1998

Cell-Cell Interactions in the Development of the Vascular System of Xenopus

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Cell-Cell Interactions in the Development of the Vascular System of *Xenopus.*

A Thesis

Presented to

The Faculty of the Department of Biology

The College of William and Mary

In Partial Fulfillment

Of the Requirements for the Degree of

Masters of Arts

by

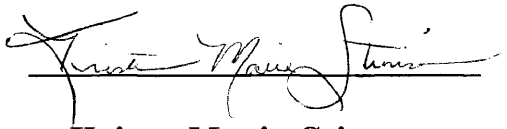
Krista Marie Stimson

1998

Approval Sheet

This thesis is submitted in partial fulfillment of the requirements for the degree of

Master of Arts



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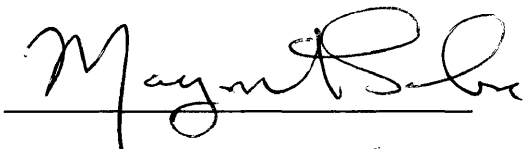
Approved, June 1998



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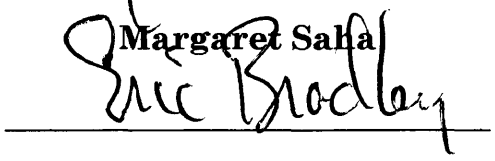

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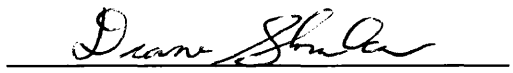


Diane Shakes

Table of Contents

Acknowledgments

First, I would like to thank Dr. Margaret Saha for her support and guidance not only in regards to my research but also future career. The time and energy she spends in pursuit of science is awe inspiring. I would like to thank Dr. Diane Shakes for her careful editing of my thesis. Many thanks to Dr. Eric Bradley for becoming a last minute addition to my committee, for his help in editing my thesis and his congenial presence in the laboratory over the past years.

I would like to thank the inhabitance of 301 and 316 for moral support, and very interesting additions to the quote board. Wenda Ribeiro was instrumental in technical assistance for both commuter scanning, and in preparing DNA clones. Robert Jackson showed great patience in teaching me many of the techniques necessary for the completion of this thesis. Last but not least, the frogs for helping me to earn the title "Queen of Matings."

List of Tables

List of Figures

Abstract

Little is known about the earliest interactions that are necessary for the formation of the vascular system. Historically, it has been thought that the blood and the vascular endothelium have a common precursor in the ventral mesoderm called a hemangioblast. However, based upon the expression pattern of a vascular endothelial marker gene, *XAngio* it was hypothesized that a broad region of the mesoderm is initially competent to form vascular endothelium and that this competence is restricted throughout development. *Xenopus* embryos are ideal for examining process during the blastula and gastrula stages because of their large size and the distribution of nutrients within each individual embryonic cell. Dispersion (without normal cell-cell contact) and disassociation (with cell-cell contact but without normal cell-cell adhesion) experiments were performed to assess the competence of mesodermal cells dispersed at blastula and gastrula stages to later express the vascular endothelial marker gene *XAngio*. The *in situ* hybridization results show that *XAngio* is widely expressed in both the dispersed and disassociated embryos. The expression pattern of *XAngio* was more widespread than the expression patterns of the regional marker genes *GATA-2* (ventral mesoderm) and *actin* (equatorial mesoderm). *XAngio* showed widespread coexpression with the pan mesodermal marker *Xbra*. This suggests that the manipulations inhibited the restriction of *XAngio* expression not the induction, which supports the hypothesis. The 'default' state of mesodermal tissue might be vascular endothelium. *Beta-globin* (blood marker gene) expression was inhibited in both of the manipulation types. Which suggests that there is not a single lineage of mesodermal cells (a hemangioblast) that gives rise to all of the blood and vasculature.

Introduction

Prologue

The vascular system is required for continued growth and differentiation in vertebrates. This complex web of tubes and their contents must infiltrate within micrometers of every cell. The process of vascularization is of critical importance throughout development as well as in the adult organism. It functions in normal processes such as wound repair, the proliferation of the endometrial wall and corpus luteum formation as well as in pathological conditions such as tumorgenesis, rheumatoid arthritis and proliferative retinopathies (reviewed in Risau, 1991; Ferrara and Davis-Smyth, 1997). This study examines some of the earliest inductive events that lead to vascular differentiation and development, a process that begins with mesoderm induction at blastula stages.

Patterning the Blastula Embryo

The patterning of the *Xenopus* embryo is initiated before fertilization by the differential distribution of maternal determents (reviewed in Heasman, 1997). At fertilization the point of sperm entry establishes the dorsal/ventral axis (Gerhart et al., 1989; reviewed in

Guger and Gumbiner, 1995; Heasman, 1997). Before the onset of zygotic transcription (the mid-blastula transition or MBT) vegetal cells signal overlying cells of the marginal zone to become mesoderm (Slack et al., 1992; Dosch et al., 1997). Mesodermal patterning is hypothesized to occur through three different types of signaling first proposed by Smith (1989). Dorsal vegetal cells signal the marginal zone become dorsal mesoderm while ventral vegetal cells signal the overlying mesoderm to become ventral mesoderm. The patterning is further refined through the interaction of signal throughout the mesoderm (reviewed in Heasman, 1997).

Various molecules have been implicated in mesoderm patterning. Increasing concentrations of the maternal determinate Vg1 or activin induce a pattern of mesodermal tissue along the dorsal ventral axis in undifferentiated animal cap tissue (reviewed in Dosch, 1997). BMP-4 (bone morphogenetic protein) will ventralize mesoderm unless antagonized by chordin and noggin which have a dorsalizing effect on mesodermal tissue (Smith and Harland, 1992; Smith et al., 1993; Harland, 1994; Dosch et al., 1997). Beta-catenin also acts to dorsalize mesoderm (Charabarti et al., 1992; Moon et al., 1993; Guger and Gumbiner, 1995; Dosch et al., 1997). Once patterned along the dorsal/ventral axis, mesoderm will give rise to many tissues including;

notochord (most dorsal), muscle, pronephros, and blood (most ventral) (Dale and Slack, 1987).

When and how the mesoderm gains the competence to form these various differentiated tissues is not well understood. The erythropoietic potential of the ventral mesoderm can be induced by contact with animal pole tissue (Maeno et al., 1994). BMP-4 acts as a morphogen in blood formation by inducing ventral mesoderm which is then fated to give rise to primary erythropoietic cells (Dosch et al., 1997). However, the relationship between the induction of blood and vasculature is not well understood.

The Origins of Blood and Vasculature in the Blastula Embryo

Fate mapping experiments have been performed in which a fluorescent dye is injected into individual blastomeres of the *Xenopus* embryo so that cells derived from that individual blastomere can be traced. These experiments show that two of the thirty two blastomeres can give rise to blood (Dale and Slack, 1987). These two blastomeres had previously been shown give rise to cells occupying the ventral marginal zone (mesoderm) (Nakamura and Kishiyama, 1971). This is consistent with experiments discussed earlier which also show that ventral mesoderm has erythropoietic potential. Endothelial cells have origins in multiple blastomeres both dorsal and ventral including

the two that give rise to blood however, each only labels a fraction of the total endothelium (Rovianen, 1991; Mills and Saha unpublished data).

The Avian System

Much of the early work on vascular development was performed in the avian system. Chick and quail embryos have extra-embryonic yolk which necessitates the very early formation of blood and vasculature to circulate nutrients from the yolk to the developing embryo proper (reviewed in Noden, 1991). Murray (1932) observed the simultaneous appearance of blood and vasculature (blood islands) in the avian extra-embryonic tissue and postulated the existence of a common precursor cell to both of these lineages, a hemangioblast localized in the extra-embryonic tissue. Hematopoiesis and endothelial cells differentiation appear at the same time in the dorsal aorta (reviewed in Pardanaud et al., 1996). The hematopoietic cells of extra-embryonic origin are transient and secondary intra-embryonic differentiation events lead to adult hematopoietic lineages (reviewed in Flamme et al., 1997). In amphibians all hematopoietic and endothelial cells must have intraembryoic origins because of the absence of extra-embryonic tissue.

The fortuitous isolation of a monoclonal antibody, QH1, that recognizes quail endothelial and hematopoietic cells was used to demonstrate that endothelial cells first emerged as single cells that later organize into an interconnected vasculature (Pardanaud et. al., 1987). Because QH1 recognizes both endothelial and hematopoietic precursors it is used as evidence supporting the hemangioblast theory. Poole and Coffin (1988) using QH1 and the scanning electron microscope observed two distinct process involved in vascular development. The first is vasculogenesis, a *in situ* derivation of endothelial cells which is followed by angiogenesis the sprouting of new vessels from pre-existing vasculature. Poole and Coffin were able to differentiate between the segregation of a single cell from the lateral mesoderm or somatopleure (mesoderm plus ectoderm) which coalesced to form the dorsal aorta and cardinal veins (vasculogenesis) and the formation of the intersegmental arteries via sprouting from the dorsal aorta (angiogenesis). Pardanaud et al. (1989) performed interspecies tissues grafts using the QH1 monoclonal antibody to trace grafted tissue. In the internal organs there were *in situ* derivations of endothelial cells but there was a separate origin of their hematopoietic cells. Either splanchnopleura (mesoderm plus endoderm) or somatopleura (mesoderm plus ectoderm) tissue was transplanted between chick and quail. From the splanchnopleura vasculogeneic

cells arise and from the somatopleura angiogenic cells arise. This contradicts the exclusive vasculogenic potential of the cardinal veins (somatopleural) seen by Poole and Coffin(1988). All of the mesodermal tissue, with the exception of prechordal plate shows angiogenic potential (Noden, 1989) which explains how the same tissue type can undergo both vasculogensis and angiogenesis.

The interplay of vasculogenesis and angiogenesis is complex. Neural crest derivatives such as the face and jaw must become vascularized by exogenous endothelial precursors and tissues of paraxial mesodermal origin such as somites appear to be vascularized by ingrowth of exogenous blood vessels contain angioblasts capable of expressing endothelium. The existence of some highly invasive endothelial cells shows the use of both immigration and sprouting as mechanism of angiogenesis (Noden, 1990). Poole and Coffin (1991) noted additional highly migratory endothelial cells and subdivided vasculogenesis into two types: Type I in which the angioblasts arise in place (dorsal aorta) and Type II in which the angioblast migrate to the vessel site (endocardium and posterior cardinal veins).

The tissue graph experiments in the avian system show the angiogenic and vasculogenic potential of mesodermal tissue in conjunction with endoderm or ectodermal tissue at post gastrula stages in the chick (Pardanaud et al., 1989; Noden, 1990; Poole and Coffin

1991). This confirms the potential of mesodermal tissue at these later stages to differentiate and contribute to both the blood and vasculature. The potential of mesodermal cells at blastula and gastrula stages is unknown and the subject of this study.

Molecular Analysis

In order for mesodermal tissue to differentiate into vascular tissue or for cells to segregate from pre-existing vasculature and invade new tissue, a highly regulated system of intercellular and intracellular singling must take place. Both *In vivo* and *in vitro,* basic fibroblast growth factor (bFGF) can induce embryonic cells to undergo vasculogenesis and angiogenesis (Folkman and Klagsbrun, 1987; Risau, 1988). However, bFGF's mitogenic potential is not restricted to vascular tissue but it is essential in the formation of vascular tissue in undifferentiated cells in culture (Krah et al., 1994; Wilting et al.,1993).

A potent mitogen that is restricted to endothelial tissues is vascular endothelial growth factor (VEGF) (Leung et al., 1989). Four isoforms of VEGF can be generated from alternative splicing of a single gene (Ferrara et al., 1991). VEGF is up-regulated in both pathological angiogenesis (tumors, chronic inflammation) and during the course of normal wound repair, the inflammation response and under hypoxic conditions (Jackson et al., 1997; Lewis et al., 1997;

Gerber et al., 1997). Anti-VEGF antibodies have the potential to inhibit tumor growth in cell lines and intraocular neovascularization in animal models (reviewed in Ferrara, 1995). VEGF also stimulates the derivation and proliferation of the capillary plexus in the extra embryonic tissue of the chick (Wilting, 1993). The exogenous addition of VEGF in an avian embryonic system leads to hyper-fussed vessels with abnormally large lumens as well as neovascularization in normally avascular areas (Drake and Little, 1995). The current literature surrounding VEGF is in flux.

