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Dr. Jessica Blackburn, Major Professor

Dr. Melinda Wilson, Director of Graduate Studies

THE ROLE OF VASCULAR ENDOTHELIAL GROWTH FACTOR IN LEUKEMIA TRAFFICKING

THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
in the College of Medicine at the University of Kentucky

By

Shaw Powell

Lexington, Kentucky

Director: Dr. Jessica Blackburn, Professor of Biochemistry

Lexington, Kentucky

2021

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ABSTRACT OF THESIS

THE ROLE OF VASCULAR ENDOTHELIAL GROWTH FACTOR IN LEUKEMIA TRAFFICKING

Vascular endothelial growth factor (VEGF) is a signaling protein involved in inducing and regulating endothelial cell proliferation and function (Duffy et al 2000). VEGF is also involved in cancer progression, as it induces vascular permeability and promotes angiogenesis to tumor laden areas, giving cancer cells critical oxygen and nutrients (Hoepfner et al.,2012. Studies indicate VEGF prevents lymphoblast apoptosis, which may contribute to leukemia formation and enable the proliferation of leukemic cells (Duffy et al 2000). Ongoing research seeks to further examine VEGF in leukemia, using a *rag2:GFP-Myc* expressing transgenic zebrafish as the animal model of T-cell Acute Lymphoblastic Leukemia (T-ALL). Recent findings have concluded a relationship between VEGF expression in leukemic fish remodels the microenvironment leading to cell migration, but not through vascular restructuring, as a means to upregulate leukemic expression.

KEYWORDS: Leukemia Trafficking, Lymphocyte Migration, Vascular Endothelial Growth Factor (VEGF), Zebrafish T-ALL, Acute Lymphoblastic Leukemia

Shaw M. Powell

31 March 2021

THE ROLE OF VASCULAR ENDOTHELIAL GROWTH FACTOR IN LEUKEMIA TRAFFICKING

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I would also like to thank my lab. This was a collaborative work that would not be possible without the joint effort of many members of the Blackburn lab to include Sergei Revskoy who collected images of the animals with my assistance. While I analyzed all the images for raw data, Lucas Tomko helped with statistical analysis, and Shea Hausman and Margaret Blair helped write protocols for vessel quantification. Dr. Blackburn provided the funding, without which none of this would have been possible.

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Introduction

Zebrafish as a Leukemia Model

Acute lymphoblastic leukemia (ALL) is a cancer prone to occurring in children, but can recur or even occur for the first time in adults, particularly the elderly, with a poorer prognosis in later years (Terwilliger et al., 2017). *In vivo* models of human cancer in zebrafish (*Danio rerio*) are a growing field of study to help inform treatment and diagnosis of the disease. Zebrafish are small, reproduce quickly and are 71% of human proteins have a direct zebrafish homology (Stolotov et al, 2008). They can be housed and fed inexpensively compared to rodents and can be genetically modified in the germline in order to conduct experiments. Zebrafish embryos are translucent, and development can be wholly observed *ex vivo*, since they are not mammals. Organ development occurs within 3 days post fertilization (dpf) can be observed without sacrificing the animal (Baeten et al., 2018). They lay clutches upwards of 30 eggs at a time, which helps dull genetic diversity when conducting large scale experiments.

Mutant lines have been generated to lack pigment, which enables fluorescence microscopy of interior anatomy. The zebrafish can be viewed live, with all its organ systems intact and with no need for sectioning in order to view deeper tissue. This saves both money and time, by negating the necessity for sectioning tools and fixative and the slow application of sections to slides. Furthermore, zebrafish can be injected with human transgenes and produce human proteins that respond to drugs and assays that already exist, which enables easy investigation into treatment or analysis. There are well established fluoresced B and T cell leukemic lines (Baeten et al., 2000).

Though zebrafish undergo hematopoiesis in their kidney marrow (deep kidney tissue) and humans undergo hematopoiesis in their bones, T cell maturation occurs in the thymus of both organisms. Furthermore, many transcription factors and signaling pathways for differentiation of hematopoietic cells into their myeloid and lymphoid progenitors are very similar, resulting in overlapping genetics and molecular biology in the development of leukemia (Baeten et al., 2000). Recombination Activating Gene 2 (Rag2) is one such conserved gene across human, mouse and zebrafish, that is present only in T and B lymphoid tissue, enabling specific activation of transgenes that can induce leukemia in zebrafish (Langenau et al., 2004).

rag2:GFP-Myc Leukemia Producing Transgene

The *Myc* oncogene is conserved between zebrafish and their mammalian counterparts in the expression of lymphocytic leukemia (O'Neil et al 2007), enabling experiment among an established line of leukemic fish. In immature lymphocytes Recombination Activating Gene 2 (Rag2) plays a critical role in recombination, and a mutation of this gene affects the outcome for VDJ recombination, impacting the later outcome of these cells (Weissman 1985, Schlissel, Kaffer et al. 2006, Mijuskovic, Chou et al. 2015). Because the *rag2* promoter for the *rag2* gene is specific to lymphocytes, the *rag2* promoter can be hijacked to express *Myc*, inducing leukemia. The line of zebrafish used in this study used transposase to create a double stranded break in the zebrafish DNA in order to insert the mouse derived *Myc* transgene under the *rag2* promoter in order to limit the cancer to T and B lymphocytes (Langenau et al, 2003). *Myc* is fused with a GFP gene in order to fluoresce (making the line *rag2:GFP-Myc*), which allows experimenters to visualize, track and measure the leukemia *in vivo*. The particular line of fish in this experiment is restricted to T lymphocytes only. Conversely, the control (nonleukemic) line is *rag2:GFP* transgenic so the microenvironment without the influence of *Myc* can also be visualized.

Investigations into the activity of *Myc* under this (*rag2*) promoter are ongoing. *Myc* affects growth at several points in the cell's life cycle, including during double negative selection into double positive selection, then again during stimulation of TCR, both of which occur in the thymus (Delgado and Leon, 2010). The role of *Myc* is not limited to individual cell development within the thymus, *Myc* is involved in cell migration and invasion in epithelial mesenchymal layers (Wolfer and Ramaswamy, 2011). Furthermore, its role in solid tumor metastasis has been well documented (Kortlever, Sodik et al., 2017). Contrary to solid tumors, in which spread of metastasis indicates poorer prognosis, spread of lymphocytes into the periphery is common to both leukemic and non-leukemic fish and impact on prognosis needs further investigation (Stoletov, Montel et al., 2007). Overall, the molecular changes brought by *rag2:GFP-Myc* enable a stable leukemic fish line.

