THE ROLE OF *SON* IN BLOOD DEVELOPMENT: AN ANALYSIS OF VERTEBRATE HEMATOPOIETIC PROLIFERATION AND DIFFERENTIATION

A Thesis/Project

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in

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by

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by

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Summer 2018

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DEDICATION

To Jackson, Addison, and Spencer

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. David Stachura for his continued encouragement, guidance, and knowledge. He has made me a better scientist and has pushed me to accomplish more than I thought I was capable of achieving.

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I also thank my family for their support and encouragement.

TABLE OF CONTENTS

LIST OF TABLES

TABLES PAGE

FIGURES

ABSTRACT

THE ROLE OF *SON* IN BLOOD DEVELOPMENT: AN ANALYSIS OF VERTEBRATE HEMATOPOIETIC PROLIFERATION AND DIFFERENTIATION

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Zebrafish (*Danio rerio*) is an excellent model organism for studying embryonic vertebrate development due to their conserved genome with humans, external development, and ease of observation under the microscope. The gene *SON* is found on the twenty-first chromosome of humans and is often overexpressed in patients with leukemias, particularly acute megakaryoblastic leukemia. Previously, we showed that mutations in the mRNA splicing co-factor gene *SON* cause malformations in human and zebrafish spines and brains. We performed these studies by knocking down the expression of the zebrafish homolog of *SON* in zebrafish at the single-cell developmental stage with specific morpholinos (MOs). In addition to the brain and spinal malformations we also observed abnormal blood cell levels with *son* knockdown. We then investigated how blood production was altered when levels of *son* were lowered. Decreased levels of *SON* resulted in impaired blood flow and lower amounts of red blood cells when visualized with *hbaa:GFP* transgenic fish. There was also a reduction in thrombocytes seen with *cd41:GFP* fish and verified with flow cytometry, and myeloid cells, as seen with *mpx:GFP* fish. We also saw a

significant decrease in the quantity of T cells, visualized with *lck:GFP* fish. However, when we plated the hematopoietic stem and progenitor cells (HSPCs) from zebrafish with reduced levels of *son*, we saw no difference in colony forming capability. Further investigation of *son* and its effect on blood development should establish how misexpression of this gene negatively impacts human health.

CHAPTER I

INTRODUCTION

Blood development (hematopoiesis) is the process by which blood cells acquire specific characteristics through carefully regulated gene expression (Shivdasani and Orkin, 1996). All blood cells arise from hematopoietic stem cells (HSCs) (Lemischka, 1991), which are adult stem cells capable of self-renewal and differentiation into more developmentally restricted blood progenitor cells (Cumano and Godin, 2007). The HSC then further differentiates into either a common myeloid progenitor (CMP) or a common lymphoid progenitor (CLP). The CMP is capable of producing all erythromyeloid cells, which are red blood cells, thrombocytes, granulocytes, and monocytes (Akashi and Traver, 2000; Manz *et al.*, 2001). The CLP is capable of producing lymphoid cells, such as B cells, T cells, and NK cells (Galy *et al.*, 1995; Kondo *et al.*, 1997). In essence, red blood cells (which carry oxygen to tissues), granulocytes (which fight infections), thrombocytes (which prevent bleeding), and lymphocytes (which confer long-term immunity) are very different mature blood cells with diverse functions that all started out as an HSC (Shivdasani and Orkin, 1996) (Fig. 1). Many factors affect the differentiation of blood cells by changing the gene expression of progenitor cells, and this control is essential- if the wrong numbers or wrong types of mature blood cells are produced it can have serious clinical effects on an individual (Shivdasani and Orkin, 1996). Understanding the molecular control of these processes is essential for understanding blood development and treating human diseases. With the advent of gene therapy, discovering mutant genes associated with abnormal blood development will also allow us to correct them, providing new treatments for blood diseases.

Our gene of interest, *SON*, is found on human chromosome 21 and may play a role in the blood disease acute megakaryoblastic leukemia (AMKL). Patients with trisomy 21, a third copy

of chromosome 21, are much more likely (93%) to have AMKL (Reinhardt *et al.*, 2005) than patients with two copies of chromosome 21 (Gassmann and Loffler, 1995). AMKL is characterized by an overproduction of megakaryocyte-erythroid progenitors (MEP) (Mirchandani and Palutke, 1982), a progenitor downstream from the CMP that produces erythrocytes and thrombocytes. *SON* codes for a protein that forms an RNA-splicing molecule important for processing other genes during transcription (as reviewed in Lu *et al.*, 2014). *SON* was initially discovered in 1988 as a partial clone called *SON3* and described as a DNA binding protein due to its relatedness to chicken and human DNA binding proteins (as reviewed in Lu *et al.*, 2014). It was later shown that *SON* is located on chromosome 21 in humans and chromosome 16 in mice (Cheng *et al.*, 1993). The full-length gene was mapped in 2000 (Wynn *et al.*, 2000). *SON* was also described as a negative regulatory element binding protein (NREBP) due to its transcriptional repression of human hepatitis B virus genes (Sun *et al.*, 2000). *SON* was later classified as a regulator of pre-mRNA splicing and a nuclear speckle protein (Sharma *et al.*, 2010). In 2011, it was further shown that *SON* functioned in pre-mRNA splicing (Ahn *et al.*, 2011; Sharma *et al.*, 2011). *SON* also has a role in regulating the pluripotency of human embryonic stem cells (as reviewed in Lu *et al.*, 2014) and is highly expressed in HSCs and downregulated during hematopoietic differentiation (as reviewed in Lu *et al.*, 2014). Previous work that I conducted with collaborators indicate that mutations in *SON* are linked to spinal and brain malformations in patients (Kim *et al.*, 2016). Due to the ability of *SON* to regulate other genes and its expression in HSCs, we hypothesized that *SON* played a role in regulating blood development.