VEGF has two endothelial specific tyrosine kinase receptors, *flt-1* (Shibuya et al., 1989) and *flk-1/KDR* (Matthews et al., 1991; Yamaguchi et al., 1993). Tyrosine kinase receptors often have transforming capabilities and are involved in growth and differentiation (reviewed in Mustonen and Alitalo, 1995). These receptors are differentially regulated at the transcription level, *flt-1* is up-regulated by hypoxic conditions without similar up-regulation in *flk-1/KDR* (Gerber et al., 1997). Mouse knockouts have shown that neither VEGF nor its receptors are essential for initial endothelial differentiation but rather for later processes (Carmeliet et al., 1996; Fong et al., 1995; Shalaby et al., 1997). *Flt-1* is essential for the organization of embryonic vasculature and assembly of vascular channels but not essential for endothelial differentiation (Fong et al.,

1995). *Flk-1/KDR* and *flt-1* are expressed in the mesodermal cells of the chick embryo prior to any morphological evidence of endothelial differentiation (Yamaguchi et al., 1993; Shalaby et al., 1997). Mice lacking the *flk-1/KDR* receptor die without mature endothelial or hematopoietic cells (Shalaby et al., 1997). VEGF deficient mice show abnormal vascular development. Organization is severely impaired but not deficient which suggests another unknown ligand for the flk-1/KDR receptor (Carmeliet et al., 1996).

The zebrafish gene *cloche* appears to act up-stream of flk-1 to delay its expression, preventing blood formation and impairing the formation of organized vasculature throughout most of the embryo (Liao, 1997). The few cells that express flk-1 in the *cloche* mutants fail to further differentiate to express the downstream receptor tie (1997). *Tie-1* and *tek* (also referred to as *tie-2*) are additional members of the receptor tyrosine kinase family and are expressed in some hematopoietic cells, mature endothelial cells and endothelial precursors (Dumont et al., 1994; Sato et al., 1995; Partanen et al., 1996). *Tie-1* is essential to maintain the structural integrity of the endothelial wall, however, it does not play a role in vasculogenesis or angiogenesis (Sato et al., 1995; Partanen et al., 1996). *Tie-2* is involved in angiogenesis and is essential for the formation of the vascular network (Sato et al., 1995). Angiopoietin-1 is a ligand that

binds and causes the phosphorylation the tie-2 receptor (Davis et al., 1996). However, it does not directly initiate the growth of endothelial cells in culture but tie-2 mediates reciprocal interactions between the endothelium and surrounding mesenchyme and matrix (Suri et al., 1996). Angiopoietin-2 is an antagonist for the tie-2 receptor and is a promising candidate for disruption of angiogenesis in adult tissue undergoing pathological angiogenesis (Maisonpierre et al. 1997).

The Vascular Marker Gene *XAngio*

In this study the major vascular marker gene used is called *XAngio*. *XAngio* (or *XMR)* is a novel G-protein couple receptor (Drysdale et al., 1997; Devic et al., 1996). It was independently cloned in the process of screening a *Xenopus* stage 42 neural cDNA library for possible neurotransmitter receptors (Devic, et al., 1996; . The clone is in the range of 2.1kb with a coding region of 1.1kb encoding a protein of 362 amino acids. Sequence analysis shows that *XAngio* encodes a novel G-protein-coupled receptor which is distinguishable through the characteristic conserved seven transmembrane region. *XAngio* is related to the human angiotensin receptor (APJ) gene. At the amino acid level *XAngio* shows only 48 percent identity to the human APJ gene. The next closest identity is 30-35 percent to the *Xenopus* angiotensin II receptor. RNAse protection assays show *XAngio* is

initially transcribed in the late blastula embryo. At hatching stages (stages 29-31) there are peak levels of transcription. Shortly after the initial formation of blood vessel (the mid to late thirties) *XAngio* is down-regulated. *In situ* hybridization shows that at early stages *XAngio* is expressed throughout a broad region of the mesoderm, excluding the most dorsal regions (presumptive notochord). At the time of hatching expression is limited to the vascular endothelium (endocardium, aortic arches, the dorsal and intersomitic arteries and veins and the head vasculature) and tailbud. *XAngio* is not expressed in the ventral blood islands. It is the first marker gene cloned in *Xenopus* restricted to vascular endothelial cell precursors.

XAngio expression can be induced in animal cap tissue by the mesoderm inducer, activin (unpublished data). It expression is not inhibited by UV-irradiation (a ventraling signal) nor lithium treatment (a dosalizing signal) (unpublished data). Debbie Kruep (Honors Thesis, 1997) performed a series of explant experiments on gastrula and neurula stage *Xenopus* embryos. Six segments of mesodermal tissue were dissected from the embryo and allowed to culture until hatching stages. *XAngio* and *beta-globin* (blood marker gene) expression in the explants was determined using *in situ* hybridization (Harland et al., 1991). These experiments show that at stage 11.5 (late gastrula) and stage 14 (neural plate) all regions of

mesoderm when excised and cultured could express the endothelial marker gene, *XAngio*. However, only the ventral explants expressed the blood marker, *beta-globin*, which is consistent with the previously discussed literature. At the onset of this study these explant results were confirmed through an additional replication of a similar explant experiment.

The *XAngio* expression pattern, which initially covers a broad region of mesoderm then becomes restricted to endothelial precursors, in conjunction with the previous experiments which show its persistence in mesodermal tissue despite excision from the rest of the embryos suggests a hypothesis. It is hypothesized that a broad region of mesodermal tissue is competent to become vascular tissue and that in the absence of restriction of this potential that tissue will continue to express that vascular marker gene *XAngio*. If a positive induction event is necessary at blastula and early gastrula stages for the mesoderm to gain the competence to form vasculature then embryos without normal cell/cell interactions at these stage will not express the appropriate vascular markers. If however, a restriction event is necessary then these manipulated embryos will retain their competence, even after manipulation and continue to express vascular marker genes.

Study Overview

The stage of development at which mesodermal cells gain the competence to form endothelial and hematopoietic precursors is unknown. In the avian and mammalian system it is very difficult to work on the development of the pre-neurula embryo. The necessity for an exogenous nutrient supply either from the yolk (avian) or placenta (mammals) and impossibly microscopic embryonic size precludes performing embryonic manipulations on the blastula stage embryos. *Xenopus laevis* embryos are relatively large (about a millimeter) and each individual cell contains it own yolk supply. This allows the individual cells to survive for days cultured only in a buffered salt solution. There are also a significant number of cloned marker genes available to determine the differentiation state of manipulated tissue.

The goal of this study is to examine the early inductive events necessary for the formation of the vascular system. The vascular system has been shown to have its origin in mesodermal tissue which is induced at blastula stages as discussed earlier. At blastula and gastrula stages cell/cell adhesion can be disturbed through the removal of calcium and magnesium from the media. Cell/cell adhesion at these stages is mediated through cadherin binding which requires extracellular calcium (Scheinder et al. 1993). In order to assay the competence of mesodermal cells to give rise to specific tissue type,

disassociation experiments are performed. Gurdon et al., (1984) performed cell dispersion experiments on *Xenopus* embryos by culturing embryos in a calcium and magnesium free salt solution (CMFM) to disturb cell/cell adhesion. Miyahara et al., (1982) showed that late blastula and gastrula cells cultured under these conditions continued to divide and did not aggregate. It was also demonstrated that these cells continued to undergo normal levels of transcription and synthesis and that cell contact is not required for normal ribosomal RNA synthesis (Shiokawa et al., 1981; Miyahara et al., 1982). Experiments which inhibit cell/cell interactions for critical periods of development are used to determine the competence of cells at the time of the dispersion to later undergo differentiation. Sato and Sargent (1989) used this method to determine that cell/cell interactions are not necessary during blastula and gastrula stages for the ectoderm to have to competence to later differentiate into neural tissue.

In this study two types of embryonic manipulations were performed, dispersions and disassociations. For disassociation experiments embryos were placed in the CMFM at the two cell stage. They were cultured in CMFM until gastrula or until late neurula stages at which time the salts were re-administered. The embryonic cells remain in close contact with each other however, because of the

cation deficiency, no normal cell migration can take place. In this type of experiment, the embryo appears to be just a "bag of cells" however, when the salts are re-administered the cells re-aggregate and undergo some cell movement. In dispersion experiments the vitelline membrane was removed of embryos at blastula stages (during the MBT) and at early gastrula stages. The embryos were then cultured in CMFM and agitated frequently to assure cell dispersion. Salts were later re-administered and the cells allowed to re-aggregated. This type of experiment allows for the assessment of the ability of these manipulated cells to differentiate in the absence of normal cell/cell interactions for critical periods of development.

Marker Genes

The mid-blastula transition (MBT) is the onset of zygotic transcription. Many genes are transcribed that can be used as markers for the patterning and differentiation state of specific tissues with in the embryo. As discussed previously, the vascular system arises from mesodermal tissue. It is essential to have the ability to assay for the presence of mesodermal tissue. *Xbra* (*Xenopus brachyury*) encodes a DNA binding protein and is expressed throughout mesodermal tissue at blastula and gastrula stages. By late gastrula stages it expression is restricted to the notochord and

ventrolateral mesoderm (Smith et al., 1991). *Xbra* is used as a pan mesodermal marker (Smith et al., 1991). The vertebrate homeobox gene *goosecoid* is involved in dorsal/ventral patterning of the mesoderm and is expressed in the dorsal lip of mesodermal tissue (Cho et al., 1991; Niehrs et al., 1994).

Lemaire and Gurdon (1994) performed a series of disassociation experiments in which embryos were placed in the CMFM at the two cell stage and were completely dissociated from their neighbors at the 32 cell stage, then subsequently assayed for expression of various genetic markers (without re-aggregation) at gastrula stages. Mesoderm induction had been disrupted as indicated by the absence of *Xbra* expression, however *goosecoid* was expressed. This suggests that the differential distribution of maternal determinates leads to the induction cascade of *goosecoid* (Lemaire and Gurdon 1994). The other mesodermal derivatives such as muscle actin are also not expressed in embryos manipulated under these conditions (1994). If mesoderm induction is permitted to occur and cells are not dissociated until the later blastula stages then *actin* is transcribed at the normal time (Gurdon et al., 1984). *Actin* is a marker for equatorial mesoderm and is only expressed in its derivatives even after disassociation has occurred (Gurdon et al., 1984).

It has been shown that blood originates in ventral mesoderm (reviewed in Kelly et al., 1994). A standard marker gene for ventral mesoderm is embryonic *beta-globin* (Meyerhof et al., 1984; Kruep, 1997). Larval globin is first detectable by *in situ* hybridization at *Xenopus* stage 26, by hatching stages (stage 32) it marks the entire ventral blood island (Kelley et al. 1994). *GATA-2* is a zinc-finger transcription factor expressed in the ventral blood island precursors prior to differentiation and expression of *beta-globin* (Kelly et al., 1994; Walmsley et al., 1994). *GATA-2* expression occurs by default in mesodermal cells in the absence of dorsalizing signals (Kelly et al., 1994; Walmsley et al., 1994). *GATA-2* is expressed at low levels as a maternal transcription factor and later defines hematopoietic potential in ventral mesodermal cells (Kelly et al., 1994; Walmsley et al., 1994; Partington et al. 1997). *GATA-1*, a member of the same family is an erythroid specific transcription factor and is essential to differentiation, proliferation and survival of red blood cells and magakaryocytes (Pevny et al., 1991; Shivdasani et al., 1997; reviewed in Long et al., 1997 and Yamamoto et al., 1997). For the proliferation and survival of early hematopoietic cells *GATA-2* is required (Fong-Ying and Orkin, 1997). *GATA-2* is essential for the terminal differentiation mast cells but not of erythroid cells (Fong-Ying and

Orkin, 1997). In this study *GATA-2* is used as a marker of hematopoietic progenitors.

Marker genes specific to endothelial lineages are scarce. *Xl-fli* is a transcription factor in the *ets* family and is expressed in several lineages of migratory cells including endothelial cells (Meyer et al., 1993; Remy et al., 1996). The *Xl-fli* transcription factor is postulated to have a role in cell adhesion and matrix interactions (Meyer et al., 1993). The molecular markers genes *Xbra, goosecoid, actin, GATA-2, beta-globin, Xl-fli* and *XAngio* are used to assess the differentiation states of the mesodermal tissue of the manipulated *Xenopus* embryos in this study.

Hypothesis

Based upon the expression pattern of *XAngio* and some of the initial experiments it is hypothesized that a broad region of the mesoderm at blastula and gastrula stages is initially competent to form vascular endothelial tissue and that this competence is gradually restricted during development. There are two possible results of dispersion and disassociation experiments. If there were a positive induction event necessary at blastula and early gastrula stages for the mesoderm to gain the competence to form vasculature then embryos without normal cell/cell interactions at these stage will not express the

appropriate vascular markers. If however, in support of the hypothesis, at these stages a broad region of the mesoderm is already competent to give rise to vasculature and a restriction event is necessary then these manipulated embryos will retain their competence, even after manipulation and continue to express vascular endothelial marker genes.

