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Nkx3.2 regulates zebrafish vascular stabilization through recruitment of mural cells

by

Peter James Spice

A THESIS

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Abstract

Vascular stabilization is the process during vascular development where the immature endothelial cell tube gains structural support through the recruitment and adhesion of perivascular mural cells; pericytes and vascular smooth muscle cells (vSMC). When vessels are not well supported by mural cells hemorrhage can occur. *Nkx3.2* deficient zebrafish embryos hemorrhage, have reduced expression of a mural cell marker, and the endothelial layer makes less contact with mural cells compared with controls. I found that *nkx3.2* regulates the expression of two genes with known roles in neural crest migration and specification; *pdgfrα* and *sox10*. Further, *nkx3.2* functions autonomously in neural crest cells, where knockdown of *nkx3.2* causes abnormal migration of neural crest cells in the head. These results show that *nkx3.2* regulates genes involved in neural crest migration and in turn supports recruitment of mural cells to mediate vascular stabilization.

Acknowledgements

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List of Symbols, Abbreviations and Nomenclature

acta2	alpha smooth muscle actin
BC	blood cells
CADASIL	cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy
cdh2	n-cadherin
cDNA	complementary deoxyribonucleic acid
CtA	central artery
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleotide 5'-triphosphate
EC	endothelial cell
EDTA	disodium ethylenediaminetetraacetic acid
igu	iguana
IOC	inner optical circle
ISH	in situ hybridization
hpf	hours post-fertilization
LDA	lateral dorsal aorta
LiCl	lithium chloride
MC	mural cell
MO	morpholino
mRNA	messenger ribonucleic acid
NC	neural crest
nkx3.2	NK3 homeobox 2
OA	optic artery
OV	optic vein
paa	pharyngeal arch artery
pdgf	platelet-derived growth factor
pdgfr	platelet-derived growth factor receptor
PHBC	primordial hindbrain channel
PICA	primitive internal carotid artery
pvm	perivascular mesenchyme
RT-PCR	reverse-transcription polymerase chain reaction
shh	sonic hedgehog
TEM	transmission electron microscopy
TL	Tupfel long fin
vegfa	vascular endothelial growth factor
vSMCs	vascular smooth muscle cell

1 CHAPTER ONE: INTRODUCTION

1.1 General Introduction

1.1.1 Vascular Development in Zebrafish

The circulatory system is one of the first organ systems to develop in vertebrates, and is essential for delivering nutrients and oxygen to the embryo and removing waste products (Baldessari & Mione, 2009). In zebrafish, unlike other vertebrates such as mice or humans, oxygen can diffuse throughout the embryo during the first week of development without functional vasculature. This, along with simple and efficient breeding and transparent embryonic development has made zebrafish a very useful tool for the study of vascular development and defects in vessel formation or stabilization (Stainier, 2001). Accordingly, zebrafish research has contributed greatly to understanding the molecular mechanisms responsible for vascular development, beginning with initial endothelial tube formation and angiogenesis, and ending with the stabilization of vessels by association of the perivascular mural cells; pericytes and vascular smooth muscle cells (vSMCs)(Adams & Alitalo, 2007; Shawber & Kitajewski, 2004).

Progenitor cells called haemangioblasts are formed during gastrulation from the mesoderm and initiate the process of vasculogenesis when they differentiate into endothelial precursors called angioblasts and blood cell precursors called haemotopoetic stem cells (Vogeli *et al.,* 2006; Hirashima 2009). Angioblasts then respond to signalling from the adjacent endoderm that secretes vascular endothelial growth factor A (VEGFA) and migrate toward the embryonic midline. Here angioblasts differentiate into the vascular endothelial cells that make

up the first primitive vessel, the dorsal aorta, and lumenize (Tjwa *et al.,* 2003; Vokes *et al.,* 2004; Lawson *et al.,* 2002). Sonic hedgehog (Shh) expressed from the notochord and floorplate induces VEGFA expression in the somites and regulates *notch3* signalling to promote artery formation (Lawson *et al.,* 2002; Hirashima 2009; Lamont & Childs 2006). This signalling is required for artery-vein specification and involves mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways downstream of kinase insert domain receptor like (Kdrl), the main receptor for VEGFA (Hong *et al.,* 2006; Lamont and Childs 2006).

kdrl expression is one of the earliest and most reliable markers of endothelial cells (Hirashima 2009) and a transgenic line (Tg(kdrl:eGFP)) allows us to view the developing vasculature in real time. I focused my work on the head vasculature. At 30 hours post fertilization (hpf) two major vessels are visible in the zebrafish ventral head: the primordial hindbrain channel (PHBC) and the lateral dorsal aorta (LDA)(Isogai *et al.,* 2001: Figure 1.1). During this time period blood is supplied to the head by a primitive network of arteries beginning in the heart, exiting the bulbous arteriosus (BuA) into the ventral aorta, travelling dorsally through the pharyngeal arch arteries (paa) into the dorsal aorta where it splits into the anterior-supplying primitive internal carotid artery (PICA) and the LDA which flows posteriorly. The optic artery and optic vein (OA & OV) are also visible as a cluster in the inner eye. Notable vascular events occur in the next 18 hours as secondary vessels sprout from these existing ones and vascular beds remodel into different networks. Vascular remodelling in embryonic development refers to pattern changes through growth, division and coalescence of vessels as new tissues form in the embryos (Ellertsdóttir *et al.,* 2010). Central arteries (CtAs) extend from

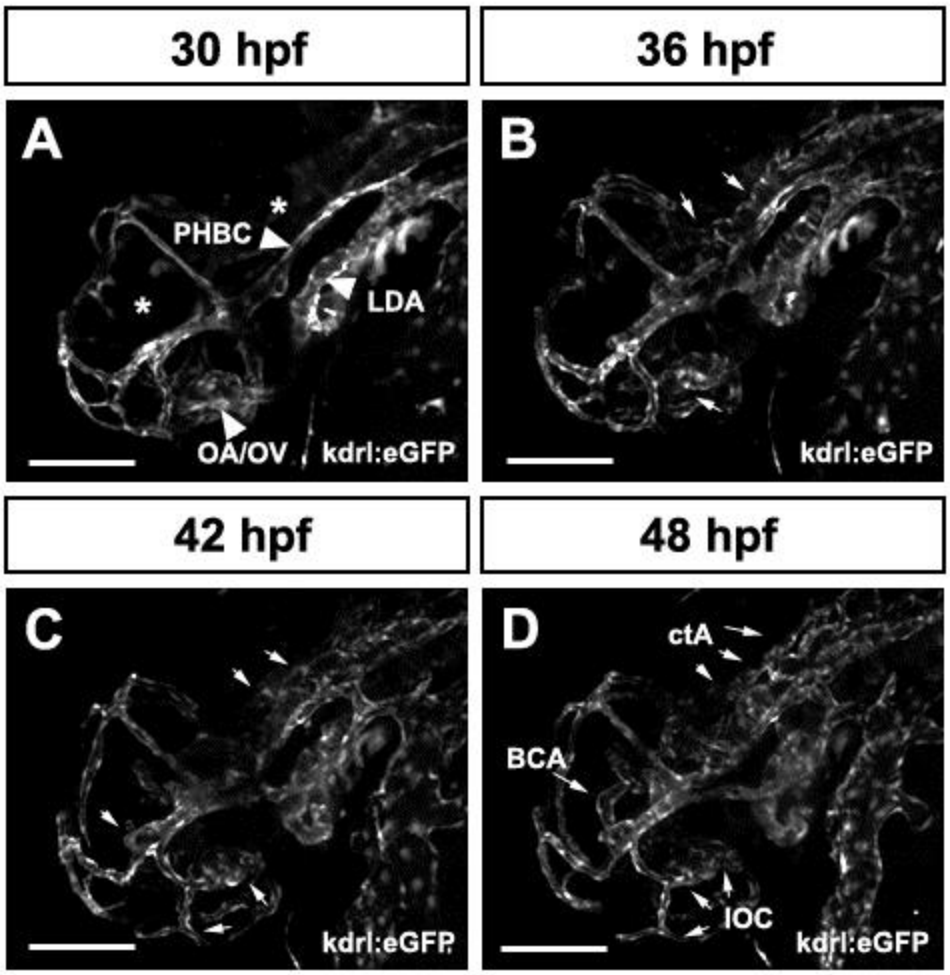


Figure 1.1: Time-lapse confocal microscopy of 30-48 hpf tg(kdrl:GFP) embryos reveals extensive vascular growth and organization in wild type embryos.

The time between 30 hpf and 48 hpf is a period of growth and reorganization of vasculature in the zebrafish head. (A) The primordial hindbrain channel (PHBC), the lateral dorsal aorta (LDA) and the optic artery and vein (OA/OV) are observed at 30 hpf. The forebrain and hindbrain contain very few vessels. (B,C) Sprouting from the PHBC and LDA occurs, and the OA/OV cluster begins to spread apart and reorganize. (D) By 48 hpf the central arteries (CtAs) populate the zebrafish hindbrain and the basal communicating artery (BCA) has extended into the forebrain. The OA/OV cluster has given rise to a thin circle surrounding the lens of the eye called the inner optical circle (IOC). Arrowheads highlight the major vessels, regions void of vasculature are labelled by an asterisk, small arrows indicate sprouting and reorganization of smaller vessels. Scale bars = 100µm

the PHBC into the brain, the inner optical circle (IOC) is remodelled from the OA/OV, the LDA extends anteriorly into the head (this branch becomes the PICA) where the basal communicating artery connects the left and right carotid arties near the forebrain (Isogai *et al.,* 2001: Figure 1.1). By 48 hpf the once avascular regions of the zebrafish head such as the forebrain and hindbrain contain many new vessels.

The subsequent process, vascular stabilization, occurs via association of mural cells, the precursors of pericytes and vascular smooth muscle cells, to the endothelium in order to create a stronger vessel wall capable of withstanding and regulating blood pressure (Adams and Alitalo, 2007; Santoro, et al., 2009). Cardiovascular defects, including hemorrhage and aneurysm, occur when this process is disrupted. The characterization of the genes and pathways required for vascular stabilization in model organisms allows us to understand the molecular basis of cardiovascular disease. The purpose of my research is to study a pathway that contributes to mural cell recruitment and vascular support, and to develop an understanding of the origin of vascular mural cells, their role in stabilization, and the genetic pathways that dictate this process.

1.1.2 Tools for genetic research in zebrafish

Zebrafish are an excellent model system for the study of vascular development and hemorrhage. A single mating pair is capable of producing hundreds of embryos at a time, embryos are transparent, and they have relatively simple genetics that allow for forward genetic screens, mutant fish, morpholino knockdowns, and transgenics (Butler *et al.,* 2011). We possess a number of transgenic fish, in which fluorescent proteins are driven by tissue specific

promoters. Certain genes make good candidates for use as molecular markers if they are well characterized and have specific expression in a relevant cell type. In this thesis, examples include the VEGF receptor *kdrl* to view endothelial cells (Hong *et al.,* 2006; Choi *et al.,* 2007), the vascular smooth muscle marker α-smooth muscle actin (*acta2*; Whitesell *et al.,* 2014) to view mural cells, the erythrocyte transcription factor GATA binding protein 1a (*gata1;* Yaqoob *et al.,* 2009) to view blood cells, and the cranial neural crest specification gene sex determining region Y-box 10 (*sox10*) to view neural crest (NC) cells (Wada *et al.,* 2005). Transgenic lines can be used individually or crossed to one another, allowing the identification of different cell types in real-time in wide variety of experimental contexts including knockdown or overexpression of genes involved in vascular development.

Knockdown of genes is easily accomplished using morpholino antisense technology (Nasevicius & Ekker, 2000). Here, either an ATG-site or a splice-site can be targeted by a specific morpholino sequence, where disruption of either site accomplishes knockdown of the gene. In this project morpholinos (MOs) used were either previously published or were verified for successful knockdown by reverse-transcription polymerase chain reaction (RT-PCR). There are some caveats to morpholino use, and to ensure specificity, multiple morpholinos targeting the same gene but different sites are used (Robu *et al.,* 2007). Overexpression of genes can be done via injection of a DNA plasmid containing a promoter which drives expression of the gene of interest, and a reporter to ensure that transformation has taken place. We use the Tol2Kit; a transposon from Medaka modified for Gateway cloning to express genes in developing fish with co-injection of transposase, which is injected into fertilized embryos at the one cell stage (Kwan *et al.,* 2007; Kikuta & Kawakami, 2009). The ease by which knockdown and/or overexpression of

zebrafish genes can be accomplished is another advantage of vascular research in this model organism and allows the assessment of gene function through observing aberrant signalling (Butler *et al.,* 2011).

1.2 Vascular Myogenesis: Migration of mural cell precursors

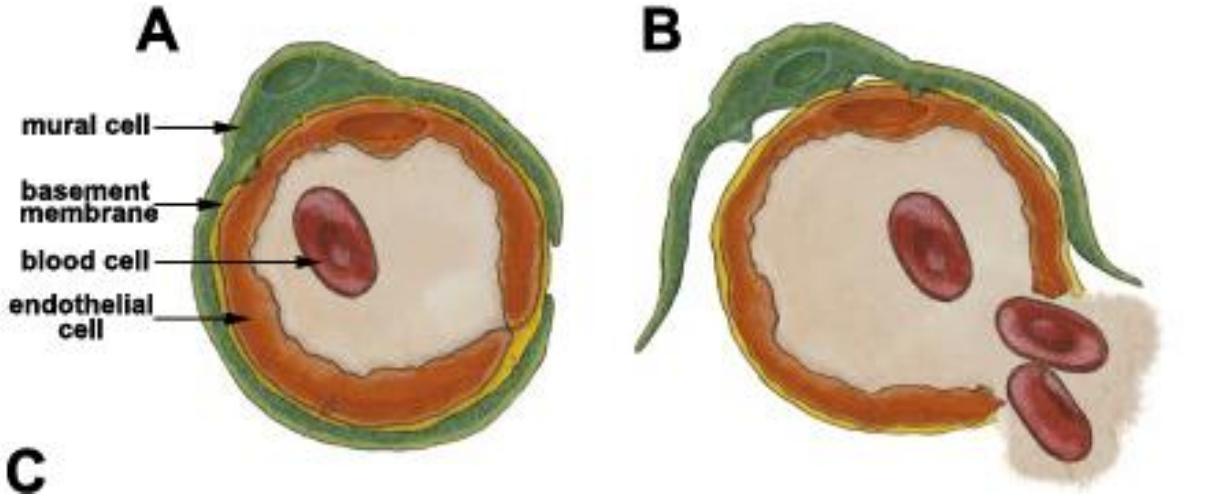
The overall process of vascular stabilization involves the recruitment of mural cells, the precursors of pericytes and vascular smooth muscle cells to blood vessels (vSMCs) (Adams & Alitalo, 2007; Santoro *et al.,* 2009). This recruitment, termed vascular myogenesis, leads to the maturation and specialization of the cardiovascular system and proper stabilization of nascent vessels (Santoro *et al.,* 2009; Carmeliet, 2000). The result of mural-endothelial interaction is the inhibition of endothelial proliferation and migration and the secretion of extracellular matrix proteins, which creates stable vessels that are resistant to hemorrhage and other vascular anomalies (Carmeliet, 2000).

Several families of signalling molecules have been implicated in the initial stages of vascular myogenesis including platelet derived growth factor (PDGF), notch and angiopoietin (Carmeliet, 2000). A key factor in mammalian vascular myogenesis is platelet-derived growth factor B (PDGRF-B) secreted from endothelial cells, which acts as a chemoattractant for mural cells. Here, it interacts with the receptor PDGRFβ on mural cells in order to establish an endothelial-mural cell connection (Lindhal *et al*., 1997, Lindhal *et al.,* 1998, Kim & Lassar, 2003). A role for the molecule in pericyte recruitment in zebrafish is not yet defined, although it is expressed in zebrafish brain pericytes (Wang *et al.,* 2014). The secreted glycoprotein angiopoietin-1 (Angpt1) functions in perivascular support cells where it interacts with the

Tie2/TEK tyrosine kinase on endothelial cells through paracrine signalling (Dumont *et al.,* 1994; Suri *et al.,* 1996). Disruption of this interaction in mice lacking Angpt1 results in a failure of endothelial cells to adhere with the surrounding cells and endothelial apoptosis (Gale & Yancopoulos, 1999). Additionally, our lab identified a role of Angpt1 in zebrafish, where it functions downstream of Sonic Hedgehog (Shh) as an important factor in vascular stabilization downstream of the *iguana* mutation, the same mutant that was used to identify the gene on which my project is based (Lamont *et al.,* 2010).

1.3 Vascular myogenesis: Adhesion and Maturation

Once mural cells have made contact with the endothelial layer, a number of cell-signalling events take place which lead to the adhesion, maturation and differentiation of the cells. An important factor in blood vessel maturation and stabilization, as well as artio-venous specification, is mural cell-expressed *notch3* (Domenga *et al.,* 2004; Liu *et al.,* 2010; Lawson *et al.,* 2001). Here, cell-cell contact activates Notch3 on mural cells, leading to physiological changes including maturation and adhesion of mural and endothelial cells through focal contacts (Liu *et al.,* 2010; Pajaniappan *et al.,* 2011). In mammals, stereotypical Notch signalling involves one of the 4 Notch receptors (Notch1-4) binding one of the 5 membrane-bound Notch ligands (Jagged1-2, Delta-like 1-4) which effects transcription of different genes in the nucleus (reviewed in Gridley, 2007; Liu *et al.,* 2010). While Notch signaling is involved in several processes of vascular development, its role in vascular stabilization through endothelial-mural interactions involves only the receptor Notch3 which is exclusively expressed in mural cells (Villa *et al.,* 2001; Liu *et al.,* 2010). Here Notch3 depends on the endothelial membrane-bound



Gene	Expression	Role in Development	Mode of action	Ref.
pdgfb	endothelial cells	myogenesis	chemoattractant for mural cells	Lindhal et al., 1997; Lindhal et al., 1998
pdgfrβ	mural cells	myogenesis	binds *pdgfb* to establish mural-endothelial contact	Lindhal et al., 1997; Lindhal et al., 1998
angpt1	mural cells	myogenesis	ligand for *tek*	Gale & Yancopoulos, 1999
tek	endothelial cells	myogenesis	binds *angpt1*to establish mural-endothelial contact and endothelial survival	Gale & Yancopoulos, 1999
notch3	mural cells	mural maturation, mural-endothelial adhesion	artery specification, formation of mural-endothelial focal contacts	Lawson *et al.,* 2001; Liu *et al.,* 2010
s1pR1	endothelial cells	mural maturation	trafficking and activation of adhesion molecules	Liu *et al.,* 2000; Paik, *et al.,* 2004
cdh2	mural cells and endothelial cells	mural-endothelial adhesion	form adherens junctions to bind cells together	Li *et al.,* 2011

Figure 1.2: Schematic of endothelial/mural interaction in vascular development.

(A) A cross-section of a blood vessel shows an inner endothelial layer forming a lumen which contains the blood. Surrounding the endothelium is a perivascular mural cell, which is tightly associated with the endothelial layer along its basement membrane. (B) When some aspect of vascular stabilization is disrupted, much as the recruitment, contact, maturation, adhesion or differentiation of mural cells, hemorrhage occurs and vessels rupture under hemostatic stress. (C) A selection of important signalling genes for various aspects of vascular stabilization as identified in mammals. The list includes *pdgfb, pdgfrβ, angpt1, tek, notch3, s1pr1,* and *cdh2.* Here, the location of expression, role in development, and mode of action are briefly summarized. *Adapted from Winkler et al., 2011 with copyright permission from Elsevier.*

receptor Jagged1 to carry out endothelial-mural communication ultimately resulting in mural cell maturation and adhesion of the two cell types (Liu *et al.,* 2009; Liu et al., 2010; Pajaniappan et al., 2011). A role for *notch3* has not been well characterized in zebrafish, although fish *notch3* is expressed in pericytes and has a role in regulating pericyte number (Wang *et al.,* 2014), and this recent work may suggest a similar mechanism in fish. Interestingly, human Notch3 is directly linked to a hereditary adult-onset disorder known to cause stroke and dementia called CADASIL syndrome where mutations in Notch3 cause vascular instability due to vessel degeneration and loss of vascular smooth muscle cells (Joutel et al., 1996; Liu *et al.,* 2010).

Another example of cell-signalling resulting in mural cell maturation and adhesion involves the platelet-derived sphingolipid sphingosine 1-phosphate (s1p). While zebrafish research in sphingolipid signalling is currently lacking, the receptors are well conserved in the species, and knockdown of sphingosine 1-phosphate receptor 1 (*s1pR1)* causes widespread vascular defects (Mendelson *et al.,* 2013). In mammalian models where the system has been better characterized, sphingosine signalling is required for mural cell maturation as impaired S1P signalling causes deficient vascular smooth muscle and pericyte formation (Liu *et al.,* 2000). Under normal conditions S1P signaling acts by trafficking and activating the cell adhesion molecule N-Cadherin (CDH2), and loss of this signalling results in impaired cell-cell interactions and poor vascular stabilization (Liu *et al.,* 2000; Paik, *et al.,* 2004).

Recent research has identified N-cadherin (*cdh2*) as a crucial molecule for the adhesion of perivascular mural cells to the endothelial cell layer in vessels (Li *et al.,* 2011; Winkler *et al.,* 2011). Work by Li *et al.* (2011) found that disruption of Smad4 in mouse led to poor coverage of

pericytes and hemorrhage in the mouse head, which occur due to a large decrease in *cdh2* expression leading to poor adhesion of supportive cells.

In summary, under normal conditions, mural cells migrate to the endothelial layer, establish a connection, undergo maturation, and develop strong adhesion to the endothelium. Each of these processes is required for vascular integrity and subsequent differentiation and specialization into pericytes and vascular smooth muscle cells. While some of the secreted molecules and receptors that mediate these events are known, little is known about the intrinsic cellular mechanisms driving vascular myogenesis.

