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The Origins of Hematopoietic and Endothelial Development in Zebrafish
by

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DISSERTATION

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For My Parents

Acknowledgments:

I would like to thank my family for their unwavering support over the past five years, especially when it wasn't clear exactly where graduate school was taking me. I would also like to thank Richard Harland, Ray Keller, and John Wallingford for providing me with the inspiration to get my Ph.D.; their work embodies what developmental biology should be. While in the lab, Ben Yu and Suk-Won Jin were indispensable by providing me with advice and access to their broad scientific knowledge. My thesis research would not have been the same if not for the independence and freedom provided by my advisors, Gail Martin and Didier Stainier. Lastly, I would like to thank Jeff Tweedy and Radiohead for making the music that has allowed me to keep a modicum of sanity throughout this process.

The Origins of Hematopoietic and Endothelial Development in Zebrafish

Kevin Vogeli

Abstract

It has been thought for over 85 years that blood and blood vessels share a common developmental origin. Early experiments inferred the presence of a progenitor cell that could give rise to both blood and blood vessels called the hemangioblast. Modern genetic and cell culture experiments have provided supporting evidence; however, the presence of the bipotential hemangioblast had not been demonstrated in a developing embryo. With this in mind we constructed single-cell resolution fate maps of the early zebrafish embryo. By labeling individual cells in the zebrafish embryo and then assessing their developmental potential, we were able to demonstrate the presence of hemangioblasts. These hemangioblasts localize to a specific region of the embryo and may provide a link between primitive and definitive hematopoiesis. The first two chapters of this study are describing these findings. The last chapter of this work focuses on the mechanism of pectoral fin development in zebrafish. While these studies are more preliminary, they provided the technical groundwork for the single cell labeling experiments conducted elsewhere.

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Chapter 1. Introduction

Stem Cells and Early Development

The ability of a single cell to give rise to all lineages within a developing embryo is a phenomenon that has long fascinated biologists. How the progenitors of this single, totipotent cell distinguish themselves from each other is a well-studied process, but even more intriguing is the process by which some of these daughter cells retain their totipotency throughout development. This process was first observed at the organismal level over a century ago when the amazing regenerative properties of planarians were studied[1]. Planarians could by dissected and bisected and yet still regenerate entire organisms from the pieces. Higher organisms possess some of these properties as it was found that the limbs of newt could fully and faithfully regenerate upon amputation[2]. Which cells in the organism still had the capacity to generate all other cells? The idea of stem cells had been proposed, but it was not until the work of McCulloch and Till in the early 1960s that it was directly demonstrated that such stem cells were present. They were able to demonstrate that bone marrow cells could form heterogeneous colonies of cells within the spleen of an irradiated mouse[3]. This seminal work demonstrated that stem cells were present even in the adult organism.

Although these experiments demonstrated that there were multipotent cells in adult mammals, the demonstration of totipotent and self-renewing cells within the embryo did not occur until experiments conducted by two separate groupsMartin and Evans in 1981. These two groups simultaneously demonstrated that cells derived from the inner cell mass of an early mouse embryo could be cultured to form all cell types[4,5]. This seminal discovery not only illuminated the most primitive cell type, but also

demonstrated the potential of these cell types. Now that it had been demonstrated that there was a totipotent, self-renewing population of cells within the embryo, the onus of research shifted to determining the order of differentiation and hierarchy of the stem cell derivatives and the factors responsible for controlling such decisions. The search was on for the tissue specific stem cells and the genes that controlled their development.

Hematopoietic Stem Cells

One of the most intensively studied systems for tissue specific stem cells was that of the Hematopoietic Stem Cell (HSC), a cell that could give rise to and continuously replenish all types of blood. The clinical relevance of such a cell is obvious as the illumination of HSC differentiation underlies the etiology of many blood disorders such as α–or β–thalassemia and as well as many blood cancers[6]. One of the key functions of HSCs during development is the generation of red blood cells (erythrocytes) for the purpose of delivering oxygen to nascent embryonic tissues. As it matures, the embryo must produce circulating blood to nourish tissues while at the same time generate cells that will lead to the continued production of blood in the adult organism. These two separate modes of blood production are referred to as primitive (embryonic) and definitive (adult) hematopoiesis. These two types of hematopoiesis can be distinguished from each other by the type of hemoglobin present in the erythrocytes. Additionally, in mammals the blood produced by primitive hematopoiesis is nucleated while the blood produced by difinitive hematopoiesis is enucleated 7. Because of these two distinct requirements for early hematopoiesis (immediate function vs. long-term renewal) as well as the production of different cell types, it has been a long-standing question as to

whether primitive and definitive hematopoiesis share the same embryonic origins. Directly stated: Do HSCs arise once or multiple times during development?

Some of the earliest experiments done to address these types of questions were conducted in parabiotic chicken embryos. In these experiments, one male and one female embryo were connected via their yolk sac vasculature and the embryos were allowed to develop together for 7-13 days[8,9]. Subsequently, the embryos were fixed and chromosome spreads were performed to distinguish the male (ZZ) cells from the female (ZW) cells. From this information the degree of chimerism in the embryos was determined. Tissue from the bone marrow, spleen, and especially the thymus was found to be chimeric for both genotypes[8,10]. These simple experiments demonstrated that cells circulating in the blood stream were able to integrate into developing tissue of the embryo. This led Moore and Owen to hypothesize that there were "circulating stem cells" that could integrate into nascent organs and develop into different tissues based on the environment they encountered[11]. These early results seem to support the idea that all blood is derived from a single origin. That is that HSCs were able to move through the circulating blood and seed other organs. While this early study provided intriguing results, more stringent and direct experiments were necessary before a definite conclusion could be drawn.

Experimental Demonstration of the Origin of HSCs-Chicken

Early experiments using grafting techniques in the chicken embryo gave a more thorough insight into the embryonic origins of hematopoiesis. Through the use of chick/quail chimera and intraspecific grafts between different chicken strains, the initial

location of hematopoietic cells and the timing of HSC development were determined. Early experiments using chick/quail chimeras demonstrated that initial HSC production began at Hamilton-Hamburger stage 8 (HH8, 26-29hpf) in the extraembryonic yolk sac. Quail embryos grafted onto the blastodisc of a chicken embryo are initially populated with blood cells entirely derived from the host chicken embryo. Later in development these chimeras contain blood derived from both host and donor tissue[12]. Because of certain irregularities in the rate of hematopoiesis in these chimeric embryos, it was impossible to determine the percentage of contribution to hematopoiesis from the donor embryos. Thus, it was impossible to determine the timing of the switch between extra-embryonically produced blood and blood derived from the embryo proper.

As a result these experiments were repeated using chicken embryos of different histocompatability groups whose cells could be distinguished from each other via antibody staining. This was accomplished by grafting chicken embryos of B2 subtype onto chicken B15 subtype blastodiscs. The chimeric embryos resulting from these experiments developed normally and yielded similar results to the chick/quail chimeras. These grafts indicated that the erythrocytes are initially derived from extra-embryonic tissue; however, as the embryos reached day 7 (HH 31) the blood being produced is derived from embryonic tissue[13].

From this set of results a new model was proposed, wherein there are two distinct sites of hematopoiesis in the embryo. Initially, the extra embryonic yolk sac produces erythrocytes and then the site of production shifts to an area within the embryo for the remainder of development. Further grafting experiments in the chicken determined this site to be the region containing the dorsal aorta[14]. In a very elegant experiment using

acetylated LDL DiI to label the endothelial cells, it was demonstrated that the cells on the ventral wall of the dorsal aorta can produce red blood cells[15]. These studies showed directly what had been hypothesized for many years as early embryologists observed that "aortic cell clusters" were closely associated with the blood vessels in this region [16].

Taken in combination with the grafting experiments it can be inferred that there are two distinct sites of hematopoiesis during development and strongly suggest that these sites arise independently from each other. These results contradict the initial model of a single source of circulating HSCs that can populate the hematopoietic organs. Although these experiments demonstrated that embryonic tissue is a major source of definitive blood, it is not the only source as it has been recently demonstrated that the allantois is also a significant source of adult blood cells in the chicken[17]. It remains a possibility that the extra-embryonic tissue is a source for some adult hematopoiesis.

Experimental Demonstration of the Origin of HSCs-Mouse

Although the data from chick strongly point to the idea of two sources of HSCs arising independently from one other, the data taken from subsequent studies in mouse are less conclusive. As in the chicken, the initial site of primitive hematopoiesis is found in the extra embryonic yolk sac during gastrulation at embryonic day (E)7.5. It has been demonstrated that cells from this region grafted into lethally irradiated mice are competent to give rise to definitive blood in adult mice18. The site of definitive blood production originates in an area known as the Aorta-gonad-mesonephros (AGM) beginning at E10. This tissue is homologous to the ventral wall of the dorsal aorta previously mentioned in the chicken embryo and demonstrated to be hematopoietic in

vivo. Consistent with these results, it was demonstrated that cells grafted from the AGM region of day E9 embryos into lethally irradiated mice were sufficient to give rise to all blood lineages in the adult19. Even though this experiment would point toward an independent origin of the definitive blood, it is not possible to rule out the idea that cells from the site of primitive hematopoiesis in the yolk sac migrate into the AGM region to produce the definitive blood. The grafting experiments from Medvinsky19 were conducted with cells derived from E10 embryos after the onset of circulation in the mouse embryo at E8.5. Taken together with the data from Nishikawa18, it would seem that it is possible that all HSCs in the mouse are derived from a single origin that migrates to different tissues during development.

HSC Development in Zebrafish

As there seems to be discordance between the results obtained in chicken and mouse, it is necessary to assay a different system to determine what is stereotypical and what can be attributed to interspecies differences. The zebrafish embryo provides the ideal model for studying hematopoiesis and the origin of HSCs20. It develops externally and is amenable to classical techniques such as lineage tracing and grafting; yet it is also genetically manipulatable through methods such as mutagenesis, transgenesis, and morpholino knock-down. Thus the fish embryo provides a combination of in vivo techniques available in chick and the genetic manipulations in the mouse.

The onset of hematopoiesis in zebrafish begins in a bilateral region of the lateral plate mesoderm along the anterior-posterior axis beginning at the 2-somite stage (10hpf). This process is first evident by the expression of the Stem Cell Leukemia gene (scl) at 10hpf and subsequently by the expression of the transcription factor gata1. Both of these

genes have been demonstrated to be essential for red blood cell development in zebrafish and mouse21,22. The hematopoietic tissue migrates to the midline of the embryo at 16hpf into an extra-embryonic region beneath the notochord called the intermediate cell mass (ICM). Because of its location and function of producing primitive red blood cells, this region is thought to be homologous to the yolk sac blood islands that exist in mouse and chicken. As the fish develops vasculature at 24hpf, blood cells enter circulation. At 36hpf cells can be seen associated with the dorsal aorta in the trunk region. These are thought to be the aortic cell clusters first described by Jordan; this region is thought to be homologous the AGM region of the mouse and chicken. It should be noted that no direct experiments have been done to test the hematopoietic potential of this region, but one line of genetic evidence suggests it. The transcription factors runx1 and c-myb are expressed in the ventral wall of the dorsal aorta, thought to be the location of initial definitive blood development. Disruption of runx1 leads to loss of all definitive blood in zebrafish as well as in the mouse 23,24.

At day 5 it is thought that definitive blood production begins in earnest as the location of HSC production shifts to the kidney20. Although transplant experiments such as those in mouse have not been conducted, all hematopoietic cell types have been detected within the kidney during this stage. The mutant, bloodless, provides additional evidence for day 5 as the start of definitive hematopoiesis. Bloodless mutants are defective for primitive hematopoiesis, but they begin recovery at day 5 and are viable as homozygous mutant adults indicating that definitive hematopoiesis is relatively unaffected25. Early transfusion experiments indicate a similar pattern of timing for definitive hematopoiesis as well. At 36hpf all the blood from a rodamine-dextran labeled

embryo is transfused into an unlabelled host embryo. These labeled donor blood cells would represent the primitive HSCs. The levels of rodamine labeled primitive blood cells do not significantly change until day 425. These experiments suggest that there are two sites of hematopoiesis within the zebrafish embryo corresponding to the yolk sac blood islands and AGM region that exist in the chicken and the mouse.

A key question is whether the initial site of primitive HSC development in the ICM seeds the second site of definitive hematopoiesis in the axial vessels and eventually the kidney. This question can be addressed through careful lineage tracing studies in the early embryo. Although the origin of HSCs has been the focus of this study so far, it would be impossible to discuss the development of these cells without mentioning the development of the cells that comprise the circulatory system, endothelial cells. Investigation of the relationship between hematopoietic and endothelial cells will yield additional insight into the origin of HSCs in the embryo.

Endothelial Cell development

The circulatory system is the first organ to develop in the embryo, as its function is important for the delivery of blood to the other developing organs. In the chicken embryo, this process begins as the yolk sac endothelium forms at HH8 in the extra embryonic tissue. A similar process occurs in the mouse embryo as vascular precursors form in the extra embryonic yolk sac starting at E7.5. Subsequent endothelial cells in the embryo proper are derived from the lateral plate mesoderm. In addition to their hollow tube-like morphology, endothelial cells can be distinguished by the expression of the VEGF receptors flk1 and flt1 (see26 for review).

In the zebrafish, angiogenesis begins at 12hpf with the onset expression of the VEGF receptor, flk-1, in bilateral stripes within the lateral plate mesoderm. These cells then migrate to the midline ventral to the notochord and form a hollow tube that becomes the axial vessel just before the onset of circulation at 24hpf27. From the midline axial vessel subsequent intersomitic vessels sprout in between the somites and eventually form the rest of the vascular network.

The Hemangioblast

Because the blood, heart, and blood vessels need to be present for circulation to function properly, it follows that these tissues should develop in concert with one another. One method for ensuring such coordination is to have the tissues develop in conjunction with each other in the same compartment. Both endothelial and HSCs emerge at the same time in the same location within the embryo. In the mouse and chicken this region is within the extra-embryonic yolk sac blood islands. In the zebrafish this region is in the posterior ICM region, dorsal to the yolk extension. The juxtaposition of these two tissue types in several different species during early development suggests that a common precursor for these two tissues exists. This idea was first proposed by Florence Sabin after she observed that endothelium and red blood cells could develop from the same piece of chicken embryo when cultured in vitro28. Her methods were refined and her results were repeated by Murray, who in turn called these bipotent cells, hemangioblasts29.

As more modern molecular techniques were developed to analyze embryos the amount of evidence for the hemangioblast grew. The developing blood and endothelial

cells share the expression of a number of genes including flk1, flt1, tie1, tie2, tal/scl, and runx126. Additional genetic evidence was provided by the targeted deletions of flk1, scl, and flt1 in the mouse which all have effects on both hematopoietic and endothelial development 30-32. While this is a strong indication that these tissues may arise from a common origin, these genes could be affecting both processes in parallel.

Perhaps the most compelling evidence for the hemangioblast was provided by cell culture experiments done on mouse embryonic stem cell derived embryoid bodies (EBs) and embryos. In these experiments individual cells from either EBs or embryos were induced to form colonies in cell culture. These colonies were then able to form both hematopoietic and endothelial cells under the appropriate cell culture conditions 33,34as assayed by western blot or RT-PCR. While these results are robust and have been repeated there is still a question of whether these experiments accurately reproduce what occurs in vivo. The most telling in vivo experiment demonstrating the relationship between hematopoiesis and endothelial development was performed in the chicken embryo. The circulatory system of a chicken embryo was labeled with acetylated-LDL DiI and then allowed to develop for 24hr. After fixation and staining for the blood marker CD45, co-localization between the DiI and CD45 was found adjacent to the ventral wall of the dorsal aorta 15. These results show that blood may develop from endothelium and direct evidence for the hemogenic endothelium described by Jordan16; however, it does not indicate that they share a common progenitor.