Materials and Methods

Animals and Matings

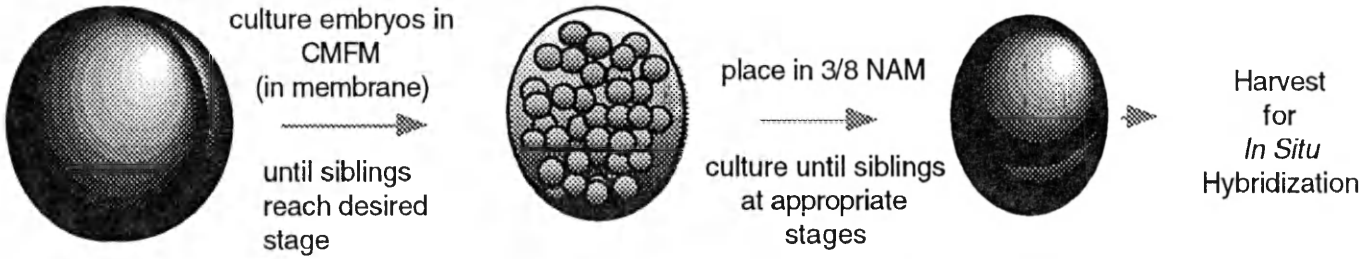
Albino *Xenopus laevis* frogs obtained from Xenopus I (Ann Arbor MI) are kept in sex segregated tanks at 20° C. Males were injected in the dorsal lymph sack with 400u and females with 600u of human chorionic gonadotrophic hormone (Sigma and Steris) in order to stimulate amplexus and fertilization of the ova. Embryos were collected 8-12 hours after injection of the adults. The embryos were then dejellied with a 2% cysteine 1M NaOH solution, rinsed three times and cultured in a 1/10 normal amphibian medium (NAM) which included 500ul/ml gentamicin sulfate solution (Slack, 1984). The embryos were cultured in glass petri dishes at 14° C or 16°C. The embryos were staged as described in Nieuwkoop and Faber (1967). All research involving animal subjects was approved by the Institutional Animal Care and Use Committee at the College of William and Mary.

Disassociation and Dispersions

The disassociations (Figure 1 A) were performed by taking embryos at the two cell stage and placing them into a calcium magnesium free solution (CMFM)(4M NaCl, 1M KCl, 0.1M $NaHCO_3$, 1MTris pH 7.6) to inhibit cell adhesion (Gurdon et al., 1984). Embryos

were cultured at 16°C until stage 10 (early gastrula) or until stage 18 (late neurula). Calcium and magnesium were re-administered by the addition of 3/8 NAM. Embryos were cultured until siblings reached stages appropriate for the expression of the desired genetic marker. The embryos to be assayed for expression of *Xbra* (Smith et. al., 1991), a pan mesodermal marker, and *goosecoid (*Hemmati-Brivanlou et al., 1990; Niehrs et al., 1994), a dorsal mesodermal marker, were fixed at late gastrula and early neurula stages (st. 11.5 - 13.5). *GATA-2* (Walmsley et al., 1994) is a marker expressed early in the presumptive ventral blood islands. Embryos to be assayed for *GATA-2* expression were fixed at tailbud stages (st. 24-26). *XAngio (XMR*, Devic et al., 1996; Drysdale et al., 1997) is a marker for presumptive endothelial cells and is optimally expressed at hatching stages (st. 29-31). *Xl-fli* (Meyer et al., 1995) is also a marker for (but not restricted to endothelial cells) and is expressed at hatching stages (st. 29-31). *Muscle actin* (Gurdon et al., 1984, Hemmati-Brivanlou et al., 1990) is synthesized by those cells whose origin is equatorial mesoderm and is expressed strongly at hatching stages (st. 29-34) in somitic mesoderm. The embryonic blood marker *beta-globin* (Kelly et al., 1994) is expressed in the ventral blood islands at late tailbud stages (st. 33-34). The origins of the blood islands are predominately ventral mesoderm.

DISASSOCIATION

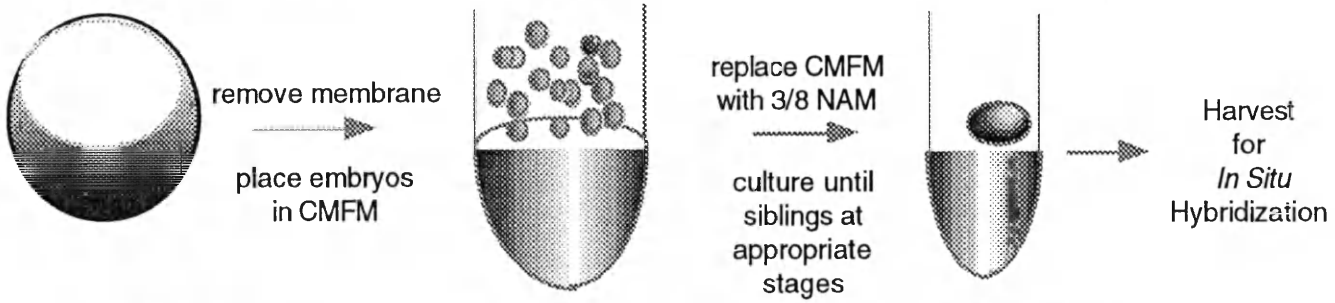


Two Cell Stage
St. 2

Gastrula/Neurula Stages
St. 10 and 18

Neurula/Hatching Stages
St. 12.5, 24, 30, 34

DISPERSION



Blastula/Early Gastrula Stages
St. 7 and 10

Gastrula/Neurula Stages
St. 11 and 12.5

Neurula/Hatching Stages
St. 12.5, 24, 30, 34

Figure 1

If the manipulated embryos were to be assayed for the expression of two of the above marker genes (two color *in situ* hybridization) then they were fixed at stages optimal for the detection of the RNA transcripts to be used. Embryos to be assayed for *XAngio* and *actin* were fixed at hatching stages (st. 29-31). Those assayed for *XAngio* and *GATA-2* or *actin* and *GATA-2* were fixed at late organogenesis (st. 24-26). Those assayed for *XAngio* and *Xbra* were fixed at late gastrula stages (st. 11.5-12). Those used for an assay of actin and beta-globin were fixed at early tailbud stages (st. 33-34). Embryos were fixed for in situ hybridization in 1X MEMFA (0.1M MOPS, pH 7.4, 2 mM EGTA, 1mM MgSO4, 3.7% formaldehyde) for 1-2 hours and stored in 100% EtOH at -20° C for 1-2 weeks before use in *in situ* hybridization.

Dispersions (Figure 1 B) were performed by taking embryos at stage 7 (blastula) and stage 10 (gastrula) and removing the vitelline membrane with fine forceps. The embryos were placed individually into eppendorfs tubes with a lining of 100*ul* of 1% agarose equilibrated with CMFM and 500*ul* of CMFM solution. The embryos were cultured from stages 7-11 (blastula to gastrula) or st.10-12.5 (gastrula to neurula) and agitated frequently to assure cell dispersal. The tubes were then spun at 600-800rpm in a standard clinical centrifuge for 3

minutes to concentrate cells at the bottom of the tube. Salts were then added to bring to a concentration of 3/8 NAM. Aggregates were cultured until stages when siblings expressed appropriate genetic markers as specified above. Embryos were fixed for *in situ* hybridization in 1X MEMFA for 1-2 hours and stored in 100% EtOH at -20^0 C for 1-2 weeks before use in *in situ* hybridization.

In Situ Hybridization

In situ hybridization was performed essentially as described in Harland (1991). Modifications include: precipitation rather than column purification, omission of the hydrolysis step, and replacement of ^{32}P with tritium labeled UTP. Plasmid DNA was isolated by centrifugation, alkaline lysis and PEG precipitation from positive bacterial colonies grown at 37^0 C in a culture of LB and 50*u*l/ml ampicillin as described in Sambrook et al.(1989). The plasmid DNA was linearized by restriction digest with BamH1 (*XAngio, Beta-globin*), EcoR1 (*Goosecoid, Actin*), EcoRV (*Xbra*), HindIII (*Gata-2*) and Sma1 (*Xfli*). Antisense RNA probes were synthesized using RNA polymerase T7(Promega) for *goosecoid, XAngio, beta-globin,* and *Xbra*. RNA polymerase T3 (Promega) was used for *GATA-2* and *Xfli,* and SP6 (Promega) was used for *actin* as described in Melton et al., 1985. Incorporation of 3H-UTP was determined using Whatman DE-81

filters in a liquid scintillation counter assay. Digoxygenin- 11-UTP or fluorescein-12-UTP (Boehringer Mannheim, Germany) was used in probe synthesis for later antibody recognition.

For the detection of a single specific RNA transcript (one color *in situ* hybridization) two substrates were used simultaneously in the color reaction. Nitro blue tetrazolium (NBT) (Sigma) and 5-bromo-4-choloro-3-indolyl-phosphate (BCIP) (Sigma) produce a dark purple stain when catalyzed by the alkaline phosphatase couple to the anti-digoxigenin or anti-fluorescein antibody. For the detection of two different RNA transcripts (double color *in situ* hybridization) , one probe was synthesized with a digoxygenin- 11-UTP and the other with a fluorescein-12-UTP. Both probes were added following the prehybridization at a concentration of 2ug/ml. The first antibody incubation was performed with the anti-digoxigenin or anti-fluorescein that corresponded to the least abundant of the two transcripts. The first of the two color reactions used 3.5ul BCIP (5-bromo-4-choloro-3-indolyl-phosphate; 50 mg/ml in 100% dimethyl formamide)/ml of alkaline phosphatase (AP) buffer (100mM Tris, pH9.5, 50mM $MgCl_2$, 100mM NaCl, 0.1% Tween20, and 5mM Levamisol). The substrate BCIP produces a turquoise color. After signal became visible the embryos were fixed 6 hours in 1X MEMFA. This was followed by three fifteen minute washes in phosphate buffered saline (PBS) and

one thirty minute wash in 1X PBS at 65°C. Embryos were washed twice for fifteen minutes in malic acid buffer (MAB, 100mM maleic acid, 150mM NaCl, pH 7.5), fifteen minutes to one hour in MAB and 2% Boehringer Mannheim Blocking Reagent (BMB). This was followed by an incubation for one hour in MAB, 2% BMB and 20% lamb serum as described in the one color protocol. A 1/2000 dilution of anti-digoxigenin or anti-fluorescein (the antibody not used for the first color reaction) was added to a MAB, 2% BMB and 20% lamb serum solution and incubated overnight at 4°C. The excess antibody was removed by at least five one-hour washes in MAB. The embryos were then washed twice in AP buffer and incubated at 37°C in 3.5 ul 5-bromo-6-chloro-3-indolyl phosphate (magenta-phosphate; Molecular Probes) 50mg/1000ul formamide/ml of AP buffer until color appeared. Magenta- phosphate produces a pink color. They embryos were then fixed in 1X MEMFA for 6 hours and stored at 4°C in 1X PBS. Sibling control embryos were placed in the same tubes as the manipulated embryos as an additional level of control.

Embryos were dehydrated in methanol and cleared in a 2:1 benzyl benzoate: benzyl alcohol solution and photographed using Ektachrome 160T slide film (Kodak) on an Olympus ST-PT dissecting microscope. All work involving radioactive materials was approved by the Radiation Safety Officer at the College.

Results

The Mesodermal Marker Xbra

The manipulated embryos were assayed for the expression of the pan mesodermal marker *Xbra* (figure 2) to confirm the presence of mesodermal tissue after manipulation. Embryos dispersed from stages 7 through 11 (figure 2, B and C) as well as from stages 10 through stage 12.5 (figure 2, C and D) show a widespread punctate expression pattern of the pan mesodermal marker *Xbra*. Embryos disassociated from stage 2 to stage 10 (figure 2, F and G) show high levels of *Xbra* stain in a pattern localized to a region of the embryo. This confirms the presence of mesodermal tissue in these manipulated embryos. Embryos disassociated from 2 through 18 were not assayed using this marker because *Xbra* expression pattern is restricted at these later stages to the notochord and ventrolateral mesoderm (Smith et al., 1991).

Regional Mesodermal Markers *Goosecoid, Actin* and *GATA-2*

Goosecoid, actin and *GATA-2* show more restricted patterns of expression and were used to assay for the presence of regional mesodermal tissue. *Goosecoid* (Figure 3 A) is a marker restricted to the most dorsal mesoderm. Embryos dispersed from stage 7 through

11 (Figure 3, B and C) show expression of this marker. Embryos dispersed from stage 10 through stage 12.5 (Figure 3, D and E) show a lighter pattern of expressing in a few regions of the aggregate. Embryos disassociated from stage 2 through stage 10 (Figure 3, F and G) show expression of *goosecoid* that appears to be limited to a smaller area of the embryos than *Xbra*, according to its more regionalized specificity. *Goosecoid* expression in these embryos is localized in a capping pattern.

Actin (figure 4 A) is a derivative of equatorial mesoderm and is expressed in somites. *Actin* is expressed in a punctate pattern in the embryos dispersed from stage 7 through stage 11(Figure 4, B and C) as well as the embryos dispersed from stage 10 through 12.5 (Figure 4, D and E). Embryos disassociated from stage 2 through stage 10 (Figure 4, F and G) show very strong actin expression often expressed in one or two bands along one side of the manipulated embryo, reminiscent of somite formation. Embryos disassociated from stage 2 through 18 (Figure 4, H and I) show *actin* expression in localized regions of the embryo.