To understand the effects of *SON* in spine and brain development, I used zebrafish (*Danio rerio*) as a model system. These experiments are not possible to conduct on humans, and zebrafish have many advantages over other animal models. Zebrafish have a well conserved genome when

compared to humans (Driever *et al.*, 1996) and they undergo rapid development (Gaiano *et al.*, 1996). It is difficult to study blood development in mammals because they develop in utero, but zebrafish develop externally and are optically transparent at the larval stage (Brownlie and Zon, 1999), aiding in their visualization during embryonic development. Zebrafish have been widely established as a model organism to study blood development (Ransom *et al.*, 1996; Weinstein *et al.*, 1996). To study the effects of *son* on blood development I used transgenic zebrafish that have blood-specific gene promoters driving green fluorescent protein expression in specific blood cell types. These fluorescently labeled cells allow for easy *in vivo* visualization of their formation in developing embryos under a microscope and it allows the use of flow cytometry to interrogate and quantify the numbers of these cells present in an animal. To understand how each branch of hematopoiesis is affected by *son*, this study used zebrafish that have fluorescently labeled red blood cells (Traver, 2003), thrombocytes (Lin *et al.*, 2005), myeloid cells (Renshaw *et al.*, 2006), macrophages (Ellett *et al.*, 2011), and T cells (Langenau *et al.*, 2004).

This investigation utilized morpholinos (MOs) to study the role of the zebrafish Son protein in blood development. A MO is a specific antisense oligonucleotide that binds to *son* mRNA; this binding prevents its translation, reducing the levels of Son protein in the developing fish (Summerton, 1999). Zebrafish are excellent model organisms to use with morpholinos, since they have been established as an effective method of knocking down translation of a specific gene in zebrafish (Nasevicius and Ekker, 2000).

Through the knockdown of *son* and the resulting observations of mature blood cells, I have gathered greater insight into how Son directs the development of blood cells during development. After injecting single-cell embryos with *son* MO, we saw a significant decrease in the amount of red blood cells, thrombocytes, myeloid cells, and T cells. However, we did not see a difference in

the quantity of hematopoietic stem and progenitor cells (HSPCs), indicating that *son* is crucial for proper blood maturation, but not formation. While we don't yet understand the specific molecular mechanism by which this happens, this study offers insight into an important gene that regulates normal blood cell development that is altered during disease. In essence, it will offer new avenues for treatment of AMKL.

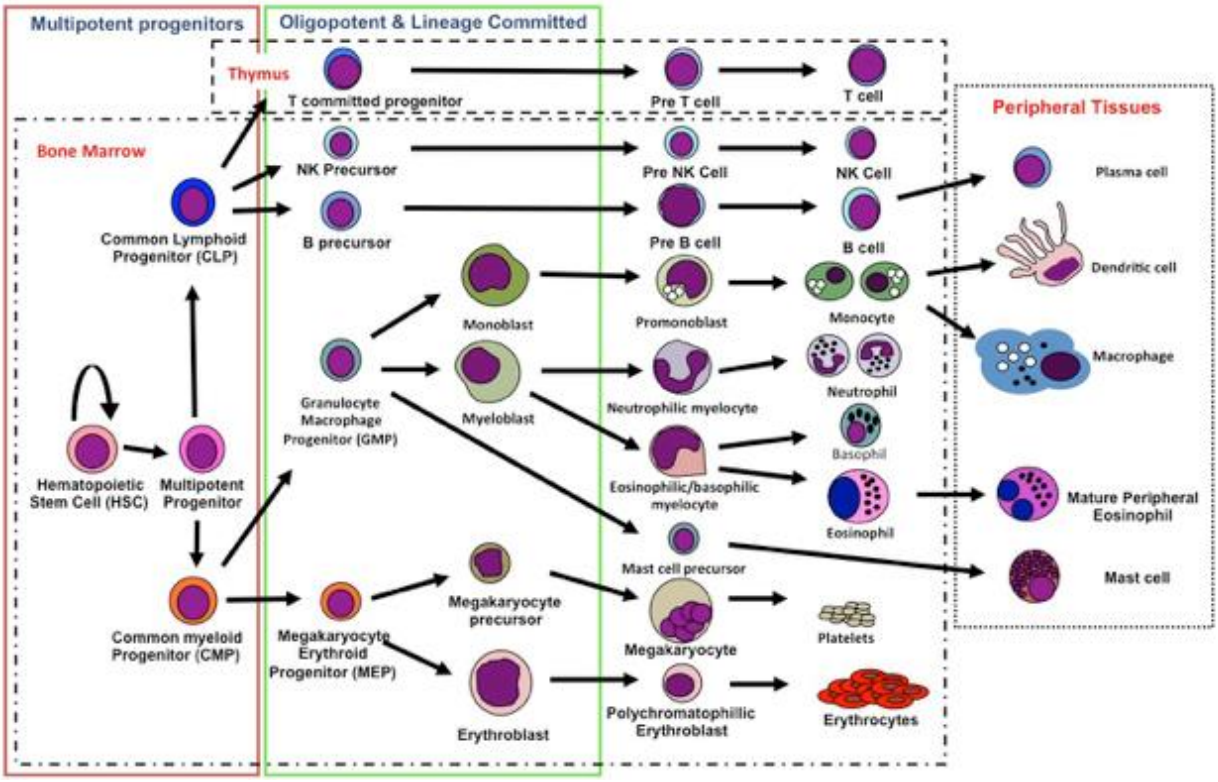

Figure 1. Chart of hematopoiesis in vertebrates (from Stachura and Traver, 2016). Hematopoiesis begins with the hematopoietic stem cell (HSC), which further differentiates to produce all mature blood cells in the body.

CHAPTER II

MATERIALS AND METHODS

Zebrafish

Wildtype and transgenic zebrafish lines (*hbaa*:GFP, *mpx*GFP, *cd41*:GFP, *lck*:GFP, and *mpeg*:GFP) used in these studies were raised and maintained in accordance with California State University, Chico IACUC guidelines. All experiments were approved by the IACUC committee before being performed.