The Search for the Hemangioblast

As a result of the dearth of in vivo evidence for the hemangioblast, many basic and fundamental questions need to be addressed. First, does the hemangioblast actually exist in vivo or is it an artifact produced by cell culture? Second, if hemangioblasts do exist what proportion of blood and endothelial cells are derived from them? Are all blood and endothelial cells derived from hemangioblasts? If not all hematopoietic and endothelial cells are descended from the hemangioblast, then are the hemangioblast derived blood and endothelial cells distinct from their non-hemangioblast derived neighbors? Is all blood derived from endothelium? Are all HSCs derived from the hemangioblast? The best method to address these questions is to label individual cells in an embryo and to assess there developmental potential within the hematopoietic and angiogenic tissues. As demonstrated in the next chapter, we used the zebrafish to perform these experiments.

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Chapter 2 Experimental Evidence for the Hemangioblast

Introduction

It has been hypothesized that hematopoietic and endothelial cells share a common progenitor, termed the hemangioblast. This idea was conceived as a result of the observation that these two cell types develop in close proximity to each other within the embryo[1,2]. Support for this hypothesis has been provided by studies on single-cell derived colonies that can produce both hematopoietic and endothelial cells *in vitro*[3-7]. Although these data point towards the existence of a common progenitor for these two lineages, the presence of a bipotential progenitor cell has yet to be demonstrated *in vivo*. Through the construction of single cell resolution fate maps of the zebrafish late blastula and gastrula, we demonstrate that individual cells can give rise to both hematopoietic and endothelial cells. These bipotential progenitors arise along the entire extent of the ventral mesoderm and contribute solely to hematopoietic and endothelial cells. We also find that only a subset of hematopoietic and endothelial cells arise from hemangioblasts. Interestingly, the endothelial descendants of the hemangioblasts all clustered in a specific region of the axial vessels regardless of the location of their progenitors. Our results provide the first *in vivo* evidence for the existence of the hemangioblast and reveal distinct features of this cell population.

To investigate whether hematopoietic and endothelial cells arise from a common precursor, a system needed to be devised to label individual cells and asses their contribution to the blood or vascular network later in development. Because of the reagents and techniques currently available, the zebrafish embryo was the only system in which this experiment could be performed. The first component of this experiment was

accomplished through the laser activation of DMNB-caged fluorescein (FITC) in single cells during late blastula (5hpf) or early gastrulation (6hpf) in zebrafish. The caged FITC is non-fluorescent until it is illuminated with sub-UV light (<400nm). With this technique it is possible to fluorescently label individual cells (fig. 2.1a, c, d), allowing a single-cell resolution fate map to be constructed. The second component of the system was the double transgenic line *flk1*:eGFP, *gata1*:DsRed which expresses eGFP in the endothelial cells and DsRed in the erythrocytes. Single cells were labeled in this line and then assayed for co-localization with the transgenes one day later at 30hpf. (fig. 2.1b) In this manner, it became much easier to find the origin of hematopoietic and endothelial cells in the early embryo.

Results

Single Cell Resolution Shield Stage Fate Map

In order to determine the location of the precursors of the blood and endothelial cells and test for the presence of the hemangioblast, the ventral portion of gastrula stage zebrafish embryos were labeled via laser activation of caged fluorescein (FITC). Earlier fate maps constructed by Rachel Warga by injection of fluorescein or rodamine dextran demonstrate that precursors of the blood are predominately located in the ventral mesoderm at shield stage. These maps also reveal that endothelial cells arise along the entire circumference of the embryo within the mesoderm. For these reasons we chose to focus our mapping studies on the ventral mesoderm. As described above one-cell stage embryos were injected with DMNB-caged FITC for ubiquitous distribution and then single cells were activated with a UV laser at shield stage (6hpf). The position of the labeled cell was immediately recorded digitally from lateral and animal views (fig.

Figure 2.1

Single Cell labeling in Early Gastrula Stage Embryos

Embryos were injected with caged fluorescein dextran at the 1-cell stage, and a single cell activated at shield stage (6 hpf). Epi-fluorescence pictures showing the location of a single labeled cell. (**a**) Lateral view to determine cell diameters from the margin. (**a'**) Animal pole view showing degrees from dorsal as determined by the distance from the shield. Embryos were allowed to develop until 30 hpf and then scored for co-localization of activated FITC with EGFP or DsRed. (**b**) Brightfield picture of 30 hpf embryo. Single cells were labeled at 6hpf and then fixed immediately and processed for immunohistochemistry with an antibody against activated fluorescein (**c-d**) Fluorescent images showing FITC staining of individual cells at shield stage.

Fig. 2.1

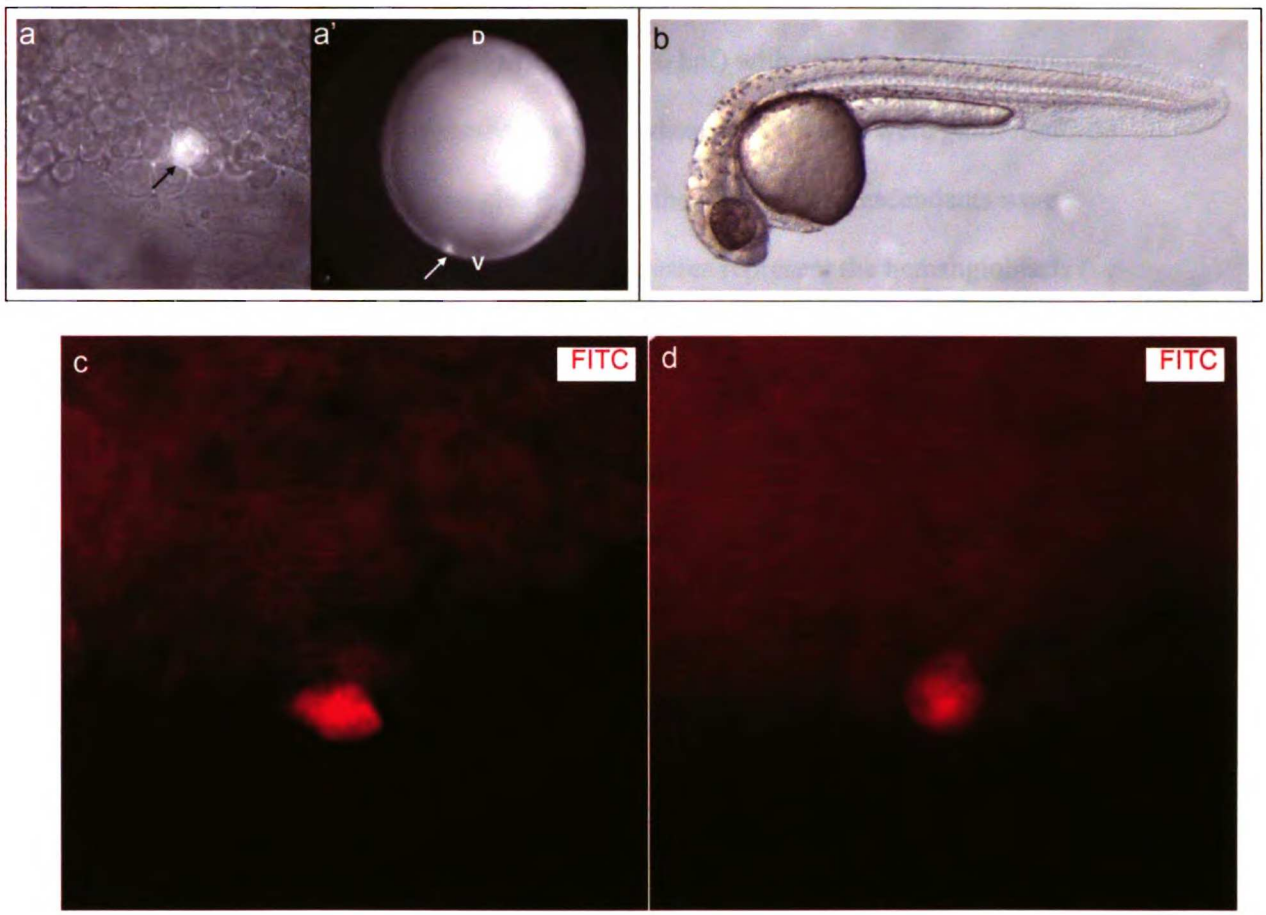
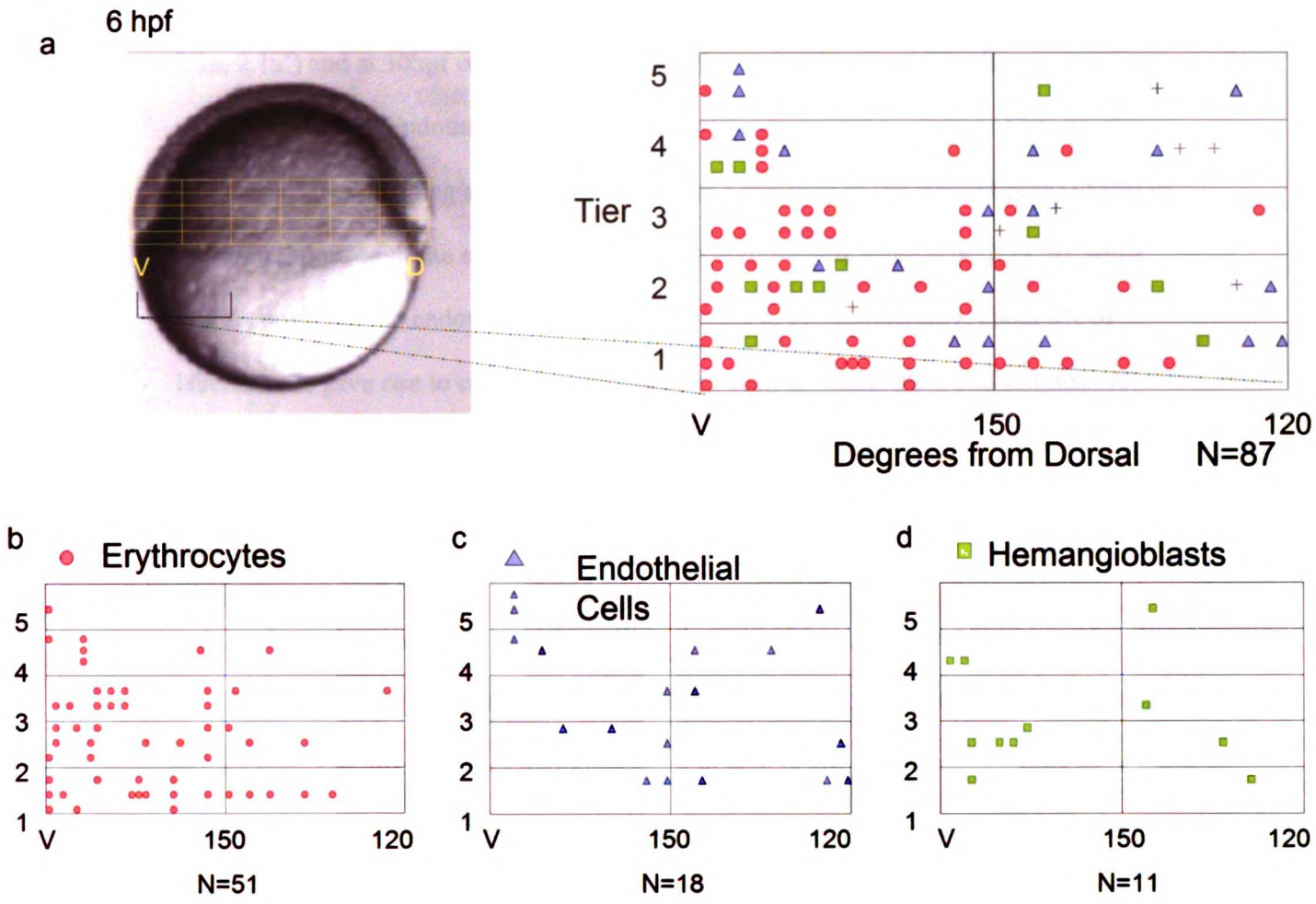


Figure 2.2
Single cell resolution fate map at shield stage
(**a**) Fate map of all cells labeled at shield stage (6 hpf) with each shape representing a single labeled cell. Circles represent the cells whose descendants were *gata1*:DsRed+ erythrocytes only (52 cells), triangles represent the cells whose descendants were *flk1*:EGFP+ endothelial cells only (18 cells), squares represent the hemangioblasts (i.e. cells whose descendants were both *gata1*:DsRed+ and *flk1*:EGFP+ cells) (11 cells), crosses represent cells whose descendants contributed to somites or pectoral fin but were not erythrocytes or endothelial cells (7 cells); n=88. (**b**) Location of all FITC-labeled progenitors of *gata1*:DsRed+ cells; (**c**) Location of all FITC-labeled progenitors of *flk1*:EGFP+ cells; (**d**) Location of all FITC-labeled hemangioblasts.

Fig. 2.2


a
6 hpf
V
D
Tier
5
4
3
2
1
V
150
120
Degrees from Dorsal
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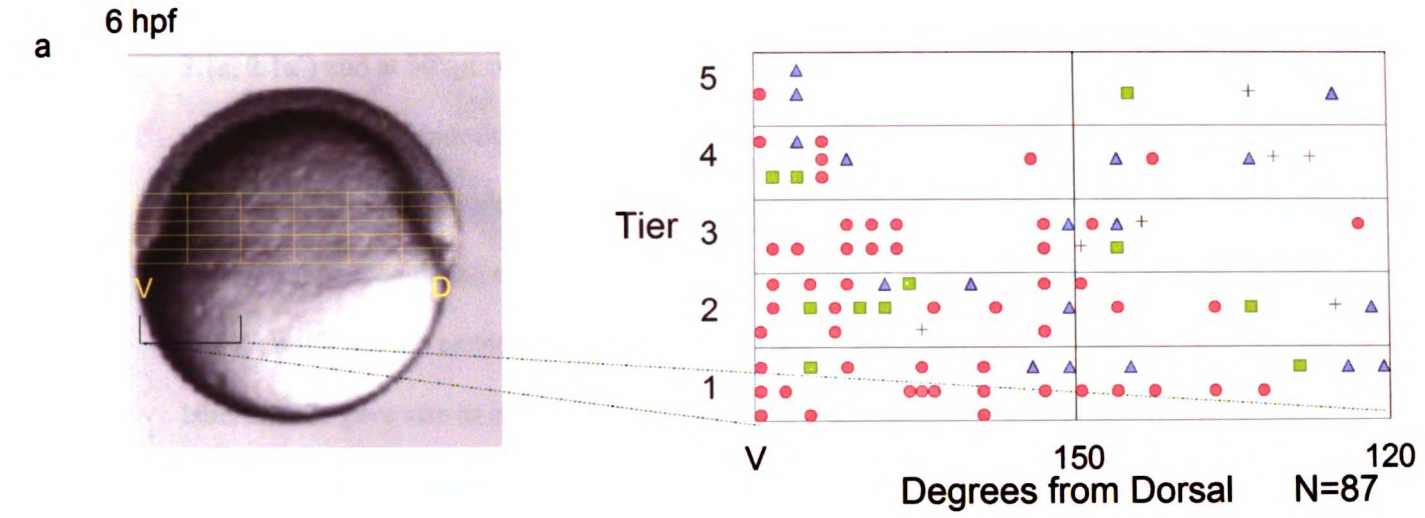
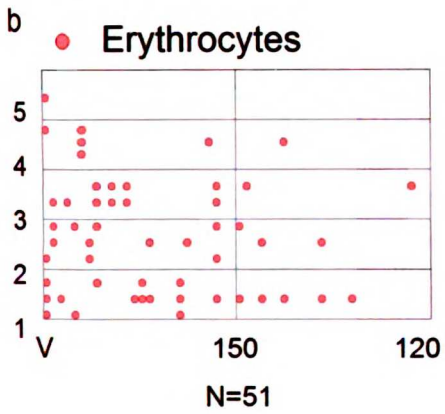
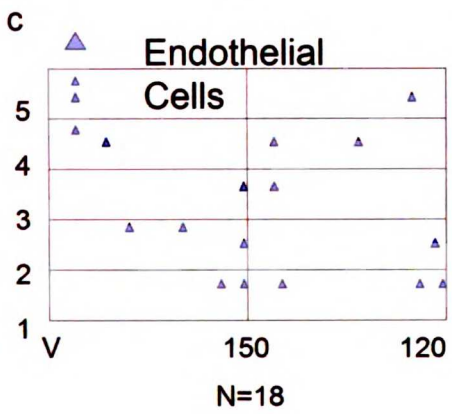
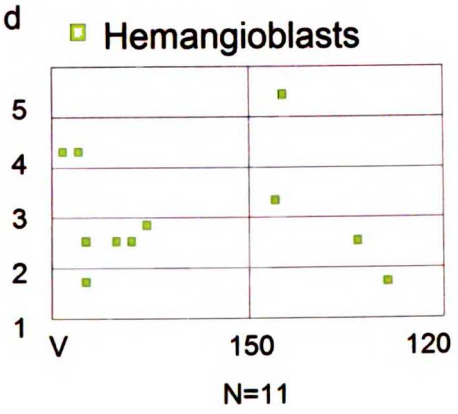
b
Erythrocytes
N=51
c
Endothelial Cells
N=18
d
Hemangioblasts
N=11

