miyamoto, 1995; Hynes and Bader, 1997). Much attention has been focused on the integrin $\alpha_v\beta_3$ dimer, expression of which is upregulated during both vascular assembly and angiogenesis (Brooks *et al.*, 1994; Drake *et al.*, 1995). A neutralizing antibody to this integrin specifically inhibits the attachment of endothelial cells to fibrinogen, vitronectin and the von Willebrand factor (Cheresh, 1987), all of which are expressed embryonically and may be involved in embryonic vascular adhesion. Indeed, the application of this monoclonal antibody to avian embryos disrupted lumen formation, suggesting that the $\alpha_v\beta_3$ integrins play a direct role in mediating lumen formation (Drake *et al.*, 1995). Given this result, it was surprising that mice in which the α_v gene had been ablated, and therefore lacked all α_v type integrins, developed a relatively normal vascular system (Bader *et al.*, 1998). It remains possible that some additional member of the integrin α family partially rescued α_v function in the mutant mice, but at present, the precise role that $\alpha_v\beta_3$ integrins play in lumen formation is unclear. It has also proven difficult to determine how other integrins, such as those which bind fibronectin, are involved in vascular assembly because a considerable amount of functional redundancy exists between integrin family members. One reasonably clear example, however is integrin α_5, ablation of which causes multiple development defects, including problems with vascular assembly (Yang *et al.*, 1993). Efforts to analyze embryos lacking two different

integrins has proved frustrating. For example, mice lacking function of both the α_v and α_5 integrin subunits die very early in development due to gastrulation defects (Yang *et al.*, 1999), thereby precluding an analysis of their vascular phenotype.

In addition to structural proteins, regulatory molecules also play an important role during vascular assembly. The VEGF signaling pathway, which is absolutely required for vascular cell development, is also involved in the correct assembly of endothelial cells into lumenated vessels. Mice lacking the VEGF receptor, VEGFR1, develop abnormal vascular channels containing internalized endothelial cells. The phenotype is apparently due to an overproliferation of endothelial cells caused by excess VEGF (Fong *et al.*, 1995, 1999) (see above). This suggests that one of the important steps in vessel formation is the down-regulation of endothelial cell mitosis. Indeed, when large amounts of excess VEGF ligand are added to avian embryos, the results include both hypervascularization and also abnormally large vascular lumens (Drake and Little, 1995). Conversely, insufficient VEGF activity results in the failure of angioblasts to form endothelial tubes (Damert *et al.*, 2002).

1.3.3. Notch signaling

Until very recently, Notch signaling was thought to play a comparatively minor role in vascular development, mainly in vascular remodeling. Within the last two years, however, the Notch signaling pathway has been suggested to play

a pivotal role in determining whether an angioblast acquires an arterial or venous fate. Although all nascent endothelial tubes are superficially similar, they contain genetic differences. For example, the presence of ephrinB2 marks future arteries, while EphB4 marks future veins in zebrafish (Lawson *et al.*, 2001). Zebrafish embryos deficient in Notch signaling have a loss of artery-specific markers with a concomitant gain in venous markers. Furthermore, activated Notch signaling results in the repression of venous markers (Lawson *et al*, 2001). This data is corroborated by a recent expression survey of Notch signaling components in mice that indicates that Notch expression is limited to arterial vessels (Villa *et al.*, 2001). The genetic network involving Notch arterial specification is controversial. One study in zebrafish argues that both Shh and VEGF lie downstream of Notch in arterial specification (Lawson *et al.*, 2002). However, this data is hard to reconcile with an overwhelming amount of genetic data indicating that VEGF is essential for the development of all blood vessels.

Gene ablation experiments in mice have also demonstrated a requirement for Notch signaling in vascular development. Mice deficient in Notch1 exhibit defects in vascular assembly and vascular remodeling (Swiatek *et al.*, 1994). While the Notch4 knockout is completely viable (Krebs *et al.*, 2000), embryos that are homozygous double mutants for Notch4 and Notch1 show a more severe vascular phenotype than Notch1 mutants alone, suggesting that the two receptors play partially overlapping roles during vascular remodeling (Krebs *et al.*, 2000). Additionally, the expression of a constitutively active Notch4 protein in

developing endothelial cells results in lethality by E10. While the large vessels of the embryo initially form, the mice have defects associated with vascular patterning and remodeling (Uyttendaele *et al.*, 2001). Further evidence for the role of the Notch signaling pathway in blood vessel development is provided by experiments showing defects in the cranial vasculature in mice embryos deficient in Jagged 1, a gene encoding a Notch ligand (Xue *et al.*, 1999). Overall, these genetic studies suggest that Notch is involved in vascular remodeling and angiogenesis. However, none of these experiments addressed the role of Notch signaling in arterial-venous specification. In the future, it will be interesting to examine whether the knockout phenotypes are caused by erroneous venous specification.

1.3.4. The Ephrin signaling pathway

The Eph/ephrins constitute a large family of tyrosine kinase receptors and their cognate ligands. The terminology for this family of molecules is quite confusing, but in all cases the name of the ligand, ephrin, commences with a lower case letter, while the name of the receptor, Ephrin, commences with a capital and is usually abbreviated to Eph. Unlike many ligand-receptor interactions, ephrin ligands must remain cellularly bound to elicit a response. Thus, for signaling to occur, the ephrin ligand must be present on the surface of a cell juxtaposed with a cell containing an Ephrin receptor. To date, at least 14 different receptors and 8 ligands comprising two subfamilies have been identified, making Ephrins the largest known family of receptor tyrosine kinases (Van der

Geer *et al.*, 1994). Because Ephrin signaling can only take place at regions where cells are contacting each other, the system potentially provide a very precise mechanism for control of cellular boundaries. Eph/ephrins have been implicated in the guidance of axonal growth cones, segmentation of the somites and rhombomeres, retinotectal patterning, and cellular migration (reviewed in Holder and Klein, 1999). In addition, Ephrin signaling plays an essential role in early vascular development.

The Ephrin receptors and their ligands are divided into two classes based on the structure of their ephrin ligands. Class A ephrins are attached to the membrane via a glycosylphosphatidylinositol linkage, while class B ephrins are transmembrane proteins. As a general rule, class A ephrins bind class A receptors and class B ephrins bind class B receptors. The only known exception to this pattern of class-specific segregation is EphA4, which binds several ephrin B ligands in addition to class A ephrins (Gale *et al.*, 1996). One of the unique characteristics of Ephrin signaling is that it occurs bidirectionally. For example, binding of either ephrinB1 or ephrinB2 ligands to the EphB2 receptor not only causes tyrosine phosphorylation of the receptor, but also of the cognate ligand molecules (Holland *et al.*, 1996). Studies using zebrafish embryos show that Ephrin receptors and their ligands restrict cell mixing and cell communication *in vivo* and that bidirectional signaling is indeed important for this function (Mellitzer *et al.*, 1999).

While previous experiments had demonstrated a role for ephrinA1 and EphA2 in pathogenic angiogenesis (Pandey *et al.*, 1995), the significance of Ephrin signaling in embryonic vascular development was first revealed by gene ablation studies in mouse. Embryos lacking ephrinB2 function displayed a severe vascular phenotype (Wang *et al.*, 1998), including gross abnormalities in the formation of the circulatory system as well as a decrease in the size of the heart and reduced myocardial trabeculation. The mutant embryos died by E11. While the primary vascular system developed relatively normally, there was an absence of internal carotid arterial branches and the mice exhibited malformed capillary beds in the head as well as vascular defects in the yolk sac. A closer examination of the normal expression pattern of ephrinB2 in the embryo revealed that expression is restricted to those endothelial cells that will contribute to the future arteries and that this arterial specific expression is present from the earliest stages of vasculogenesis (Wang *et al.*, 1998). In addition, an Ephrin receptor, EphB4, is expressed in a reciprocal pattern in the developing venous network. This striking pair of observations leads to the possibility that endothelial cells that are fated to contribute to veins or arteries are genetically distinct from a very early developmental stage. Furthermore, these studies implicate ephrinB2/EphB4 signaling in establishment of the boundaries between the arterial and venous networks of the embryo.

Subsequent studies show that the role of Ephrin signaling during vascular development is more complicated than it first appears. Other Ephrin signaling

molecules are also found in developing blood vessels. For example, ephrinB1 is expressed at high levels in both arteries and veins, and EphB3 is expressed in developing veins and aortic arches (Adams *et al.*, 1999). Another receptor, EphB2 is present in the mesenchyme adjacent to the umbilical vein. Furthermore, ephrinB2, in addition to its previously described expression in arterial endothelial cells, is also expressed in the mesenchyme adjacent to intersomitic vessels. These observations suggest that Ephrin signaling may also be involved in blood vessel development at the interface between endothelial cells and the adjacent mesenchyme. Nonetheless, conditionally ablating ephrin B2 specifically in the endothelium phenocopies the cardiovascular effects of the ephrin B2 knockout (Gerety and Anderson, 2002). A possible biological role for these additional Ephrin receptors and ligands is suggested by *in vitro* studies which show that both ephrinB1 and ephrinB2 are capable of inducing capillary sprouting (Adams *et al.*, 1999). Further evidence is provided by the demonstration that embryonic misexpression of either the ephrin B ligand, or a dominant negative EphB4 receptor, results in abnormal growth of intersomitic blood vessels (Helbling *et al.*, 2000; Oike *et al.*, 2002). Overall, it is clear that Ephrin signaling plays an important role in delineating the vascular system, and the reciprocal expression of EphB4 and ephrinB2 on veins and arteries respectively makes it tempting to speculate that these molecules, at least, are involved in conferring an arterial or venous fate. In addition, other members of the Ephrin signaling family appear to be involved in regulation of vascular sprouting and morphogenesis.

1.3.5. Hedgehog signaling

Very recent data has implicated hedgehog signaling in vascular development. All vertebrates express three hedgehog genes, which are homologues of the single hedgehog (Hh) gene in Drosophila. These genes are Shh, Indian hedgehog (Ihh) and Desert hedgehog (Dhh). These share similar biochemical activities (Pathi *et al.*, 2001), and they share a common set of signal transduction molecules. The hedgehog receptor Patched (Ptc), which is upregulated in response to Shh does not directly mediate hedgehog signaling. Rather, in the absence of a Hh ligand, Ptc represses signaling by indirectly blocking the activity of Smoothened (smo), another transmembrane protein. Hh binding to Ptc relieves its inhibition on Smo, allowing it to activate downstream targets in a manner that is still poorly understood (rev. in Ingham and McMahon, 2001).

In mice, embryoid bodies derived from ES cells lacking either the global hedgehog transducer smoothened or Ihh initially express endothelial cells markers, however they are unable to form blood islands (Byrd *et al.*, 2002). Furthermore, Indian hedgehog can activate ectopic vasculogenesis in prospective neurectoderm (Dyer *et al*, 2001). The *in vivo* implications for this particular data are unclear, however, because angioblasts are still specified in smoothened deficient embryoid bodies that lack all hedgehog signaling (Byrd *et al.*, 2002). Sonic hedgehog has also been implicated as an indirect angiogenic factor in postnatal mice (Pola *et al.*, 2001). Finally, zebrafish shh mutants, such as the

sonic-you mutations, do not form vascular tubes in the trunk region of the embryo, although angioblasts are still present (Brown *et al*., 2000). One recent study has suggested that in combination with VEGF, Shh acts upstream in the specification of the dorsal aorta in zebrafish (Lawson *et al*., 2002). Overall, these studies suggest important, but undefined roles for hedgehog signaling in vascular development.

1.3.6. VE-cadherin

The calcium mediated cell adhesion protein, VE-cadherin (also known as cadherin-5) is endothelial specific (Lampugnani *et al*., 1992; Breier *et al*., 1996), and gene ablation experiments indicate that VE-cadherin plays a vital role in mediating vascular assembly. Mouse embryos lacking VE-cadherin express many endothelial markers, but these cells fail form patent vessels in the anterior portion of the body, and the embryos die by E11.5 of severe vascular defects (Gory-Fauré *et al*., 1999). The defects are even more spectacular in the extraembryonic yolk sac and in embryoid bodies of mutant embryos, which contain no organized vascular pattern (Vittet *et al*., 1997; Gory-Fauré *et al*., 1999). Taken together, these results support the idea that VE-cadherin plays a pivotal role in vascular assembly. However, the persistence of primary blood vessels in the posterior regions of the embryo suggests that other cell adhesion molecules must have overlapping roles in vascular assembly.

Characteristic of cadherin proteins, VE-cadherin consists of an extracellular domain involved in cell adhesion and an intracellular component that mediates intracellular signaling. The importance of the intracellular domain is underscored by studies of mice genetically manipulated to express only a truncated form of VE-cadherin that lacks the signaling domain (but still maintains cell adhesion). In these mice, assembly of the initial vascular network was not affected, however, the endothelial cells failed to respond to anti-apoptotic signaling mediated by VEGF and mice died at E9.5 due to vascular insufficiency caused by increased apoptosis (Carmeliet *et al.*, 1999b). This interesting link between VEGF signaling and VE-cadherin may help to explain the increased size of blood vessels lumens that resulted when VEGF was overexpressed in chick embryos (Drake and Little, 1995) (see above).

1.3.7. Angiogenesis and vascular remodeling

The preceding sections have primarily addressed the formation of the primary vascular plexus from individual angioblasts. After this initial assembly, the vascular network becomes greatly elaborated during a series of events termed vascular remodeling. This term encompasses a variety of processes in which the vascular system expands and acquires the vast heterogeneity seen in mature blood vessels. Examples of this diversity range from the dense microcapillary networks in the developing lungs to the large vessels such as major veins and arteries that carry blood throughout the embryo (Evans, 1909). It is important to note that the process of vascular remodeling greatly alters the topography of pre-existing blood

vessels and these changes continue throughout development to accommodate the changing needs of the embryo. The alterations not only include the development of new vessels but also the regression of existing vessels. For example, loss of blood vessels is particularly prominent in the aortic arch region of the embryo. The six pairs of symmetrical vessels present at various stages of development undergo a programmed series of ablations and regressions such that only the third, fourth and sixth pairs ultimately contribute to the adult vasculature. In addition to remodeling of the overall vascular architecture, the blood vessels themselves physiologically mature. Initially the vessels consist only of a tube of endothelial cells, but during subsequent development they acquire layers of vascular smooth muscles cells (or similar cells known as pericytes around capillaries), connective tissues such as collagen and elastin, and a basement membrane. The blood vessels also acquire the physiological properties characteristic of either veins or arteries.

Angiogenesis is defined as the sprouting or splitting of blood vessels from pre-existing blood vessels. The sprouting and splitting processes are achieved by two quite different and distinct mechanisms. In the embryo, sprouting angiogenesis (Hertig, 1935; Clark and Clark, 1939; Ausprunk and Folkman, 1977) is responsible for formation of the intersomitic vessels, vascularization of the developing brain, growth of blood vessels into developing limbs and vascularization of numerous other embryonic tissues (Coffin and Poole, 1988; Risau, 1997). Embryonic sprouting angiogenesis consists of several phases

(Fig.4A). Initially, endothelial cells detach and migrate out from the pre-existing endothelial tube, in response to an angiogenic stimulus. The cells continue to extend further from the original vessel as sprouting continues. In blood vessels in the mature organism this process is known to involve degradation of the basement membrane or ECM surrounding the endothelial cells prior to cell outgrowth (Hiraoka *et al*., 1998). However, this is unlikely to be the case in early embryonic angiogenesis, where the basement membranes and ECM are still developing (Wagner, 1980; Wilting and Christ, 1996). Endothelial cell migration is followed by lumen formation, which originates from the parent vessel and proceeds in a proximal to distal direction along the branch. In addition, the sprouting process involves endothelial cell proliferation, which also proceeds in a proximal to distal pattern (Ausprunk and Folkman, 1977; Wilting and Christ, 1996).

The second major type of embryonic angiogenesis is called intussusceptive growth, the final result of which is expansion of microcapillary beds. This type of angiogenesis was first characterized during scanning electron microscope studies on the developing capillaries in rat lungs (Caduff *et al*., 1986; Burri and Tarek, 1990). Subsequent studies have shown that the intussesceptive growth occurs during blood vessel development in the avian chorioallantoic membrane, in addition to sprouting angiogenesis (Patan *et al*., 1993; Patan *et al*., 1996). The process of angiogenesis by intussusceptive growth has been divided into four stages (Fig. 4B). In the first phase, the opposite walls of a vessel come into contact with each other, forming an 'interendothelial bridge.' In the second

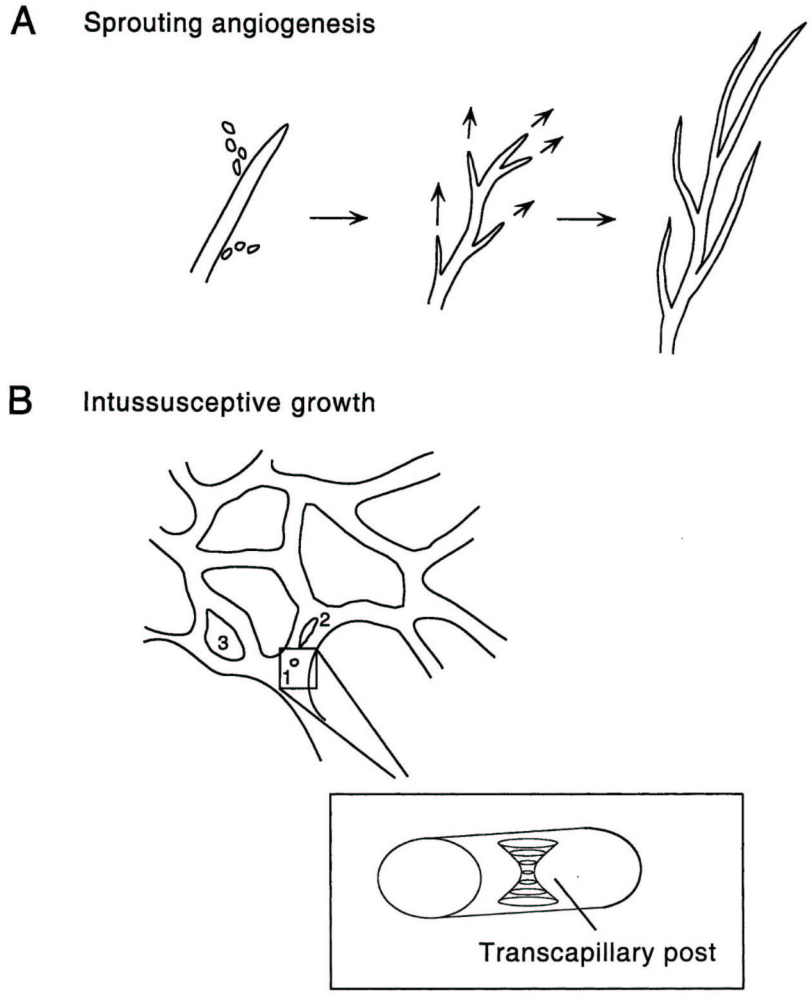

Figure 4. The two major forms of angiogenesis.

(A) Sprouting angiogenesis is the most common mechanism for generating new blood vessels in the embryo. Endothelial cells migrate away from the parent vessel, proliferate and form a vascular branch. (B) Intussusceptive angiogenesis is primarily a mechanism for increasing the vascular density of capillary beds. In this mechanism, the opposite walls of a vessel come together and split the capillary into two portions to create a transcapillary post of tissue (shown in the inset), resulting in a splitting of the parent capillary. The numbers indicate progressive increase in the size of the intussusceptive split.

phase, as endothelial cells reorganize, the capillary is actually divided into two portions by formation of a transcapillary post of tissue (somewhat analogous to a pillar separating the floor and ceiling). During phases three and four, this post is stabilized by the addition of pericytes and connective tissue and grows in length, gradually splitting the capillary into two daughter vessels. The final result is the creation of two adjacent vessels from a single original capillary. Problems with the process of intussceptive growth have been implicated at the cause of defects in the intraembryonic vascular network of tie-1 and tie-2 mutant mice (Patan, 1998) (See Angiopoietin Signaling).

1.3.8 Angiopoietin Signaling

1.3.8.1. Tie Receptors

Angiopoietins and their receptors Tie-1 and Tie-2 (Tek) comprise another developmentally essential signaling system involved in vascular formation. Unlike VEGF and its tyrosine kinase receptors that act early in vasculogenesis to specify the initial pattern of the vascular plexus and endothelial sprouting and proliferation, the angiopoietin system acts later, after the primary vascular plexus has been established. Both Tie receptors are tyrosine kinases. The Tie-2 receptor is bound either agonistically or antagonistically by angiopoietins, while currently, no ligands for Tie-1 have been identified. Analysis of the signaling pathway downstream of the Tie receptors is an area of active investigation, but is beyond the scope of this chapter (for a review see Partanen and Dumont, 1999).

