Establishing a Zebrafish Model for Venous Malformations

A Major Qualifying Project
Submitted to the Faculty of
WORCESTER POLYTECHNIC INSTITUTE
In partial fulfillment of the requirements for the
degree of Bachelor of Science in Biochemistry

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Date: April 27, 2023

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Abstract

Venous malformations are the most common type of vascular malformation. There is currently no cure and few treatment options for venous malformations. Because they can cause significant impacts on patient quality of life and cause complications leading to death, identifying potential therapeutics is extremely important. A common somatic mutation causing venous malformations is TIE2-L914F, which has the zebrafish homologue TIE2-L906F. In order to study venous malformations caused by this mutation, zebrafish embryo microinjections were utilized to create a stable transgenic zebrafish line by using a Cre-dependent FLEX approach for a conditionally activated allele. Although establishment of the stable transgenic line was successful, there was no phenotypic evidence in the F1 generation of venous malformations or other defects following activation of the transgene during embryonic development. Further analysis and troubleshooting is necessary to validate these results, and future experiments should include repeating the microinjections with variable doses of Cre recombinase for transgene activation, assaying to determine if the transgene is creating the desired effect on the TIE2 signaling pathway, and redesigning the transgenic construct.

Acknowledgements

I would like to thank Professor Destin Heilman for all his help and guidance throughout this project. He has been invaluable as a mentor, and he has been tremendously patient and supportive during this entire process.

I would like to thank Dr. Nathan Lawson for allowing me to have the incredible opportunity to complete my MQP in his lab at UMass Chan Medical School over the past year. I have learned so much and grown tremendously as a scientist. I would also like to thank Dr. Sarah Oikemus for her mentorship and assistance for the duration of the project, which is what made this project possible. Finally, I would like to thank the other lab members in the Lawson Lab, including Aliece Goodman, Amy Kolb, Dr. Masahiro Shin, Feston Idrizi, and Ryan Singer for their continual support.

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Introduction

Congenital vascular malformations are structural abnormalities resulting from errors during embryonic vascular morphogenesis.[1] The most prevalent type of congenital vascular malformations are venous malformations. These malformations are localized defects and are categorized as slow-flow vascular lesions.[2] These lesions are characterized by venous channels with enlarged endothelial lumen that are lined unevenly with endothelial cells, have disorganized extracellular matrices, and are surrounded by a thin, irregular, or absent smooth muscle wall.[1,2] Typically, venous malformations occur in the skin, mucosa, and subcutaneous tissue, although they may also appear in other structures such as internal organs, muscle, and bone.[2,3] The appearance of venous malformations can vary, but they generally appear as soft, compressible blue or purple lesions that vary in size and grow proportionately with the child.[2,3] Venous malformations may cause significant disfigurement, pain, impact organ function, spontaneously thrombose, and cause complications leading to death (Figure 1).[2] Currently, there are limited options for treatment which include sclerotherapy and surgical resection, but these methods can be painful, invasive, and ineffective in the long-term.[4] For this reason, identifying potential therapeutic treatments is imperative.

Recent research has suggested that venous malformation pathogenesis is frequently associated with genetic mutations. Specifically, the majority of responsible mutations occur in the endothelial tyrosine kinase receptor TIE2/TEK gene.[4] TIE2 plays an integral role in the PI3K/AKT/mTOR signal transduction pathway; in addition to its roles in cell cycle regulation, is also involved in regulation of angiogenesis and vascular tone.[5] The TIE2 gene is involved in a variety of processes and has important roles in angiogenesis, maintenance of vessel stability, and vascular quiescence, among other roles.

The mutations in the TIE2 gene resulting in venous malformations are nearly all caused by gain-of-function mutations. In sporadic venous malformations, the most common mutation is the somatic mutation L914F. Generally, upon binding of angiopoietin-1 to TIE2 in endothelial cells, increased activation of AKT in the PI3K/AKT/mTOR pathway occurs, resulting in reduced PDGF-B (platelet-derived growth factor-B) levels[1,6]. In vitro studies demonstrated that all venous malformation-causing TIE2 mutations caused a ligand-independent hyperphosphorylation of TIE2.[7] This constitutive overactivation causes significantly decreased PDGF-B levels causing irregular and/or absent smooth muscle cells surrounding the venous channel.[1]

Ligand-independent phosphorylation of TIE2 is highest in the L914F mutation and occurs most frequently of any somatic mutation, making it a good target for research. A recent study utilized the TIE2-L914F mutation to create a venous malformation model using human umbilical vein endothelial cells expressing the mutation and injecting them into mice. Upon successful establishment of the model, the efficacy of rapamycin as a therapeutic to treat venous malformations was also demonstrated in the mouse model as well as in preliminary clinical trial.[8] In this trial, rapamycin exhibits strong potential as a therapeutic to treat venous malformations since administration of the drug decreased lesion growth. Although rapamycin treatment was efficacious, there are many possible side effects and the long-term effects are unknown, so further testing is necessary to understand how it affects venous malformations.[8] Therefore, further investigation of venous malformations resulting from the L914F mutation in model organisms is necessary to conduct research on both the venous malformations themselves and potential therapeutics. While the previous TIE2-L914F was the first of its kind in creating a

human venous malformation model in mice, this study aims to do the same in zebrafish to further current understanding of these venous malformations and potential therapeutics.

The zebrafish is an ideal model organism to study vascular disease. Zebrafish develop extremely quickly and externally, and their embryos are easily manipulated. In addition, their embryos and larvae are optically transparent, making imaging vasculature anywhere in the developing zebrafish possible. This allows for imaging of vasculature in zebrafish with fluorescently-tagged vessels or structures possible in transgenic fish.[9] The zebrafish circulatory system is also highly conserved compared to humans. Although it is much simpler, they also have a closed circulatory system, a heart with the same specialized cell types, structures, and signaling pathways. Vasculature between zebrafish and other mammals is also highly conserved, including vasculogenesis, angiogenesis, vascular remodeling, and signaling pathways.[10] A critical advantage of using a zebrafish model is that the zebrafish embryo receives oxygen via passive diffusion from the water throughout the first several days of development, allowing lethal mutant phenotypes and other experimentation to be observed in vivo.[10]

Moreover, humans and zebrafish share many genetic similarities – 82% of all known human disease-related genes have zebrafish orthologues.[10] In humans, a missense mutation in the endothelial cell tyrosine kinase receptor TIE2 is known to cause venous malformations. Among somatic alleles, the L914F mutation causes the greatest increase in TIE2 activation.[11] In zebrafish, the homologous mutation is L906F, and the other amino acids in the region are highly conserved in comparison to the human allele. This makes the zebrafish an ideal model organism to study venous malformations caused by this mutation.

