Title
Discovery of novel mechanisms for HSC specification by reverse and forward genetics

Permalink
https://escholarship.org/uc/item/8c4114h6

Author
Manegold, Jennifer Erin

Publication Date
2012

Peer reviewed|Thesis/dissertation
Discovery of Novel Mechanisms for HSC Specification by Reverse and Forward Genetics

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Biology by Jennifer Erin Manegold

Committee in charge:
David Traver, Chair
Elvira Tour
Deborah Yelon

2012
The Thesis of Jennifer Erin Manegold is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

__________________________
Chair

__________________________

University of California, San Diego

2012
DEDICATION

In appreciation of their love and support, I dedicate this thesis to my mom and dad and my brothers, Billy and Jimmy.
EPIGRAPH

“For me, I am driven by two main philosophies: know more today about the world than I knew yesterday and lessen the suffering of others. You'd be surprised how far that gets you.”

— Neil deGrasse Tyson
# TABLE OF CONTENTS

Signature page.......................................................................................... iii
Dedication...................................................................................................... iv
Epigraph....................................................................................................... v
Table of Contents......................................................................................... vi
List of Figures.............................................................................................. vii
Acknowledgments......................................................................................... viii
Abstract........................................................................................................ ix

I. Regulation of HSC Specification by Fgf Signaling................................. 1
   Introduction............................................................................................... 2
   Materials and Methods............................................................................ 7
   Results...................................................................................................... 9
   Discussion............................................................................................... 25

II. Forward Mutagenesis to Discover Novel Genes in Hematopoiesis......... 30
   Introduction............................................................................................... 31
   Materials and Methods............................................................................ 33
   Results...................................................................................................... 35
   Discussion............................................................................................... 38

References.................................................................................................... 39
LIST OF FIGURES

Figure 1. Zebrafish hematopoiesis is conserved with the mammalian system……………3

Figure 2. Blocking Fgf signaling inhibits definitive HSC formation without alteration of vasculature formation or primitive hematopoiesis…………………………………….10

Figure 3. HSC formation requires Fgf signaling during early somitogenesis……………..12

Figure 4. Ectopic activation of Fgf signaling rescues pharmacological inhibition by SU5402..14

Figure 5. Transient expression of constitutively-active form of Fgfr1 in lmo2-positive mesoderm does not rescue runx1 expression with SU5402 treatment……………16

Figure 6. Fgf target, pea3, is expressed in the somites but is not localized in PLM at 13 or 15 hpf……………………………………………………………………………………..18

Figure 7. Knockdown of each of the four Fgf receptors leads to loss of HSCs……………..20

Figure 8. fgfr1 is expressed in the somites but not in lmo2+ mesoderm at 13 or 15 hpf……22

Figure 9. fgfr4 is expressed in posterior region but not in lmo2+ mesoderm at 13 or 15 hpf……………………………………………………………………………………23

Figure 10. fgfr2 and fgfr3 are expressed in the anterior region but not in the posterior region at 15 hpf…………………………………………………………………………24

Figure 11. A well-characterized ENU mutagenesis screen in zebrafish is designed to identify recessive mutations………………………………………………………………33

Figure 12. Two mutants with defects in runx1 and cd45 expression have been found………36

Figure 13. Four mutants with defects in runx1 expression have been found………………37
ACKNOWLEDGEMENTS

I thank David Traver for giving me the opportunity to join his lab and for his support throughout my undergraduate and graduate education.

I thank Yoonsung Lee for his excellent mentorship and critical review of this manuscript. 감사합니다! Yoonsung Lee is the primary investigator for sections IA and IB.

I would also like to thank Deborah Yelon for her guidance and expertise in conducting a forward genetic screen and the entire screen team, including Christine Hochmuth, Jun Hwa Hong, Kate McDaniel, Lauren Pandolfo, and Joanne Petrivelli.

Finally, I would like to thank my fellow Beta lab members, especially Clyde Campbell, Raquel Espin Palazon, Albert Kim, and Dave Stachura, as well as the whole Traver lab for their invaluable support.
Hematopoietic stem cells (HSCs) both self-renew and give rise to all blood cell lineages over an individual’s lifetime. A greater understanding of the molecular
pathways that regulate HSC development is necessary to advance the goals of regenerative medicine which aims to replenish damaged tissues via cell replacement therapy. Zebrafish have evolutionarily conserved hematopoietic genes and regulatory networks and possess all mature blood cell lineages found in mammals. We utilize the experimental advantages of zebrafish, such as its visual accessibility and applications in forward and reverse genetics, to study hematopoiesis. Our preliminary findings indicate that fibroblast growth factor (Fgf) signaling regulates HSC specification between 13 and 15 hours post fertilization (hpf). To determine if Fgf signaling regulates HSC specification in the pre-HSC population, we performed double staining of Fgf genes and HSC precursors between 13 and 15 hpf. We observed expression of Fgf receptors and the Fgf target gene, pea3, in the somites, but did not find colocalization with HSC precursors, suggesting a potential environmental requirement for Fgf signaling. We confirmed this by plasmid injection, showing that transient activation of Fgf signaling in HSC precursors does not rescue HSC depletion from pharmacological inhibition of Fgf signaling. These experiments suggest that Fgf may have a cell non-autonomous role in HSC specification. For long term studies, we have initiated a forward mutagenesis screen in zebrafish. This unbiased approach will allow us to discover novel genes required for HSC formation. Together these studies will shed light on the genetic requirements for HSC development.
I. Regulation of HSC Specification by Fgf Signaling
INTRODUCTION