Gene ablation studies in mouse demonstrate that the Tie-2 receptor is essential for vascular patterning and remodeling in the embryo. Dumont *et al* (1994) analyzed tie-2 deficient mice and discovered that, although initial vascular development was normal, there was a dramatic decrease in endothelial cells as development continued. Day E8.5 embryos contained approximately 30% fewer endothelial cells than wild type, with a 75% reduction in endothelial cell number by E9.0. The embryos exhibited massive vascular hemorrhaging from both the embryonic and yolk sac vasculature. For example, in E9.5 embryos, the dorsal aorta is often collapsed and blood cells may be detected in the adjacent mesenchyme. The embryos also exhibited defects in heart development, suggesting that Tie-2 function may be required for interactions between the myocardial and endocardial cell layers. No embryos survived beyond E9.5. Function of the tie-2 gene was independently ablated by Sato *et al* (1995). Some intriguing differences were observed in the mutant phenotype. In contrast to the previous studies (Dumont *et al*., 1994) which reported that vascular development was normal until E8.5, Sato *et al* (1995) observed distinct abnormalities in vascular formation, primarily in the head and extraembryonic vasculature. In these regions the blood vessels were homogenous in size, rather than displaying the full range of different blood vessel diameters. Presumably, this effect is due to problems with remodeling of the primary vascular plexus. Subsequent studies have revealed that Tie-2 deficient blood vessels lack associated smooth muscle cells (Patan, 1998). Significantly, these studies of the tie-2 mutant phenotype have direct relevance to human disease. Vascular dysmorphogenesis, a human

vascular malformation caused by diminished or absent smooth muscle in some vascular channels, is caused by a mutation in the kinase domain of Tie-2 (Vikkula *et al*., 1996).

Mice lacking Tie-1 receptor function die between E13.5 and birth. While the embryos initially have a normal vascular pattern, they develop edema and localized hemorrhaging, resulting from leaky endothelial cells (Puri *et al*.; 1995, Sato *et al*., 1995). These endothelial cells exhibit abnormally thin extensions, suggesting that the leaky phenotype may result from excessive endothelial cell stretching (Patan, 1998). Chimeric studies indicate that mutant endothelial cells are initially comparable to wild type cells in their ability to populate the embryonic blood vessels. However by E15.5, there is a strong bias against the tie-1 mutant endothelial cells in regions such as the intestinal and midbrain capillary plexi, which are primarily vascularized by intussusceptive angiogenesis (Burri and Tarek, 1990; see section an Angiogenesis and vascular remodeling). On the other hand, large blood vessels like the aorta, which is formed by vasculogenesis, still contain an equal representation of mutant cells (Partanen *et al*., 1996). Because tie-1 mutant embryos actually display denser capillary plexi than wildtype embryos, it has been proposed that the phenotype may be due to an increase in intussusceptive angiogenic growth resulting from more elastic endothelial cells (Patan, 1998).

The fact that both Tie-1 and Tie-2 are present in vascular endothelial cells suggests that they may play partially redundant functions. To address this question, mice lacking activity of both the Tie-1 and Tie-2 receptors have been generated (Puri *et al.*, 1999). These embryos survive at least until E9.5 and possess an intact vascular system. The overall phenotype of the double mutant was quite similar to that of embryos mutant for tie-2 alone, although the double mutants also exhibited somitic defects. Chimeric analysis of early stage embryos indicates a strong reduction in the contribution of mutant cells to the endocardium, but not to other regions containing endothelial cells. However, by E15.5, mutant cells are excluded from virtually all of the vascular system. Overall, the exclusion of vascular endothelial cells mutant for both Tie-1 and Tie-2 occurs much earlier and is more comprehensive than that observed for cells mutant for Tie-1 alone (see above). Based on the genetic studies and also on detailed histological analysis, it is proposed that the major function of the Tie-2 receptor is to mediate interactions between endothelial cells and the extracellular matrix (Patan, 1998). In contrast, the primary function of Tie-1 appears to involve inhibition of endothelial cell stretching, an activity which may help to explain the leaky cell phenotype seen in the knockout (Patan, 1998).

1.3.8.2. Angiopoietins

Angiopoietins are the ligands for the Tie receptors. Angiopoietin-1 (Ang-1), binds specifically to Tie-2, but not Tie-1 (Davis *et al.*, 1996). Ang-1 is expressed in mice starting at E9.0, at which stage it is strongly expressed in the

myocardium. During later development, Ang-1 becomes prominently expressed in the mesenchyme surrounding maturing blood vessels. On the other hand, the Ang-1 receptor, Tie-2 is expressed in the endocardium and in endothelial cells themselves. The ligand and the receptor therefore, exhibit approximately complementary expression patterns suggesting a paracrine signaling pathway. Unlike VEGF however, Ang-1 does not elicit a proliferative response in endothelial cells (Davis *et al.*, 1996). Ablation studies of the Ang-1 gene in mice strongly suggest that Ang-1 is the principal biological ligand for Tie-2 because the mutant phenotype recapitulates most aspects of the Tie-2 knockout phenotype. Notably, Ang-1 $^{-/-}$ embryos die by E12.5, apparently due to heart defects that closely resemble those observed in the Tie-2 knockout. Furthermore, Ang-1 $^{-/-}$ embryos also display defects in both vascular branching and vessel size, strikingly similar to those in the Tie-2 mutant. These vessels lack closely associated periendothelial cells (Suri *et al.*, 1996). Overall, the defects in embryos lacking Ang-1 function are slightly less severe than the Tie-2 knockout, an observation that may be explained by the presence of other angiopoietins which partially compensate the vascular defects (see below). In addition to the gene ablation studies, transgene approaches have been used to overexpress Ang-1 in the skin of mice, under control of the keratin-14 promoter. The skin capillaries in these mice were larger, more numerous and more highly branched than those of control embryos (Suri *et al.*, 1998), further supporting a role for Ang-1 signaling in vascular remodeling.

Like Ang-1, Angiopoietin-2 (Ang-2) is expressed throughout the embryonic vasculature, although in this case the pattern appears to be more punctate. Ang-2 binds to Tie-2, but shows no affinity for the Tie-1 receptor. In contrast to Ang-1, despite the fact that Ang-2 binds to Tie-2, it does not induce phosphorylation (Maisonpierre *et al.*, 1997; Kim *et al.*, 2000). Additional experiments show that an excess of Ang-2 is capable of blocking Tie-2 phosphorylation by Ang-1, implying that Ang-2 may act as a natural antagonist of Tie-2 mediated signaling. Transgenic mice have been generated in which Ang-2 is expressed under control of the Tie-2 promoter, thereby ensuring that Tie-2 and Ang-2 are expressed in the same cells. These animals died at E9.5-10.5 as the result of vascular defects very similar to those observed in the Tie-2 knockout (Maisonpierre *et al.*, 1997). This result clearly supports the hypothesis that the normal function of Ang-2 is to antagonize Tie-2 signaling.

Recently, several additional angiopoietin or angiopoietin related molecules have been isolated (Kim *et al.*, 1999a; Kim *et al.*, 1999b; Nishimura *et al.*, 1999; Valenzuela *et al.*, 1999). Of these molecules, human Ang-4 has been shown to bind and activate the Tie-2 receptor, while mouse Ang-3 binds to Tie-2 but acts as an antagonist. Surprisingly, none of these new angiopoietins are capable of binding Tie-1. It will be necessary to further characterize the new angiopoietins, both biochemically and at the level of embryonic expression, before it will be possible to suggest a potential function for these molecules during early blood vessel development.

1.3.9. Transcription factors

Surprisingly little is known about the transcription factors involved in regulation of early blood vessel development. Indeed, even those transcription factors that are known to be expressed in vascular endothelial cells usually show expression in additional embryonic tissues. A rather typical example is the homeodomain transcription factor hex. Hex is expressed in the embryonic endoderm, the developing liver and also in endothelial precursor cells (Newman *et al.*, 1997; Thomas *et al.*, 1998; Yatskievych *et al.*, 1999). In the *Xenopus* embryo, overexpression of hex causes an increase in the number of endothelial cells (Newman *et al.*, 1997) suggesting a role in differentiation or proliferation. Ablation of hex function in mouse, produces liver and anterior patterning defects, consistent with its known embryonic expression domains, but no discernible vascular phenotype, possibly because other factors compensate for its absence in endothelial cells (Barbera *et al.*, 2000).

Another transcription factor implicated in vascular development is *gridlock*. The *gridlock* (*gr1*), mutation in zebrafish was originally recognized because it causes a blockage in circulation to the posterior trunk and tail due to an obstruction in the aorta (Weinstein *et al.*, 1995). The *gr1* gene has been positionally cloned and encodes a novel transcription factor of the bHLH family that is expressed in the heart region and dorsal aorta (Zhong *et al.*, 2000). Related gene members of this family acts as transcriptional repressors downstream of

Notch signaling in mice (Nakagawa *et al*., 2000). A recent study reports that gr1 is essential for arterial specification in zebrafish, acting as a downstream effector of Notch signaling (Zhong *et al*., 2001). However, this data conflicts with another study that found that blocking Notch signaling in an identical fashion did not cause a change in *grl* expression, although the expression of the arterial marker *ephrinB2* was diminished (Lawson *et al*., 2001). This suggests that grl may not be as important for arterial specification as originally claimed.

Members of the Ets family of transcription factors are widely expressed in vascular endothelial cells and have been implicated in the regulation of vascular genes. In particular, specific Ets binding sites have been identified in the promoter regions of many endothelial genes, including VEGFR1 (Wakiya *et al*., 1996) and also members of the matrix metalloproteinase family, which are involved in vascular remodeling (Yamamoto *et al*., 1998; Hiraoka *et al*., 1998). However, the high redundancy within the family makes assessment of the role of specific Ets family proteins difficult. For example, genetic ablation of Ets-1 itself produces viable offspring with no vascular defects, presumably due to the rescuing function of other Ets family genes (Barton *et al*., 1998). Some members of the Ets family are more clearly involved in endothelial cell gene regulation. In the case of fli1, which shows high levels of expression in embryonic angioblasts (Brown *et al*., 2000), genetic ablation leads to impaired hematopoiesis and also widespread hemorrhaging, including leakage from the dorsal aorta (Spyropoulos *et al*., 2000). The Ets transcription factor TEL, is expressed in a range of different

embryonic tissues and mouse embryos lacking TEL function display defects in yolk sac angiogenesis (Wang *et al.*, 1997). The Ets family sequence erg is also expressed at high levels in the embryonic vasculature but a specific function remains to be determined (Baltzinger *et al.*, 1999).

The transcription factor SCL/Tal is expressed in both endothelial and hematopoietic progenitor cells (Kallianpur *et al.*, 1994; Drake *et al.*, 1997; Gering *et al.*, 1998; Drake and Fleming, 2000). The primary role of SCL seems to be in specifying blood development since targeted disruption of the gene results in embryos that lack blood but still contain endothelial tubes (Shivdasani *et al.*, 1995). However, conditional SCL mutants, in which blood formation has been rescued, die from defects in remodeling of yolk sac blood vessels (Visvader *et al.*, 1998), clearly indicating a role for SCL in embryonic vascular development.

1.4. CONCLUSION

In 1922, the pioneering embryologist Florence Sabin remarked on the origins of blood vessels that "We know just how blood-vessels begin" (Sabin, 1922). As it turned out, her brilliant insights into vascular development represented only the beginnings of the journey towards understanding fomation of the vascular system. Over the last decade in particular, there has been an explosion in our knowledge of the cellular and molecular mechanisms underlying all facets of vascular development. Perhaps the most obvious conclusion from these recent studies is that the process of vascular development is inherently

complex. While a significant amount is known about some of the signaling pathways, structural proteins and transcription factors involved in blood vessel formation, other important molecules remain essentially uncharacterized. Now that complete genome sequences are becoming available and high throughput analysis of gene expression patterns is routine, identification of yet more molecules involved in vascular development can be expected to proceed at an unprecedented rate. The placement of these new molecules within genetic hierarchies and signaling pathways and their integration into existing regulatory networks will represent the next great challenge on the road leading towards the understanding of vascular development.

Chapter 2: Endoderm is Required for Vascular Endothelial Tube Formation, but not for Angioblast Specification[2]

2.1. INTRODUCTION

The primary network of blood vessels in the embryo is formed by the process of vasculogenesis, which is defined as the de novo formation of blood vessels by the aggregation of individual angioblasts. At a fundamental level, the first step in vasculogenesis involves the specification of endothelial cell precursors (angioblasts) from mesoderm. Subsequently, the angioblasts proliferate and coalesce into cords that then form continuous strands of endothelial cells. These cells then form tubular vascular structures. The process of tube formation is initiated when a 'slit-like' space opens up between two angioblasts. These spaces enlarge, combining with other such spaces to form a hollow endothelial tube (Houser *et al.*, 1961). At least in some cases, lumen formation precedes the formation of a continuous endothelial network (Hirakow and Hiruma, 1983; Drake and Jacobson, 1988; reviewed in Risau and Flamme, 1995; Wilting and Christ, 1996; Roman and Weinstein, 2000; Vokes and Krieg, 2001). Subsequent elaboration of the vascular network occurs via angiogenesis,

---

[2] This chapter has been previously published under the title "Endoderm is required for vascular endothelial tube formation, but not for angioblast specification" in the journal Development 129: 775-785 (2002). Reproduced with permission by the Company of Biologists Ltd. Plastic sections and electron microscopy were performed by Peggy McCuskey and Gina Zhang. Janet Heasman and Matt Kofron provided VegT cDNA samples, *Xenopus* bFGF was provided by David Kimelman. Tatiana Yatskievych assisted with the quail experiments.

which is the growth and extension of vessels from the pre-existing vascular network (for a recent review, see Carmeliet, 2000).

A number of signaling pathways are known to play regulatory roles during embryonic vasculogenesis. At the earliest stages of vascular development, the VEGF signaling pathway is essential for blood vessel formation (Shalaby *et al.*, 1995; Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). The VEGF ligand is bound by two high affinity receptors, VEGFR2 (Flk-1/KDR) and VEGFR1 (Flt-1), both of which belong to the tyrosine kinase receptor family. Flk-1 is expressed exclusively in vascular endothelial cells, and represents the earliest known specific marker of endothelial cells. In addition to its role as a mitogen, VEGF also acts as a chemoattractant for endothelial cells (Waltenberger *et al.*, 1994; Cleaver and Krieg, 1998; Ash and Overbeek, 2000), and is also involved in the correct assembly of endothelial cells into lumenated vessels (Drake *et al.*, 2000). Ablation of VEGF expression results in an almost complete block to vascular development (Carmeliet *et al.*, 1996, Ferrara *et al.*, 1996). On the other hand, expression of excess VEGF ligand in the embryo results in both hypervascularization and also formation of abnormally large vascular lumens (Drake and Little, 1995; Flamme *et al.*, 1995b; Cleaver *et al.*, 1997). Following the formation of the original vascular network, numerous other growth factor signaling pathways are involved in the subsequent remodeling and maturation of the vascular system (reviewed in Yancopoulos *et al.*, 2000).

In amniotes, the formation of primary vascular networks occurs in two distinct regions. Extraembryonic vasculogenesis is observed in the yolk sac blood islands, while intraembryonic vasculogenesis occurs within the developing embryo itself. Classical embryological experiments have demonstrated that formation of the two vascular systems is not developmentally linked since assembly of the intraembryonic vascular network is completely independent of extraembryonic vasculogenesis (Hahn, 1909; Miller and McWhorter; 1914; Reagan, 1915). On the other hand, in organisms such as teleosts (bony fishes) and amphibians, all vasculogenesis occurs intraembryonically (Stockard, 1915b). A major difference between extraembryonic angioblasts and intraembryonic angioblasts lies in their organization. Extraembryonic angioblasts originate in blood islands, containing an outer layer of endothelial cells and an inner layer of red blood cells. In contrast, intraembryonic endothelial precursors are almost always first observed as solitary angioblasts (Risau, 1995) and these can arise in any mesodermal tissue in the embryo with the exception of the prechordal mesoderm (Noden, 1989; Wilms *et al.*, 1991; Wilting *et al.*, 1995). Only in certain specific, rare, instances are these intraembryonic angioblasts closely associated with blood cells (Cormier and Dieterlen-Lièvre, 1988, Olah, *et al.*, 1988; Jaffredo *et al.*, 1998; Ciau-Uitz *et al.*, 2000). Based on the remarkable ability of diverse mesodermal tissues to form angioblasts, it appears that the tissue environment in and around a specific region of mesoderm is responsible for regulating vascular endothelial cell specification and commitment (Noden, 1989; Pardanaud *et al.*, 1989; Pardanaud and Dieterlen-Lièvre, 1999; Cox and Poole,

2000). Although both intraembryonic and extraembryonic angioblasts are of mesodermal origin, the different environments in which they arise and the differences in the fate of associated cells raises the possibility that the two populations may be specified by different mechanisms.

At present, the precise origin of the embryonic angioblast lineage is uncertain. Numerous anatomical studies have shown that angioblasts in the extraembryonic blood islands, and also in the earliest intraembryonic blood vessels, arise in close proximity to endoderm (Mato *et al*., 1964; Gonzalez-Crussi, 1971; Mobbs and McMillan, 1979; Meier, 1980; Kessel and Fabian, 1985; Pardanaud *et al*., 1989). Based on these observations, Wilt (1965) proposed that direct interactions between the endoderm and mesoderm might be required for angioblast induction, and this possibility has been investigated in a number of different studies carried out using the avian embryo (Wilt, 1965; Miura and Wilt, 1969; Pardanaud *et al*., 1989; Pardanaud and Dieterlen-Lièvre, 1993). In chick tissue culture experiments, when specific portions of the area vasculosa that form the extraembryonic blood islands were separated into the mesectodermal and endodermal components, the mesectodermal component failed to generate detectable endothelial cell enclosed blood islands (Wilt, 1965). Endothelial cell differentiation could be restored if the mesectoderm was recombined with endoderm. This suggests that an endodermally derived inductive signal is necessary for extraembryonic endothelial cell formation, at least in the context of blood island formation. This result was corroborated in a subsequent study

(Miura and Wilt, 1969). While these studies implied that endoderm is required for blood island formation, in the absence of molecular markers it was not possible to identify individual angioblasts prior to blood vessel formation, and so the results are not necessarily conclusive.

In studies of intraembryonic vasculogenesis, Pardanaud *et al* (1989) also proposed that interactions between mesodermal and endodermal tissues are necessary for vasculogenesis. Once again, this proposal was based on the fact that vasculogenic mesoderm is always observed in the immediate vicinity of endoderm. This hypothesis was extended in a subsequent study showing that, when grafted onto chick limb buds, quail splanchnopleuric mesoderm (which is in contact with endoderm) generated greatly more endothelial cells than somatopleuric mesoderm (not in contact with endoderm). On the basis of this result, it was concluded that an endodermal factor is necessary to promote the emergence of endothelial cells (Pardanaud and Dieterlen-Lièvre, 1993). More recently, it has been argued that an indian hedgehog signal from the visceral endoderm is necessary for specifying endothelial cell fate in mouse embryos (Belaoussoff *et al.*, 1998; Dyer *et al.*, 2001). Overall these studies imply that interactions between endoderm and mesoderm are required for vascular endothelial cell specification. Notwithstanding a large number of assumptions and a relative paucity of experimental support, this relationship is routinely stated in the literature and has largely assumed the status of dogma (Wilt, 1965; Miura and Wilt, 1969; Gonzalez-Crussi, 1971; Augustine, 1981; Kessel and Fabian,

1985; Pardanaud *et al.*, 1989; Pardanaud and Dieterlen-Lièvre, 1993; Risau and Flamme, 1995; Sugi and Markwald, 1996; Belaoussoff *et al.*, 1998; Waldo and Kirby, 1998; Cleaver and Krieg, 1999; Roman and Weinstein, 2000; Dyer *et al.*, 2001; Poole *et al.*, 2001).

Despite the widespread acceptance of a role for endoderm in angioblast specification, a number of experiment results using several different organisms have called this conclusion into question (see Discussion). It is important to acknowledge however, that none of these studies had been designed to specifically address the requirement of endoderm for angioblast formation and so none of the studies were fully controlled. To formally address this question, we have used a combination of molecular and classical embryology techniques to examine the role of endodermal tissues during vasculogenesis. We find that large numbers of angioblasts are formed in frog embryos that contain no detectable endoderm. However, angioblasts in these endoderm depleted embryos fail to assemble into endothelial tubes. This observation was confirmed in complementary experiments using avian embryos. In summary, our studies indicate that endoderm is indeed important for vascular development, not for angioblast specification, but for the formation of tubular blood vessels.