Role of Vascular Endothelial Growth Factor in Immunity

During this maturation process, some cells will undergo clonal expansion, increasing the odds that a cell with the correct antigen receptor will encounter the pathogen presenting the

reciprocating antigen (Hess et al 2012). In leukemic cells, the maturation process is mutated between the selection processes, so propensity for cells to expand uncontrollably within and out of the thymus is possible and this has been suggested as a means for leukemic cell dissemination from the thymus (Feng et al. 2010)

We know that solid tumors remodel their local vascular environment in order to transport nutrients and oxygen into the area, particularly through the use of VEGF to induce angiogenesis (Kortlever et al 2017). Prior studies in mouse models indicate VEGF induced microvasculature remodeling was also associated with leukocyte and mast cell recruitment (Detmar et al 1998). In other forms of leukemia, particularly acute myeloid leukemia (AML) and chronic lymphoblastic leukemia (CLL), plasma VEGF levels are markedly higher than in non-leukemic patients (Aguayo et al., 2000). The same study also found a relationship between increased vascularization of the bone marrow, coinciding with increased VEGF expression and poorer prognosis in AML and CLL patients (Aguayo et al., 2000). Using our zebrafish model, I will be able to visualize leukemia progression *in vivo*, particularly as it relates to T cell dissemination from the thymus to other areas of the fish's body and identify what role VEGF may play in that dissemination. I will be able to see patterns of vascular recruitment as VEGF changes the microenvironment surrounding the thymus. This would not be possible in a mouse model because of size constraints of the fluorescence microscope and depth of mouse tissue. A mouse model would have to be sacrificed and sectioned in order to be wholly observed. Fish have the advantage of being small enough that their vasculature and organ systems can be imaged in three dimensions while the animal is still alive.

Using the zebrafish model, we can elucidate the role VEGF plays in leukemia progression and angiogenesis among endothelial (Flk1a), VEGFR-2 (Flk) and lymphatics (Lyve) fluorescently tagged vasculature. While there are four kinds of VEGF (VEGF-A through VEGF-D) and three receptors, the ones analyzed in this experiment will be VEGF-A and VEGFR-2, which regulates angiogenesis and vascular permeability (Wang et al 2020). Additionally, VEGF expression can be controlled in animals via heat shock promoter (hsp), to determine the role that VEGF plays in leukemia progression. These fish with VEGF induction via heat shock promoter are also positive for fluorescent Flk1a vasculature (*Flk1a:RFP*).

My hypothesis is that I will see an increase in VEGF-R vasculature (Flk) remodeling in the local area surrounding the thymus in leukemic (*rag2:GFP-Myc*) fish, and I expect the vascular

density to increase as leukemia progresses, possibly providing routes for leukemic dissemination. I also expect to see an increase in thymus volume as the leukemia progresses, in keeping with suggestions by prior literature that leukemia expands within the thymus before progression into the bloodstream. Furthermore, I expect that under increased VEGF protein expression through use of a heat shock promoter, these features will be exacerbated in leukemic (*rag2:GFP-Myc*), but not control (*rag2:GFP*), non-leukemic fish.

Methods

Zebrafish Husbandry

All experimental procedures involving zebrafish were approved by the University of Kentucky's Institutional Animal Care and Use Committee, protocol number 2015-2225. Transgenic lines used in this study are shown in Table 1. Animals were not excluded from experiments based on sex and were between 13-30 days post-fertilization for these studies.

Light Sheet Fluorescence Microscopy

To prepare fish for imaging, each fish was anesthetized and then transferred to a 2 mL microcentrifuge tube, removing excess water afterward. 300 μ L of 0.004% tricaine (MS-222, Western Chemical Inc) in E3 media was added to each tube and fish were kept at rest for 5 minutes. 200 μ L of a solution of 2% NuSieve GTG low-melt agarose (Lonza, cat. no. 50081) was added to the tubes for a final agarose concentration of 0.8%. Tubes were mixed by inversion and then the fish was loaded, tail first, into a glass capillary using a custom plunger (Zeiss). The E3/agarose was then allowed to cool and solidify for five minutes. Agarose-constrained fish were imaged using a Zeiss Light Sheet Z.1 dual-illumination microscope system and Zen imaging software (ZEN 2014 SP1 Black Edition) at the University of Kentucky Arts & Sciences Imaging Center. Fish \leq 21 dpf were imaged into a Zeiss file, then converted to Imaris files. 20x objective lens (P/N 421452-9700-000) and fish \geq 21dpf were imaged with a Zeiss 5x objective lens (P/N 420330-8210-000).

To make E3: 14.6g NaCl, 0.65g KCl, 2.20g CaCl and 4.05g MgSO₄ were combined in a 1L bottle and topped off to 1L with ddH₂O. The liquid made 50x concentration, which must be diluted to a 1X concentration and buffered to pH=7. 50ul Methylene Blue (an antifungal) per 1L of media was then added to yield the final 1X E3 media.

Table 1. Zebrafish Lines Used

Zebrafish Line Name	Transgene	Expression Site	Obtained From
Flk:RFP	<i>Tg(kdrl:RFP)</i>	VEGFR+vasculature	ZIRC (Huang, Zhang et al. 2005)
Fli1a:RFP	<i>Tg(fli1a:RFP)</i>	VEGFR- endothelial vasculature	Laboratory of Protein Signaling and Interactions (Martin et al, 2013)
Lyve:dsRED	<i>Tg(-5.2Lyve1b:dsRED)</i>	Lymphatics	Crosier Lab (Okuda, Astin et al. 2012)
Rag2:GFP	<i>Zrag2:GFP</i>	Lymphocytes	Look Lab (Anderson, Li et al. 2016)
Rag2:GFP-Myc	<i>Zrag2:GFP-Myc</i>	Lymphocytes/Leukemia	Look Lab (Langenau, Traver et al. 2003)
hsp:VEGF	<i>h70-mC-hVEGF</i>	Global	Mukhopadhyay Lab (Hoepfner et al., 2012)

Table 1: Zebrafish Lines: “zebrafish line name” indicates the shorthand name for the transgene of interest, while “transgene” indicates the scientific nomenclature of the transgene. Expression site indicates what will be fluoresced. “Obtained from” is the name of the lab of origin for the transgene.

Image Processing and Data Analysis

The images were analyzed using Imaris 9.2 (Bitplane) or FIJI. Files were converted to .IMS files using Imaris File Converter 9.3.0. The Imaris surface creation software was used to enhance the clarity of the *rag2:GFP-Myc* and *rag2:GFP* cells by applying a mask over all GFP+ cells. Images were saved as .tifs and GIMP photo editing software was used to de-convolute and crop images.

For Volume: Using Imaris, the thymus volume was calculated by selecting the volume tool and restricting the parameters to a box encompassing only the thymus. I selected the green (GFP) field with a smoothness setting between 3 and 6. I completed the automated steps but toggled the surfaces in the final step so only the thymus is selected, if multiple surfaces were detected by the automated algorithm. The surface's volume was then available to view under the 'statistics' tab.

For measuring vascular density using Imaris, each thymus length was first measured in pixels, then that length was doubled to create a region of interest that is proportional to the thymus. The region of interest (ROI) was converted to μm by using Edit> image properties> 'look at x, y, z dimensions' and then all axes' pixel values were multiplied by their respective conversion factor. The ROI was reported in μm^3 and the percentage vascular density was calculated by dividing the ROI vascular volume by the total ROI volume. Then I divided the sum volume by the ROI and get a percent.

For measuring cell-vessel proximity using FIJI, all image stacks and channels were adjusted for optimal signal:noise background reduction. Using the 'points' tool and ensuring 'stack position' was selected in the measurement options, cells were measured from the center of their mass to the nearest vessel, whether that happened to be in plane or in a nearby plane. 30-50 cell-vessel measurements were made for each image volume included in this study. After all points were reported, the values were moved to a spreadsheet, subtracted for all x-, y-, and z-dimensions and then calculated using a simple, 3-dimensional, vector calculation between two points. All calculated cell-vessel distances were averaged before reporting a value for each animal, maintaining statistical independence of results.