GATA-2 is a marker for ventral mesoderm and is expressed in the ventral region of the control embryo (Figure 5 A). *GATA-2* is expressed in a diffuse punctate pattern in the embryos dispersed from both stages 7 through 11 (Figure 5, B and C) as well as 10 through

12.5 (Figure 5, D and E). Expression is also shown in the embryos disassociated from stages 2 though 10 (Figure 5, F and G) as well as stages 2 through 18 (Figure 5, H and I).

In dispersed embryos the mesoderm is distributed in a punctate pattern throughout the aggregate. However, in disassociated embryos the mesoderm is restricted to a region of the manipulated embryo in a capping pattern. The expression of these mesodermal markers in both dispersion manipulation types (7-11 and 10-12.5) as well as both disassociation manipulation types (2-10 and 2-18) shows that mesoderm is induced (*Xbra* expression) and to some degree patterned (*GATA-2* ventral marker, *actin* equatorial marker and *goosecoid* dorsal marker) in these manipulated embryos.

The Vascular Marker Genes *XAngio* and *Xl-fli*

XAngio (Figure 6 A) is the endothelial precursor marker gene and is expressed in the developing vasculature of the hatching stage embryo. Embryos dispersed from stage 7 through stage 11 (Figure 6, B and C) as well as embryos dispersed from stage 10 to stage 12.5 (Figure 6, D and E) show high levels of punctate *XAngio* expression. Embryos disassociated from stage 2 to stage 10 (Figure 6, F and G) as wells as from stage 2 through stage 18 (Figure 6, H and I) show high levels of *XAngio* stain in a localized in a capping pattern. The levels of staining appear to encompass a significantly greater proportion of the

embryos than the levels of staining in the sibling control (Figure 6 A) in which the staining is restricted to the developing vasculature. The absence of normal inductive interactions has not lead to the inhibition of the expression of the vascular marker gene *XAngio.*

Xl-fli (Figure 7) is an addition vascular marker gene but is not restricted to vascular tissue. There is a capping expression pattern in the embryos disassociated from stage 2 though 10 (Figure 7, F and G) a well as those from stage 2 though 18 (Figure 7, H and I). *Xl-fli* is a non-abundant transcription factor. The punctate pattern of expression seen in the dispersed embryos both stages 7 through 11 (Figure 7, B and C) as well as stages 10 through 12.5 (Figure 7, D and E) more diffuse with significantly higher levels of back ground than the pattern seen with *XAngio*. *XAngio* expression creates a strong signal and is specific to endothelial precursors unlike *Xl-fli*, thus for future experiments *XAngio* the most appropriate choice for a vascular marker gene.

The Blood Marker Gene *Beta-globin*

Beta-globin is expressed at very high levels in the ventral blood islands of *Xenopus* embryos (Figure 8 A). Dispersed embryos, from both manipulation either dispersions stages 7 through11 (Figure 8 B) and stages 10 though 12.5 (Figure 8 C) or disassociations from stages 2

through 10 (Figure 8 D) or 2 though 18 (Figure 8 E), assayed for *beta-globin* expression have no detectable signal. The absence of the *beta-globin* signal suggests that there are different inductive event necessary for the formation of the blood and endothelial precursors.

Two Color *In Situ* Hybridizations

Two color *in situ* hybridizations were performed in order to assess the spatial relationship between the expression patterns of gene combinations. *GATA-2* and *actin* as well as *beta-globin* and *actin* show discrete expression patterns in control embryos. *Actin* is specific to the developing in the somites which have an equatorial mesodermal origin. *GATA-2* and *beta-globin* are specific to areas of ventral mesodermal origin, the presumptive and realized ventral blood islands respectively. The expression patterns of *XAngio* and *actin* overlap. The dispersed embryos, stages7 through 11 (Figure 9 B) and stages 10 though 12.5 (not pictured) show punctate expression patterns for both *XAngio* and *actin*. However, *XAngio's* pattern is more widespread than *actin*. The disassociated embryos (stage 2 though 10) show a broad capping patterning of *XAngio* expression (turquoise) with a region within the *XAngio's* region strongly expressing actin (magenta). *Actin* is not expressed in areas of the embryos devoid of *XAngio* expression. These results suggest that in these manipulated embryos

that *XAngio's* expression pattern has remain broad (relative to *actin*) and has not been restricted as it has been in the sibling control.

Similar results are seen with the expression pattern of *XAngio* and *GATA-2*. *GATA-2* and *XAngio* both show punctate expression patterns in dispersed embryos (Figure 9 E) but *XAngio* (turquoises) is more broadly expressed with *GATA-2* restricted to regions of coexpression with *XAngio*. These regions appear in a dark blue color which is mix of *XAngio's* turquoise and *GATA-2's* magenta. In the disassociated embryos (Figure 9 F) *XAngio* (turquoise) is expressed though the capping region. With the broad *XAngio* region there is a localized region of coexpression with *GATA-2*. Once again *XAngio's* expression pattern remains widespread with in the manipulated embryos and showing regional colocalization with a regional mesodermal marker.

The two regional mesodermal markers used (*GATA-2* and *actin*) do not show coexpression. The dispersed embryos (stages 10 through 12.5) show the punctate patterns of expression for both GATA-2 and actin (Figure 9 H) as seen earlier in the single gene assays. However, both of the signals the actin (magenta) and the GATA-2 (turquoise) remain distinct. In the disassociated embryos (stage 2 though 10; figure 9 I) there is the capping pattern seen earlier however, the *GATA-2* (turquoise) signal is adjacent to the *actin* signal

(magenta). This suggests that the region specificity is maintained in the manipulated embryos.

Beta-globin and *actin* are two abundant genes expressed in discrete regions of the tailbud stage embryos (Figure 9 M). Both the dispersed embryos (stages 7 though 11; Figure 9 N) as well as the disassociated embryos (stages 2 though 10; Figure 9 O) shows *actin* expression seen in previous experiments, however, *beta-globin* is again not expressed. In two individuals (not shown), one dispersed from stages 10 through 12.5 and one disassociated from stages 2 though 10 a *beta-globin* signal (magenta) was seen adjacent to an *actin* signal (turquoise). These results suggest that dispersion and disassociation affects the normal expression of the blood marker *beta-globin* without inhibiting *actin* expression.

XAngio and *Xbra* have similar specifies in the gastrula stage embryo. The control embryo (Figure 9 J) shows the *Xbra* stain in magenta and *XAngio* stain in turquoise. The expression pattern of these two genes over lap through out most of the mesoderm which creates a dark blue color. However, *Xbra* is exclusively expressed in the most dorsal mesoderm (presumptive notochord) which creates a magenta crescent around the blastopore (see arrow on Figure 9 J). Coexpression of the two genes is seen the dispersed embryos (stages 7 through 11; Figure 9 K) which appears in a dark blue stain throughout

the aggregate. Coexpression is also seen in the disassociated embryos (stages 2 though 10; Figure 9 L) in the dark purple staining localized in the same capping pattern seen earlier in the assays for individual gene expression.

XAngio's expression pattern remains widespread throughout the mesoderm despite it restriction in sibling control. These results support the prediction that a broad region of mesoderm is competent to become vascular endothelium and that in the absence of the normal inductive interactions that lead to the restrictions of this potential, the mesodermal tissue retains this competence. There also appear to be distinct inductive events necessary for the formation of the endothelial cells and the blood cells.

Figure 2. *In situ* hybridization assaying for the expression of the pan mesodermal marker gene *Xbra*.

A Sibling control stage 13.5 (neural plate stages). *Xbra* signal is seen in the notochord (indicated by the arrow) and the ventrolateral mesoderm. Anterior is to the upper left and posterior to the lower right (40X). **B, C.** Embryos dispersed from stage 7-11 assayed for *Xbra* expression. Dark purple staining is seen in a broad punctate pattern (B, 40X. C, 30X). **D, E.** Embryos dispersed from stage 10-12.5. Staining is seen in a broad punctate pattern. (D, 40X. E, 30X) **F, G.** Embryos disassociated from stage 2-10. Staining is seen in the a localized capping pattern (indicated by the arrow). Embryos in **G** are seen at various orientations (F, 40X. G, 25X).

XBra

CONTROL
STAGE 13.5

DISPERSION
STAGE 7-11

DISPERSION
STAGE 10-12.5

DISASSOCIATION
STAGE 2-10

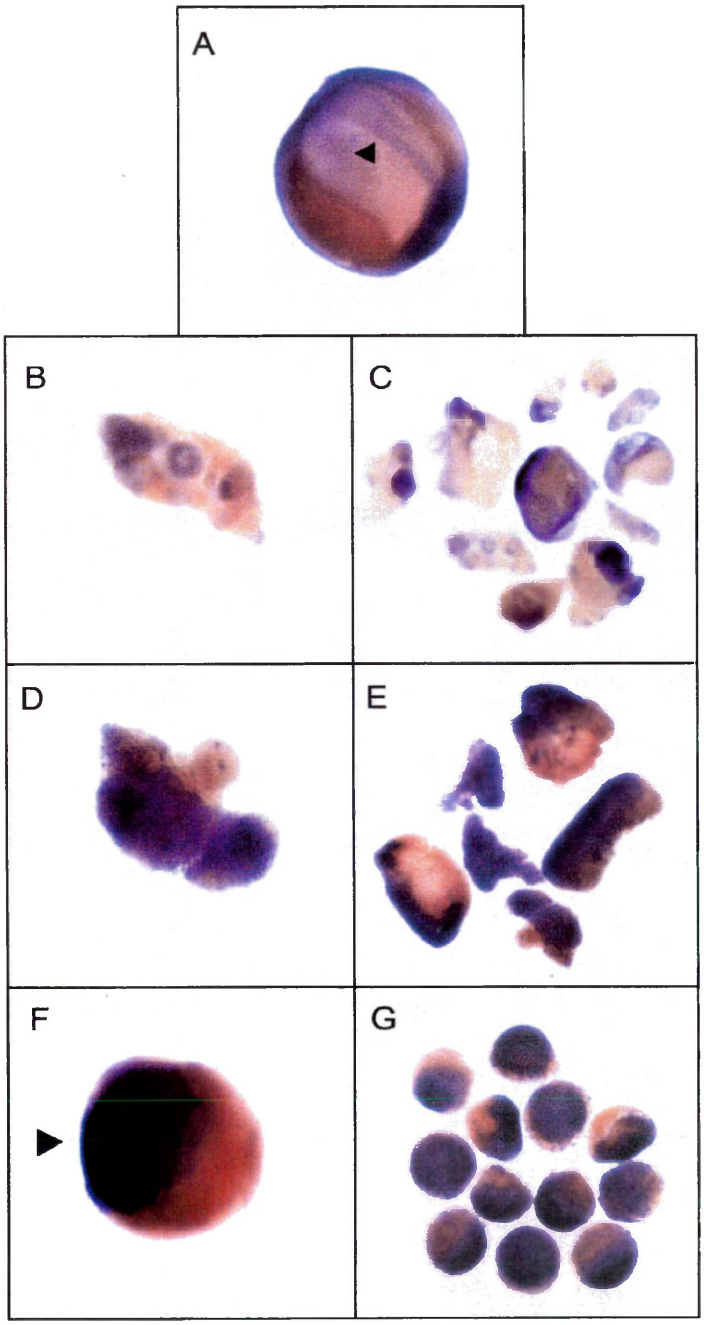


Figure 3. *In situ* hybridization assaying for the expression of the dorsal mesodermal marker gene *goosecoid.*

A. Sibling control stage 12.5 (neural plate stage). Signal is seen localized in the dorsal mesoderm (indicated by the arrow) (40X). **B, C**. Embryos dispersed from stage 7-11. Staining is seen in a punctate pattern throughout the aggregate (B, 40X. C, 35X). **D, E.** Embryos dispersed from stage 10-12.5. Very light signal is apparent in a punctate pattern throughout the aggregate (indicated by the arrow) (D, 40X. E 30X). **F, G**. Embryos disassociated from stage 2-10. Staining is seen in the a localized capping pattern. Embryos in **G** are seen at various orientations (F, 40X. G, 30X).