Hematopoiesis is the vital process of blood formation, in which self-renewing hematopoietic stem cells (HSCs) give rise to all mature blood cell lineages over the lifetime of an individual. A greater understanding of the mechanisms that regulate HSC development is necessary to advance the goals of regenerative medicine, which aims to replenish damaged tissues via cell replacement therapy. In this approach, multipotent, tissue-specific stem cells are formed from induced pluripotent cells (iPSCs) and embryonic stem cells (ESCs) by recapitulating endogenous mechanisms. Current methodologies have not successfully generated HSCs from iPSCs or ESCs, thus necessitating a greater understanding of how HSCs are formed endogenously during embryonic development.

The zebrafish system serves as a powerful model for studying HSC development in vertebrates. The hematopoietic genes and regulatory networks of zebrafish are evolutionarily conserved with the mammalian system. Furthermore, zebrafish and mammals share all the same progenitors and mature lineages in blood (Traver et al., 2003). Additionally, the zebrafish have many unique experimental advantages which make it a useful tool for studying development. For instance, fertilization occurs externally in zebrafish, so we can observe the resulting embryo starting from the one-cell stage and throughout development. Other strengths of the zebrafish system are the easy visualization of blood cells in the translucent embryo as well as its applications in both large-scale forward genetic screens and reverse transgenesis. Together, these experimental advantages make the zebrafish a powerful...
Figure 1. Zebrafish hematopoiesis is conserved with the mammalian system. (Stachura and Traver, 2009).
research tool and effective model that complements the mammalian system for studying hematopoiesis and its underlying mechanisms.

Understanding the genetic pathways that regulate HSC formation is critical to instructing the derivation of HSCs from pluripotent progenitors. HSCs are first specified in the post lateral mesoderm and later emerge from hemogenic endothelium in the dorsal aorta (Bertrand et al., 2010; Patterson et al., 2007). These processes are regulated by molecular signaling pathways and downstream intracellular transduction networks that are vital to maintain blood and immune cell homeostasis throughout the body. There are many known regulatory inputs required for HSC formation. The hedgehog (Hh) signaling pathway is required for formation of the dorsal aorta and for HSC formation (Gering and Patient, 2005; Wilkinson et al., 2009). The bone morphogenetic protein (Bmp) pathway induces the hematopoietic stem cell program in the dorsal aorta (Wilkinson et al., 2009). The vascular endothelial growth factor (Vegf) signaling pathway is critical for development of the dorsal aorta from which HSCs derive (Cleaver and Krieg, 1998). The notch signaling pathway is required for HSC specification but not for other hematopoietic precursors (Bertrand et al., 2010; Burns et al., 2005). Studies suggest that the fibroblast growth factor (Fgf) signaling pathway is also involved in regulation of hematopoiesis, but its role remains largely unexplored.

Fibroblast growth factors (Fgfs) are a member of the receptor tyrosine kinase family that play an important role in the differentiation and proliferation of various cell types and tissues during development. The Fgf signaling pathway is an expansive network comprised of four receptors in vertebrates with 23 identified ligands in
mammals and 27 ligands in zebrafish (Itoh and Ornitz, 2004). Upon binding of an Fgf ligand, the Fgf receptors dimerize, and their intracellular receptor tyrosine kinase domains become phosphorylated. This activates many downstream signaling pathways that regulate a variety of developmental processes, including wound healing and regeneration.

Previous studies suggest that Fgf signaling also has a role in regulation of hematopoiesis. Studies in murine bone marrow culture have shown that treatment with the ligand FGF-1 causes expansion of transplantable HSCs (de Haan et al., 2003). This indicates that Fgf signaling is essential for HSC development in vitro, but the role of Fgf signaling in vivo remains largely unknown. Knockdown of the ligand, Fgf21, in zebrafish embryos did not result in a decrease in HSCs or hemangioblasts (the multipotent progenitors from which blood cells derive), but did result in a significant reduction in erythroid and myeloid cells (Yamauchi et al., 2009). This suggests that Fgf21 is required in determining cell fate during hematopoiesis. In the chick embryo, Fgf receptor 2 was determined to play a role in inhibiting primitive erythroid differentiation and promoting endothelial development (Nakazawa et al., 2006). A recent study in mice has shown that treatment with the ligand FGF-2 expands hematopoietic stem and progenitor cells (Itkin et al., 2012). Thus, Fgfs have been demonstrated to play essential roles in hematopoiesis, but further studies are necessary to characterize the role of Fgf signaling in HSC development.