Morpholino Injection:

To examine the specific function of *son*, I injected 1 nL of *son* morpholino (MO) (Gene Tool, LLC, Philomath, OR), a molecule that blocks the production of *son*, at 6.25 μM into single cell zebrafish embryos, resulting in a total injection of 6.25 ng of the MO into each individual. This amount was used due to previous work that showed that 6.25 ng of *son* MO is an effective quantity (Kim *et al.*, 2016). The *son* morpholino sequence is 5'-TGGTCCTGGATATAACAGACAGATT-3' (Kim et. al, 2016).

Microscopy:

hbaa:GFP and *mpx*:GFP embryos were sorted at 24 hours post fertilization (hpf). At 48 hpf *hbaa*:GFP and *mpx*:GFP embryos were observed. *hbaa*:GFP embryos were immediately sorted into phenotypic groups and counted. Images of *mpx*:GFP embryos were taken at 48 hpf and the number of fluorescent cells per embryo were counted using the images. *lck*:GFP embryos were observed at 5 days post fertilization (dpf) and images were taken of each embryo. *cd41*:GFP embryos were observed at 72 hpf. All observations were made with a Leica M165C microscope

and pictures were taken with a Leica DFC295 camera. Images of *lck*:GFP embryos were analyzed with ImageJ (https://imagej.nih.gov/ij/) for pixel density of each thymus.

Methylcellulose Assay:

To determine the effect of reduced or increased *SON* expression methylcellulose assays were performed. This allowed for quantification of HSPCs from whole embryos by observing the colonies formed (Berrun and Stachura, 2018). The HSPCs were grown in methylcellulose media as described (Berrun and Stachura, 2018) with carp serum, Gcsf, and Epo added to stimulate myeloid and erythroid differentiation (Stachura *et al.*, 2013; Stachura *et al.*, 2011). Samples were incubated at 5% carbon dioxide at 32C for 7 days. Each sample was observed at 40x with an Olympus IX53 inverted microscope (Olympus Life Science, Waltham, MA) and colonies were counted for each sample.

Flow Cytometry:

At 48 hpf *hbaa*:GFP and *mpeg*:GFP embryos were sorted. The chorions were removed, and the embryos were moved to microcentrifuge tubes in equal numbers. The samples were rinsed with sterile E3, then incubated in E3 with DTT. Next, the samples were rinsed with DPBS (containing Ca^{+2} and Mg^{+2}) before adding 5 uL of 5 mg/mL (26 U/mL) Liberase (Roche, Indianapolis, IN) to 500 uL of the sample. The embryos were incubated for approximately 1 hour shaking gently at 37C, until the embryos were dissociated. Each sample was then passed through a filter and transferred to a round bottom tube. The samples were then centrifuged at 300 x g for 5 minutes. The supernatant was removed and the cell pellet was resuspended. Sytox red (Thermo Fisher, Waltham, MA) was then added to each sample and they were individually run though BD Accuri

C6 flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed using FloJo software (FloJo LLC, Ashland, Oregon) to quantitate total percentage of positive fluorescent cells.

Quantitative Reverse Transcriptase PCR (qRT-PCR):

RNA was extracted with the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) from control embryos and those injected with *son* MO at 24 hpf, 48 hpf, 72 hpf, 5 dpf, and 7 dpf. iScript (Bio-Rad, Hercules, CA) was used to generate cDNA from each sample. Each cDNA sample was made by pooling 5 whole embryos per condition. Samples were then run on an Eppendorf realplex^2 with primers for *ef1α*, *band3*, *cmpl*, and *mcsfr* (Table 1). Data were analyzed for relative expression change with *ef1α* as the standard gene. ΔΔCt was calculated by comparing the expression of the *son* MO injected embryos to both the uninjected embryos and to the standard gene, *ef1α*. 48 hpf cDNA was also run on a Bio-Rad T100 thermocycler with *son* primers (Table 1). The denaturation temperature of 95C was run for 30 seconds, and annealing temperature of 55C for 30 seconds, and an extension temperature of 72C for 2 minutes. The previous three steps were repeated 34 times. Afterwards, the extension temperature ran for an additional 5 minutes before holding at 4C. The sample was then run on a 1% agarose gel with EtBr in TAE. The gel was photographed with a Kodak Gel Logic 100 imaging system.

Table 1. Primers used for qRT-PCR and RT-PCR.

Gene	Primer Sequence	Citation
ef1α	FWD: 5'-GAGAAGTTCGAGAAGGAAGC-3'	Bertrand, 2008
	REV: 5'- CGTAGTATTTGCTGGTCTCG -3'	
band3	FWD: 5'- CAACTTCCAGAAGAAGATG -3'	Wolf *et al.*, 2017
	REV: 5'- TTGACCATACCACCAAATG -3'	
cmpl	FWD: 5'- CGCCAACCAAAGCCAGAGTTA -3'	Bertrand, 2008
	REV: 5'- ACTTTTCAACAGGTGCATCCCA -3'	
mcsfr	FWD: 5'- TCGGTCTTGCTAGAGACATC -3'	Wittamer *et al.*, 2011
	REV: 5'- ATGACCAGACATCACTTTGG -3'	
SON (0A/12)	FWD: 5'- ATGGAGAAAATCCAACTGTG -3'	Kim *et al.*, 2016
	REV: 5'- GACCTTAAAGAGGAAGTTC -3'	