To create a transgenic zebrafish line bearing the TIE2-L906F mutation, the mutant allele must be silent in adult fish before they pass it on to their progeny, so they are not affected by

potential vascular deformities or other arising phenotypes. In order to achieve conditional expression of the TIE2-L906F transgene in zebrafish endothelial cells, a Cre-dependent FLEX approach is utilized by incorporating both loxP and lox5171 sites in the plasmid construct, flanking inverted exons in the TIE2 coding sequence where the L906F mutation is located. Cre recombinase can recognize these lox sites, and the inverted coding sequence between them will be flipped, correcting the inverted exons. The TIE2 coding sequence containing the mutation will remain in this orientation, allowing for transgene expression. Thus, transgene expression will only occur when Cre recombinase mRNA is co-injected along with the transgenic construct, and a nonfunctional transcript will be produced in the absence of Cre. This transgenic construct was injected into zebrafish embryos in order to generate zebrafish bearing the conditional allele.

After establishing a stable line of the TIE2-L906F transgenic zebrafish, founders for the next generation of transgenic fish were selected based on transmission rate of the transgene onto their progeny. Following several rounds of Cre recombinase injections into F1 generation embryos, no phenotypic evidence was observed indicating the presence of vascular defects as a result of transgene activation during embryonic development.

Materials and Methods

Plasmid Construction

The expression plasmid to generate a TIE2 transgenic zebrafish line was constructed by utilizing a multisite Gateway reaction. In designing the plasmid, the transgene sequence was flanked with two sets of lox sites: loxP and lox5171 (Figure 2). Due to the size of the transgene, the lox sites flanked an inverted cassette containing only some of the exons. The entire transgene was cloned into the "middle entry" plasmid to be used in the Gateway reaction. The middle entry plasmid, 5′ entry plasmid containing the actb2p promoter, 3′ entry plasmid containing sfGFP, and a destination plasmid containing a cryaa promoter driving EGFP expression (causing GFP lens expression) were all placed in a microcentrifuge tube overnight with LR Clonase II at room temperature according to Thermofisher Scientific's Multisite Gateway® Three Fragment Vector Construction Kit LR recombination protocol. The resulting plasmid was transformed into CC 10-bata NEB commercial *E. coli* cells using a standard transformation procedure. The transformation was plated on LB-amp agar plates and incubated overnight. The resulting colonies were inoculated in LB-amp media and cultured overnight. The cultures were spun down and their DNA was collected using a Qiaprep Spin Miniprep Kit following the manufacturer's Quick-start protocol. The identity of the plasmid was confirmed through diagnostic restriction enzyme digests and visualizing the results on an agarose gel (Figure 3).

After the DNA identity was confirmed, it was purified via phenol: chloroform purification. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the DNA in nuclease-free water. The mixture was vortexed until completely homogenized and subsequently spun down at top speed for two minutes. The aqueous layer was transferred to a new tube, an equal volume of chloroform was added, then the mixture was vortexed and

centrifuged for two minutes. The aqueous layer was transferred to a new tube, and the chloroform step was repeated. After transferring the aqueous layer again, 0.1 volumes of sodium acetate (pH 5.2) and two volumes of ice cold 100% ethanol was added to the tube. The mixture was vortexed and precipitated at -20°C overnight. Then, it was centrifuged at full speed at 4°C for thirty minutes. The supernatant was removed, and the pellet was washed with ice cold 70% ethanol, then centrifuged for 10 minutes. The wash step was repeated, all the ethanol was removed, the pellet was left to air dry, and was resuspended in nuclease-free water.

Transient Injections with zfCre-ERT2

The purified DNA construct was injected in zebrafish embryos at the one-cell stage with zfCre-ERT2 mRNA, which is zebrafish codon optimized Cre recombinase fused to a mutated estrogen receptor, making it inducible with tamoxifen (Figure 4). The injection mix contained 2.5µL of the 40ng/µL construct, 1µL 125ng/µL Tol1 transposase, 1µL 15.625ng/µL zfCre-ERT2 mRNA, 1µL phenol red, and 4.5µL nuclease-free water. Another injection mix was also utilized that did not contain any zfCre-ERT2. Each embryo was injected with 2nL of the injection mix directly into the one-cell, puncturing through the chorion and the yolk. The embryos were incubated at 28°C in a solution (blue egg water) containing methylene blue, salt, and RO water. The embryos injected with zfCre-ERT2 were treated with tamoxifen (1:2000 dilution) when they reached the 18-somite stage. At one day post fertilization, the embryos were scored for survival and mutations. Any dead embryos or embryos with mutant phenotypes (tail that is too short or too long, abnormally shaped head, etc.) were removed. At three days post-fertilization, the embryos were evaluated for GFP and cryG expression. The embryos injected with and without zfCre-ERT2 were scored for expression and sorted accordingly, then harvested.

To confirm the presence of the transgene in the injected embryos, RNA was isolated from the harvested embryos. This was accomplished by following the "tissues" procedure according to Invitrogen's TRIzol Reagent User Guide. Ten embryos were used for each sample, and they were dissolved in TRIzol using an electric homogenizer. The resulting RNA was purified via phenol: chloroform purification according to the same procedure used for the DNA purification. Glycogen was added during the ethanol precipitation to visualize the RNA pellet more easily. The purified RNA was used to synthesize cDNA by RT-PCR. Invitrogen's Superscript III First Strand RT-PCR Kit was used, and the manufacturer's protocol was followed. Then, the cDNA was used for a PCR using Kapa 2G HS Polymerase. The PCR included the embryo cDNA, primers, and nuclease-free water. Several sets of primers were utilized to verify the sequence at critical locations, such as splice junctions and the sfGFP cassette (Figure 5).