Cell Counts

In Imaris, a surface mask was used to ignore the GFP+ thymus in each image series. Using the 'spots' tool, the GFP fluorescence channel was selected and then the diameter of a cell was measured; then used as the estimated XY diameter. After the software has selected all spots that meet the XY criteria, a manual deselection of cells in the thymus that were not previously masked is carried out. From there, the cell count for each image can be copied from the 'statistics' tab into a spreadsheet for further analysis.

RNA Isolation

Zebrafish bearing heat-shock promoter VEGF-A in the *rag2:GFP-Myc* genetic background were raised until 21 dpf. Fish were separated into two groups, one remaining at 28°C and the other to be maintained at 36°C for 3 days. Fish transferred to tanks for heat shock activation at 36°C were acclimated at 2°C increments daily until reaching 36°C. After 3 days of heat shock treatment, all fish were sacrificed by placing in containers of aquarium water with lethal dosage of tricaine supplemented prior to freezing in micro-centrifuge tubes on dry ice. All fish were stored at -80°C until RNA isolation with TRIzol Reagent (Fisher Scientific, cat. no. 15596026). After thawing, animals were disintegrated into 150 µL of TRIzol in a micro-centrifuge tube while using a disposable pestle for each fish. After disintegration, 350 µL of TRIzol was added to each tube, was mixed by flicking and inversion, with the remainder of the manufacturer's protocol for RNA isolation followed. Recovered RNA was quantified for each sample using Qubit RNA HS Assay Kit (Fisher Scientific, cat. no. Q32852) and the Qubit 4 fluorometer system (Thermo Fisher).

Reverse Transcription and qPCR

1 µg of RNA from each sample in the previous step was reverse transcribed using the iScript cDNA synthesis kit (BioRad, cat. no. 1708891) and following the manufacturer's instructions. The list of primers used were as follows: vegfaa FWD: GAG CTG CTG GTA GAC ATC ATC; vegfaa REV: TTC GAG CGC CTC ATC ATT AC; ef1a FWD: ATG GCA CGG TGA CAA CAT GCT; ef1a REV: CCA CAT TAC CAC GAC GGA TG; rplp0 FWD: GCG TCC CTA CCG TGA GAT TTT; rplp0 REV: CGC CCA CGA TGA AAC ACT TG. qPCR samples were run in duplicate using iTaq Universal SYBR Green Supermix (BioRad, cat. no. 1725121) in 96-well plates on C1000 Touch thermal cycler (Bio-Rad) with standard, 2-step cycling parameters and 50°C annealing/extension. Results

were analyzed using comparative ΔCt analysis in spreadsheet software. Results were exported to GraphPad Prism v7.04 and Mstat 6.6.2 (McArdle Laboratory, University of Wisconsin-Madison) for analysis and graphical representation of the results.

Statistical Analysis

For all multi-category comparisons, the Kruskal-Wallis (K-W) test was first used to determine if any category's values attained statistical significance from the other groups. For all two-sample comparisons, the Mann-Whitney U(MWU)/Wilcoxon Rank Sum (WRS) statistical tests were employed. C.I.'s were 0.95 and significance was defined as $p \leq 0.05$. Graphical representations of data were generated in GraphPad Prism v7.04.

Results

Leukemic Cell Dissemination Follows a Pattern

Using the fish in Figure 1A to orient ourselves to Figure 1B and 1E, we visualized the cell dissemination, in leukemic fish (*rag2:GFP-Myc*), as it migrated from the thymus, caudally toward the kidney head (Figure 1E). We saw that migration occurred in a predictable, stepwise manner and did not occur in the non-leukemic (*rag2:GFP*) fish. This is important because such a pattern means the microenvironment surrounding the thymus (place of migration initiation) and along the route of travel are being remodeled, perhaps in the same manner as solid tumors remodel their local microenvironment in order to recruit vessels for nutrients and oxygen. While fish younger than 14dpf did not appear to express leukemia regardless of Myc (or VEGF, as we will get into) expression, by 21dpf, the leukemic fish (*rag2:GFP-Myc*) display a consistent exodus of lymphocytes from the caudal tip of the medulla of the thymus that was not found in the non-leukemic (*rag2:GFP*) fish lines at any time period (Figure 1C). The dissemination direction was significantly caudal in leukemic fish (*rag2:GFP-Myc*), with no particular direction of dissemination in non-leukemic (*rag2:GFP*) fish (Figure 1D). Not only do more cells migrate caudally, but a greater number of cells, overall, begin their journey (Figure 1C and 1D) among the leukemic fish than among non-leukemic fish. I postulate that some cells will always be present throughout the zebrafish, since lymphocytes are necessary responders to infection and daily maintenance of the fish's adaptive immunity, so non-leukemic cell dissemination will not be zero.