CHAPTER III

FINDINGS AND RESULTS

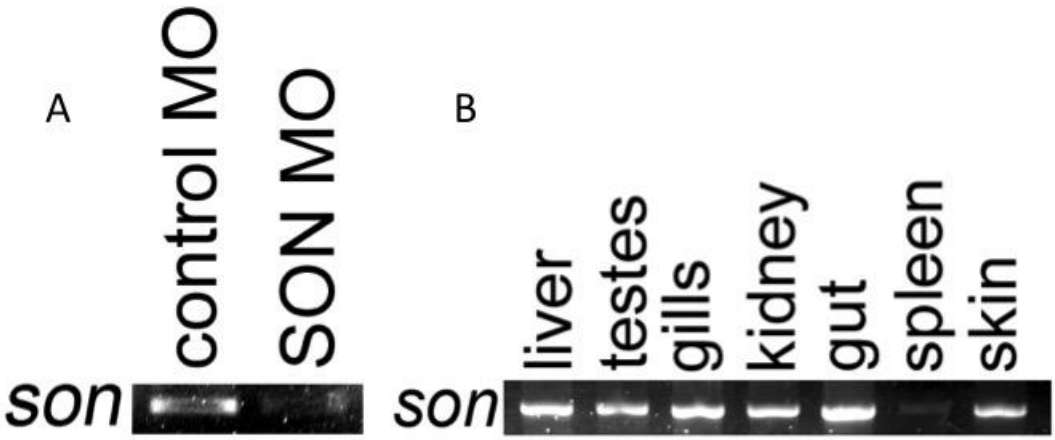

Figure 2. Injection of *son* MO into single cell embryos reduces *son* expression. (A) *son* transcript was measured from control MO injected (left) and *son* MO injected (right) zebrafish analyzed at 48 hpf. (B) *son* transcript was also measured from cDNA collected from different tissues of adult zebrafish.

To understand the role of *son* in blood development, we used a *son*-specific morpholino (MO) (Kim *et al.*, 2016) to knock down its expression at the single-cell stage in zebrafish embryos. This allowed us to observe phenotypic changes caused by a reduction in *son* levels. To ensure that our MO was successfully knocking down *son* expression, we isolated mRNA from injected embryos at 48 hpf and performed RT-PCR for *son*. As expected, we saw a reduction in the band intensity of *son* only in embryos injected with *son* MO (Fig. 2A). After seeing no phenotypic difference between embryos injected with the control MO and those that were not injected, we

used uninjected embryos as the control for the remaining experiments. We also investigated the expression level of *son* in various tissues of adult zebrafish (Fig 2B). Interestingly, we saw that *SON* was expressed in all tissues, although to a lesser extent in the spleen. It is important to note that *son* is expressed in the kidney, which is the site of hematopoiesis in zebrafish. The expression of *son* in other tissues suggests that it is important in many tissues; in addition to blood. This is supported by its role in brain and spinal development (Kim *et al.*, 2016).

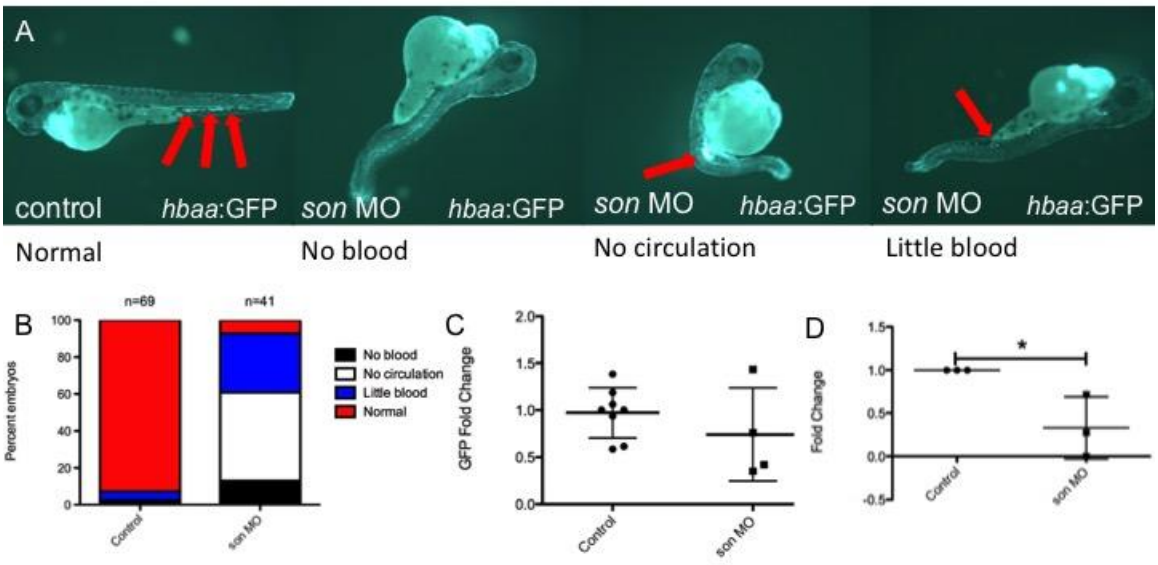

Figure 3. Reduction in *son* results in impaired erythropoiesis. (A) Representative images of the four common phenotypic categories. Category titles are listed underneath each image. Red arrows indicate locations of red blood cells, as seen with green fluorescence. (B) Percentage of embryos that fit into each of the four phenotypic categories, compared between control and those injected with *son* MO. (C) The results of flow cytometry measurements for both control and *son* MO injected embryos ($p=0.27$). (D) Expression level changes in *band3* with a reduction in *son* as measured with qRT-PCR. * indicates $p<0.05$. Middle bar represents mean and whiskers represent SD. Data were analyzed with a Student's t-test with the change in gene

expression and the percentage of GFP cells as the dependent variables and the injection category as the independent variable.

Once we confirmed that our MO reduced *son* levels, we observed altered phenotypes that accompanied *son* reduction. First, we examined erythrocytes utilizing a transgenic zebrafish that have the alpha globin (*hbaa*) promoter driving GFP expression. *hbaa*:GFP transgenic zebrafish only express GFP in red blood cells (Ganis *et al.* 2012). Therefore, every GFP+ fluorescent cell was a red blood cell (Fig. 3A). Reducing *son* resulted in a significant decrease in the amount of red blood cells in circulation (Fig. 3A, 3B). While we didn't measure a statistically significant decrease in the number of fluorescent cells measured by flow cytometry in the 48 hpf embryos when *son* was reduced (Fig. 3C), qRT-PCR showed a significant decrease in the expression of *band3*, a marker of erythrocytes, when *son* MO was injected compared to control embryos (Fig. 3D). Together, these data indicate that decreased *son* impairs successful erythropoiesis.