1.4 Pericytes and vSMCs

Vascular smooth muscle cells and pericytes differ in their morphology, the markers they express, and the types of vessels they are associated with, even though at the molecular level they are hard to distinguish. Vascular smooth muscle cells tend to associate with larger vessels such as arteries and arterioles, where they form a strong supportive layer surrounding the endothelium (Fuxe *et al.,* 2011). Conversely, pericytes tend to associate with the vascular endothelium in a more punctate manner, and are often found in smaller vessels such as brain capillaries (Fuxe *et al.,* 2011; von Tell *et al.,* 2006). Pericytes are embedded in the basement membrane of the vascular endothelium where they provide support and contractility, and regulate the blood-brain barrier (Armulik *et al.,* 2005; Daneman *et al.,* 2010). The distinction between pericytes and vSMCs is not as clear in embryonic zebrafish as in mouse or chick. Furthermore, pericytes and smooth muscle cells come from the same lineage in birds and mammals (Etchevers *et al.,* 2001). Our lab hypothesizes that pericytes and smooth muscle cells

may exist on a continuum based on the degree of maturation rather than being distinct, specified cell types. In fact, our preliminary experiments reveal colocalization of some pericyte and vSMC markers in vessels of the zebrafish head. Pericytes have also recently been shown to possess certain stem cell characteristics, such as the ability to act as progenitors for muscle, bone, or fat following injury (Dar *at al.,* 2012). The "stemness" of these cells suggests that they may act as precursors for the more mature perivascular cells; vascular smooth muscle cells. Unpublished data from our lab supports this theory, as an inhibitor of platelet-derived growth factor (PDGF) signalling, a pathway essential for pericyte formation (Lindhal *et al.,* 1997), virtually abolishes expression of the vSMC marker *acta2*.

The timing of mural cell recruitment and maturation has not been fully characterized in zebrafish, though recent results from our lab and others have aided the process. In mouse, pericytes are present on small vessels of the brain at E11.5 and clear vascular smooth muscle is seen on larger blood vessels of the brain at E17.5 (Hellström *et al.,* 1999). In fish, transmission electron microscopy (TEM) from Lamont *et al.* (2010) and other unpublished data from our lab has revealed that perivascular mural cells are present on the dorsal aorta, internal carotid and other head vessels at 48 hours post fertilization (hpf) prior to expression of any molecular mural cell markers. Recently, Wang *et al.* (2014) eloquently presented the first evidence of *notch3* and *pdgfrβ* expressing brain pericytes in early zebrafish embryos, when cells began expressing the pericyte marker *pdgfrβ* at 56 hpf and *notch3* at 72 hpf. Further, our lab developed the tg(acta2:EGFP) transgenic zebrafish in order to view vascular smooth muscle cells in developing zebrafish embryos, where mural cells of the head begin expressing the vSMC marker α-smooth muscle actin (*acta2*) at 84 hpf (Whitesell, *et al,* 2014). While *acta2* is a marker of vSMCs in other

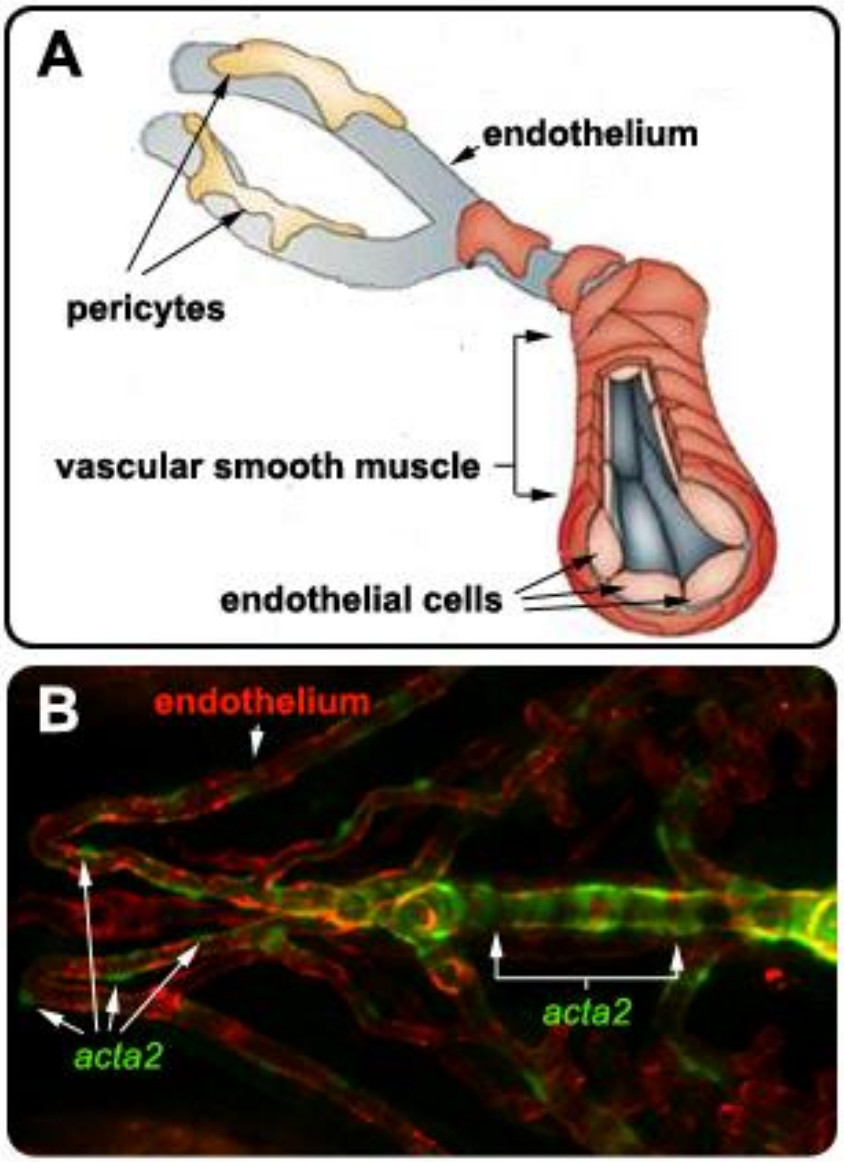


Figure 1.3: Schematic of pericyte and vascular smooth muscle specification and an application in zebrafish.

(A) A schematic illustrates features that distinguish pericytes and vascular smooth muscle cells. Pericytes are typically found on smaller vessels, where they adhere to the endothelium in a punctate manner. Vascular smooth muscle cells form a tight layer around the endothelial cells. (B) Vascular stabilization by mural cells in the zebrafish head. *Acta2,* a vascular smooth muscle cell marker in other model organisms, is expressed in a punctate manner on smaller vessels and in a continuous layer on larger ones. *Figure adapted from Adams & Alitalo, 2004 with copyright permission from Nature Publishing Group; and Whitesell et al., 2014.*

systems, the expression of the marker in embryonic zebrafish can take on either a stereotypical vSMC pattern or a pericyte-like pattern, where acta2-positive cells are sometimes present along vessels in a continuous sheet and sometimes individually in a punctate pattern. The distinction between pericytes and vascular smooth muscle cells in zebrafish cannot currently be made based on the few available markers. The current understanding of mural cell recruitment and differentiation in zebrafish is characterized by the presence of early mural cells undetectable by any of the current markers, the emergence of stereotypical brain-pericytes shortly after, and the later appearance of vSMC-marker expressing cells that sometimes resemble the pericytes observed in other model systems. This data suggests that a continuum of mural cell specification may be occurring based on the vascular context and maturity of the cells, and much more research is required before each individual cell type can be considered wholly distinct from the other. My research, while acknowledging the diversity of vascular support-cells, will consider any perivascular support-cell of the embryonic zebrafish head a mural cell.

1.5 Mural Cell Origins

In zebrafish the origins of pericytes and vascular smooth muscle cells (vSMCs) are not well understood, although much work has been done in other vertebrates. Neural crest tissue is a specialized population of cells arising from the ridge of ectoderm along the developing neuroepithelium (Le Douarin *et al.,* 2007). Here, cells delaminate as they undergo an epithelial-mesenchymal transition and become migratory (Theveneau & Mayor, 2012). Cranial neural crest cells migrate collectively and then into discrete streams where they give rise to cells of the nervous system, craniofacial bones and cartilage, pigment cells, and, in murine and avian systems, perivascular mural cells (Le Douarin *et al.,* 2007; Theveneau & Mayor, 2012; Dupin *et*

al, 2006). We use Sox10 as a marker of this lineage because it is a strong, fairly specific neural crest marker (Wada *et al.,* 2005). Though the origins of zebrafish mural cells are unclear, chick and mouse pericytes and vSMCs of the head have been found to be derived from the ectomesenchymal lineage of the neural crest, a population of cells that contributes to the development of facial bones and cardiac tissue (Whitesell *et al.,* 2014; Etchevers *et al.,* 2001; Mundell & Labosky, 2011; Olesnicky Killian *et al.,* 2009).

While the origins of zebrafish pericytes and vascular smooth muscle cells remain disputed, there is evidence that normal neural crest migration is required for vascular stabilization. Work by Eberhart *et al.* (2008) identifies *pdgfrα* as a crucial molecule for the guidance of migrating neural crest cells. While the primary purpose of their research involved palatogenesis, a fascinating secondary result was that loss of *pdgfrα* led to neural crest migratory defects and hemorrhage. These results support a hypothesis that neural crest-derived mural cells are present in zebrafish and are required for vascular stability, where *pdgfrα* signaling drives their early migration. The results also coincide with mouse data implicating PDGFRα as a regulator of vascular integrity and stroke (Su *et al.,* 2008).

Conversely, data from quail-chick chimera research reveals that mural cells of the trunk arise from compartments of the mesoderm-derived somites (Pouget *et al,* 2008). This indicates that the lineage of perivascular murals cells differs depending on the region of the embryo that the cells are present. This anterior-posterior disparity in contributing mural cell lineage may be conserved in zebrafish as neural crest tissue of the zebrafish trunk lacks the ectomesenchymal population (Lee, *et al., 2013*). It appears likely that zebrafish mural cells may arise from multiple

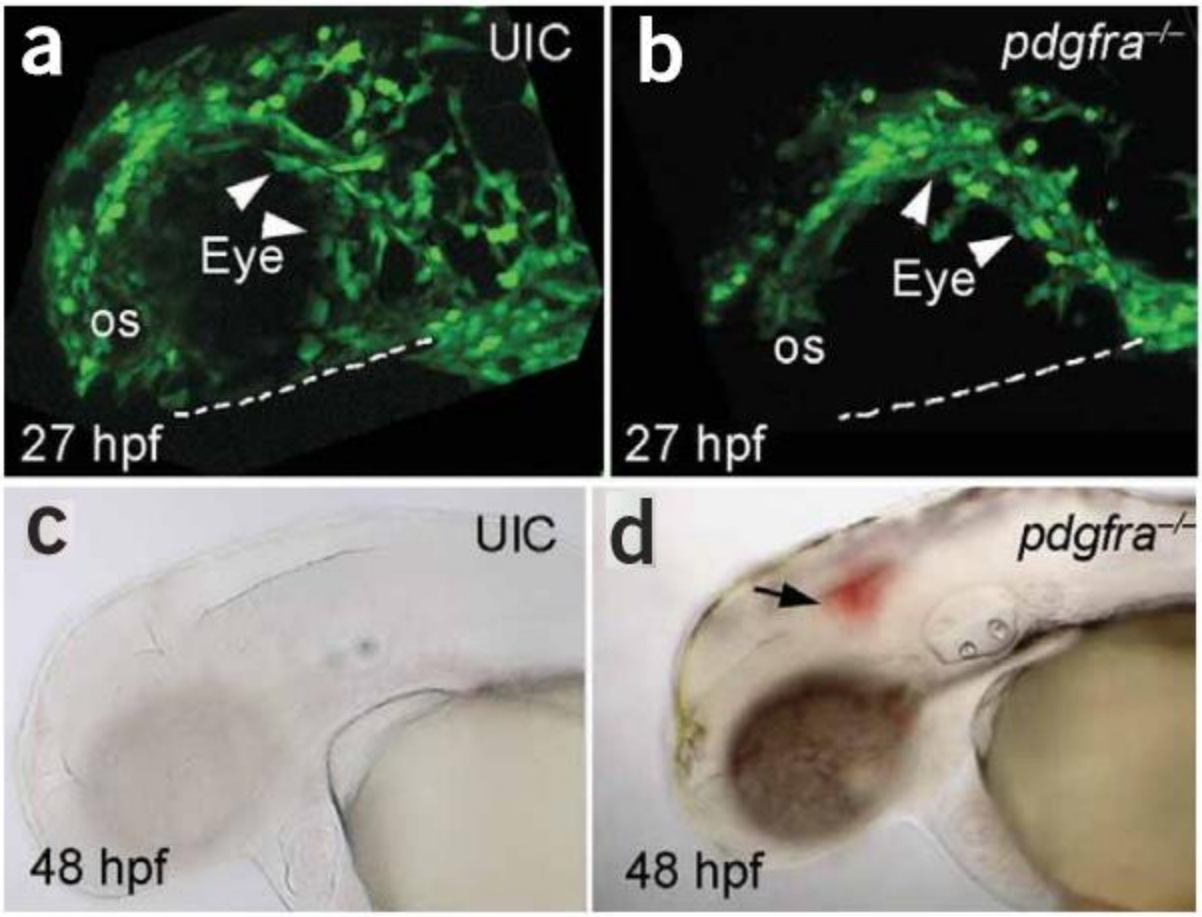


Figure 1.4: *pdgfrα* is required for neural crest migration and vascular stabilization.

(A) Fluorescent imaging of sox10:EGFP transgenic zebrafish embryos reveals migrating neural crest cells. The neural crest cells of wildtype embryos are spaced apart and have migrated beyond the optic stalk by 27 hpf. (B) In contrast, the neural crest cells of *pdgfrα* null mutant embryos are clumped together and have not migrated beyond the optic stalk by 27 hpf. (C) Accordingly, wildtype embryos appear normal at 48 hpf, (D) whereas *pdgfrα* mutants hemorrhage. *Figure adapted from Eberhart et al., 2008 with copyright permission from Nature Publishing Group.*

lineages, and a secondary goal of my research is to explore possible contributing cell populations as they apply to vascular stabilization.

1.6 Insights from the *Iguana* zebrafish genetic mutant

The sonic hedgehog signalling pathway influences development of many different body systems at different stages in development including the limbs, central nervous system, gut, face, and notably the vasculature (Nagase *et al*, 2006). In vasculogenesis, Shh aids in patterning early vessels by signalling from the endoderm and notochord (Vokes *et al.,* 2004) inducing expression of VEGF in the trunk (Siekmann & Brand, 2005), and is important in maintaining normal mural cell interactions with the endothelial layer (Passman *et al.,* 2008). Loss of Shh signaling in developing mouse embryos results in defects in vascular remodelling and hemorrhage (Kolesová *et al.,* 2008). Our lab also identified a role for hedgehog signalling in vascular stabilization where disrupted Shh signalling by either the *iguana* mutation or cyclopamine treatment results in hemorrhage (Lamont *et al.,* 2010). The genetic mutant *iguana* has a mutation in the DAZ interacting zinc finger protein 1 (*dzip1*) gene, which causes a ciliary formation defect. As Shh uses the cilia to transduce the signal, this results in defective Shh signalling. Cilia are required for the translocation of the de-repressed smoothened receptor and the transcription factor Gli (Sekimizu *et al.,* 2004; Kim *et al.,* 2010). Under normal circumstances, the secreted sonic hedgehog ligand binds a membrane-bound receptor called Patched, which is then inactivated. Inactivation of Patched releases its inhibition of Smoothened, which then activates Protein Kinase A and initiates a phosphorylation cascade (Wolff *et al.,* 2003; Ingham & Kim, 2005). This involves phosphorylation of Gli transcription

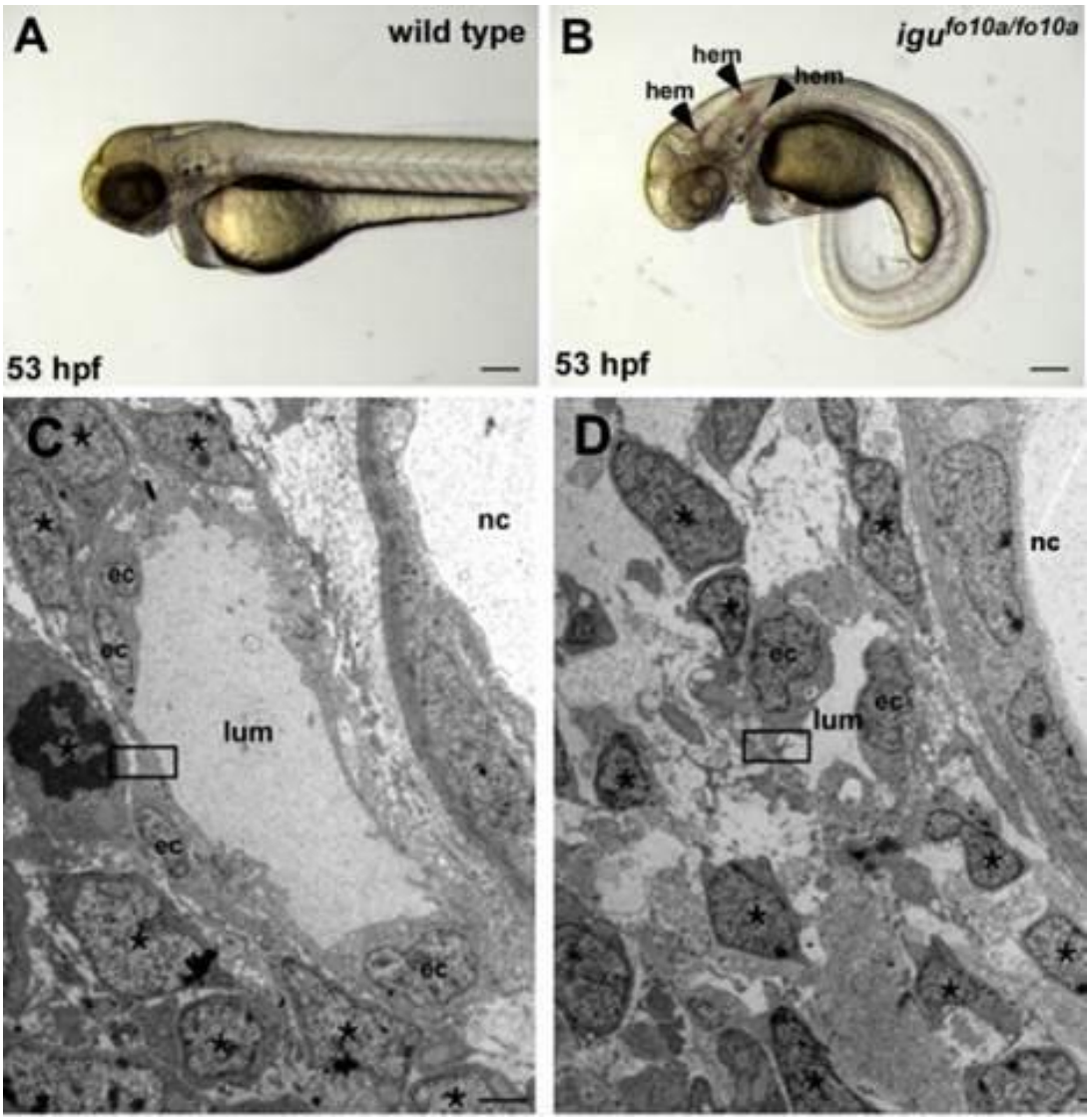


Figure 1.5: iguana mutant zebrafish hemorrhage due to poorly supported vessels.

(A) wildtype 53 hpf zebrafish embryos compared with (B) 53 hpf *iguana* mutant fish. *Iguana* mutant fish have multiple hemorrhages in various regions of the head. (C) A cross section of a wildtype vessel reveals tight association of mural cells (*) to endothelial cells (ec), whereas the vessels of *iguana* mutant embryos (D) have much less support. The lumen of the mutant vessel is smaller, less mural cells are making contact with the endothelium, and more loose interstitial space is present surrounding the endothelium. *Figure adapted from Lamont et al., 2010 with copyright permission from Elsevier.*

factors, which are translocated to the nucleus and can act as either repressors or activators of transcription of downstream genes and ultimately determine the transcriptional program downstream of hedgehog signalling (Wong & Reiter, 2008). In *iguana* mutant zebrafish, where this signalling cascade is disrupted by loss of the cilia, the fish exhibit a phenotype characterized by a curved body axis and hemorrhage in the head. Because hemorrhage indicates a defect in vascular stabilization, this mutant is of great interest to our laboratory. Further, electron microscopy of vessels in *iguana* mutant zebrafish revealed normal endothelial-endothelial cell connections but poor endothelial-mural cell connections (Lamont *et al.,* 2010). Very often, zebrafish hemorrhage phenotypes have been shown to be the direct result of defective vascular integrity due to poor endothelial-endothelial junction formation or disrupted cell signalling between endothelial cells (Wilson *et al.,* 2013; Fish *et al.,* 2008; Kwon *et al.,* 2012). The *iguana* mutant provides a model where vascular stabilization defects downstream of sonic hedgehog signalling, one of the best understood morphogen pathways, appear to be caused by defects in mural cell recruitment, maturation or adhesion. An ongoing challenge in our laboratory is to define the molecular processes downstream of *iguana* that mitigate vascular stability.