The goal of my research is to better understand and characterize the role of Fgf signaling in HSC formation in zebrafish. Using transgenic animals, we performed loss of function studies blocking Fgf signaling during development (Lee et al., 2005). Our
preliminary findings indicate that global genetic blockade of Fgf signaling inhibits HSC formation. We found that Fgf signaling between 13 and 15 hours post fertilization (hpf) is critical for HSC specification. We were then interested in determining where the signal acts during this time frame. Our preliminary findings suggest that Fgf signaling is not active in the HSC precursors during early somitogenesis. Fgf receptors do not seem to be localized in the HSC precursors between 13 and 15 hpf, suggesting that the Fgf signal is not transduced in this tissue during this time window. Furthermore, transient activation of Fgf signaling in HSC precursors was not sufficient to rescue pharmacological inhibition of Fgf signaling between 13 and 15 hpf. Taken together, our findings suggest a possible cell non-autonomous role for Fgf signaling in HSC formation.
MATERIALS AND METHODS

Zebrafish strains and maintenance

Adult fish were maintained in accordance with the guidelines provided by the UCSD IACUC. Matings were set up, and embryos were collected and staged as previously described (Westerfield, 2004). Wildtype AB* fish and transgenic lines Tg (hsp70:dn-fgfr1) and Tg (hsp70:ca-fgfr1) were used (Lee et al., 2005; Marques et al., 2008). For heat shock assays embryos were harvested, grown until 12 hpf, then placed in a 38°C waterbath for 20 minutes.

Whole-mount RNA in situ hybridization

Embryos were dechorionated using pronase (Sigma, St. Louis, MO) and treated with 0.003% 1-phenyl-2-thiourea (PTU, Sigma, St. Louis, MO) to prevent pigmentation for optimal visualization. Dechorionated, PTU-treated embryos were fixed at 13 hpf, 14 hpf, 15 hpf, 20 hpf, 26 hpf, and 36 hpf. Digoxygenin- and fluorescein-labeled antisense RNA probes were synthesized using a digoxygenin (DIG) RNA Labeling Kit (SP6/T7 Roche). Whole-mount in situ hybridization (WISH) was performed as described (Thisse et al., 1993) using antisense probes against ca-fgfr1, cmyb, efnb2a, fgfr1, fgfr2, fgfr3, fgfr4, gata1, l-plastin, kdrl, lmo2, pea3, and runx1. Fluorescent in situ hybridization (FISH) analysis was conducted with the NEL TSA Plus system (Perkin-Elmer).
Confocal microscopy

Confocal images were acquired on a Leica SP5 microscope and processed with Volocity software (Perkin-Elmer).

**SU5402 treatment**

WT embryos and *hsp70:ca-fgfr1* embryos were treated with 5 µM SU5402 (Mohammadi et al., 1997) (Calbiochem) between 13 and 15 hpf. Embryos were incubated at 28°C and fixed at appropriate stages. Control embryos were treated with an equal volume of DMSO added to fish water.

**Creation of lmo2:ca-fgfr1 construct and microinjection**

The *lmo2:ca-fgfr1* construct contains a constitutively active form of *Xenopus* Fgfr1 controlled by the Lmo2 2.5 kb upstream regulatory sequence (Marques et al., 2008). For transient expression of the transgene, WT AB* embryos were injected with 25 µg/ml of plasmid at one-cell stage of development.
RESULTS

IA. Genetic blockade of Fgf signaling blocks HSC formation

We first wanted to determine if Fgf signaling is required for HSC formation. We performed loss of function studies by genetically blocking Fgf receptor (Fgfr) function the hsp70:dn-fgfr1 transgene (Figure 2). This dominant negative (DN) transgene contains a truncated form of Fgfr1, in which the 3’ intracellular signaling domain is replaced with eGFP for visualization by fluorescent microscopy (Figure 2A). Inducing expression of this transgene by heat shock effectively blocks Fgf signaling throughout the embryo (Lee et al., 2005). Transgenic animals heat-shocked at 12 hpf, however, had a specific loss of the HSC markers, runx1 and cmyb, in the dorsal aorta at 26 and 36 hpf, respectively (Figure 2B-E).

To determine if the loss of HSCs in hsp70:dn-fgfr1 embryos was caused defects in other mesodermal tissues, we examined gene expression in these tissues (Figure 2F-M). Transgenic animals had normal expression of the endothelial marker, kdrl (also called flk1) and of the aortic marker, efnb2a (Figure 2F-I), which indicates that the observed HSC defects was not due to incorrect aorta formation. Expression of the primitive erythroid maker, gata1, and the primitive myeloid marker, l-plastin, was normal in hsp70:dn-fgfr1 animals (Figure 2J-M), suggesting that primitive blood is not affected by induction of the transgene. Together, these data suggest that inhibition of HSC formation by induction of the hsp70:dn-fgfr1 transgene is not secondary to defects in specification of other mesodermal tissues.

Yoonsung Lee is the primary investigator of the work described in this section.
Figure 2. Blocking Fgf signaling inhibits definitive HSC formation without alteration of vasculature formation or primitive hematopoiesis. (A) A cartoon of a transgenic construct of hsp70:dn-fgfr1. (B-I) Inhibition of FGF signaling following induction of hsp70:dn-fgfr1 at 12hpf leads to loss of the HSC markers runx1 and cmyb in aortic hemogenic endothelium (C, E) compared to wild-type embryos (arrowheads in B, D). Markers of endothelium (kdrl) and dorsal aorta (efnb2a) are normal in hsp70:dn-fgfr1 embryos (F-I). (J-M) Identical induction of hsp70:dn-fgfr1 does not alter expression of either the primitive erythroid marker, gata1 (J, K) or primitive myeloid marker, l-plastin (L, M).
IB. Fgf signaling during early somitogenesis is essential for HSC formation

To determine the specific time window in which Fgf signaling is required for HSC formation, we conducted temporal studies (Figure 3). First we performed a time course to determine when the *hsp70:dn-fgfr1* transgene must be induced to block HSC formation. Embryos heat-shocked at 8 hpf had global morphological defects (data not shown). Induction of the transgene at 12 hpf led to a severe loss of HSCs (Figure 2B-E), but induction at 15 hpf had no effect on HSC formation as observed in WISH (data not shown).