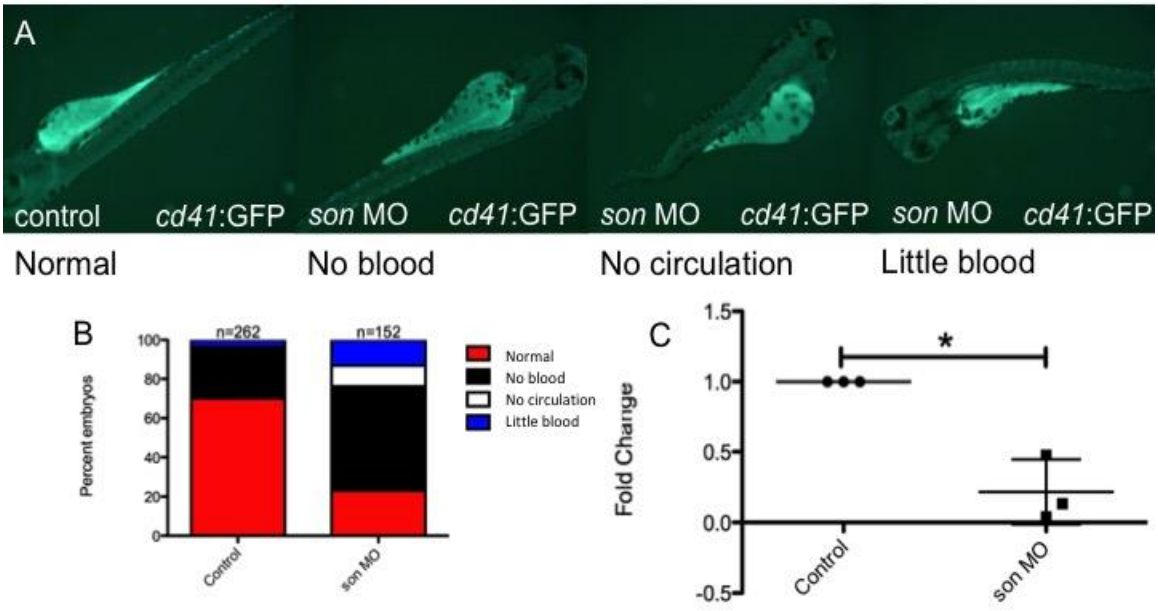

Figure 4. Reduced *son* expression causes a reduction in thrombocytes. (A) Representative images for each of the four observed phenotypic categories, with the category titles listed underneath each image. (B) The percentage of embryos in each of these categories when developing normally or injected with the *son* MO shown in (A). (C) qRT-PCR results for *cmpl* at 5 dpf are shown. * indicates $p<0.05$. Middle bar represents mean, and whiskers represent SD. Data were analyzed with a Student's t-test with the relative gene expression as the dependent variable and the injection category as the independent variable.

We next examined thrombocytes, cells responsible for blood clotting, by utilizing *cd41*:GFP embryos, which have GFP+ thrombocytes (Lin *et al.* 2005) (Fig. 4A). These embryos showed a significant decrease in *cd41*:GFP fluorescent cells when injected with *son* MO when examined at 72 hpf (Fig. 4B). We also performed qRT-PCR on these embryos and observed a significant decrease in the expression level of *cmpl*, a thrombocyte marker, at 5 dpf for the injected

embryos when compared to the control embryos (Fig. 4C). Together these data indicate that *son* is also important for normal thrombocyte production.

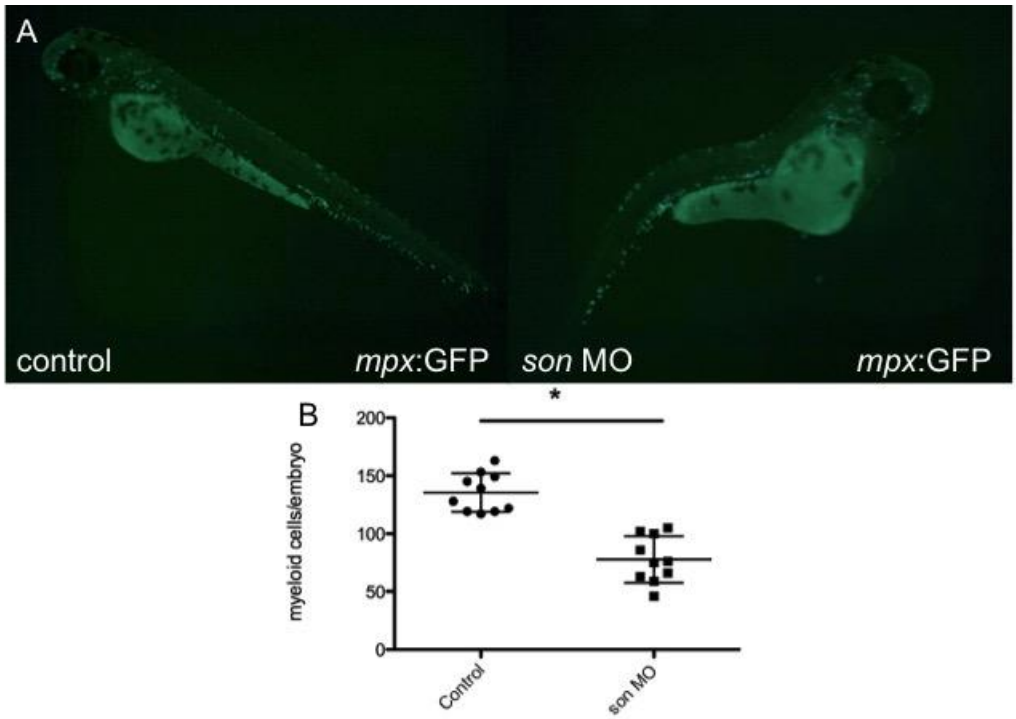

Figure 5. *son* knockdown causes a reduction in myeloid cells. (A) Representative images of *mpx*:GFP embryos that were used to count the total number of myeloid cells per individual. (B) The resulting counts from (A) (* indicates $p<0.05$). Middle bar represents mean and whiskers represent SD. Data were analyzed with a Student's t-test with the number of myeloid cells per embryo as the dependent variable and the injection category as the independent variable.