Fig. 2.2









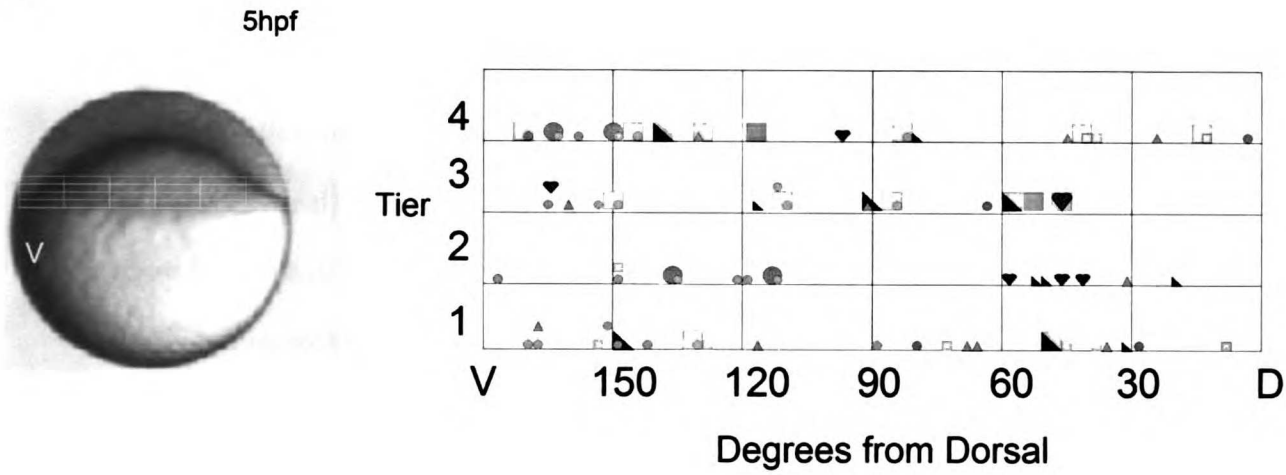
2.1a, 2.1a') and at 30hpf embryos were scored for co-localization of the lineage tracer with the *flk1*:GFP (endothelium) or gata1:DsRed (erythrocyte) transgene. Consistent with earlier fate mapping studies[8], we found that a majority of the labeled cells (~58%) in the ventral portion of the embryo contributed to erythrocytes alone (Fig. 2.2a, b), while ~20% contributed to endothelial cells alone(Fig. 2.2 a, c). Additionally, about 8% of labeled cells gave rise to other type of mesoderm such as lateral plate and somites. A significant number of individual cells (12.6%) specifically gave rise to both lineages (Fig. 2.2a, d) consistent with the definition of the hemangioblast. It is important to note that these bipotent cells gave rise to blood and endothelial cells only. Confocal microscopy was performed on these embryos to confirm these results and clearly demonstrated that the FITC (fig. 2.4f, j) localized to the endothelium (fig. 2.4g, k) and the blood (fig. 2.4h, l) only (fig. 2.4i, m). Another important point to note is that the hemangioblasts were the only cell type we observed giving rise to more than one lineage, indicating that at this stage most cells in the mesoderm are fated to become a single tissue. These hemangioblasts derived from positions all around the ventral margin of the embryo and were interspersed among cells fated to become blood or endothelial cells alone.

40% Epiboly Fate Map

In order to further test whether all endothelial and blood cells arise from hemangioblasts, and also to investigate the presence and prevalence of hemangioblasts at earlier developmental stages, we performed fate-mapping experiments at 40% epiboly (5 hpf). At this stage, one cannot distinguish dorsal from ventral, leading us to survey the entire margin of the embryo rather than just the ventral mesoderm as in the shield stage

Figure 2.3
Single cell resolution fate map at 40% epiboly
(**a**) Fate map of all labeled cells at 40% epiboly (5 hpf) with each shape representing a single labeled cell. Positions on the map containing two different shapes (one larger, one smaller) indicate the locations of single cells whose descendants were found in two lineages.

Fig. 2.3



Erythrocytes	24	Somites	19	Heart	6
Endothelial Cells	11	Pectoral Fin	11	Notochord	3
Hemangioblasts	2	Gut/endoderm	9	Pronephros	1

N=68

fate map. Consistent with previous fate mapping data [8,9], cells at this stage were not entirely restricted to a single lineage. At 40% we found many single cells giving rise to more than one lineage (~30%, fig 2.3). For example, cells giving rise to blood or endothelial cells were often found to give rise to other cell types, sometimes apparently even to endodermal cells (fig. 2.3). Hemangioblasts were found only rarely (2.9%) suggesting that they might be localized in parts of the embryo we were not able to survey for technical reasons.

As with the shield stage fate map, confocal microscopy was used to confirm the presence of hemangioblasts via co-localization with the transgenes. Our scans clearly demonstrate the FITC (fig. 2.4b) co-localized with the endothelium (fig. 2.4c) and blood (fig. 2.4d) in the same focal plane (fig. 2.4e). Because of the gastrulation movements that occur between 40% epiboly (5hpf) and shield stage (6hpf) the cells that we assayed in this fate map have mostly involuted by shield stage. Thus we were not looking at an entirely overlapping population of cells in the two fate maps, allowing for the discrepancy in the percentage of hemangioblasts observed at each stage. Importantly, our fate mapping data at this earlier stage clearly show that not all endothelial and blood cells arise from a common progenitor. This suggests that the hemangioblast is a specialized cell type, perhaps with characteristics that set it apart from other endothelial and blood cells.

Molecular Control of the fate decision in the Hemangioblast

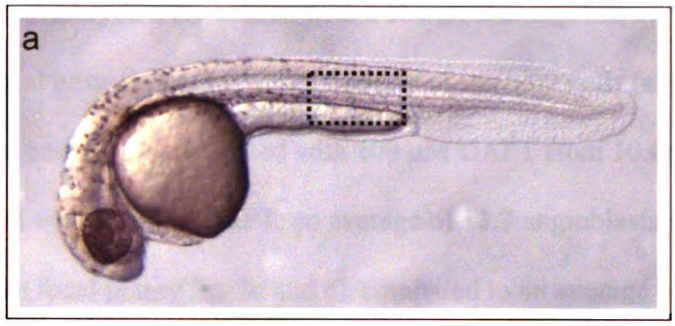
In many biological systems, Notch activity functions to set apart cells from an equipotent precursor population, allowing them to adopt specific fates [10,11]. Based on results recently obtained in *Drosophila* and zebrafish hematopoiesis[12,13], we sought to

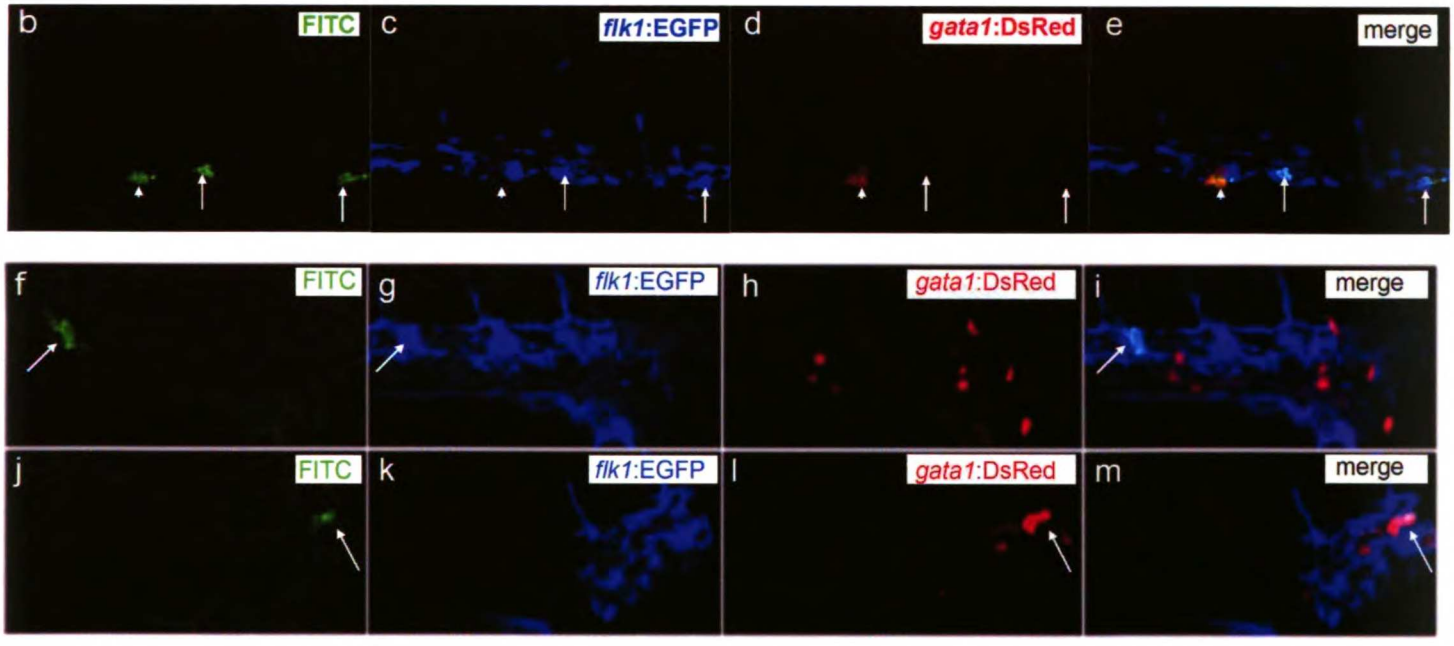
Figure 2.4

Descendents of a single cell give rise to both Blood and Endothelium

Lateral confocal sections of a 30 hpf embryo in which a single cell had been labeled. Scans were performed within the region defined by the box in (**a**) (**b-e**) Single lateral confocal section of the axial vessels of a 30 hpf embryo showing the descendants of a single cell labeled at 40% epiboly. The embryo was imaged with a 40x objective with an optical section thickness of 1.9 μm. (**b**) descendants of a single FITC labeled cell. (**c**) *flk1*:EGFP transgene showing expression in the endothelium. (**d**) *gata1*:dsRed transgene showing expression in the erythrocytes. (**e**) merge of all channels showing co-localization of activated FITC with *flk1*:EGFP (arrows) and *gata1*:DsRed (arrowhead). (**f-i and j-m**) Two lateral confocal sections of a 30 hpf embryo within the region defined by the box in (**a**). Embryos were imaged using a 40X objective with an optical section thickness of 0.58 μm. (**f, j**) Activated FITC showing the descendants of a single labeled cell. (**g, k**) *flk1*:EGFP marking endothelial cells. (**h, l**) *gata1*:DsRed marking erythrocytes. (**i**) Merge of **f-h**, arrow indicates labeled endothelial cell. (**m**) Merge of **j-l,** showing a different cell in the same embryo as that shown in **f-h,** only in a more superficial focal plane; arrow indicates a labeled erythrocyte.

Fig. 2.4


a


b
FITC
c
flk1:EGFP
d
gata1:DsRed
e
merge
f
FITC
g
flk1:EGFP
h
gata1:DsRed
i
merge
j
FITC
k
flk1:EGFP
l
gata1:DsRed
m
merge

determine whether Notch signaling is involved with the fate decision of the hemangioblast. Embryos were treated with a chemical antagonist of Notch activity, DAPT, starting at gastrula stages[14]. The number of vascular cells (angioblasts) greatly increased when embryos were treated with 100 μM DAPT from 10 to 18 hpf (fig. 7a). In embryos treated with 100 μM DAPT, an average of 13.7 angioblasts were present in any given transverse focal plane (fig. 7c and e), compared to an average of 6.7 angioblasts in DMSO-treated embryos (fig. 7b and d).

In addition to chemical inhibition of notch signaling, we also looked at the number of angioblasts in the notch pathway mutant mindbomb. In mibta52b embryos we saw an average of 9 angioblasts per section compared to an average of 6 in wild type (fig. 7a and f) at 18hpf. These results suggest that Notch activity negatively regulates angioblast formation during early development. To distinguish whether these ectopic angiogenic cells were formed as a result of excess proliferation, BrdU incorporation assays were performed on DAPT treated embryos in two-hour intervals from 12hpf to 18hpf. There was no detectable difference between DMSO (fig. 8a) and 100 μM DAPT (fig. 8b) treated embryos, indicating that the ectopic angioblasts did not arise as a result of increased rate of cell division. This result led us to hypothesize that these angioblasts were developing at the expense of another tissue.

Due to the close developmental relationship between hematopoietic and endothelial cells, we sought to determine if markers of hematopoietic development were down regulated upon the disruption of Notch signaling. Expression of the hematopoietic markers *gata1* and *draculin* was evaluated in embryos that had reduced levels of Notch

signaling. Embryos treated with the chemical inhibitor DAPT or injected with a morpholino against the Notch signaling component *gridlock* showed a pronounced

Figure 2.5

Notch signaling negatively regulates angioblast formation

(**a**) The number of endothelial nuclei per focal plane was counted in embryos treated with DMSO as a control (n=15), and 100 μM DAPT (n=18); phenotypic wild-type siblings (n=6) and *mib*^{ta52b} mutant embryos (n=13). Epi-fluorescence micrographs of 18 hpf embryos treated with DMSO (**b**) or 100 μM DAPT (**c**) from 10 hpf to 18 hpf. White lines mark the area where the transverse sections were imaged. Transverse sections of 18 hpf embryos (**d**) WT (**e**) *mib*^{ta52b} (**f**) 100 μM DAPT, visualized for β-catenin (red), *flk1*:EGFP (green), and TOPRO nuclear stain (gray). Reduction in Notch signaling in *mib*^{ta52b} embryos or by DAPT treatment led to an increase in the number of endothelial precursors in the trunk region.

