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microRNAs (miRNAs), ~21-24 nucleotide-long RNAs that post-transcriptionally regulate gene expression, have rapidly become one of the most extensively studied mechanisms of the past decade. Since their discovery as temporal regulators of post-embryonic development in *C. elegans*, miRNAs have been functionally implicated in almost every cellular process investigated to date. miRNAs are integral to the complex biological processes of embryonic development and aging. In this research, we sought to determine whether misregulation of miRNAs could be responsible for eliciting adverse effects during these two distinct developmental stages. First, to uncover the potential role of miRNAs in teratogenicity, we investigated whether miRNAs were involved in regulation of retinoic acid (RA) induced vertebrate axis defects. Global miRNA expression profiling revealed that RA exposure suppressed the expression of miR-19 family members during zebrafish somitogenesis. Bioinformatics analyses predict that miR-19 targets cyp26a1, a key RA detoxifying enzyme, and a physiological reporter assay confirmed that cyp26a1 is a bona fide target of miR-19. Transient knockdown of miR-19 phenocopied RA-induced body axis defects. In gain-of-function studies, exogenous miR-19 rescued the axis defects caused by RA exposure. Our findings indicate that the teratogenic effects of RA exposure result, in part, from repression of miR-19 and the subsequent misregulation of cyp26a1. This highlights a previously unidentified role of miR-19 in facilitating vertebrate axis development. Next, to explore whether age-related changes in miRNAs trigger deficits in regeneration capacity, we performed mRNA and small RNA sequencing on regenerating and non-regenerating caudal fin tissue from aged, adult and juvenile zebrafish. An unbiased approach identified cbx7 as

the most abundant transcript with significantly increased expression in regenerative-competent adult and juvenile tissue and decreased expression in regenerative-compromised aged tissue. While cbx7 is a known regulator of aging, this is the first report of its role in tissue regeneration. A computational approach was used to discover mRNAs expressed during regeneration, which are potential targets of the significantly expressed miRNAs in regenerating tissue. miR-21 was one of the most abundant and significantly increased miRNAs in regenerating tissue and exhibited an aberrant age-dependent expression profile. Bioinformatics predicts miR-21 to target the 3' UTR of cbx7 and a reporter assay confirmed that miR-21 targets cbx7 *in vivo*. Transient knockdown of miR-21 inhibited tissue regeneration, suggesting a role for miRNA mediated regulation of cbx7 during regeneration. These findings reveal a novel, age-dependent regenerative function of cbx7 and emphasize the importance of miR-21 as a master regulator of vertebrate regenerative responses. This research, when combined, underscores the negative consequences misregulation of miRNAs has on embryonic development and aging.

Consequences of miRNA Misregulation on Embryonic Development and Aging

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I dedicate this dissertation in loving memory of my dear friend Meeten Chauhan, inspiring scientist and even better friend, who taught me the importance of a work – life balance in graduate school. Sadly, life balanced against him, thwarting the fulfillment of his doctoral degree. I got this one to share with you, Meeten.

Chapter 1 – Introduction

Since the discovery of microRNAs (miRNAs) in 1993, these small non-coding RNAs have rapidly become one of the most extensively studied regulatory mechanisms of the past decade (1). miRNAs are integrally involved in complex biological processes such as animal development and aging (2-6). While embryonic development is inherently dynamic and aging is a more gradual process, miRNAs play a pivotal role in each of these life stages through the post-transcriptional regulation of genes that dictate the formation or degradation of cells, tissues and organs (5, 6). In this research, we explored how misregulation of miRNAs could be responsible for undesirable effects such as teratogenicity in developing animals (Chapter 2) and a declined ability in aging animals to maintain and restore tissue (Chapter 3).

microRNAs

MicroRNAs (miRNAs) are ~21-24 nucleotide-long RNAs that post-transcriptionally regulate gene expression (7). Since their original discovery as temporal regulators of post-embryonic development of *C. elegans* (8, 9), post-transcriptional regulation by miRNAs have been functionally implicated in almost every cellular process investigated to date (10). miRNAs are predicted to control ~60% of mammalian protein coding genes (10). Hence, the effects of miRNA-mediated gene expression are widespread. This prompted our investigation of the role of miRNAs in embryonic development and aging, using zebrafish as a model to understand how misregulation of miRNAs contributes to teratogenicity and age-related declines in tissue maintenance and repair.