Next, we investigated the effects of *son* reduction on myeloid cells using *mpx*:GFP embryos, which label neutrophils (Renshaw *et al.* 2006). We photographed sixteen 48 hpf embryos from both the control and *son* MO injected groups and counted the number of fluorescent cells per embryo (Fig. 5A). The control embryos had an average of 155.75 ± 44.4 myeloid cells and the embryos injected with *son* MO had 106.25 ± 44.2 myeloid cells per embryo (Fig. 5B), indicating

a significant decrease in the number of myeloid cells correlated with a reduction in *son*. These data indicate that *son* is also crucial for myeloid cell development.

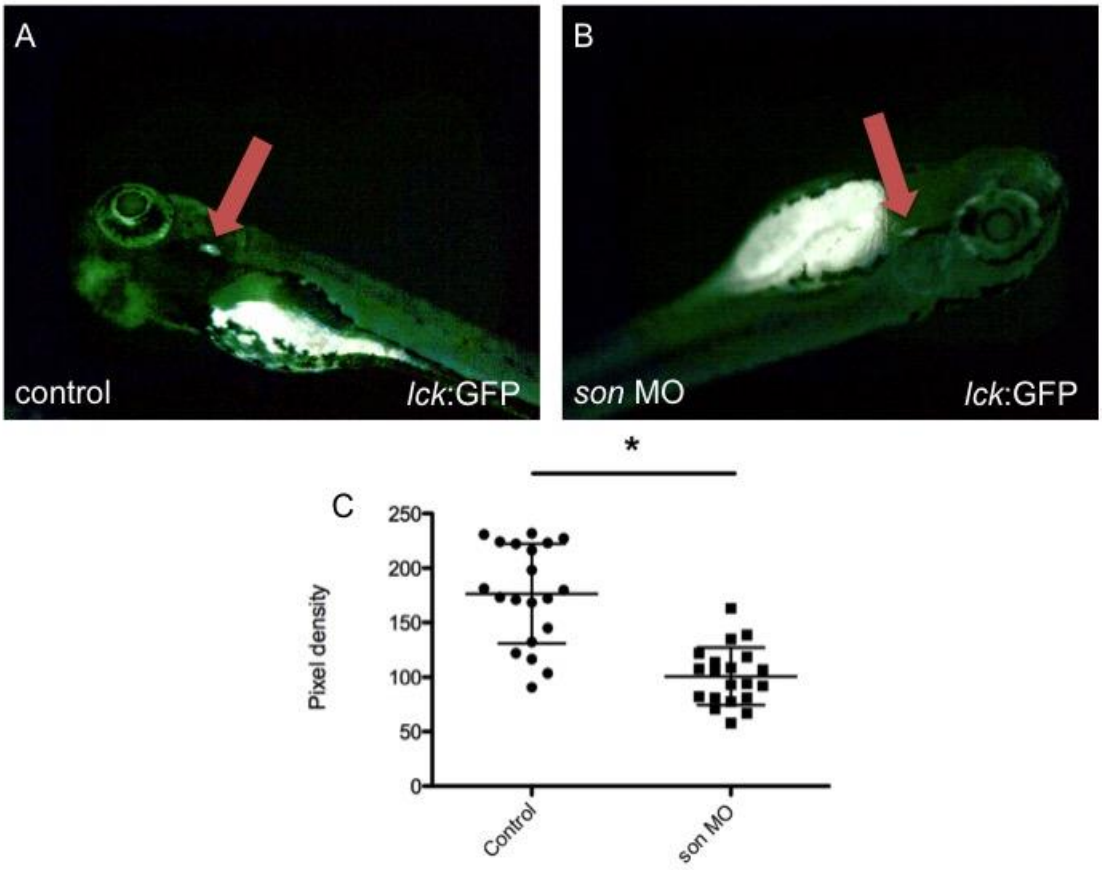

Figure 6. *son* reduction decreases T cells. (A, B) Representative images used to calculate thymic density. Each thymus is indicated by a red arrow. (C) The pixel density as calculated with ImageJ (* indicates $p<0.05$). Middle bar represents mean, and whiskers represent SD. Data were analyzed with a Student's t-test with the pixel density as the dependent variable and the injection category as the independent variable.

Next, we investigated if *son* perturbation negatively affected the number of T cells with *lck*:GFP embryos (Langenau *et al.*, 2004). Since T cells develop in the thymus, we imaged each

fluorescent thymus from twenty individuals at the same exposure time from both the control and *son* MO injected groups at 5 dpf (Fig. 6A, 6B). We attempted to maintain a consistent focal plane to minimize outside effects on the magnitude of fluorescence detected. We then used ImageJ to calculate the pixel density of each thymus. The control embryos had an average thymus with a pixel density of 160.68 ± 43.8 and the *son* MO injected embryos had an average density of 100.73 ± 26.5 (Fig. 6C). These data indicate that *son* is necessary for proper T cell development.

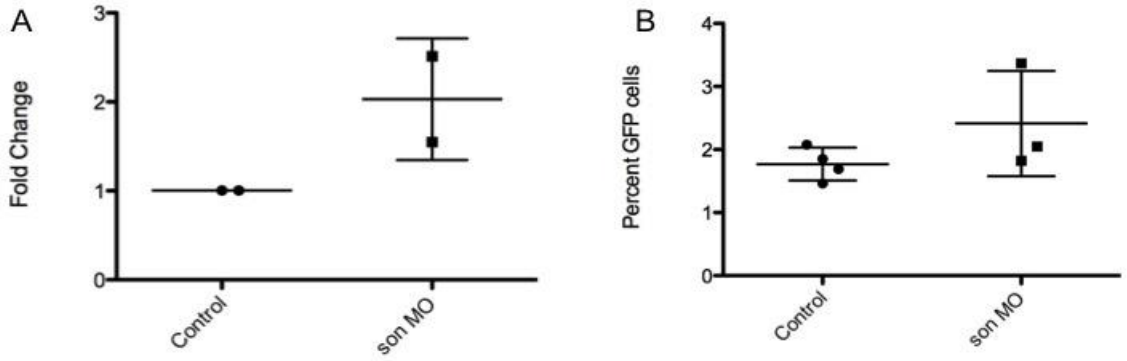

Figure 7. *son* knockdown slightly increases macrophage numbers. (A) qRT-PCR results for *mcsfr* at 5 dpf (p=0.167). (B) The results from flow cytometry on 48 hpf *mpeg*:GFP embryos (p=0.197). Middle bar represents mean and whiskers represent SD. Data were analyzed with a Student's t-test with the change in gene expression and the percentage of GFP cells as the dependent variables and the injection category as the independent variable.