2.2. THE ROLE OF ENDODERM IN VASCULAR SPECIFICATION

2.2.1. Angioblast formation after manual removal of endoderm

Using the frog embryo, we have carried out a series of experiments to test whether interactions between endodermal and mesodermal tissues are required for the formation of angioblasts. Our initial experiments used standard embryological techniques to physically remove the vast majority of endoderm from the gastrula stage *Xenopus* embryo. This dissection is closely modeled on methods previously described by Cooke (1989) and Nascone and Mercola (1995). Both of these studies showed that endoderm acts as a permissive signal that is essential for cardiac development. We used tungsten needles and hair loops to carefully remove all detectable endoderm from stage 10 embryos (Fig. 5A) and then allowed the manipulated embryos to develop until control embryos showed the presence of a beating heart (about stage 34). As expected, none of the endoderm-depleted embryos (0/24) showed the presence of beating cardiac tissue (data not shown) thereby indicating successful removal of endoderm (Cooke, 1989; Nascone and Mercola, 1995). Apart from a loss of a large proportion of the total tissue mass, endodermless embryos exhibited a generally normal overall body pattern, including segmented somites and morphologically intact notochords and neural tubes. As described by Cooke (1989), the ventral region of the embryos consisted primarily of "lateroventral mesoderm," although the precise nature of this tissue is uncertain. Endoderm depleted embryos, at the equivalent of stage 34, were assayed for the presence of angioblast cells by in situ hybridization using several distinct angioblast marker probes, including *X-msr*,

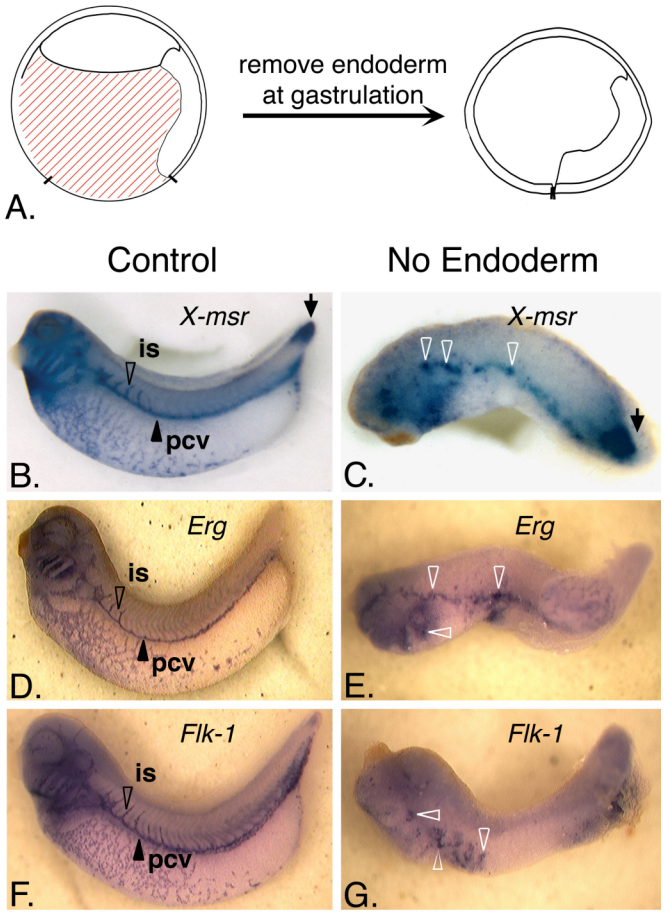

Figure 5. Endoderm is not necessary for *in vivo* angioblast specification.

(A) Schematic of the dissection used to remove endoderm. The vegetal core (red), comprising future endoderm, was removed from embryos at the onset of gastrulation, and the resulting endoderm depleted embryos were incubated until stage 34. (B, D, F) Control embryos assayed with *X-msr*, *erg* and *flk-1* probes respectively. These show elaborate vascularization, including posterior cardinal veins (pcv; closed arrowhead), intersomitic vessels (is; open arrowhead), and a ventrolateral vascular plexus. (C, E, G) Endoderm depleted embryos, assayed with *X-msr*, *erg* and *flk-1* probes respectively, contain angioblasts (white open arrowheads), but these are not organized into patent blood vessels.

flk-1 and *erg*. Using the in situ method, expression of these markers is first detected in developing vascular tissues at the late neurula stage (approx. stage 18) (Cleaver *et al.*, 1997; Baltzinger *et al.*, 1999). Surprisingly, all endoderm depleted embryos examined showed the presence of significant numbers of angioblasts. This was particularly evident in lateral regions of the embryos which showed strong expression of *X-msr* (14/14 embryos; Fig. 5C), *erg* (5/5 embryos; Fig. 5E) and *flk-1* (5/5 embryos; Fig. 5G). In order to confirm, and control, these in situ hybridization observations, endodermless embryos were assayed for vascular markers and a number of endodermal markers by RT-PCR analysis (Fig. 6). In this analysis, thepresence of angioblasts was assessed using *erg* and *flk-1*. We did not use *X-msr* in the RT-PCR assays because this gene is expressed in an additional, apparently non-endothelial, domain at the tip of the tail (Fig. 5B, C) that might confuse interpretation of the results. As shown in Fig. 6, RT-PCR analysis indicates that angioblast markers *flk-1* and *erg*, and the angioblast/hematopoietic cell marker, *SCL/tal-1* (Mead *et al.*, 1998) are expressed at significant levels in endoderm depleted embryos. To determine the efficiency with which endodermal cells were eliminated by physical dissection, RT-PCR analysis was carried out on RNA samples from the same manipulated embryos, using a number of different markers of endodermal tissue. This assay reveals that expression of the definitive endodermal markers *insulin*, *IFABP* and *xlhbox8*, is almost completely eliminated in manipulated embryos, indicating that removal of endoderm, although not complete, has been very effective. Another commonly

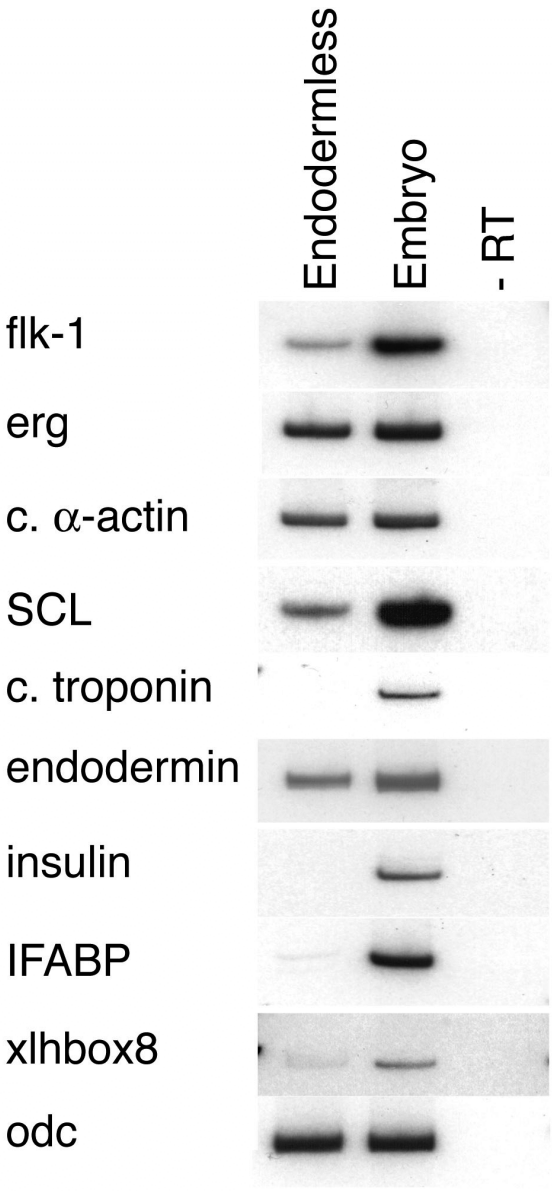

Figure 6. Endodermless embryos show a marked reduction in expression of endodermal markers but still express endothelial markers.

RT-PCR was performed on total RNA from a stage 34 endodermless embryo. Expression levels of the endodermal markers *insulin*, *IFABP* and *xlhbox8* are either severely reduced or eliminated relative to unmanipulated controls, while the vascular markers *flk-1* and *erg* and the angioblast/hematopoietic cell marker *SCL/tal-1*, are still present.

used endodermal marker, *endodermin*, is detected at quite high levels in endoderm-depleted embryos, but this is presumably because of its additional expression domain in the paraxial mesoderm, especially the notochord (Sasai *et al*., 1996 and data not shown). Note also that the general muscle marker, *cardiac α-actin* is expressed at normal levels in the manipulated embryos while, as expected, expression of the heart specific marker *cardiac troponin I* is undetectable (Fig. 6). We believe that the slightly reduced levels of angioblast markers in endoderm-depleted embryos may be due to reduced angioblast proliferation, because in normal embryos the endoderm expresses substantial amounts of VEGF (Cleaver *et al*., 1997), which is a potent mitogen for angioblasts (Keyt *et al*., 1996). Overall, these dissection experiments indicate that angioblasts are specified at significant levels in embryos from which endoderm has been greatly depleted or eliminated

2.2.2. Angioblast formation in embryos with reduced-VegT function.

VegT function is essential for endoderm formation in the frog embryo and has recently been shown to be the crucial initiating molecule underlying all endoderm specification (Xanthos, *et al*., 2001). Treatment of embryos with VegT antisense oligonucleotides results in abolition of all detectable endodermal tissue (Zhang *et al*., 1998) and, at higher doses can cause elimination of as much as 90% of mesodermal tissue (Kofron *et al*., 1999). To complement our studies in which endoderm was physically removed, we have assayed for angioblast formation in embryos treated with VegT antisense phosphorothioate oligonucleotides at levels

sufficient to eliminate endodermal tissue (5-8 ng per embryo). Since the antisense oligonucleotide acts prior to fertilization, optimally treated embryos should not contain endoderm at any stage of development. Analysis of treated embryos by RT-PCR shows severe reduction or elimination of expression of all endodermal markers tested, including endodermin, *IFABP*, *Xlhbox8* and *insulin* (Fig. 7). In this experiment, we believe that *endodermin* expression is completely absent because VegT depletion also results in down regulation of many mesodermal genes (Kofron *et al.*, 1999). Importantly however, the experimental embryos continue to show expression of the vascular-specific markers *flk-1 and erg*, and the angioblast/hematopoietic cell marker *SCL/tal-1*, although at somewhat reduced levels compared to wild type embryos. Complete rescue of embryos, by injection of *VegT* mRNA, restores *flk* and *erg* expression to normal levels. When antisense VegT-treated embryos are partially rescued by microinjection with *eFGF* mRNA, which restores ventrolateral mesodermal levels to wild-type while specifically excluding endoderm (Kofron *et al.*, 1999), expression of the vascular markers *flk-1* and *erg* is restored to wild type levels (Fig. 7). No expression of endodermal markers is detected in the *eFGF*-rescued embryos. Taken together, these experiments using VegT-depleted embryos strongly imply that formation of embryonic angioblasts is not dependent on the presence of endodermal tissue.

2.2.3. Angioblasts form in FGF-treated animal caps that contain no endoderm

The experiments described in the preceding sections do not formally preclude the possibility that very small amounts of endoderm are sufficient for the induction of angioblasts or, in the case of the embryonic dissection experiments,

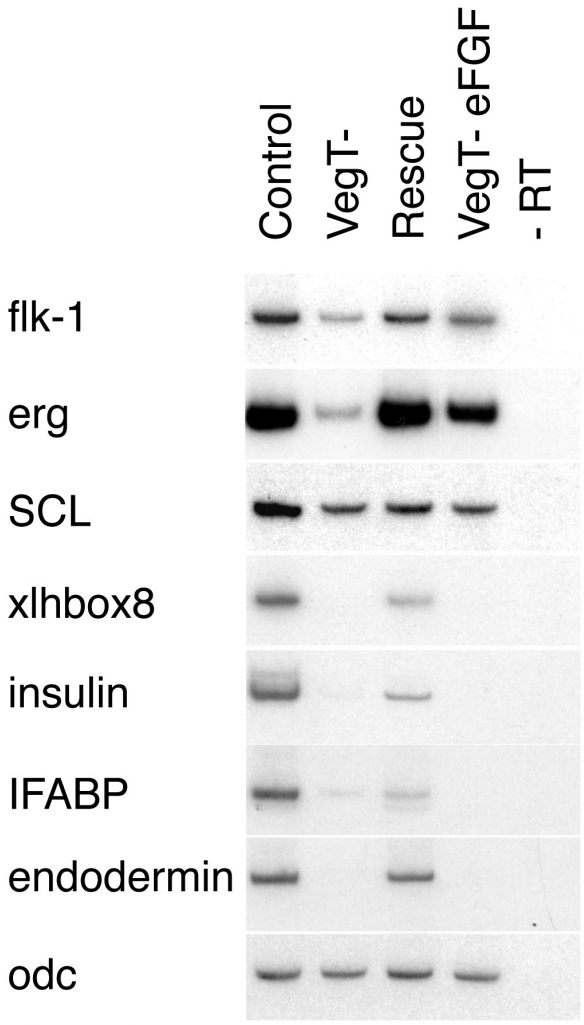

Figure 7. Embryos depleted of endoderm by treatment with VegT antisense oligonucleotides continue to express vascular markers.

RT-PCR analysis of RNA from stage 34 embryos shows a lack of endodermal markers in VegT treated embryos (labeled VegT-), while vascular markers are still present. Rescue by co-injection of VegT mRNA (labeled Rescue) restores both endodermal and mesodermal markers to control levels, whereas partial rescue with eFGF (labeled VegT-eFGF) restores mesodermal marker expression but has no effect on endodermal markers (Kofron *et al.*, 1999).

that a transient interaction of mesoderm and endoderm prior to stage 10.5, is adequate to specify the lineage. To address these two possibilities, we have employed animal cap techniques to generate mesodermal tissue that has never come into contact with endoderm. Specifically, we treated animal caps with basic fibroblast growth factor (bFGF) under conditions that generate mesoderm completely free of endoderm (see materials and methods; Cornell *et al.*, 1995; Gamer and Wright, 1995). At the equivalent of stage 30, the caps were assayed using RT-PCR for expression of a range of endothelial, mesodermal and endodermal tissue markers. Stage 30 was chosen because all endothelial markers are expressed at significant levels in wild type embryos at this time. As shown in Fig. 8A animal caps treated with 100ng/ml of bFGF express the endothelial markers *flk-1* and *erg*, and the angioblast/hematopoietic cell marker *SCL/tal-1*, as well as the general mesodermal marker *cardiac α-actin*. There is however, no detectable expression of the endodermal markers *endodermin*, *Xsox17-α, insulin*, *IFABP* and *xlhbox8*. While endodermin is detected in manually dissected endodermless embryos, probably due to its expression in paraxial mesoderm, it is not present in bFGF induced animal caps (Sasai *et al.*, 1996). This is most likely because bFGF does not induce the expression of genes representing more dorsal mesodermal tissues such as notochord (Green *et al.*, 1990). We also note that the early endodermal marker *mixer* is not present in stage 30 control embryos in agreement with its published expression pattern (Henry and Melton, 1998). To ensure that endodermal tissue was not transiently present soon after bFGF treatment, animal caps were also assayed for marker expression at the late

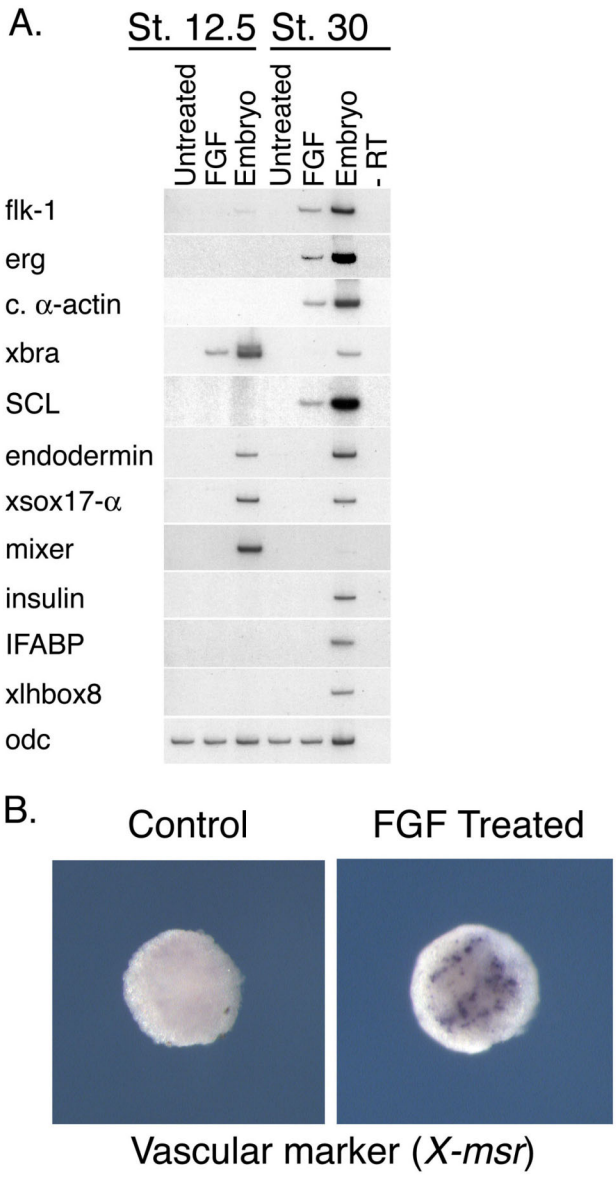

Figure 8. Animal caps treated with bFGF form mesoderm containing endothelial markers in the absence of detectable endoderm.

(A) Animal caps were incubated in bFGF and cultured until the appropriate stage (either 12.5 or 30). Caps were then assayed for early or late markers of endoderm and for endothelial markers using RT-PCR. While the animal caps show expression of both endothelial and mesodermal markers, there is no detectable expression of endodermal markers at either stage. Note that significant expression of endothelial markers is not expected in the stage 12.5 samples. (B) Stage 30 animal caps treated with bFGF express the vascular marker *X-msr* in discrete patches when assayed by in situ hybridization.

gastrula stage (Stage 12.5). Whereas treated caps express *Xbra*, indicating the presence of early mesodermal tissue, they do not express the early endodermal markers *endodermin*, *Xsox17-α* and *mixer*. The vascular markers *flk-1* and *erg* are also absent at this stage, in agreement with their known embryonic expression profiles (Cleaver *et al*., 1997; Baltzinger *et al*., 1999). In order to determine the distribution of endothelial cells in FGF treated caps, we examined the stage 30 animal caps for the presence of vascular markers by in situ hybridization. As shown in Fig. 8B, the vascular marker *X-msr* reveals the presence of individual angioblasts in treated caps, but not in untreated control caps.

2.3. ENDODERM IS REQUIRED FOR ENDOTHELIAL TUBULE ASSEMBLY

We have carried out three independent sets of experiments, physical dissection, VegT ablation and induction of mesoderm in animal caps, all of which suggest that angioblast specification is independent of interactions with endoderm. Does this imply that endoderm plays no role at all in the development of the embryonic vascular system? In fact, results presented below strongly support a role for endoderm during assembly of angioblasts into patent vascular tubes.

As described in the preceding section, embryos from which endoderm had been physically dissected at stage 10.5 showed the presence of an abundance of aggregated cords of angioblasts during later development (stage 34) (Figs. 5C, 1E

and 1G). In no case however, did we observe angioblasts assembling into the patent blood vessels visible in the control embryos. In order to ensure that this was not merely the consequence of a developmentally delayed phenotype, endoderm-depleted embryos were incubated until stage 37. At this stage, all embryos contained dark eye pigment and melanocytes, clear indications that they had developed past the stage when blood vessel tube formation would normally occur (about stage 34; Cleaver *et al.*, 1997). When these endoderm-depleted embryos were assayed by in situ hybridization for the vascular marker *X-msr*, angioblasts, but no endothelial tubes, were visible in wholemount embryos (Figs. 9B and 9C). In sectioned embryos, thick assemblages of angioblasts were visible in lateral regions of the embryo (Fig. 9F). However, despite the presence of large numbers of angioblasts, none of the endoderm-depleted embryos (0/21) contained any detectable vascular tubes. On the other hand, patent vessels were readily visible in all control embryos (15/15 examined; Fig. 9E).

To ensure that the absence of tube formation by angioblasts in endoderm depleted embryos was indeed due to the absence of endoderm rather than a dissection artifact, we carried out a rescue experiment. In this experiment, stage 10 embryos from which endoderm had been removed were implanted with a small amount of vegetal core tissue from a sibling embryo. We estimate that approximately 20% of the normal amount of endodermal tissue was restored to the embryo. In all cases (11/11), the rescued embryos exhibited much improved overall morphology and also a substantial degree of vascular assembly and tube

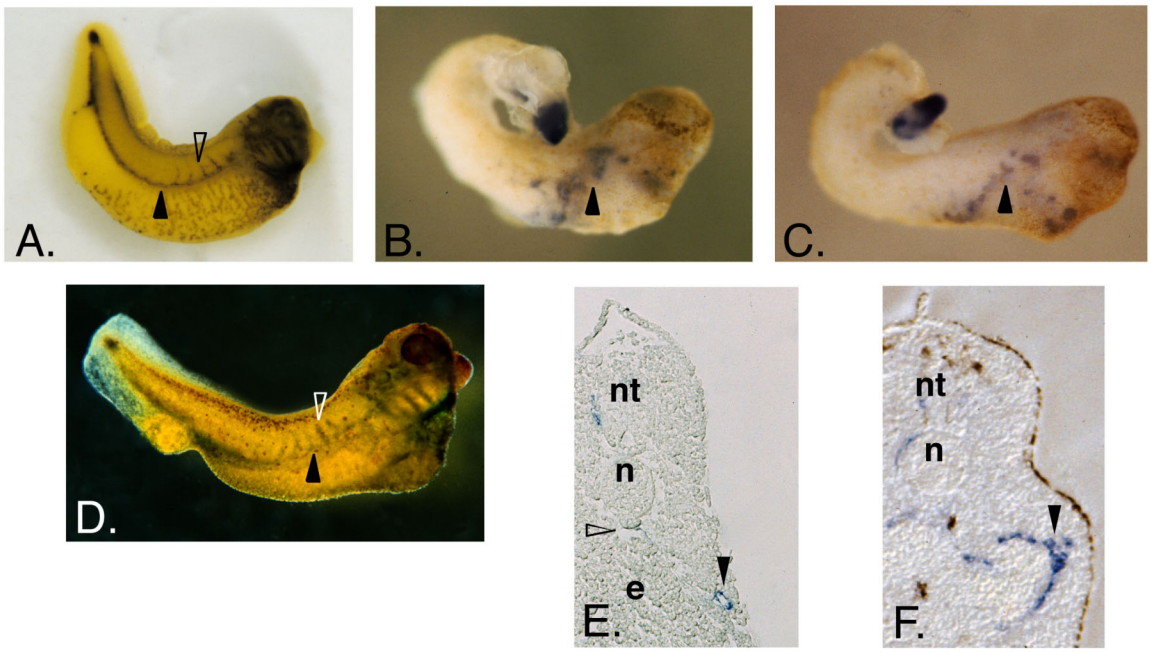

Figure 9. Endoderm is required for endothelial tube formation.