miRNA biogenesis:

Primary miRNAs (pri-miRNAs) genes are transcribed in the nucleus by RNA polymerases II or III (11, 12). Pri-miRNAs range in length from hundreds to thousands of nucleotides (13). Approximately half of mammalian miRNAs are located in the intronic regions of coding genes or exons of non-coding RNAs (14). Additionally, miRNAs can be clustered relatively close within the genome and co-transcribed as polycistronic transcripts (13). The remainder are located in intergenic regions, exclusive from protein coding genes (13). Similar to coding

genes, miRNA transcription is tightly regulated by both transcription factors and epigenetic mechanisms which control tissue and cell specific expression patterns (1).

In the nucleus, Drosha, a nuclear RNase III, in association with DGCR8, cleaves the ends of the pri-miRNA stem-loop leaving behind a ~70 nucleotide stem loop structure, called precursor miRNA (pre-miRNA) (15-18). Processing by Drosha results in a 2 nucleotide overhang on the 3' end at the cleavage site (19). Following cleavage, the pre-miRNA is translocated into the cytoplasm by Exportin 5, a Ran-GTP dependent nucleo/cytoplasmic cargo transporter (20). The stem loop of the pre-miRNA is then cleaved by Dicer in complex with TAR RNA binding protein (TRBP) yielding a ~21-24 nucleotide mature miRNA duplex with a 2-nucleotide long 3' overhang (13, 21).

One strand of miRNA/miRNA* (guide strand/star strand) duplex is embedded with one of four Argonaute proteins (Ago 1-4) and incorporated into the RNA induced silencing complex (RISC), while the other strand is selectively degraded (22, 23). In most cases, the guide strand is retained by RISC as it generally has the less stably base-paired 5' end (10). Yet, there are numerous documented instances in vertebrates when the miRNA* strand is loaded into RISC and participates in post-transcriptional gene regulation (24). The RISC complex guides the loaded miRNA to target mRNA to inhibit translation or evoke mRNA degradation (21), as described in the following section.

miRNA-mRNA interactions:

miRNAs exert post-transcriptional control of target mRNA by binding with near perfect complementarity to target recognition sequences within the 3' UTR of mRNA. The binding requires Watson-Crick pairing of nucleotides 2-8 on the 5' end of the miRNA, known as the "seed" sequence, with cognate mRNA recognition sequences (7). In animals, imperfect pairing of miRNAs to mRNAs inhibits target protein synthesis by translational repression or mRNA deadenylation and decay (10). Translational inhibition can occur at the initiation phase through repression of cap recognition (25-27) or at the elongation phase causing either slowed elongation or ribosome 'drop off' (28-30). Additionally, deadenylation of mRNA can result in subsequent decapping and degrading of transcripts (31-34).

Members of a miRNA family share the same seed sequence, and, in turn, a majority of the same targets (7). Additionally, an individual miRNA can interact with hundreds of target mRNAs and a single mRNA can be regulated by several miRNAs (35). As a result, miRNA gene regulatory networks are highly complex. Fine-tuning of gene expression has been suggested as the fundamental role of miRNA through temporal regulation of tissue and cell specific protein output (7). Hence, we sought to determine how misregulation of these global 'micromanagers' could negatively impact embryonic development and the ability to maintain and repair tissue.

Retinoic acid

To explore the role of miRNAs in teratogenicity, we used retinoic acid (RA), as a model teratogen, since it is well known for its multifarious, deleterious developmental effects. It has been over 50 years since the teratogenic influences of aberrant RA signaling during development were first documented in rodents (36). Since that time, numerous studies in various different models have contributed to a clearer picture of the molecular mechanisms that underlie RA signaling during development (36). Yet, given the diverse role of RA in a vast number of tissue and cell types, questions still remain about the etiology of RA induced teratogenicity. Since developmental RA abundance requires strict spatiotemporal regulation (as reviewed in (37, 38)) and miRNAs are known to absolve transcriptional programs from previous developmental stages (as reviewed in (39-41)), we sought to determine whether dysregulation of miRNA signaling resulted in the hallmark curved body axis phenotype triggered by RA exposure (Chapter 2).