Following the cDNA PCR, the resulting PCR product was purified via gel extraction and purification. The PCR product was run on a 1% TAE gel and excised. The Zymoclean Gel DNA Recovery Kit was used according to the manufacturer's protocol. The PCR product was eluted in 8uL of nuclease-free water. All of the resulting volume was used in a blunting reaction, as Kapa 2G HS Polymerase makes sticky ends. The Thermofisher Scientific cloneJET PCR Cloning Kit was used for the following blunting and ligation reactions, and the manufacturer's protocol for the kit was followed. The PCR product was ligated with the pJet 1.2 cloning vector and T4 DNA ligase. The ligation reactions were transformed into TOP10 CC E. coli, according to standard transformation protocol. The transformation was plated on LB-amp agar plates and incubated overnight. The resulting colonies were screened using a colony PCR. At least eight colonies from each transformation were picked using a micropipette tip. The tip was scraped on the sides of labelled PCR tubes on ice while using aseptic technique. The pJet primers from the cloneJET

PCR Cloning Kit were used to create a master mix, along with Taq polymerase and 10X HM Buffer. The master mix was added to each tube containing the colonies. To identify potential positive clones, 5uL of the reaction was run on an agarose gel. Any clones with a fragment that looked like the correct size were sent out for sequencing.

Generating a Stable Transgenic Line

After obtaining positive sequencing results, the construct containing the transgene was injected into wild-type zebrafish embryos to create a TIE2 transgenic zebrafish line. The same injection mix and protocol was utilized, except no zfCre-ERT2 was injected. The injected embryos were incubated at 28℃ in blue egg water. At one day post fertilization, dead embryos and embryos with mutant phenotypes were removed. At three days post-fertilization, the embryos were examined for cryG expression (Figure 6). The embryos with strong cryG expression were sorted out, and the rest were discarded. The remaining cryG+ embryos were incubated and placed in a fish tank with water at five days post-fertilization. At three months of age, the transgenic fish reached maturity.

Screening for Founders

Each transgenic fish grown to maturity was individually screened to determine if it could be used as a founder for the next generation. Each transgenic fish was crossed with a transgenic fish, and the resulting clutch of embryos was screened at three days post-fertilization for cryG expression. Those fish that transmitted the transgene onto the next generation, which was identified by the presence of cryG+ embryos, were identified as founders. Fish that did not

transmit the transgene at all or transmitted it at a rate lower than 5% were euthanized, and the rest were kept for future experiments (Figure 7).

zfCre Injections for Transgene Activation

Identified founders were out-crossed to wild-type zebrafish and their embryos were collected. Microinjections using zfCre mRNA (zebrafish codon optimized Cre recombinase mRNA) were performed in order to activate the TIE2 transgene in the embryos that inherited the gene. The injection mix contained 2.5µL of 5.25 ng/µL zfCre mRNA, 1µL phenol red, and 6.5µL nuclease-free water, and each embryo was injected with 2nL of the injection mix directly into the one-cell. Half of the collected embryos from each cross were injected, while the other half were left uninjected for use as a control. Embryos were evaluated each day until five days post-fertilization for possible phenotypes such as hemorrhage, abnormal circulation, edema, vascular defects, etc. At three days post-fertilization, embryos were screened and separated based on presence of cryG expression (Figure 8). Larvae from each group (injected/cryG+, injected cryG-, uninjected cryG+, uninjected cryG-) from each founder were collected and RNA was isolated from each group. The RNA was used for RT-PCR to create cDNA, and the cDNA was used for cDNA PCR using the same primers flanking critical locations in the construct. cDNA PCR results were visualized on an agarose gel. The PCR product was used for shotgun cloning and PCR product from positive clones were sent out for sequencing.

Results

Plasmid Construction and Verification

The plasmid containing the TIE2 transgene was deliberately designed to incorporate several key elements to make establishing a stable transgenic line possible, as shown in Figure 2. To successfully create a conditional allele, the Cre-lox system was utilized. Two pairs of lox sites (loxP and lox5171) flanked an area of the TIE2 coding sequence where the mutation was located. The sequence was designed so this region would be inverted, and any transcript produced without first modifying the transcript would be nonfunctional. This would allow for zebrafish to carry the transgenic construct without causing transgene expression. In the presence of Cre recombinase, the lox sites are recognized by the enzyme and the inverted region between them is flipped into the correct orientation. The plasmid was also designed with a strong constitutive promoter driving transgene expression (actb2p) to ensure transgene expression would be robust. Another promoter, cryaa, was also included in the plasmid to drive EGFP expression in the lens of the fish. This was necessary to create as a visual marker for successful integration of the plasmid into the genome, as well as to differentiate between transgenic and non-transgenic fish.

To construct the desired plasmid containing the TIE2 transgene, LR recombination was performed. Utilizing LR recombination allowed for each desired component of the transgenic plasmid to be present in the necessary order for transgene expression. The components that were needed to make the transgenic construct existed within several other plasmids. A 5′ entry plasmid containing the actb2 promoter was used in conjunction with a 3′ entry plasmid, a middle entry plasmid containing the TIE2 transgene with lox sites, and a destination plasmid containing the cryaa promoter and EGFP cassette. This system allows for easy manipulation of elements in an expression plasmid. Each entry plasmid contained recombination sites which the enzyme in the

LR reaction could recognize, and the plasmids were cleaved at those locations. The corresponding recombination sites from each plasmid were recognized by the enzyme and the DNA fragments were covalently attached in the desired order, generating the desired plasmid.