We next examined macrophages. Surprisingly, when we performed qRT-PCR on these embryos we saw a small insignificant increase in the expression levels of *mcsfr*, a marker for macrophages at 5 dpf (Fig. 7A). We then investigated *mpeg*:GFP embryos, which have GFP-labelled macrophages (Ellett *et al.* 2011). When measured with flow cytometry we saw a small increase in the number of macrophages present in *son* MO injected embryos as compared to the control (Fig. 7B). These data indicate that *son* knockdown does not cause significant changes in macrophage numbers. Additional investigation of macrophages needs to be conducted to further understand the effects of *son* on macrophage development.

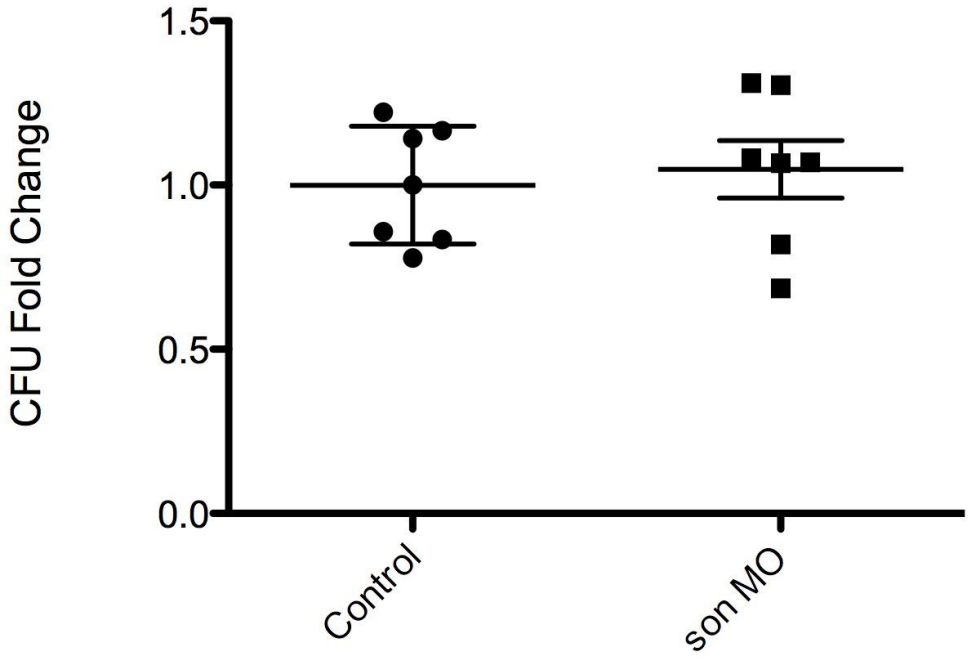

Figure 8. *son* knockdown does not affect HSPC numbers. The fold change in the number of colony forming units (CFUs) as compared to the average number of control colonies from 48 hpf complete embryos. Middle bar represents mean and whiskers represent SD. Data were

analyzed with a Student's t-test with the number of CFUs as the dependent variable and the injection category as the independent variable.

After seeing a decrease in mature blood cell types, we turned our attention to enumerating hematopoietic stem and progenitor cells (HSPCs). We used a methylcellulose assay to enumerate the amount of HSPCs in each embryo (Berrun and Stachura, 2017). With this assay embryos were grouped by treatment and equal numbers at 48 hpf were enzymatically digested (Berrun and Stachura, 2017). The single cells were plated in methylcellulose medium along with cytokines and other supportive factors. The HSPCs divide and proliferate, but the medium does not allow the cells to migrate throughout the plate, so each colony that is seen represents one HSPC that was present in the original embryo. The number of colonies were then counted under a microscope. Interestingly, we saw no significant difference between the number of colonies from control and *son* MO injected embryos (Fig. 8). These data indicate that *son* is not crucial for HSPC production.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

We have previously shown that zebrafish are an effective model organism for investigating the effects of *son* knockdown with *son* MO (Kim *et al.*, 2016). We showed that *son* is necessary for proper brain and skeletal formation (Kim *et al.*, 2016). Here we established that *son* is necessary for proper blood formation through an analysis of mature blood cell and HSPC numbers. We saw a decrease in the number of red blood cells, thrombocytes, myeloid cells, and T cells; however, we did not see a change in the number of HSPCs. Further studies are warranted to establish the molecular mechanism by which *SON* directs and manages blood cell differentiation.

While zebrafish are an excellent organism for studying blood development, there are some limitations. Common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs) have been identified in mice and humans, however they are not clearly defined in zebrafish. The site of hematopoiesis and role of some blood cells also differs between humans and zebrafish (as reviewed in Chen and Zon, 2009), making it more difficult to expand our understanding of zebrafish hematopoiesis to human hematopoiesis. Because of this reason we did not restrict ourselves to studying only the erythrocytes and thrombocytes, the blood cells affected by AMKL. We wanted to have a broader understanding of how *son* affects all of blood development, not only cells arising from the megakaryocyte erythroid progenitor (MEP), so we investigated mature blood cells that arise from different progenitor cells. In addition, it has been shown that human patients with *SON* deficiency have recurrent infections, low immunoglobulin levels, and clotting issues, suggesting that these patients have issues with hematopoiesis (Tokita *et al.*, 2016). This supports our study since we investigated *son* knockdown, as seen in this human study, instead of the overexpression seen in AMKL patients.