In order to identify the downstream factors involved in mediating vascular stabilization by association of mural cells to the endothelium, a Nimblegen gene expression microarray surveying >30000 genes was conducted (Arnold *et al.,* submitted). We compared gene expression in WT and *iguana* mutant zebrafish as well as embryos treated with the small molecule cyclopamine, a Shh antagonist (Incardona *et al.,* 1998), and DMSO treated controls. We chose to investigate gene expression levels downstream of both cyclopamine and *iguana,* as each method disrupts *Shh* signalling indirectly, and therefore likely also cause disrupted

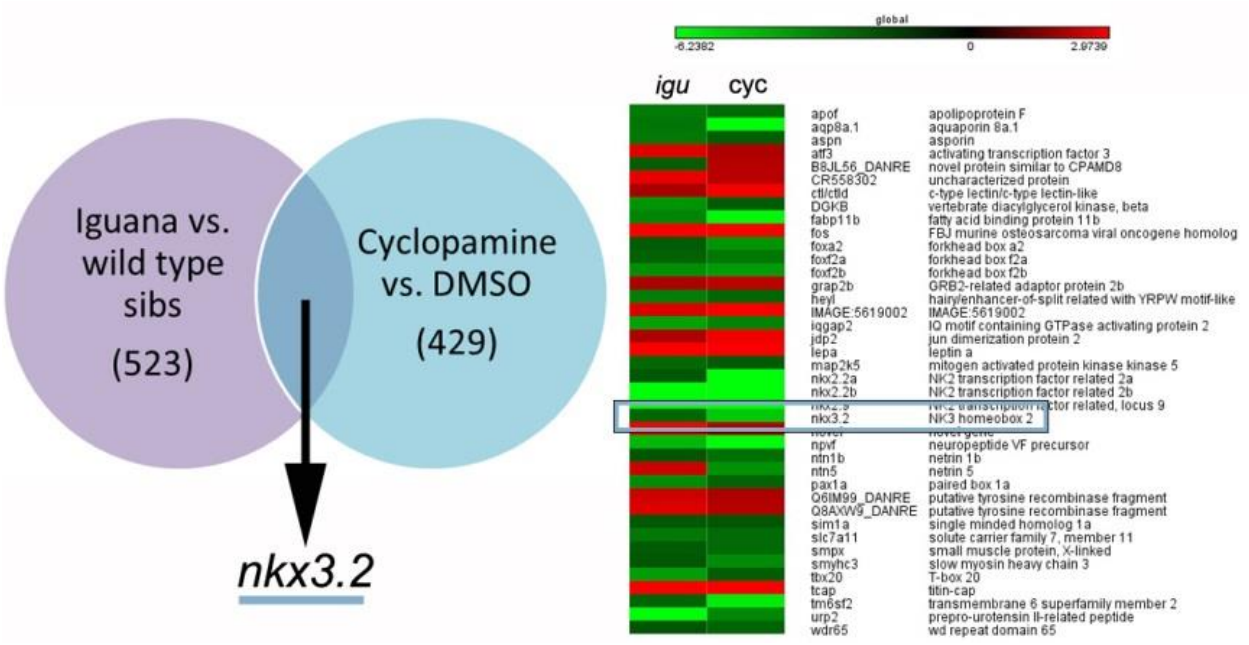


Figure 1.6: A microarray identifies genes up or down regulated by iguana and cyclopamine-treated zebrafish embryos.

(A) A Venn diagram shows the total number of genes significantly up or downregulated in *iguana* mutant (523 genes) and cyplopamine-treated (429 genes) zebrafish embryos at 30 hpf. 40 genes were overlapping between the two groups. (B) A heat map of the 40 genes differentially expressed by the two groups shows up-regulation in red and down-regulation in green. The scale bar can be used to estimate scale-change based on colour, between a range of 6.2 fold down-regulation and 2.1-fold upregulation. *nkx3.2,*the subject of my research, was downregulated 2.4-fold in *igu* mutants and 4.9-fold in cyclopamine-treated embryos. *Figure adapted from Arnold et al., 2014 with copyright permission from John Wiley and Sons.*

signalling in other pathways. *Iguana* mutant embryos have defective primary cilia, and thus lose an important signalling centre for Shh and other signalling molecules (Wolff *et al.,* 2003). Cyclopamine is an antagonist of hedgehog receptor *smoothened* and influences the balance of the active and inactive forms of the receptor (Chen *et al.,* 2002). Our goal was to filter our results to genes downstream of *Shh* by looking at expression changes that were up or down regulated in both *iguana* and cyclopamine-treated fish, two distinct means by which *Shh* signalling is disrupted. Several of the genes currently being investigated by our lab are *pim1*, a cell-cycle regulator and a proto-oncogene with a known role in vascular remodelling (Moloche *et al.,* 2011); and *foxf2b*, a forkhead transcription factor with a role in extracellular matrix secretion in gut visceral smooth muscle specification (Ormestad *et al.,* 2006; Jakobsen *et al.,* 2007). The purpose of my research is to study the role of *NK3 homeobox 2 (nkx3.2)*, in vascular stabilization and mural cell recruitment in developing zebrafish embryos.

1.7 The homeobox-containing transcription factor Nkx3.2

Nk3 homeobox 2 (*nkx3.2*) encodes a homeodomain containing transcription factor (Yoshiura & Murray, 1997). We are interested in this transcription factor because we found it had significantly reduced expression in both *iguana* mutant (2.4-fold) and cyclopamine treated zebrafish (4.9 fold). Previous research by Verzi *et al.* (2009) has indicated a role for *nkx3.2* in the mouse gut, where it is involved in visceral smooth muscle differentiation and recruitment. *Nkx3.2* has also been found to be expressed in vascular smooth muscle in chicken, which may suggest a dual role for *Nkx3.2* in visceral and vascular smooth muscle (Nishida *et al.,* 2002). If *Nkx3.2* is necessary for mural cell recruitment, this may fully or partially explain the vascular

stability defects observed in *iguana* zebrafish, where Shh signalling is disrupted. In support, *NKX3.2* expression is modulated downstream of Shh in chick (Murtaugh *et al.,* 2001).

One goal of my research is to explore the origins of mural cells through analysis of *nkx3.2* expression and function. Mice express *Nkx3.2* in the sclerotomes, which are compartments of the somites and are mesodermally derived (Lefebvre & Smits, 2005). *Nkx3.2* mouse mutants exhibit diverse defects associated with defective mesodermal differentiation including impaired sketelal, gut and spleen development (Akazawa *et al.,* 2000). Included in the skeletal defects are craniofacial abnormalities indicative of the role of *Nkx3.2* in promoting chondrogenesis from sclerotome (Lefebvre & Smits, 2005; Yamashita *et al.,* 2009). In support of this, data from the chick has identified that some vascular mural cells are derived from sclerotomal tissue (Pouget *et al.,* 2008), indicating a role in mesoderm.

On the other hand, in the head *nkx3.2* has a role in ectomesenchymal neural crest. Knockdown of *nkx3.2* in zebrafish results in an identifiable facial bone phenotype characterized by a loss of structures including the jaw-joint (Miller *et al.,* 2004). While some facial abnormalities may be due to defects in chondrogenesis from mesodermal tissues, many also have routes in neural crest migration and differentiation. As mural cells are derived from ectomesenchymal neural crest in other systems, a role for *nkx3.2* in ectomesenchymal proliferation or differentiation could have major implications for mural cell recruitment (Etchvers *et al.,* 2001). Part of the goal of my research is to better understand the role for *nkx3.2* signaling in cells from a cranial neural crest lineage, and to determine if *nkx3.2* functions autonomously in these cells only. If *nkx3.2* plays a role in mural cell recruitment, it is important

to understand what tissues the gene is active in. Through this approach I can provide evidence into the origins of zebrafish mural cells and better understand whether these cells come from a neural crest or a mesodermal lineage.

Nkx3.2 collaborates with its close paralog *barx1/nkx3.1*, which exhibits redundancy with *nkx3.2* through its role in facial bone development (Winslow & Burke, 2010). *nkx3.1* is required for proliferation of the ectomesenchymal lineage of neural crest, and knockdown of the gene causes loss of facial bone structures similar to the defects observed in *nkx3.2* loss of function experiments (Sperber *et al.,*2008; Winslow & Burke, 2010). Interestingly, *nkx3.1* and *nkx3.2* have a previously documented relationship in a signalling pathway related to visceral smooth muscle differentiation (Verzi *e al.,* 2009). I have not considered a role for *nkx3.1* in this thesis, but it remains possible that it may be partially redundant with *nkx3.2.* Nkx3.2 also participates in a transcriptional repressor complex with the intracellular proteins Smad 1 and Smad 4 in cultured cells (Kim & Lassar, 2003). Work by Li *et al.* (2011) reveals that this interaction may be relevant to vascular stabilization as loss of Smad4 in mice causes hemorrhage and reduced adhesion of pericytes to endothelial cells due to impaired levels of the cell-adhesion molecule n-cadherin (Li *et al.,* 2011; Winkler *et al.,* 2011). One goal of my work is to explore these potential interactions by examining *nkx3.2* gene regulation of relevant candidates.

In this thesis I examine the expression, function and mode of action of *nkx3.2* in the context of vascular stabilization by mural cells. Here, I first confirm that *iguana* regulates *nkx3.2* by conducting expression analysis and by performing genetic rescue of hemorrhage in *iguana* mutant embryos by overexpression. I then focus on deficient *nkx3.2* signalling via morpholino

knockdown and find that *nkx3.2* knockdown results in hemorrhage due to a reduction in the expression of mural cell markers and observe vessels with fewer contacts to mural cells. Finally, I attempt to characterize the mode of action of *nkx3.2* signalling, first by identifying targets of Nkx3.2 through differential expression of various genes in knockdown fish. I find that Nkx3.2 regulates the expression of the neural crest guidance factor *pdgfrα* and the general neural crest marker *sox10.* I discover that *nkx3.2* functions autonomously in *sox10* expressing cells, where it regulates *pdgfrα* and mediates neural crest migration. The general hypothesis I study in this thesis:

***nkx3.2* regulates vascular stabilization by mediating recruitment of perivascular mural cells to the endothelial layer**

2 CHAPTER TWO: METHODS

Reagents were acquired through Sigma, St. Louis, MO unless otherwise specified.

2.1 Embryo handling and fixation

Embryos were raised at 28.5°C in E3 media (Figure 2.1) and 0.003% of the pigmentation blocker 1-phenyl 1-2-thiourea added at 24 hpf. They were staged according to Kimmel *et al.,* 1995 and then fixed using 4% paraformaldehyde in PBS. Embryos were then washed briefly in PBT and stored in 100% methanol at 20°C for later use in *in situ* hybridization or antibody staining experiments. Fish used included wild type Tupfel long fin (TL) zebrafish or *Tg(kdrl:mcherry)*^{ci5} (Proulx *et al.,* 2010), *Tg(kdrl:EGFP)*^{la116} Choi *et al.,* 2007), *Tg(acta2:EGFP)*^{Ca7} (Whitsell *et al.,* 2014), *Tg(gata1:dsRed)*^{sd2} (Traver *et al.,* 2003), or *Tg(-4.9sox10:egfp)*^{ba2} (Carney *et al.,* 2006)(Table 2.4). All animal protocols were approved by the University of Calgary Animal Care Committee.

2.2 RNA Expression

Gene expression was documented using *in situ* hybridization (ISH). Before probes could be synthesized, whole embryo zebrafish RNA was isolated and used to make cDNA. RNA was isolated by homogenizing 50-100 zebrafish embryos with 600 µL of RLT buffer from the RNeasy Mini Kit (Qiagen, Mississauga, ON) and 6 µL of beta-mercaptoethanol in a tube using a 22 gauge needle and a syringe. The RNeasy Mini Kit and the manufacturer's instructions were used for the rest of the procedure. To make the cDNA, 14.4 µL of 200-500 ng/µL total RNA was incubated at room temperature with 1.8 µL DNase I reaction buffer (Thermo Scientific, Wilmington, DE) and 1.8 µL DNAse I (Thermo) for 15 minutes, and was then inactivated by

adding 1.8 µL 25mM EDTA and placing the mixture at 65°C for 10 minutes. To synthesize the cDNA, 2 µL of oligo $(dT)_{20}$(50µM) (Invitrogen, , Carlsbad, CA) and 4 µL of dNTPs (10mM, Invitrogen) were added to the tube and incubated at 65°C for 5 minutes. The tube was placed on ice and then 8 µL of 5X cDNA synthesis buffer, 2 µL of DTT (0.1), 2 µL of RNAseOUT (40U/µL, Invitrogen), 2 µL of RNase-free water, and 2 µL Superscript III (Invitrogen) were added and the mixture was incubated at 50°C for 1 hour. The reaction was stopped by incubation at 85°C for 5 minutes, 2 µL of RNAse H was added, and the mixture was incubated at 37°C for 20 minutes. The cDNA was stored at -20°C.

PCR was used to generate probes for ISH, where Taq DNA polymerase (0.25 µL; New England Biolabs, Ipswich, MA), 5x buffer (2µL), dNTP (1µL, 10mM; Invitrogen), and 1 µL of each the forward and reverse primers (University of Calgary DNA services) were used to amplify varying volumes of whole-embryo cDNA. Typically, reverse primers contained an additional T7 promoter binding site which allowed for synthesis of RNA probes from the resultant template DNA. This template was separated using gel electrophoresis in 1% agarose in TBE buffer to verify accurate fragment size, the product was purified using a QIAGEN PCR purification kit and the manufacturer's instruction, and the concentration was measured using a Nanodrop spectrophotometer (Thermo Scientific).

Probe synthesis was conducted by *in vitro* transcription using 0.1µg of template for every 200 bps of the fragment size and T7 RNA polymerase (2µL; Promega), 5X buffer (4uL; Promega), 100mM DTT (2µL; dithiothritol; Promega), 10X DIG RNA labeling mix (2µL; Roche Applied Science, Laval, QC), and RNAsin (1µL; Promega) to a final volume of 20 µL, which was incubated

for 2 hours at 37°C. The DNA template was removed by incubation with 2 µL of RNase-free DNase (Promega) for 15 minutes at 37°C, and then the reaction was stopped by adding 2µL of 0.2M EDTA (pH8). To precipitate the RNA probe, 4M LiCl and ethanol were added to the tube at -20°C, and the mixture was left overnight at -80°C. The following day the mixture was centrifuged at 13000 rpm at 0°C for at least 30 minutes. The supernatant was discarded and the pellet resuspended in 20 µL of RNase-free water. 1 µL of the probe mixture was run on a 1% agarose gel to verify the presence of an RNA fragment, and the remaining probe was further diluted in 50% hybridization buffer (Table 2.1).

Whole-mount *in situ* hybridization of zebrafish embryos was conducted using a modified protocol described by Lauter *et al.* (2011). Fixed embryos were first treated with 3% H_2O_2 in methanol for 20 minutes followed by rehydrated using 25%, 50% and 75% serial dilutions of PBT in methanol respectively. Embryos were rinsed several times in PBT and were then permeabilized by incubation with Proteinase K (Promega). The Proteinase K reaction was stopped with a short rinse in 5% glycine in PBT followed by a 15 minute fixation with 4% PFA, and then 3, 5 minute washes in PBT. The embryos then underwent pre-hybridization in 50% hybridization buffer at 65°C for 3-6 hours. Next, pre-hybridization buffer was removed and replaced with probe in a 1:100 dilution of hybridization buffer containing 5% dextran sulfate. Hybridization occurred overnight at 65°C. The following day, probe and buffer was removed and the embryos were washed twice for 5 min with a 50% fomamide, 2x SSC, 0.1% Tween solution at 60°C. This was followed by a 15 min wash in 2xSSC at 60°C, and by 2 x 30 minute washes in 0.2x SSC at 60°C. Embryos were then transferred to PBT at room temperature.

In order to detect the probe, embryos underwent a blocking procedure in 10% sheep serum in PBT for 1 hour at room temperature. Next, embryos were rocked at room temperature for 2 hours in 10% sheep serum in PBT containing a 1/5000 dilution of anti-digoxigenin alkaline phosphatase-congugated antibody (Roche). The embryos were then rinsed quickly two times with PBT and then washed overnight. The colour reaction took place the following day, where embryos were first equilibrated to a higher pH through 3 x 5 min washes in NTT solution (Table 2.1). Next, the embryos were stained in 1 mL of NTT containing 4.5 µL NBT (Roche) and 3.5 µL BCIP (Roche). The embryos were allowed to react in the dark and took a variable amount of time, ranging from about an hour, to almost 4 days. Once the desired depth of staining had been reached, the reaction was stopped with a 15 minute rinse in 4% PFA in PBS, and 2 quick rinses in PBT. Embryos were typically stored in PBT at 4 °C for imaging.

2.3 Morpholino knockdown

Morpholinos were obtained from Gene Tools LLC (Corvallis, OR) and were diluted in water to a stock concentration of 2mM (Table 2.3). Zebrafish embryos were collected before the 8 cell stage and had their chorions removed using pronase for 1.5 minutes. Embryos were injected using a Femtojet injector (Eppendorf AG, Hamburg, Germany) and visualized under a dissection microscope (Leica Microsystems, Richmond Hill, ON). Morpholino dose was calculated by measuring the diameter of the injection liquid at a specific magnification and using volume injected and concentration to convert the value to ng. Embryos were raised at 28.5 °C in E3 according to standard zebrafish protocol before undergoing various experiments including hemorrhage counts, ISH and TEM. For hemorrhage counts, 48 hpf live zebrafish embryos were viewed under white light and any embryos without circulating blood were

omitted. Each fish was observed and the proportion of embryos with hemorrhages was recorded.

To verify that the morpholino was successful in knocking down endogenous *nkx3.2* signal, a splice site-targeting morpholino was used. Here, a RT-PCR using the same conditions previously for probe synthesis was conducted using both wildtype and morphant cDNA. Primers in Exon1 and Exon2 flanked the targeted splice site between Exon1 and Intron1. When the DNA product visualized by gel electrophoresis revealed an intron inclusion, this was indicative of successful morpholino knockdown whereas a smaller band was indicative of the normal endogenous product.

For overexpression of *nkx3.2,* DNA constructs had been synthesized previously by Jing Liu and Jae-Ryeon Ryu in the Tol2 vector by Gateway cloning as previously described (Kwan *et al.,*2007; Kikuta & Kawakami, 2009). Here, LR cloning was used to incorporate the desired promoter-gene-reporter into a plasmid backbone. For hsp70:nkx3.2;myc, a heat-shock promoter, full length nkx3.2, a myc tag and a poly-A tail were incorporated into the pDestTol2 plasmid backbone (Kwan *et al.,* 2007). For the sox10:nkx3.2;myl7:GFP construct, the sox10 promoter, full length nkx3.2, and a poly-A tail were incorporated into the pDestTol2CG2 backbone (Kwan *et al.,* 2007) which contains a *cmlc2*-EGFP-pA mini-gene used for identification of successful transformation by observing GFP in cardiac tissue. DNA was stored in aliquots of 25-50 ng/µL, and 4 µL were combined with 1 µL of transposase immediately prior to injection. The final injected dose was ≈40 picograms (pg) per embryo.

2.4 Antibodies

In order to detect transgenes colorimetrically, an anti-GFP antibody was used with DAB staining. Embryos which had previously been stained for gene expression using *in situ* hybridization, or un-stained, fixed embryos could be used. Embryos were rinsed 2-3 times quickly in 100% methanol, and then treated with pure acetone for 30 minutes at -20°C. They were then rinsed 3 times in PBT for 5 minutes each and blocked for 1 hour with 10% sheep serum in PBT. The primary antibody (mouse JL-8; Clontech Laboratories Inc.) was diluted 1:500 in PBT and incubated with the embryos at room temperature for 2 hours. The embryos were then washed 4 times (or more) for 15 minutes each in PBT. Next, the embryos were incubated with the secondary antibody (anti-mouse biotin-conjugated; Vector) in a 1:400 dilution in PBT for 2 hours at room temperature. The embryos were then washed 4 times (or more) for 15 minutes each in PBT. During the last few washes of secondary antibody, a mixture of 10 µL vectastain A (Vector Laboratories Inc., Burlingame, CA), 10 µL vectastain B, and 1 mL of PBT was rocked for 30 minutes, then added to each tube of embryos and they were rocked for 30 minutes. The embryos were washed 4 times (or more) for 15 minutes each in PBT and then transferred to 24 well plate and detected using DAB (VectorDAB, Vectorlabs). Solution was added to embryos in the 24 well plate and the reaction was monitored at room temperature under a dissection microscope. Once the desired level of staining had been reached, the reaction was stopped by removing the DAB solution, rinsing several times with PBT, and treating the embryos for 15 minutes with 4% PFA. The dish was stored in darkness at 4°C for further imaging or sectioning. If sectioning was done, embryos were embedded in JB4 medium (Polysciences, Inc, Warrington, PA) according to the manufacturer's instructions, and were then

moved into the proper orientation as the solution solidified. They were sectioned the following day at 7 µm using a Leica microtome.

2.5 Confocal microscopy

For high resolution imaging, a Zeiss LSM700 confocal microscope was used. Live embryos were mounted in 0.75% low melt agarose in E3 and positioned into the proper orientation. For timelapse images, the embryo in agarose were covered with E3 containing 0.04% buffered Tricaine in order to prevent movement and desiccation. Images were taken using either the 10x or 20x objective lens, and imaging typically involved compiling several z-stack layers to achieve image depth. Image analysis was performed using Zeiss Zen software, where adjusting levels, compiling z-layers, and producing video was possible. All cell counts and size measurements were done using ImageJ software (Schneider *et al.,* 2012).

2.6 Transmission electron microscopy

For transmission electron microscopy of zebrafish embryos, embryos were fixed in 0.1M sodium cacodylate (pH=7.4) containing 2% glutaraldehyde for 1 hour at room temperature, before 3x5 minute washes with 0.1M sodium cacodylate. A postfix step was conducted, where the fixed embryos were treated with 1% osmium tetroxide in 0.1M sodium cacodylate for 1 hour on ice followed by another 3 washes with sodium cacodylate for 5 minutes each at room temperature. A serial dehydration was done using 5 minute washes with 25%, 50%, 70%, and 95% high-grade ethanol. Two final 30 min washes in 100% anhydrous ethanol were then done to ensure complete dehydration. Embryos were then washed 3 times in propylene oxide; twice for an hour and once overnight. Next, the embryos were embedded using serial dilution of resin

in propylene oxide. Resin contained 10mL EmBed 812 resin, 8mL DDSA, 4 mL NMA, and 0.33mL DMP-30 (Polysciences, Inc). Infiltration of resin was done by rocking embryos in a 3:1 dilution of propylene oxide and resin for an hour or longer followed by a 1:1 propylene oxide-resin ratio for an hour or longer. Next the embryos rocked overnight in a 1:3 ratio of propylene oxide-resin, which was followed by 24 hours in 100% resin. Embryos were positioned carefully in a rubber block and left to polymerize in a vacuum oven for 48 hours at 60°C. Sectioning and post-staining were conducted by Wei-Xiang Dong from the University of Calgary Microscopy and Imaging Facility (MIF) by standard methods. Imaging was conducted using a Hitachi model H-7650 transmission electron microscope.