Next we wanted to determine the kinetics of the dominant negative transgene. Though heat induction at 12 hpf of *hsp70:dn-fgfr1* blocks HSC formation, we did not know when the transgene is first expressed or when Fgf signaling is effectively blocked following heat shock. To determine the kinetics of the transgene, we took advantage of the eGFP fusion protein that is expressed along with the DN-FGFR1 transgene. We used confocal microscopy to visualize when the transgene becomes localized to the plasma membrane (Figures 3A-C). One hour post heat shock (hpHS), we could first see expression of eGFP started to become localized to the membrane, and by 3 hpHS, membrane localization of eGFP was complete (Figure 3A-C). To determine when Fgf signaling activity is blocked following heat induction, we examined expression of the Fgf target, *pea3*, by WISH. At 2 hpHS, *pea3* expression was greatly reduced in induced *hsp70:dn-fgfr1* animals compared to wildtype and was completely lost by 3 hpHS (Figure 3E-F). This is consistent with our confocal microscopy data and suggests that Fgf signaling is required between 13-15 hpf.
Figure 3. HSC formation requires Fgf signaling during early somitogenesis. (A-C) Confocal microscopy images of hsp70:dn-fgfr1-EGFP embryos heat-shocked at 12 hpf. GFP expression starts to localize in the plasma membrane from 13 hpf (A, B) through 15 hpf (C). (D) Whole-mount in situ hybridization of Fgf target pea3 expression in hsp70:dn-fgfr1 embryos heat-shocked at 12 hpf. The activity of FGF signaling is robustly diminished at 2 hours post heat-shock (hpHS) and completely depleted by 3 hpHS. (E, F) Pharmacological inhibition of FGFRs using SU5402 during 13-15 hpf leads to a complete loss of the HSC marker, runx1 (F), whereas embryos treated with vehicle alone (DMSO 0.05%) show no effects (E).
To confirm this time window, we used a pharmacological inhibitor of Fgf signaling, SU5402. SU5402 is a small molecule inhibitor that binds the kinase domain of the FGFRs, thus preventing activation of the Fgf signaling cascade (Mohammadi et al., 1997). Embryos treated with SU5402 between 13 to 15 hpf had no expression of the HSC marker, runx1, at 26 hpf (Figure 3E, F). This confirmed the temporal window established by our transgenic loss of function studies.

Although SU5402 is a known inhibitor of Fgf signaling, it also has reported effects on the kinase activity of VEGF receptors which are critical to vascular and hematopoietic cell development (Mohammadi et al., 1997; Shalaby et al., 1997; Shalaby et al., 1995) To determine if the observed loss of HSCs following SU5402 treatment was due to loss of Fgf signaling activity, we designed a rescue experiment (Figure 4). We used a transgenic line in which expression of a constitutively active form of FGFR1 is driven by the zebrafish heat shock promoter (Figure 4A). This line expresses a version of the Xenopus FGFR1 that is autophosphorylated, independent of ligand binding. This causes ectopic activation of Fgf signaling after heat induction. Following heat induction of the transgene, we treated embryos with SU5402 between 13 to 15 hpf (Figure 4B). Heat induction of the transgene at 12 hpf rescued normal runx1 expression of SU5402 treated animals compared to animals treated with vehicle alone (Figure 4C).

Taken together, these studies indicate that Fgf signaling is required for HSC formation between 13-15 hpf.

Yoonsung Lee is the primary investigator of the work described in this section.
Figure 4. Ectopic activation of Fgf signaling rescues pharmacological inhibition by SU5402. (A) A cartoon of the transgenic construct of hsp70:ca-fgfr1. (B) Diagram of the experimental plan to recover the activity of Fgf signaling in the presence of SU5402 during 13 to 15 hpf using hsp70:ca-fgfr1 transgenic embryos. Heat-shock was administered at 12 hpf, followed by SU5402 treatment for 2 hours at 13 hpf. (C) Images of whole-mount in situ hybridization using 26hpf hsp70:ca-fgfr1 transgenic embryos from the experiment performed in (B). runx1 expression is recovered in the presence of SU5402 following ectopic activation of FGF signaling.
IC. Fgf signaling may have a cell non-autonomous role in HSC formation