Figure 1: Leukemia Cell Dissemination

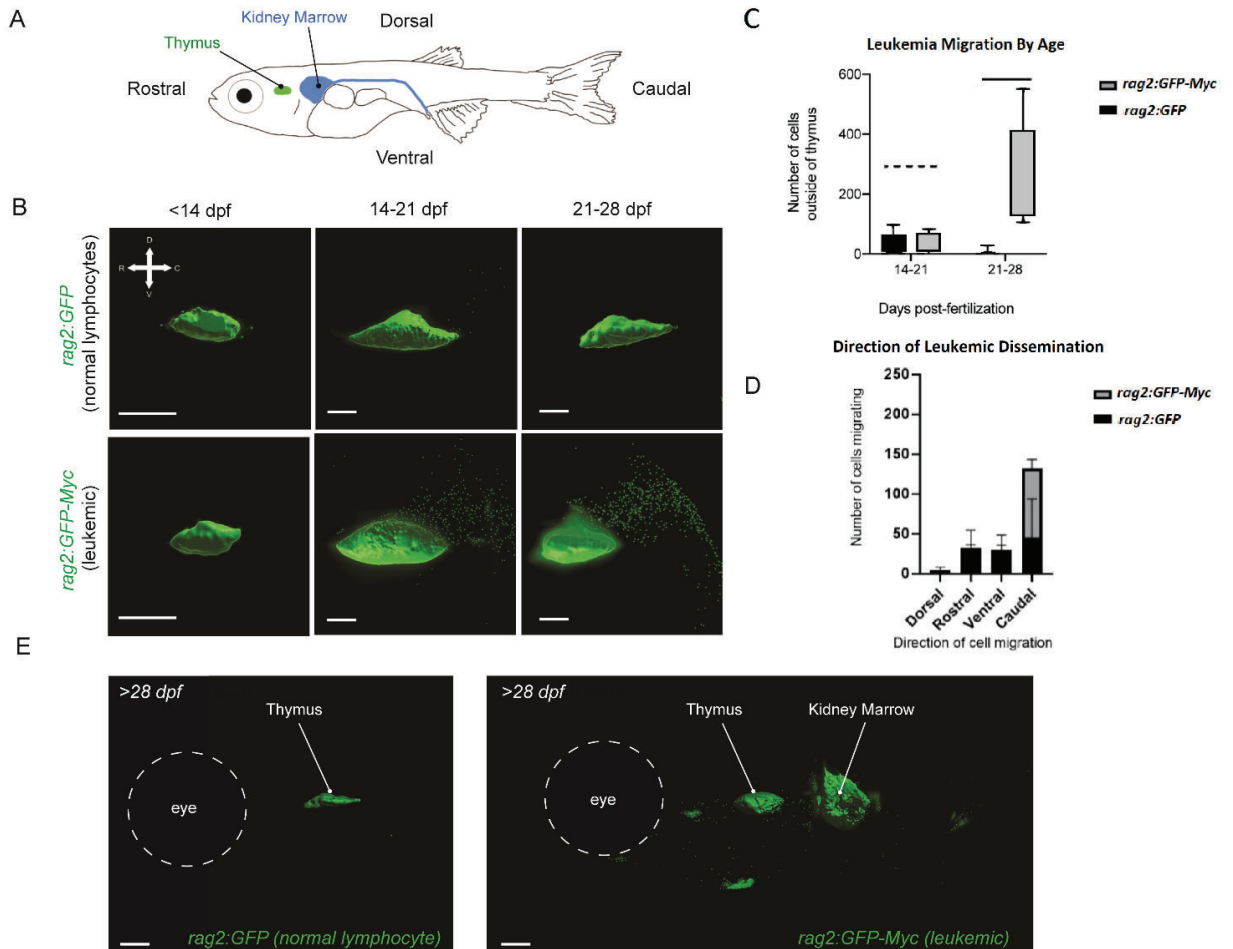


Figure 1: Leukemia Cell Dissemination: Using lightsheet microscopy and Imaris imaging analysis, lymphocyte migration was calculated as lymphocytes left the thymus between leukemic and non-leukemic fish with either a *rag2:GFP* (Wild Type) or *rag2:GFP-Myc* (leukemic) cell marker. **A)** Animation for orientation of the fish. **B)** Representative images of cell dissemination from the thymus displaying cell count significantly increased in *rag2:GFP-Myc* (leukemic) fish compared to non-leukemic (*rag2:GFP*) fish. **C)** Significantly more cells migrate in older leukemic (*rag2:GFP-Myc*) fish when compared to younger leukemic fish and non-leukemic (*rag2:GFP*) at any age. (Solid line: $p < 0.05$ dashed line: n.s.) **D)** Dissemination was also shown to occur in a predictable pattern, with most cells exiting caudally with great significance in Leukemic fish (*rag2:GFP-Myc*) ($p < 0.05$), but pattern of dissemination was not significant (n.s.) in non-leukemic (*rag2:GFP*) fish. **E)** The pattern of dissemination appears to migrate caudally, toward the kidney head, seen in this graphic.

VEGF Heat Shock Increases Dissemination

As Figure 1C indicates, leukemic fish experienced greater cell dissemination than non-leukemic fish as the fish age. Since the effect can be induced in greater number under the VEGF heat shock promoter (hspVEGF+) (Figure 2C) than without VEGF transgene (hspVEGF-) (Figure 2C), I can conclude the presence of VEGF protein provides a possible driver to heightened leukemic dissemination, though other proteins, including the fish's endogenous VEGF, which appear to already be contributing to lymphocyte dissemination. The dissemination of lymphocytes is simply lower in number prior to heat shock, and greater in number in fish that possess the VEGF transgene. I speculate that since we see a similar uptick in dissemination between among heat shocked fish with a VEGF transgene (hspVEGF+) and the RNA analysis provided by the rtPCR data, the progression is at least in part driven by VEGF (Figure 2G). We see increased lymphocyte spread from the thymus in the leukemic fish under the heat shock than the control fish under the heat shock (Figures 2D, 2E and 2F). The spread is significantly more caudal, but does have a non-significant, but trending dissemination dorsally as well (figure 2E). This implies that VEGF protein is certainly important to Leukemia trafficking, which is consistent with previous research (Dias, 2000), though the specific mechanism needs further exploration.

While the hspVEGF+ and hspVEGF- fish both experience significant cell migration caudally, cells migrate *en masse* after a heat shock, at which time dorsal migration also becomes significant in only the VEGF+ fish with the transgene ($p < 0.05$) (Figure 2E). Heat shock induced more cell exodus overall in the transgenic (VEGF+) fish, though the trend was slightly above a p value of 0.05 ($0.05 < p < 0.1$). This indicates that an endogenous mechanism of cell migration may be multifaceted but can be amplified with VEGF heat shock.

Since there is increased expression in VEGF protein expression and VEGF plays a role in angiogenesis, this experiment wanted to assess the level of VEGF-Receptor (VEGFR) expression in a selected area of the zebrafish. While levels of VEGFR do not bear significant difference between leukemic and non-leukemic samples, a follow up real time PCR (Figure 2G) indicated the VEGF protein was overexpressed in leukemic fish with a heat shock promoter after the promoter was activated.