Fig. 2.5

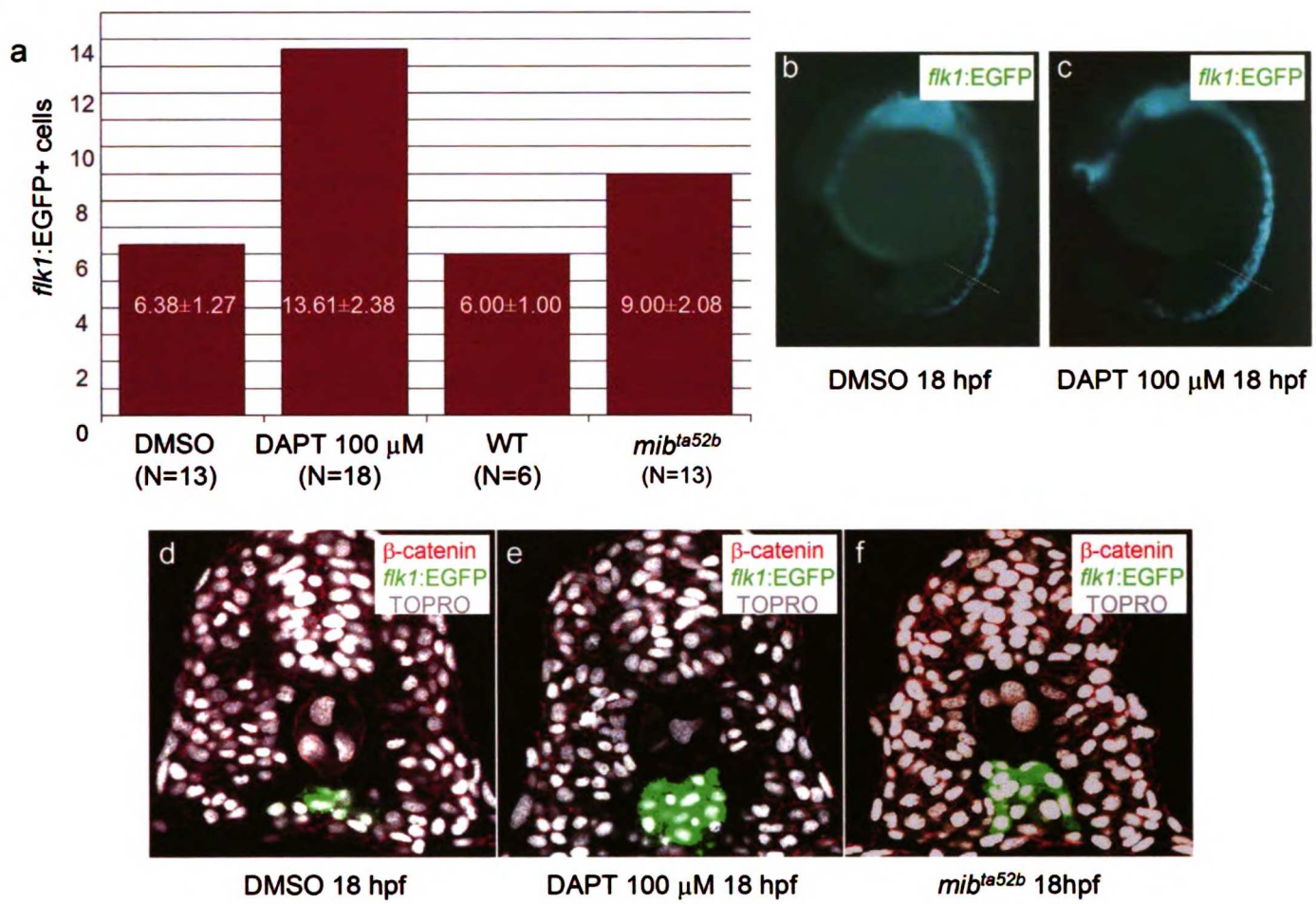
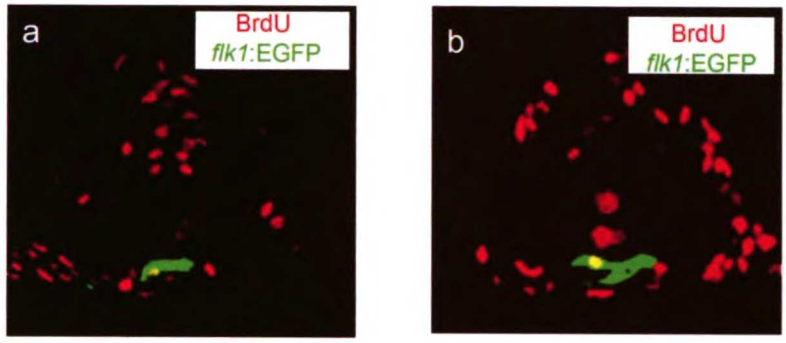

a
flk1:EGFP+ cells
0
2
4
6
8
10
12
14
6.38±1.27
13.61±2.38
6.00±1.00
9.00±2.08
DMSO (N=13)
DAPT 100 μM (N=18)
WT (N=6)
mib ta52b (N=13)
b
flk1:EGFP
c
flk1:EGFP
DMSO 18 hpf
DAPT 100 μM 18 hpf
d
β-catenin
flk1:EGFP
TOPRO
e
β-catenin
flk1:EGFP
TOPRO
f
β-catenin
flk1:EGFP
TOPRO
DMSO 18 hpf
DAPT 100 μM 18 hpf
mib ta52b 18hpf

Figure 2.6

Loss of Notch increases the number of angioblasts while inhibiting hematopoietic differentiation

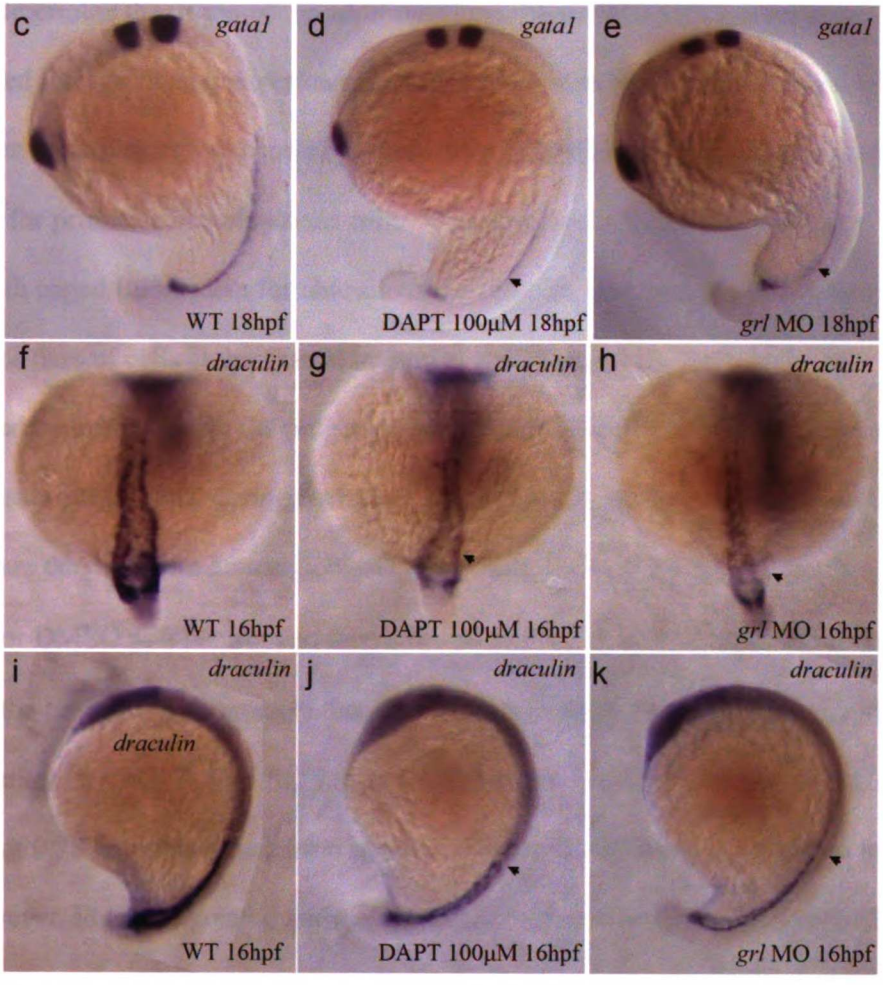
BrdU incorporation was assessed in the endothelial cells of embryos from 14pf-16hpf in DMSO treated control (**a**), and 100 μM DAPT (**b**), embryos at 18hpf. Difference in could be detected between the two. *Gata1* and *draculin* expression in WT embryos (**c**, **f**, **i**), those treated with 100 μM DAPT (**d**, **g**, **j**) or injected with *grl* MO (**e**, **h**, **k**). Arrowheads mark the reduced expression of *gata1* and *draculin* in embryos with reduced Notch activity by either chemical treatment or *grl* MO injection.

Fig. 2.6



DMSO 18 hpf

DAPT 100 μM 18 hpf



reduction in levels of both *gata1*(fig. 2.6 **d, e**) and *draculin* (fig 2.6**g,h,j,k**) as compared to untreated controls(fig2.6 **c,f,i**). Because of our data demonstrating the presence of a bi-potent precursor cell and this data showing the down regulation of hematopoietic markers, we sought to determine if the ectopic angioblasts were arising at the expense of hematopoietic cells.

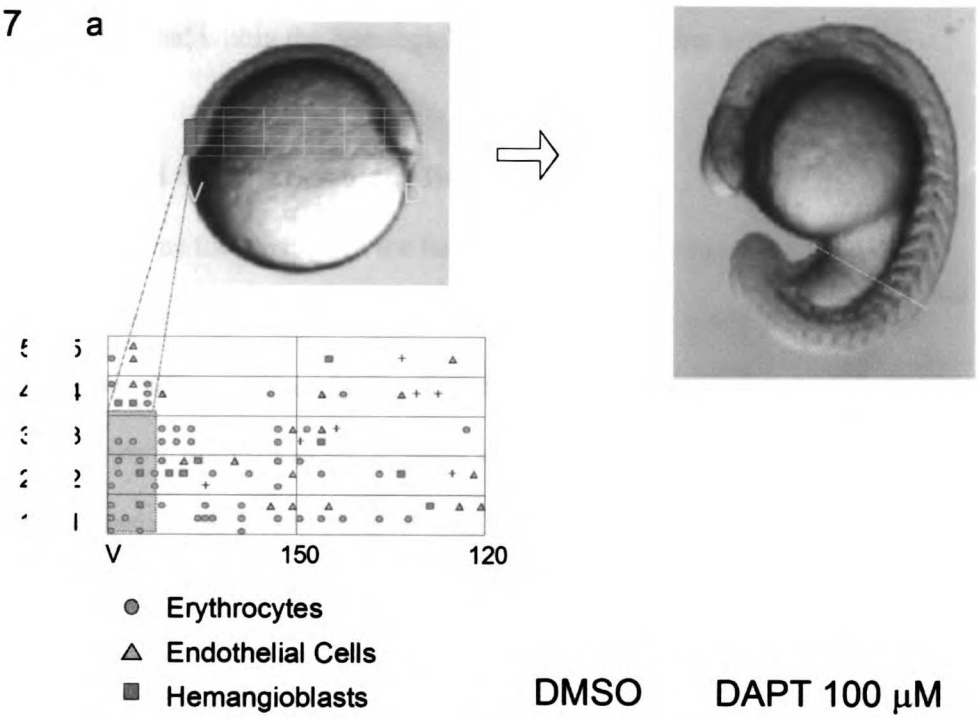
To determine the origin of the additional angioblasts in DAPT treated embryos, we performed lineage tracing experiments. As demonstrated by our fate map and by others[8,15], the ventral marginal zone of gastrula stage zebrafish embryos contains the progenitors for primitive hematopoietic cells. Therefore, we injected one-cell stage embryos with caged fluorescein for ubiquitous distribution, and uncaged fluorescein in the first three tiers of cells at the ventral margin at shield stage (fig. 9a, inset). As demonstrated by our fate map, the progeny of these cells give rise to erythrocytes and hemangioblasts only. Thus, during normal development any labeled vascular cells from this region are derived from hemangioblasts. The embryos were treated with either 100 μM DAPT or DMSO as a control and then allowed to develop until 18hpf. Transverse sections of the posterior portion were then taken and assayed for co-localization with the vascular marker *flk1*:EGFP. (see fig. 9a) In DMSO-treated control embryos, we observed an average of 0.28 angioblasts per focal plane co-localized with uncaged fluorescein (Fig. 9 b-e). However, in DAPT-treated embryos, we observed an average of 3.26 angioblasts per focal plane co-localized with uncaged fluorescein. (fig. 9 f-i). Thus, embryos with reduced Notch function showed a gain of angioblasts at the expense of cells that would have become erythrocytes in wild type embryos. These data suggest that Notch functions

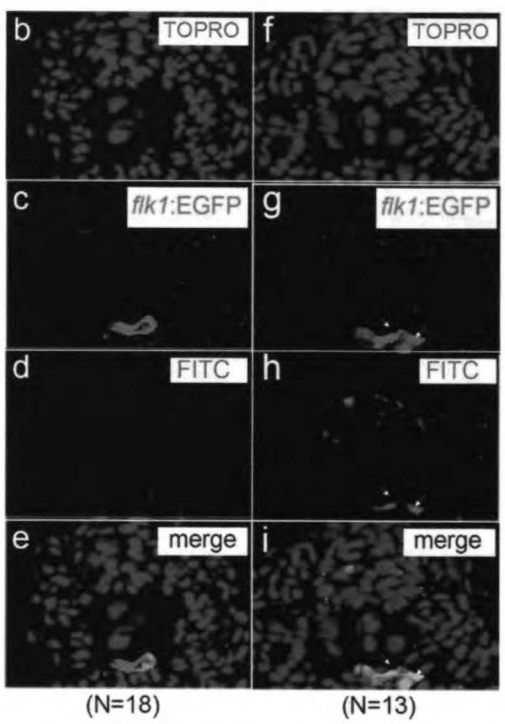
Figure 2.7

Notch signaling might function as a cell fate switch

Diagram of lineage tracing experiments (**a**). Embryos were injected with caged-fluorescein at the 1-cell stage, and at 6 hpf a group of cells within the ventral margin were uncaged. Embryos were raised until 18 hpf and transverse sections from the trunk were visualized by confocal microscopy. Confocal images of embryos treated with DMSO (**b-e**), and with 100 μM DAPT from 10 hpf to 18 hpf (**f- i**), visualized with TOPRO (**b, f**), *flk1*:GFP (**c, g**), FITC (**d, h**) and merged (**e, i**). White arrowheads point to endothelial cells that contain uncaged fluorescein, suggesting that they originated from a group of cells in the ventral marginal zone which in wild type embryos gives rise to hematopoietic cells.

Fig. 2.7 a





to restrict the endothelial within the hemangioblast in order to allow primitive hematopoiesis.

Characterization and Localization of the Hemangioblast

In order to refine the location of the hemangioblast within the embryo at 30 hpf, we performed confocal microscopy on embryos in which an individual hemangioblast was labeled by laser activation (see fig 4). The anterior-posterior position of endothelial cells has been shown to correspond to the initial dorsal-ventral position of endothelial precursors in the gastrula(R. Warga, unpublished). The more ventral an endothelial precursor is within the gastrula, the more posterior it will be upon axis formation. In our fate maps we observed similar results with labeled cells that gave rise to endothelial cells alone; however, we found that the endothelial derivatives of hemangioblast cells did not follow the same pattern. Interestingly, the endothelial derivatives of the bi-potential cells were consistently located to a specific area of the axial vessel, dorsal to the yolk extension regardless of their initial dorsal-ventral position at shield stage (fig. 2.8a). Our data reveal that the endothelial derivatives of the hemangioblasts localize to the same A/P level regardless of their initial D/V position, indicating that they are a specialized population of cells.