An additional consideration for zebrafish is the time that it takes for each blood cell type to develop. We attempted to analyze each blood cell as soon as they were formed. Red blood cells, myeloid cells, and macrophages can first be detected at 48 hpf. Thrombocytes are first seen at 72 hpf, and T cells are observed at 5 dpf (as reviewed in Chen and Zon, 2009). All images were taken when mature blood cells were first seen; however, some qRT-PCR data were collected after the mature blood cells first developed. The results were consistent at various time points between microscopy and qRT-PCR analyses, indicating that the observed *son* MO induced phenotypes were not caused by a developmental delay.

As noted in our 2016 study, embryos injected with *son* MO often have a bent tail, in addition to other physical deformities (Kim *et al.*, 2016). This may cause a decreased number of cells since the body is malformed, and could explain the decrease seen in RBCs, thrombocytes, myeloid cells, and T cells. Since each blood cell type is affected in *son* MO injected embryos, and *son* MO injected embryos are smaller, there may be a decreased amount of HSPCs to create those mature cell types. However, there was not a difference in the number of CFUs between control and *son* MO injected embryos. This shows that both the control embryos and *son* MO injected embryos had equal numbers of HSPCs, so any possible change in the size of the embryo did not affect the HSPC numbers. HSPCs either proliferate or differentiate. If they proliferate, they create more cells of the same type as themselves. If they differentiate, they create more mature blood cells downstream of themselves. Therefore, any change in the amount of mature blood cells present per embryo is due to an error in differentiation, not because of a lack of HSPCs or change in size. While the methylcellulose assay is an excellent assay for evaluating the number of total HSPCs in the embryo, it does not differentiate between the different types of progenitor cells in the embryo. Therefore, a decreased expression of *son* may shift the levels of CLPs versus CMPs, but the

methylcellulose assay does not allow for us to measure this difference. Additional research needs to be conducted to understand how *son* may shift the types of progenitor cells present in the animals.

The decreased number of myeloid cells *in vivo* show that *son* is necessary for proper granulocyte development. However, by looking at *mpx*:GFP embryos to establish changes in myeloid cells in response to reduced levels of *son* we evaluated the levels of monocytes, macrophages, and neutrophils. We compared them to *mpeg:*GFP embryos, which only have fluorescent macrophages, and saw a reduction in *mpx*:GFP *son* MO injected embryos, but did not see a significant change in *mpeg*:GFP *son* MO injected embryos. These data indicate that a lack of *son* causes a decrease in the number of monocytes and neutrophils but does not affect macrophages. However, there are many types of macrophages (as reviewed in Chen and Zon, 2009) and the diagram of hematopoiesis (Fig. 1) is simplistic and does not show the various types of macrophages that occur in the body. By analyzing *mpeg*:GFP embryos we were not able to differentiate between these various macrophage types. It is possible that one, or many, types of macrophages are knocked down with a decreased level of *son* and another type is upregulated to compensate. An investigation of the various types of macrophages and how they respond to altered levels of *son* will help us gain a greater understanding of this interesting result.

Since not all mature blood cells were knocked down by a decreased expression of *son*, we were able to see that *son* does not non-specifically knockdown mature blood cell production. The specificity of which granulocytes are affected by altered levels of *son* suggests that granulocytes are held in balance through the level of *son* present. Overexpression experiments need to be conducted to understand how different levels of *son* may affect CMP differentiation. The fluorescent markers for each blood cell type may also be imprecise, so investigations based on

morphology and mRNA expression may help us have a clearer understanding of how these blood cells are affected.

SON is upregulated in hematopoietic cells and organs (Sun *et al.*, 2001), and binds to the leukemogenic protein AML1-ETO in leukemia cells, triggering signals inhibiting leukemogenesis (Ahn *et al.*, 2008). *SON* is also involved in the regulation of microRNA transcription, which influences hematopoiesis by regulating *gata2*, which is essential for HSCs (Ahn *et al.*, 2013). These previous studies viewed alongside our study shows that *SON* is crucial for proper blood maturation. To further understand how *SON* controls and effects blood development the levels of various progenitors, such as CLPs, CMPs, and MEPs, should be further studied. There should also be attention given to blood cell types that were not addressed during this study, such as B cells, NK cells, and basophils. In addition to knocking down *SON* expression, overexpression of *SON* should be studied. These experiments along with rescue experiments will confirm *SON*'s role in hematopoiesis.

We have now shown that *SON* affects brain, spine, and blood development. It is particularly interesting that both brain and blood development are affected by *SON* since the brain develops from ectoderm, but blood develops from mesoderm. One hypothesis for how one gene can affect both endoderm and mesoderm derived tissues, without affecting the entire embryo, is through niche signaling from the ectoderm to the mesoderm during HSPC development. Current research suggests that ectoderm derived neural crest cells contributes to the HSPC niche, impacting HSPC development (Damm and Clements, 2017). Trunk neural crest cells, which become sympathetic nervous system neurons, physically associate with the dorsal aorta, the location of primary blood development, prior to hematopoietic initiation. Any disruption of this interaction results in impaired hematopoiesis (Damm and Clements, 2017). Another hypothesis is that SON is

regulating brain, spinal, and blood development through alternative mRNA splicing. SON may also regulate different genes in different tissue types or regulate different genes at different developmental time points. Differential gene regulation in this way could explain how SON is able to impact a variety of cell types. It is likely that *son* is pleotropic, affecting different genes in different tissues under different conditions. A closer look into the specific genes that SON regulates may shed light on how one gene can affect the formation of many diverse tissues.

While our results differed from the conditions seen in AMKL we were able to show that *son* is necessary for proper blood development in zebrafish. This is encouraging and lays a clear foundation for the investigation of the role of *son* in AMKL. Additional research on the role of *son* in mammals can help us gain a clearer understanding of how *son* affects blood development. Data derived from this study and others will help offer a greater understanding of *son* and its role in a unique disease.

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