Figure 2: Patterned Lymphocyte Migration

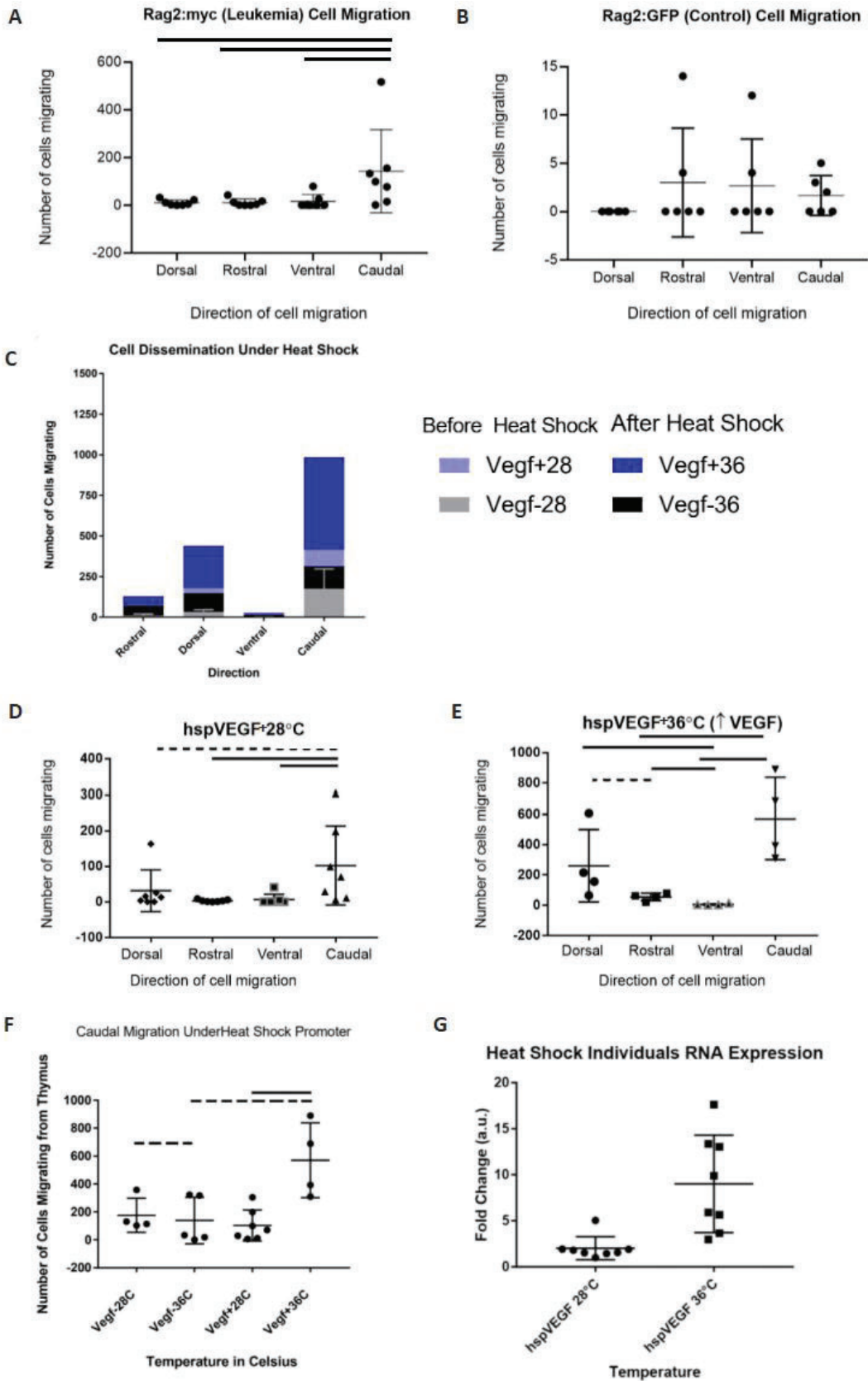


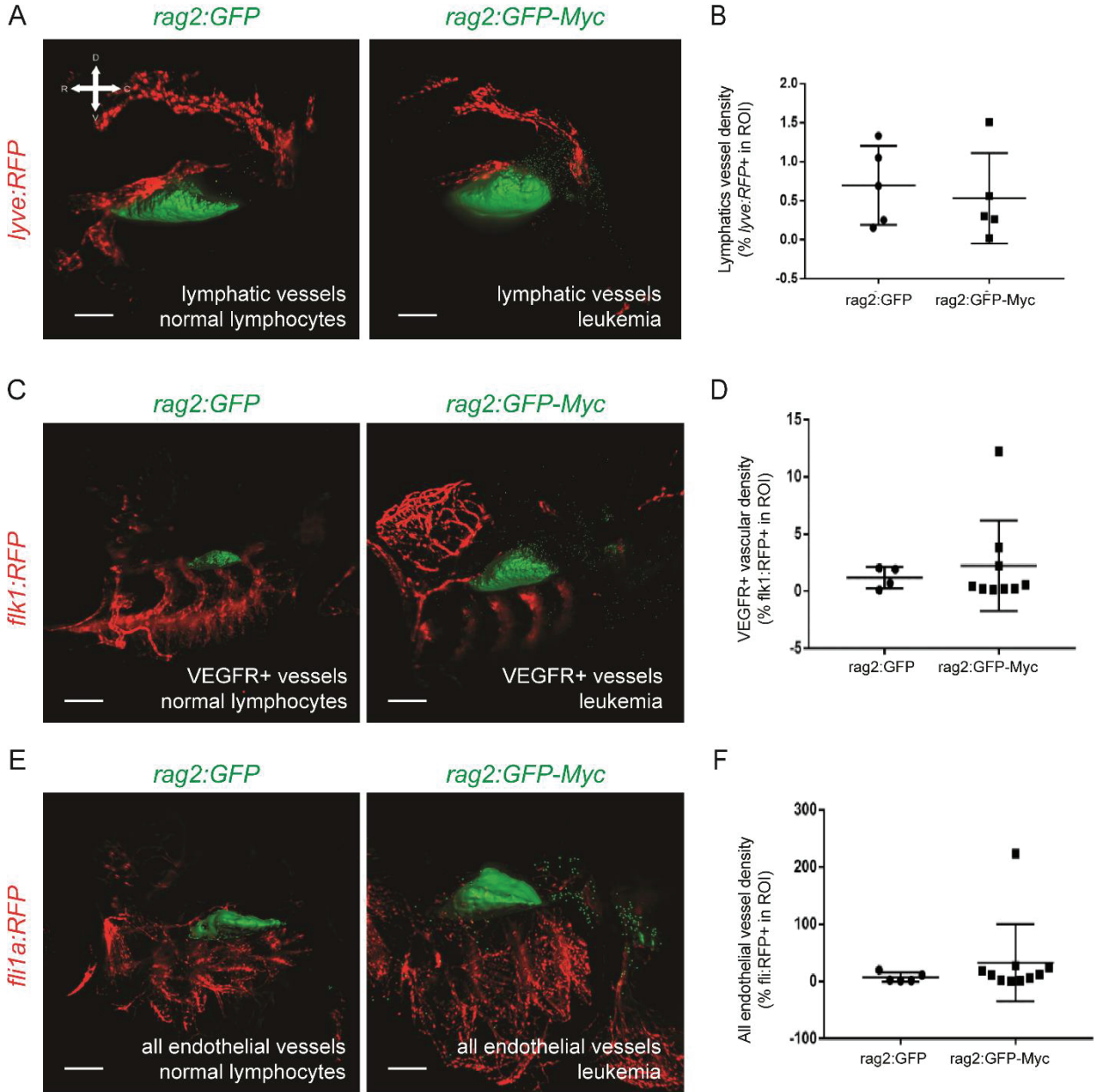
Figure 2: Patterned Lymphocyte Migration: Using light sheet microscopy and Imaris imaging analysis, the direction of lymphocyte migration was calculated as the lymphocytes left the thymus. **A)** Leukemic (*rag2:GFP-Myc*) fish dissemination was most significant caudally ($p < 0.001$ between caudal and all other directions). **B)** Non-leukemic (*rag2:GFP*) fish cell migration did not have a significant direction of cell dissemination (n.s.). **C)** Overlay of heat shocked fish, possessing the VEGF transgene (*hspVegfA,rag2:GFP-Myc, flk:RFP*) (blue), and without the transgene (*hspVegfA,rag2:GFP, flk:RFP*) (grayscale,) showed an increase in caudal and rostral cell dissemination for both transgenic and non-transgenic but the amount of cells migrating caudally in the VEGF+ transgenic fish trends much greater than the VEGF- fish, without the transgene after heat shock ($0.05 < p < 0.1$). **D)** Non-heat shocked VEGF+ fish (*hspVegfA,rag2:GFP-Myc, flk:RFP*) began to undergo caudal cell migration at a significant rate when compared to ventral and rostral directions ($p < 0.05$ for both), with dorsal migration trending similarly ($0.05 < p < 0.1$). **E)** Heat shocked VEGF+ (*hspVegfA,rag2:GFP-Myc, flk:RFP*) fish experienced significant cell dissemination caudally, compared to ventral and rostral ($p < 0.05$ both prior to and after heat shock), but only after heat shock does dorsal migration become significant when compared to ventral and rostral ($p < 0.05$ for both). **F)** Caudal cell migration differences between transgenic and non-transgenic fish indicated the greatest significance between the VEGF+ (transgenic) fish after heat shock ($p < 0.01$), but there was not significant difference between the VEGF+ and VEGF- fish after heat shock, despite trending ($0.05 < p < 0.1$), nor was there significance between the non- transgenic VEGF- before and after heat shock (n.s.) **G)** qPCR quantification (derived from cDNA made from RNA) of VEGF gene expression validated significant uptick in expression of the VEGF gene after the heat shock, but the same significance did not exist in the fish lacking the VEGF gene ($p < 0.05$)