2.7 Alcian blue staining

The Alcian blue staining procedure was simplified from methods used by Walker & Kimmel (2007). Fixed 4-day old embryos were transferred out of PBT and into a 0.1% solution of Alcian blue in 5% glacial acetic acid, 70 % pure ethanol, and 25% ddH2O. The solution was incubated overnight at room temperature and the following day was washed 5 times with 5% glacial acetic acid, 70 % pure ethanol, and 25% ddH2O. Embryos were then further cleared by placing in pure glycerol overnight, and were imaged the following day in glycerol. The absence of the jaw joint in *nkx3.2* MO injected embryos indicated the successful knockdown of the gene as observed in Miller *et al* (2004).

2.8 Neural crest migration

In order to document neural crest cell migration, timelapse confocal microscopy was conducted as previously described. Individual cells were identified and their position was marked once every hour for 21 hours manually on a separate layer in Adobe Photoshop. The 21 images could then be stacked, and the distance and course of neural crest migration was measured using ImageJ software. In addition to distance, migration speed (distance/time) and directionality (distance from starting point to end point/total distance travelled) were measured. The compilation of all available timepoints was also used to create timelapse videos using Zeiss Zen Blue software.

2.9 Scoring criteria and statistics

For hemorrhage counts and general analysis of zebrafish phenotypes, only fish with normal blood circulation were counted. In addition, morphant fish were carefully selected for developmental stage based on somite count, eye size and body-angle to ensure that any morphological changes were not due to developmental delay. Data comparing groups of morphant and wild-type fish from the same clutch of embryos was typically paired, and was analyzed as such by utilizing the Student's paired t-test. For cell migration data, individual cell migration patterns were analyzed using a standard Student's t-test.

Table 2.1: Table of reagents

Solution	Contents
E3	- 34.8g NaCl - 1.6g KCl - 5.8g $CaCl_2 \bullet 2H_20$ - 9.78g $MgCl_2 \bullet 6H_20$ - H_2O to a final volume of 2L
PBS	- 8g NaCl - 0.2g KCl - 1.44g Na_2HPO_4 - 0.24g KH_2PO_4 - H_2O to a final volume of 1L
PBT	- 8g NaCl - 0.2g KCl - 1.44g Na_2HPO_4 - 0.24g KH_2PO_4 - 1mL Tween 20 - H_2O to a final volume of 1L
TBE	- 10.8g Tris base - 5.5g Boric acid - 4mL 0.5M EDTA - H_2O to a final volume of 1L
Hybridization buffer	- 50mL formamide - 25mL 20x SSC - 500mg torula yeast RNA - 5mg heparin - 40µL Tween 20 - 25 mL RNAse-free H_2O
20x SCC	- 175g NaCl - 88.2g Sodium citrate - H_2O to a final volume of 1L
NTT	- 4mL 1M Tris (pH 9.5) - 4mL 1M NaCl - 40µL Tween 20 - 32 mL H_2O

Table 2.2: Table of primers

Gene	Forward Primer	Reverse Primer
nkx3.2	TGGACCGCGTAATCTTAACC	aatttaatacgactcactataggACGTCGCTTGGTTTTGTACC
nkx3.2 MO efficiency	TGAACGAGCTGGATGTGTGT	ACGTCGCTTGGTTTTGTACC
pdgrfα	CCCCATTCCCTGAAGTGGAC	aatttaatacgactcactataggCACAGGCTGAGAACGGCTTA
sox10	ACCGTGACACACTCTACCAAGATGACC	taatacgactcactataggCATGATAAAATTTGCACCCTGAAAAGG
foxf2a	CGAATTGATAGGTGGTCGCG	tgtaatacgactcactataccCTTGACACACTCCCTGGTGA
shha	CTACGGCAGAAGAAGACAT	aatttaatacgactcactataggTGACCGCTATCATCAACAA
ctgf	CTCCCCAAGTAACCGTCGTA	aatttaatacgactcactataggTAGTGGTACAGCCGGAAA
cdh2	CATCCCGGAGACATAGGAGA	aatttaatacgactcactataggACGTTTCGTACCGATGATCC

*****smad1 probe from McReynolds *et al.,* 2007
**ptc probe from Wolff *et al.,* 2003

Table 2.3: Table of morpholinos

Morpholino	Sequence	Target	Dosage per embryo
nkx3.2 #1	5'-GCGCACAGCCATGTCGAGCAGCACT-3'	ATG site	54 ng/embryo
nkx3.2 #2	5'-GCGGAGCATTAGGGTTAAGATTACG-3'	ATG site	3.4 ng/embryo
nkx3.2 #3	5'- AAGCTTTGAGTGGTACTCACCGGAC-3'	e1i1 splice site	3.4 ng/embryo

Table 2.4: Table of transgenic lines

Transgenic line	Transgene	Origin	Fluorophore
Tg(kdrl:EGFP)^{la116}	kdrl	Choi *et al.,* 2007	EGFP
Tg(kdrl:mCherry)^{ci5}	kdrl	Proulx *et al.,* 2010	mCherry
Tg(acta2:EGFP)^{Ca7}	acta2	Whitesell *et al.,* 2014	EGFP, mcherry
Tg(gata1:dsRed)^{sd2}	gata	Traver *et al.,* 2003	dsRed
Tg(sox10:EGFP)^{ba2}	sox10	Carney *et al.,* 2006	EGFP

3 CHAPTER THREE: RESULTS

Impaired associations between endothelial and mural cells as in *iguana* mutant zebrafish embryos lead to a lack of vascular stability and hemorrhage. My project, the study of transcription factor *nkx3.2,* provides an example of a downstream effector of Shh in vascular stabilization involved in the recruitment of perivascular mural cells. I identify *nkx3.2* as being downstream of *dzip-1* and *Shh,* and expressed in the vicinity of vessels. I show that *nkx3.2* is required for vascular stabilization by mural cells, as knockdown of the gene results in hemorrhage, low numbers of mural-marker expressing cells, and reduced coverage of mural cells adjacent to the endothelial cells of blood vessels. Hemorrhages occur in diverse regions of the zebrafish head, though they typically form in newly formed vessels or ones undergoing reorganization. *nkx3.2* negatively regulates the neural crest specification gene *sox10* and positively regulates the neural crest migration effector *pdgfrα*. The interaction of *nkx3.2* with *pdgfα* is especially significant as we show the importance of the PDGF signaling pathway in mural cell recruitment and vascular stabilization. Finally, I show that *nkx3.2* partially colocalizes with *sox10* in neural crest cells, where it functions in a cell autonomous manner to influence normal migration of neural crest derivatives including, presumably, precursors of perivascular mural cells. Together, my results present a compelling example of a transcription factor acting in a regulatory network involving neural crest transcription factors, which eventually leads to the recruitment of perivascular mural cells and the establishment of vascular support.

3.1 nkx3.2 expression is controlled by *dzip-1* and *Shh*

Iguana (*igu*^{fo10a}) is a null mutation in the zebrafish *dzip-1* gene, which results in the loss of primary cilia and thus disruption of cell signalling (Wolff *et al.,* 2003; Sekimizu *et al.,* 2004; Lamont *et al.,* 2010), including the sonic hedgehog pathway. Since both *igu* mutants and shh disrupted fish (cyclopamine treated) hemorrhage, this suggests that Shh promotes a vascular stabilization pathway (Lamont *et al.,* 2010). In order to find genes downstream of Shh involved in vascular stabilization, our lab used a microarray analysis to identify genes with changed expression when Shh signalling was deficient in both *iguana* mutant and cyclopamine treated zebrafish embryos.

My project studies the homeobox-containing transcription factor *NK3 homeobox 2* (*nkx3.2*), which was downregulated on the array. In order to verify differential *nkx3.2* gene expression downstream of Shh, I used *in situ* hybridization of an *nkx3.2* probe in 30 hours post fertilization (hpf) *iguana* genetic mutant and wildtype zebrafish embryos. I chose to examine expression at 30 hpf because it is near the onset of vascular stabilization (Lamont *et al.,* 2010) and was the timepoint from which the microarray data was gathered. I found that *nkx3.2* was downregulated in the *iguana* mutant fish at 30 hpf, the same developmental timepoint used for the microarray analysis (Figure 3.1). Under normal conditions *nkx3.2* is expressed strongly at the base of the notochord, and faintly in the brain region and ventral head at 30 hpf (Figure 3.1 A). The expression level of *nkx3.2* in *igu*^{fo10} mutant zebrafish is reduced in these regions compared to controls (Figure 3.1 B). I note that expression in the brain is faint at this timepoint, making it difficult to determine exactly which type of cells express it. In the future, once we have better markers of perivascular mural cells, we will need to undertake this analysis using

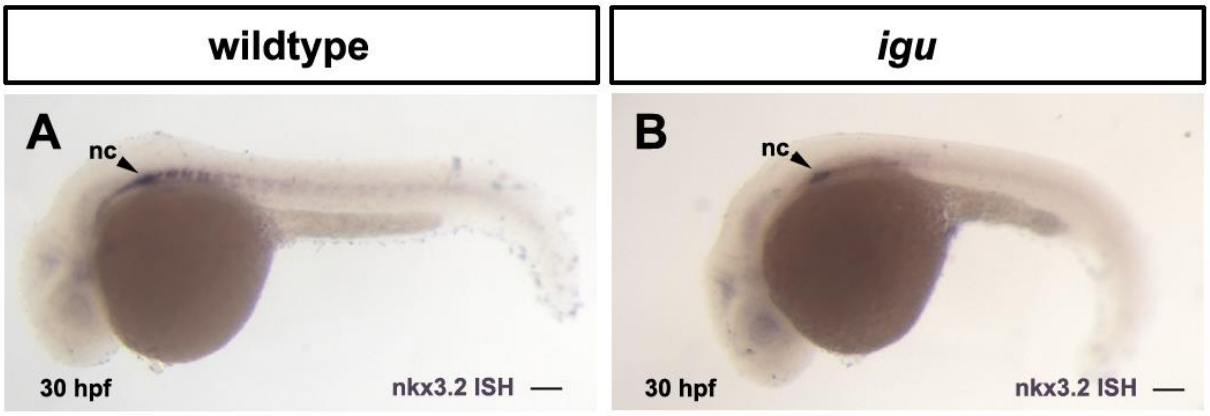


Figure 3.1: *nkx3.2* is downregulated in *igu*^{fo10} mutant zebrafish embryos.

(A) 30 hpf wildtype embryos express *nkx3.2* around the anterior tip of the notochord and peri-notochord cells in the trunk. Faint staining is also seen in the brain region. (B) *nkx3.2* is downregulated in *igu*^{fo10} mutant embryos of the same stage. Scale bars = 100µm

markers of this lineage to show co-expression.

3.2 *nkx3.2* is sufficient to stabilize blood vessels downstream of *iguana*

Genetic rescue through overexpression of potential downstream genes is a valuable tool for piecing together regulatory networks (Mahlmann *et al.,* 1998). To test whether *nxk3.2* was sufficient to stabilize blood vessels downstream of *iguana,* a former graduate student in the laboratory, Jing Liu, overexpressed *nkx3.2* under the heat shock protein promotor and tagged it with a myc epitope to follow expression (hsp70:nkx3.2myc DNA). She counted hemorrhage and compared this to non myc-positive mutants, and sorted for embryos positive for myc (red fluorescence; Figure 3.2 B), and with the *iguana* phenotype (curly down body axis). She found that upregulation of *nkx3.2* was sufficient to partially rescue the hemorrhage rate in the mutant fish (Figure 3.2 C). *Iguana* mutant zebrafish with elevated *nkx3.2* expression levels had lower hemorrhage rates (28.7%) than their un-injected counterparts (51.0%; p=0.001; as determined by the Student's paired t-test). The data supports my hypothesis that *nkx3.2* functions downstream of *iguana* in a pathway required for vascular stabilization.

3.3 *nkx3.2* is expressed adjacent to blood vessels from 24-48 hpf

To determine where and when *nkx3.2* is expressed in developing embryos, I conducted an expression analysis by *in situ* hybridization (Figure 3.3). The time period between 24 and 48 hours is an important window for vascular stabilization, and is notably relevant to the hemorrhage defects observed in *iguana* mutants as fish have hemorrhaged by 48 hpf (Lamont *et al.,* 2010). I chose to use this time period for my *nkx3.2* expression analysis, sampling

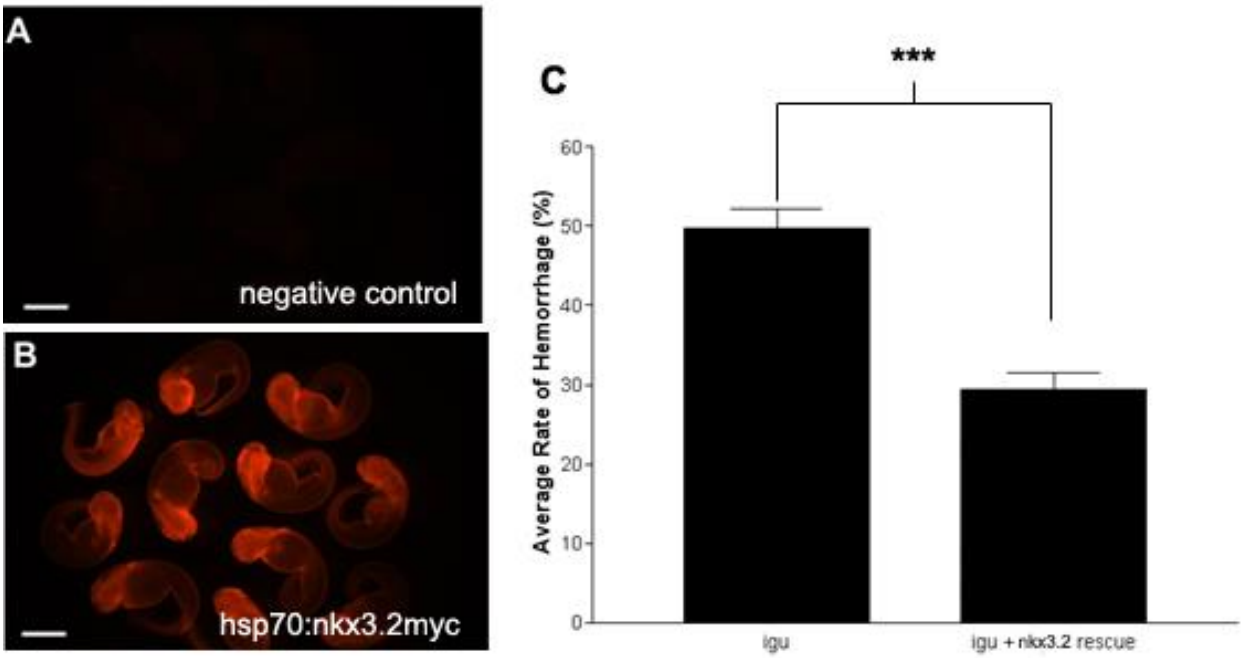
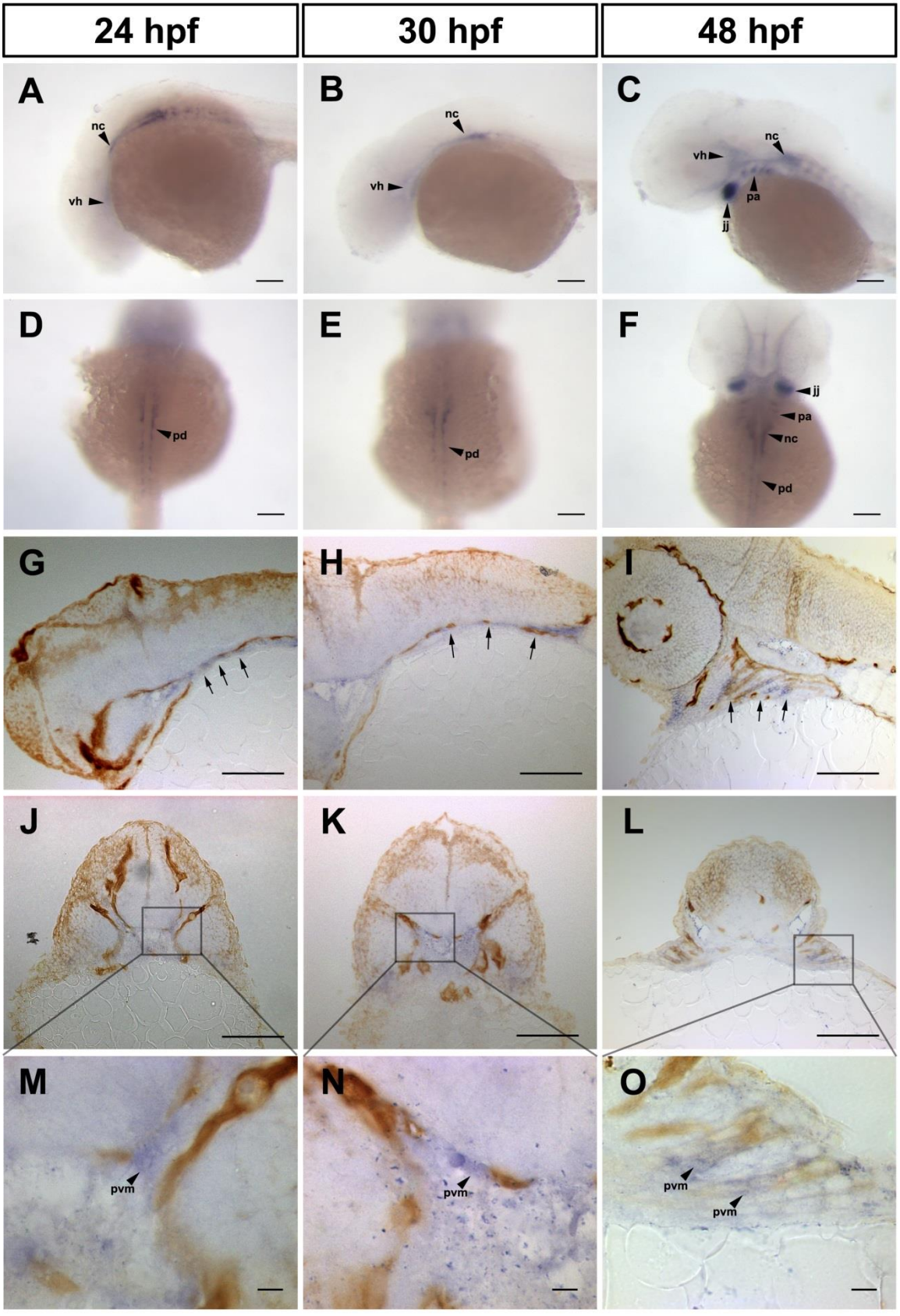


Figure 3.2: Overexpression of *nkx3.2* rescues hemorrhage in *igu*^{fo10} mutant zebrafish.

(A) Un-injected negative control embryos do not fluoresce at 54 hpf. (B) Embryos injected with the hsp70:nkx3.2myc DNA construct and activated using a heat-shock express the myc reporter as detected by antibody staining. Red fluorescent embryos with the *iguana* phenotype were included in the hemorrhage screen.(C) Hemorrhage rates were lower in *iguana* mutant embryos that had received the construct and undergone heat-shock as compared to un-injected iguana mutants. Scale bars = 500µm. *** indicates p=0.001 as determined by the Student's paired t-test. *Experiment conducted by Jing Liu.*


24 hpf
30 hpf
48 hpf
A
nc
vh
B
nc
vh
C
nc
vh
pa
jj
D
pd
E
pd
F
jj
pa
nc
pd
G
H
I
J
K
L
M
pvm
N
pvm
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pvm

Figure 3.3: *nkx3.2* is expressed in diverse tissue types of the embryonic zebrafish from 24-48 hpf, including perivascular mesenchyme.

(A-F) Wholemount *in situ* hybridization of *nkx3.2* shows expression at the base of the notochord (nc; A-C and F), in the ventral head (vh; A-C) from 24-48 hpf and in the pronephric ducts (pd) from 24-48 hpf (D-F). At 48 hpf expression is also visible in the developing jaw joint (jj) and in the pharyngeal arch region (pa) (C, F). (G-O) Sections of double-stained ISH/ anti-GFP Ab stained embryos reveal *nkx3.2* expression (blue, NTT-NBT-BCIP staining) near blood vessels (brown, anti-GFP staining). (G,H,I) In ventral areas of the head, *nkx3.2* is expressed alongside vessels at 24, 30, and 48 hpf (arrows). (J-O) Transverse sections show *nkx3.2* expression immediately adjacent to blood vessels in perivascular mesenchyme (pvm). Scale bars (A-I) = 100µm. Scale bars (M-O) = 10µm

expression at 24, 30, and 48 hpf, looking specifically for gene expression spatially in the vicinity of vessels.

From 24-48 hours post fertilization *nkx3.2* is expressed consistently at the base of the notochord and in the pronephric ducts (Serluca & Fishman, 2001; Figure 3.3 A-F). At 48 hours enriched staining appears in the ventral head region, particularly in the developing jaw joint and pharyngeal arches (Figure 3.3 C & F). In order to identify *nkx3.2* expression in or around blood vessels, the expression analysis was repeated in Tg(kdrl:GFP) fish which express GFP in blood vessels, and an anti-GFP antibody stain was used to view vessels in brown. From lateral sections I found that *nkx3.2* was expressed near blood vessels at 24, 30, and 48 hpf (Figure 3.3 G-I). I further analysed transverse sections at lower (J, K, L) and higher (M, N, O) magnifications, which revealed *nkx3.2* expression immediately adjacent to endothelial cells in perivascular mesenchyme (pvm). This data shows that *nkx3.2* is expressed in perivascular mesenchymal cells adjacent to vessels, and suggests a potential role of the Nkx3.2 transcription factor in mural cell recruitment.