(A) Wild-type stage 37 embryo showing posterior cardinal vein (closed arrowhead), intersomitic vessels (open arrowhead) and a prominent vascular plexus. (B, C) Stage 37 embryos deprived of endoderm at stage 10 contain thick assemblages of angioblasts (closed arrowheads), but do not contain endothelial tubes. (D) Stage 37 embryo deprived of endoderm at stage 10 and rescued by the addition of a vegetal plug of endoderm from a sibling embryo. Note the presence of posterior cardinal veins (closed arrowhead) and intersomitic vessels (open arrowhead). (E) Cross-section through a wild-type stage 37 embryo showing posterior cardinal vein (closed arrowhead) and dorsal aorta (open arrowhead). (F) Cross-section through a stage 37 endodermless embryo showing presence of angioblasts (closed arrowhead) but no assembly into endothelial tubes. All embryos were assayed by in situ hybridization with the vascular marker *X-msr*. Abbreviations: e, endoderm; n, notochord; nt, neural tube.

formation. Phenotypes ranged from formation of a vascular plexus, generally restricted to the ventral region of the embryo, up to an almost complete vascular network that contained paired posterior cardinal veins and intersomitic vessels (Fig. 9D). The presence of patent blood vessel morphology was examined more closely in serial, plastic, semi-thin sections from additional endoderm depleted and rescued embryos. These embryos were not assayed by in situ hybridization because we find that the in situ procedure makes the embryos brittle and compromises histological quality, especially for delicate structures like blood vessels. In this experiment, only 2/11 endoderm depleted embryos showed any discernible endothelial tubes in any section along the length of the embryo (Fig. 10B) for a total of 55 sections examined. In contrast, 6/8 rescued embryos showed the clear presence of vascular tubes (Fig. 10C). These results are statistically significant ($P < 0.05$). Representative transverse sections through posterior cardinal veins from wild type and rescued embryos were also examined by electron microscopy (Fig. 10D and 10E respectively). Based on examination of numerous sections, endothelial tube structures in the rescued embryos were morphologically indistinguishable from those in wild-type embryos.

The original experiments of Wilt and Miura suggesting a role for endoderm in angioblast specification were carried out using avian embryos (Wilt, 1965; Miura and Wilt, 1969). These experiments clearly indicated an absence of endothelium enclosed blood islands in endoderm depleted embryos but, without the aid of molecular markers, it was not possible to determine whether angioblasts

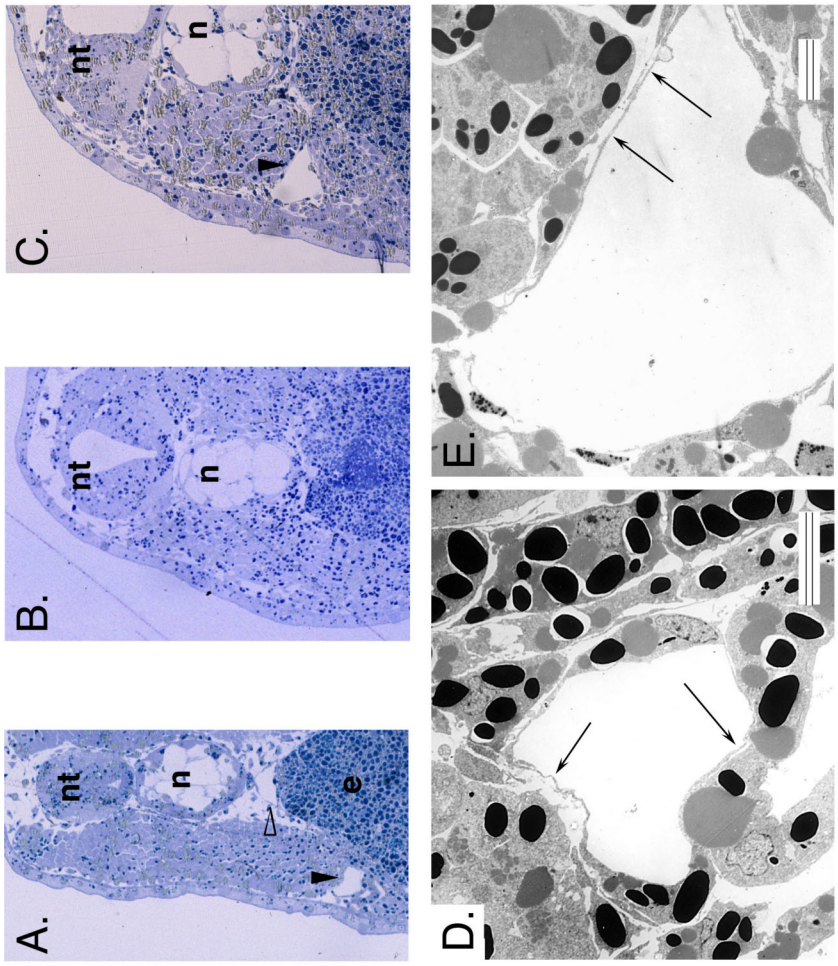

Figure 10. Embryos without endoderm lack patent blood vessels.

(A-C) 1μm plastic sections stained with toluidene blue. (A) Cross-section through a wild-type stage 37 embryo showing endothelial tubes, including a posterior cardinal vein (closed arrowhead) and dorsal aorta (open arrowhead). (B) Endothelial tubes are not present in stage 37 endodermless embryo but are present in stage 37 embryos that have been rescued by the addition of endoderm (C). (D, E) Transmission electron microscopy showing transverse sections through the posterior cardinal veins of a wild-type embryo (D) and an endodermless embryo rescued by the addition of endoderm from a sibling donor embryo (E). Arrows indicate the characteristic thin-walled endothelial cell morphology in each section. Scale bar equals 1 μm. Black dots in sections are lipid droplets generated during histological preparation. Abbreviations: e, endoderm; n, notochord; nt, neural tube.

were indeed present. To address this question, we have examined vascular development in endoderm depleted quail embryos using the angioblast marker, QH1 (Pardanaud *et al.*, 1987). For these experiments, both intra- and extraembryonic endoderm was removed from the left side of stage 5 embryos, with the unmanipulated right side serving as an internal control. Embryos were cultured for approximately 12 hours until they had approximately 6 somites (stage 9-). We assayed embryos at this stage, rather than later in development, to ensure that the vasculature was formed exclusively by vasculogenic mechanisms. Using the quail endothelial cell-specific antibody QH1, 8/8 embryos examined contained no discernible endothelial tubes on the side lacking endoderm, although all embryos had robust vascular development on the control side (Fig. 11A and 11B). This result is statistically significant ($P < 0.01$). Despite the absence of blood vessels, all embryos showed the presence of abundant QH1 positive cells on the endodermless side, indicating that angioblasts were still specified in the absence of endoderm. Basically these experiments in the quail embryo support the original observations of Wilt (1965) that endoderm is required for formation of organized endothelial structures. The underlying reason however, is not the absence of angioblasts, but the failure of these cells to assemble into patent blood vessels.

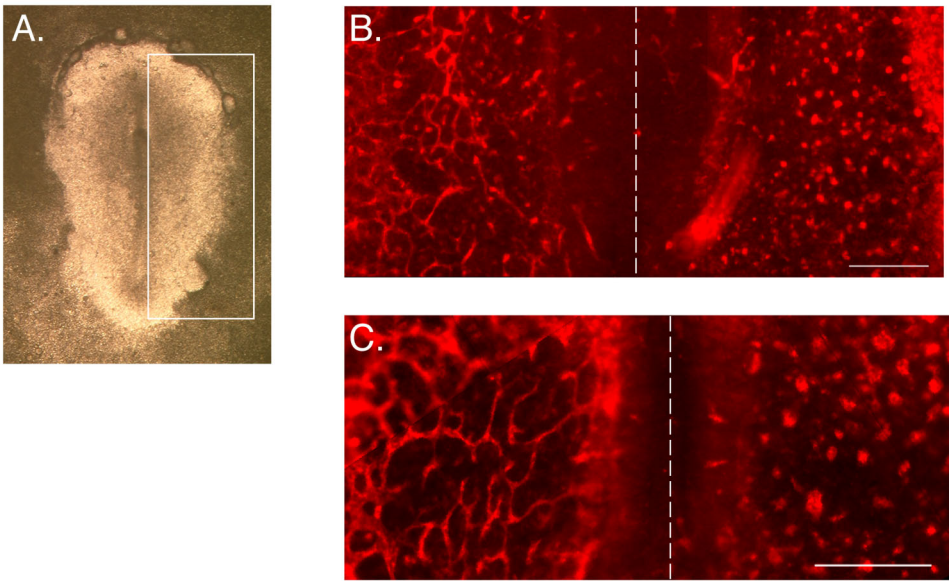

Figure 11. Removal of endoderm in quail embryos does not prevent angioblast formation.

(A). Ventral view of quail embryo showing approximate region from which endoderm was removed. (B, C) Ventral images of fluorescent staining of the endothelial cell marker, QH1 at approximately the four and six-somite stages respectively. The endoderm depleted side is on the right. Note the presence of endothelial cells within the manipulated region, but the absence of vascular assembly. The approximate midline of the embryo is indicated by the dashed line. Scale bar equals 10 μm.

2.4. DISCUSSION

2.4.1. Angioblast specification does not require endoderm

Based on three distinct experimental approaches in two different model systems, our results indicate that embryonic specification of angioblasts is independent of the presence of endoderm. Using the *Xenopus* embryo, consistent results are obtained when endoderm is removed by embryonic dissection, when endoderm is ablated using antisense VegT oligonucleotides and when mesodermal tissue is induced in animal caps in the complete absence of detectable endoderm. In the avian embryo, angioblasts still form when endoderm is physically removed. Overall, these results directly challenge the broadly cited proposition that interactions between mesoderm and endoderm are necessary to specify endothelial cells (Wilt, 1965; Miura and Wilt, 1969; Gonzalez-Crussi, 1971; Kessel and Fabian, 1985; Pardanaud *et al.*, 1989; Pardanaud and Dieterlen-Lièvre, 1993; Sugi and Markwald, 1996, Belaoussoff *et al.*, 1998; Dyer *et al.*, 2001, and stated in numerous reviews including Augustine, 1981; Risau and Flamme, 1995; Cleaver and Krieg, 1999; Roman and Weinstein, 2000; Poole *et al.*, 2001).

Although this study is amongst the first to use molecular markers to directly address the role of endoderm in angioblast specification, it is important to acknowledge that a number of previous studies, using different experimental systems, have hinted that endodermal-mesodermal interactions are not essential

for the formation of angioblasts. For example, it has been shown that mouse embryoid bodies lacking activity of the transcription factor GATA-4, fail to form extraembryonic endoderm. In the absence of endoderm, these embryoid bodies are unable to form endothelial cell enclosed blood islands. This observation is in apparent agreement with the endoderm induction model. However, use of specific markers indicated that vascular endothelial cells were still present in these embryoid body cultures (Bielinska *et al.*, 1996). Similar results were obtained embryologically by Palis *et al* (1995), who showed that murine yolk sac explants that contained extraembryonic mesoderm, but were separated from endoderm, still developed endothelial cells, but lacked organized blood vessels. In this experiment however, dissections were performed at E7.5. Since extraembryonic angioblasts are initially detected at E6.5 (Drake and Fleming, 2000), it is possible that angioblasts had already been specified prior to the separation of mesoderm from endoderm.

Further evidence that angioblasts form in the absence of endoderm is provided by a series of experiments using quail-chick heterochronic chimeras. In these experiments, quail blastoderm treated with cytochalasin B to block gastrulation was grafted to host limb buds. The presence of endothelial cells was then assessed using the antibody QH-1. Because limb buds do not contain endoderm, the presence of quail endothelial cells in these chimeras implied that the endodermal germ layer is not necessary for vascular cell specification (Christ *et al.*, 1991; von Kirschhofer *et al.*, 1994; Wilting and Christ, 1996). However,

interpretation of the limb bud experiments in the context of endothelial cell specification is difficult since they utilized an older, already specified population of mesoderm that contains a complex and specific set of growth factors involved in limb bud patterning.

Studies of zebrafish mutants that are deficient in endoderm formation also support our suggestion that endoderm is not necessary for vascular specification. For example, *one-eyed pinhead* (*oep*) mutants lack almost all endoderm (Schier *et al.*, 1997) but still contain abundant angioblasts (Brown *et al.*, 2000). In these mutants however, at least some endodermal tissue is still present and so the absolute requirement for endoderm in angioblast formation is difficult to ascertain.

Some recent molecular studies using mouse tissues would appear to directly contradict our conclusions. In particular, Belaoussof *et al* (1998) have suggested that an early signal from the visceral endoderm can respecify neurectoderm to a posterior mesodermal cell fate containing both endothelial and blood markers. It was concluded that a secreted signal from the visceral endoderm is needed to induce endothelial cell fate. Subsequent work has suggested that indian hedgehog (Ihh) is the secreted signaling factor (Dyer *et al.*, 2001). This result is challenged by gene ablation studies in mice which show that embryos lacking function of either *Ihh* or *Smoothened* (the receptor for all hedgehog proteins) still contain at least rudimentary endothelial tubes in the yolk

sac (Byrd *et al.*, in press). This result conclusively demonstrates that hedgehog signaling is not necessary for angioblast specification, at least in an *in vivo* context. The tissue recombination work (Belaoussof *et al.*, 1998) implying that visceral endoderm is required to induce endothelial cells, is a more complicated issue. However, we propose that the function of visceral endoderm in these experiments is in fact the induction of mesodermal tissue, since this is not present in the original explants. Once mesoderm is present, it is then capable of forming angioblasts, precisely as observed in our experiments. Alternatively, it is possible that the mechanism leading to specification of angioblasts in frog and avian embryos differs from that operating in the mammalian embryo.

2.4.2. Endoderm is required for endothelial tube formation

Our experiments show that angioblasts are indeed present in embryos containing no endoderm. However, these angioblasts fail to assemble into patent vascular tubes. Serial sectioning through endoderm depleted embryos shows that formation of tubular blood vessels is absent or severely reduced (Fig. 10B), although in situ hybridization indicates that angioblasts have assembled into dense, cord-like aggregations throughout the trunk of the embryo (Fig. 9B and 9C). These observations suggest that vasculogenesis in endoderm depleted embryos is interrupted at a step prior to tube formation. This view is supported by the rescue experiments in which endoderm from a donor embryo is implanted into the endoderm-depleted embryo. Despite the trauma caused by this rather crude

manipulation, the majority of rescued embryos show vascular tube formation. In the most effective cases, the rescued embryos showed clear organization of the posterior cardinal veins and intersomitic vessels. Variation in the amount of vascular structure observed in different rescued embryos is presumably due to differential healing, but we cannot exclude the possibility that pre-patterning of the endoderm has already occurred and therefore the degree of vascular rescue may be related to the orientation of the implanted endodermal tissue. In agreement with our results using *Xenopus*, we note that zebrafish *oep* mutants, which lack most endoderm, contain angioblasts but exhibit dramatic defects in axial vascular formation, and lack a functional circulatory system (Brown *et al.*, 2000), suggesting that endoderm is indeed required for vascular assembly. Likewise, murine extraembryonic mesoderm, when isolated from endoderm, forms endothelial cells that fail to assemble into vascular tubes (Palis *et al.*, 1995; Bielinska *et al.*, 1996).

The results of these experiments raise two fundamental questions relating to the mechanisms underlying vascular development. First, what is the molecular nature of the endodermal signal necessary for vascular tubulogenesis? This question will be examined extensively in the following chapter. The second question is related to the observation that endoderm is not involved in angioblast specification. This implies that any signal for angioblast specification arises within the mesoderm itself. The ectodermal germ layer, the only other theoretically possible source of inductive signals, is not likely to contribute to

vasculogenesis because it has been shown to profoundly inhibit vasculogenesis (Feinberg *et al*., 1983; Wilson *et al*., 1989; Pardanaud and Dieterlen-Lièvre, 1993; 1999). While our results suggest that the origin of the angioblast specification signal is likely to be exclusively mesodermal, the molecular nature of the signal is completely unknown. Because almost all mesoderm has the potential to express angioblasts (Noden, 1989), it is possible that angioblast specification occurs by an inherent patterning mechanism, perhaps analogous to the Delta/Notch signaling pathway responsible for neuroblast specification in *Drosophila*. Inhibitory signals from ectodermal tissues may subsequently help to determine the boundaries of the vasculogenic network.

Chapter 3: Sonic Hedgehog signaling from the endoderm is essential for the formation of endothelial tubes during vasculogenesis[3]

3.1. INTRODUCTION

Growth factors are known to be essential for proliferation of angioblasts and for the maturation of early endothelial tubes. For example, signaling by vascular endothelial growth factor A (hereafter VEGF) through its receptor VEGFR-2 (Flk-1/KDR) is essential for the formation of blood vessels, and embryos lacking either of these molecules have few (or no) angioblasts and die early in development (Shalaby *et al.*, 1995; Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). Within the embryo, initial formation of endothelial tubes always occurs in mesoderm that is tightly juxtaposed against endoderm, and signals from the endoderm are essential for the assembly of angioblasts into a functional vascular network (Vokes and Krieg, 2002a). Although the morphogenesis of endothelial tube formation has been described in some detail, little is known about the molecules that underlie this process (Houser *et al.*, 1961; Gonzalez-Crussi, 1971; Hirakow and Hiruma, 1983; Drake *et al.*, 1997).

In this study, we demonstrate the molecular identity of this inductive signal, showing that endodermally derived Sonic hedgehog is both necessary and sufficient for vascular tube formation in avian embryos. This demonstrates a

[3] Tatiana Yatskievych assisted with the embryonic manipulations, and contributed the artwork for Figures 14A,B. Parker Antin sectioned Figures 14K,I,and M.

novel role for hedgehog signaling in vascular development and provides a molecular model for vascular tube formation.

3.2. HEDGEHOG SIGNALING COMPONENTS ARE EXPRESSED IN THE ENDODERM AND ADJACENT MESODERM

In order to determine the molecular identity of the endoderm-derived signal we have used the avian embryo as a model system, first because of the ease with which the endoderm layer can be removed, and second because an endothelial cell marker antibody, QH1, is available (Pardanaud *et al.*, 1987). Our initial experiments were designed to determine the time at which endoderm signaling is required for vascular assembly. Endoderm was removed from 3–somite quail embryos, shortly before the first blood vessels form (Coffin and Poole, 1988). When these embryos were assayed for vascular tube formation at the 8-somite stage, no vascular tubes were present, although abundant unassembled angioblasts were present (12/12 embryos; Fig. 12A). Therefore, we conclude that the endodermal signal is required immediately prior to, or during, vascular tube formation.

We next examined the expression of candidate growth factors in both the mesodermal and endodermal layers of 5-somite chick embryos by RT-PCR. Of the growth factors examined, only *Sonic hedgeh*og (*Shh*) was present in the endoderm and absent from the mesoderm (Fig. 12B and data not shown). *VEGF* was also detected in the endoderm, but was present in the mesoderm in higher concentrations, and this mesodermal expression was not disrupted by the removal

of endoderm (Fig. 12B and data not shown). Because *Shh* has not previously been reported in the endoderm of such early embryos, we examined its pattern of expression by in situ hybridization. *Shh* is initially detected in low levels in the lateral endoderm of 2 and 3-somite embryos (Fig. 12C). At 4-somites, this expression domain expands to include almost all embryonic endoderm, and the level of staining is greatly intensified, a pattern that persists throughout early development (Figs. 12D,E). Additional in situ assays showed that the hedgehog signaling components *patched 1* (*ptc1*), *patched 2* (*ptc2*) and *smoothened* (*smo*) are expressed in numerous mesodermal tissues, including angioblasts (Figs. 12F-J). This is the first report of expression of hedgehog signaling molecules in the developing vasculature, although at least one previous study shows their presence in retinal angiogenesis in adult mice (Pola *et al.*, 2001).