No Significant Evidence of Vascular Remodeling

The second point to my hypothesis was that as leukemia expands outward from the thymus, it would recruit and remodel the vasculature of the local environment. Vessel density among leukemia positive specimens is of interest because tumorous tissue develops in hypoxic niches. This experiment sought to assess the role of vessel formation in leukemia to see if it was similar to that found in solid tumors. Looking at fish with red fluoresced lymphatics (Figure 3A and 3B), general endothelial (Figure 3C and 3D) and endothelial vasculature bearing a VEGF-receptor marker (Figure 3E and 3F), I compared leukemic and non-leukemic fish to see if the vascular density in the area surrounding the thymus was being remodeled. Fundamentally, I was treating the thymus like a solid tumor. Additionally, I looked at co-localization between leukemic cells and their vasculature by quantifying their overlap (Figure 3G).

I saw no evidence of co-localization between any vessel line and the lymphocytes (Figure 3G). In analyzing a region of interest twice the length of the thymus all the way around (and limited to the boundaries within the fish itself) I saw that vascular change during leukemic progression was not quite significant among any vessel type. Figures 3A,3B and 3C show that no matter the vasculature type, the vessel density within the region of interest was not significantly remodeled when compared to the control of the same vessel type. In fact, vessel densities varied widely between animals. Flk is vasculature bearing a VEGF receptor (VEGFR) (Figure 3C and 3D). I was expecting the Flk (VEGFR+) vasculature to undergo the most remodeling, since we understand that VEGF presence creates the optimum environment to increase leukemia dissemination, but as Figure 3D shows, the vessel density of VEGFR-2 tagged vasculature (Flk) is not significantly changed during leukemia migration. This allows us to conclude that unlike solid tumor formation, VEGF is not playing a role in angiogenesis and vasculature remodeling in leukemic zebrafish; it might be playing a different role, which needs further research.

Figure 3: Vessel Density and Distance



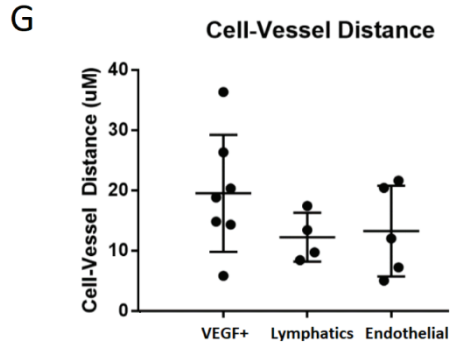


Figure 3: Vessel Density and Distance: Using light sheet microscopy and Imaris imaging analysis, a region of interest surrounding the thymus was taken. The percent of the ROI taken up by vessels was calculated and compared between Leukemic (*rag2:GFP-Myc*) and non-leukemic (*rag2:GFP*) fish. **A**) Representative imagery of Lymphatics (*Lyve:RFP*) vasculature with **B**) graphic analysis of lymphatics vasculature (n.s.). **C**) Representative imagery of VEGFR+ vasculature with **D**) graphic analysis of VEGFR+ vasculature (n.s.). **E**) Representative imagery of Endothelial (*Fli1a:RFP*) vasculature with **F**) graphic analysis of endothelial vasculature (*Fli1a:RFP*) (n.s.). **G**) Graphic analysis of co-localization was achieved using FIJI imaging software, in which co-localization of the vessels (red) and the leukemic cells (green) would have been represented in yellow, which indicated leukemic cells are not significantly close to any vasculature as (see figures 2A, 2C and 2C) (n.s.).

Thymus Volume Does Not Induce Leukemic Dissemination

Initially I hypothesized that as leukemia progressed, I would see an increase in thymus volume but the increase in thymus volume did not appear to associate significantly with leukemia progression (Figure 4). Any difference in volume could be attributed to age, which is why we see such large ranges for each subtype in Figure 4. There was a difference in thymus volume between 14dpf fish and 28 dpf fish within the same subgroup (within just leukemic fish for example), but there was no significant difference between the thymus volume of control and leukemic fish. This runs contrary to the current knowledge that leukemogenesis may evolve from a single cell dividing in the thymus into a mass of leukemic cells that push the thymic boundaries and enter the interstitial space (Hess et al., 2012). If that were the case, I would see random selection as regards to which side of the thymus experienced eruption of the leukemic cells first, but as previously explored in Figure 1 and 2, the data indicates that leukemia progression is largely predictable.

It can be noted that the Fli thymus volume has a p value that is very close to being significant ($0.05 < p < 0.1$). This is important because Fli vasculature do not wholly overlap with VEGF-R tagged (Flk) vasculature, so the driver of this trend may be something other than VEGF entirely. Further investigation is needed.

Not surprisingly, our VEGF heat shock experiment (hspVEGF) follows the trends set by the fli, flk and lyve assessments of thymus volumes. Looking at Figure 4D we see no significance in the volume of the thymus whether leukemic or control after heat shock. While both the leukemic and control experienced a change in thymus size due to the heat itself, the difference between them is non-significant and implies that VEGF did not play a role in inducing the thymus volume change.

Figure 4a represents the reality of what I see when I analyze and process the fluorescence imagery. Not only do we see how the leukemic cells exit caudally from the thymus, we see in 4b that no cells overlap or travel along any vasculature type. Since the thymocytes are green fluoresced and the vessels are red fluoresced, overlap will appear as yellow fluorescence (this is done automatically during imaging), but no such overlap occurs. This contests the theory of migration along vessels that had previously been established (Langenau et al., 2003)

Thymus volume appears to increase in size regardless of whether the fish has the VEGF gene or does not have it. The presence of VEGF does not appear to enable a significant difference in thymus size; the heat shock alone appears to be the contributing factor. Between VEGF+28 and VEGF+ 36 $p < 0.05$, and between VEGF-28 and VEGF-36 $p < 0.01$, indicating it is significant regardless, only heat matters. There is no significant change in size when both VEGF- and VEGF+ are at 28 Celsius and no difference between VEGF- and VEGF+ at 36 Celsius either.