3.4 *nkx3.2* is required for vascular stabilization

In order to determine what role *nkx3.2* has in vascular stabilization, it is important to assess the consequences of *nkx3.2* knockdown. Morpholino antisense technology is a standard technique in the field in assigning biological functions to genes in zebrafish (Ekker, S., 2000). In order to test the consequences of *nkx3.2* knockdown, I injected morpholino oligonucleotides into zebrafish embryos between the 1 and 8 cell stage (Figure 3.3). Three morpholinos were tested, and hemorrhage rates were counted in un-injected controls (UIC) and morpholino

injected (morphant; MO) embryos at 48 hpf. Morpholinos were designed with sequences specific to *nkx3.2* in the zebrafish genome, and dosages adjusted in order to minimize embryo-wide toxicity or off-target effects. Some criteria that I considered to be toxic or off-target effects, and thus necessitated a lower dosage, were reduced circulation, embryos size, eye size or embryonic lethality. The first MO, an ATG targeting MO, caused hemorrhage (UIC=3.7%; *nkx3.2* MO=36.2%; p= 0.107 as determined by the Student's paired t-test) but was only functional at a very high dose (54 ng/embryo) and so was abandoned for further experimentation (Figure 3.3 F). The second, another ATG targeting morpholino, was functional at a much lower dose (3.4 ng/embryo) and caused hemorrhage (UIC=2.67%; *nkx3.2* MO=27.6%; p=0.027 as determined by the Student's paired t-test), but was difficult to dissolve and came out of solution easily (Figure 3.3 G). Each of the ATG-targeting morpholinos have been used previously by another group and have been shown to cause facial bone defects (Miller *et al.,* 2004). The third morpholino, a splice targeting morpholino was advantageous to us in that is caused hemorrhage (UIC=5.8%; *nkx3.2* MO=31.3%; p=0.008 as determined by the Student's paired t-test) at a low dose (3.4 ng/embryo) and I was able to verify mis-splicing of the *nkx3.2* message using RT-PCR (Figure 3.3 C). The RT-PCR results for *nkx3.2* from the UIC sample show a thick band of the correct size as a properly spliced *nkx3.2* fragment. In contrast, the *nkx3.2* morpholino cDNA sample had a different set of bands, most significantly a very dark, larger band which I excised from the gel and had sequenced. This mis-spliced cDNA contained the whole intron between exon 1 and 2 (data not shown), indicating that the morpholino was successful in disrupting an mRNA splice site. Although only one of the three morpholino oligomers was selected for further experimentation, each produced a nearly identical

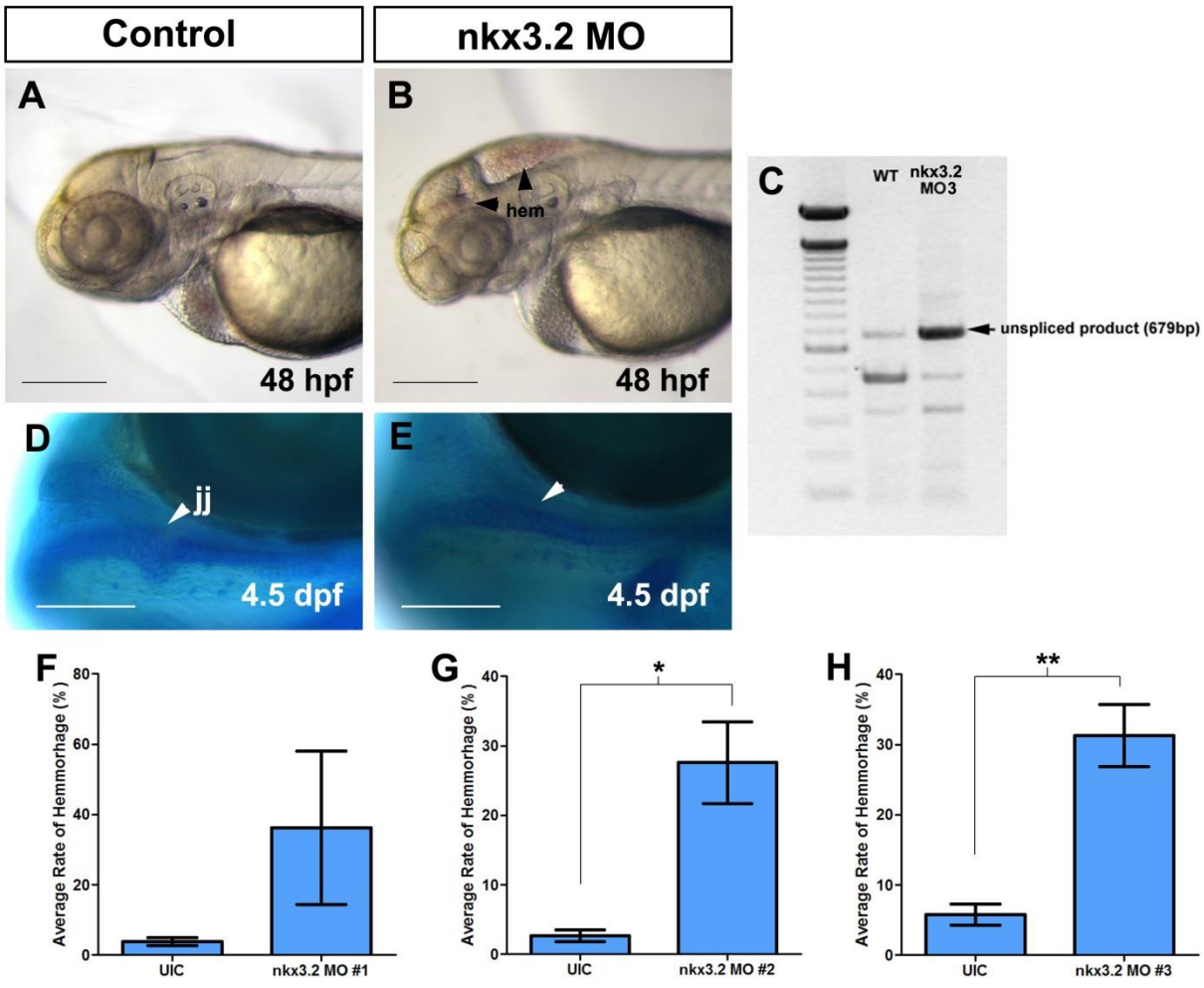

Figure 3.4: Morpholino knockdown of *nkx3.2* causes hemorrhage at 48 hpf and facial bone defects at 4.5 dpf.

(A) While uninjected embryos develop normally, (B) embryos injected with *nkx3.2* morpholino have observable hemorrhages. (C) A morpholino verification shows successful knockdown, as a larger, unspliced mRNA fragment is present in injected embryos. (D,E) 4.5 dpf embryos stained with alcian blue have an observable early jaw-joint protrusion in wild type embryos (D), but fail to form a jaw-joint when *nkx3.2* is disrupted (E). (F,G,H) Injection of three different morpholinos produced near-identical hemorrhage frequencies , though the third (splice-targeting) was the most reliable with a high hemorrhage rate, verifiability by RT-PCR (C), and an effective relatively low dose. Scale bars (A, B) = 200µm. Scale bars (D, E) = 100 µm. * indicates p= 0.027 as determined by the Student's paired t-test. ** indicates p= 0.008 as determined by the Student's paired t-test

hemorrhage rate and morphological facial defects which were detectable under bright field microscopy. In addition, I used a cartilage stain, alcian blue (Figure 3.3 D, E) to show that the jaw-joint fails to form when *nkx3.2* is deficient, replicating the published phenotype (Miller *et al.,* 2004). The occurrence of hemorrhage implicates *nkx3.2* as an effector of vascular stabilization, whereas facial bone defects are typical of neural crest developmental abnormalities.

3.5 nkx3.2 knockdown fish express lower levels of the vascular smooth muscle marker *acta2* than controls

In order to determine whether the biological function of *nkx3.2* in developing zebrafish embryos is related to the recruitment or development of perivascular mural cells, I examined the expression of the vascular smooth muscle marker *α-smooth muscle actin (acta2).* Our lab has developed a transgenic zebrafish line that drives GFP in *acta2*-expressing cells, allowing the mural cell counts and analysis of mural cell morophology in live embryos (Whitesell, *et al.,* 2014). These transgenic fish can be crossed with Tg(kdrl:mCherry) fish, producing offspring with red blood vessels and green mural cells. The only disadvantage of this model is that the transgene turns on relatively late in development. Hence I used this model to image mural cells in the ventral head region of 4 days post fertilization (dpf) fish and count mural cells on certain blood vessels, even though this was after hemorrhage occurs in *nkx3.2* morphants.

Expression of *acta2* was observed in Tg(kdrl:mCherry;acta2:GFP) fish, and individual cells were counted on pharyngeal arch arteries. I found the number of *acta2* expressing cells was reduced in *nkx3.2* morphants (*nkx3.2* MO=8.1 cells; n=9; Figure 3.5 B & C) compared to

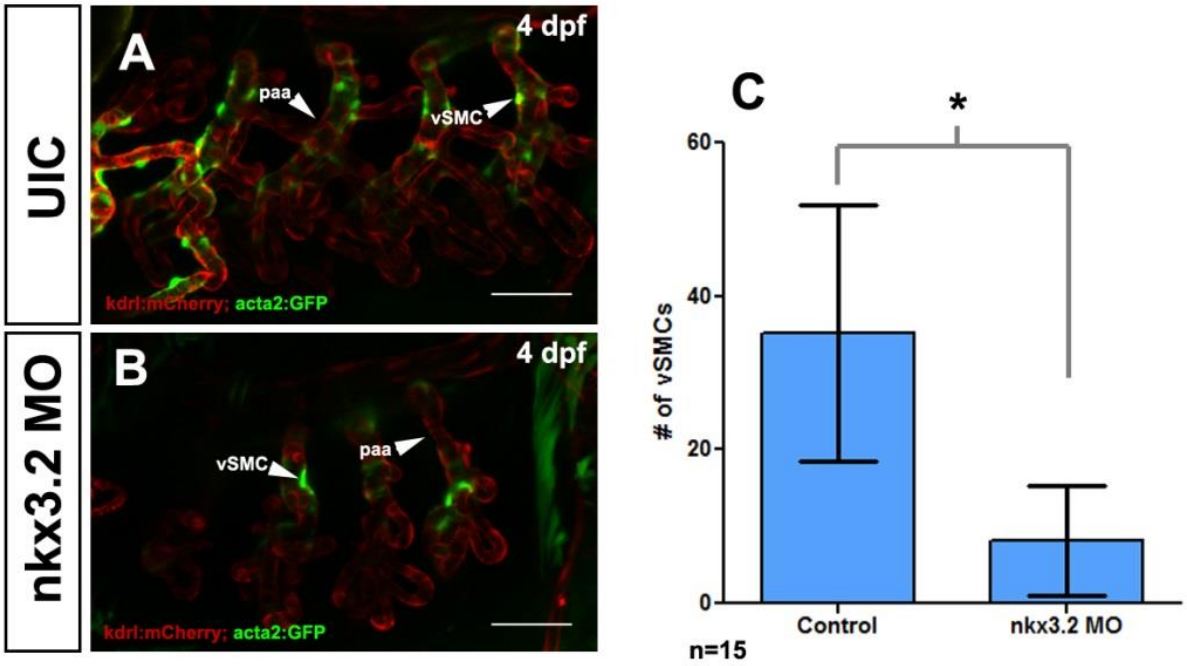

Figure 3.5: The number of cells expressing the vSMC marker *acta2* is reduced in *nkx3.2* morphant embryos.

(A) The pharyngeal arch arteries (paa) of uninjected 4 dpf Tg(kdrl:mCherry;acta2:GFP) zebrafish embryos are well covered in vSMCs, (B) while *nkx3.2* morphant embryos have fewer acta2-expressing cells on the same vessels(C). Scale bars = 50µm. * indicates p=0.0102 as determined by the Student's t-test.

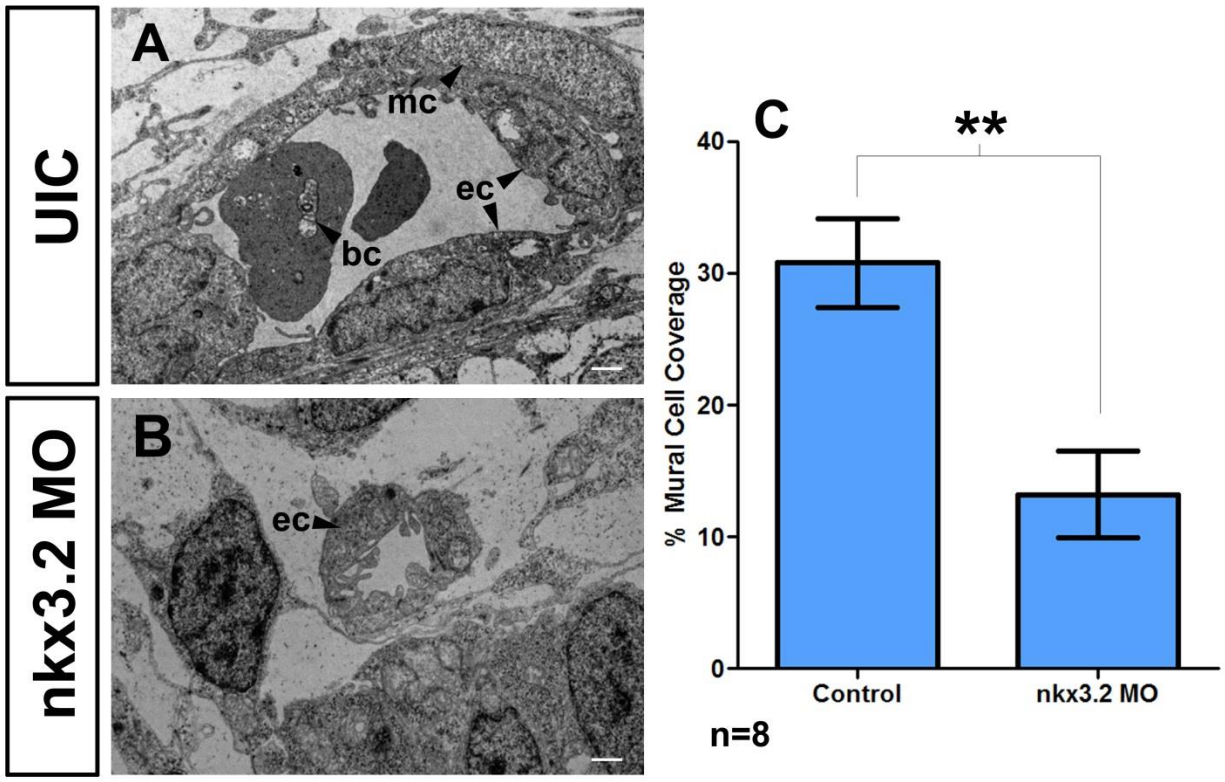

Figure 3.6: Transmission electron microscopy reveals *nkx3.2* morphant vessels have less surface contact with mural cells.

(A) A normal zebrafish lateral dorsal aorta (LDA) contains blood cells (bc) surrounded by the endothelial layer (ec). Mural cells (mc) form tight associations with the endothelium and cover a proportion of the vessel circumference. (B) *nkx3.2* morphant lateral dorsal aortas are smaller, have less blood, had fewer, if any, associated mural cells. (C) The percentage of the vessel circumference covered by mural cells was significantly reduced in *nkx3.2* morphant lateral dorsal aortas. Scale bars = 500nm. **indicates p=0.0022 as determined by the Student's t-test.

controls (UIC=35.2 cells; n=9; Figure 3.5 A & C; p=0.01 as determined by the Student's t-test). A reduction of *acta2* in mural cells suggests that the cells are either absent, or have not differentiated into mature vSMCs.

3.6 *nkx3.2* knockdown fish have lower mural cell coverage compared to controls

Because *acta2* expression does not turn on in mural cells until well after hemorrhage occurs in *nkx3.2* morphants, I chose to examine the ultrastructure of 48 hpf control and *nkx3.2* MO blood vessels using transmission electron microscopy as mural cells can be identified without a molecular marker using this method. I imaged the lateral dorsal aortas of control and *nkx3.2* MO embryos at 48 hpf and measured the circumference of the outer endothelial cell layer of each vessel, and the proportion of the circumference with membrane contact to mural cells. I found that *nkx3.2* knockdown embryos had lower coverage by perivascular mural cells (13.2% of the outer circumference; Figure 3.6 B & C) compared to controls (30.79% of the outer circumference; Figure 3.6 A & C; p=0.0022 as determined by the Student's t-test). This data suggests that early mural cell recruitment is regulated directly or indirectly by *nkx3.2*, as the vessels of morphant embryos are less covered by mural cells than controls at 48 hpf.

3.7 Real-time confocal imaging of hemorrhage identifies nascent CtAs as a frequent location of hemorrhage in *nkx3.2* morphants

In order to better characterize the location, cellular events, and timing leading up to hemorrhage, I examined hemorrhage in high definition using high resolution timelapse microscopy of *nkx3.2* MO in Tg(kdrl:GFP;GATA1:dsRed) zebrafish embryos between 30 and 49 hpf, taking images every 20 minutes. The embryos, which express GFP in blood vessels and

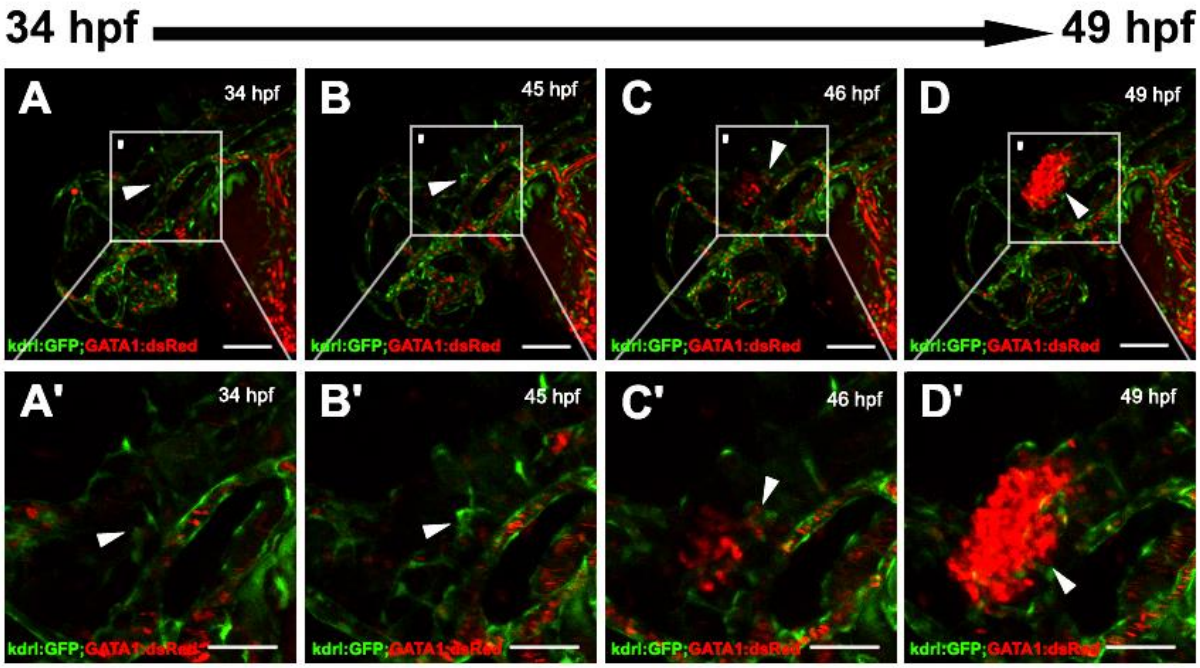

Figure 3.7: Confocal time-lapse of a Tg(kdrl:GFP;GATA1:dsRed) shows hemorrhage in the CtA vessels of an *nkx3.2* morphants.