3.3. HEDGEHOG SIGNALING IS NECESSARY FOR VASCULAR ASSEMBLY

To determine whether hedgehog signaling plays a role during embryonic vasculogenesis, embryos were treated with cyclopamine from the 2-somite stage until approximately 8-somites, by which time the majority of vessels have formed a lumen (Hirakow and Hiruma, 1983). Cyclopamine is a specific inhibitor of hedgehog biosynthesis, and is equally effective at blocking signaling by all members of the hedgehog family (Incardona *et al.*, 1998; Taipale *et al.*, 2000). Analysis of blood vessel formation by QH1 immunofluorescence showed that all embryos treated with 100μM cyclopamine (13/13) exhibited vascular abnormalities. These ranged from the presence of small, interrupted tubes, with a corresponding increase in unassembled clusters of angioblasts, to instances where

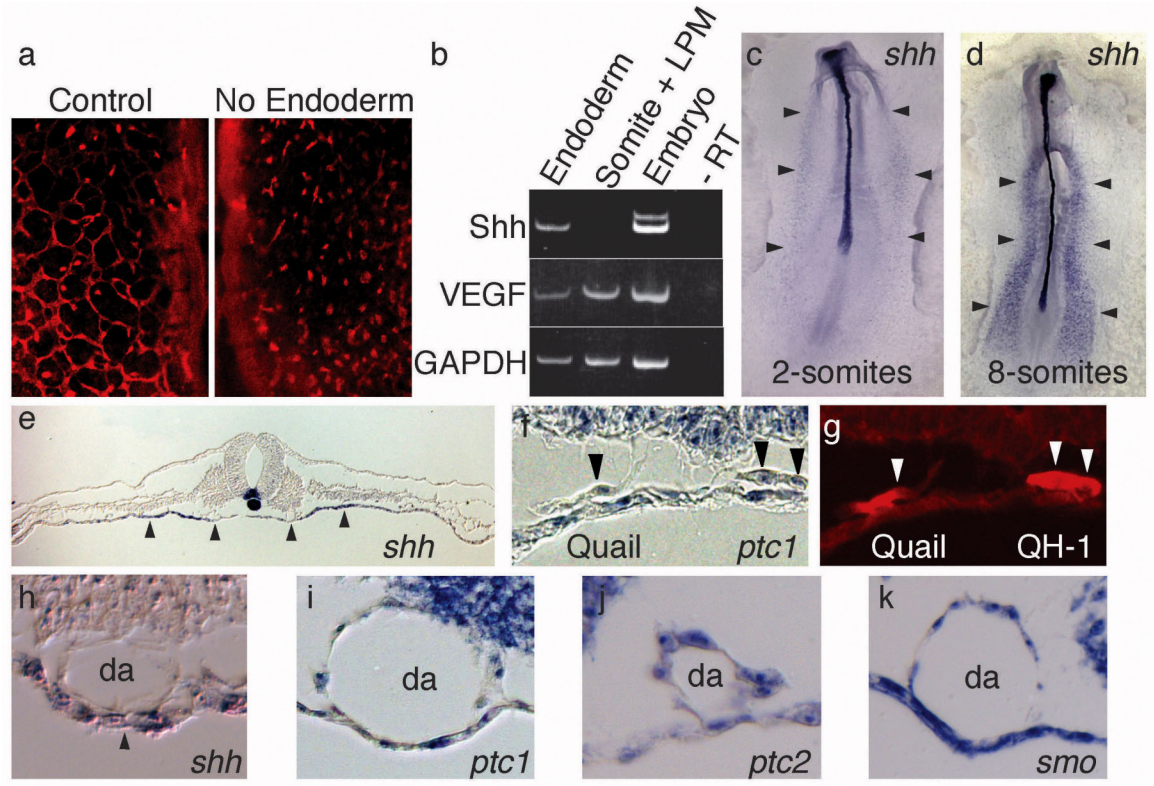

Figure 12. Expression patterns of hedgehog signaling compnonents.

(A) Endoderm was removed from one half of 3-somite embryos with the other half serving as a control. This results in failure of vascular assembly. (B) RT-PCR on 5-somite chick showing presence of *Shh* in the endoderm and absence in non-axial mesoderm. VEGF is present in both the mesoderm and endoderm. *Shh* in situ hybridizations on (C) 2-somite chick embryos showing low levels of endodermal expression and (D) 8-somite chick embryos showing greatly increased staining. (E,) Transverse section showing through 8-somite chick embryo showing *shh* expression in the endoderm. (F,G) In situ hybridization on a 6-somite quail embryo with *ptc1*(bright field) and QH-1 (fluorescence) showing that *ptc1* is present in angioblasts prior to tube formation. (H-K) Transverse sectionw through 7-somite chick embryos. Note the expression of *Shh* in the endoderm (H, filled arrowhead), but not in the dorsal aorta (da). (I-K) The hedgehog receptors *Patched 1* (*ptc1*) and *Patched 2* (*ptc2*) are present in endothelial cells of 7-somite embryos, as is *smoothened* (*smo*).

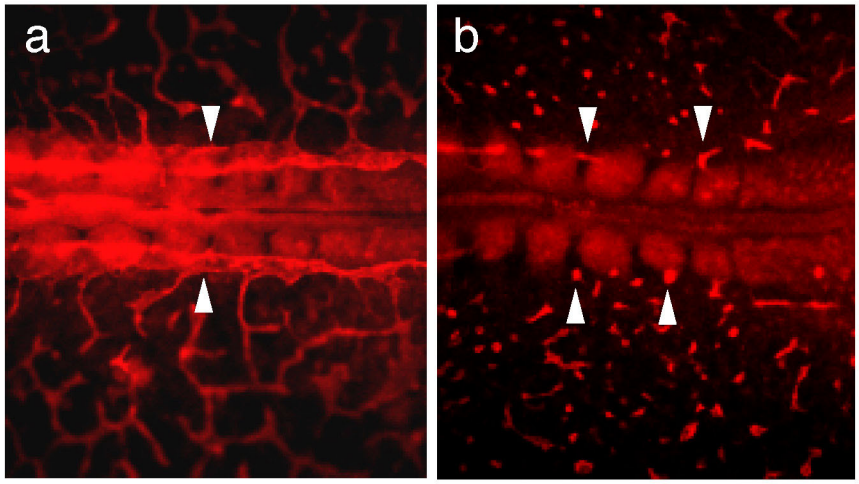

Figure 13. Hedgehog signaling is essential for vascular assembly.

Quail embryos treated with the hedgehog inhibitor cyclopamine (B) have severe deficiencies in vascular assembly. Note the lack of dorsal aortae formation (arrows) and almost complete lack of vascular assembly when compared with embryos treated with control media (A).

virtually no discernible patent vessels were detected (Fig. 13B). In the latter case, angioblasts remained abundant and were located where blood vessel formation would normally occur. No significant vascular defects were observed in control embryos treated with carrier solution alone (Fig. 13A). These experiments demonstrate that interference with hedgehog signaling, using the specific inhibitor cyclopamine, prevents angioblasts from undergoing normal vascular assembly and tube formation.

3.4. SHH SIGNALING RESCUES TUBE FORMATION IN THE ABSENCE OF ENDODERM

Is Shh signaling sufficient to rescue tube formation in the absence of endoderm? When beads carrying Shh (3μg/μl) were added to quail embryos from which the endoderm had been removed, well-formed vascular tubes were produced in the majority of cases (11/15). These tubes were typically linear vessels that formed in close proximity to the surface of the bead (Fig. 14H). Transverse sections through these embryos indicated that these vessels contained patent tubes (Fig. 14I). This effect was not seen in endodermless embryos with control beads (1/12), which lacked vascular assembly (Fig. 14E). Moreover, the punctate clusters of angioblasts present in these embryos did not form tubular structures (Figs. 14F,G). The effect of Shh on vascular assembly is highly specific since no appreciable vascular tube formation was observed when beads carrying BMP4, Activin, FGF-2, or TGFβ1 were added to endodermless embryos (data not shown). One potential explanation for our observations is that Sonic hedgehog causes increased endothelial cell proliferation, which may be a requirement of efficient tube formation. However, two independent lines of

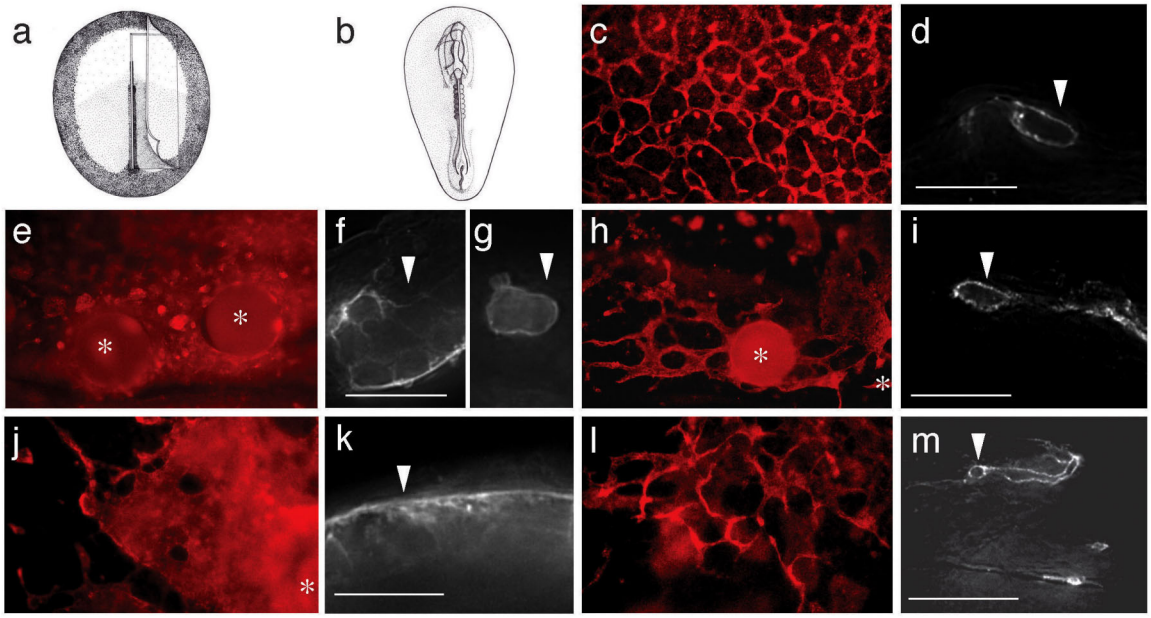

Figure 14. Sonic hedgehog signaling is sufficient to rescue vascular assembly in the absence of endoderm.

Endoderm was removed from one half of HH stage 5 quail embryos with the unmanipulated half serving as an internal control (A). The embryos were then assayed at 7-8 somites (B). The control side of embryos contains a robust vascular plexus with patent tubes (C,D). In contrast, the side lacking endoderm (asterisks indicate control beads) contains unassembled clusters of angioblasts (E-G). Beads containing Shh rescue vascular assembly (H,I). VEGF causes massive endothelial proliferation, but is not able to rescue vascular assembly (J,K). When Shh and VEGF beads (not in field of vision) are both added, a vascular plexus is formed (L,M). Note the intersection of two blood vessels in M. Scale bar, 15μm.

evidence suggest that this is not the case. First, the potent vascular mitogen VEGF is not sufficient to bring about vascular tube formation, in endodermless embryos, at any dose tested. Instead, VEGF treatment causes angioblasts to assemble into broad sheets of QH1 positive tissue in a dosage dependent manner. These sheets were never observed to form vascular tubes or organize into a vascular network in either whole embryos or sections (0/32 embryos assayed at VEGF concentrations of1ng/μl, 10ng/μl and 100ng/μl) (Figs. 14J,K). Second, hedgehog signaling does not cause proliferation of endothelial cells in culture (Pola *et al.*, 2001).

Because the endoderm contains a significant amount of VEGF (Fig. 12B) we sought to determine whether the addition of VEGF potentiated the effect of Shh on tube formation. When a combination of Shh and VEGF beads was added to endodermless embryos, a robust vascular plexus was generated (6/7 embryos). In overall appearance, the blood vessels formed in response to combined Shh/VEGF signaling appeared similar to a wild-type vascular network (Compare Figs. 14 L,M with Figs. 14C,D), containing many more blood vessels than the Shh beads alone. Based on these results, we propose that the hedgehog signaling pathway instructs angioblasts to form endothelial tubes and that it operates in parallel with VEGF (Fig. 15). In this model, VEGF is essential for the proliferation of normal numbers of angioblasts and apparently for their aggregation. However, our results demonstrate that VEGF by itself is not capable of mediating tube formation. While Shh still causes tube formation in embryos

lacking endoderm (and therefore containing a reduced dose of VEGF), the addition of exogenous VEGF and Shh causes an increase in the number of available endothelial cells, and therefore an increase in the total number of vessels that can be made. If the amount of VEGF is too low, the number of specified angioblasts would be insufficient to form vessels even in the presence of Shh. This scenario is supported by a recent study demonstrating that VEGF from the visceral endoderm is absolutely required for yolk sac vasculogenesis (Damert *et al.*, 2000). Sonic hedgehog has also been implicated as an indirect angiogenic factor in postnatal mice, achieving its effects through activation of the VEGF pathway (Pola *et al.*, 2001). However, our studies demonstrate that a simple epistatic relationship is unlikely during embryonic vasculogenesis and that both factors are required for assembly of vascular tubes (compare Figs. 14 J,K (VEGF alone) with Figs. 14 L,M (Shh/VEGF).

3.5. DISCUSSION

While this study is the first to demonstrate a specific requirement for hedgehog signaling in intraembryonic vascular assembly, several genetic studies have hinted that hedgehog signaling may be important for vascular development. For example, mouse embryos ectopically expressing Shh in the dorsal neural tube display hypervascularization (Rowitch *et al.*, 1999). Embryoid bodies derived from ES cells lacking the global hedgehog transducer, *smoothened,* initially express endothelial cells markers, however they are unable to form blood islands (Byrd *et al.*, 2002). In addition, zebrafish *Shh* mutants, such as *Sonic-you*, do not form vascular tubes in the trunk region of the embryo, although angioblasts are

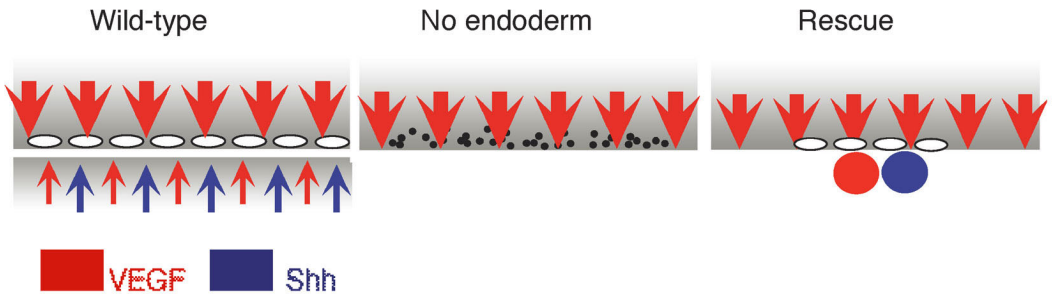

Figure 15. Schematic model for vascular assembly.

In wild-type embryos, high levels of VEGF (large red arrows) from mesoderm cause the proliferation of angioblasts within the tissue layer. In combination with Sonic hedgehog (Shh) from the endoderm (blue arrows), these angioblasts assemble into a vascular network with patent tubes. When endoderm is experimentally removed from embryos, angioblasts are still specified, but are unable to organize into a vascular network. The addition of Shh beads (blue circles) to endodermless embryos is sufficient to rescue vascular assembly. However, the addition of VEGF beads (red circles) with Shh beads causes a more complete vascular plexus, suggesting that smaller levels of VEGF in the endoderm (small red arrows) also play a role in vascular assembly.

still present (Brown *et al.*, 2000). Shh has also been shown to be an upstream factor mediating arterial identity in zebrafish (Lawon *et al.*, 2002).

At present, the mechanism by which Shh promotes vascular assembly is completely unknown. One hypothesis is that Shh mediates the expression of a cell adhesion molecule. Shh has been proposed to control cell segregation in the *Drosophila* wing imaginal disc by regulating a cell adhesion molecule (Dahmann and Basler, 2000). Alternatively, Shh signaling within dental epithelial cells was recently shown to be necessary for cell polarization (Gritli-Linde *et al.*, 2002). Shh could be effecting a similar role by causing angioblasts to become polarized, a necessary prerequisite to tube formation (Hogan and Kolodziej, 2002).

Chapter 4: Future Directions

4.1. INTRODUCTION

The preceding experiments indicate that contrary to what was previously thought, endoderm is not necessary for angioblast specification. However, endoderm does provide an inductive signal that instructs angioblasts to form tubes. Our data indicate that Sonic hedgehog (Shh) signaling represents the critical endodermal factor responsible for vascular tube formation. These results raise a number of interesting questions that are addressed below.

4.2. INTRA-MESODERMAL MECHANISMS OF ANGIOBLAST SPECIFICATION

Since endoderm does not induce angioblasts, what signal(s) is responsible for their formation? Our experiments suggest that specification of angioblasts must occur within the mesoderm itself. The mechanisms of this signal are currently unknown. The earliest known endothelial-specific gene, *flk-1*, first appears in the mesoderm shortly after the onset of somite formation. In mouse, the flk-1 promoter has been shown to contain GATA, ETS and SCL/TAL binding sites (Kappel *et al*., 1999). It has not been shown if these factors are sufficient to drive *flk-1* expression, and it appears likely that additional factors will also be required. Thus one clear set of experiments that would help clarify endothelial specification is to more thoroughly characterize the *flk-1* promoter. Recently, *Xenopus* genomic sequences from the proximal *flk-1* promoter and first intron have been shown to be sufficient to drive endothelial-specific expression of GFP

in *Xenopus* embryos (Paul Krieg, personal communication). With this tool, it is now possible to closely study the regulation of this promoter. While these experiments will give important information on how *flk-1* is regulated, it is important to emphasize that the precise role of flk-1 in endothelial specification is unknown. Furthermore, other molecules are also likely to be involved in specifying angioblasts. Thus, obtaining a comprehensive picture of how angioblasts are specified is clearly a complex question that will require an extensive amount of research.

4.3. WHICH TRANSCRIPTION FACTORS ARE MEDIATING HEDGEHOG SIGNALING IN THE DEVELOPING VASCULATURE?

Another set of questions from these results concern the mechanism by which Shh instructs angioblasts to form tubes. Depending on its cellular context, hedgehog signaling has been shown to perform a very diverse set of biological functions. As a first step towards understanding this process, it will be necessary to learn which transcription factors are mediating hedgehog signaling in the developing vasculature. Hedgehog signaling is largely mediated through the Ci/Gli family of transcription factors, consisting of one Ci protein in *Drosophila* and three homologous Gli transcription factors (Gli1-3) in vertebrates. To determine which of these genes are involved in mediating hedgehog signaling during vasculogenesis, it will be necessary to characterize the expression of these different genes in the developing vasculature of the chick or *Xenopus*. Determining the expression of these genes should be relatively straight-forward, as they are all cloned in both the chick and frog. The determination of which Gli

genes are expressed in endothelial cells is potentially important since different Gli genes as either transcriptional activators or repressors.

4.4. ARE EXPRESSION OF VASCULAR CELL ADHESION MOLECULES REGULATED BY HEDGEHOG SIGNALING?

An additional question concerning the mechanism of Shh signaling is what molecules are effecting the processes of vascular assembly. As mentioned previously, one possibility is that Shh is regulating the expression or activity of a cell adhesion molecule. One of the primary events of tubulogenesis at the cellular level, is the establishment of cell-cell junctions (Hogan and Kolodziej, 2002), the formation of which is largely mediated by cell adhesion molecules. Genetic studies of *Drosophila* reveal that at least one cell adhesion molecule (E-cadherin) is required for tracheal tubulogenesis (Lee and Kolodziej, 2002). We hypothesize that expression of endothelial cell adhesion molecules will be altered in the absence of Shh signaling. There is precedence for this hypothesis, as clonal populations of hedgehog expressing cells preferentially co-segregate in *Drosophila* abdominal segments (Lawrence *et al*., 1999). Furthermore, Hedgehog has been postulated to regulate a cell adhesion molecule that controls anterior-posterior compartment sorting in the Drosophila wing imaginal disc (Dahmann and Basler, 2000). To test this hypothesis, the expression patterns of VE-Cadherin and PECAM (another vascular cell adhesion molecule) sequences could be examined in manipulated embryos. Alteration of expression of any of these molecules when hedgehog signaling is perturbed would provide a plausible model to explain the lack of vascular tube formation.

4.5. DOES SHH EXPRESSION AT THE EMBRYONIC MIDLINE PLAY A ROLE IN DORSAL AORTA FORMATION?

The paired dorsal aortae are by far the largest vessels to assemble in the chick embryo. They are located immediately ventrolateral to the somites, on either side of the ventral midline. As shown in Figs. 12B-D, the highest areas of expression of Shh in the embryo are located in the notochord and floorplate of the neural tube. In Fig. 13, we show that blocking hedgehog activity using cyclopamine largely eliminates vascular assembly. However, if any remaining vessels are visible, they are always located adjacent to the somites, often consisting of fragments of a small dorsal aorta tube. Based on these observations, we hypothesize that Shh expression at the embryonic midline plays a role in mediating the size of the dorsal aorta. This question can be addressed experimentally with embryological approaches that utilize avian embryos to remove or displace the midline from direct contact with the adjacent somites.