Figure 4: Thymus Volume

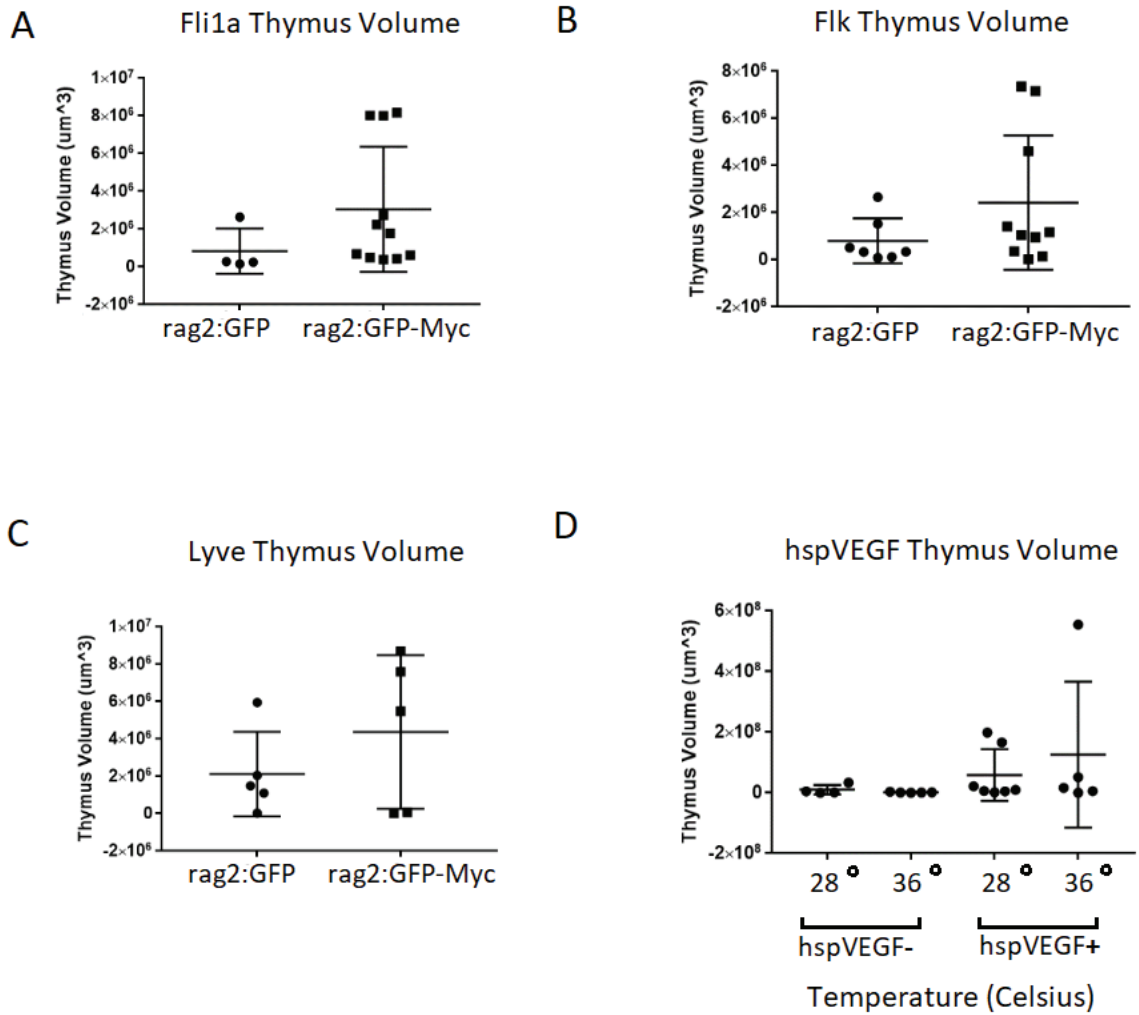


Figure 4: Thymus Volume: Using light sheet microscopy and Imaris imaging analysis, the thymus volume was calculated in its entirety between non-leukemic (*rag2:GFP*) and leukemic (*rag2:GFP-Myc*) fish with a **A**) *Fli1a:RFP* endothelial cell marker, **B**) *Flk:RFP* VEGF-receptor marker, **C**) *Lyve:RFP* Lymphatics marker and **D**) Heat shocked VEGF marker. There was no significance between the leukemic and non-leukemic thymuses in any line. P>0.05 for all (n.s.).

Discussion

Using a well-established line of zebrafish bearing Myc-induced T-cell leukemia (Langenau, Traver et al. 2003), we visualize the initial expansion of leukemia from the thymus in the context of the vascular microenvironment and enhanced VEGF expression. I compared these *rag2:GFP-Myc* transgenic, (leukemic) fish to a *rag2:GFP* control (non-leukemic) model. Both fish lines were outcrossed to a Casper strain, which is a mutation of the *mitfa* gene that causes a complete lack of melanocytes, so the fish's scales are somewhat transparent (White, Sessa et al. 2008). This allowed me to better visualize the fluorescence via light sheet microscopy. With the goal of understanding the role of Vascular Endothelial Growth Factor in angiogenesis during leukemogenesis, I identified a step-wise onset of leukemic cell migration from the caudal tip of the thymus (Figure 1 and Figure 2). Leukemia progression was not associated with any vascular remodeling, and leukemia cells did not migrate along particular vascular tracks (Figure 3). Leukemia progression was not associated with any change in thymus size, which contests earlier studies that the reason for sudden cell migration was that clonal expansion within the thymus breached the limits of the thymus (Hess et al., 2012). However, VEGF significantly accelerated leukemia progression from the caudal tip of the thymus along a predictable path toward the kidney head, indicating that VEGF may be acting as a signaling molecule directly impacting leukemia cells (Figure 2).

Our means of zebrafish husbandry required successive generations of pairing from Myc positive fish, which means that the onset of leukemia had to be later, with slow leukemia formation and spread, as the animals are maintained in a manner that enables them to reach breeding age. This may mean that my study does not have good representation of acute lymphoblastic leukemia, which would likely have killed afflicted fish prior to making it to adulthood. This slow progression bears a resemblance to chronic lymphocytic leukemia in mammals (Finola et al, 2012). The spread into the caudal area proximal to the thymus and kidney head is consistent with prior literature (Finola et al, 2012, Blackburn et al 2012). I chose to examine the microenvironment surrounding the thymus because the pattern leukemic fish undergo during cell dissemination was relatively linear (Figures 1 and 2) and I likened the thymus to a solid tumor and observed to see if it, or the leukemic cells that composed it, would remodel their surroundings in the same way.

I looked at lymphatic (Lyve), all endothelial (Fl1a) and VEGFR-tagged endothelial vasculature, though if I had been more thorough I would have analyzed other linear tissue, (such as neural tissue) but I will explore the latter briefly. The direction of cell migration does not appear to be influenced by any endothelial or lymphatics vasculature (Figure 3). This contrasts findings of earlier studies that indicate in acute lymphoblastic leukemia cells, which show an initial random migration of leukemic cells from the thymus, followed by travel along blood vessels to the kidney head (Langenau, Traver et al 2003). I further analyzed co-localization of cells and vasculature, looking for overlap, but did not find any significance (Figure 3G).

While I did not observe T-cells following along any vasculature, it is important to note that the zebrafish used in this study were F2 generation as opposed to the F0 generation in the Langenau study, which means the onset of leukemia had to be slower to enable successive generations and thus maybe more akin to chronic lymphoblastic leukemia (CLL) than the acute lymphoblastic leukemia (ALL) that appear in previous studies. The path of dissemination may vary for this reason. Of course, it is possible that since ALL is so acute, patterns of dissemination are difficult to track and the slow progression in my experiment allows time for tracking subtlety that would otherwise be missed. Variance from the previous study may also be the result of an amplifying, synergistic effect between the endogenous myc of the zebrafish and its mouse derived Myc transgene. Having two copies of the gene might influence leukemic progression. Nevertheless, the ultimate dissemination pattern resulted in the same destination for the lymphocytes as mentioned in previous literature; caudal migration appears to be heading toward the kidney (Figures 1 and 2).