Goosecoid

CONTROL
STAGE 12.5

DISPERSION
STAGE 7-11

DISPERSION
STAGE 10-12.5

DISASSOCIATION
STAGE 2-10

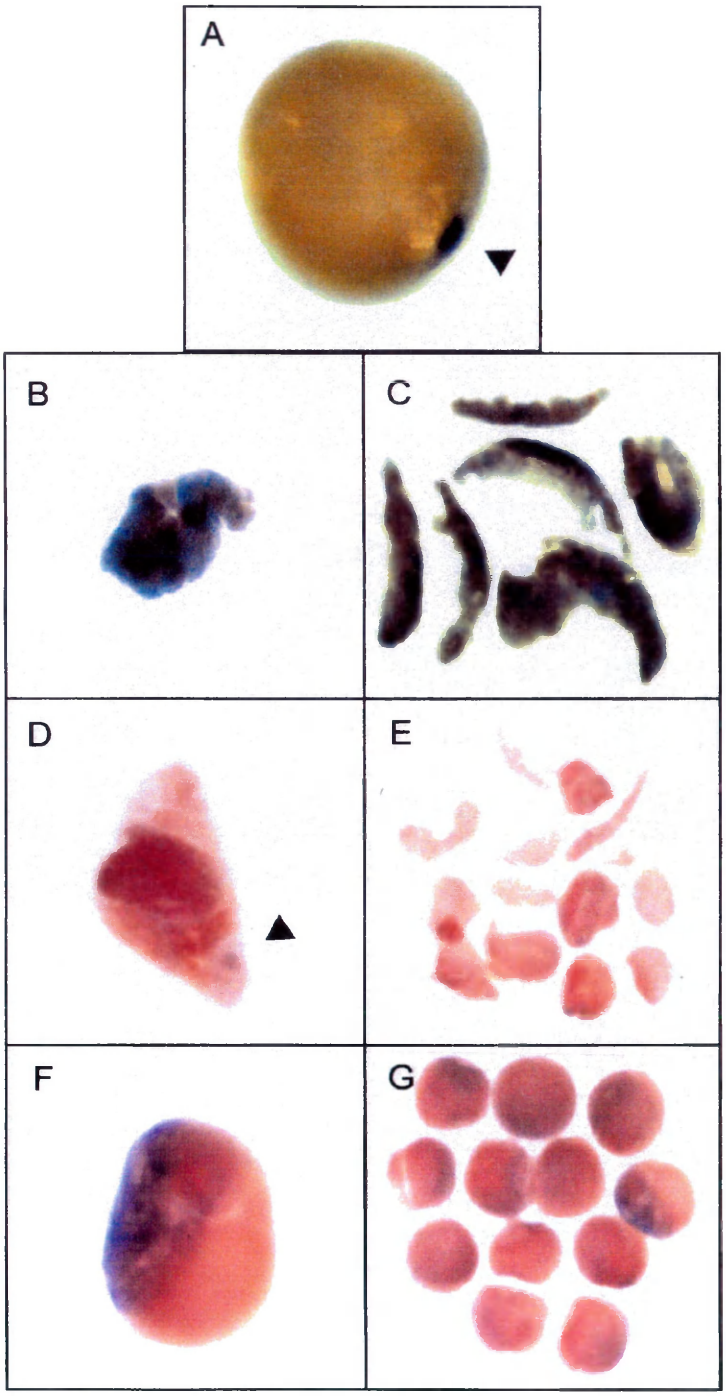


Figure 4. *In situ* hybridization assaying for the expression of the equatorial mesodermal marker gene *actin.*

A. In the sibling control (stage 30) hatching stage a signal is seen in the somites (dorsal side of the embryo is facing up) and heart (35X). **B, C.** Embryos dispersed from stage 7-11. A strong punctate signal is seen throughout the aggregate (B, 40X. C, 35X). **D, E.** Embryos dispersed from stage 10-12.5. Staining is seen in a broad punctate pattern (D, 40X. E, 35X). **F, G.** Embryos disassociated from stage 2-10. Staining is seen in a localized capping pattern in a band along one side of the embryo. Embryos in **G** are seen at various orientations (F, 40X. G 25X). **H, I.** Embryos were disassociated from stage 2-18. *Actin* staining is seen in a capping pattern in one region of the embryo. Embryos in **I** are seen at various orientations (H, 40X. I, 23X).

Actin

CONTROL
STAGE 30

DISPERSION
STAGE 7-11

DISPERSION
STAGE 10-12.5

DISASSOCIATION
STAGE 2-10

DISASSOCIATION
STAGE 2-18

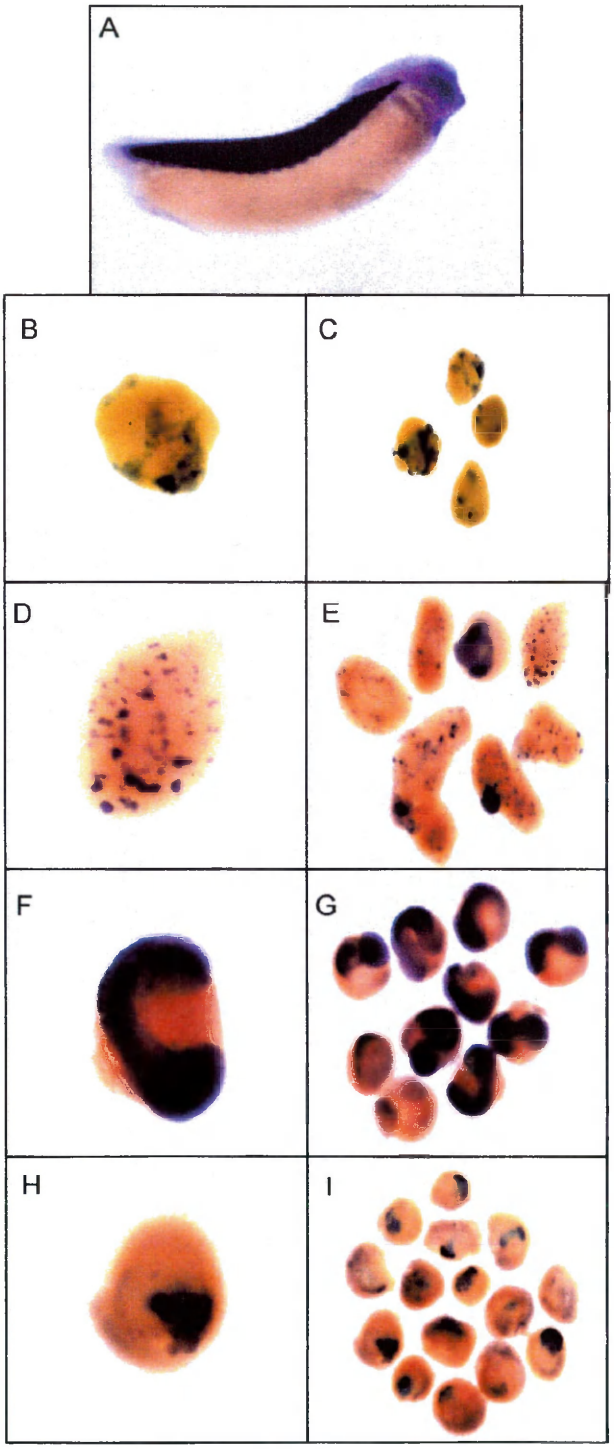


Figure 5. *In situ* hybridization assaying for the expression of the ventral mesodermal marker gene *GATA-2*

A. In the sibling control stage 25 (tailbud) the staining is seen in the ventral region (presumptive ventral blood islands which are indicated by the arrow) (38X) **B, C.** Embryos dispersed from stage 7-11. Staining is seen in a diffuse punctate pattern throughout the aggregate (B, 40X. C, 30X.). **D, E.** Embryos dispersed from stage 10-12.5. Signal is apparent in a diffuse punctate pattern throughout the aggregate (D, 40X. E, 35X). **F, G.** Embryos disassociated from stage 2-10. Staining is seen in the a localized capping pattern (indicated by the arrow). Embryos in **G** are seen at various orientations (F, 38X. G30X) **H, I**. Embryos were disassociated from stage 2-18. *GATA-2* staining is seen in a capping pattern in one region of the embryo (indicated by the arrow). Embryos in **I** are seen at various orientations (H 40X, I 30X).

GATA-2

CONTROL
STAGE 24

DISPERSION
STAGES 7-11

DISPERSION
STAGES 10-12.5

DISASSOCIATION
STAGES 2-10

DISASSOCIATION
STAGES 2-18

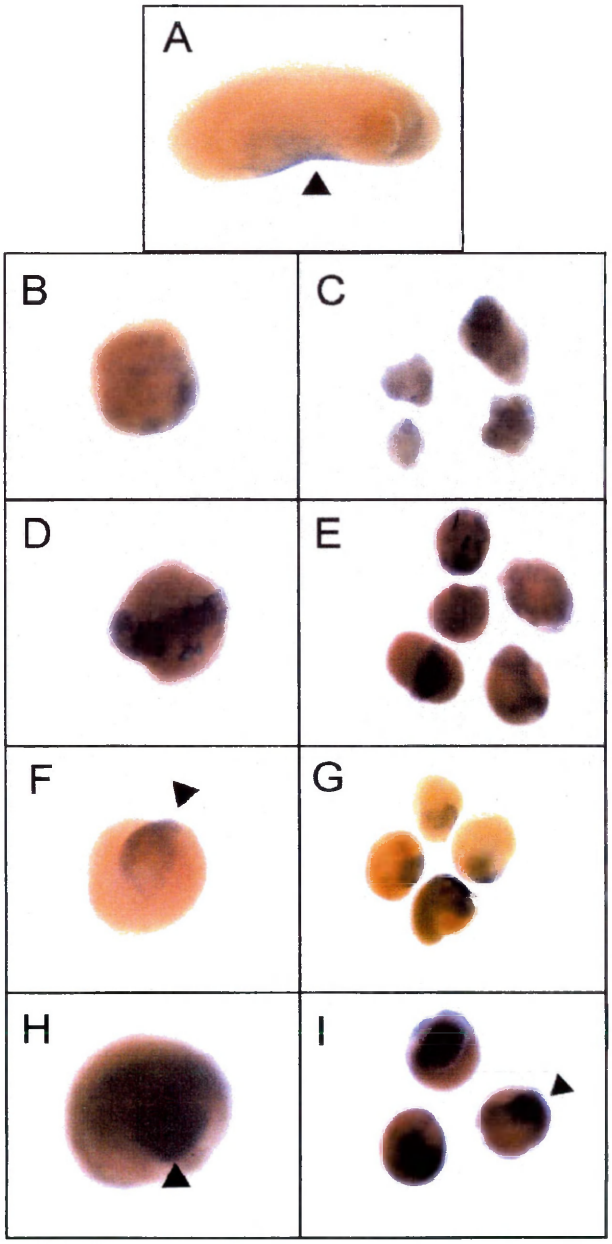


Figure 6. *In situ* hybridization using the vascular endothelial marker gene *XAngio*.

A. Sibling control stage 30 (hatching). *XAngio* staining (dark purple) seen in the tailbud (left facing), head vasculature and forming vasculature, common cardinal veins and endocardium (30X). **B, C.** Aggregates of embryos dispersed from stage 7-11 and cultured until stage 30. Staining seen throughout the aggregate in a broad dark purple punctate pattern (B, 40X. C, 35X). **D** (40X), **E** (35 X). Aggregates of embryos dispersed from stage 10-12.5. Staining seen throughout the embryo in a broad punctate pattern (D, 40X. E 35). **F, G.** Embryos were disassociated from stage 2-10. *XAngio* staining is seen in a capping pattern in one region of the embryo (F, 40X. G, 30X) Embryos in **G** are seen at various orientations. **H, I.** Embryos were disassociated from stage 2-18. *XAngio* staining is seen in a capping pattern in one region of the embryo (H, 40X. G, 30X) Embryos in **I** are seen at various orientations.