(A) A new CtA sprouts at 34 hpf. (B)The young CtA bulges as it extends dorsally. (C) The vessel ruptures. (D) The blood pools dorsally. Scale bars (A-D) = 100µm. Scale bars (A'-D') = 50 µm

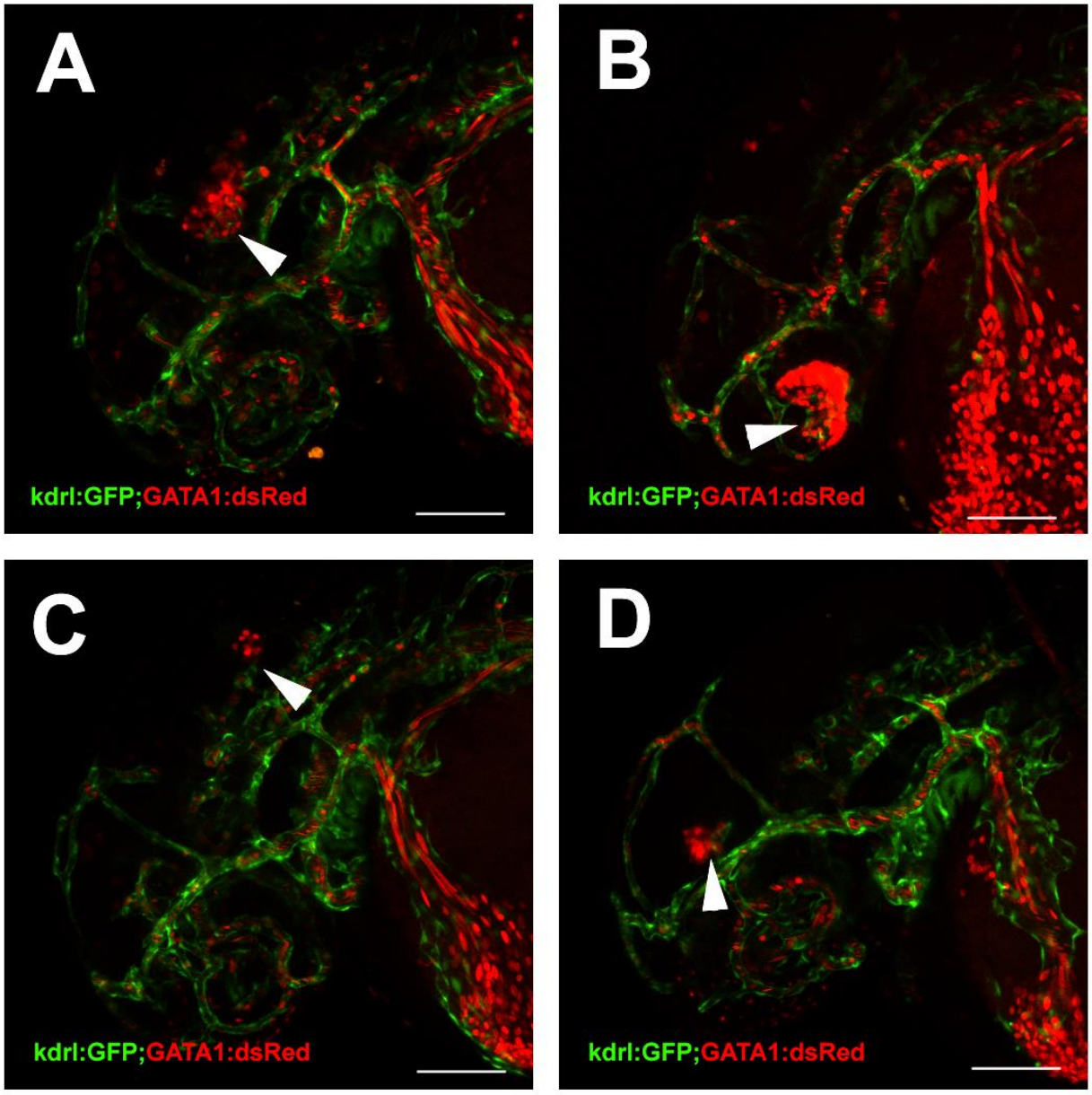

Figure 3.8: Confocal microscopy of tg(kdrl:GFP;GATA1:dsRed) embryos show diverse hemorrhage locations in *nkx3.2* morphants.

(A) A hemorrhage originating from an anterior CtA. (B) A hemorrhage originating from the inner optical circle (IOC). (C) A hemorrhage originating from a posterior CtA. (D) A hemorrhage originating from a vessel of the forebrain. Scale bars = 100µm.

DsRed in blood, enabled me to pinpoint the origins of hemorrhages to individual blood vessels at precise times. I found that hemorrhages caused by *nkx3.2* knockdown often occur in the central arteries (CtAs), at a rate of 6/8 embryos tested. Here, hemorrhages typical arise from vessels that have only recently sprouted from the main artery (Figure 3.7 A). In one example, before hemorrhage occurs, a bulge in the vessel can be seen (Figure 3.7 B) followed by the rupture of the vessel (Figure 3.7 C). Blood then leaks from the opening and pools, usually moving dorsally into surrounding tissues and the ventricle of the brain (Figure 3.7)

3.8 Real-time imaging of hemorrhage also identifies IOC and anterior head vessels as locations of hemorrhage in *nkx3.2* morphants

While CtA hemorrhages (Figure 3.7 and Figure 3.8 A&C) were the most commonly observed origin of hemorrhage in Tg(kdrl:GFP;GATA1:dsRed) zebrafish embryos, hemorrhages do arise from several other locations. These include the anterior head in the forebrain region (Figure 3.8 D; 1/8), and in the inner optical circle of the eye (IOC; Figure 3.8 B; 1/8). The CtAs undergo a period of growth during this time window, whereas the IOC primarily underwent vascular remodeling (Figure 1.1). Taken together, these results suggest that vascular beds undergoing active angiogenesis or remodeling are vulnerable to hemorrhage when *nkx3.2* is knocked down.

3.9 Testing potential nkx3.2 targets or target pathways

To identify the mechanism by which nkx3.2 modulates vascular stability I carried out expression analysis of genes with known roles in the recruitment, maturation, differentiation or adhesion of perivascular mural cells (Figure 3.9) with the hypothesis that these could be

potential targets of *nkx3.2*. Candidate *smad1*, a cofactor of *nkx3.2* in other systems, was found to be expressed in the brain and along the developing neural tube of 30 hpf embryos, and was not differentially expressed in un-injected or *nkx3.2* knockdown fish (Figure 3.9 A & E). I examined expression of the *Shh* pathway components *shha* and its receptor *ptc1* to test whether there was any potential feedback of *nkx3.2* with the up-stream signalling pathway. The ligand *shha* was expressed in the brain and along the floorplate in a very specific pattern at 30 hpf, and expression levels appeared to be very similar in both control and *nkx3.2* MO fish (Figure 3.9 B, F). The receptor *ptc1* was expressed in the head and throughout the trunk of the 30 hour fish embryos and head expression did not appear to change between control and *nkx3.2* morphants, though expression in the trunk appeared to be upregulated in *nkx3.2* morphants (Figure 3.9 C, G). Expression of *foxf2a,* a gene from our microarray that also appears to be downstream of *iguana,* was observed in the pharyngeal arch region of the zebrafish ventral head in both control and *nkx3.2* MO fish at similar levels (Figure 3.9 D, H). Expression of the growth factor connective tissue growth factor (*ctgf*) was observed along the floor plate at similar expression levels in control and *nkx3.2* MO embryos (Figure 3.9 I, M). The expression of the cell adhesion gene n-cadherin (*cdh2*) was observed in neural and mesodermal tissue of the head in control and *nkx3.2* MO embryos at 48 hpf and was found to be expressed in similar levels in each case (Figure 3.9 J, N). In summary, this directed candidate screen did not identify a clear target of nkx3.2, indicating that *nkx3.2* may not significantly regulate any of the tested sonic hedgehog components, genes isolated from the same microarray where nkx3.2 was identified, growth factors, or vascular adhesion genes.

In Contrast, I found large differences in expression of neural crest genes. For instance, I found that expression of the neural crest gene *sox10 (sex determining region Y-box 10*) was upregulated in *nkx3.2* MO embryos. Expression of *sox10* is necessary for neural crest (NC) fate specification and for the development of cell derivatives such as pigment cells and sensory neurons (Carney *et al.,* 2006). *Tg(sox10:GFP)* is also an established marker of zebrafish NC cells where migration and can be used to track development of individual cells in real-time (Matthews *et al.,* 2008). I observed that ISH staining of *sox10* in neural crest tissue including the pharyngeal arch region of the zebrafish head was much darker in *nkx3.2* morphant embryos compared to controls (Figure 3.9 K, O), suggesting that *nkx3.2* either directly or indirectly negatively regulates *sox10* under normal conditions.

I also found that expression of the platelet-derived growth factor receptor *pdgfrα* was downregulated in *nkx3.2* MO embryos. Expression of *pdgfrα* is required for normal neural crest (NC) migration and palatogenesis (Eberhart *et al.,* 2008). I observed that ISH staining of *pdgfrα* in neural crest-derived tissue including the pharyngeal arch region of the zebrafish head was very faint or even absent in *nkx3.2* morphant embryos compared to controls (Figure 3.9 L, P), suggesting that *nkx3.2* either directly or indirectly positively regulates *pdgfrα* under normal conditions.

3.10 The functional role of PDGF signalling in vascular stabilization

Platelet-derived growth factor (PDGF) signaling is important for the development of perivascular mural cells, specifically in the recruitment of brain pericytes in mouse (Betsholtz, *et al.,* 2005; Bondjers *et al.,* 2003). In order to test the effects of interfering with the PDGF

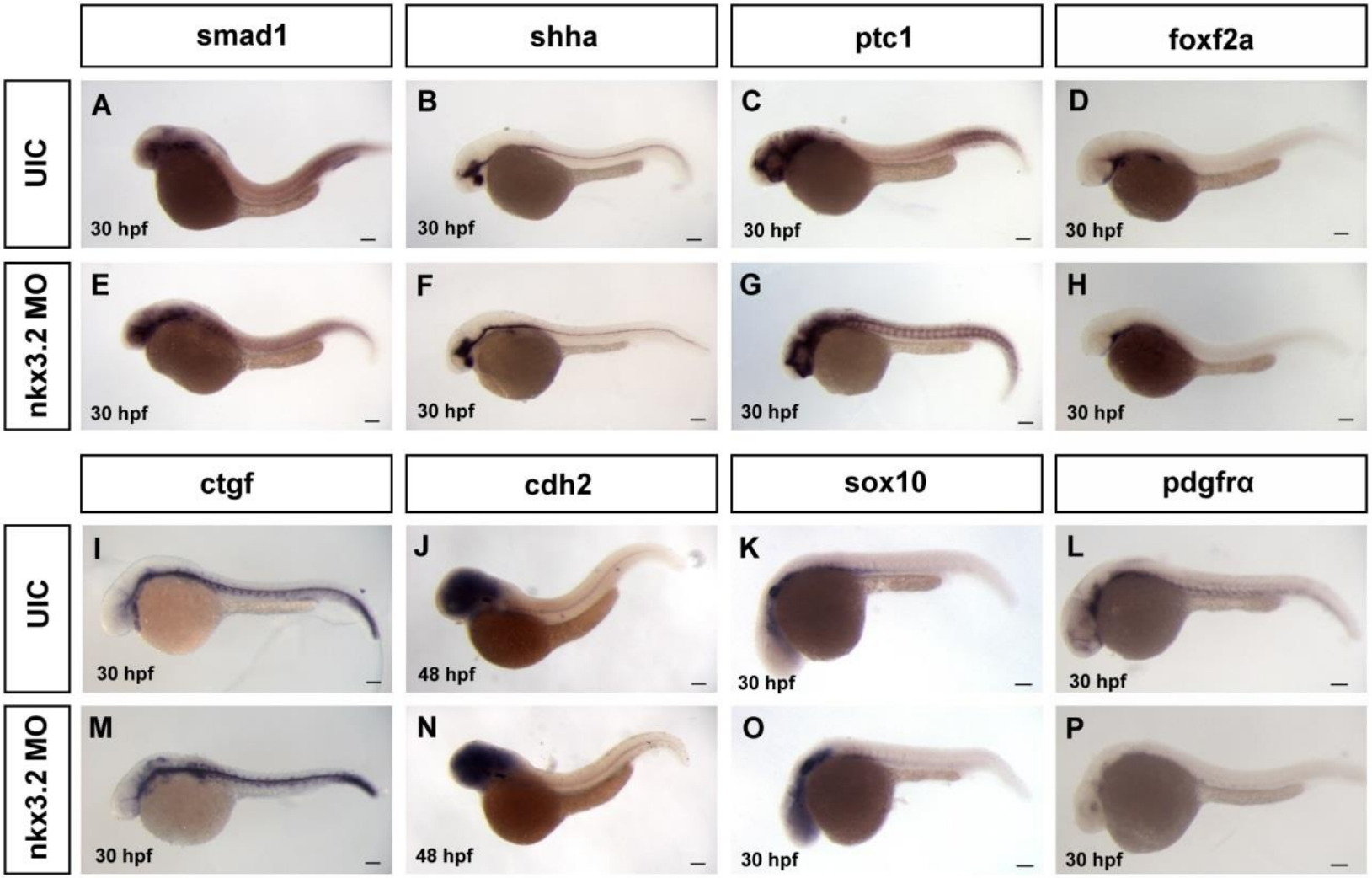
smad1
shha
ptc1
foxf2a
UIC
nkx3.2 MO
A
B
C
D
E
F
G
H
30 hpf
ctgf
cdh2
sox10
pdgfrα
I
J
K
L
M
N
O
P
48 hpf

Figure 3.9: Expression analysis of possible *nkx3.2* targets.

In order to identify targets of *nkx3.2*, expression analysis of known cofactors (*smad1*: A, E), sonic hedgehog components (*shha*: B, F; *ptc1*: C, G), genes from the microarray (*foxf2a*: D, H), growth factors (*ctgf*: I, M) vascular adhesion genes (*cdh2*: J, N), neural crest transcription factors (*sox10*: K, O), and growth factor receptors (*pdgfrα*: L, P) was conducted in un-injected and *nkx3.2* morphant embryos. Most of these potential targets yielded very similar expression patterns and levels in control vs. morphant embryos. Exceptions were *sox10* and *pdgfrα*, where *sox10* expression was increased and *pdgfrα* expression was decreased compared to un-injected controls. Scale bars = 100µm.

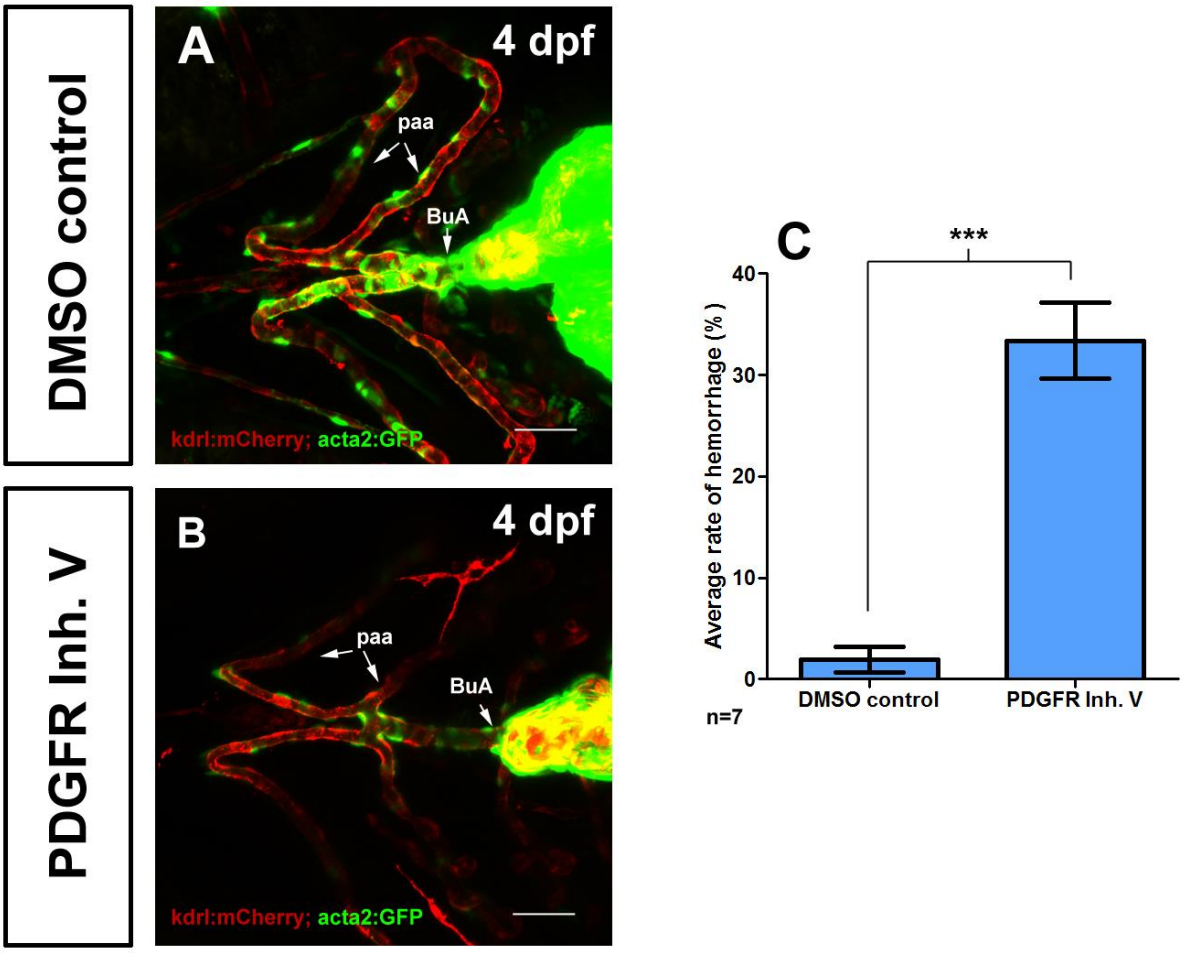

Figure 3.10: Disrupting PDGF signalling causes a loss of vSMCs and hemorrhage.

(A) Under normal conditions, a ventral view of the ventral aorta and pharyngeal arch arteries (paa) of a 4 day Tg(kdrl:mCherry; acta2:GFP) is rich in *acta2* expression. The outflow tract (bulbous arteriosus; BuA) is nearly completely covered in vSMCs and the other ventral vessels have many mural cells associated with the endothelial layer. (B) When PDGF signalling is disrupted with PDGFR inhibitor V, there is a reduction of *acta2* expression in the outflow tract and in the other ventral vessels of the zebrafish head. (C) In addition to a reduction in mural cell number, embryos treated with the PDGFR inhibitor hemorrhage at a very significant rate as compared with DMSO treated controls. Scale bars = 50µm. ***indicates $p<0.0001$ as determined by the Student's t-test. *Experiment conducted by Dr. Sarah Childs.*

signalling pathway on mural cell recruitment and vascular stabilization my supervisor, Dr. Sarah Childs, treated zebrafish embryos with 0.25 µM of PDGF receptor tyrosine kinase inhibitor V or with DMSO as a control as conducted by Wiens *et al.,* 2010 (Figure 3.10). PDGFR inhibitor V inhibits the activity of the PDGF receptor tyrosine kinases *pdgfrα* and *pdgfrβ* (Wiens *et al.,* 2010). Tg(kdrl:mCherry; acta2:GFP) embryos under normal conditions possess ventral head vessels with many *acta2* expressing cells. The outflow tract has a nearly complete sheath of vSMSc, and the neighboring ventral vasculature also has many mural cells associated with the endothelial layer (Figure 3.10 B). In contrast, the ventral aspect of PDFGR inhibitor V treated embryos had sparse coverage by perivascular mural cells. The lack of *acta2* expression could indicate poor recruitment of the cells, or a failure of the cells to normally mature and express vSMC markers. We also quantitated hemorrhage rates in PDGFR inhibitor V treated embryos to assess the importance of PDGF signalling on vascular stability. As expected, the treated embryos had a higher hemorrhage rate (33.4 %) than DMSO treated controls (1.9%; $p<0.0001$ as determined by the Student's t-test: Figure 3.10 C). The requirement of PDGR signalling for vascular stabilization and mural cell recruitment, the knowledge that *pdgfrα* regulates neural crest migration, and the fact that *nkx3.2* appears to regulate *pdgfrα*, all suggest a role for *nkx3.2* in an upstream pathway involving neural crest migration and mural cell recruitment.

3.11 Expression of nkx3.2 partially overlaps with sox10 expression

To test whether nkx3.2 is expressed in the neural crest derivatives, I looked for colocalization of sox10 and nkx3.2 using *in situ* hybridization (Figure 3.11 A) and detection of Sox10:GFP using an anti-GFP antibody stain in Tg(sox10:GFP) (Figure 3.11 B). I found that the two genes partially colocalize in a number of locations. A lateral section of the head of the 48

hpf zebrafish shows *nkx3.2* expression within individual *sox10* expressing cells around various facial bone tissues including near the jaw-joint; a neural crest derived tissue where *nkx3.2* has been previously shown to be active (Figure 3.11 C, C', C"). These tissues of the ventral head are also typically rich in vasculature and I have previously identified *nkx3.2* expression in this region immediately adjacent to vessels (Figure 3.3). Transverse sections of these double-stained embryos reveal additional sites of colocalization of the two genes surrounding the entire eye and in tissues of the ventral head near facial bones (Figure 3.11 D, D', D"). Tissues near the eye and bones of the ventral head are also highly vascular during this time. It is noteworthy that colocalization of the two genes is not complete, and so the interaction of the two factors is likely limited to regions of overlap. Because *sox10* is a general neural crest marker but does not identify individual lineages of the neural crest, the partial colocalization of *nkx3.2* could represent a distinct role of this transcription factor in a subset of NC cells (Carney *et al.,* 2006). Additional research is required in order to find the significance of this partial colocalization in the context of NC specification into distinct cell types. The data provides evidence that *nkx3.2* is expressed in some *sox10* expressing neural crest cells in vascular regions of the 48 hpf zebrafish head, where partial colocalization is observed.