Dorsal migration also appears to have some significance, though to a much less significant degree than caudal migration (Figure 2E). This may be due to my own imperfect measuring the sections of the fish. Looking at Figure 1B and 1E cell dissemination is in a somewhat dorsal-caudal pattern, but in my to avoid counting the same cells twice, I had to make a very hard cut off for what qualified as dorsal and what qualified as caudal. I could have simply divided the fish into caudal and rostral halves, but four directions more accurately represented a three-dimensional animal. Nonetheless, whether the path of dissemination is distinctly caudal or somewhat dorsal, vessels were not the tract along which the cells were traveling (Figure 3G).

If vessels are not the track leukemic cells follow, I speculated that something else about the thymus might induce the leukemic migration. Keeping with the theory that T-cell clonal expansion was the culprit, I observed the thymus volume. I found the thymus increased in size significantly whether the fish had leukemia or not (Figure 4). It seemed increase in thymus volume was a function of growth over time. It is worth noting the Fli1a fish line was very close to significance, but it fell short. It could be anomalous, or there could be an increase in the thymus of those Fli1a fish, but perhaps more research is needed.

With vascular remodeling and changes to the thymus not fully explaining what summoned the thymocytes from the thymus, there may be some kind of chemoattractant gradient stemming from an unseen source that I have not yet measured. Studies in mice identify VEGF receptor (VEGF) on the surface of leukemic cells. Furthermore, increased human VEGF protein appears in mice xenografted with human leukemia (Dias et al., 2000). Murine Vegf is not elevated in these xenografted mice, indicating that it is the leukemic cells, themselves, that are the potential producer of the human VEGF protein, which may be acting in a paracrine or autocrine manner (Dias et al, 2000). The leukemia is potentially releasing VEGF protein, which in turn acts on either the cell itself or its neighboring cells. If I apply this logic to my experiment, its possible that leukemic cell migration is activated by the leukemia at a certain time, since we see that the *en masse* migration begins in older rag2:GFP-Myc fish (Figure 1C). This migration might be induced by the first cell(s) to turn on VEGF, which would explain why we see so much more dissemination caudally under a heat shock promoter for Vegf (Figure 2). During the heat shock experiment I noticed that lymphocyte migration did not begin *en masse* until after the heat shock (Figure 2F) when VEGF expression is upregulated (Figure 2G); it did not occur with any significance in the heat shocked control fish (Figure 2F). Increased intracellular VEGF expression is associated with poorer prognosis in chronic lymphoblastic leukemia and CLL was also found to have no significant change to the vasculature (Aguayo et al. , 2000). This supports my postulation that it is the leukemia itself using VEGF as a signal to migrate *en masse*. Investigation into why it migrates in such a linear fashion from the caudal tip of the thymus toward the kidney head needs further investigation.

Future Directions

Paracrine and autocrine signaling do not fully explain why the leukemic cells travel *en masse* along the same route of dissemination from the caudal tip of the thymus toward the

kidney head. If the leukemia is acting in a paracrine or autocrine manner, it could be activated at any place within the thymus and therefore disseminate from all sides. Non-leukemic fish experienced random dissemination patterns (Figure 2B), so it stands to reason that the thymocyte passage through the thymus cortex to medulla for maturation is not responsible for the caudal exodus point. It should be noted that leukemic cells are abnormal, so further investigation is needed to rule this out. I cannot rule out the kidney head as the chemoattractant secreting organ, but since the path of dissemination was limited only to the side of the organ proximal to the thymus, I propose its worth looking at what is special about what is between the two organs. Since the route between thymus and kidney head is such a linear tract, it is worth examining linear tracts of tissue. Since I have ruled out lymphatics and endothelial vasculature, including endothelial vasculature bearing the VEGF receptor, it might be worth looking into nervous tissue. Nervous tissue have linear tracts and the kidney head is highly innervated.

Looking at pathologies across central nervous tissue VEGF has been shown to be upregulated when cerebellar granule neurons (and astrocytes, to a lesser degree) are subjected to slightly hypoxic conditions. The study found the presence of VEGF bound to its receptor provided protection to the local neurons, but when unbound that protection is lost, further supporting the paracrine/autocrine hypothesis (Wick et al., 2002). Hypoxic tumors are also known to secrete VEGF in order to promote vascularization of the tumor in order to deliver oxygen and nutrients as the tumor expands (Casey et al., 2015). From this I might speculate that hypoxia could be playing a role to induce the VEGF signaling in leukemia, but further investigation is needed.

Previous studies on the effects of double stranded breaks and faulty repair mechanisms in *Rag2:Myc* murine model indicate that not only does V(D)J recombination enable leukemogenesis in lymphocyte progenitors, but also that there is a high incidence of comorbidity with severe neural pathology. While this study analyzed the phenomena in pro-B cell leukemia, it compared its findings to leptomeningeal leukemia and terminal acute lymphoblastic leukemia (Gladdy et al., 2003). Patients experience a poorer prognosis once the leukemia spreads to the central nervous system. Future research might elucidate the role hypoxic neurons might play in VEGF signaling. There may be a link to the leukemia trafficking we have seen, but I will have to look at hypoxia, VEGF levels and fluorescently tagged neurons.

Limitations of Experiment

One of the shortcomings in this set of experiments is the limitation on the number of animals included. While more than 50 animals were studied, significance could be vastly improved upon with more animals at specific age brackets, and more per fish line. In fact, the Fli vessel density was trending very close to significance that analysis of 5 or 10 more fish may have been able to slide the significance to be more representative of the reality. Animal studies are limited by time constraints. This study also had to contend with the fact that the microscopy and RNA extractions were terminal for all the fish.

In conclusion, consistent, predictable trafficking among *rag2:GFP-Myc* induced leukemia may be the result of some kind of microenvironment change that is influenced by VEGF. The trafficking does not appear related to nearby endothelial (including VEGFR-tagged) vasculature or lymphatics, but investigation into the roles of neuron VEGF secretion and hypoxia is needed as the trafficking is linear and predictable. I now hypothesize that VEGF protein and VEGFR-receptor are acting together in a paracrine/autocrine manner. The role of zebrafish as a model for leukemia can be further explored with further focus on other linear tissues, such as neuronal and more focus on molecular mechanisms in those tissues, particularly in adult fish, whose leukemia may be more akin to chronic lymphoblastic leukemia. Understanding these mechanisms may provide therapeutic drug targets that could be modified to benefit human patients.

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PUBLICATIONS

Pending submission of “Intravital imaging reveals VEGF-A signaling, but not vessel contact guidance, directs the early progression of lymphoid leukemia,” to Plos One. Collaboration with Sergei Revskoy, Lucas Tomko, Shea Hausman, Margaret Blaire and Jessica Blackburn.

PROFESSIONAL AFFILIATION

Zebrafish Disease Models Society, 2020- Present

University of Kentucky LGBTQ Advisory Committee 2020-Present