XAngio

CONTROL
STAGE 30

DISPERSION
STAGE 7-11

DISPERSION
STAGE 10-12.5

DISASSOCIATION
STAGE 2-10

DISASSOCIATION
STAGE 2-18

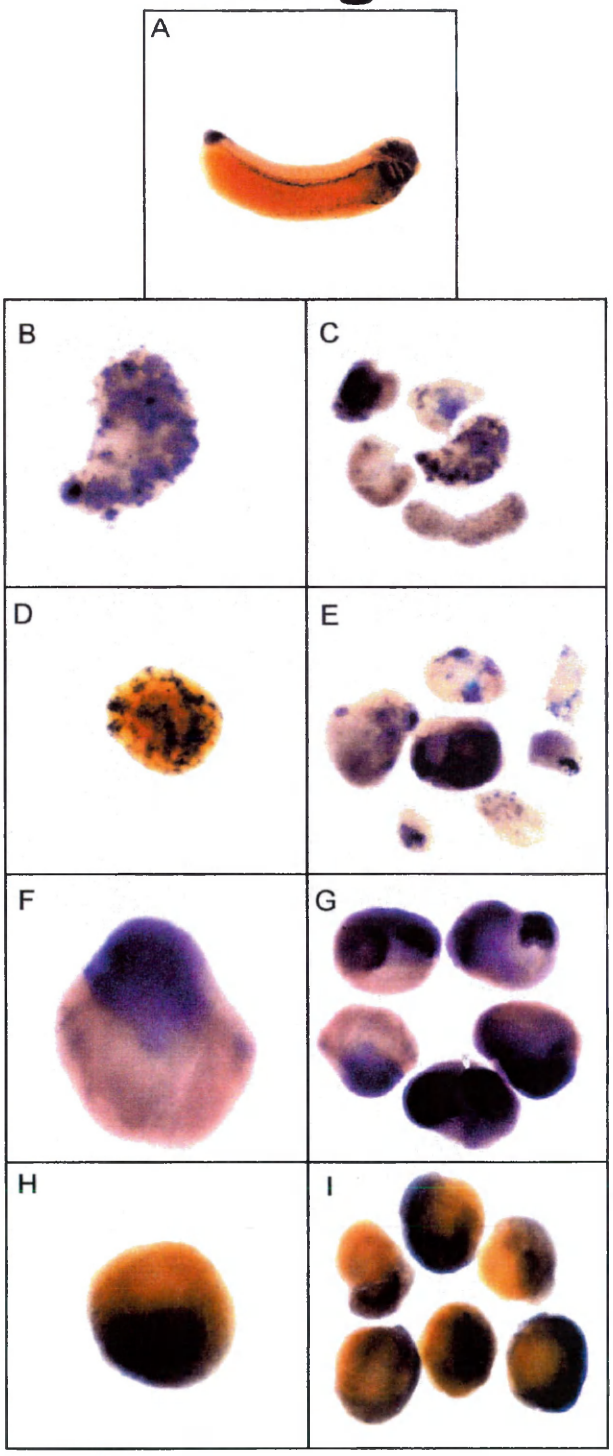


Figure 7. *In situ* hybridizations assaying for the expression of the vascular marker gene *Xfli.*

A. In the sibling control stage 30 (hatching stages) the signal is seen in the presumptive vasculature (35X). **B, C.** Embryos dispersed from stage 7-11. Staining is seen in a diffuse punctate pattern throughout the aggregate (B, 40X. C, 30X). **D, E**. Embryos dispersed from stage 10-12.5. Signal is apparent in a punctate pattern throughout the aggregate (D, 40X. E, 30X). **F, G**. Embryos disassociated from stage 2-10. Staining is seen in the a localized capping pattern. Embryos in **G** are seen at various orientations (F, 35X. G, 20X) **H, I.** Embryos were disassociated from stage 2-18. *Xfli* staining is seen in a capping pattern in one region of the embryo. Embryos in **I** are seen at various orientations (H, 40X. I 25X).

XFli

CONTROL
STAGE 30

DISPERSION
STAGE 7-11

DISPERSION
STAGE 10-12.5

DISASSOCIATION
STAGE 2-10

DISASSOCIATION
STAGE 2-18

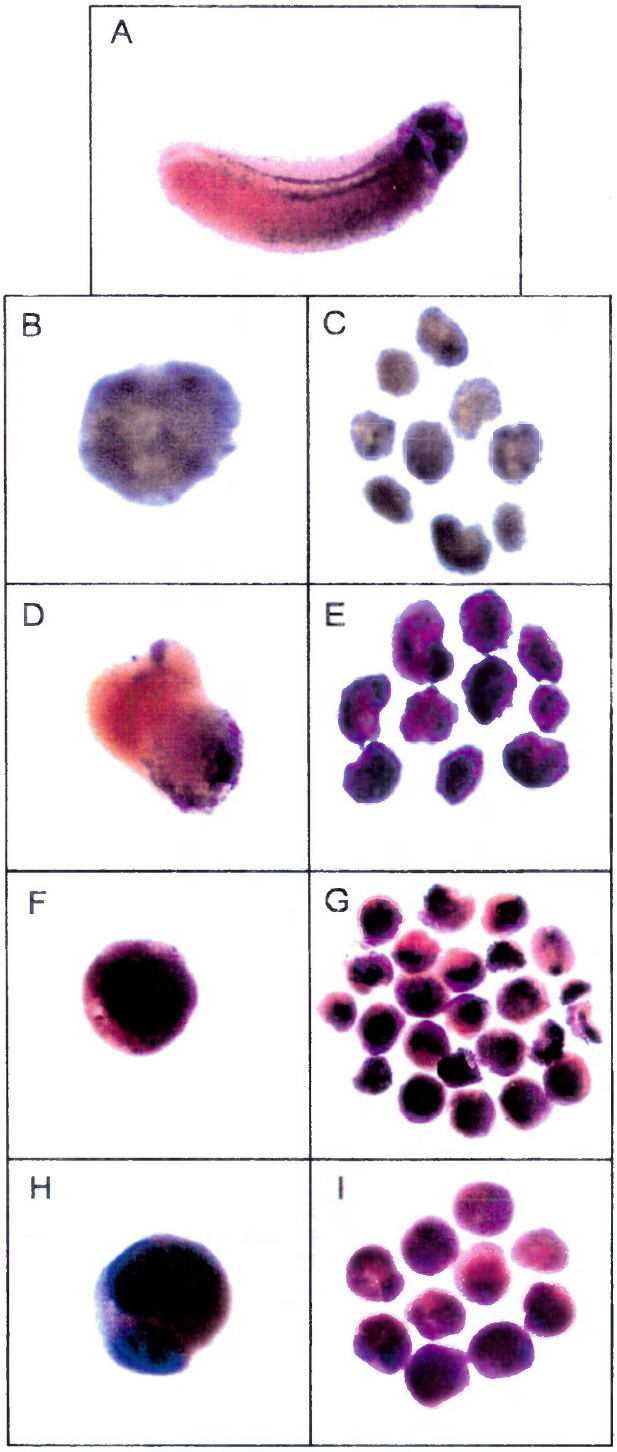


Figure 8. *In situ* hybridization assaying for the expression of the blood marker gene *beta- globin.*

A. In the sibling control (stage 34) the staining is seen in the ventral blood islands (38X). **B**. Embryos dispersed from stage 7-11. No signal is detected (35X). **C.** Embryos dispersed from stage 10-12.5. No signal is detected (30X). **D.** Embryos disassociated from stage 2-10. No signal is detected (30X). **F.** Embryos were disassociated from stage 2-18. No signal is detected (27X).

Beta-globin

CONTROL
STAGE 35

DISPERSION
STAGE 7-11

DISPERSION
STAGE 10-12.5

DISASSOCIATION
STAGE 2-10

DISASSOCIATION
STAGE 2-18

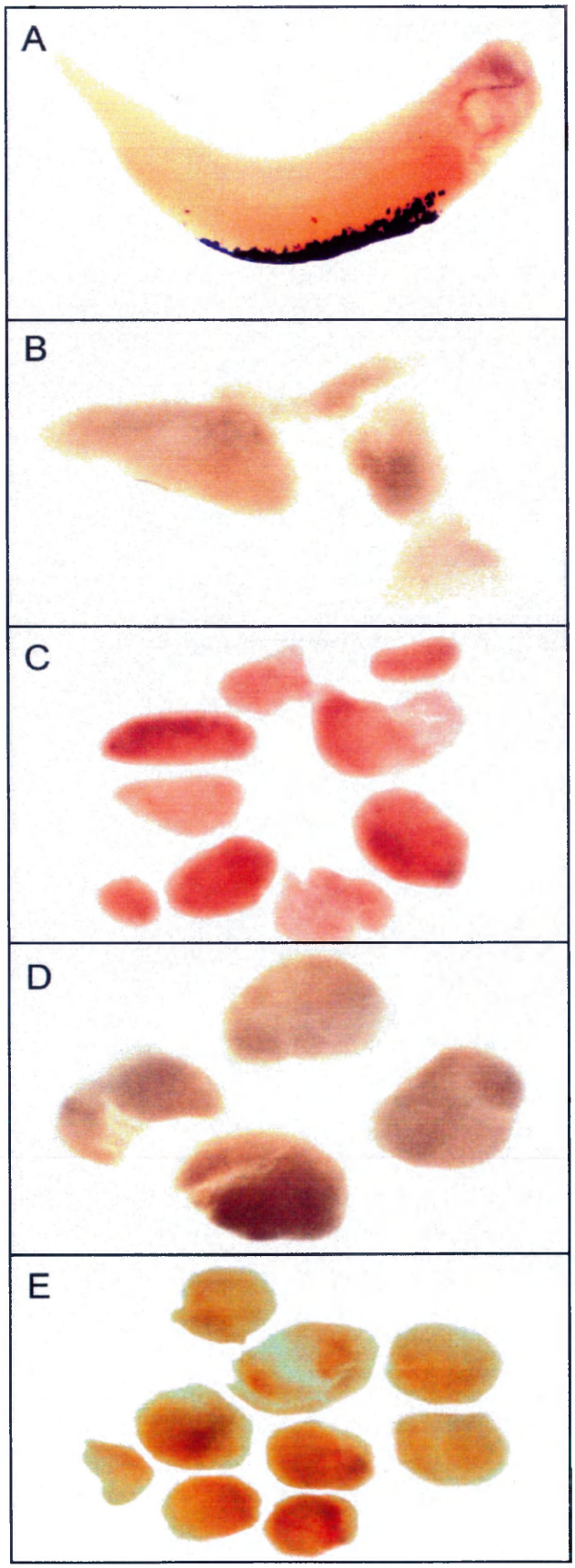
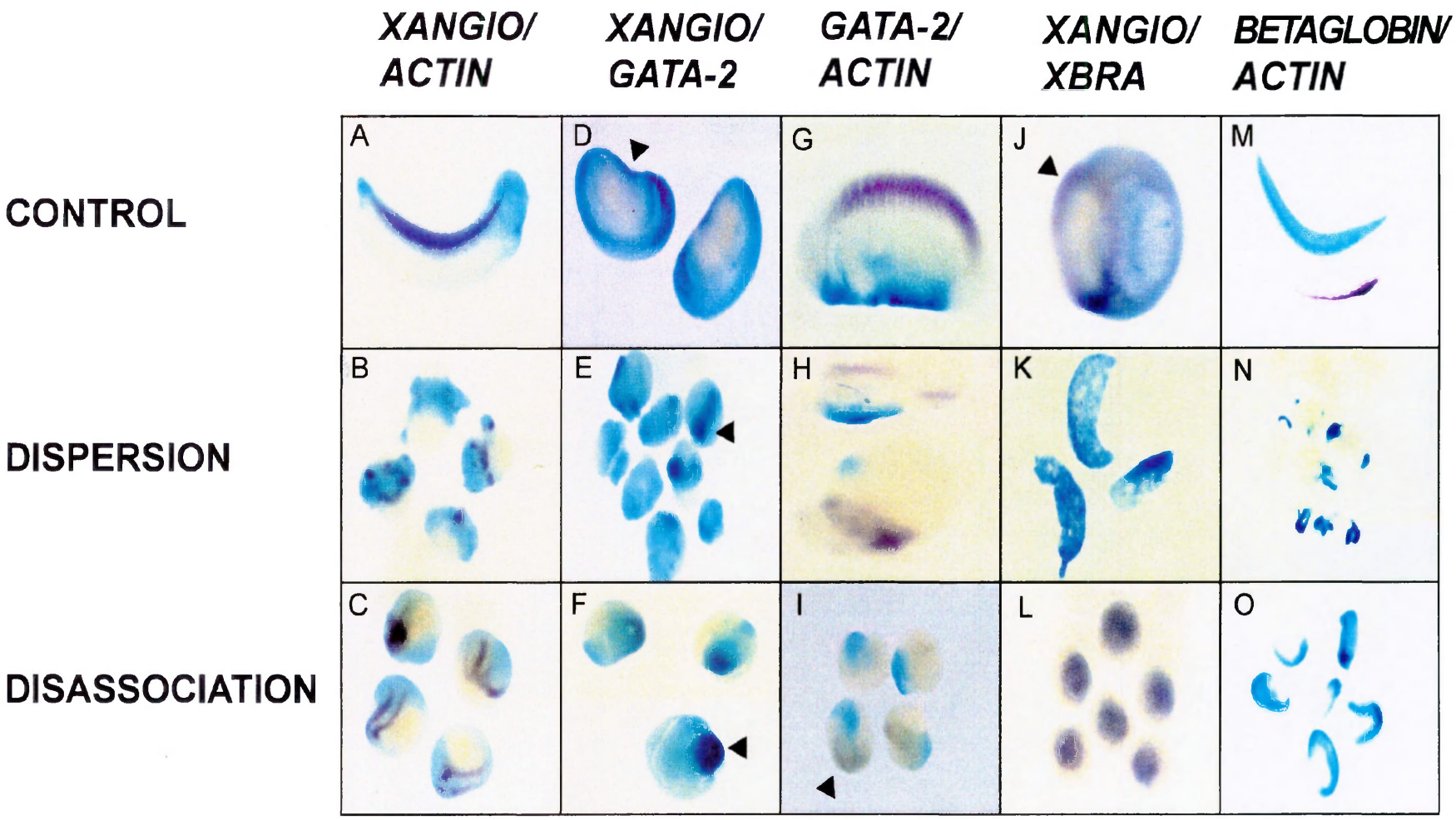


Figure 9. *In situ* hybridization assaying for the expression of two different transcripts.