In addition to the A/P position, we wanted to know if the hemangioblast was localized to a particular axial vessel. As mentioned in the introduction, it has been reported in mouse and chicken that the dorsal aorta is hematopoietic in an area known as the AGM. The area in which the hemangioblasts were observed corresponds to the area thought to be equivalent to the AGM in zebrafish. Although this area is defined by the expression of the transcription factor *runx1* in the dorsal aorta, it has not been directly

demonstrated that this area produces erythrocytes in zebrafish. To accomplish this we sectioned embryos in which we had labeled a single hemangioblast and then analyzed these sections via confocal microscopy (see fig. 2.8a, b). In the sections we observed, the endothelial derivatives of the hemangioblast were located in both the dorsal aorta and the posterior cardinal vein (arrowheads fig. 2.8c). While this is not conclusive evidence it does indicate that the hemangioblast may be the link between primitive and definitive hematopoiesis. Additional experiments need to be conducted to directly demonstrate that the axial vessels in the zebrafish are hematopoietic themselves. While the results of this experiment were preliminary, future experiments will demonstrate the fate and potential of these endothelial cells.

Discussion

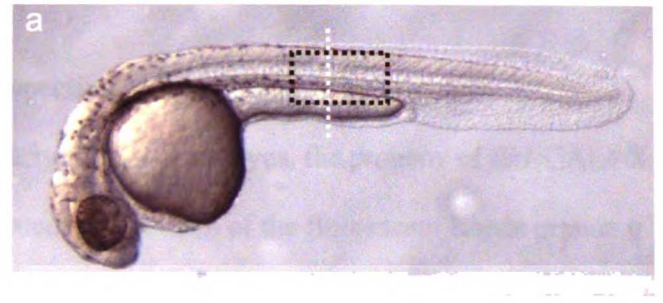
Our data provide the first *in vivo* evidence for the existence of the hemangioblast. While the existence of this cell type has been hypothesized for over 85 years, our data are novel in that they demonstrate conclusively that hemangioblasts exist in live embryos. Our fate mapping studies reveal that at late blastula and early gastrula stage these bi-potential cells are interspersed between cells that give rise to blood and endothelial cells alone. Interestingly, we found that the endothelial derivatives of the hemangioblast consistently locate to a stereotyped position within the vasculature of the embryo. Not only do these data indicate that the hemangioblast represents a distinct, specialized cell type, but also may provide a link between primitive and definitive HSC

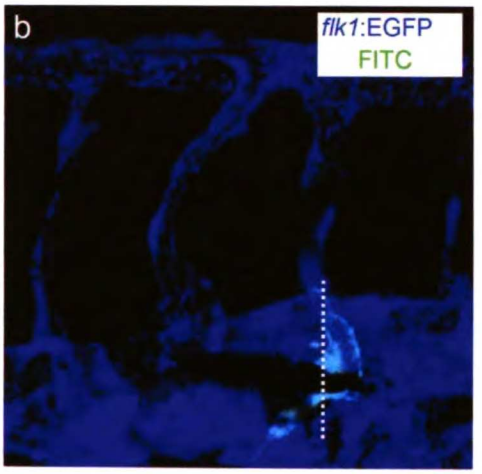
Figure 2.8

Location of Endothelial Derivatives of the Hemangioblast

A/P position of endothelial derivatives of the hemangioblast shown in the box in (**a**). (**b**) **Lateral** confocal view of the location of the endothelial descendants of a single hemangioblast. (**c**) Transverse vibratome section of (**b**) showing labeled cells within the dorsa aorta (DA) (solid arrowhead) and the posterior cardinal vein (PCV) (open arrowhead).

Fig. 2.8





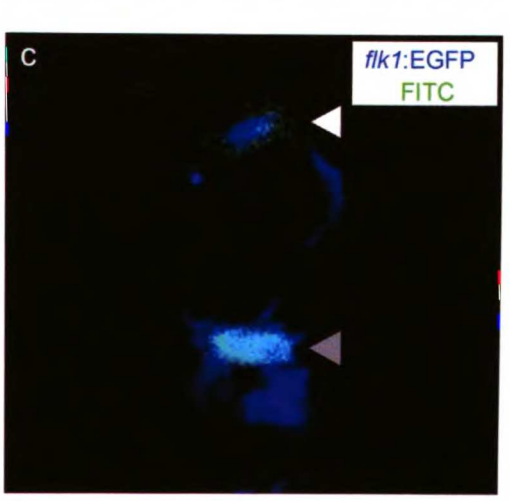
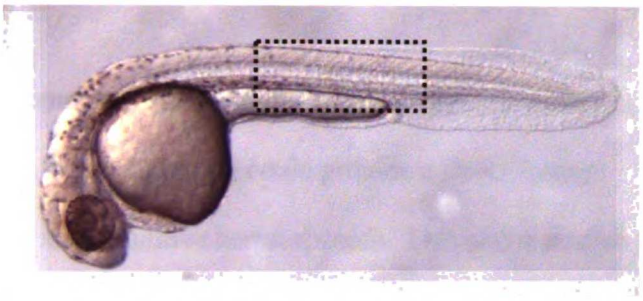


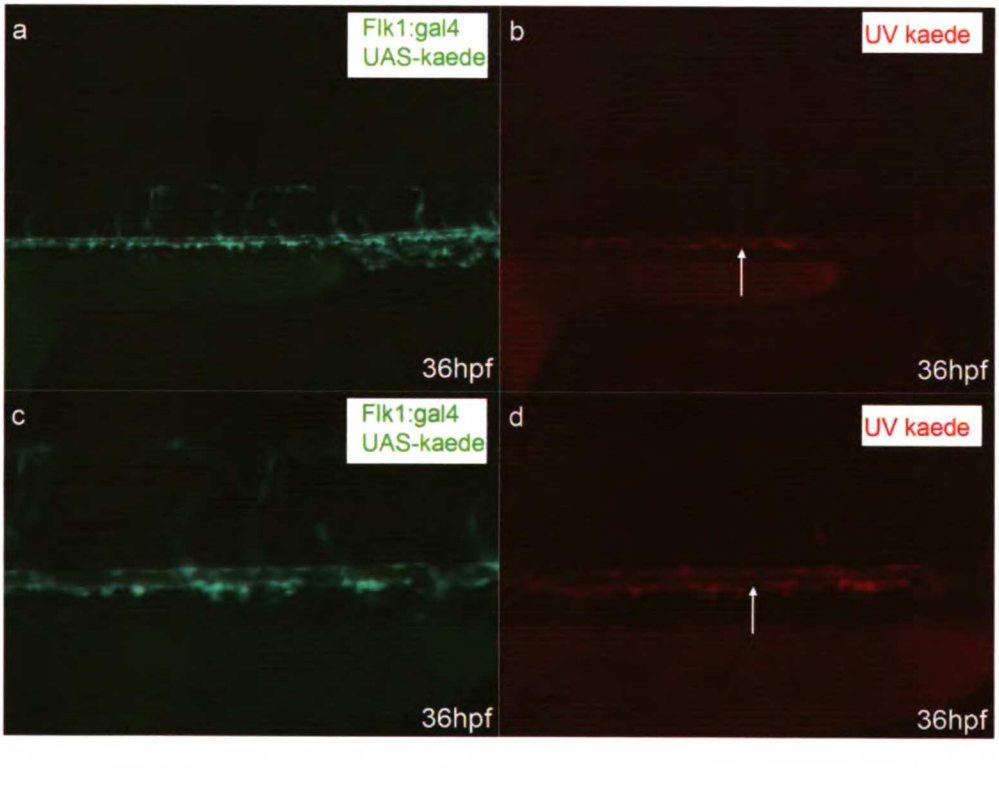
Figure 2.9

Genetic Marking of the Prospective AGM region

To label the axial vessels in early zebrafish embryos, the progeny of *flk1*:GAL4 **X** UAS:Kaede cross were generated. Expression of the fluorescent Kaede protein in the endothelial cells allowing a subset of these cells to be labeled via UV light. **(a,c)** Fluorescent images of 36hpf *flk1*:GAL4 **X** UAS:Kaede embryos prior to exposure to UV light. **(b, d)** Fluorescent images of the prospective AGM region marked by the photo conversion of Kaede by UV light.

Fig. 2.9




a
Flk1:gal4
UAS-kaede
36hpf
b
UV kaede
36hpf
c
Flk1:gal4
UAS-kaede
36hpf
d
UV kaede
36hpf

development. If we could demonstrate that the endothelial derivatives of the hemangioblast localized to the AGM region we could provide a direct lineage relationship between primitive and definitive hematopoiesis. This could resolve the apparent discrepancy in the literate regarding the number of times hematopoietic stem cells arise in the embryo.

As mentioned in the discussion, the data from mouse and chicken present conflicting views on the origins of HSC within the early embryo. The data from chicken point toward two separate origins, while the experiments done in mouse cannot distinguish between multiple or a single origin. Our cell tracing experiments show that a single cell can give rise to a primitive erythrocyte and an endothelial cell that localizes to the prospective area of definitive hematopoiesis. We know that the labeled erythrocytes are primitive due to the nature of the *gata1*:dsRed transgene used in the experiments. Because the DsRed protein takes so long to fold, transgene expression is not visible until 30hpf almost 20 hours after mRNA transcripts can be detected. Thus at 30hpf, the DsRed positive erythrocytes observed are primitive as definitive hematopoiesis does not begin until at least 24hpf. Upon sectioning these embryos in which we had labeled a hemangioblast we were able to show that the endothelial derivatives were localized to the axial vessels at an A/P level thought to be the site of definitive hematopoiesis. Based on these data, we hypothesize that the hemangioblast first divides to produce a primitive erythrocyte and a specialized endothelial cell. This endothelial cell then becomes part of the axial vessel dorsal to the yolk extension where it produces definitive erythrocytes. By demonstrating that the hemangioblast produces primitive erythrocytes and then endothelial derivatives that localize to the site of putative definitive hematopoiesis in the

dorsal aorta, we believe we have provided evidence in zebrafish for a single origin of HSCs. These data could potentially resolve the question of single or multiple origins of HSCs in vertebrate embryos. Further data on the developmental potential of the endothelial cells in the axial vessels is needed to solidify this hypothesis.

Future Directions

One such method for conducting this experiment would be to genetically label the axial endothelial cells using the gal4-UAS system now available in zebrafish. Using the *flk1* promoter driving gal4 in one line crossed to a UAS-kaede line would lead to the expression of the fluorescent kaede protein in the endothelial cells (fig 2.9a, c) Briefly, kaede is protein originally found in rock coral that is initially fluorescent in the FITC channel (~ab480nm, em 520nm), but can be irreversibly and stably converted to become fluorescent in the TRITC channel (~ab520nm, em 560nm) when exposed to UV light (<400nm). Using this protein it is be possible to mark a subset of the endothelial cells at a chosen point in development. \ Using this system we were able to label the axial vessels corresponding to the location of the hemangioblast using expression of kaede and UV light (fig. 2.9b, d). If one were to perform this experiment in embryos that contained the *gata1*:dsRed transgene, one could look for co-localization between the activated kaede and DsRed to show that the endothelial cells from a selected area and time were producing erythrocytes. Even though the activated kaede and DsRed are in the same fluorescent channel, one could possibly fix the embryos and then antibody stain against the two fluorescent proteins and use secondary antibodies in different fluorescent channels. If this method is not possible (the antibodies to distinguish between the different forms of kaede are not available at this date), the experiment could be done

without the *gata1*:dsRed transgene and one would just look for labeled red fluorescent cells in the circulation of a live embryo.

In addition the question of the role the hemangioblast plays in definitive hematopoiesis, the molecular nature of hemangioblast differentiation should be an area of intense future investigation. The fate maps presented in the data section only represent two early stages of wild type development. In order to gain a fuller understanding of the developmental potential of the hemangioblasts, single cell resolution fate maps should be constructed at later stages (*i.e.* late gastrulation, tailbud). From such data it should be possible to track the number and location of hemangioblasts over time. Additionally, these data may provide us with an idea of how many times the hemangioblast divides during early development as well as the order and fate of its descendents.

In addition to a more comprehensive set of wild type lineage data, fate maps should be constructed in mutant embryos known to have abnormal hematopoiesis and angiogenesis. These mutants include *cloche*, *bloodless*, and embryos that contain altered levels of Notch signaling. *Cloche* embryos completely lack primitive hematopoietic cells and have a severely reduced number of endothelial cells at 18hpf. Sometime after 24hpf, the endothelial cells begin to recover in an anterior to posterior fashion (S.W. Jin and D.Y. Stainier, unpublished). A day later, hematopoiesis recovers in *cloche* mutants. Because there is no ectopic cell death in *cloche* embryos, it would be very interesting to ascertain the fate of single cells. What is the fate of cells that would normally become blood and endothelium? Are there any hemangioblasts left in *cloche* embryos? One hypothesis is that hemangioblasts are the only hematopoietic and endothelial cells in *cloche* embryos and that they repopulate the embryo after 24hpf. In *bloodless* mutants

primitive hematopoiesis does not occur, but definitive hematopoiesis allows the embryos to recover around day 3 in development. It would be interesting to determine the fate of the cells that would normally become primitive blood as well as to see the effects on the number of hemangioblasts and angioblasts. As shown in figure 2.7, the inhibition of notch signaling leads to ectopic endothelial cells and a reduced number of blood cells. Even though some primitive fate mapping experiments were done in embryos with compromised notch signaling, it would be informative to repeat the experiments using single cell fate mapping. In order to achieve this, the amount of DAPT must be titrated so that and effect is still seen and the embryos survive until 30hpf (to be scored). With these data in hand one could definitively make the statement that notch signaling regulates the fate decision within the hemangioblast.

Materials and Methods

Zebrafish husbandry, microinjection, and chemical treatment

Zebrafish (Danio rerio) embryos were obtained from a mixed wild-type strain and raised at 28°C as previously described[16]. Hemizygous *Tg(flk1:*EGFP*)*^{s843} [17] and double hemizygous *Tg(flk1:*EGFP*)*^{s843}*; Tg(gata1:DsRed)*[18]embryos were used. Microinjections of Morpholinos (MO) were performed as previously described [19]. The sequence of the *grl* MO used is: 5'-CGCGCAGGTACA GACACCAA AAACT-3' [20]. One-cell or two-cell stage embryos were injected with 4ng of MO and were raised at 28 C° until harvested. Embryos were treated with 100 μM of the γ-secretase antagonist DAPT (CalBiochem) as previously described [14]. As a control, embryos from the same batch were treated with DMSO. Embryos were treated from 10 hpf until either 14 hpf or before fixation at 18 hpf.

Immunohistochemistry and in situ hybridization

Immunohistochemistry was performed as previously described [21,22]. . Processed samples were mounted in Vectashield (Vector Laboratories) and the images were acquired using a Zeiss LSM5 Pascal confocal microscope.

Immunohistochemistry

Immunohistochemistry was performed as previously described[21]. The following antibodies were used at the following concentrations: mouse IgG anti-β-catenin (Sigma) [22], Polymerized actin was visualized by phalloidin (Molecular Probes) at 1:100 [21,22]. Nuclei were visualized with TOPRO (Molecular Probe) at 1:10000 [23], Anti-FITC goat IgG (Molecular Probes) at1:100, anti-GFP mouse Ig2a (Molecular Probes) at 1:500, and anti-DsRed rabbit IgG (BD Biosciences) was used at 1:200. Processed samples were mounted in Vectashield (Vector Laboratories) and imaged using a Zeiss LSM5 Pascal confocal microscope.

RNA *in situ* hybridization

Whole mount in situ hybridization was performed as previously described [24]. Riboprobes for *gata1* [25]and *draculin* [26] were prepared with Ambion mMessage Machine. Embryos were mounted in benzylbenzoate:benzyl alcohol and documented with Zeiss Axiocam.