After determining the temporal window in which Fgf signaling is required for HSC formation, we addressed issues of cell autonomy. HSCs arise from the posterior lateral mesoderm (PLM) (Bertrand et al., 2010; Patterson et al., 2007). We wanted to determine if Fgf signaling in HSC precursors regulates HSC formation. To test this, we designed a rescue experiment similar to the one from Figure 4B. Instead of using the hsp70:ca-fgfr1 transgene, Fgf signaling was ectopically activated specifically in HSC precursors. The zebrafish lmo2 gene is widely expressed in the vascular precursors of HSCs, and its 2.5 kb upstream regulatory sequence (URS) recapitulates endogenous expression in germline and transient transgenics (Zhu et al., 2005). We designed an lmo2:ca-fgfr1 transgenic construct, in which expression of the ca-fgfr1 transgene is driven by the lmo2 promoter for ectopic activation in lmo2 tissue (Figure 5A). Embryos were injected with lmo2:ca-fgfr1 plasmid at the one-cell stage then treated with SU5402 from 13 to 15 hpf. Successful plasmid injection was evaluated by WISH at 12 hpf (Figure 5B-E). After SU5402 treatment between 13 to 15 hpf, un.injected controls had a complete loss of runx1 expression at 26 hpf, as expected, and runx1 expression was not recovered in lmo2:ca-fgfr1 injected embryos (Figure 5F). It seems that transient activation of Fgf signaling in lmo2 tissue does not rescue loss of runx1 expression caused by SU5402 treatment. This result suggests that Fgf signaling may not regulate HSC formation cell-autonomously.

This striking result led us to ask where the Fgf signal is active during the time window in which Fgf signaling is required for HSC formation. To determine if the Fgf signal is active in the PLM during the critical window for HSC specification, we
Figure 5. Transient expression of constitutively-active form of Fgfr1 in lmo2-positive mesoderm does not rescue runx1 expression with SU5402 treatment. (A) lmo2:ca-fgfr1 plasmid was injected into 1-cell stage zebrafish embryos. (B-E) Transient expression of lmo2:ca-fgfr1 can be detected in the PLM after plasmid injection at 12 hpf by WISH using antisense probe against ca-fgfr1 transgene. Two representative images of lmo2:ca-fgfr1 expression in the PLM (D, E). (F) WISH images of 26hpf lmo2:ca-fgfr1 injected embryos with SU5402 treatment. runx1 expression is not recovered in the presence of SU5402 following transient activation of Fgf signaling in lmo2 cells.
examined expression of *pea3*, a known target of Fgf signaling, and *lmo2* by FISH of wildtype embryos at 13 and 15 hpf (Figure 6). At both time points, we saw expression of *pea3* in the somitic region of the embryo but observed no colocalization of *pea3* and *lmo2*.

This result suggests that Fgf may not be active in the PLM between 13 to 15 hpf. However, since *pea3* is just one of many Fgf target genes, it may be necessary to examine several other Fgf targets, such as *spry4*, and *erm*, to confirm whether they are coexpressed in the PLM by FISH. We will examine other Fgf targets, such as *spry4* and *erm*, to determine if they are coexpressed in the PLM by FISH. As a parallel strategy, we will create double transgenic animals using the *dusp6:dGFP* reporter line, which marks another Fgf target, with the *lmo2:mCherry* reporter. This transgenic approach provides another means for assessing if Fgf activity is localized in the PLM when the signal is required for HSC formation.
Figure 6. Fgf target, *pea3*, is expressed in the somites but is not localized in PLM at 13 or 15 hpf. (A, B) Double fluorescence whole-mount in situ hybridization using probes against *lmo2* and *pea3*. *lmo2* (red) and *pea3* (green) at 13 hpf (A). *lmo2* (green) and *pea3* (red) at 15 hpf.
As an alternative approach to determine which tissues experience Fgf signaling, we also investigated the individual Fgf receptors. Fgf signaling is transduced by four receptors in vertebrates (Itoh and Ornitz, 2004). We first wanted to determine if each Fgf receptor plays an individual role in HSC formation. Individual Fgf receptors can be knocked down by morpholino (MO) injection (Nakayama et al., 2008). Compared to uninjected wildtype control embryos, animals injected with MOs against \textit{fgfr1}, \textit{fgfr2}, \textit{fgfr3}, or \textit{fgfr4} between 1- to 4- cell stage exhibited a decrease in \textit{runx1} expression at 26 hpf (Figure 7). This indicates that each Fgf receptor is required for HSC formation.
Figure 7. Knockdown of each of the four Fgf receptors leads to loss of HSCs. Compared to expression of *runx1* in uninjected control animals at 26 hpf, (A), inhibition of each single Fgf receptor leads to loss of HSCs (B-E). Each antisense MO was injected into WT embryos at the 1- to 4-cell stage.
Because each of the four Fgf receptors seems to be required for HSC formation, we next wanted to determine where these receptors are localized during the 13 to 15 hpf time window that is critical for HSC formation. We used double FISH to determine if each fgfr is expressed in lmo2+ cells at 13 hpf and 15 hpf (Figures 8, 9, 10). By FISH, we observed expression of fgfr1 in the posterior and presomitic region at both 13 and 15 hpf, consistent with single WISH data (Figure 8). Interestingly, however, we did not observe colocalization of fgfr1 in lmo2+ mesoderm at these timepoints (Figure 8B, C). fgfr4 was expressed posteriorly, and we observed a striking banded pattern at both 13 and 15 hpf by FISH and at 15 hpf by single WISH (Figure 9). Again, however, we did not observe colocalization of fgfr4 and lmo2 at these timepoints (Figure 9B, C). Expression of fgfr2 and fgfr3 was concentrated in the anterior region with little to no expression in the posterior region at both 13 and 15 hpf, consistent with single WISH data (Figure 10, data not shown). We did not observe coexpression of either fgfr2 or fgfr3 in lmo2+ cells at 13 or 15 hpf (Figure 10A, B; data not shown). From these data, it appears that none of the Fgf receptors are localized in the PLM during the time frame in which Fgf signaling is critical for HSC formation. Thus it seems that the Fgf signal which regulates HSC specification is not transduced in the HSC precursors. This suggests that the Fgf receptors may be required environmentally for regulation of HSC formation. Taken together, our data suggest a possible cell non-autonomous role for Fgf signaling in HSC formation.
Figure 8. fgfr1 is expressed in the somites but not in lmo2+ mesoderm at 13 or 15 hpf. (A) WISH of wildtype embryo at 15 hpf using antisense probe against fgfr1. (B, C) Double fluorescence in situ images using probes against lmo2 (green) and fgfr1 (red) at 13 hpf (B) and 15 hpf (C).
Figure 9. *fgfr4* is expressed in posterior region but not in *lmo2*+ mesoderm at 13 or 15 hpf. (A) WISH of wildtype embryo at 15 hpf using antisense probe against *fgfr4*. (B, C) Double fluorescence in situ images using probes against *lmo2* and *fgfr4*. *lmo2* (red) and *fgfr4* (green) at 13 hpf (B). *lmo2* (green) and *fgfr4* at 15 hpf (C).
Figure 10. *fgfr2* and *fgfr3* are expressed in the anterior region but not in the posterior region at 15 hpf. (A) WISH of wildtype embryo at 15 hpf using antisense probe against *fgfr2*. (B) Double fluorescence in situ images using probes against *lmo2* (green) and *fgfr2* (red) at 15 hpf. (C) WISH of wildtype embryo at 15 hpf using antisense probe against *fgfr3*. (D) Double fluorescence in situ images using probes against *lmo2* (green) and *fgfr3* (red) at 15 hpf.
DISCUSSION