The LR recombination reaction containing the transgenic expression clone was transformed into chemically competent *E. coli* and plated on LB-amp agar plates to ensure that only colonies with the plasmid, which contained an ampicillin resistance cassette, would thrive. After transformation of the plasmid, the DNA was collected from the resulting colonies and screened using a diagnostic restriction digest to confirm the presence of the correct, intact plasmid. Figure 3 illustrates the restriction digest using XhoI and XbaI to determine if the DNA collected from the transformation contained the entire plasmid with all of the intended features. The first lane of the gel in Figure 3 shows the undigested DNA, which did not run very far on the gel. This indicates that it is circular and very large, which is expected. The third lane of the gel has four distinct bands with sizes around 5.8kb, 3.8kb, 3.3kb, and 1.8kb. These were the expected band sizes for this digest, with the exception of the absence of another band around 220bp, but it is safe to assume this band is hard to visualize on a gel because of its small size. After confirming the bands observed on the gel matched those which were anticipated, it was safe to assume that the DNA collected was most likely the desired plasmid. After the DNA identity was confirmed, it was ready to be purified for future injections. Although the DNA isolated from the colonies using a mini prep was relatively pure, further purification was necessary to avoid lethality when injecting it into developing embryos.

Transient Injections with zfCre-ERT2

After the DNA was purified by phenol:chloroform purification, it was injected into wild-type zebrafish embryos at the one-cell stage, along with Tol1 transposase, which is responsible for inserting the plasmid into the genome (Figure 4). Injecting at the one-cell stage allows for the greatest chance of efficient integration into the genome. Half the embryos were also injected with zfCre-ERT2 mRNA, and half of the zfCre-ERT2 injected embryos were treated with tamoxifen at the 18-somite stage. zfCre-ERT2 is zebrafish codon-optimized Cre recombinase that is fused to a mutated estrogen receptor that binds tamoxifen with a very high affinity.[12] Because it is fused to a mutated estrogen receptor, it is sequestered in the cytoplasm until tamoxifen is introduced, at which point it translocates to the nucleus and proceeds to carry out recombination.[12] Once in the nucleus, Cre recombinase can recognize the lox sites flanking the transgene in these embryos. One pair of lox sites is first recognized by Cre, and the region between the sites is inverted. This first inversion causes two identical lox sites to form direct repeats on one side of the inverted region. This causes Cre to excise the segment between the direct repeats, leaving only one lox site behind on either side of the inverted region. After this occurs, the flanked region will remain in the correct orientation and transgene expression is activated. By injecting only half the embryos with Cre and treating only half of the Cre-injected embryos with tamoxifen, this allowed for utilization of a control group that was injected with Cre-ERT2 but still had the inactive transgene.

Embryos from each group were evaluated for cryG and GFP expression. These embryos were identified by examining them under a fluorescent microscope using a GFP filter. Those embryos with cryG expression could be identified by the lens in their eyes glowing bright green due to GFP localization. Embryos with GFP expression were examined in the same manner but

were identified by somatic GFP expression. Although there was minimal GFP expression observed, cryG expression was present in many Cre-injected embryos.

Confirmation of Successful Plasmid Integration

Embryos with and without cryG expression from each population were harvested for RNA collection. Embryos with cryG expression indicated possible successful integration of the injected plasmid, as the cryaa promoter included in the construct drives EGFP lens expression. The embryos were dissolved, and RNA was isolated from them. The resulting isolated RNA from each population of embryos was used to synthesize cDNA via RT-PCR. Because cDNA is produced from the RNA template, it should lack introns, regulatory elements, and in the presence of Cre, the transgene should be inverted. This cDNA was utilized for several PCR reactions using primers anchored in critical locations in the plasmid sequence, such as splice junctions. The PCR product was visualized on a gel to confirm the amplification of these fragments occurred and were approximately the correct size. Primers for PCR were designed with one primer inside the inverted region of the construct. Without inversion of this region, there would be two forward primers, and amplification would not occur. However, if inversion occurred and the inverted region flipped, the primer anchored in this region would become a reverse primer, allowing for amplification. Amplification occurred only in the population injected with zfCre-ERT2 and treated with tamoxifen, indicating that only this population contained the active transgene with the inverted region flipped into the correct orientation (Figure 5). The presence of a strong band in only the PCR product from cryG+ embryos injected with Cre and treated with tamoxifen is indicative of inversion occurring. To verify whether inversion of the coding region flanked by dual lox sites was successful and if the transcript was properly spliced in the

population treated with tamoxifen, the PCR product was used for shotgun cloning. Thus, the PCR product from this gel was gel purified, blunted and ligated, and cloned into a plasmid vector to be transformed into *E.coli*. The resulting colonies were screened and potential positive clones, indicated by correct PCR fragment size visualized on a gel, were sent out for sequencing. Alignment of sequence results with the transgene plasmid map revealed a positive match in some of the clones, indicating the transgene sequence inverted into the desired orientation upon tamoxifen treatment. These sequencing results served to confirm that the construct was properly integrated, spliced, and inverted when appropriate. Obtaining these positive sequence results was an important milestone because the plasmid design worked as intended, and therefore was ready to be injected into embryos that would mature into adulthood and serve as the first generation of the transgenic line.

Generating a Stable Transgenic Line

After confirmation of successful plasmid integration into the zebrafish genome, a stable transgenic line of zebrafish could be established. To establish a stable transgenic line, wild-type embryos were injected with the construct and Tol1 transposase, without any zfCre-ERT2. This allowed the inactive transgene to be integrated into the genome without causing lethality and allowing the fish to transmit the gene to their progeny. Injected embryos were screened at three days post fertilization and those that were positive for cryG were separated and allowed to develop into adulthood (Figure 6). These fish were selected because the presence of cryG expression is indicative of integration of the transgenic construct into the genome. After the fish reached maturity at nearly three months of age, potential founders for future generations and screening could be identified.