Lineage tracing

Our lineage tracing strategy was adapted from those previously described[27,28]. Briefly, double hemizygous *Tg(flk1:*EGFP*)*^{s843}*;Tg(gata1:DsRed)* fish were intercrossed and the resulting embryos injected with 4.6 nl of 0.2% DMNB-caged, biotinylated, lysine fixable fluorescein dextran (10 kD) (Molecular Probes) in 0.2M KCl and allowed to develop until 40% epiboly (5 hpf) or shield stage (6 hpf). Fluorescein was activated in a

single cell in each embryo using pulses from a 365 nm laser (Photonic Instruments) focused through a 40X objective on a Zeiss Axioskope 2 microscope. The laser was calibrated in the z-plane and centered in the field of vision by puncturing test holes (approximately 1 μm in diameter) in a foil-coated slide. Additionally, the intensity of the laser was regulated through the use of a neutral density filter. The focus and intensity of the laser were adjusted such that it was only able to puncture a small point in the foil, and only in the plane of focus. After laser activation of a single cell, each embryo was assessed for supernumerary labeled cells, in the same or different focal planes. Embryos in which more than one cell had been labeled were discarded. The position of the cell was then recorded digitally using a Spot camera. In the case of cells activated at 40% epiboly, the embryos were allowed to develop until shield stage so that their dorsal/ventral position could be determined. Embryos at 30 hpf were fixed overnight in 4% PFA and then permeabilized in 100% methanol. In order to detect FITC, EGFP and DsRed, embryos were incubated in anti-FITC goat IG, anti-GFP mouse Ig2a, and anti-DsRed rabbit IG followed by anti-goat Alexa Fluor 568, anti-mouse Alexa Fluor 488, and anti-rabbit Alexa Fluor 648 (Molecular Probes).

In order to assess the effects of Notch signaling, homozygous *Tg(flk1:EGFP)*^{s843} were intercrossed. These embryos were injected with DMNB-FITC at the one cell stage and at shield stage the cells located in first three vegetal tiers of the ventral margin were activated using pulses from a 365 nm laser focused through a 20X objective. Embryos were then treated with 100μM DAPT or 1% DMSO and fixed at 18hpf in 4% PFA overnight. To detect activated fluorescein and distinguish it from GFP, embryos were

incubated in biotinylated rabbit IgG anti-FITC antibody followed by Alexa Fluor 568 Streptavidin (Molecular probes).

BrdU Incorporation

Homozygous *Tg(flk1:EGFP)*^{s843} embryos were treated with 100 μM DAPT or 1% DMSO from 10 hpf to 18 hpf and also incubated in 10 mM BrdU in 15% DMSO from 12 hpf to 18 hpf in two hour intervals. At 18 hpf, embryos were fixed in 4% PFA overnight and then incubated in rat monoclonal anti-BrdU followed by anti-rat Alexa Fluor 568.

Kaede Activation

flk1:GAL4, UAS:Kaede embryos were generated and allowed to develop until 36hpf. Those with bright fluorescence were selected and mounted in 3% methyl cellulose on a Zeiss Axiocam. The Kaede protein was photo converted by reducing the pinhole of the UV lamp to its smallest setting and then exposing the embryo to UV light using the DAPI filter set for ~5 seconds.

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Chapter 3 The mechanism of pectoral fin bud formation

Introduction

The vertebrate limb has provided an excellent system for the study of the cellular and molecular aspects of pattern formation during development. Classic embryological experiments in the chicken embryo and genetic manipulations in the mouse have greatly enhanced our knowledge of how growth and signaling combine to pattern the limb bud [1]. While the usefulness of these two systems for the study of limb development is unquestionable, neither one boasts the availability of both genetic manipulation and *in vivo* analysis. The zebrafish embryo provides a unique system in vertebrate development which combines the accessibility of external development and the power of genetics. The pectoral fin of the zebrafish has been demonstrated to be structurally homologous to the vertebrate forelimb, as many genes known to be involved in limb development are also expressed in similar patterns in the fin bud: the genes of the Hoxd cluster and *shh* are expressed in the posterior fin bud mesenchyme, *fgf-8* and *fgf-4* are expressed in the AER-like apical fin fold at the proximal edge of the bud, and *tbx5*, and *fgf-10* are expressed throughout the mesenchyme of the fin bud [2-6]. Functionally, mutations in some of these genes display the same phenotype as the corresponding mouse knockouts *e.g.* the fin buds of the Shh mutant *sonic you* have defects in A/P polarity, tbx5 mutant *heartstrings* completely lacks fin buds, and the fgf-10 mutant *daedelus* displays truncated fin buds reminiscent of the mouse knockout phenotype. [7-9] [6,10,11] For these reasons, the zebrafish fin bud is the ideal model to study the mechanisms of appendage development.

Pectoral Fin Bud Development

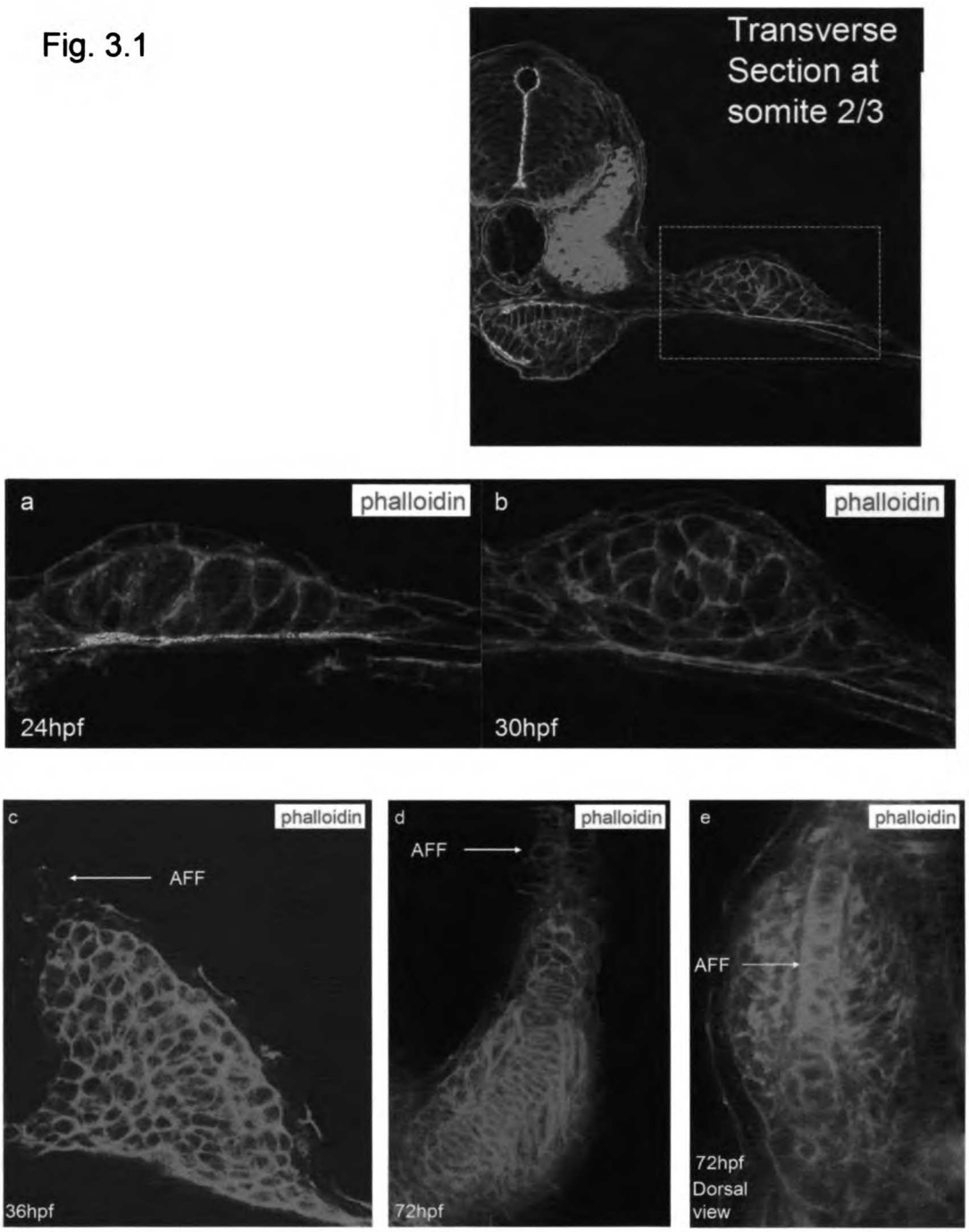
The pectoral fin bud initially arises from the lateral plate mesoderm adjacent to somites 2

Figure 3.1

Early Development of the Pectoral in Zebrafish

Transverse Sections of Phalloidin stained zebrafish embryos at the level of somite 2/3 showing the progression of pectoral fin development. (**a**) 24hpf section when the fin bud is only 1-2 cell layers thick and not morphologically distinct. (**b**) 30hpf section showing the thickening of lateral plate. (**c**) 34hpf section showing a much more advanced fin bud with an apical fin fold (AFF see arrow) at its distal tip. (**d**) Transverse section of a day 3 fin bud showing a much larger AFF. (**e**) Dorsal view of a day 3 fin bud showing a different view of the AFF

Fig. 3.1


Transverse Section at somite 2/3
a
phalloidin
24hpf
b
phalloidin
30hpf
c
phalloidin
AFF
36hpf
d
phalloidin
AFF
72hpf
e
phalloidin
AFF
72hpf
Dorsal view

and 3 and the overlying ectoderm[12]. At 23 hours post fertilization (hpf) fin bud development begins with a thickening of the lateral plate mesoderm until the fin bud becomes morphologically distinct at 26hpf [12](fig 3.1a). Subsequently, the apical fin fold, thought to be analogous to the tetrapod Apical Ectodermal Ridge (AER), forms at 34hpf as the distal ectoderm overlying the lateral plate mesenchyme folds back onto itself (see figure 3.1c). Both the AFF and the mesenchyme underlying the AFF continue to proliferate and the fin bud increases substantially in size (fig. 3.1d, e). By day 7, the AFF makes up most of the external, visible portion of the fin (fig 3.2a, b). Subsequently, the mesodermally derived mesenchyme condenses to form the skeletal elements that make up the pectoral girdle while the AFF itself grows from the dermal fin rays that make up most of the visible external pectoral fin in the adult (fig 3.2c).

What is the Mechanism of Pectoral Fin Formation?

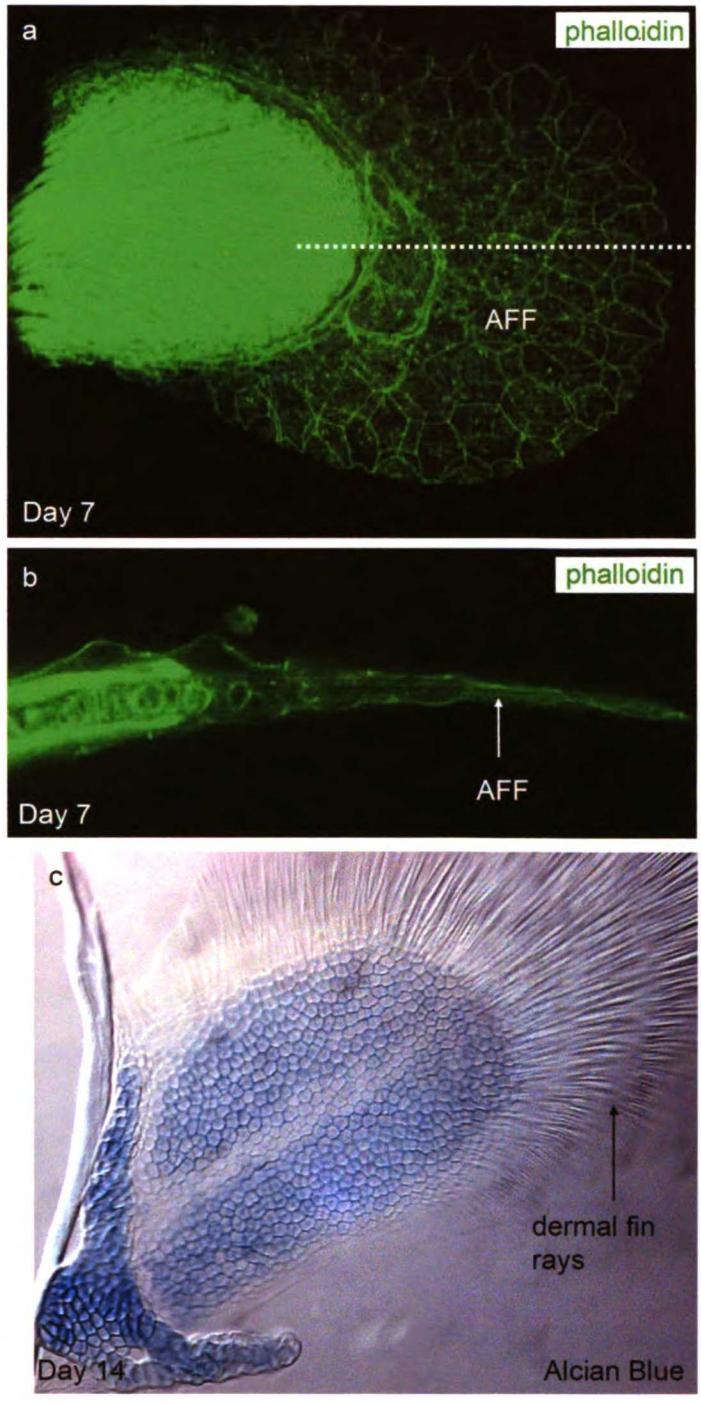
While many studies have been conducted to investigate the molecular mechanisms of patterning the pectoral fin in zebrafish, little has been done to study the pectoral fin before the formation of the AFF. Unlike Tetrapods in which the formation of the limb bud and the presence of the AER (or its molecular components) are nearly concomitant, the zebrafish pectoral fin arises and develops for almost 12 hours before the formation of the AFF (23hpf-34hpf). How does this tissue arise and how is it patterned? Is the pectoral fin bud formed via the condensation of the lateral plate? The dynamic pattern of expression of tbx55 in the lateral plate during these stages would seem to indicate this.If so what is the Anterior-Posterior extent of this contribution to the fin bud? Does the pectoral fin arise from in increase in cell proliferation? Early studies of

Figure 3.2

Later Development of the Pectoral Fin Bud

Wholemount confocal and brightfield pictures of late larval stage fin buds. (**a**) Confocal projection of a day7 fin bud stained with phalloidin. Note the flat, elongated cells of the AFF. (**b**) Confocal section of day7 fin bud in the plane shown by the line in (**a**) arrow shows AFF. (**c**) Brightfield picture of day14 pectoral fin skeletal prep. The alcian blue stain marks the condensing skeletal elements formed by the lateral plate mesoderm, while ectodermally derived dermal fin rays (arrow) remain translucent.

Fig. 3.2


a
phalloidin
AFF
Day 7
b
phalloidin
AFF
Day 7
c
dermal fin
rays
Day 14
Alcian Blue

the chicken limb bud indicate a difference in proliferative index between flank and limb bud lateral plate underlies the formation of the limb bud[13]. We sought to uncover the mechanism by which the pectoral fin forms and how this may differ from limb formation in tetrapods.