Fgf signaling is critical for HSC formation between 13 to 15 hpf

We have shown that genetic blockade of Fgf signaling inhibits HSC formation. Animals with induced expression of the dominant negative form of Fgfr1 (dn-fgfr1) have decreased expression of the HSC markers, runx1 and cmyb, in the dorsal aorta. These induced transgenic animals showed no alteration in expression of vascular and aortic markers, flk1 and efnb2a, and the integrity of vascular structures was preserved. Thus, the HSC defect observed in animals in induced hsp70:dn-fgfr1 animals was not due to a defect in the formation of the vasculature. Expression of the primitive erythroid marker, gata1, and of the primitive erythroid marker, l-plastin, was also normal in in induced hsp70:dn-fgfr1 embryos. Thus, the inhibition of HSC formation was not a secondary effect of defects in mesodermal development. This indicates that Fgf signaling is critical for HSC formation.

After demonstrating that genetically blocking Fgf signaling inhibits HSC formation, we wanted to ascertain the exact time window in which Fgf signaling is required for HSC formation. We found that induction of the hsp70:dn-fgfr1 transgene at 12 hpf specifically blocked HSC formation, while induction at 15 hpf had no effect on HSC expression. We then wanted to address the kinetics of the hsp70:dn-fgfr1 transgene. This transgene contains a GFP fusion, thus allowing easy visualization of transgene expression. By confocal microscopy, we observed the hsp70:dn-fgfr1 transgene first start to localize at the plasma membrane one hour following heat shock (13 hpf) with complete localization by three hours following heat shock (15 hpf).
Next, we performed an assay for Fgf activity by observing expression of the Fgf target, pea3, following heat induction of the hsp70:dn-fgfr1 transgene. Consistent with our confocal microscopy data, we found that pea3 expression was severely reduced two hours after heat shock (14 hpf), and expression was completely lost by three hours after heat shock (15 hpf). These experiments suggest a time window between 13-15 hpf in which Fgf signaling is critical for HSC formation.

We confirmed this time window using a pharmacological inhibitor of Fgf signaling, SU5402. Consistent with our results from genetically blocking Fgf signaling, we found that treating embryos with SU5402 between 13 and 15 hpf resulted in a robust decrease in runx1 expression. We found that global ectopic activation of Fgf signaling could rescue the loss of runx1 expression due to SU5402 treatment. This demonstrated that the observed inhibition of runx1 expression in SU5402 treated animals was caused by a blockade of Fgf signaling and not due to off-target effects. Taken together, our data suggest that Fgf signaling is required between 13 and 15 hpf for HSC specification.

**Fgf signaling may have a cell non-autonomous role in HSC formation**

After determining the temporal requirement for Fgf signaling in HSC formation, we focused on the issue of cell autonomy. We wanted to determine if Fgf signaling acts in the HSC precursors to regulate HSC formation. We designed the lmo2:ca-fgfr1 transgenic construct to ectopically activate Fgf signaling in lmo2-specific mesoderm. We found that transient activation of the constitutively active Fgfr1 in lmo2 cells was not sufficient to rescue loss of runx1 expression by SU5402
treatment. This result suggests that Fgf signaling may not be acting cell autonomously to regulate HSC formation.