Screening for Founders

To identify if each fish was a founder, and thus if they were transmitting the transgene to their progeny, each fish was outcrossed individually to a wild-type fish. Each clutch of embryos was collected and grown until three days post-fertilization, then evaluated for cryG expression. Embryos with cryG expression indicated that the transgene was being transmitted to the next generation, and these fish were identified as founders (Figure 6). More than thirty fish were identified as potential founders, and twelve of those fish had transmission rates greater than 5% (Figure 7). Because it would not be feasible for fish with transmission rates lower than 5% to produce enough embryos with the transgene, they were euthanized. The twelve founders with higher transmission rates would be used for further experiments.

Transgene Activation

Several of the identified founders were out-crossed to wild-type fish, and the resulting embryos were injected with zfCre mRNA for transgene activation. Embryos were evaluated every day until five days post-fertilization for potential phenotypes such as hemorrhage, abnormal circulation, edema, and other vascular deformities. Both injected and uninjected embryos were evaluated to differentiate between phenotypes arising from the activated transgene and normal phenotypes. At three days post-fertilization, embryos were screened and sorted based on cryG expression in order to differentiate between those embryos with and without the transgene in both injected and uninjected pools. There were no obvious phenotypes that were prevalent amongst the injected, cryG+ embryos that should have the activated transgene when compared to the controls. Larvae collected from each group from each founder were used to

collect RNA which was used to make cDNA, which was subsequently used for cDNA PCR. The results of the cDNA PCR showed amplification only in injected embryos that were cryG+, confirming inversion of the floxed region of the transgene (Figure 9). These are the same results as observed in the transient injections, and again confirmed that the transgenic construct was behaving as expected.

Discussion and Future Directions

Successful establishment of a TIE2 transgenic zebrafish line was achieved and founders for the next generation of transgenic zebrafish were identified as a result of these experiments. This is a critical step towards creating a zebrafish model for venous malformations resulting from the most common somatic mutation of TIE2 in humans.

Although there have not yet been any observed phenotypes prevalent among the embryos injected for transgene activation, this data is very preliminary and further testing is necessary to validate these findings. Results from cDNA PCR using RNA from zfCre injected embryos for transgene activation suggest recombination is occurring as expected, and thus a functional protein should be produced from the resulting transcript. There are many reasons why no phenotype has yet been observed. Further experiments should include doing injections for transgene activation on embryos from every founder and varying the dose of zfCre mRNA in embryo microinjections. It is possible that overall transgene expression is low in the founders that have been tested thus far, and a phenotype may be observed in founders with higher transgene expression. Variable splicing also may be occurring, which could result in not every transcript produced from the transgene generating a functional protein. If no phenotype is observed following further testing, protein assays may be performed to determine if activation of TIE2 is occurring. Additionally, the same experiments can be repeated using a redesigned construct in order to achieve a phenotype resulting from transgene activation.

After embryonic vascular phenotypes are achieved and characterized in TIE2 transgenic zebrafish, small molecule inhibitors with downstream targets of TIE2 should be administered to determine if the activated phenotype is being observed, and inhibitor efficacy as a treatment option should be explored.

Creating a successful model of human venous malformations in zebrafish would advance vascular disease research and understanding of venous malformations tremendously. The vast majority of venous malformations are caused by mutations in TIE2, and the majority of these are caused by the exact missense mutation used in this model. Developing a zebrafish model of venous malformations caused by the TIE2-L914F mutation would represent the only model of human venous malformations that allows for in vivo imaging of vasculature throughout embryogenesis, creating a unique opportunity to observe disease progression and vascular morphogenesis. Moreover, this exact genetic mutation is prevalent in patients with venous malformations. This model would be ideal for testing potential therapeutics to treat venous malformations, paving the way for identification of pharmacological treatments that could be life-changing for some individuals. Therefore, this research remains extremely relevant, as these defects are not well understood, and treatment options are limited and largely ineffective in the long term.

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Appendix of Figures

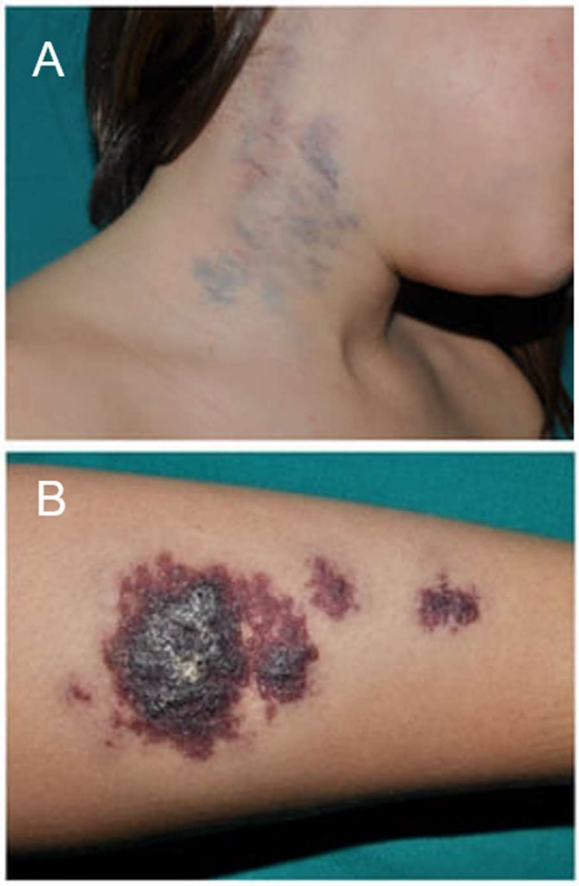

FIGURE 1. **Venous malformations**. *A*, venous malformation on the neck. *B*, verrucous venous malformations on the lower limb caused by a mutation downstream of the TIE2 signaling pathway.[2]