Chapter 5: Materials and Methods

5.1. EMBRYOLOGICAL MANIPULATIONS.

Xenopus embryos were staged according to Nieuwkoop and Faber (1994). Animal caps were dissected from stage 8 embryos and cultured in 50% NAM containing 0.1% BSA and Penicillin-Streptomycin until sibling embryos were at stage 30. When applicable, caps were cultured in media containing 100 ng/ml *Xenopus* bFGF (a gift from David Kimelman) at 13°C overnight (until sibling embryos were at stage 12.5), and then transferred 50% NAM to generate a population of mesoderm completely devoid of endoderm. While activin treatment is a more routine method for generating mesodermal populations in animal caps and is effective in the induction of endothelial cell markers, it also induces the expression of endodermal genes (data not shown), making these experiments uninterpretable. Embryological manipulations were performed using electrolytically sharpened tungsten needles and hair loops in 75% NAM. Embryos were subsequently incubated in 50% NAM until the appropriate stage. Presumptive endoderm was removed from stage 10 embryos as described by Nascone and Mercola (1995). In the rescued endodermless embryos, endoderm was removed as above, and a small core of vegetal mass from a sibling embryo was inserted into the embryo. Embryos were then allowed to heal under glass bridges overnight.

Stage 5 quail embryos (unless specified) were placed on plastic rings and endoderm was removed from one half of the embryo using tungsten needles (Fig. 14A). No enzymatic treatment was used with stage 5 embryos, but older embryos were dissected in media containing 0.01% trypsin, which was subsequently inactivated with 0.02% trypsin inhibitor. Embryos were then incubated on New Cultures (New, 1955) at 37° until the appropriate stage (usually 7-8 somites) (Fig. 14B). When necessary, heparin acrylic beads (Sigma) were implanted immediately after endoderm removal. In these experiments, heparin beads were rinsed in PBS and soaked for one hour or more in the appropriate concentration of growth factor on ice. These beads were then briefly rinsed in PBS before being implanted in embryos. For hedgehog inhibition experiments, embryos at 1-2 somites were incubated as New cultures (New, 1955) immersed in DMEM containing 0.5% ethanol and 100μm cyclopamine (Toronto Research Chemicals Inc.) or DMEM containing 0.5% ethanol for controls and incubated at 37° in 95% oxygen until approximately 8 somites.

5.2. VEGT ANTISENSE-TREATED EMBRYOS.

cDNA from VegT antisense oligonucleotide treated embryos was generously provided by Matt Kofron and Janet Heasman. The samples, obtained following the host-transfer technique, are identical to those used in Kofron *et al.* (1999), and represent oocytes injected with 5-8 ng of phosphorothioate antisense VegT oligos and subsequently implanted into host females prior to fertilization. Embryos were harvested at stage 34 for RT-PCR analysis.

5.3. RT-PCR.

5.3.1. RT-PCR Conditions.

For experiments involving animal caps, approximately 8 animal caps were harvested for each sample. For experiments involving chick embryos, approximately 8 embryos worth of endoderm, or of lateral plate mesoderm and somites was collected. Total RNA was prepared using a standard SDS-Proteinase K method. cDNA samples were prepared from one-half of the total RNA (with the other have serving as a –RT control) and radioactive RT-PCRs (Chapter 2) were performed using 1/25th of the cDNA reaction as template and 0.3 μCi of [^{32}P]dATP in a 50μl reaction. The number of cycles for each primer was empirically determined so that they would be in the linear range of amplification. PCR samples were run on non-denaturing 5% acrylamide gels. In Chapter 3, the conditions were the same as those above, except that non-radioactive nucleotides were used in the reactions and the products were electrophoresed on 2% agarose gels.

5.3.2. *Xenopus* RT-PCR Primers

Cardiac α-actin (Niehrs *et al.*, 1994) (Tm = 63°).

Cardiac Troponin I:

Forward: 5'TCGGTCCTATGCCACAGAACCAC3',

Reverse: 5'TTTTGAACTTGCCACGGAGG3' (Tm = 63°).

Endodermin:

Forward: 5'GAGACTTGGCTTTGGGACCTTGTTG3',

Reverse: 5'CCATTTCCTGCGAGCACAGTAACC3' (Tm = 62°).

Erg (Detects both isoforms):

Forward: 5'CCTCAACAAGACTGGCTCTCACAG3',

Reverse: 5'TGCTCCACAAAGTAGGGTCAGC3' (Tm = 66°).

Flk-1:

Forward: 5'AAGAGGGAACAAGAATGAGGGC3',

Reverse: 5'TGCTGCTGCTGTGAAGAAACC3' (Tm = 64°).

IFABP: (Henry *et al.*, 1996) (Tm = 60°).

Insulin (Henry *et al.*, 1996) (Tm = 63°).

Mixer:

Forward: 5'GCTTTGTTCAGAATCCACCTACGC3',

Reverse: 5'AGTGATGGTCTTGTTGGGAGGG3' (Tm = 61°).

Ornithine Decarboxylase (ODC) (Bouwmeester *et al.*, 1996) (Tm = 64°).

SCL/tal-1:

Forward: 5'CCCAAATGAAAGGCAAACGG3',

Reverse: 5'CAGTTCTGTGGCTGGTGTCAAAG3' (Tm = 64°).

Xbra:

Forward: 5'GGAGTAATGAGTGCGACCGAGAGC3',

Reverse: 5'GCCACAAAGTCCAGCAGAACCG3' (Tm = 60°).

Xlhbox8:

Forward: 5'AAGGACAGTGGACAGATG3',

Reverse: 5'GGATGAAGTTGGCAGAGG3' (Tm = 65°).

Xsox17- α:

Forward: 5'TGCCAATAATGATGACTGGACTCG3',

Reverse: 5'TCTTCACCTGTTTCCTCCTGCG3' (Tm = 61°).

5.3.3. Chicken RT-PCR Primers.

GAPDH:

Forward: 5'CAGGTGCTGAGTATGTTGTGGAGTC3',

Reverse: 5'TCTTCTGTGTGGCTGTGATGGC3' (Tm=62°C).

Sonic Hedgehog (Shh):

Forward: 5'ATCTCGGTGATGAACCAGTGGC3',

Reverse: 5'TTTGACGGAGCAGTGGATGTGC3' (Tm=58°C).

VEGF (core sequence common to all isoforms):

Forward: 5'CAAATTCCTGGAAGTCTACGAACG3',

Reverse: 5'AATTCTTGCGATCTCCATCGTG3' (Tm=62°C).

5.4. IN SITU HYBRIDIZATION AND HISTOLOGY.

Digoxigenin-labeled RNA probe was transcribed using MEGAscript (Ambion). Chick, quail and *Xenopous* embros were assayed by in situ hybridization as previously described (Gerber *et al* 1999) and developed in either BM-Purple (Roche) or NBT-BCIP (Roche). In situ hybridizations to sections

(Chapter 3) were performed as described by Grapin-Botton *et al* (2001). Paraffin sections on embryos assayed by wholemount in situ hybridization (Chapter 2) were carried out by dehydrating the embryos in a graded ethanol series, washing twice for 10 minutes each in xylene, and then three times in paraplast at 60°C for a total of 2 hours. Embryos were then embedded in paraplast and sectioned at a thickness of 12 μm. Slides were dewaxed in xylene and viewed by DIC optics. For plastic sections, embryos were fixed in 1/2 strength Karnovsky's solution in 0.1 M cacodylate buffer, embedded in Spurr resin, post-fixed in 2% OsO_4, sectioned at a thickness of 1 μm and stained with toluidine blue (semi-thin histological sections) or 3μm (in situ hybridized sections). For electron microscopy imaging, thin sections (approximately 0.06 μm) were stained with uranyl acetate and lead citrate and imaged on a Philips CM12 transmission electron microscope.

5.5. IMMUNOHISTOCHEMISTRY.

Quail endothelial cells were detected with the QH1 monoclonal antibody (Pardanaud *et al.*, 1987; Developmental Studies Hybridoma Bank). The procedure was performed as described by Sugi and Markwald (1996) except that embryos were blocked in 5% Normal donkey serum and a donkey anti-mouse Texas Red-conjugated IgG secondary antibody (Jackson ImmunoResearch) was used at a 1:500 dilution. Immunostained sections were generated by embedding previously stained embryos in 30% gelatin and fixing overnight in 4% paraformaldehyde. Specimens were vibratome sectioned at a thickness of 40μm.

Sections were then imaged using deconvolution microscopy. To detect colocalized QH1 immunostaining and *ptc1* expression, in situ hybridization was performed on 10μm paraffin sections of 6-somite quail embryos, which were post-fixed prior to antibody staining using standard conditions.

References

Adams, R., Wilkinson, G., Weiss, C., Diella, F., Gale, N., Deutsch, U., Risau, W. and Klein, R. (1999). Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. *Genes. Dev.* **13**, 295-306.

Akiri, G., Nahari, D., Finkelstein, Y., Le, S., Elroy-Stein, O. and Levi, B. (1998). Regulation of vascular endothelial growth factor (VEGF) expression is mediated by internal initiation of translation and alternative initiation of transcription. *Oncogene* **17**, 227-236.

Ash, J. and Overbeek, P. (2000) . Lens-specific VEGF-A expression induces angioblast migration and proliferation and stimulates angiogenic remodeling. *Dev. Biol.* **223**, 383-398.

Augustine, J. (1981). Influence of the entoderm on mesodermal expansion in the area vasculosa of the chick. *J. Embryol. Exp. Morphol.* **65**, 89-103.

Ausprunk, D. and Folkman, J. (1977). Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumor angiogenesis. *Microvasc. Res.* **14**, 53-65.

Azar, Y. and Eyal-Giladi, H. (1979). Marginal zone cells-the primitive streak inducing component of the primary hypoblast in chick. *J. Embryol. Exp. Morph.* **52**, 79-88.

Bader, B., Rayburn, H., Crowley, D. and Hynes, R. (1998). Extensive vasculogenesis, angiogenesis, and organogenesis precede lethality in mice lacking all α_v integrins. *Cell* **95**, 507-519.

Baltzinger, M., Mager-Heckel, A. and Remy, P. (1999). Xl erg: Expression pattern and overexpression during development plead for a role in endothelial cell differentiation. *Dev. Dynamics* **216**, 420-433.

Barbera, J., Clements, M., Thomas, P., Rodriguez, T., Meloy, D., Kioussis, D. and Beddington, R. (2000). The homeobox gene Hex is required in definitive endodermal tissues for normal forebrain, liver and thyroid formation. *Development* **127**, 2433-2445.

Barton, K., Muthusamy, N., Fischer, C., Ting, C., Walunas, T., Lanier, L. and Leiden, J. (1998). The Ets-1 transcription factor is required for the development of natural killer cells in mice. *Immunity* **9**, 555-563**.**

Belaoussoff, M., Farrington, S. and Baron, M. (1998). Hematopoietic induction and respecification of the A-P identity by visceral endoderm signaling in the mouse embryo. *Development* **125**, 5009-5018.

Bielinska, M., Narita, N,. Heikinheimo, M., Porter, S. and Wilson, D. (1996). Erythropoiesis and vasculogenesis in embryoid bodies lacking visceral yolk sac endoderm. *Blood* **88**, 3720-3730.

Bouwmeester, T., Kim, S., Sasai, Y., Lu, B. and De Robertis, E. (1996). Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. *Nature* **382**, 595-601.

Breier, G., Breviario, F., Caveda, L., Berthier, R., Schnürch, H., Gotsch, U., Vestweber, D., Risau, W. and Dejana, E. (1996). Molecular cloning and expression of murine vascular endothelial-cadherin in early stage development of cardiovascular system. *Blood* **87**, 630-641.

Breier, G., Albrecht, U., Sterrer, S. and Risau, W. (1992). Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. *Development* **114**, 521-532.

Brooks, P., Clark, R. and Cheresh, D. (1994). Requirement for vascular integrin $\alpha_v\beta_3$ for angiogenesis. *Science* **264**, 569-571.

Brown, L., Rodaway,, A., Schilling,, T., Jowett, T., Ingham, P., Patient, R. and Sharrocks, A. (2000). Insights into early vasculogenesis revealed by expression of the ETS-domain transcription factor Fli-1 in wild-type and mutant zebrafish embryos. *Mech. Dev*. **90**, 237-252.

Burri, P. and Tarek, M. (1990). A novel mechanism of capillary growth in the rat pulmonary microcirculation. *Anat. Rec*. **228**, 35-45.

Byrd N, Becker S, Maye P, Narasimhaiah R, St-Jacques B, Zhang X, McMahon J, McMahon A. and Grabel, L. (2002). Hedgehog is required for murine yolk sac angiogenesis. *Development* **129**, 361-372.

Caduff, J., Fischer, L. and Burri, P.P. (1986). Scanning electron microscope study of the developing microvasculature in the postnatal rat lung. *Anat. Rec.* **216**, 154-164.

Carmeliet, P. (2000). Mechanisms of angiogenesis and arteriogenesis. *Nature Med.* **6**, 389-395.

Carmeliet, P., Ng, Y., Nuyens, D., Theilmeier, G., Brusselmans, K., Cornelissen, I., Ehler, E., Kakkar, V., Stalmans, I., Mattot, V., Perriard, J., Dewerchin, M., Flameng, W., Nagy, A., Lupu, F., Moons, L., Collen, D., D'Amore, P. and Shima, D. (1999a). Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. *Nat. Med.* **5**, 495-502 .

Carmeliet, P., Lampugnani, M., Moons, L., Breviario, F., Compernolle, V., Bono, F., Balconi, G., Spagnuolo, R., Oosthuyse, B., Dewerchin, M., Zanetti, A., Angellilo, A., Mattot, V., Nuyens, D., Lutgens, E., Clotman, F., de Ruiter, M., Gittenberger-de Groot, A., Poelmann, R., Lupu, F., Herbert, J., Collen, D. and Dejana, E. (1999b). Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell* **98**, 147-157.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kleckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C., Declercq, C., Pawling, J., Moons, L., Collen, D., Risau, W. and Nagy, A. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* **380**, 435-438.

Chapman, W. (1918). The effect of heart-beat upon the development of the vascular system of the chick. *Am. J. Anat.* **23**, 175-203.

Cheresh, D. (1987). Human endothelial cells synthesize and express an Arg-Gly-Asp-directed adhesion receptor involved in attachment of fibrinogen and von Willebrand factor. *Proc. Natl. Acad. Sci. USA* **84**, 6471-6475.

Choi, K., Kennedy, M., Kazarov, A., Papadimitriou, J. and Keller, G. (1998). A common precursor for hematopoietic and endothelial cells. *Development* **125**, 725-732.

Christ, B., Poelmann, R., Mentink, M. and Gittenberger-de Groot, A. (1990). Vascular endothelial cells migrate centripetally within embryonic arteries. *Anat. Embryol.* **181**, 333-339.

Christ, B., Grim, M., Wilting, J., von Kirschhofer, K. and Wachtler, F. (1991). Differentiation of endothelial cells in avian embryos does not depend on gastrulation. *Acta Histochem.* **91**, 193-199.

Ciau-Uitz, A., Walmsley M. and Patient, R. (2000). Distinct Origins of Adult and Embryonic Blood in *Xenopus*. *Cell* **102**, 787-796.

Clark, E. and Clark, E. (1939). Microscopic observations on the growth of blood capillaries in the living mammal. *Am. J. Anat.* **64**, 251-301.

Clauss, M., Weich, H., Breier, G., Kniest, U., Röckl, W., Waltenberger, J. and W. Risau (1996). The vascular endothelial growth factor receptor Flt-1 mediates biological activities. *J. Biol. Chem.* **271**, 17629-17634.

Cleaver, O. and Krieg, P.A. (1999). Molecular Mechanisms of Vascular Development. In: Heart Development (eds. Harvey, R. and Rosenthal, N.), pp. 221-252. San Diego, CA: Academic Press.

Cleaver, O. and Krieg, P.A. (1998). VEGF mediates angioblast migration during development of the dorsal aorta in *Xenopus*. *Development* **125**, 3905-3914.

Cleaver, O., Tonissen, K., Saha, M. and Krieg, P.A. (1997). Neovascularization of the *Xenopus* embryo. *Dev. Dynamics* **210**, 66-77.

Coffin, J. and Poole, T. (1988). Embryonic vascular development: immunohistochemical identification of the origin and subsequent morphogenesis of the major vessel primordia in quail embryos. *Development* **102**, 735-748.

Coffin, J., Harriison, J., Schwartz, S. and Heimark, R. (1991). Angioblast differentiation and morphogenesis of the vascular endothelium in the mouse embryo. *Dev. Biol.* **148**, 51-62.

Cooke, J. (1989). *Xenopus* mesoderm induction: evidence for early size control and partial autonomy for pattern development by onset of gastrulation. *Development* **106**, 519-529.

Cormier, F. and Dieterlen-Lièvre, F. (1988). The wall of the chick embryo aorta harbours M-CFC, G-CFC, GM-CFC and BFU-E. *Development* **102**, 279-285.

Cornell, R., Musci, T. and Kimelman, D. (1995). FGF is a prospective competence factor for early activin-type signals in *Xenopus* mesoderm induction. *Development* **121**, 2429-2437.

Cox, C . and Poole, T. (2000). Angioblast differentiation is influenced by the local environment: FGF-2 induces angioblasts and patterns vessel formation in the quail embryo. *Dev. Dynamics* **218**, 371-382.

Dahmann, C. and Basler, K. (2000). Opposing transcriptional outputs of hedgehog signaling and engrailed control compartmental cell sorting at the *Drosophila* A/P boundary. *Cell* **100**, 411-422.

Damert, A., Miquerol, L., Gertsenstein, M., Risau, W. and Nagy, A. (2002). Insufficient VEGFA activity in yolk sac endoderm compromises haematopoietic and endothelial differentiation. *Development* **129**, 1881-1892.

Dantschakoff, W. (1908). Untersuchungen über die entwickelung des blutes und bindegewebes bei den vögeln. 1. Die erste entstehung der blutzellen beim hühnerembryo und der dottersack als blutbildendes organ. Anatomische Hefte. *Erste Abteilung, Arbeiten Aus Anatomischen Instituten* **37**, 471-589.

Davis, S., Aldrich, T., Jones, P., Acheson, A., Compton, D., Jain, V., Ryan, T., Bruno, J., Radziejewsk, C., Maisonpierre, P. and Yancopoulos, G. (1996). Isolation of angiopoietin-1, a ligand for the TIE2 receptor by secretion-trap expression cloning. *Cell* **87**, 1161-1169.

Del Amo, F., Smith, D., Swiatek, P., Gendron-Maguire, M., Greenspan, ,R., McMahon, A. and Gridley, T. (1992). Expression pattern of Motch, a mouse homolog of *Drosophila* Notch, suggests an important role in early postimplantation mouse development. *Development* **115**, 737-744.

Dieterlen-Lièvre, F. and Martin, C. (1981). Diffuse intraembryonic hemopoiesis in normal and chimeric avian development. *Dev. Biol.* **88**, 180-191.

Drake, C., LaRue, A., Ferrara, N. and Little, C. (2000). VEGF regulates cell behavior during vasculogenesis. *Dev. Biol.* **224**, 178-188.

Drake, C. and Fleming, P. (2000). Vasculogenesis in the day 6.5 to 9.5 mouse embryo. *Blood* **95**, 1671-1679.

Drake, C. and Little, C. (1999). VEGF and vascular fusion: implications for normal and pathological vessels. *J. Histochem. Cytochem.* **47**, 1351-1355.

Drake, C. and Little, C. (1998). The morphogenesis of primordial vascular networks. In: Little C., Mironov V, Sage E. (eds) Vascular Morphogenesis. In vivo, in vitro, in mente. Boston, Birkhauser pp 3–21.

Drake, C., Brandt, S., Trusk, T. and Little, C. (1997). TAL1/SCL is expressed in endothelial progenitor cells/angioblasts and defines a dorsal-to-ventral gradient of vasculogenesis. *Dev. Biol.* **192**, 17-30.

Drake, C. and Little, C. (1995). Exogenous vascular endothelial growth factor induces malformed and hyperfused vessels during embryonic neovascularization. *Proc. Natl. Acad. Sci. USA* **92**, 7657-7661.

Drake, C., Cheresh, D. and Little, C. (1995). An antagonist of integrin $\alpha_v\beta_3$ prevents maturation of blood vessels during embryonic neovascularization. J. *Cell. Sci.* **108**, 2655-2661.

Drake, C. and Jacobson, A. (1988). A survey by scanning electron microscopy of the extracellular matrix and endothelial components of the primordial chick heart. *Anat. Rec.* **222**, 391-400.

Dumont, D., Fong, G., Puri, M., Gradwohl, G., Alitalo, K. and Breitman, M. (1995). Vascularization of the mouse embryo: a study of flk-1, tek, tie, and vascular endothelial growth factor expression during development. *Dev. Dynamics* **203**, 80-92.