Though these preliminary data are striking, further studies must be performed to confirm this result. We would also like to study the effects of specifically inhibiting Fgf signaling in \textit{lmo2} tissue. To do this, we will inject our \textit{lmo2:dn-fgfr1-egfp} transgenic construct into the \textit{lmo2:mCherry} reporter line. We will then sort embryos with double positive cells at 13 hpf to identify animals with transgenic expression in the PLM. We will analyze these embryos for \textit{runx1} expression at 26 hpf to determine if genetic inhibition of Fgf signaling in the PLM has an effect on HSC formation.

We will also perform dosage experiments to determine if we observe an effect with a higher dosage of \textit{lmo2:ca-fgfr1} plasmid. If a higher dosage of \textit{lmo2:ca-fgfr1} transgene can rescue loss of \textit{runx1} expression with SU5402 treatment, this would indicate that Fgf signaling is in fact required cell autonomously. If, however, no dose of \textit{lmo2:ca-fgfr1} is capable of rescuing the SU5402 phenotype, this would suggest a possible cell non-autonomous role for Fgf signaling HSC formation regulation.

To strengthen these tissue-specific studies, we will take advantage of the GAL4-UAS system available in zebrafish. This method exploits the yeast transcriptional activator GAL4 and the enhancer UAS (Upstream Activating Sequence). GAL4 specifically binds UAS, thus activating gene transcription. We will generate \textit{UAS:dn-fgfr1} and \textit{UAS:ca-fgfr1} transgenic lines. This will allow us to control expression of the \textit{dn-fgfr1} and \textit{ca-fgfr1} transgenes only in vascular precursors by using the \textit{fli1:Gal4} transgenic line. The zebrafish \textit{fli1} gene is expressed in the PLM during early somitogenesis and in the vasculature in later stages of development (Brown et
al., 2000). These germline transmitted transgenics will allow us to observe the effects of genetically blocking or ectopically activating Fgf signaling specifically in HSC precursors.

Though it seems that Fgf signaling may not be required cell autonomously for regulation of HSC formation, we wanted to determine where Fgf signaling is active in the critical 13 to 15 hpf time window. To address this, we examined expression of the Fgf target, *pea3*, and *lmo2*+ mesoderm in wildtype embryos at 13 and 15 hpf. We found that *pea3* is expressed in the posterior and somitic region at 13 and 15 hpf, but we observed no coexpression with *lmo2* cells. This suggests that Fgf signaling may not be active in HSC precursors during the window in which Fgf signaling is required for HSC formation. *Pea3* is only one of many Fgf target genes, however. To confirm this finding, we will examine expression of other known targets of Fgf signaling, including *erm* and *spry4* by in situ at 13 and 15 hpf. We will also use the *dusp6:GFP* transgenic reporter line which marks the Fgf target, *dusp6* (also called *mkp3*), in conjunction with PLM-specific reporter lines, such as, *lmo2:mCherry*.

As an alternative approach to determine where Fgf signaling acts between 13 and 15 hpf, we studied the four Fgf receptors. We found that knockdown of individual receptors led to decreased *runx1* expression. This suggests that each Fgf receptor has a role in HSC formation. To determine where the Fgf signal is transduced, we then observed expression of these receptors at 13 and 15 hpf by FISH. We found that *fgfr1* and *fgfr4* were both expressed in the posterior and somitic regions of the embryo at 13 and 15 hpf, but we did not observe colocalization of these receptors with *lmo2* at these stages. We observed expression of *fgfr2* and *fgfr3* in the anterior region of the embryo
at 13 and 15 hpf, but there was almost no expression of either receptor in the posterior region, and there was no colocalization with *lmo2*. It seems that none of the receptors are expressed in the PLM between 13 and 15 hpf. Thus, it seems that the Fgf signal is not transduced in HSC precursors during the critical time window for HSC specification.

Taken together, our findings suggest that Fgf signaling may regulate HSC specification cell non-autonomously. Based on expression patterns of the Fgf target, *pea3*, and of the individual Fgf receptors, it seems that there may be an environmental requirement for Fgf signaling in regulation of HSC development. To test this hypothesis, we will perform further tissue-specific loss and gain of function studies. Because we observed that the Fgf target, *pea3*, as well as *fgfr1* and *fgfr4* are expressed in the somites, we will use somitic drivers of GAL4 in conjunction with the *UAS:dn-fgfr1* line to determine if somitic Fgf signaling is required for HSC formation.

To further characterize the role of Fgf signaling in HSC specification, we want to determine the epistatic relationships between Fgf and downstream target genes. It will be important to examine the interactions between Fgf signaling and other signaling pathways with known roles in HSC development, such as Notch, Hh, Vegf, and Bmp. A complete understanding of the molecular signals that regulate HSC development will be necessary to generate HSCs from iPS or ES cells.
II. Forward Mutagenesis to Discover Novel Genes in Hematopoiesis
INTRODUCTION

To discover novel genes required in hematopoiesis in an unbiased manner, we are taking advantage of the well-defined forward mutagenesis screen in zebrafish.

One of the primary advantages of the zebrafish model system is its application in forward mutagenesis screens. Its small size makes it easy to manage in large numbers in the laboratory. Compared to other vertebrates, its relatively short generation time (about three months) make it possible to create multiple generations in a reasonable time period. Large clutch sizes and the developing embryos transparency allow for efficient screening of potential recessive mutants.