RA synthesis and gene regulation:

During development, maternal Vitamin A is the major source of retinoids to placental embryos. Oviparous species derive RA from Vitamin A stored in the egg yolk (37). Most adult species cannot synthesize retinoids *de novo*. Therefore, it must be consumed in the diet (42). RA is synthesized intracellularly via a two-step process. First, vitamin A is converted to

retinaldehyde in a rate-limiting reaction by microsomal retinol dehydrogenases (RDHs) and cytosolic alcohol dehyrogenases (ADHs). Retinaldehyde dehydrogenases (RALDH1, RALDH2 and RALDH3) then generate RA via an irreversible oxidation step (37). RA is then either transported to the nucleus by cellular RA binding proteins (CRBPs) or metabolized by the cytochrome P450 26 (CYP26) family of enzymes. In the nucleus, RA interacts with the ligand-inducible heterodimers of RA nuclear receptors (RARs) and retinoid X receptors (RXRs) to initiate the transcription of target genes (43). Members of the RAR family (RAR α, β, γ) are activated by all-trans-RA and 9-cis-RA, the most active biological retinoids, and 4-oxo-RA, 3,4-didehydro-RA and 4-hydroxy-RA. The RXR family (RXR α, β, γ) is only activated by one RA metabolite, 9-cis-RA. However, this metabolite is not detected endogenously in embryonic or adult tissue due to its rapid conversion to all-trans-RA (37).

RAR-RXR heterodimers bind to DNA responsive elements of target genes known as RA response elements (RAREs). The majority of RAREs contain two direct repeats of the canonical nucleotide sequence (A/G)G(G/T)TCA separated by 1-5 nucleotides (44, 45). RAR-RXR dimers can bind RAREs by a ligand-independent mechanism. This leads to transcriptional repression upon recruitment of co-repressors, SMRT (silencing mediator for retinoid and thyroid receptors) and NCoR (nuclear receptor corepressor), histone deacetylases and methyltransferase complexes (37, 42, 46). Upon ligand binding, a conformational change occurs in the RAR ligand binding domain; in turn, the co-repressors are released which allows the recruitment of co-activators (37, 46). Over the last 30 years, more than 530 genes have been identified as putative regulatory targets of RA (44). Yet, only ~30 have been proven to contain functional RAREs. The majority are indirect targets, indicating the actions of intermediate transcription factors, associations with other proteins or distant signaling pathways (44). The expansive nature of the RA regulatory network underscores the criticality of maintaining proper RA signaling during development.

RA metabolism:
Cyp26 enzymes convert all-trans-RA into more polar metabolites, primarily 4-hydroxy-RA, which is further oxidized to 4-oxo-RA (38). Other metabolites include 18-hyrdoxy-RA and 5,8-epoxy-RA (47). All the metabolites are then subject to further conjugation and

elimination (37). Although there is debate about whether the CYP26 metabolites of RA are biologically active, evidence suggests that those produced via Cyp26a1 are not (37, 46). The Cyp26 family of enzymes includes Cyp26a1, Cyp26b1 and Cyp26c1, each with its own distinct expression domains during development (38). These enzymes all play an important role in regulating RA levels to prevent inappropriate RA signaling (43, 46). Targeted manipulation of Cyp26a1 and Cyp26b1 results in abnormalities that mimic the teratogenic effects of excess exogenous RA exposure (48-51) suggesting that the developmental role of RA is strongly related to the enzymatic regulation of its synthesis and tissue specific metabolism (as reviewed in (37)). Therefore, we chose to explore, specifically, how miRNAs might regulate enzymes responsible for RA abundance that, in turn, affect the expression of RA-responsive genes.

RA induced teratogenicity:

Imbalances in RA abundance, either deficiency or excess, are known to cause teratogenicity (37, 42). Vitamin A deficiency (VAD) induces congenital malformations in the cardiac, respiratory, ocular and urogenital systems (52, 53). Deficient RA signaling during development has implications on human spinal column birth defects, such as scoliosis. Excess amounts of Vitamin A can cause abnormal morphological development of the central nervous system, skeletal system, liver and skin (42, 54). In our research, we focus on the role of RA-induced miRNAs in eliciting developmental axis defects through the misregulation of RA signaling during somitogenesis.