A. The sibling control stage 30 is assayed for the expression of *XAngio* (turquoise) and *actin* (magenta). *XAngio* (turquoise) is seen in the head vasculature and tailbud. *Actin* (magenta) is seen in the somites (35X). **B.** Embryos dispersed from stage 7-11. *XAngio* (turquoise) expression is seen in a broad punctate pattern. *Actin* (magenta) expression is seen in a similar but not as wide spread punctate pattern showing coexpressed with *XAngio* (35X) **C.** Embryos disassociated from stage 2-10. *Actin* (magenta) expression is seen in bands or in a capping pattern. *Actin* (magenta) is coexpressed within the broader expression region of *XAngio* (turquoise) (30X). **D**. Sibling controls stage 24. Expression of *XAngio* (turquoise) is seen in a broad range of mesodermal derivated; tailbud, head mesoderm, lateral plate mesoderm. *GATA-2* (magenta, indicated by the arrow) expression is seen in the ventral region of the embryos (ventral is facing up) (35X). **E**. Embryos dispersed from stage 10-12.5. *XAngio (*turquoise) staining is seen a broad punctate pattern. *GATA-2* (magenta) and *XAngio* (turquoise) are coexpressed in various area of the aggregate creating a dark blue signal (indicated by the arrow) (33X). **F.** Embryos disassociated from stage 2-10. *XAngio* (turquoise) staining is seen in a broad capping pattern. *GATA-2* (magenta) is coexpressed with *XAngio* (turquoise) in a localized region creating a dark blue signal (indicated by the arrow) (30X). **G**. Sibling control stage 24. *Actin (*magenta) staining is seen in the developing somites. *GATA-2* (turquoise) is seen in the ventral regions (40X). **H.** Embryos dispersed form stage 10-12.5. *Actin* (magenta) signal is seen in a punctate pattern. *GATA-2* signal is seen in a punctate pattern not coexpressed with *actin* (40X). **I.** Embryos disassociated from stage 2-10. *GATA-2* (turquoise) expression is seen in a localized capping pattern. *Actin* (magenta) expression is seen adjacent to but not colocializaed with the *GATA-2* signal (indicated by the arrow) (30X). **J.** Sibling control stage 11.5. *Xbra* (magenta) expression is seen in a cresant shape around the dorsal portions of the blastopore (facing right, indicated by the arrow). XAngio (turquoise) expression is colocalized with the *Xbra* expression throughout the rest of the mesoderm creating a dark blue signal (40X). **K.** Embryos dispersed from stage 7-11. XAngio (turquoise) and *Xbra* (magenta) expression are coexpressed in a broad punctate pattern throughout the aggregate creating a dark blue signal (35X). **L.** Embryos disassociated form stage 2-10. *XAngio* (turquoise) and *Xbra* (magenta) are coexpressed throughout a large region (capping pattern) of the embryo creating a dark purple signal (30X). **M**. Sibling control stage 33. *Actin* (turquoise) signal is seen in the somites. *Beta-globin* (magenta) is seen in the ventral blood islands. **N**. Embryos dispersed from stage 10-12.5. *Actin* (turquoise) expression is seen in a punctate pattern throughout the aggregate. No *beta-globin* signal is detected (35X). **O.** Embryos disassociated form stage 2-10. *Actin* (turquoise) is seen in a band localized to a region of the embryo. No *beta-globin* signal is detected (30X).


XANGIO/ ACTIN
XANGIO/ GATA-2
GATA-2/ ACTIN
XANGIO/ XBRA
BETAGLOBIN/ ACTIN
CONTROL
DISPERSION
DISASSOCIATION
A
B
C
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Gene	Manipulation	Type of Staining	# Experiments	# Positive/Total per experiment	Total	% Positive
XAngio	Dispersion st. 7-11	Punctate	4	2/2, 3/3, 5/5,17/17	37/37	100
	Dispersion st. 10-12.5	Punctate	6	2/2, 5/5, 4/5, 6/7, 16/16, 38/38	71/73	97
	Disassociation st. 2-10	Continuous	4	3/3, 5/5, 7/7, 25/25, 49/49	89/89	100
	Disassociation st. 2-18	Continuous	4	6/6, 8/8, 9/10, 30/30	53/54	98
Actin	Dispersion st. 7-11	Punctate	6	4/4, 6/6, 9/9, 9/9,14/14, 17/17	50/50	100
	Dispersion st. 10-12.5	Punctate	4	5/5, 8/8, 15/15, 17/17	45/45	100
	Disassociation st. 2-10	Continuous	5	4/4, 5/5, 10/10, 10/10, 22/22	51/51	100
	Disassociation st. 2-18	Continuous	3	7/7, 10/10, 36/39	53/56	95
Beta-Globin	Dispersion st. 7-11	None	4	0/3, 0/4, 0/6, 0/10	0/23	0
	Dispersion st. 10-12.5	None	3	1/8, 0/15, 0/17	1/40	2.5
	Disassociation st. 2-10	Punctate	4	0/3, 0/5, 1/14, 0/49	1/ 71	1
	Disassociation st. 2-18	None	3	0/12, 0/33, 0/39	0/84	0
Xfli	Dispersion st. 7-11	Punctate	3	6/6, 7/7, 30/30	43/43	100
	Dispersion st. 10-12.5	Punctate	4	12/12, 16/16, 16/16, 25/25	69/69	100
	Disassociation st. 2-10	Continious	3	10/10, 21/21, 30/30, 43/43	104/104	100
	Disassociation st. 2-18	Continuous	3	36/36, 38/40, 28/34	102/110	92
Gata-2	Dispersion st. 7-11	Punctate	2	28/28, 29/29	57/57	100
	Dispersion st. 10-12.5	Punctate	2	18/18, 37/37	55/55	100
	Disassociation st. 2-10	Continious	3	12/12, 28/28, 32/32	72/72	100
	Disassociation st. 2-18	Continuous	2	24/24, 26/26	26/26	100
Xbra	Dispersion st. 7-11	Punctate	3	12/12, 14/14, 22/22	48/48	100
	Dispersion st. 10-12.5	Punctate	1	6/6	6/6	100
	Disassociation st. 2-10	Continuous	4	5/5, 10/10, 12/12, 13/13	40/40	100
Goosecoid	Dispersion st. 7-11	Punctate	3	7/7, 6/6, 16/16	29/29	100
	Dispersion st.10-12.5	Light Puctate	1	1012	10/12	83
	Disassociation st. 2-10	Continuous	3	10/12, 9/11, 30/33	51/51	95
XAngio/Actin	Dispersion st.7-11	Punctate	1	19/19	19/19	100
	Dispersion st.10-12.5	Punctate	2	26/26, 30/30	26/26	100
	Disassociation st. 2-10	Continuous	1	24/24	24/24	100
XAngio/GATA	Dispersion st.10-12.5	Punctate	1	27/27	27/27	100
	Disassociation st. 2-10	Continuous	1	16/16	16/16	100
XAngio/Xbra	Dispersion st. 7-11	Punctate	1	8/8	8/8	100
	Disassociations st. 2-10	Continuous	1	21/21	21/21	100
GATA-2/Actin	Dispersions st. 10-12.5	Punctate	2	12/12, 15/15	27/27	100
	Disassociation st. 2-10	Continuous	2	14/14, 26/26	40/40	100

Table 1

Discussion

Vasculature must infiltrate all of the tissues of the developing embryo. If a broad range of mesodermal tissues are competent to form vasculature this allows candidate cells to be available in the many areas of the embryo. The dispersion and disassociation experiments performed had two possible outcomes. If a positive induction event is necessary at blastula and early gastrula stages for the mesoderm to gain the competence to form vasculature then embryos without normal cell/cell interactions at these stage will not express the appropriate vascular markers. If however, a restriction event is necessary then these manipulated embryos will retain their competence, even after dispersion or disassociation and continue to express vascular marker genes. The later of these is supported by the results of the dispersion and disassociation experiments reported here. This helps to elucidate three majors areas of investigation in the study of vascular development including: the competence of mesodermal tissue to form vasculature, the existence of the hemangioblast and the induction of blood and vasculature.

The Competence of Mesoderm to form Vasculature

Disassociations

We predicted that if the mesodermal tissue was competent at early blastula and gastrula stages to give rise to vascular precursors, that disturbing normal cell/cell interactions would not prevent expression of the vascular marker gene *XAngio*. The results of the disassociation experiments support this prediction. In the disassociation experiments mesodermal tissue was localized to a region of the manipulated embryos. Impairing cell/cell adhesion without dispersing embryos does not prevent mesoderm induction or broad patterns of regionalization because mesoderm induction does not require cell/cell adhesion but intracellular signaling through secreted molecules and differential distribution of maternal determents. These disassociated embryos strongly express the pan mesodermal marker *Xbra* as well as show more localized expression of the other regional mesodermal markers *GATA-2* and *actin*. *GATA-2* and *actin* do not show coexpression, confirming the maintenance of their regional specificity throughout the course of the disassociation manipulation.

The expression of the vascular marker *XAngio* is not inhibited by these manipulations. It is coexpressed with *Xbra* through the entire capping region which is localized to one side of the embryo. *XAngio*

expression is maintained throughout the capping region and coexpressed in more restricted areas with the regionalized markers *GATA-2* and *actin*. These disassociation experiments show that disassociation of *Xenopus* embryos from the two cell stage through early gastrula (stage 10) as well as disassociation from the two cell stage through the neural tube (stage 18) does not inhibit the competence of a broad region of mesodermal cells to express that vascular endothelial marker gene *XAngio*.

Disassociation experiments in which the embryos were placed in the CMFM and allowed to culture without removal of the outer membrane have not been performed by previous investigators. These experiments were designed to assess the necessity of cell/cell adhesion from first cleavage through gastrulation or through neural tube stages. As a result of the contact between the embryonic cells, inductive interaction were not prevented but formation of normal structures through critical process such as gastrulation were prevented. The embryos while in the CMFM culture appear to be a "bag of cells" with no distinguish characteristics . Upon the readdimistration of the salt media the cell reaggregate and attempt some cell movements. The result of these movements is a lumpy embryo. The mesoderm is induced under these conditions as evident by the expression of the pan mesodermal marker *Xbra* as well as the more regional markers *actin*,

goosecoid and *GATA-2*. The mesoderm is localized to a region of this embryo. Many more experiments can been conducted to investigate the differentiation state of the other tissue types present in these disassociated embryos.

Dispersions

We predicted that if the mesodermal tissue was competent at early blastula and gastrula stages to give rise to vascular precursors, that disturbing normal cell/cell interactions would not prevent expression of the vascular marker gene *XAngio*. The results of the dispersion experiments support this prediction. Both those embryos dispersed from stages 7 though 11 as well as those dispersed from stages 10 though 12.5 show a broad punctate expression pattern of *XAngio*. In these manipulated embryos *XAngio*'s expression pattern is not restricted as it is in sibling controls. In un-manipulated embryos *XAngio* expression is initiated at the onset of zygotic transcription in all but the most dorsal mesoderm. However, treatment of embryos with lithium and activin (both act to dorsalize) does not affect *XAngio* expression (Saha, unpublished data). By the neural tube stages *XAngio's* expression is progressively restricted to the vascular endothelium (Figure 10). In dispersed embryos this restriction event is inhibited. Experiments employing two color *in situ* hybridizations show that *XAngio*'s expression is maintained in dispersed embryos in

essentially all mesodermal derivatives as demonstrated by its coexpression with the pan mesodermal marker *Xbra*. *XAngio* is maintained in a broader expression pattern than the regional mesodermal markers *GATA-2* (ventral) and *actin* (equatorial). *GATA-2* and *actin* do not show coexpression, confirming the maintenance of their regional specificity throughout the course of the dispersion manipulations. These dispersion experiments show that dispersion of *Xenopus* embryos from blastula (stage 7) through gastrula (stage 11) as well as from early gastrula (stage 10) through late gastrula (stage 12.5) does not inhibit the competence of mesodermal cells to express that vascular endothelial marker gene *XAngio* at later stages.

Previous investigators have used dispersion experiments as indicators cell autonomy. During development there are distinct ways in which cells obtain information that lead to their differentiation. Zygotic differentiation is regulated by inductive interaction with surrounding cells and by inheritance of cell autonomous factors. True cell autonomy is the ability of a cell differentiate in the absence of any cell/cell communication. When embryonic cells are dispersed, the normal inductive interactions are disturbed which allows for the assessment of the role of cell autonomous factors and the competence of the cells to differentiate. The stages at which dispersions take place are critical. Dispersion experiments can determine the competence of

the cells only at the time of dispersion to later differentiate. Gurdon et al., (1984) used this technique to show that cell/cell contact is not essential during cleavage stages for the transcription of the *actin* gene. *Actin* is normally transcribed even after dispersion during cleavage stages only in mesodermal tissue of equatorial mesodermal origin. *Goosecoid* as well, has been shown to be cell autonomous (Lemaire and Gurdon, 1994). Dispersion of embryos from fertilization to stage 10.5 (gastrulation) without reaggragation does inhibit *Xbra* and *actin* expression. *Xbra* and *actin* are expressed if the embryos are allowed to re-aggregate (Lemaire and Gurdon, 1994). The dispersion experiment that were performed were designed to test the necessity of normal cell/cell interaction from blastula to gastrula stage (stage-11) as well as from early gastrula stages through late gastrulation (stage 10-12.5) on expression of genes as far downstream as hatching stages (stage 29-31) and tail bud stages (stages 33-35) which are days later. These experiments were not designed to assess true cell autonomy but mesodermal competence. These previous researchers have found that the markers *actin, goosecoid* and *Xbra* should be expressed in these aggregates (Gurdon et al., 1984; Lemaire and Gurdon, 1994), which was confirmed through the course of this study. The expression of the vascular marker gene *XAngio* shows that the conditions of the dispersion experiment does inhibit it's expression. Dispersion appears

to affect the other inductive interactions that would be taking place to restrict the vascular potential of the mesoderm as other mesodermal tissues differentiate, as well as erythropoiesis in the ventral mesodermal tissue.