The well-characterized three generation screen is designed to discover recessive mutations that lead to defects in the development of HSCs as well as defects in differentiated lineages. Previous mutagenesis screens in zebrafish have identified hematopoietic mutants, such as moonshine (in which normal erythroid differentiation is blocked) and vampire (a mutant that cannot generate blood cells) (Ransom et al., 1996; Weinstein et al., 1996).

This forward mutagenesis screen will lead to the discovery of new genes which are required for HSC formation and for differentiation, thus allowing us to genetically dissect the pathways involved in hematopoiesis.
Figure 11. A well-characterized ENU mutagenesis screen in zebrafish is designed to identify recessive mutations. (Patton and Zon, 2001)
MATERIALS AND METHODS

Zebrafish strains

Adult fish were maintained in accordance with the guidelines provided by the UCSD IACUC. Matings were set up, and embryos were collected and staged as previously described (Westerfield, 2004). Wildtype AB* fish and golden (gol -/-) mutants were used. Gol -/- fish have a single point mutation that affects pigment.

ENU treatment

Twenty AB* males were subjected to three treatments with N-ethyl-N-nitrosourea (ENU), a chemical mutagen which introduces single point mutations to male spermatogonia (Solnica-Krezel et al., 1994).

Screen strategy

ENU-treated males were crossed to gol(-/-) females to generate F1 heterozygous individuals. Use of gol(-/-) females enabled confirmation of mutagenesis efficiency. 10 golden mutants in 6389 F1 embryos were identified. F1 heterozygous progeny are crossed to siblings to generate the F2 generation. Half of the fish from one F2 family are carriers of a given mutation, and the other half are wildtype. F2 sibling incrosses are set up. Crosses between two heterozygous F2 fish produce a clutch of F3
embryos with 25% wildtype (+/+), 50% heterozygous (+/-), and 25% recessive (-/-) for a given mutation.

Whole-mount RNA in situ hybridization

Embryos were dechorionated using pronase (Sigma, St. Louis, MO) and treated with 0.003% 1-phenyl-2-thiourea (PTU, Sigma, St. Louis, MO) to prevent pigmentation for optimal visualization. Dechorionated, PTU-treated embryos were fixed at 30 hpf. Digoxygenin- and fluorescein-labeled antisense RNA probes were synthesized using a digoxygenin (DIG) RNA Labeling Kit (SP6/T7 Roche). Whole-mount in situ hybridization was performed as described (Thisse et al., 1993) using antisense probes against cd45 and runx1.
RESULTS

To date, 90 F2 families of a total of 150 families have been successfully screened by whole-mount in situ hybridization (WISH). F3 embryos are fixed at 30 hours post fertilization (hpf), and expression patterns of the hematopoietic genes, runx1 and cd45, are observed. The transcription factor, Runx1, is critical for HSC production and marks HSCs in the floor of the dorsal aorta between 26 to 48 hpf (North et al., 1999; North et al., 2002; Burns et al., 2005; Chen et al., 2009). The Cd45 gene is a pan-leukocytic marker expressed in primitive myeloid cells and in definitive cells first born in the posterior blood island (PBI) between 30 to 36 hpf.

Six hematopoietic mutants have been identified (Figure 12). Two of these mutants have deficiencies in both runx1 and cd45 (Figure 12B, D, F) compared to wildtype siblings (12A, C, E). Four of these mutants have deficiencies only in runx1 (Figure 13B, D, F, H) compared to wildtype siblings (Figure 13A, C, E, G).
Figure 12. Two mutants with defects in *runx1* and *cd45* expression have been found. F3 embryos were obtained from F2 in crosses then fixed at 30 hpf and analyzed by whole mount in situ hybridization. (A, B) WISH images from one clutch of F3 embryos treated with a cocktail of antisense probes against *runx1* and *cd45*. (C-F) WISH images from one clutch of F3 embryos treated with antisense probe against either *runx1* (C, D) or *cd45* (E, F). Compared to wildtype siblings (A, C, E), mutant embryos showed no expression of either *runx1* or *cd45* (B, D, F).
Figure 13. Four mutants with defects in runx1 expression have been found. F3 embryos were obtained from F2 in crosses then fixed at 30 hpf and analyzed by whole mount in situ hybridization. (A-D) WISH images of F3 embryos treated with antisense probe against runx1. (E-H) WISH images of F3 embryos treated with antisense probes against runx1 and cd45. Compared to wildtype siblings (A, C, E, G), mutant embryos have decreased or complete loss of expression of runx1 at the dorsal aorta (B, D, F, H).
DISCUSSION

Approximately 60 families remain to be screened by WISH to complete a total of 150 F2 families screened. After finding mutants with deficiencies in \textit{runx1} and/or \textit{cd45}, mutants will be further characterized by WISH using other hematopoietic and vascular markers. Mutants with hematopoietic specific defects will be analyzed by linkage analysis to identify the chromosomal location of the mutation. Mutants and wild type siblings will be submitted for deep sequencing analysis to identify the exact mutation which causes the observed phenotype. These studies will allow us to discover novel genes required for hematopoiesis.
REFERENCES