3.12 *nkx3.2* functions autonomously in *sox10* expressing cells

To demonstrate that nkx3.2 is required in sox10 expressing cells, we made a construct expressing nkx3.2 under the sox10 promoter. The construct also carries a transgenesis marker myl7:GFP. Single cell embryos were injected with the DNA construct and were screened for transgenesis after 24 hpf by observing green hearts, and only these animals were counted (Figure 3.12 A). I found that upregulation of *nkx3.2* in *sox10* expressing cells was sufficient to

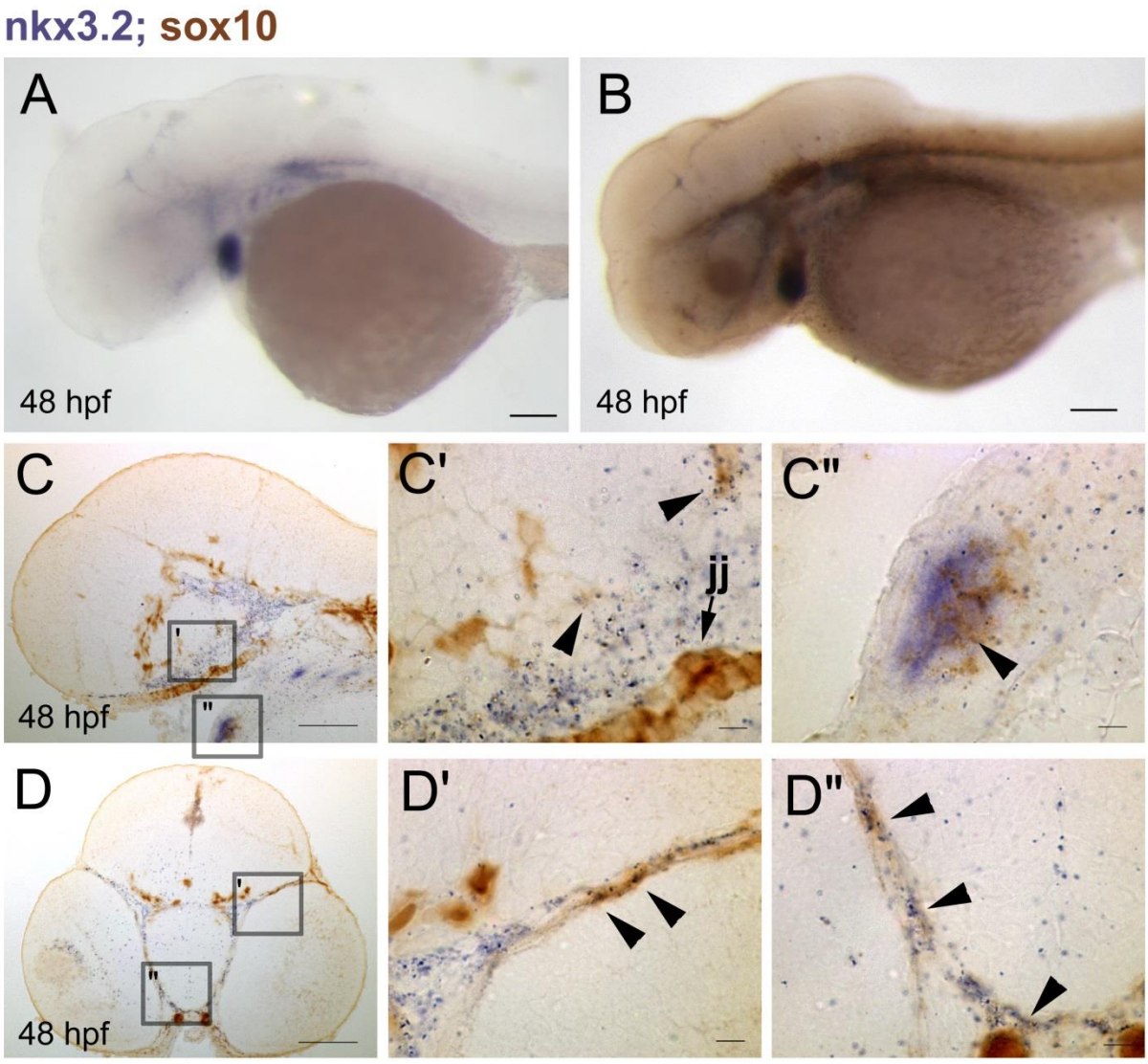

Figure 3.11: *sox10* partially colocalizes with *nkx3.2* in the zebrafish head at 48 hpf.

(A) An *nkx3.2 in situ* hybridization and (B) anti-GFP antibody stain in Tg(sox10:GFP) shows colocalization of the two markers at 48 hpf. (C)Lateral and (D) transverse sections reveal *nkx3.2* and *sox10* expression in similar tissues. (C', C") The lateral section viewed at a higher magnification shows colocalization (arrowheads) of the two genes in individual cells near the jaw-joint (jj) and other facial bones. (D', D") The tranverse section viewed at a higher magnification shows colocalization (arrowheads) of the two genes in individual cells surrounding the entire eye and ventrally near bone. Scale bars (A-D) = 100µm. Scale bars (C',C",D',D") = 10µm.

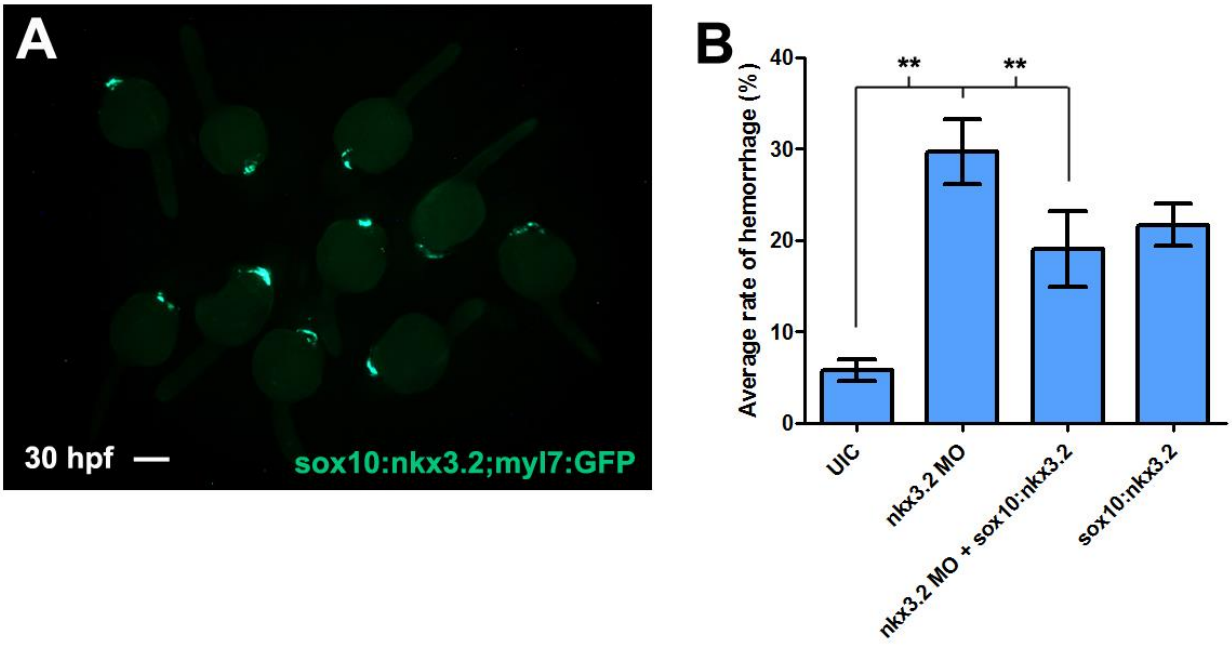

Figure 3.12: *nkx3.2* functions autonomously in *sox10* expressing cells.

(A) Transient transgenic expression of the sox10:nkx3.2;myl7:GFP DNA construct shows expression of GFP in embryo hearts. The sox10 promoter drives the nkx3.2 gene while myl7:GFP acts as a transgenesis marker. (B) By sorting embryos expressing the transgenesis marker, I show that sox10:nkx3.2 injection partially rescues hemorrhage in *nkx3.2* morphant zebrafish. The upregulation of *nkx3.2* in *sox10* expressing cells alone causes a higher hemmorhage rate compared to controls. Scale bars = 500µm. **(UIC vs. nkx3.2 MO) indicates p=0.003 as determined by the Student's paired t-test. ** (nkx3.2 MO vs. nkx3.2 MO + sox10:nkx3.2) indicates p=0.0073 as determined by the Student's paired t-test.

partially rescuing the hemorrhage rate caused by *nkx3.2* morpholino knockdown (Figure 3.12). I note that injection of the sox10:nkx3.2;my17:GFP construct alone caused significant hemorrhage compared to controls (UIC=5.8% vs. sox10:nkx3.2;my17:GFP = 21.7%; p=0.0035 as determined by the Student's paired t-test). Embryos injected with *nkx3.2* morpholino hemorrhaged at a rate of 29.7% compared to 5.8% in controls (p=0.003 as determined by the Student's paired t-test: Figure 3.12 B). Embryos injected with both *nkx3.2* morpholino and the sox10:nkx3.2;my17:GFP DNA construct had a reduced hemorrhage rate compared to *nkx3.2* morpholino alone (*nkx3.*2MO+ sox10:nkx3.2;my17:GFP = 19% vs. *nkx3.*2MO = 29%; p= 0.0073 as determined by the Student's paired t-test: Figure 3.12 B). This data suggests that *nkx3.2* functions in a cell autonomous manner in *sox10* expressing neural crest cells to mediatevascular stabilization, as the upregulation of *nkx3.2* in this cell type was sufficient in reducing the hemorrhage rate cause by ubiquitous knockdown of the gene.

3.13 *nkx3.2* morphant embryos have abnormal neural crest migration

nkx3.2 appears to negatively regulate *sox10,* a NC specification gene and NC marker (Carney *et al.,* 2006), and positively regulate *pdgfrα,* an effector of NC migration (Eberhart *et al.,* 2008); either directly or indirectly. I chose to further examine the biological function of *nkx3.2* and study the migration of *sox10* positive cells in *nkx3.2* morphants. In order to determine the effect of *nkx3.2* knockdown on the migration distance, speed and direction of *sox10* expressing neural crest cells, Tg(sox10:GFP) embryos were injected with *nkx3.2* morpholino and individual cells were traced through 21 hours of development using a confocal timelapse (Figure 3.13). Images taken at each hour mark were stacked atop one another and the migration routes of individual cells were traced for the duration of the time-lapse.

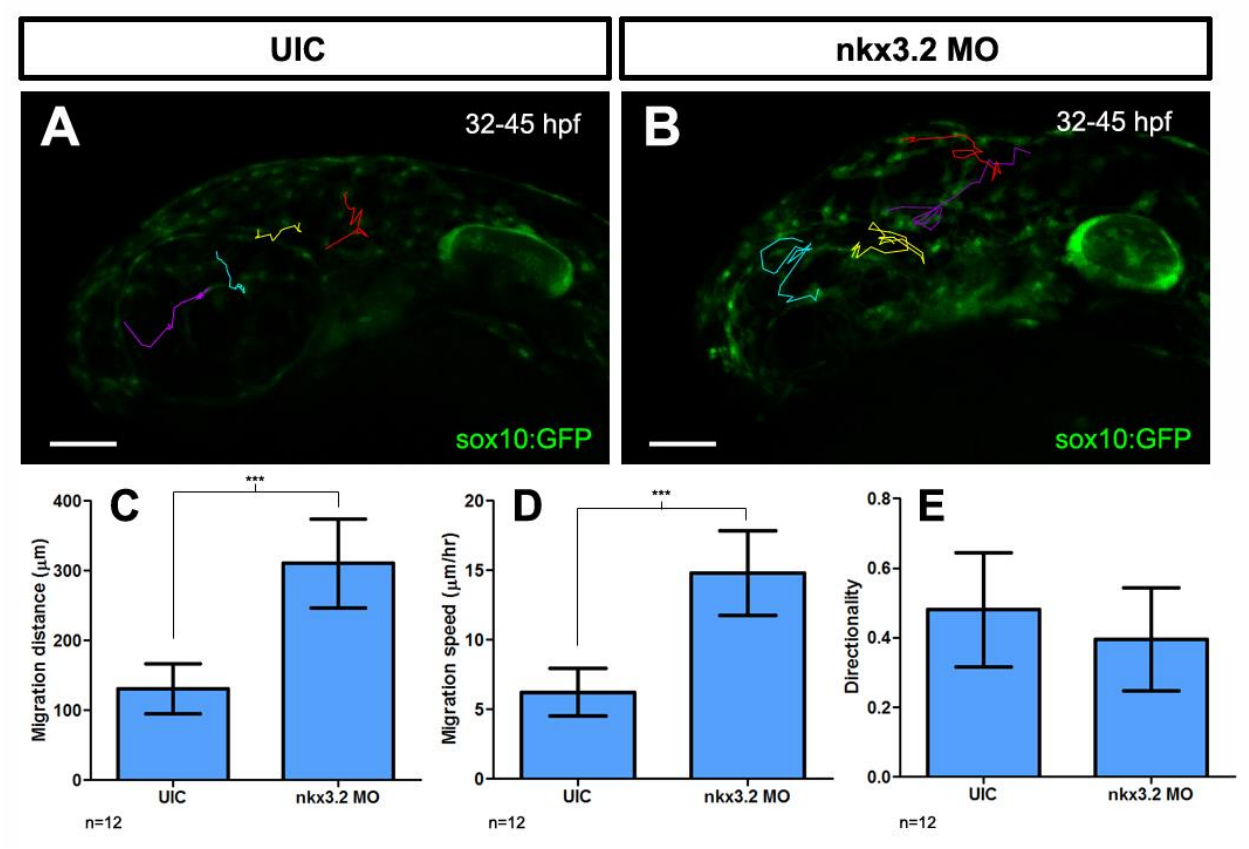

Figure 3.13: *nkx3.2* morphant embryos have abnormal neural crest cell migration.

(A, B) Using Tg(sox10:GFP) transgenic embryos, time-lapse confocal microscopy reveals the path of migrating neural crest cells from 32-45 hpf. (A) Under normal conditions, the cells migrate a relatively short distance and settle into position. (B) The neural crest cells of *nkx3.2* morphant embryos appear to migrate a greater distance and remain restless for the duration of the time-lapse. Following individual cell migration patterns I find that *nkx3.2* morphant cells migrate further (C) and faster (D), though no difference in overall migration direction is observed (E). Scale bars = 100µm. ***(C and D) indicates $p<0.0001$ as determined by the Student's t-test.

Migration distance was calculated by measuring the distance that each cell travelled in µm. I found that the migration distance of *nkx3.2* morphant neural crest cells was significantly longer than those of un-injected controls (*nkx3.2*MO= 310.6µm vs. UIC= 130.9µm; $p<0.0001$ as determined by the Student's t-test: Figure 3.13 C). Next, the individual distances were divided by the 21 hour time-lapse to determine the average migration speed of the cells. The neural crest cells of *nkx3.2* morpholino injected embryos migrate at a faster speed over the documented timeframe (*nkx3.2* MO= 14.79 µm/hour vs. UIC = 6.23 µm/hour; $p<0.0001$ as determined by the Student's t-test; n=12 cells (4/embryo); Figure 3.13 D). In order to determine if the overall migration direction is affected by *nkx3.2* knockdown, the ratio of the total distance to the distance between the start and end points of the migration path was calculated. In this case, directionality was not significantly different between *nkx3.2* morphant and un- injected Tg(sox10:GFP)embryos (UIC = 0.48 vs. *nkx3.2* MO = 0.39; $p=0.197$ as determined by the Student's t-test: Figure 3.13 E). Taken together, these results indicate that *nkx3.2* is required for normal neural crest migration, as cells migrate further and faster, but not in a different direction, when *nkx3.2* function is disrupted.

4 CHAPTER FOUR: DISCUSSION

4.1 Major findings

4.1.1 Nkx3.2: an effector of vascular stabilization downstream of iguana

In my project, I sought to identify genes that functioned downstream of the *iguana* mutation in a developmental pathway required for vascular stabilization. I first identified *nkx3.2* on a microarray in which genes with differential expression in both *iguana* mutant and cyclopamine-treated zebrafish were considered as possible effectors of vascular stabilization. Cyclopamine, a chemical antagonist of *sonic hedgehog* signalling, was included in the array because the *iguana* mutation is known to disrupt *hedgehog* signalling through the loss of primary cilia where *Shh* signaling typically occurs (Incardona *et al.,* 1998; Sekimizu *et al.,* 2004). While microarrays are useful tools for identifying potential candidate genes is it important to back the results up using another method such as *in situ* hybridization (Suzuki & Motokawa, 2004). Through this method I was able to show downregulation of *nkx3.2* in *iguana* mutants, confirming that nkx3.2 is downstream of *hedgehog* signalling in zebrafish. Further, very recent work by my co-worker Corey Arnold also shows *nkx3.2* expression reduced in cyclopamine-treated zebrafish embryos, further supporting this finding.

Because cyclopamine is a general antagonist of all *hedgehog* signalling (Incardona *et al.,* 1998) and *iguana* disrupts primary cilia and likely a variety of signalling molecules (Tay *et al.,* 2010), it is not certain that *nkx3.2* is directly downstream of *Shh*. To test this, one could assess expression levels of *nkx3.2* in $Shh^{-/-}$ mutant zebrafish as in Prykhozhij 2010, or detect Gli binding to the *nkx3.2* promoter, suggesting direct activation. Interestingly in embryonic mouse

chondrocytes, Nkx3.2 protein levels are negatively regulated by the morphogen Indian Hedgehog (Ihh) (Choi *et al.,* 2012) in contrast to our finding that during development, hedgehog promotes *nkx3.2* transcription (microarray data & Arnold *et al.,* submitted). It is also noteworthy that *dzip1* (the disrupted gene in *iguana* mutants) has bipartite roles in regulation of Hedgehog signalling; where positive regulation of *Shh* signalling occurs through cilia formation, and negative regulation of *Shh* occurs through regulation *dzip1* regulation of Gli protein turnover (Schwend *et al.,* 2013). Thus control of *nkx3.2* gene expression is complex and has tissue and timing-dependent differences in regulation at both the transcriptional and protein levels in response to hedgehog.

A second major finding from this group of experiments was that *nkx3.2* activity downstream of *iguana* was very likely to be involved in a pathway involving vascular stabilization. Genetic rescue of hemorrhage established this potential role for *nkx3.2,* as ubiquitous overexpression of *nkx3.2* in *iguana* mutant fish was sufficient in significantly reducing hemorrhage rates compared to *iguana* alone. The discovery of an effector of vascular stabilization downstream of *iguana* was a critical step in forming the hypothesis that *nkx3.2* is involved in the recruitment of mural cells to develop supported vessels. Literature citing *nkx3.2* as a transcriptional regulator in visceral smooth muscle development in mouse (Verzi *et al.,* 2009), and expression of the gene in vascular smooth muscle cells of chick (Nishida *et al.,* 2002) further supported this early hypothesis. These findings were paramount in establishing *nkx3.2* as a strong candidate for being an effector of vascular stability and enabled my eventual finding that *nkx3.2* modulates the migration of neural crest progenitors of mural cells through *pdgfrα* leading to normal mural cell recruitment and vascular support.

Nkx3.2 has known roles in visceral and smooth muscle in other systems and has been shown to be a regulator of palatogenesis and chondrogenic maturation of the facial bones (Verzi et al., 2009; Nishida *et al.,* 2002; Miller *et al.,* 2004; Kawato *et al.,* 2012). Despite the fact that facial bones and perivascular mural cells come from the same progenitors, the cranial neural crest (Whitesell *et al.,* 2014; Etchevers *et al.,* 2001; Mundell & Labosky, 2011; Olesnicky Killian *et al.,* 2009), my research is the first to demonstrate a potential connection between mural cell and facial bone formation by *nkx3.2* regulation of neural crest development.

My findings suggest it will be important to look into other known cofactors or targets of *nkx3.2* in the future as potential mediators of vascular stability. An example is the previously documented interaction between Nkx3.2 and Smads 1 and 4 as cofactors in a transcriptional repressor complex in cultured cells (Kim & Lassar, 2003). More recent findings demonstrate the requirement for Smad4 in adhesion of brain pericytes to endothelial cells through N-cadherin in mouse (Li *et al.,* 2011; Winkler *et al.,* 2011). My finding that *nkx3.2* is required for vascular stabilization in zebrafish through regulation of neural crest cells, together with the finding that Nkx3.2 directly interacts with Smad4, and that Smad4 is required for vascular stabilization in mouse makes Smad4 an important gene to characterize for protein and genetic interactions with nkx3.2. Through this strategy of revisiting known interactions in the context of more recent findings we are able better characterize the molecular regulation of vascular stabilization and gain insights into the factors responsible for development and disease.

4.1.2 nkx3.2 is expressed near blood vessels of the zebrafish head

In support of my hypothesis that *nkx3.2* is an effector of vascular stabilization through mural cell recruitment I found that *nkx3.2* is present near blood vessels during an important window of vascular development. Faint, ubiquitous *nkx3.2* expression is visible throughout the zebrafish head from 24-48 hpf, and very specific staining of the jaw joint and pharyngeal arches appeared at 48 hpf. The staining in the developing jaw joint on each lateral aspect of the embryo was consistent with literature documenting the gene as an effector of chondrogenic maturation in the developing facial bones (Miller *et al.,* 2004). This staining, as well as the less-specific expression found throughout the head were of great interest, as the ventral head is populated by many vessels including major pharyngeal arch arteries that parallel developing bone (Isogai *et al.,* 2001). The lineage of these facial bone structures is also relevant to my project as mural cells and facial bone cells share a common lineage, at least in avian and mammalian models (Whitesell *et al.,* 2014; Hunt *et al.,* 1991; Etchevers *et al.,* 2001; Mundell & Labosky, 2011; Olesnicky Killian *et al.,* 2009). Specifically, my results show *nkx3.2* expression in tissues known to be derived from the cranial neural crest. If zebrafish mural cells originate from neural crest cells as they do in other organisms, *nkx3.2* localization in neural crest-derived tissues implicates a potential role of the gene in some process in their early development.

In order to verify whether *nxk3.2* expressing cells are proximate to blood vessels, as would be expected for mural cells, I conducted double staining. I was able to show that not only was expression of *nkx3.2* in the proximity of vessels, it was actually directly adjacent to them. While these results may indicate that *nkx3.2* is specifically expressed in perivascular mural cells, the cell type cannot be confirmed until we co-localize *nkx3.2* with a mural cell marker or rescue

experiments using a mural-specific promoter are conducted. One potential future experiment would involve conducting double fluorescence *in situ* hybridization of *nkx3.2* alongside a mural cell marker such as *pdgfrβ* as in Wang *et al.* (2014). In fact, this is one of the most critical future experiments stemming from my work; to definitively identify the cell types in which *nkx3.2* is expressed. The technology available to study perivascular support through mural-endothelial interactions was limited by available markers until recently, with the best marker for zebrafish vascular mural cells of the head during my project being *acta2,* which turns on a 4 day in development. The use of earlier mural markers such as *pdgfrβ* opens the door into visualizing mural cells at a timepoint closer to 48 hpf where hemorrhage occurs in *nkx3.2* knockdown fish. A related question, and one that is also very important going forward, is whether we can conclude definitively whether *nkx3.2* is expressed only adjacent to the endothelial layer or is also expressed within endothelial cells. If *nkx3.2* is also expressed in endothelial cells, the vascular defects I observe following *nkx3.2* knockdown could be related to endothelial development or adhesion with one another. Indeed, many occurrences of vascular instability have been linked to endothelial cell maturation, signalling, or adhesion (Wilson *et al.,* 2013; Fish *et al.,* 2008; Kwon *et al.,* 2012). While my data does not suggest the presence of an endothelial defect, it will be important to rule it out by conducting a tissue specific rescue of hemorrhage driven by an endothelial promoter. Here, my hypothesis would be that overexpression of *nkx3.2* in endothelial cells only in *nkx3.2* knockdown fish would not be sufficient in reducing the hemorrhage rate.