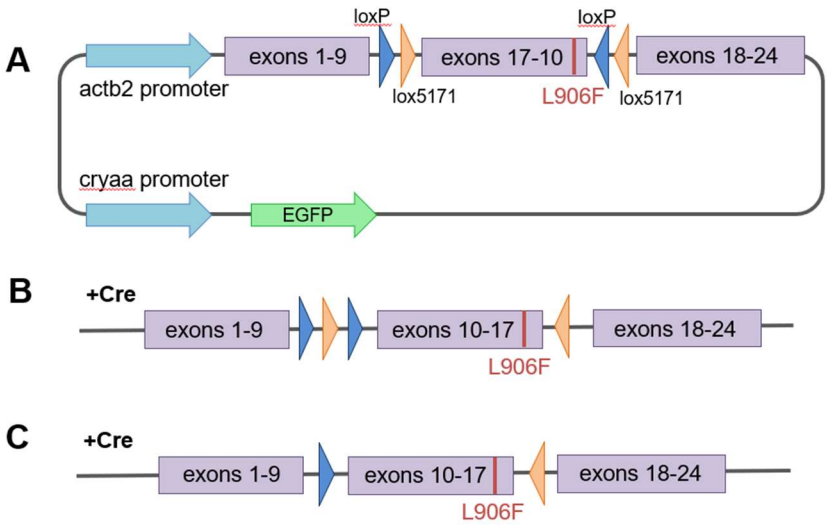

FIGURE 2. **Plasmid map containing TIE2 transgene**. *A*, This is a simplified schematic of the plasmid construct used to generate zebrafish bearing the L906F mutation. The actb2 promoter was used because it is a strong constitutive promoter that drives high levels of transgene expression. The cryaa promoter was included along with the coding sequence for EGFP to drive GFP lens expression to serve as a marker for successful integration of the plasmid into the genome and to differentiate between fish with and without the transgene. To allow for conditional allele activation, the Cre-lox system was utilized in building the construct. Two pairs of lox sites (loxP and lox5171) flank exons (exons 10-17) in the middle of the TIE2 gene where the mutation is located. Because the coding sequence for the TIE2 gene was long, only exons 10-17 were inverted and flanked by the lox sites to ensure recombination would be successful. *B*, In the presence of Cre recombinase, the inverted exons flip into the correct orientation. Because the pairs of lox sites are oriented as indirect repeats, one pair of lox sites are first inverted along with the transgene in the middle. *C*, The new direct repeat lox sites are excised, leaving only one of each loxP and lox5171 sites. The now inverted exons will remain in the correct orientation, allowing for expression of the transgene.

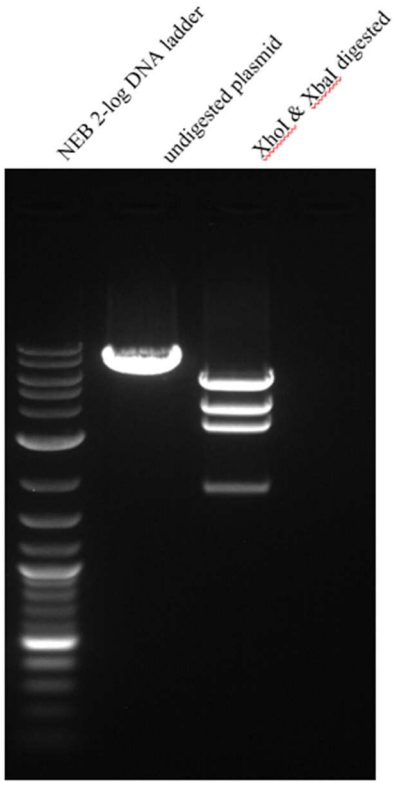

FIGURE 3. **Diagnostic restriction enzyme digest of plasmid**. LR recombination was utilized to generate the transgenic construct with the desired features. Multiple plasmids were used to create the construct including the actb2 promoter in the 5′ entry plasmid, the TIE2 transgene in the middle entry plasmid, and the cryaa promoter and EGFP in the destination plasmid. After the LR recombination was complete, the plasmid was transformed into chemically competent *E. coli*, and DNA from the resulting colonies was collected and purified. The identity of the plasmid was confirmed using diagnostic restriction enzyme digests. The restriction enzymes XhoI and XbaI were used and the resulting bands on the gel matched the expected sizes for the transgenic construct. After the identity of the plasmid was confirmed and the DNA was purified, it was ready to be injected into zebrafish embryos.