Cell Migration Underlies Pectoral Fin Bud Formation

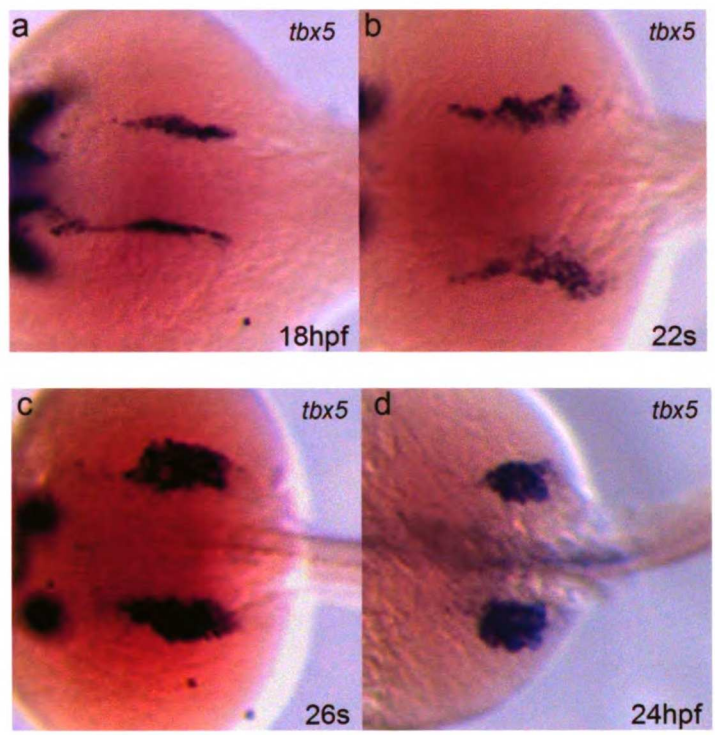
In order to determine the extent of the lateral plate along the antero-posterior (A/P) axis that contributes to the fin bud, I conducted lineage analysis on pre-fin bud stage embryos. Some data suggest that cell migration does play a role in this process. Mice that lack Fgf8 form limb buds that are smaller than their wild type counterparts without a difference in cell proliferation or cell death [14]. Additionally, zebrafish embryos without *tbx5* signaling do not form a fin bud and display a migration defect in the lateral plate mesoderm that otherwise would have formed the bud[15]. Initially, the *tbx5* expressing lateral plate cells extend from the tissue adjacent to somites 1-4 at 18hpf (fig. 3.3a) and then these cells condense (fig 3.3b,c) until they are only in the area adjacent to somites 2 and 3 at 24hpf (fig3.3d). Specifically, the cells of the lateral plate that express *tbx5* condense along the A/P axis during wild type fin bud development[5], but in absence of *tbx5* expression these lateral plate cells do not condense and actually become more diffuse. Because this evidence relies upon the expression pattern of an RNA transcript, it is possible that the dynamic pattern is only due to a variance in the size of the field of cells that express *tbx5* rather than cell migration. Because of the nature of these data, I investigated the role that cell migration plays in the initial formation of the fin bud in zebrafish through the use of cell labeling and lineage tracing.

Figure3.3

Dynamic Expression Pattern of Tbx5 in the Anterior Lateral Plate

Wholemount RNA *in situ* hybridization showing the expression pattern of *tbx5* in the pectoral fin forming anterior lateral plate. Dorsal view with anterior to the left. (**a**) 18hpf, (**b**) 20hpf, (**c**) 22hpf, (**d**) 24hpf

Fig. 3.3



Results

The Migration of the lateral Plate is necessary for fin bud formation

Because the expression of *tbx5* is initially diffuse in the pectoral fin bud forming region of the lateral plate at 18hpf and then condenses at 24hpf when the fin bud becomes visible[5], I conducted cell labeling experiments starting prior to 18hpf and then assessed their contribution to the fin bud after 24hpf. Zebrafish embryos were injected at 1-cell stage with DMNB-FITC for ubiquitous distribution and then allowed to develop in the dark until 16hpf (fig. 3.4a). Cells in the lateral plate were then labeled through photo activation of the FITC with a 365nm laser focused through a 20x objective on a compound microscope. Embryos were then allowed to develop until 28hpf and then fixed and processed for immunohistochemical staining against FITC to amplify the signal. Using the extent of *tbx5* expression at 18hpf in the lateral plate as a guideline, cells in the lateral plate adjacent to specific somites were labeled. We found that cells in the lateral plate adjacent to somites 1-4 contributed to the mature pectoral fin bud (see fig. 3.5). Labeled cells adjacent to somite 1 were found in the pectoral fin bud at 28hpf 73% of the time while cells adjacent to somite 2 were found 56% of the time, somite 3 53%, and somite 4 50%. We also found that labeled cells outside of this region such as adjacent to somite 5 never contributed to the fin bud.

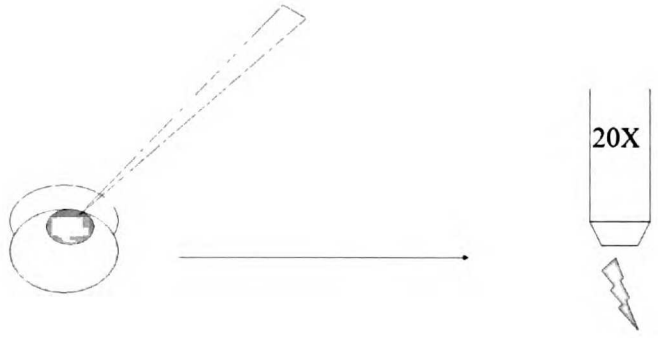
Similar labeling experiments were conducted in *tbx5* morpholino injected embryos, which do not form fin buds[15,16]. In these experiments, labeled cells from the lateral plate next to somites 1-4 were never found in the presumptive pectoral fin region (fig 3.4c), and these labeled cells were often found on the ventral side of the yolk. While the pectoral fin arises at the A/P level of somites 2 and 3, we were able to

Figure 3.4

Schematic for Labeling Cells in the Lateral Plate Prior to Fin Bud Formation

Clumps of cells in the anterior lateral plate were labeled with activated FITC at 16-18hpf based on their A/P position using the somites as a guideline. At 30hpf the contribution of these cells to the fin bud was assessed. (**a**) Dorsal view of 18hpf embryo with FITC labeled cells in the lateral plate (arrow). (**b**) Lateral view of WT 30hpf embryo showing labeled cells in the pectoral fin bud (arrow). (**c**) Lateral view of tbx5 MO injected embryo showing labeled cells scattered across the yolk. (**d**) Lateral view of *dHand* mutant embryo showing labeled cells in the fin bud (arrow).

Fig. 3.4



DMNB-FITC injection into embryos at 1-cell stage

Activate FITC in lateral plate at specific somite level at 16-18hpf

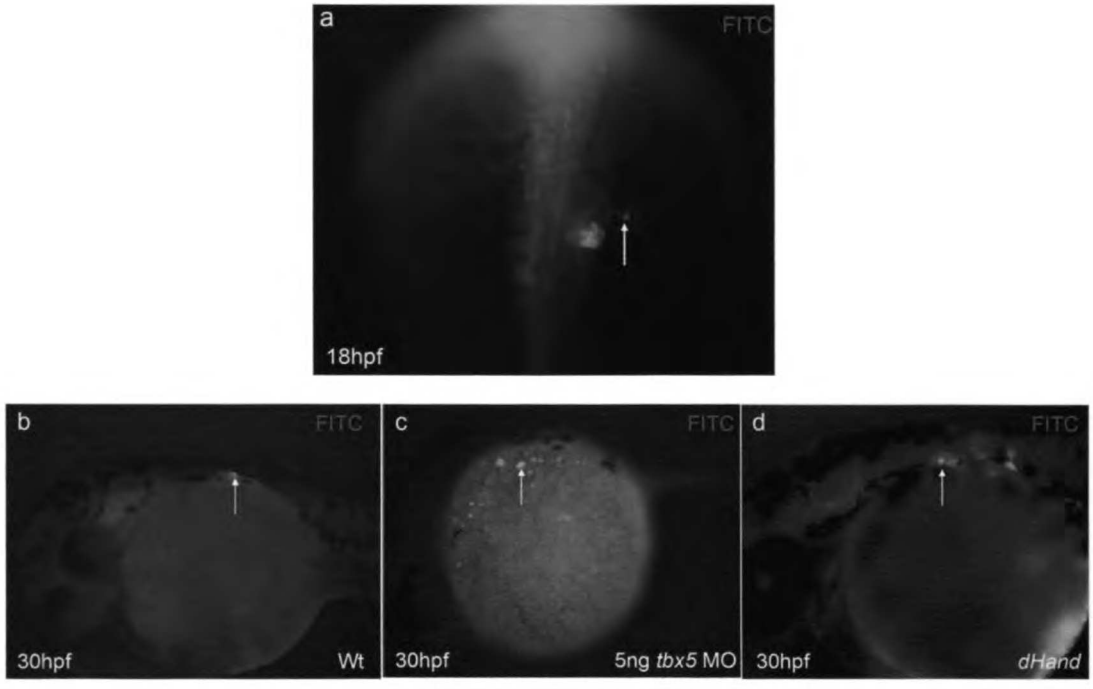


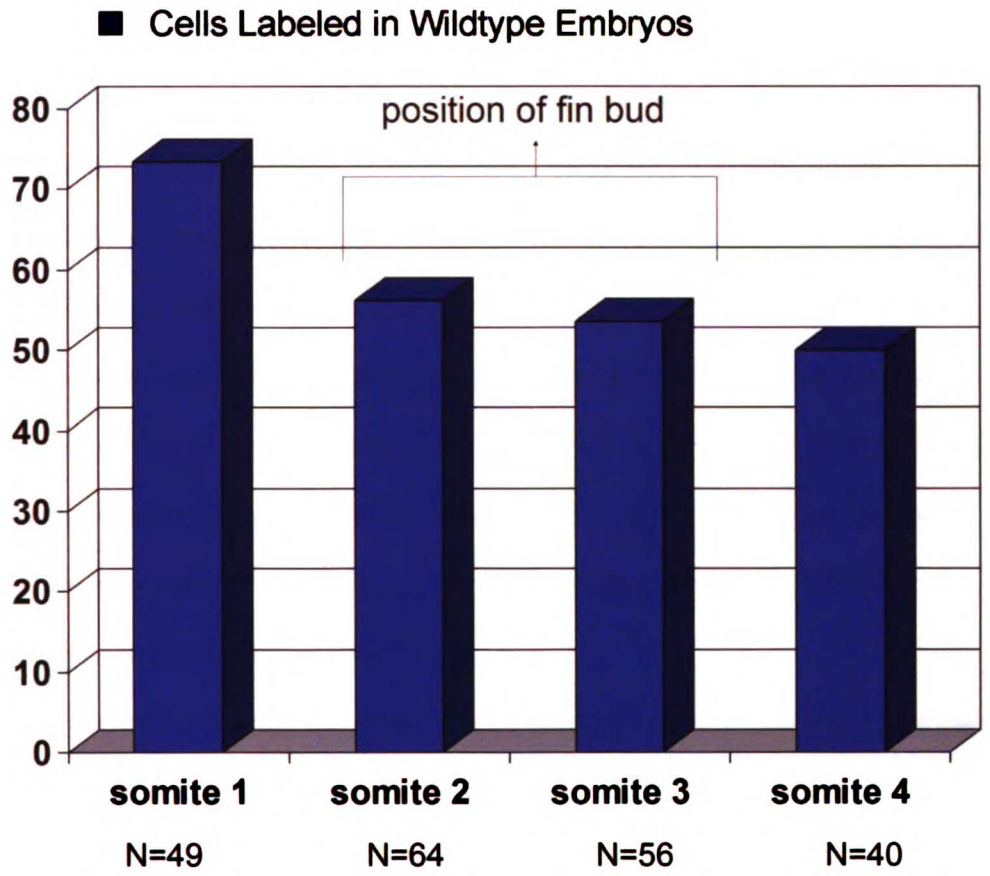
Figure 3.5

Summary of Lineage Tracing Results in the Lateral Plate

This graph represents all of the results of the lateral plate lineage tracing conducted. The percentage depicted in the y-axis represents the embryos in which the lateral plate adjacent to a specific somite contributed to the pectoral fin at 30hpf.

Fig. 3.5

Percentage of Lateral Plate Cells Contributing to the Pectoral Fin



demonstrate that the lateral plate adjacent to somites 1-4 contributes to some extent to pectoral fin. These results suggest that the cells of the anterior lateral plate migrate and condense between 18hpf and 24hpf during the formation of the pectoral fin.

Cell Proliferation contributes to fin bud outgrowth subsequent to migration

While many studies have been done to address the induction, outgrowth, and patterning of the prospective limb/fin field, relatively little has been done to investigate the mechanism of initial limb/fin bud formation. It has been hypothesized that differences between cell proliferation in the lateral plate mesoderm of the prospective limb field and the flank initially gives rise to the limb bud [1]. Previously, it has been demonstrated in the developing chicken embryo that there is a difference in mitotic index between the flank and the prospective limb field before the appearance of the a limb bud [13]. Because we had previously demonstrated that cell migration was a fundamental mechanism of pectoral fin bud formation, we sought to determine whether cell proliferation might also play in this process.

In order to determine the extent of cell proliferation in the lateral plate during pectoral fin bud formation, BrdU incorporation experiments were performed at fin bud forming stages. Embryos were subjected to two-hour BrdU pulses at intervals between 16hpf and 26hpf; BrdU was removed by washing the embryos, which were then allowed to develop until 36hpf. After fixation, these embryos were stained for BrdU incorporation, sectioned, and subjected to confocal analysis. We detected BrdU positive cells in the pectoral fin forming lateral plate from 16hpf-18hpf and then again from 24hpf-26hpf. Interestingly, we did not see any significant incorporation

Figure 3.6

Cell Proliferation in the Lateral Plate of WT and *dHand* Embryos

BrdU incorporation assays were performed over 2-hour intervals during the time of initial fin bud formation (18hpf-24hpf) in WT (**a-e**) and *dHand* (**f-j**) sibling embryos. After the 2hr BrdU pulse the embryos were washed and allowed to develop until 36hpf. They were processed for immunohistochemistry using antibodies against BrdU and β-catenin (as a counterstain) and then sectioned for analysis. WT embryos demonstrate proliferation in the pectoral fin forming lateral plate between 16-18hpf (**a**), but this proliferation does not continue through 18-22 hpf (**b-d**). The proliferation resumes at 24hpf (**e**). Initially, *dHand* mutant embryos demonstrate proliferation similar to WT at 16-18hpf (**f**). *dHand* embryos do not proliferate from 18-22 hpf (**g-i**) like their WT siblings. Unlike WT embryos, *dHand* mutant embryos fail to resume proliferation at 24hpf (**j**).