Another critical experiment made possible by the discovery of a reliable early mural cell marker (Wang *et al.,* 2014) would be to use the *pdgfrβ* promoter to drive the overexpression of

nkx3.2. Here, we may be able to determine whether *nkx3.2* functions autonomously in mural cells by co-injecting this mural-specific overexpression construct in *nkx3.2* morphants to assess whether upregulating *nkx3.2* in these cells only is sufficient to reduce hemorrhage rates.

4.1.3 Knockdown of nkx3.2 causes hemorrhage due to abnormal mural cell recruitment

Once I had established that *nkx3.2* functioned downstream of *iguana* as an effector of vascular stabilization and was expressed near blood vessels during a relevant time in vascular development, I began to analyze *nkx3.2* knockdown fish in greater detail. My goals for this section were to identify that *nkx3.2* was required for normal vascular stabilization, to view the expression of mural cell markers in *nkx3.2* knockdown fish, to observe the ultrastructure of these vessels by transmission electron microscopy, and to view high definition confocal images of hemorrhage occurring in real time. These experiments were successful in demonstrating the consequences of *nkx3.2* dysfunction in multiple levels of detail; hemorrhage counts at whole organism level, reduced mural marker expression at the tissue level, and reduced mural-endothelial contact at the cellular level. I was also able to pinpoint individual ruptured vessels in real time and in high definition in order to put together a more complete description of vascular instability.

I used three different morpholino oligomers to knockdown *nkx3.2* and found that each produced hemorrhage and a facial phenotype that was identical. Hemorrhages were visible under brightfield and hemorrhage counts from all three morpholinos yielded very similar numbers. This finding was paramount in my study, as many inferences regarding the function of a gene can be made from the defects caused by loss of function. Here, the highly

reproducible occurrence of hemorrhage has major implications for the requirement of *nkx3.2* in vascular stabilization.

Two of the morpholinos were ATG-targeting morpholinos which I did not use for experiments beyond the initial phenotype screen because the first only caused a phenotype using a high-volume injection, and the second came out of solution in storage. The third morpholino worked well at low concentrations and because of its ability to target a splice-site, I could also verify efficiency using a simple RT-PCR. cDNA synthesized from mRNA from wildtype and morpholino-injected fish was used for this analysis, where the morphant cDNA yielded an observable band by electrophoresis that included an intron indicating the successful blockage of a splice site by the morpholino. This data supports the use of morpholino oligomers as an effective means of gene knockdown in zebrafish as it is very unlikely that three identical phenotypes produced by three independent morpholinos be caused by morpholino toxicity or off-target effects. These findings come at a critical time in zebrafish research as some groups are moving away from the use of morpholinos in favour of site-directed mutagenesis by TALEN or CRISPR technology (Bedell *et al.,* 2012). I was able to quickly identify an identical vascular phenotype using three different MOs, two of which were previously published (Miller *et al.,* 2004) and one that was verified by RT-PCR. My results present a very strong case for the continued use of morpholino oligomer technology.

In order to further characterize the *nkx3.2* knockdown phenotype with a focus on cell morphology and ultrastructure I hypothesized that vascular stabilization defects are caused by mural cell recruitment problems and were not due to some error in endothelial layer formation

or patterning. This was guided by the nature of the vascular stability defects in the original observations in the *iguana* mutant that has normal endothelial interactions but impaired mural:endothelial interactions, and because initial screening of *nkx3.2* knockdown fish revealed no apparent endothelial formation or patterning defects. I confirmed my hypothesis by first analyzing the expression of the mural cell marker *acta2* in *nkx3.2* knockdown Tg(acta2:mCherry;kdrl:eGFP) fish at 4 dpf, where I found reduced acta2:GFP expression in pharyngeal arch arteries. From this data I cannot conclude whether the expression of the marker is decreased in cells, or if there are actually fewer mural cells present. Furthermore, the timing of this experiment was difficult because the *acta2* marker is not expressed in perivascular mural cells until 4 days post fertilization, 2 days after hemorrhage had occurred. Though *acta2* was the best available tool at the time of this experiment, in the future it may be useful to consider other mural cell markers such as *pdgfrβ,* which has expression in mural cells in the brain region beginning around 56 hpf (Wang *et al.,* 2014).

The comparison of wildtype and *nkx3.2* morphant blood vessel ultrastructure via transmission electron microscopy does not rely on molecular markers and was useful in quantifying mural cell recruitment a second way, and thereby further supporting my hypothesis that vascular instability was caused by defective mural cell recruitment. The endothelial layer of *nkx3.2* knockdown zebrafish embryos contacted mural cells significantly less than in controls at 48 hpf. An important control in this experiment was selecting the same vessels for analysis between different embryos. I chose to analyse the lateral dorsal aorta and was able to locate this individual vessel in each case by identifying easily recognizable structures such as the notochord and by examining other zebrafish TEM data (Eriksson & Löfberg, 2000). The

advantage of looking at these vessels is that they were relatively easy to locate due to their size and location. As the dorsal aorta is a large vessel of the head running from the pharyngeal arch arteries into the trunk, it is present across a long anterior-posterior distance in the dorsal-ventral position in embryos. One challenge encountered during this experiment was identifying mural cells based on cell morphology and proximity to vessels and not by expression of particular markers. The endothelial layer of the LDA vessels of control embryos often had several perivascular support cells very tightly bound to it, which is an important finding as no current mural cell marker is known to label the cells of this region, except perhaps *pdgfrβ* which has not been well studied. Staining for this marker is visible in only a few cells at 48 hpf and is not expressed to a significant degree in the dorsal aorta until 72 hpf (Wang *et al.,*2014), though further work may identify signalling earlier than this. My research provides evidence of well supported vessels by 48 hpf in wildtype embryos despite the lack of reliable mural cell markers at this time, and illustrates a role for *nkx3.2* in mediating these endothelial-mural interactions as these embryos had less endothelial-mural contact.

I set out to examine the precise location and time of hemorrhages by imaging Tg(kdrl:GFP;GATA1:dsRed) *nkx3.2* knockdown fish in real time between 30 and 48 hpf and found that hemorrhages occur in a number of different vessel types. In the CtAs, hemorrhage occurs around 36 hpf, where very newly formed vessels are subject to rupture. I also identified hemorrhages in the anterior head and in the inner optical circle of the eye. The IOC is a hotspot for vascular remodeling, since during this time frame vessels reorganize from a cluster into a well-organized circle with outward spokes. The occurrence of hemorrhage in new vessels or those undergoing remodelling is understandable as new vessels have been shown to be more

vulnerable to cardiovascular defects including hemorrhage (Khurana *et al.,* 2005). New vessels or those undergoing many changes may hemorrhage more frequently because they have had less time in development to establish strong mural-endothelial interactions or deposit extracellular matrix. Although it seems likely that the CtAs or IOC have defective endothelial:mural cell interactions, the data I collected using transgenic markers or looking at ultrastructure comes from large blood vessels such as the pharyngeal arch arteries or the lateral dorsal aorta due to the late coverage of only relatively large blood vessels (*acta2*) or the inability to identify smaller vessels reliably (TEM). Instead, I must rely on extrapolation of the data found in larger vessels to infer that defects in mural-endothelial interactions or mural cell recruitment itself are also occurring on newer vessels. It is also possible that hemorrhage is a readout for systemic vascular defects. For example, if embryonic blood pressure were altered by a vessel stability defect in a large vessel such as the dorsal aorta, hemorrhage may occur in smaller, weaker vessels far from the site of the original defect. In this manner, hemorrhage could also be an indicator for general vascular defects rather than only as an indicator of a local problem.

4.1.4 nkx3.2 regulates the neural crest genes pdgfrα and sox10 and modulates neural crest migration

I undertook a search for genes regulated by *nkx3.2* through an *in situ* hybridization screen of potential candidates and discovered that *pdgfrα* was positively regulated by *nkx3.2* and *sox10* was negatively regulated by *nkx3.2.* Of the genes I tested, I did not encounter any expression differences in growth factors, other genes from the microarray, *Shh* signalling pathway genes, genes involved in mural-endothelial adhesion, or known co-factors of *nkx3.2.*

As *nkx3.2* is a transcription factor, one would hypothesize that it regulates a number of other genes and could be an effector of vascular development by modulating several processes, however at this point, we do not know transcriptional targets in vascular stabilization. Indeed, other homeo-box transcription factors have been shown to regulate many genes and effect development, growth and maintenance of tissues in a complex manner (Luh & Traber, 1996). Performing chromatin immunopreciptiation assays-sequencing (ChIP-Seq) with *nkx3.2* would be a next step to identifying direct transcriptional targets.

The platelet-derived growth factor receptor *pdgfrα* is a very interesting hit as the PDGF signalling family is involved in several areas of vascular development. One role of the other receptor in this signaling network, *pdgfrβ,* involves mural cell recruitment to the endothelium as demonstrated in mouse (Lindhal *et al.,* 1997*;* Lindhal, Lindhal *et al.,* 1998; Meinecke *et al.,* 2012). The role of *pdgfrα* in zebrafish development has been partially characterized during normal neural crest migration (Eberhart *et al.,* 2008). Mural cells differentiate from neural crest cells, at least in other organisms, and thus the link between a neural crest migratory factor and vascular stability is compelling (Etchevers *et al.,* 2001; Mundell & Labosky, 2011; Olesnicky Killian *et al.,* 2009). I tested *pdgfrα* levels in *nkx3.2* knockdown zebrafish by gel electrophoresis (data not shown), and confirmed downregulation in *nkx3.2* knockdown embryos, indicating that *nkx3.2* positively regulates *pdgfrα* directly or indirectly under normal conditions. This discovery spurred the investigation of other genes involved in neural crest development.

The second gene of interest from the expression screen was *sox10,* a neural crest marker and neural crest specification gene (Carney *et al.,* 2006). *Sox10* is expressed early in

neural crest development and its expression is maintained during migration. It also plays a role in differentiation of cells in different lineages (reviewed in Sauka-Spengler & Bronner-Fraser, 2008). Sox10 was a good candidate because of its profound influence on neural crest specification throughout all stages of neural crest development and migration. The gene was upregulated in *nkx3.2* fish, which I was able to confirm by in situ hybridization and by using the sox10:eGFP transgenic zebrafish line indicating that *nkx3.2* positively regulates *sox10* under normal conditions.

Finding that two neural crest-specific genes were regulated by *nkx3.2* pointed to the idea that *nkx3.2* might act in developing neural crest cells themselves. I took advantage of the marking capabilities of *sox10* and tested whether *nkx3.2* was found in *sox10* expressing cells. I found that *nkx3.2* and *sox10* sometimes colocalized, though their expression patterns were quite different, and *nkx3.2* turns on much later than *sox10*. *nkx3.2* expression posterior to the eye was found in a punctate pattern covering a large region. Specific expression was also enriched in key areas such as the pharyngeal arches and jaw joint as previously mentioned. While some regions of *sox10* expression were rich in *nkx3.2* colocalization, others appeared to be free of *nkx3.2* altogether. I can conclude that *nkx3.2* is found in a subset of neural crest cells, possibly in a lineage that will develop into one or only a few specific NC-derived tissues. As we know, neural crest cells give rise to a diverse array of cells including neural tissue, pigment cells, mural cells, cartilage and bone (Le Douarin *et al.,* 2007; Theveneau & Mayor, 2012; Dupin *et al,* 2006), and it will be important to closely characterize *nkx3.2* co-localization with these lineages. To accomplish this, *nkx3.2* expression later in development could be documented alongside markers of neural crest lineage-specific markers.

One interesting inference I can make from the earlier *nkx3.2* expression data is that *nkx3.2* staining becomes stronger and more specifically localized later in the developmental window of interest. Notably, *nkx3.2* expression in the pharyngeal arches and jaw joint, tissues derived from the cranial neural crest (Le Douarin *et al.,* 2007; Theveneau & Mayor, 2012), is visible beginning at about 48 hpf. This stage is also the timeframe when *sox10* and *nkx3.2* sometimes colocalize, and is a period of specification and development of zebrafish neural crest cells into a variety of craniofacial structures (Schwend & Ahlgren, 2009). I can speculate that the presence of *nkx3.2* during this stage in development near the end of neural crest migration might be coupled with the process of specification into different cell types. This hypothesis could be further tested by screening for other genes downstream of *nkx3.2* involved in neural crest migration and/or specification and assessing whether *nkx3.2* positively or negatively regulates any of them. For instance, I found that *nkx3.2* negatively regulates the neural crest migration gene *pdgfrα,* a finding that correlates with my later discovery that *nkx3.2* morphants have abnormal CNC migration. The role of *nkx3.2* near the end of neural crest migration when cells are specified has not been characterized as my work is the first of its kind to identify *nkx3.2* regulation of neural cells as a required process in mural cell recruitment.

A genetic rescue of *nkx3.2* knockdown-induced hemorrhage was conducted through upregulation of *nkx3.2* in neural crest cells. This was accomplished by driving *nkx3.2* with the *sox10* promoter. This experiment confirmed that *nkx3.2* functions autonomously in neural crest cells, as cell injected with both the *nkx3.2* morpholino and the sox10:nkx3.20;myl7:GFP rescue contruct had a significantly lower rate of hemorrhage than embryos with the *nkx3.2* morpholino alone. One difficulty I encountered during this experiment was managing the

toxicity of injecting emrbyos with both the morpholino and the DNA construct. While injection of either alone did not produce significant embryo loss, the combination of both tended to result in high numbers of embryos with no blood circulation and a high rate of embryonic lethality. A number of "morphological monsters" (severely disfigurted embryos lacking normal heads and tails) were present in the samples, though these are not uncommon when conducting zebrafish injections of more than one reagent (Halbig *et al.,* 2012). Despite the high level of toxicity this method is the best currently available method for determining cell autonomy, though additional supportive experiments may be necessary in the future.

The final and perhaps the most important finding of my research is a mechanism by which *nkx3.2* regulates vascular stabilization. Because I found that *nkx3.2* functions in neural crest cells and regulates *pdgfrα* I hypothesized that the gene may modulate neural crest migration. As previously mentioned, homozygous mutants for *pdgfrα* hemorrhage and have defective neural crest migration (Eberhart *et al.,* 2008). I used the sox10:eGFP transgenic fish and timelapse confocal microscopy to track the migration of individual neural crest cells in real time between 30 and 48 hpf. I found that while directionality was unchanged, migration speed and distance was significantly increased in *nkx3.2* knockdown embryos. This mechanism provides an explanation for the vascular stabilization defects observed in *nkx3.2* knockdown fish as mural cell precursors would migrate aberrantly and not reach their intended location in this model. Further, my research supports the hypothesis that zebrafish mural cells of the head are neural crest-derived like in other vertebrates (Etchevers *et al.,* 2001; Mundell & Labosky, 2011; Olesnicky Killian *et al.,* 2009), as the observed migration problems in *nkx3.2* knockdown

embryos precede mural cell coverage deficiencies observed at 48 hpf by TEM and *acta2* marker expression at 4 dpf.

4.2 Summary and Conclusions

My project provides the first evidence of a vascular stabilization pathway involving the homeobox gene *nkx3.2,* and suggests a role for the gene in modulating genes responsible for neural crest migration and eventual mural cell recruitment to vessels. The experiments presented here are a window into a novel pathway that is likely far more complex than the single storyline that has been uncovered.

The project began by examining the molecular effectors of vascular stabilization downstream of the *iguana* mutation. A microarray served as a stepping-off point, from which I was able to select *nkx3.2* as a potential candidate for vascular stabilization through its published roles in visceral smooth muscle and formation of neural-crest derived tissues (Verzi *et al.,* 2009; Miller *et al.,* 2004). I was able to verify that *nkx3.2* functions downstream of *Iguana* by documenting reduced expression of *nkx3.2* in *igu*^{fo10} mutant zebrafish and by partially rescuing *iguana* hemorrhage by upregulating *nkx3.2* in the mutants (latter experiment by Jing Liu). This section of experiments provided the first evidence of an effector of vascular stabilization from the microarray data. The interaction between *nkx3.2* and the upstream components of this signalling pathway such as Shh, and any interactions with other potential effector genes from the array remain a subject of study in our lab.

The phenotype of the *nkx3.2* knockdown zebrafish provides a strong platform for the study of defects in vascular stabilization. The three morpholinos used produced consistently

identical phenotypes characterized by craniofacial abnormalities and hemorrhage while displaying no other major developmental problems. The fish tend to hemorrhage from certain vessels, notably the ctAs and the IOC, both of which are small and newly formed at the time of hemorrhage. This imaging of hemorrhage, conducted in real time using high definition confocal microscopy, was an effective and innovative mean of studying this vascular defect. Hemorrhage location and timing could be precisely identified, which allowed the identification of particularly weak vessels and time points in development during which vessels were particularly vulnerable to rupture. This imaging accomplishes a higher standard of documentation of hemorrhage than the previous methods of more simple hemorrhage counting.

My research is the first of its kind to document a role for *nkx3.2* in mural cell recruitment. I utilized the Tg(acta2:GFP) zebrafish created in our laboratory to observed mural cell expression levels in the pharyngeal arch region of developing zebrafish embryos, and found a reduction in expression in *nkx3.2* knockdown fish. I also used transmission electron microscopy to observe endothelial-mural cell contacts at 48 hpf and found a reduction in mural cell coverage in the *nkx3.2* knockdown fish. These results documented defective mural cell recruitment in *nkx3.2* knockdown zebrafish by two separate methods in two different windows in development.

The finding that *nkx3.2* regulates two neural crest-specific genes, *pdgfrα* and *sox10*, was a breakthrough in my project. The major implication of *nkx3.2* functioning in neural crest cells is support of a model of zebrafish mural cell recruitment where mural cells differentiate from neural crest. I used colocalization of *nkx3.2* with *sox10* and genetic rescue of hemorrhage in

sox10 expressing cells to provide evidence of *nkx3.2* function autonomously in the neural crest. In addition, I was able to identify a potential mechanism by which *nkx3.2* modulates mural cell recruitment by identifying a neural crest migration defect when *nkx3.2* signalling is impaired. The migration of neural crest cells is an important step in development and is required for the later specification of the cells into a broad range of cell types (Noisa *et al.,* 2014; reviewed in Sauka-Spengler & Bronner-Fraser, 2008). A major defect in migration of these cells would have consequences on downstream specification of mural cells if the mural cells do indeed originate from the neural crest. A single example of *nkx3.2* function in neural crest cells and the associated vascular stability defects is not grounds for concluding that zebrafish head mural cells are derived from neural crest, though it does provide a compelling example in support of this hypothesis.

Some questions regarding the causality of defects in vascular stabilization were not addressed in my research. There is a correlation between *nkx3.2* activity in neural crest cells and impaired mural cell recruitment in *nkx3.2* knockdown fish, though this does necessarily indicate that one is the result of the other. Expression of *nkx3.2* in neural crest cells could have implications in a number of diverse cell types. As I have previously mentioned, craniofacial structures are neural crest-derived, and *nkx3.2* knockdown zebrafish have abnormalities in these tissues. The activity of *nkx3.2* in the neural crest could be distinct from its role in mural cell recruitment, although rescue of hemorrhage by upregulation of *nkx3.2* in neural crest cells suggests an autonomous relationship. In order to directly connect neural crest migratory defects to mural cell recruitment defects new mural cell markers must be developed. Identifying colocalization of neural crest and mural markers is paramount in both the general

characterization of mural cell origins in zebrafish, and in linking phenotypic evidence such as the migratory defect described here and the mural cell recruitment defects highlighted by the *acta2* expression results and TEM.

4.3 Future directions

This project has opened a window into a novel molecular pathway for vascular stabilization through the actions of the transcription factor *nkx3.2* in neural crest migration and mural cell recruitment. Much research must be conducting in order to fully characterize the molecular events leading to vascular support.

It is very likely that *nkx3.2* functions in several ways, possibly in overlapping molecular pathways in development. A great challenge moving forward is to identify direct evidence of a mechanism in development of a particular cell type. Part of the challenge of this goal is developing new tools and markers for investigating mural cell development. Only very recently has a new marker been successfully used; the labelling of apparent brain pericytes by *pdgfrβ* (Wang *et al.,* 2014). It will be critical to further study the origins of mural cells from neural crest cells as much of these results and data from other organisms support this notion.

Other mechanisms for mural cell development and recruitment were not addressed by these experiments. The relationship between *nkx3.2* and other key genes in vascular myogenesis (*tek, angpt1, pdgfrβ* (Gale & Yancopoulos, 1999; Lindhal et al., 1997, Lindhal et al., 1998Wang *et al.,* 2014)) or mural maturation (*notch3, s1pR1, cdh2* (Lawson *et al.,* 2001; Liu *et al.,* 2010; Paik *et al.,* 2004; Li *et al.,* 2011)) should be examined by examining expression of these regulators of vascular support.

In zebrafish research it is still unclear whether mural cells can be conclusively divided categorically into pericytes and vascular smooth muscle cells. A role for *nkx3.2* in mural cell development could be specific to both or just one of these cell types. I found high hemorrhage levels in brain vessels, which are thought to be covered by pericytes and not smooth muscle cells. Additionally, if pericytes and vascular smooth muscle are molecularly distinct it is important to study the genes responsible for their specification in order to better understand mural cell recruitment as a whole.

Finally, as the study of vascular development in zebrafish evolves, new technologies become available and require testing and troubleshooting. The largest change in the study of molecular regulation in zebrafish comes from the ability to produce mutants for nearly any gene via TALEN or CRISPR technology. It will be important to verify the phenotype and interaction of *nkx3.2* with *pdgfrα* and *sox10* by creating *nkx3.2* mutants by germline mutagenesis.

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