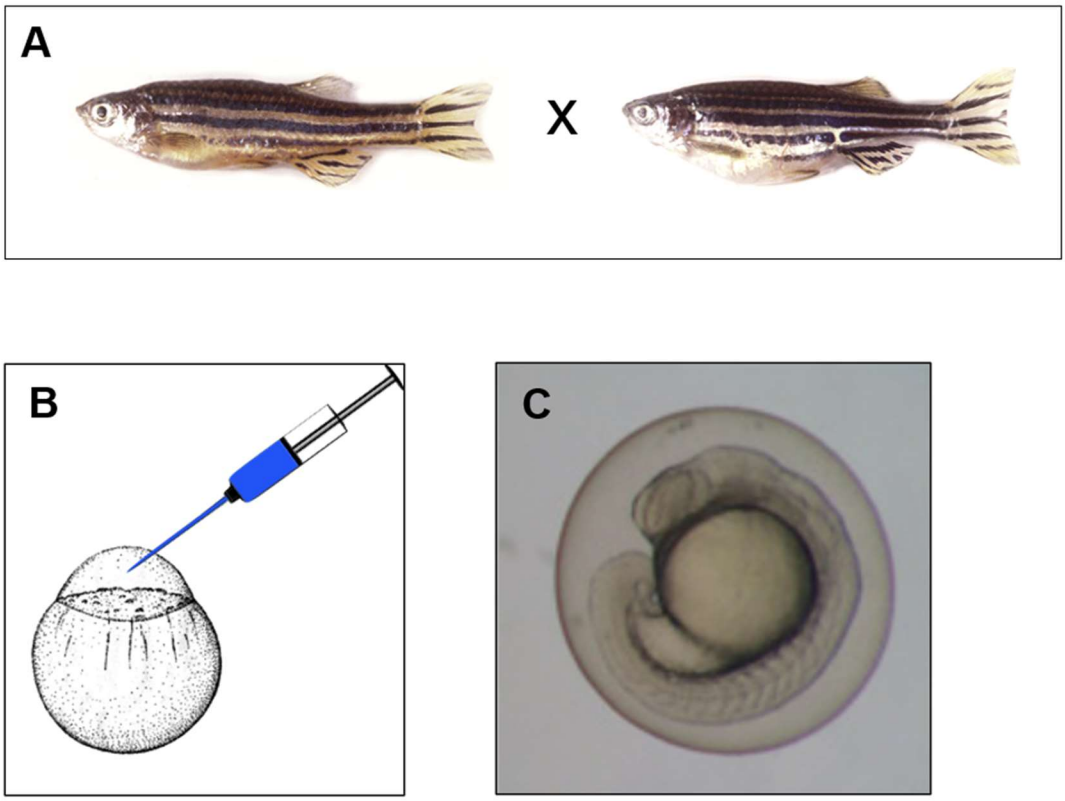

FIGURE 4. **Wild-type zebrafish in-cross for test injections**. A, To determine if the construct would successfully integrate and splice correctly, test injections were performed on embryos from wild-type in-crosses. The injections included zfCre-ERT2 mRNA, Tol1 transposase, and the TIE2 transgenic construct. Tol1 transposase allows for the integration of the construct into the genome, and zfCre-ERT2 is zebrafish codon-optimized Cre that is fused to a mutated estrogen receptor and can only be released into the nucleus in the presence of tamoxifen, a synthetic estrogen receptor antagonist. *B*, Embryos were injected during the one-cell stage of development, immediately after fertilization. Each embryo was injected through the chorion and yolk, directly into the one-cell. This allowed for the greatest chance of integration into the genome. *C*, At the 18-somite stage of development, half of the Cre-injected embryos were treated with tamoxifen to activate Cre recombinase.[13] At 3dpf, embryos were screened for cryG expression (expression of GFP in the lens) to determine if the construct integrated.

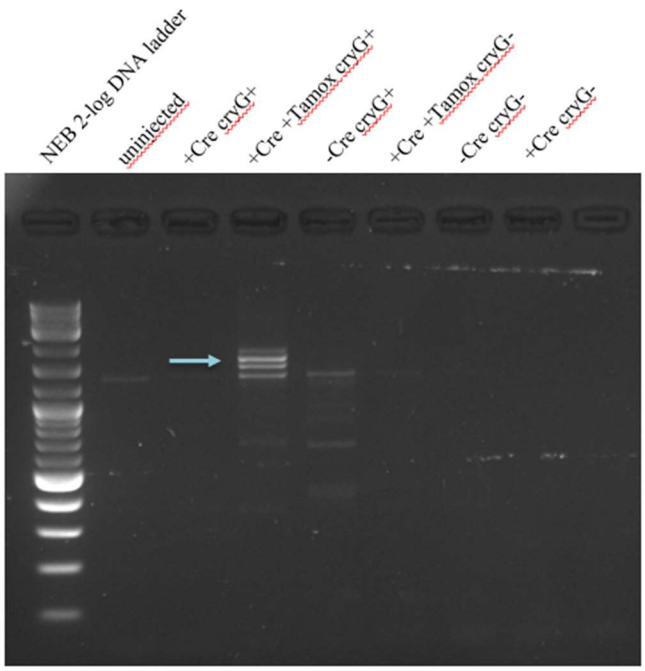

FIGURE 5. **cDNA PCR using RNA extracted from injected embryos**. Embryos from each group (uninjected, injected with/without zfCre-ERT2 mRNA, treated or not treated with tamoxifen, and cryG+/-) were collected for RNA isolation. RT-PCR was performed to create cDNA from the RNA in order to perform a cDNA PCR utilizing primers (11475/11521) flanking critical locations in the construct, such as splice junctions. Analysis of the PCR product on an agarose gel revealed strong bands in only the group injected with Cre and treated with tamoxifen that exhibited cryG expression. This indicated that proper splicing may be occurring only in this group. The resulting PCR product from that group was used for shotgun cloning and sent out for sequencing. Sequencing results revealed proper splicing was occurring in the sequenced areas, confirming the results seen on the cDNA PCR gel.

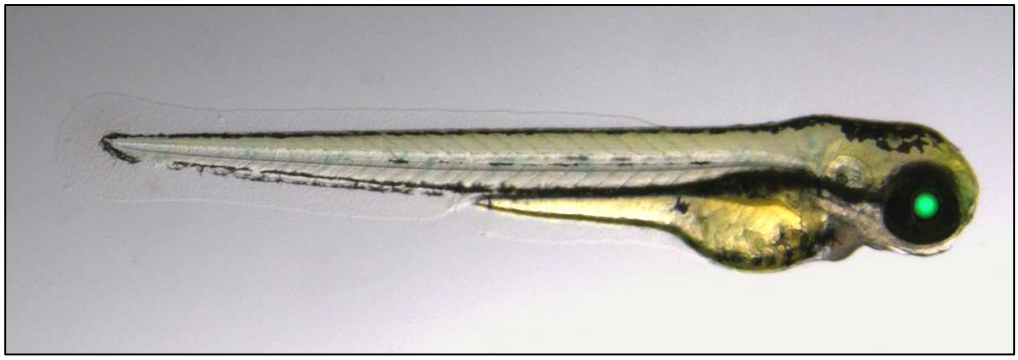

FIGURE 6. **Zebrafish larva exhibiting cryG expression**. After confirmation that the transgenic construct was working properly, more embryos were injected from wild-type crosses. These embryos were injected with only Tol1 transposase and the TIE2 transgenic construct so the transgene would remain inactive. At 3dpf, larvae were screened for cryG expression. The larvae without cryG expression were removed, and those exhibiting cryG expression were allowed to mature into adulthood. These fish would become the F0 generation for the TIE2 transgenic line.