Fig. 3.6

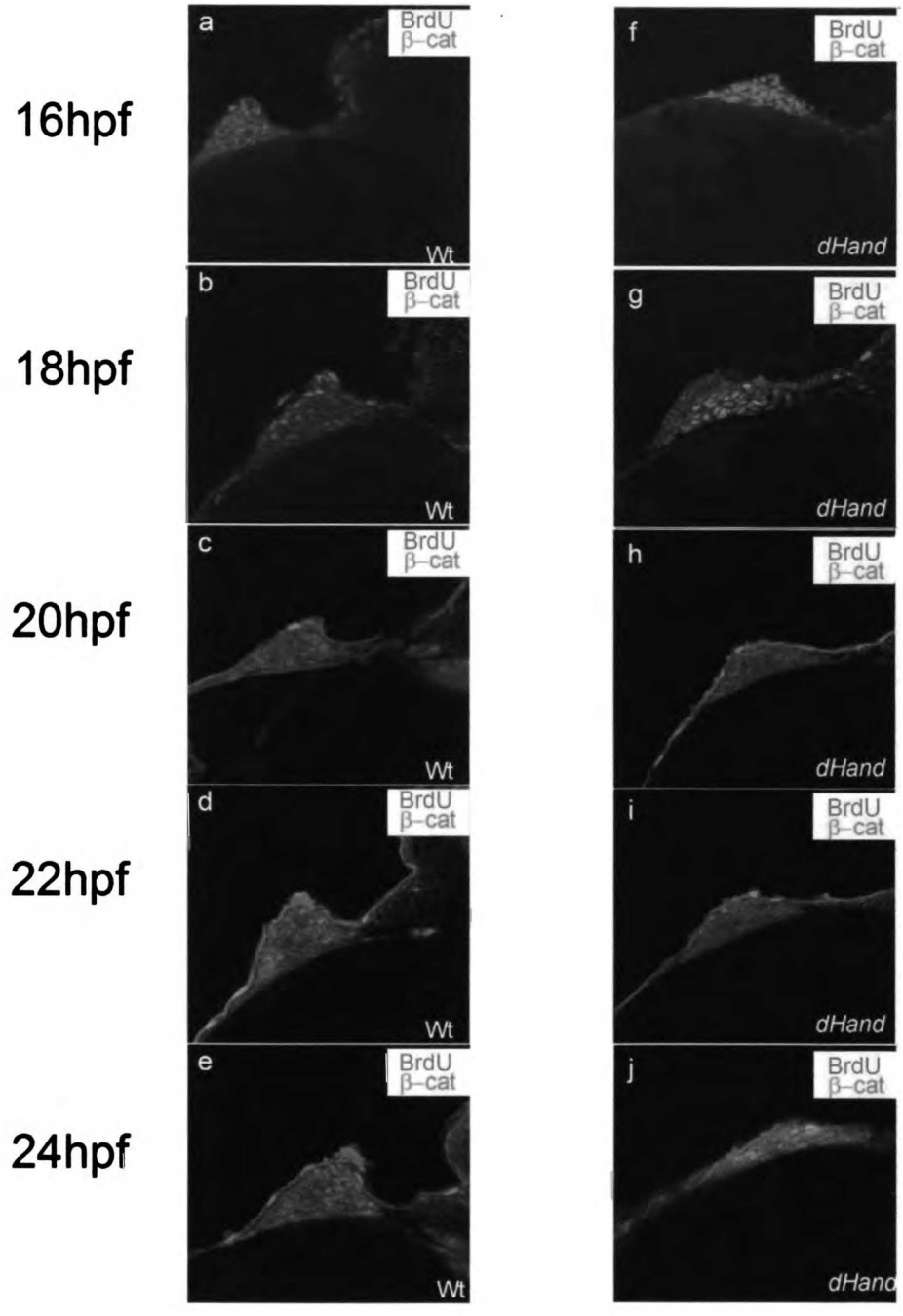

16hpf
18hpf
20hpf
22hpf
24hpf
a
BrdU
β-cat
Wt
b
BrdU
β-cat
Wt
c
BrdU
β-cat
Wt
d
BrdU
β-cat
Wt
e
BrdU
β-cat
Wt
f
BrdU
β-cat
dHand
g
BrdU
β-cat
dHand
h
BrdU
β-cat
dHand
i
BrdU
β-cat
dHand
j
BrdU
β-cat
dHand

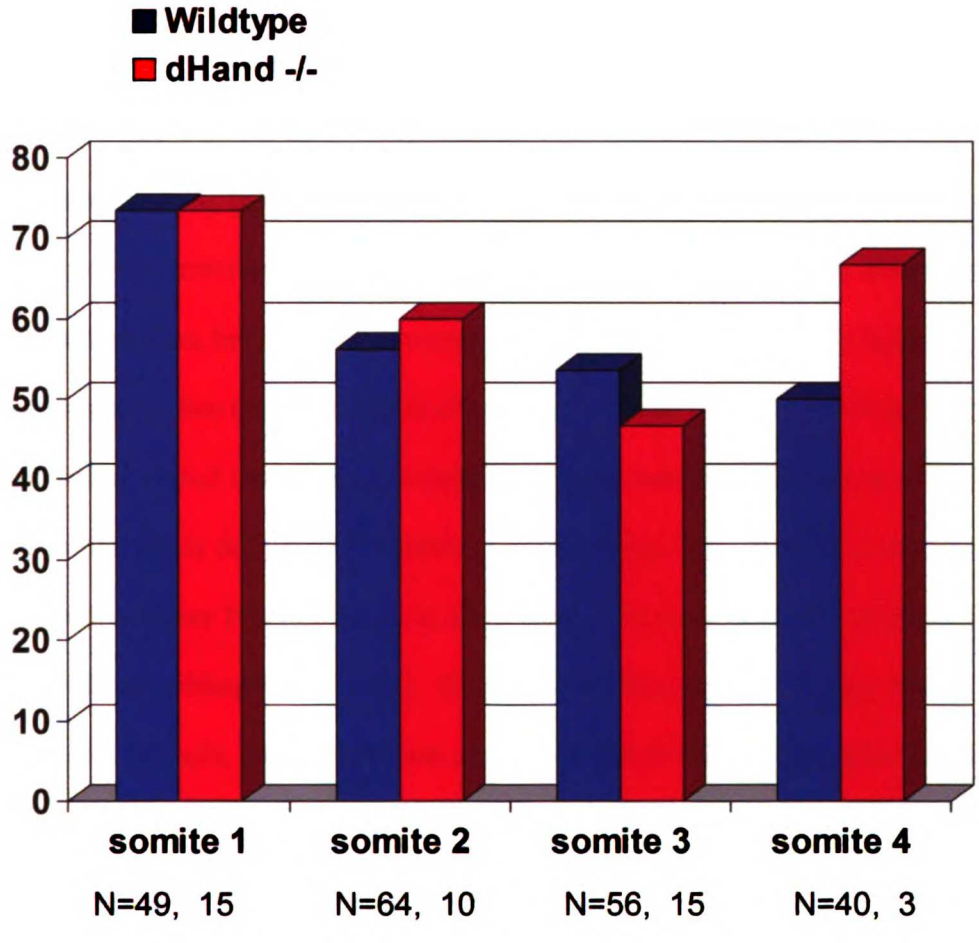
Figure 3.7

Summary of Lineage tracing Results in dHand Embryos

A graph summarizing the lineage tracing experiment performed in the lateral plate of *dHand* mutant embryos compared to those performed in WT. The y-axis represents the percentage of labeled embryos containing cells that contribute to the pectoral fin. The lateral plate in *dHand* embryos contributes to the fin bud in a similar manner as in wild type indicating that cell migration does not underlie the phenotype. Wild Type in Blue, *dHand* in Red.

Fig. 3.7

Percentage of Lateral Plate Cells Contributing to the Pectoral Fin



between 18hpf-24hpf. This corresponds to the window of time when the lateral plate in condensing to form the lateral plate.

An Absence of Cell Proliferation Underlies the *dHand* Phenotype

Now that we had demonstrated that cell migration and proliferation were occurring during initial pectoral fin bud formation and growth, we sought to undercover the relationship between these two processes. This was done through the investigation of the role of the basic helix-loop-helix transcription factor *dHand* which is expressed throughout the anterior lateral plate and later the pectoral fin. Zebrafish *dHand* mutants form pectoral fins, but these fins are severely growth retarded and never form the AFF[17]. To demonstrate that the phenotype in *dHand* mutants was not due a defect in cell migration as in *tbx5* morphants, we conducted lineage-tracing experiments in the lateral plate as previously described. Labeled cells in the lateral plate of *dHand* embryos adjacent to somites 1-4 contributed to the pectoral fin bud at nearly the same percentage as in wild type siblings (see fig 3.7). Even though *dHand* mutants display smaller and dismorphic fin buds, the condensation of the lateral plate to form the initial fin bud still occurred. Because of this phenotype and previous studies showing that *dHand* can be necessary for proliferation, we hypothesized that the fin bud phenotype of *dHand* mutants was due to a defect in the proliferation of the lateral plate. In experiments parallel to those conducted in wild type embryos, BrdU incorporation assays were performed on embryos mutant for *dHand*. We found that cell proliferation occurred from 16-18 hpf in the lateral plate as in wild type; however, *dHand* mutants did not show any significant

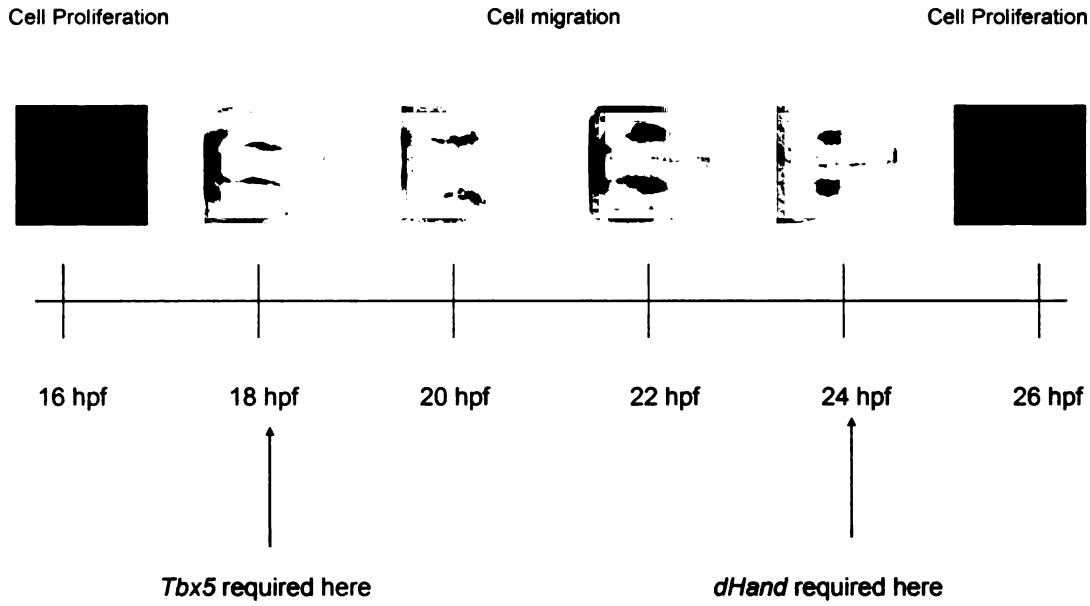
Figure 3.8

Summary of Results of Pectoral Fin Studies

Diagram showing the timing of cell proliferation and cell migration in the lateral plate during the time of pectoral fin bud formation. Based on our studies *tbx5* is required at 18hpf for the cell migration in the lateral plate, while *dHand* is required at 24hpf to re-initiate proliferation.

Fig. 3.8

Temporal Sequence of Pectoral Fin Development in the Anterior Lateral Plate



proliferation between 24-26 hpf. The timing of this lack of cell proliferation corresponds perfectly with the phenotype seen in the embryo, as the pectoral fin bud does form initially but never elongates as seen in wild type.

Discussion

Cell migration and Proliferation act in Concert to in the Nascent fin bud

In our studies of wild type, *tbx5,* and *dHand* embryos we were able to demonstrate that both cell migration and proliferation were essential for the formation of the pectoral fin in zebrafish. These analyses also revealed the timing and hierarchy of these two processes: 1. Beginning at 18hpf, the anterior lateral plate adjacent top somites 1-4 condenses to the area of somites 2 and 3 forming the fin bud. 2. While these cells are migrating very little cell proliferation occurs, but upon the completion of this condensation at 24hpf the cells begin to rapidly proliferate leading to further fin bud growth. *Tbx5* is essential for the first step of this process, while *dHand* is required for the second step. Thus, it appears that cell migration and proliferation are acting in concert within the lateral plate to form the early pectoral fin bud. Although these two processes are coordinated, our studies did not reveal the nature of their relationship and this remains an area for further future investigation.

Future Directions

While a great deal of effort has been put forth to uncover the molecular nature of the genetic signaling required for the formation and patterning of the vertebrate limb, very little is known about the mechanism and cell behaviors underlying this process. These studies, while preliminary, were one of the few attempts to describe appendage development at the cellular level. In subsequent studies it will be interesting to determine

the specific cell movements required for the lateral plate to condense to form the pectoral fin between 18hpf and 24hpf. This could be done through the observation of this condensation process via time-lapse confocal microscopy of the anterior lateral plate. This observation of the anterior lateral plate could be conducted by expressing a membrane targeted fluorophore such as GFP or RFP within the tissue. This could be done by simple mRNA injection into 1 or 2 cell embryos or by the construction of a transgenic line expressing a fluorescent protein under the control of a lateral plate specific promoter such as dHand or tbx5 (Repeated attempts to amplify the promoters of these genes by genomic PCR were not successful). It would be interesting to see which cell behaviors are necessary for this migration and to what extent they are disrupted with the loss of *tbx5* function.

The second aspect of this work that would be interesting to study is the molecular control of the cell proliferation that occurs in the lateral plate. Our data show that cell proliferation in the lateral plate is abundant until it is severely reduced at 18hpf and then resumes at 24hpf. As stated earlier, this is the time period which corresponds to the cell migration of the lateral plate. While our work demonstrated that *dHand* was essential for the resumption of cell proliferation at 24hpf, it is unclear what the molecular signal to stop cell proliferation at 18hpf is and how this is coordinated with cell migration. Perhaps the same signals that govern the initiation of the fin bud and the migration of the lateral plate control the rate and timing of cell proliferation. Because cell migration and cell proliferation are coordinated in the lateral plate, it is likely that the phenotype of an embryo that did not stop its cell proliferation would be the same as in an embryo that could not undergo cell migration. If one were to find mutation in a gene that controlled

this aspect of cell proliferation the phenotype would likely be a complete loss of fin bud. Thus the best method in which to find a gene that controlled this aspect of cell proliferation would be to conduct BrdU incorporation assays in the lateral plate of embryos with disrupted signaling in *wnt2b*, *fgf10*, or *fgf24* (either in mutant or Morpholino injected embryos). Embryos lacking function in these genes do not form fin buds and are good candidates for controlling this process.

Materials and Methods

Immunohistochemistry

Immunohistochemistry was performed as previously described[18]. The following antibodies were used at the following concentrations: mouse IgG anti-β-catenin (Sigma), Polymerized actin was visualized by phalloidin (Molecular Probes) at 1:100, Rat anti-BrdU (Allied) and anti-FITC alexa flour 488 (Molecular Probes) at 1:200. Embryos were mounted in Vectashield (Vector Labs) and imaged using a Zeiss LSM5 confocal microscope.

RNA *in situ* hybridization

Whole mount in situ hybridization was performed as previously described[19]. Riboprobe for *tbx5*[5] was prepared with Ambion mMessage Machine. Embryos were mounted in benzylbenzoate:benzyl alcohol and documented with Zeiss Axiocam.

Lineage Tracing

Embryos were injected with 20nl of 2% DMNB-caged, lysine fixable, Fluorescein dextran (Molecular Probes) diluted in 0.2M KCl. Embryos were allowed to develop in the dark until 16hpf then they were mounted in methyl cellulose and then groups of

lateral plate cells adjacent to somites1-4 were activated with pulses from a 365nm laser (Photonic Instruments) focused through a Zeiss Axioskop 2. At 30hpf the embryos were fixed in 4%PFA and then stained with Anti-FITC alexa flour 488 (Molecular Probes) to amplify the signal.

BrdU Incorporation

Embryos from Heterozygous *had*^{t617} fish were treated with 100μM BrdU in 15% DMSO at two hour intervals from 16hpf to 26hpf. After the initial pulse the embryos were thoroughly washed with egg water and then allowed to develop until 36hpf. The embryos were then fixed in 4%PFA and stained with anti-BrdU and anti-β-catenin. The embryos were then sectioned transversely through the fin bud region and the sections were analyzed on the Zeiss LSM5 confocal microscope.

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For reference

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