Dumont, D., Gradwohl, G., Fong, G., Puri, M., Gertsenstein, M., Auerbach, A. and Breitman, M. (1994). Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. *Genes Dev.* **8**, 1897-1909.

Dyer, M.A., Farrington, S.M., Mohn, D., Munday, J.R. and Baron, M.H. (2001). Indian hedgehog activates hematopoiesis and vasculogenesis and

can respecify prospective neurectodermal cell fate in the mouse embryo. *Development* **128**, 1717-1730.

Eriksson, U. and Alitalo, K. (1999). Structure, expression and receptor-binding properties of novel vascular endothelial growth factors. In: Claesson-Welsh (ed) Vascular growth factors and angiogenesis. Springer-Verlag, Berlin Heidelberg New York pp 41-57.

Evans, H. (1909). On the development of the aortae, cardinal and umbilical veins, and the other blood vessels of vertebrate embryos from capillaries. *Anat. Rec.* **3**, 498-518.

Faloon, P., Arentson, E., Kazarov, A., Deng, C., Porcher, C., Orkin, S. and Choi, K. (2000). Basic fibroblast growth factor positively regulates hematopoietic development. *Development* **127**, 1931-1941.

Feinberg, R., Repo, M.A. and Saunders, J. (1983). Ectodermal control of the avascular zone of the peripheral mesoderm in the chick embryo. *J. Exp. Zool.* **226**, 391-398.

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K., Powell-Braxton, L, Hillan, K. and Moore, M. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* **380**, 439-442.

Flamme, I., Breier, G. and Risau, W. (1995a). Vascular endothelial growth factor (VEGF) and VEGF Receptor 2 (flk-1) are expressed during vasculogenesis and vascular differentiation in the quail embryo. *Dev.. Biol.* **169**, 699-712.

Flamme, I., von Reutern, M., Drexler, H., Syed-Ali, S. and Risau, W. (1995b). Overexpression of vascular endothelial growth factor in the avian embryo induces hypervascularization and increased vascular permeability without alterations of embryonic pattern formation. *Dev. Biol.* **171**, 399-414.

Folkman, J. and D'Amore, P. (1996). Blood vessel formation: what is the molecular basis? *Cell* **87**, 1151-1155.

Fong, G., Rossant, J., Gertsenstein, M. and Breitman, M. (1995). Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* **376**, 66-69.

Fong, G., Zhang, L., Bryce, D. and Peng, J. (1999). Increased hemangioblast commitment, not vascular disorganization, is the primary defect in flt-1 knock-out mice. *Development* **126**, 3015-3025.

Fouquet, B., Weinstein, B., Serluca, F. and Fishman, M. (1997). Vessel patterning in the embryo of the zebrafish: guidance by the notochord. *Dev. Biol.* **183**, 37-48.

Fuh, G., Garcia, K. and de Vos, A. (2000). The interaction of Neuropilin-1 with vascular endothelial growth factor and its receptor Flt-1. *J. Biol. Chem.* **275**, 26690-26695.

Gale, N., Holland, S., Valenzuela, D., Flenniken, A., Pan, L., Ryan, T., Henkemeyer, M., Strebhardt, K., Hirai, H., Wilkinson,, D., Pawson, T., Davis, S. and Yancopoulos, G. (1996). Eph receptors and ligands comprise two major specificity subclasses and are reciprocally compartmentalized during embryogenesis. *Neuron* **17**, 9-19.

Gamer, L. and Wright, C. (1995). Autonomous endodermal determination in *Xenopus*: regulation of expression of the pancreatic gene XlHbox 8. *Dev. Biol.* **171**, 240-251.

Gengrinovitch, S., Berman, B., David,, G., Witte,, L., Neufeld, G. and Ron, D. (1999). Glypican-1 is a VEGF165 binding proteoglycan that acts as an extracellular chaperone for VEGF165. *J. Biol. Chem.* **274**, 10816-10822.

Gerber, H., Hillan, K., Ryan, A., Kowalski, J., Keller, G., Rangell, L., Wright, B., Radtke, F., Aguet, M. and Ferrara, N. (1999). VEGF is required for growth and survival in neonatal mice. *Development* **126**, 1149-1159.

Gerber, W.V., Yatskievych, T., Antin, P., Correia, K., Conlon, R. and Krieg, P.A. (1999). The RNA-binding protein gene, *hermes*, is expressed at high levels in the developing heart. *Mech. Dev.* **80**, 77-86.

Gerety, S.S. and Anderson, D.J. (2002). Cardiovascular ephrinB2 function is essential for embryonic angiogenesis. *Development* **129**, 1397-1410.

Gering, M., Rodaway, A., Göttgens, B., Patient, R. and Green, A. (1998). The SCL gene specifies haemangioblast development from early mesoderm. *EMBO J.* **17**, 4029-4045.

Gilbert, S. (2000). Developmental Biology. 6th Edition. Sinauer Associates Inc., Sunderland.

Gluzman-Poltorak, Z., Cohen, T., Herzog, Y. and Neufeld, G. (2000). Neuropilin-2 and Neuropilin-1 are receptors for VEGF165 and PLGF-2, but only neuropilin-2 functions as a receptor for the 145-amino acid form of VEGF. *J. Biol. Chem.* **275**, 18040-18045.

Gonzalez-Crussi, F. (1971). Vasculogenesis in the chick embryo, an ultrastructural study. *Am. J. Anat.* **130**, 441-460.

Gory-Fauré, S., Prandini, M., Pointu, H., Roullot, V., Pignot-Paintrand, I., Vernet, M. and Huber, P. (1999). Role of vascular endothelial-cadherin in vascular morphogenesis. *Development* **126**, 2093-2102.

Goss, C. (1928). Experimental removal of the blood island of amblystoma punctatum embryos. *J. Exp. Zool.* **52**, 45-63.

Grapin-Botton, A. Majithia, A.R. and Melton, D.A. (2001). Key events of pancreas formation are triggered in gut endoderm by ectopic expression of pancreatic regulatory genes. *Genes. Dev.* **15**, 444-454.

Green, J., Howes, G., Symes,, K., Cooke, J. and Smith, J. (1990). The biological effects of XTC-MIF: quantitative comparison with *Xenopus* bFGF. *Development* **108**, 173-183.

Gritli-Linde, A., Bei, M., Maas, R., Zhang, X.M., Linde, A., and McMahon, A.P. (2002). Shh signaling within the dental epithelium is necessary for cell proliferation, growth and polarization. *Development* **In Press**.

Hahn, H. (1909). Experimentelle studien uber die entstehung des blutes und der ersten Gefabe beim huhnchen. *Archiv für Entwicklungsmechanik Der Organismen* **27**, 337-433.

Hamburger, V. and Hamilton, H. (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* **88**, 49-93.

He, Z. and Tessier-Lavigne, M. (1997). Neuropilin is a receptor for the axonal chemorepellent semaphorin III. *Cell* **90**, 739-751.

Helbling, P., Saulnier, D. and Brändli, A. (2000). The receptor tyrosine kinase EphB4 and ephrin-B ligands restrict angiogenic growth of embryonic veins in *Xenopus laevis*. *Development* **127**, 269-278.

Henry, G. and Melton, D.A. (1998). Mixer, a homeobox gene required for endoderm development. *Science* **281**, 91-96.

Henry, G., Brivanlou, I., Kessler, D., Hemmati-Brivanlou, A. and Melton, D.A. (1996). TGF-β signals and a prepattern in *Xenopus laevis* endodermal development. *Development* **122**, 1007-1015.

Hertig, A. (1935). Angiogenesis in the early human chorion and in the primary placenta of the macaque monkey. *Contrib. Embryol. Carnegie Inst.* **25**, 39-81.

Hirakow, R. and Hiruma, T. (1981). Scanning electron microscopic study on the development of primitive blood vessels in chick embryos at the early somite-stage. *Anat. Embryol.* **163**, 299-306.

Hirakow, R. and Hiruma, T. (1983). TEM-studies on development and canalization of the dorsal aorta in the chick embyro. *Anat. Embryol.* **166**, 307-315.

Hiraoka, N., Allen, E., Apel, I., Gyetko, M. and Weiss, S. (1998). Matrix metalloproteinases regulate neovascularization by acting as perricellular fibrinolysins. *Cell* **95**, 365-377.

Hiratsuka, S., Minowa, O., Kuno, J., Noda, T. and Shibuya, M. (1998). Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proc. Natl. Acad. Sci. USA* **95**, 9349-9354.

His, W. (1901). Lecithoblast und angioblast der wirbelthiere: histogenetische studien. *Abhandlungen Der Mathematisch-Phpysischen Classe Der Königlich Sächsischen Gesellschaft Der Wissenschaften* **14**, 171-328.

His, W. (1868). Untersuchungen über die erste anlage des wirbelthierleibes. Verlag von F.C.W. Vogel, Leipzig.

Hogan, B.L. & Kolodziej, P.A. (2001). Molecular mechanisms of tubulogensis. *Nat. Rev. Genet.* **3**, 513-523.

Holash, J., Wiegand, S. and Yancopoulos, G. (1999). New model of tumor angiogenesis: dynamic balance between vessel regression and growth mediated by angiopoietins and VEGF. *Oncogene* **18**, 5356-5362.

Holder, N. and Klein, R. (1999). Eph receptors and ephrins: effectors of morphogenesis. *Development* **126**, 2033-2044.

Holland, S., Gale, N., Mbamalu, G., Yancopoulos, G., Henkemeyer, M. and Pawson, T. (1996). Bidirectional signalling through the EPH-family receptor Nuk and its transmembrane ligands. *Nature* **383**, 722-725.

Houck, K., Ferrara, N., Winer, J., Cachianes, G., Li, B. and Leung, D. (1991). The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. *Mol. Endocrinol.* **5**, 1806-1814.

Houck, K., Leung, D., Rowland, A., Winer, J. and Ferrara, N. (1992). Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. *J. Biol. Chem.* **267**, 26031-**26037.**

Houser, J., Ackerman, A. and Knouff, R. (1961). Vasculogenesis and erythropoiesis in the living yolk sac of the chick embryo. *Anat. Rec.* **140**, 29-43.

Huez I., Creancier L., Audigier S., Gensac M. and Prats, H. (1998). Two independent internal ribosome entry sites are involved in translation initiation of vascular endothelial growth factor mRNA. *Mol Cell Biol.* **18**, 6178-6190

Hynes, R. and Bader, B. (1997). Targeted mutations in integrins and their ligands: their implications for vascular biology. *Thromb. Haem.* **78**, 83-87.

Ingham, P.W. and A.P. McMahon (2001). Hedgehog signaling in animal development: paradigms and principles. *Genes Dev.* **15**, 3059-3087.

Ikeda, E., Achen, M., Breier, G. and Risau, W. (1995). Hypoxia-induced transcriptional activation and increased mRNA stability of vascular endothelial growth factor in C6 glioma cells. *J. Biol. Chem.* **270**, 19761-19766.

Incardona, J.P., Gaffield, W., Kapur, R.P. and Roelink, H. (1998). The teratogenic Veratrum alkaloid cyclopamine inhibits Sonic hedgehog signal transduction. *Development* **125**, 3553-3562.

Jaffe, E., Hoyer, L. and Nachman, R. (1973). Synthesis of anti-hemophilic factor antigen by cultured human endothelial cells. *J. Clin. Invest.* **52**, 2757-2764.

Jaffredo, T., Gautier, R., Eichmann, A. and Dieterlen-Lièvre, F. (1998). Intraaortic hemopoietic cells are derived from endothelial cells during ontogeny. *Development* **125**, 4575-4583.

Kallianpur, A., Jordan, J. and Brandt, S. (1994). The SCL/TAL-1 gene is expressed in progenitors of both the hamatopoietic and vascular systems during embryogenesis. *Blood* **83**, 1200-1208.

Kappel, A., Ronicke, V., Damert, A., Flamme, I., Risau, W. and Breier, G. (1999). Identification of vascular endothelial growth factor (VEGF) receptor-2 (Flk-1) Promoter/Enhancer Sequences. *Blood* **93**, 4284-4292.

Kawasaki,T., Kitsukawa, T., Bekku, Y., Matsuda, Y., Sanbo, M., Yagi, T. and Fujisawa, H. (1999). A requirement for neuropilin-1 in embryonic vessel formation. *Development* **126**, 4895-4902.

Keck, P., Hauser, S., Krivi, G., Snazo, K., Warren, T., Feder, J. and Connolly, D. (1989). Vascular permeability factor, and endothelial cell mitogen related to PDGF. *Science* **246**, 1309-1312.

Kessel, J. and Fabian, B. (1985). Graded morphogenetic patterns during the development of the extraembryonic blood system and coelom of the chick blastoderm: a scanning electron microscope and light microscope study. *Am. J. Anat.* **173**, 99-112.

Keyt, B., Berleau, L., Nguyen, H., Chen, H., Heinsohn, H., Vandlen, R. and Ferrara, N. (1996). The carboxyl-terminal domain (111-165) of vascular endothelial growth factor is critical for its mitogenic potency. *J. Biol. Chem.* **271**, 7788-7795.

Kim, I., Kim, J., Ryu, Y., Jung, S., Nah, J. and Koh, G. (2000). Characterization and expression of a novel alternatively spliced human angiopoietin-2. *J. Biol. Chem.* **275**, 18550-18556.

Kim, I., Kwak, H., Ahn, J., So, J., Liu, M., Koh, K. and Koh, G. (1999a). Molecular cloning and characterization of a novel angiopoietin family protein, angiopoietin-3. *FEBS Lett*. **443**, 353-356.

Kim, I., Moon, S., Koh, K., Kim, H., Uhm, C., Kwak, H., Kim, N. and Koh, G. (1999b). Molecular cloning, expression, and characterization of angiopoietin-related protein. *J. Biol. Chem*. **274**, 26523-26528.

Knower, H. (1907). Effects of the early removal of the heart and arrest of the circulation on the development of frog embryos. *Anat. Rec*. **1**, 161-165.

Kofron, M., Demel, T., Xanthos, J., Lohr, J., Sun, B., Sive, H., Osada, S., Wright, C., Wylie, C. and Heasman, J. (1999). Mesoderm induction in *Xenopus* is a zygotic event regulated by maternal VegT via TGFβ growth factors. *Development* **126**, 5759-5770.

Krah, K., Mironov, V., Risau, W. and Flamme, I. (1994). Induction of vasculogenesis in quail blastodisc-derived embryoid bodies. *Dev. Biol*. **164**, 123-132.

Krebs, L., Xue, Y., Norton, C., Shutter, J., Maguire, M., Sundberg, J., Gallahan, D., Closson, V., Kitajewski, J., Callahan, R., Smith,, G., Stark, K. and Gridley, T. (2000). Notch signaling is essential for vascular morphogenesis in mice. *Genes Dev*. **14**, 1343-1352.

Labastie, M., Poole, T., Pèault, B. and Le Douarin, N. (1986). MB1, a quail leukocyte-endothelium antigen: partial characterization of the cell surface and secreted forms in cultured endothelial cells. *Proc. Natl. Acad. Sci. USA* **83**, 9016-9020.

Lampugnani, M., Resnati, M., Raiteri, M., Pigott, R., Pisacane, A., Houen, G., Ruco, L. and Dejana, E. (1992). A novel endothelial-specific membrane protein is a marker of cell-cell contacts. *J. Cell Biol*. **118**, 1511-1522.

Lawrence, P.A., Casal, J. and Struhl, G. (1999). The hedgehog morphogen and gradients of cell affinity in the abdomen of *Drosophila*. *Development* **126**, 2441-2449.

Lawson, N.D., Scheer, N., Pham, V.N., Kim, C., Chitnis, A.B., Campos-Ortega, J.A., and Weinstein, B.M. (2002). Notch signaling is rquired for

arterial–venous differentiation during embryonic vascular development. *Development* **128**, 3675-3683.

Lawson, N.D., Vogel, A.M., and Weinstein, B..M. (2002). sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation. *Dev. Cell* **3**, 127-36.

Lee, S. and Kolodziej, P.A. (2002). The plakin Short Stop and the RhoA GTPase are required for E-cadherin-dependent apical surface remodeling during tracheal tube fusion. *Development* **129**, 1509-1520.

Leung, D., Cachianes, G., Kuang, W., Goeddel, D. and Ferrara, N. (1989). Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* **246**, 1306-1309.

Liang, D., Xu, X., Chin, A., Balasubramaniyan, N., Teo, M., Lam, T., Weinberg, E. and Ge, R. (1998). Cloning and characterization of vascular endothelial growth factor (VEGF) from zebrafish, *Danio rerio*. *Biochim. Biophys. Acta* **1397**, 14-20.

Liao, E., Paw, B., Oates, A., Pratt, S., Postlethwait, J. and Zon, L. (1998). SCL/Tal-1 transcription factor acts downstream of *cloche* to specify hematopoietic and vascular progenitors in zebrafish. *Genes. Dev.* **12**, 621-626.

Liao, W., Bisgrowve, B., Sawyer, H., Hug, B., Bell, B., Peters, K., Grunwald, D. and Stainier, D. (1997). The zebrafish gene *cloche* acts upstream of a *flk-1* homologue to regulate endothelial cell differentiation. *Development* **124**, 381-389.

Liu, Y., Cox, S., Morita, T. and Kourembanas, S. (1995). Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells, identification of a 5' enhancer. *Circ. Res.* **77**, 638-643.

Maisonpierre, P., Suri, C., Jones, P., Bartunkova, S., Wiegand, S., Radziejewski, C., Compton, D., McClain, J., Aldrich, T., Papadopoulos, N., Daly, T., Davis,, S., Sato, T. and Yancopoulos, G. (1997). Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* **277**, 55-60.

Mato, M., Aikawa, E. and Kishi, K. (1964). Some observations on interstice between mesoderm and endoderm in the area vasculosa of the chick blastoderm. *Exp. Cell Res.* **35**, 426-428.

Mead, P., Kelley, C., Hahn, P., Piedad, O. and Zon, L. (1998). SCL specifies hematopoietic mesoderm in *Xenopus* embryos. *Development* **125**, 2611-2620.

Meier, S. (1980). Development of the chick embryo mesoblast: pronephros, lateral plate, and early vasculature. *J. Embyol. Exp. Morph.* **55**, 291-306.

Mellitzer, G., Xu, Q. and Wilkinson, D. (1999). Eph receptors and ephrins restrict cell intermingling and communication. *Nature* **400**, 77-81.

Miller, A. and McWhorter, J. (1914). Experiments on the development of blood vessels in the area pellucida and embryonic body of the chick. *Anat. Rec.* **8**, 203-227.

Miquerol, L., Gertsenstein, M., Harpal, K., Rossant, J. and Nagy, A. (1999). Multiple developmental roles of VEGF suggested by a lacZ tagged allele. *Dev. Biol.* **212**, 307-322.

Miura, Y. and Wilt, F. (1969). Tissue interaction and the formation of the first erythroblasts of the chick embryo. *Dev. Biol.* **19**, 201-211.

Mobbs, I. And McMillan, D. (1979). Structure of the endodermal epithelium of the chick yolk sac during the early stages of development. *Am. J. Anat.* **155**, 287-309.

Murray, P. (1932). The development in vitro of the blood of the early chick embryo. *Proc. R. Soc. B London, Ser. B* **111**, 497-521.

Nakagawa, O., McFadden, D.G., Nakagawa, M., Yanagisawa, H., Hu, T., Srivastava, D. and Olson, E.N. (2000). Members of the HRT family of basic helix-loop-helix proteins act as transcriptional repressors downstream of Notch signaling. *Proc. Natl. Acad. Sci. USA* **97**, 13655-13660.

Nascone, N. and Mercola, M. (1995). An inductive role for the endoderm in *Xenopus* cardiogenesis. *Development* **121**, 515-523.

New, D. (1955). A new technique for the cultivation of the chick embryo in vitro. *J. Embryol. Exp. Morph.* **3**, 326-331.

Newman, C., Chia, F. and Krieg, P.A. (1997). The *Xhex* homeobox gene is expressed during development of the vascular endothelium: overexpression leads to an increase in vascular endothelial cell number. *Mech. Dev.* **66**, 83-93.

Niehrs, C., Steinbesser, H. and De Robertis, E. (1994). Mesodermal patterning by a gradient of the vertebrate homeobox gene *goosecoid. Science* **263**, 817-820.

Nieuwkoop, P.D., and Faber, J. (1994). Normal Table of *Xenopus laevis* (Daudin). New York, NY: Garland Publishing, Inc.

Nishimura, M., Miki, T., Yashima, R., Yokoi, N., Yano H., Sato, Y. and Seino, S. (1999). Angiopoietin-3, a novel member of the angiopoietin family. *FEBS Lett.* **448**, 254-256.

Noden, D. (1989). Embryonic origins and assembly of blood vessels. *Amer. Rev. Respir. Dis.* **140**, 1097-1103.

Oike, Y., Ito, Y., Hamada, K., Zhang, X.Q., Miyata, K., Arai, F., Inada, T., Araki, K., Nakagata, N., Takeya, M., Kisanuki, Y.Y., Yanagisawa, M., Gale, N.W.. and Suda, T. (2002). Regulation of vasculogenesis and angiogenesis by EphB/ephrin-B2 signaling between endothelial cells and surrounding mesenchymal cells. *Blood* **100**, 1326-1333.