Founder #	% embryos cryG+	Founder #	% embryos cryG+
1	7%	14	49%
5	7%	20	11%
6	19%	25	21%
7	7%	26	34%
10	18%	27	17%
12	29%	29	27%

FIGURE 7. **Founder screen results**. After three months, the F0 fish bearing the transgene reached maturity and were able to breed. Each fish was out-crossed to a wild-type fish in a single cross, and the embryos were collected. At 3dpf, the clutch of embryos from each potential founder was screened to identify the presence of embryos with cryG expression. If the embryos resulting from the out-cross exhibited cryG expression, this meant that the fish was transmitting the transgene to next generation, and therefore was identified as a founder. The transmission rate was recorded as the percent of cryG+ embryos per clutch, and the founders were designated with unique numbers.

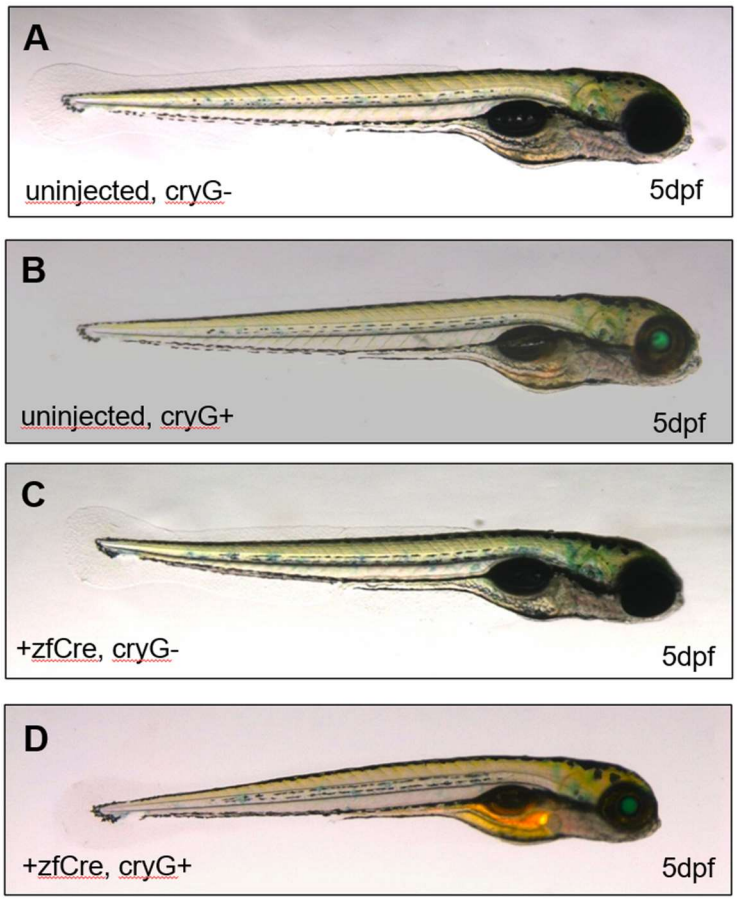

FIGURE 8. **Evaluating larvae phenotypes following transgene activation.** The identified founders were outcrossed to wild-type fish and the resulting F1 embryos were used for injections. *A, B*, Half of the embryos collected from each cross were not injected in order to use as a control. At 3dpf, larvae were screened and separated based on presence of cryG expression. *C, D*, The remaining half of the embryos collected were injected with just zfCre, and the larvae were screened and separated based on presence of cryG expression at 3dpf. Larvae were evaluated each day until 5dpf for possible phenotypes such as hemorrhage, abnormal circulation, edema, vascular defects, etc. No obvious phenotypes were prevalent between zfCre injected cryG+ embryos and negative controls.

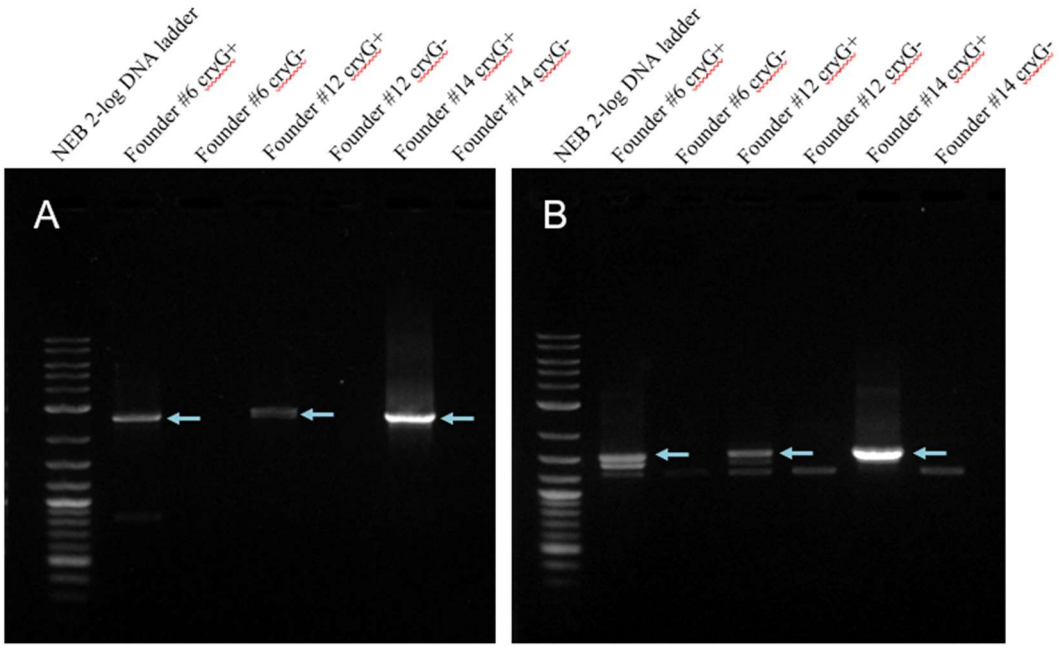

FIGURE 9. **F1 generation cDNA PCR**. Larvae from each group (cryG +/-) from each founder were collected and RNA was isolated from each identified group. The RNA was used to create cDNA to be used for cDNA PCR using primers that flanked critical locations in the transgenic construct. cDNA PCR results revealed that the construct was only present in the cryG+ embryos. *A*, Primers 11554/11559. *B*, Primers 11475/11521.