Debbie Kreup (Honor's Thesis, 1997) showed that explants of ventral mesodermal tissue at stage 11.5 (gastrula) and at 14 (neural plate) are competent to give rise to *beta-globin*. Only two individual embryos in over two hundred assayed, showed *beta-globin* staining. One embryo was a dispersion from stages 10-12.5 and the other disassociated from stages 2-10. All other manipulated embryos did not have *beta-globin* signal. These results indicate that *beta-globin* expression is severely inhibited by both manipulation types. *Beta-globin* is used as a marker for ventral mesoderm. However, the appearance of the *GATA-2* single confirms the presence of ventral mesodermal tissue. This suggests that a ventral fate is insufficient to induce the competence of mesodermal cells to give rise to blood.

The Hemangioblast

The hemangioblast, a common precursor of all blood and vasculature tissue has been a difficult lineage to isolate. Evidence supporting the hemangioblast existence has come from the existence of markers which are present on both vascular and hematopoietic

lineage's (e.g. *QH1, flt* and *flk).* Mouse knockout experiments have been used as evidence supporting the hemangioblast theory. Knockouts of VEGF and its receptors (*flt* and *flk)* impair both vascular and hematopoietic lineage's. However neither VEGF nor its receptors are essential for initial endothelial differentiation but rather for later processes (Carmeliet et al., 1996; Fong et al., 1995; Shalaby et al., 1997). The same pathways, processes and genes can be essential to different lineages depending upon the context in which they function. A specific receptor that in endothelial cells is essential for the structural integrity of the endothelial wall could function in blood cells for adhesion in clotting processes. Though these commonalties between the markers present on blood and vascular cells suggests the existence of a hemangioblast, they are not definitive proof of its existence.

Endothelial cells have been show to have a common origin with many mesodermal derivatives. In avian systems Eisenberg and Bader (1995) have show that myocardial and endothelial cells have a common precursor. Cultures of quail blastoderm cells have been show to be puripotent being able to give rise to myocardium, endothelial and blood cells (Eisenburg and Markwald, 1997). Asahara et al., (1997) have recently found punitive endothelial progenitors in the blood of adults. Paradanaud et al., (1996) suggest that in the chick there are

two distinct lineages of endothelial cells. The first of the lineages originates in the paraxial mesoderm giving rise to endothelial cells and the other lineage originates in the splachnopleural mesoderm which gives rise to endothelial cells as well as vasculature. This finding is not surprising when viewed in conjunction with our results. We suggest that there is a singular hematopoietic lineage induced in ventral mesodermal derivatives. Endothelial precursors cells, however, arise from a broad region of mesodermal tissue and do so in the absence other restriction signals. There are not two distinct lineages but a singular induced hematopoietic lineage and a endothelial 'default' in mesodermal derivatives.

These results are confirmed by fate mapping experiments currently being conducted. Kenna Mills (Honor's Thesis, 1998) has shown that endothelial cell can arise from any of the blastomeres of a 16 cell embryo, however that blood is restricted to the ventral blastomeres. Ventral blastomeres are common precursors therefor, for both the vasculature endothelium and hematopoietic lineages. Ventral mesoderm cell lineages that give rise to both cell types do exist they are not however progenitors of all of the vascular endothelial lineages, therefor they are not hemangioblasts.

Induction of Blood and Vasculature

Our results suggest the existence of distinct inductive events for the blood and vasculature. The first induction events is initiated at the onset of mesoderm induction and gives a broad range of mesodermal cells the competence to become vascular endothelium. This is supported by the broad expression of *XAngio* and its coexpression with the pan mesodermal marker *Xbra*. The second induction event is the regionalization of the mesoderm. Neither the dispersion experiments nor the disassociation experiments inhibit regionalization as exhibited by continued expression of the ventral marker *GATA-2* and equatorial marker *actin*. The absence of *beta-globin* signal suggests that, the presence of the ventral tissue does not necessarily lead to the formation of blood cells.

Many experiments have shown that ventral mesoderm gives rise to blood and that the ventralization of mesodermal tissue leads to the formation of blood. As early as 1926 (Federici) and 1928 (Goss) removed ventral regions from neurula stage embryos and showed that the resulting embryos lack blood within their circulatory systems (reviewed in Kelly et al., 1994). UV treated embryos (ventralized) have an abundance of blood around the entire circumference of the embryo while lithium treated embryos (dorsalized) lack blood (reviewed in Slack et al., 1992). Explants of ventral tissue taken from mid-blastula embryos form blood in the absence of signaling from the

rest of the embryo (reviewed in Slack et al., 1992). Primary erythropoiesis and *beta-globin* expression in explanted mesoderm can be stimulated by animal cap tissue (gastrula stages and later) and by distinct concentrations of the morphogen BMP-4 (Maeno et al., 1994; Dosch et al., 1997). It has also been shown that *beta-globin* signal (blood marker gene) is not inhibit by explanting ventral mesodermal tissue at later stages (11.5, gastrula and 14 neural plate) (Kruep, Honors Thesis, 1997). *Beta-globin* signal is only inhibited through dispersions (stage 7-11 and 10-12.5), disassociations (stages 2-10 and 2-18) or dorsalization of mesodermal tissue (Slack et al., 1992; reviewed in Kelly et al., 1994).

The inhibition of *beta-globin* in the dispersion and disassociation experiments is not a result of the dorsalization of the embryo. This is demonstrated by the maintenance of the ventral marker gene *GATA-2*. *GATA-2* is involved in the maintenance and proliferation of hematopoietic lineages but is not indicative of their induction (Tsai and Orkin, 1997). UV-irradiated embryos show a radially symmetrical expression patterns of GATA-2 however, its expression is prevented in lithium treated embryo, demonstrative of its ventral lineage (Kelly et al., 1994). *GATA-2* signal is present in the dispersed and disassociated embryos and its regional identity is maintained. The absence of *beta-globin* signal in conjunction with the

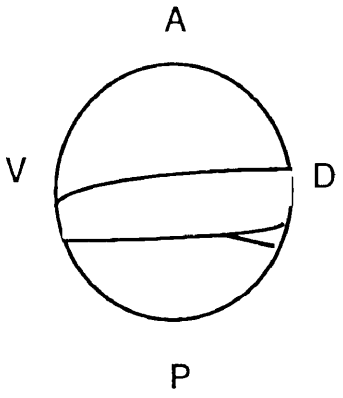
maintenance of the regional *GATA-2* signal and the broad expression of *XAngio* suggests that the presence of ventral mesodermal tissue is not necessarily indicative of the induction of red blood cells while endothelial potential is maintained in all mesodermal derivatives.

Future Directions

Induction of Hematopoietic Potential

These results suggest that there are different inductive events necessary for the formation of the blood versus the vasculature. The next step in this investigation will be to refine the period of development in which the ventral mesodermal tissue gains the competence to give rise to the hematopoietic lineage. Dispersion at blastula (stage 7 though 11) and gastrula stages (stage 10 through 12.5) inhibits the formation of *beta-globin* expressing cells while explants from gastrula stage tissue (11.5) does not inhibit this expression. Future dispersion experiment should be performed at slightly later stages to see if the potential to express *beta-globin* is maintained these embryos. Dispersions from stages 11.5 though 13 and from stage 13 though 14 should be performed. If the potential is maintained in either of these manipulations it would help to determine the time at which hematopoietic potential is induced.

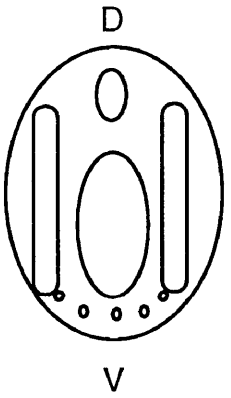
Blastula



All but the most dorsal mesoderm has the competence to form vascular endothelium

Neural Tube

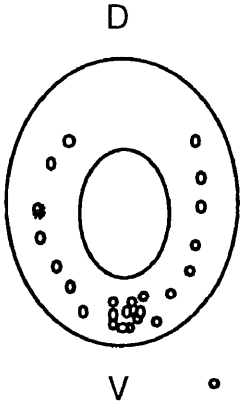
Ventral anterior section



Vascular competence remains in the majority of mesodermal derivatives

Tailbud

Sectioned through the heart



At tailbud stages the vascular competence is tighting to regions of presumptive vasculogenesis and is eventually restricted to endothelial precursors

Figure 10

GATA-1 is a erythroid specific transcription factor and is essential for the differentiation, proliferation, and survival of red blood cells (Peveny et al., 1991; Shividasi et al., 1997; reviewed in Long et., 1997 and Yamamoto et al., 1997). The dispersion and disassociation experiments that were performed here should be repeated and assayed for *GATA-1*. The explant experiments that were performed earlier by Debbie Kruep (1997) should also be repeated and assayed for *GATA-1* expression. If the dispersions and disassociations lack the expression of *GATA-1*, and the explants maintained GATA-1 expressions, it would explain the inconsistency in *beta-globin* expression between the two manipulations types. Dispersion and disassociations would lack expression of *beta-globin* because expression because without *GATA-1* the red blood cells would never differentiate to express *beta-globin*. If the dispersed embryo do express GATA-1 then some other type of inductive interaction has been disturbed.

Restriction of Vascular Competence

The potential of mesoderm to give rise to endothelial cells appears to be restricted during neural tube stages. This restriction signal could have its origins in the notochord. The notochord has been implicated in various other patterning events and is prominent at neural tube stages. During neural tube stages it is possible to dissect

the notochord from a embryo. Placing notochord (from a florescently labeled embryo to enable tracking of tissue) on mesodermal tissue dissected from a gastrula stage embryo could restrict the expression of *XAngio* in that tissue. Experiments could be further refined by dissecting out anterior versus posterior notochord and co-culturing it with early mesodermal tissue. In addition, it has been established that after dispersions that aggregates show a broad expression pattern of *XAngio*. Co-incubation of an aggregate with a sibling neural tube stage notochord could inhibit that expression of *XAngio* if the notochord is the origin of the inhibition signal. This type of manipulation could be used to increase our understanding of the early tissue interactions necessary for the formation of the vascular system.

XAngio's Role

XAngio's role in vascular development is not well understood. It is know that it is expressed in a broad region of mesodermal tissue exclusive of the most dorsal notochord. *XAngio* expression is neither inhibited by lithium treatment (dorsalizing factor) nor by UV irradiation (ventralizing factor). It would be informative to perform experiments to overexpress the known ligands involved in mesodermal patterning. Microinjection experiments could be performed in which one and two cell embryos are injected with noggin (dorsalizing) or with

BMP-4 (ventralizing). *Beta-globin* or *GATA-2* expression would be a used as a positive control for BMP-4 and a negative control for *noggin*. Injection of the embryos with *noggin* could inhibit *XAngio* expression given that *XAngio* is not expressed in the most dorsal mesodermal tissue. However, all mesodermal tissue might have the competence to form vascular endothelial tissue and the differentiation of presumptive notochord could reflect the first restriction of the mesoderm.

VEGF and it receptors (*flt* and *flk*) are known to have a roles in vascular development. Dissections of animal cap tissue exposed to VEGF might show *XAngio* expression if *XAngio* is involved in endothelial differentiation and development. Exposure of embryos to VEGF in the tailbud stages when the vascular system is forming could lead to the maintained expression of *XAngio* and higher levels of vascularization or vascularization of previously a vascular areas. This process, may or may not, involve up-regulation of *XAngio*.

Our current understanding of vasculogenesis and hematogenesis is imperfect. The experiments performed here suggest that a broad region of mesodermal tissue is initially competent to form vasculature endothelial tissue and that this competence is gradually restricted during development. Through the dispersion and disassociation manipulations at blastula and gastrula stages the competence of a broad region of mesodermal cell to express the vascular endothelial

marker gene *XAngio* was not inhibited. This suggests an inhibition of a restriction event not the inhibition of an induction event. Future studies might confirm vascular endothelium as the 'default' state for mesodermal tissue.

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