Olah, I., Medgyes, J. and Glick, B. (1988). Origin of aortic cell clusters in the chicken embryo. *Anat. Rec.* **222**, 60-68.

Palis, J., McGrath, K. and Kingsley, P. (1995). Initiation of hematopoiesis and vasculogenesis in murine yolk sac explants. *Blood* **86**, 156-163.

Pandey, A., Shao, H., marks, R., Polverini, P. and Dixit, V. (1995). Role of B61, the ligand for the Eck receptor tyrosine kinase, in TNF-α-induced angiogenesis. *Science* **268**, 567-569.

Pardanaud, L. and Dieterlen-Lièvre, F. (1999). Manipulation of the angiopoietic.hemangiopoietic commitment in the avian embryo. *Development* **126**, 617-627.

Pardanaud, L., Luton, D., Prigent, M., Bourcheix, L., Catala, M. and Dieterlen-Lièvre, F. (1996). Two distinct endothelial lineages in ontogeny, one of them related to hemopoiesis. *Development* **125**, 1363-1371.

Pardanaud, L. and Dieterlen-Lièvre, F. (1993). Emergence of endothelial and hemopoietic cells in the avian embryo. *Anat. Embryol.* **187**, 107-114.

Pardanaud, L., Yassine, F. and Dieterlen-Lièvre, F. (1989). Relationship between vasculogenesis, angiogenesis and haemopoiesis during avian ontogeny. *Development* **105**, 473-485.

Pardanaud, L., Altmann, C., Kitos, P., Dieterlen-Lièvre, F. and Buck, C. (1987). Vasculogenesis in the early quail blastodisc as studied with a monoclonal antibody recognizing endothelial cells. *Development* **100**, 339-349.

Park, J., Keller, G. and Ferrara, N. (1993). The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. *Mol Biol. Cell* **4**, 1317-1326.

Parker, ,L. and Stainier, D. (1999). Cell-autonomous and non-autonomous requirements for the zebrafish gene cloche in hematopoiesis. *Development* **126**, 2643-2651.

Partanen, J. and Dumont, D. (1999). Functions of Tie1 and Tie2 receptor tyrosine kinases in vascular development. In: Claesson-Welsh (ed) Vascular growth factors and angiogenesis. Springer-Verlag, Berlin pp 159-172.

Partanen, J., Puri, M., Schwartz, L., Fischer, K., Bernstein, A. and Rossant, J. (1996). Cell autonomous functions of the receptor tyrosine kinase TIE in a late phase of angiogenic capillary growth and endothelial survival during murine development. *Development* **122**, 3013-3021.

Patan, S. (1998). Tie1 and Tie2 receptor tyrosine kinases inversely regulate embryonic angiogenesis by the mechanism of intussusceptive microvascular groowth. *Microvasc. Res.* **56**, 1-21.

Patan, S. ,Haenni, B. and Burri, P. (1996). Implementation of intussusceptive microvascular growth in the chicken chorioallantoic membrane (CAM). *Microvasc. Res.* **51**, 80-98.

Patan, S., Haenni, B. and Burri, P. (1993). Evidence for intussusceptive capillary growth in the chicken chorio-allantoic membrane (CAM). *Anat. Embryol.* **187**, 121-130.

Pathi, S., Pagan-Westphal, S., Baker, D.P., Garber, E.A., Rayhorn, P., Bumcrot, D., Tabin, C.J., Pepinsky, R.B. and Williams, K.P. (2001). Comparative biological responses to human Sonic, Indian and Desert hedgehog. *Mech. Dev.* **106**, 107-117.

Pèault, B., Thiery, J. and Le Douarin, N. (1983). Surface marker for hemopoietic and endothelial cell lineages in quail that is defined by a monoclonal antibody. *Proc. Natl. Acad. Sci. USA* **80**, 2976-2980.

Persico, M., Vincenti, V. and DiPalma, T. (1999). Structure, expression and receptor-binding properties of placenta growth factor (PlGF). In: Claesson-Welsh (ed) Vascular growth factors and angiogenesis. Springer-Verlag, Berlin Heidelberg New York pp 41-57.

Pola, R., Ling, L.E., Silver, M., Corbley, M., Kearney, M., Pepinsky, R.B., Shapiro, R., Taylor, F.R., Baker, D.P., Asahara, T. and Isner, J.M. (2001). The morphogen Sonic hedgehog is an indirect angiogenic agent upregulating two families of angiogenic growth factors. *Nature Med.* **7**, 706-711.

Poole, T. and Coffin, J. (1991). Morphogenetic mechanisms in avian vascular development. In: Stolte, H, Kinne, R., Bach, P. (eds) The development of the vascular system. Issues in Biomedicine 14. S. Karger, Basil, pp 25-36.

Poole, T., Finkelstein, E. and Cox, C. (2001). The role of FGF and VEGF in angioblast induction and migration during vascular development. *Dev. Dynamics* **220**, 1-17.

Puri, M., Partanen, J., Rossant, J. and Bernstein, A. (1999). Interaction of the TEK and TIE receptor tyrosine kinases during cardiovascular development. *Development* **126**, 4569-4580.

Puri, M., Rossant, J., Alitalo, K., Bernstein, A. and Partanen, J. (1995). The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells. *EMBO J*. **14**, 5884-5891.

Reagan, F. (1915). Vascularization phenomena in fragments of embryonic bodies completely isolated from yolk-sac blastoderm. *Anat. Rec*. **9**, 329-341.

Risau, W. (1995). Differentiation of endothelium. *FASEB J*. **9**, 926-933.

Risau, W. and Flamme, I. (1995). Vasculogenesis. *Annu. Rev. Cell Dev. Biol*. **11**, 73-91.

Risau, W. (1997). Mechanisms of angiogenesis. *Nature* **386**, 671-674.

Roman, B. and Weinstein, B.M. (2000). Building the vertebrate vasculature: research is going swimmingly. *BioEssays* **22**, 882-893.

Rowitch D.H., St-Jacques B., Lee, S.M., Flax, J.D., Snyder, E.Y., and McMahon, A.P. (1999). Sonic hedgehog regulates proliferation and inhibits differentiation of CNS precursor cells. *J. Neurosci*. **19**, 8954-8965.

Rückert, J. and Mollier, S. (1906). Die erste entsehung der gefässe und des blutes bei wirbeltieren. In: Hertwig, O. (ed) Handbuch der vergleichenden und experimentellen entwickelungslehre der wirbeltiere. Verlag von Gustav Fischer, Jena, pp 1019-1297.

Sabin, F. (1920). Studies on the origin of blood vessels and of red blood corpuscles as seen in the living blastoderm of chicks during the second day of incubation. *Contrib. Embryol. Carnegie Inst*. **9**, 213-226.

Sabin, F. (1922). Direct growth of veins by sprouting. *Contrib. Embryol. Carnegie Inst*. **14**, 1-11.

Sasai, Y., Lu, B., Piccolo, S. and DeRobertis, E. (1996). Endoderm induction by the organizer- secreted factors chordin and noggin in *Xenopus* animal caps. *EMBO J*. **15**, 4547-4555.

Sato, T., Tozawa, Y., Deutsch, U., Wolburg-Buchholz, K., Fujiwara, Y., Gendron-Maguire, M., Gridley, T., Wolburg, H., Risau, W. and Qin, Y. (1995). Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* **376**, 70-74.

Schier, A., Neuhauss, S., Helde, K., Talbot, W. and Driever, W. (1997). The one-eyed pinhead gene functions in mesoderm and endoderm formation in zebrafish and interacts with no tail. *Development* **124**, 327-342.

Shalaby, F., Ho, J., Stanford, W., Fischer, K., Schuh, A., Schwartz, L., Bernstein, A. and Rossant, J. (1997). A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. *Cell* **89**, 981-990.

Shalaby, F., Rossant, J., Yamaguchi, T., Gertsenstein, M., Wu, X., Breitman, M. and Schuh, A. (1995). Failure of blood-island formation and vasculogenesis in Flk-deficient mice. *Nature* **376**, 62-66.

Shivdasani, R., Mayer, E. and Orkin, S. (1995). Absence of blood formation in mice lacking the T-cell leukemia oncoprotein tal-1/SCL. *Nature* **373**, 432-434.

Soker, S., Takashima, S., Miao, H., Neufeld, G. and Klagsbrun, M. (1998). Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* **92**, 735-745.

Stainier, D., Weinstein, B., Detrich III, H., Zon, L. and Fishman, M. (1995). *cloche*, an early acting zebrafish gene, is required by both the endothelial and hematopoietic lineages. *Development* **121**, 3141-3150.

Stein, I., Itin, A., Einat, P., Skaliter, R., Grossman, Z. and Keshet, E. (1998). Translation of vascular endothelial growth factor mRNA by internal ribosome entry, implications for translation under hypoxia. *Mol. Cell Biol.* **18**, 3112-3119.

Stockard, C. (1915a). A study of the wandering mesenchymal cells on the living yolk-sac and their developmental products: chromatophores, vascular endothelium and blood cells. *Am. J. Anat.* **18**, 525-594.

Stockard, C. (1915b). The Origin of blood and vascular endothelium in embryos without a circulation of the blood and in the normal embryo. *Am. J. Anat.* **18**, 228-326.

Sugi, Y. and Markwald, R. (1996). Formation and early morphogenesis of endocardial endothelial precursor cells and the role of endoderm. *Dev. Biol.* **175**, 66-83.

Sumoy, L., Keasey, J., Dittman, T. and Kimelman, D. (1997). A role for notochord in axial vascular development revealed by analysis of phenotype and the expression of VEGR-2 in zebrafish *flh* and *ntl* mutant embryos. *Mech. Dev.* **63**, 15-27.

Suri, C., Jones, P., Patan, S., Bartunkova, S., Maisonpierre, P., Davis, S., Sato, T. and Yancopoulos, G. (1996). Requisite role of angiopoietin-1, a ligand for the TIE2 receptor during embryonic angiogenesis. *Cell* **87**, 1171-1180.

Suri, C., McClain, J., Thurston, G., McDonald, D., Zhou, H., Oldmixon, E., Sato, T. and Yancopoulos, G. (1998). Increased vascularization in mice overexpressing angiopoietin-1. *Science* **282**, 468-471.

Swiatek, P., Lindsell, C., del Amo, F., Weinmaster, G. and Gridley, T. (1994). Notch1 is essential for postimplantation in mice. *Genes Dev.* **8**, 707-719.

Syropoulos, D., Pharr, P., Lavenburg, K., Jackers, P., Papas, T., Ogawa, M. and Watson, D. (2000). Hemorrhage, impaired hematopoiesis, and lethality in mouse embryos carrying a targeted disrupton of the fli1 transcription factor. *Mol. Cell Biol* **20**, 5643-5652.

Taipale, J., Chen J.K., Cooper, M.K., Wang, B., Mann, R.K., Milenkovic, L., Scott, M.P. and Beachy, P.A. (2000). Effects of Oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine. *Nature* **406**, 1005-1009.

Tatemoto, K., Hosoya, M., Habata, Y., Fujii, R., Kakegawa, T., Zou, M., Kawamata, Y. Fukusumi, S., Hinuma, S., Kitada, C., Kurokawa, T., Onda, H. and Fujino, M. (1998). Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. *Biochem. Biophys. Res. Commun.* **251**, 471-476.

Thomas, P., Brown, A. and Beddington, R. (1998). Hex: a homeobox gene revealing peri-implantation asymetry in the mouse embryo and an early transient marker of endothelial cell precursors. *Development* **125**, 85-94.

Thompson, M., Ransom, D., Pratt, S., MacLennan, H., Kieran, M., Detrich III, H., Vail, B., Huber, T., Paw, B., Brownlie, A., Oates, A., Fritz, A., Gates, M., Amores, A., Bahary, N., Talbot, W., Her, H.., Beier, D., Postlethwait, J. and Zon, L. (1998). The *cloche* and *spadetail* genes

differentially regulate hematopoiesis and vasculogenesis. *Dev. Biol.* **197**, 248-269.

Uyttendaele, H., Ho, J., Rossant, J. and Kitajewski, J. (2001). Vascular patterning defects associated with expression of activated Notch4 in embryonic endothelium. *Proc. Natl. Acad. Sci. USA* **98**, 5643-5648.

Valenzuela, D., Griffiths, J., Rojas, J., Aldridch, T., Jones, P., Zhou, H., McClain, J., Copeland, N., Gilbert, D., Jenkins, N., Huang, T., Papadopoulos, N., Maisonpierre, P., Davis, S. and Yancopoulos, G. (1999). Angiopoietins 3 and 4: diverging gene counterparts in mice and humans. *Proc. Natl. Acad. Sci. USA* **96**, 1904-1909.

Van der Geer, P., Hunter, T. and Lindberg,R. (1994). Receptor protein-tyrosine kinases and their signal transduction pathways. *Annu. Rev. Cell. Biol.* **10**, 251-337.

Van der Stricht, O. (1895). De la première origine du sang et des capillaires sanguins dans l'aire vasculaire du lapin. *Comptes rend. D. l. Soc. d. Biol.*, t. 12, **10**, P181-185.

Vernon, R., Lara, S., Drake, C., Iruela-Arispe, M., Angello, J., Little, C., Wight, T. and Sage, E. (1995). Organized type I collagen influences endothelial patterns during "spontaneous angiogenesis in vitro". planar cultures as models of vascular development. *In vitro Cell. Dev. Biol.* **31**, 120-131.

Vikkula,, M., Boon, L., Carraway, K., Calvert, J., Diamonti, A., Goumnerov, B., Pasyk, K., Marchuk, D., Warman, M., Cantley, L., Mulliken, J. and Olsen, B. (1996). Vascular dysmorphogenesis caused by an activating mutation in the receptor tyrosine kinases TIE2. *Cell* **87**, 1181-1190.

Villa, N., Walker, L., Lindsell, C.E., Gasson, J., Iruela-Arispe, M., Weinmaster, G. (2001). Vascular expression of Notch pathway receptors and ligands is restricted to arterial vessels. *Mech. Dev.* **108**, 161-164.

Visvader, J., Fujiwara, Y. and Orkin, S. (1998). Unsuspected role for the T-cell leukemia protein SCL/tal-1 in vascular development. *Genes. Dev.* **12**, 473-479.

Vittet, D., Buchou, T., Schweitzer, A., Dejana, E. and P. Huber (1997). Targeted null-mutation in the vascular endothelial-cadherin gene impairs the organization of vascular-like structures in embryoid bodies. *Proc. Natl. Acad. Sci. USA* **94**, 6273-6278.

von Kirschhofer, K., Grim, M., Christ, B. and Wachtler, F. (1994). Emergence of myogeneic and endothelial cell lineages in avian embryos. *Dev. Biol.* **163**, 270-278.

Vokes, S.A. and Krieg, P.A. (2002a). Endoderm is required for vascular endothelial tube formation, but not for angioblast formation. *Development* **129**, 775-785.

Vokes, S.A. and Krieg, P.A. (2002b). Molecular vascular embryology. In PanVascular Medicine (eds. Lanzer, P., Topol, E.J.), pp. 18-35. Berlin Heidelberg New York, Springer-Verlag.

Wagner, R. (1980). Endothelial cell embryology and growth. *Adv. Microcirc.* **9**, 45-75.

Wakiya, K., Begue, A., Stehelin, D. and Shibuya, M. (1996). A cAMP response element and an Ets motif are involved in the transcriptional regulation of flt-1 tyrosine kinase (Vascular endothelial growth factor receptor 1) gene. *J. Biol. Chem.* **271**, 30823-30828.

Waldo, K. and Kirby, M. (1998). Development of the great arteries. In Living Morphogenesis of the heart (ed. M.V. de la Cruz and R. Markwald), pp. 187-217. Boston: Birkhäuser.

Waltenberger, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M. and Heldin, C. (1994). Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *J. Biol. Chem.* **269**, 26988-26995.

Wang, H., Chen, Z. and Anderson, D. (1998). Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. *Cell* **93**, 741-753.

Wang, L., Kuo, F., Fujiwara, Y., Gilliland, D., Golub, T. and Orkin, S. (1997). Yolk sac angiogenic defect and intra-embryonic apoptosis in mice lacking the Ets-related factor TEL. *EMBO J.* **16**, 4374-4383.

Weinstein, B.M. (1999). What guides early embryonic blood vessel formation? *Dev. Dynamics* **215**, 2-11.

Weinstein, B., Stemple, D., Driever, W. and Fishman, M. (1995). Gridlock, a localized heritable vascular patterning defect in the zebrafish. Nature Med. **1**, 143-147.

Wilms, P., Christ, B., Wilting, J. and Wachtler, F. (1991). Distribution and migration of angiogenic cells from grafted avascular intraembryonic mesoderm. *Anat. Embryol.* **183**, 371-377.

Wilson, D., McNeill, J. and Hinchcliffe, J. (1989). Posterior half amputation of the chick wing bud: the response of the developing vasculature, and subsequent wound healing. *J. Anat. Embryol.* **180**, 383-391.

Wilt, F. (1965). Erythropoiesis in the chick embryo: the role of endoderm. *Science* **147**, 1588-1590.

Wilting, J., Birkenhager, R., Eichmann, A., Kurz, H., Martiny-Baron, G., Marme, D., McCarthy, J., Christ, B. and Weich, H. (1996). VEGF 121 induces proliferation of vascular endothelial cells and expression of flk-1 without affecting lymphatic vessels of the chorioallantoic membrane. *Dev. Biol.* **176**, 76-85.

Wilting, J., Brand-Saberi, B., Huang, R., Zhi,Q., Köntges, G., Ordahl, C. and Christ, B. (1995). Angiogenic potential of the avian somite. *Dev. Dynamics* **202**, 165-171.

Wilting, J. and Christ, B. (1996). Embryonic angiogenesis: a review. *Naturwissenschaftenaufsätze* **83**, 153-164.

Wolpert, L., Beddington, R., Brockes, J., Jessel, T., Lawrence, P. and Meyerowitz, E. (1998). Principles of Development. Oxford University Press, Oxford.

Xanthos, J., Kofron, M., Wylie, C. and Heasman, J. (2001). Maternal VegT is the initiator of a molecular network specifying endoderm in *Xenopus laevis*. *Development* **128**, 167-180.

Xue, Y., Gao, X., Lindsell, C., Norton, C., Chang, B., Hickss, C., Gendron-Maguire, M., Rand, E., Weinmaster, G. and Gridley, T. (1999).

Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. *Hum. Mol. Genet.* **8**, 723-730.

Yamada, K. and Miyamoto, S. (1995). Integrin transmembrane signaling and cytoskeletal control. *Curr. Opin. Cell Biol.* **7**, 681-689.

Yamaguchi, T., Dumont, D., Conlon, R., Breitman, M. and Rossant, J. (1993). flk-1, an flt-related receptor tyrosine kinase is an early marker for endothelial cell precursors. *Development* **118**, 489-498.

Yamamoto, H., Flannery, M., Kupriyanov, S., Pearce. J., McKercher, S., Henkel, G., Maki, R., Werb, Z. and Oshima, R. (1998). Defective trophoblast function in mice with a targeted mutation of Ets2. *Genes Dev.* **12**, 1315-1326.

Yancopoulos, G., Davis, S., Gale, N., Rudge, J., Wiegand, S. and Holash, J. (2000). Vascular-specific growth factors and blood vessel formation. *Nature* **407**, 242-248.

Yang, J., Bader, B., Kreidberg, J., Ullman-Cullerè, M., Trevithick, J. and Hynes, R. (1999). Overlapping and independent functions of fibronectin receptor integrins in early mesodermal development. *Dev. Biol.* **215**, 264-277.

Yang, J., Rayburn, H. and Hynes, R. (1993). Embryonic mesodermal defects in α_5 integrin-deficient mice. *Development* **119**, 1093-1105.

Yatskievych, T., Pascoe, S. and Antin, P. (1999). Expression of the homeobox gene Hex during early stages of chick embryo development. *Mech. Dev.* **80**, 107-109.

Zhang, J., Houston, D., King, M., Payne, C., Wylie, C. and Heasman, J. (1998). The role of maternal VegT in establishing the primary germ layers in *Xenopus* embryos. *Cell* **94**, 515-524.

Zhong, T.P., Rosenberg, M., Mohideen, M., Weinstein, B.M. and Fishman, M. (2000). gridlock, an HLH gene required for assembly of the aorta in zebrafish. *Science* **287**, 1820-1824.

Zhong, T.P., Childs, S., Leu, J.P. and Fishman, M. (2001). Gridlock signaling pathway fashions the first embryonic artery. *Nature* **414**, 216-220.

Vita

Steven Alexander Vokes was born in San Antonio, Texas on November 9, 1974, the son of Carol Cooper Vokes and Robert Emmett Vokes. After graduating from Saint Mary's Hall in 1993, he attended Swarthmore College, receiving a Bachelor of Arts in Biology in June, 1997. In September 1997 he enrolled in the Graduate School of the University of Texas at Austin. He was awarded the degree of Doctor of Philosophy in December, 2002.

Permanent address: 215 W. Mulberry Ave, San Antonio, Texas, 78212

This dissertation was typed by the author.