Role of RA in somitogenesis and axial patterning:

There are numerous RA-dependent events during development whose disparate outcomes rely on the delicate interplay of RA synthesis and metabolism (37). Segmental patterning of the vertebrate axis during somitogenesis is one of these events (37, 38, 55, 56). The most important tissues for RA production during embryonic development in vertebrates include the presomatic mesoderm (PSM) and somites. The PSM is mesenchymal tissue that parallels both sides of the neural tube, and somites are epithelial spheres of mesoderm derived from the PSM that give rise to the vertebrae and skeletal muscles (45, 56-59). RA signaling regulates the directional, periodic, and synchronous segmentation of the PSM into somites

by two key modes of action: (1) control of the anterior-posterior (AP) body axis of the traveling determination front that defines the positioning of the somite (57, 60-62) and (2) symmetric formation of somites along the left-right (LR) body axis (58, 59, 63-66). Perturbations of RA signaling during somitogenesis result in the desynchronization of somite formation in mouse, chicken and zebrafish (58, 59, 64). The observed phenotypes reflect human defects of the vertebral column (67, 68). RA is proposed to have a pivotal role in vertebrate segment formation by antagonizing the FGF/WNT signaling gradient that controls the somite positioning (69).

RA is also critical in protecting the paraxial mesoderm from molecular signals directing LR patterning and, thus, maintaining symmetry in the PSM (55, 58, 59, 64). Asymmetric somite formation results from aberrations in RA signaling (64). Interestingly, when researchers investigated the mechanism by which RA signaling becomes lateralized in response to the LR information cascade, obvious LR asymmetries were not observed in the transcript expression of key RA synthesizing and metabolizing enzymes as hypothesized (64). The conclusion was that control of RA signaling by the LR information cascade during somitogenesis is likely to be post-transcriptional (64).

Role of miRNAs in somitogenesis:

miRNA-meditated post-transcriptional regulation of gene expression was demonstrated to influence vertebrate axis patterning and development (70-77). Defects in somitogenesis, gastrulation, and heart and brain development are observed in maternal zygotic *dicer* zebrafish mutants that lack the *dicer* enzyme necessary to generate mature miRNAs (71). Reduced axis extension resulted in a truncated tail phenotype in mutants (71). Similarly in mice, loss-of-function of *Dicer* during mesoderm development caused reduction in somite size, a reduced AP axis, and caudalization of somites (77). Other studies have revealed the functional role of miRNAs in somite-derived sclerotome and dermomyotome development in mice (78-80), chicken (81, 82), zebrafish (70, 83, 84), and xenopus (85). These results suggest the necessity for miRNA-mediated regulation of the precisely timed events that control somite formation.

Several reports describe a mechanistic link between miRNAs, early embryonic patterning and somitogenesis (73, 74, 86). Additional studies demonstrated the role of miRNAs in modulating the expression of Hox cluster genes that participate in the patterning of the AP axis (72, 75, 87-89). RA directly regulates the transcription of several Hox genes (90-92). This brings to light the broad network of RA-mediated miRNA regulation during somitogenesis.

Role of miRNAs in RA signaling:

Beyond axis patterning and formation, numerous other studies have investigated the role of miRNAs in RA-dependent biological processes. Post-transcriptional regulation is implicated in RA signaling during carcinogenesis (93-98), cardiac development (99), spinal cord development (77), appendage development (72), eye function (100), spermatogenesis (101), muscle cell differentiation (102), and stress-induced premature cellular senescence (103). A clear understanding of the post-transcriptional effects of miRNAs on the delicate balance of RA synthesis and metabolism, critical to early developmental patterning, has yet to be elucidated.

Aging

One of the hallmarks of aging, across all species, is a decline in the ability to maintain tissue function or restore damaged tissue (104-108). Many similarities exist in molecular mechanisms that regulate development and regeneration (109). Regeneration requires the reactivation of fundamental developmental signaling pathways in order to restore damaged or missing structures (109). Both development and regeneration require precise control over the spatiotemporal gene expression necessary to initiate and terminate molecular signaling cascades that guide cells to either create or recreate new tissue architecture(109). Given the known role of miRNAs in fine-tuning gene expression patterns (3), we chose to investigate whether age-related changes in miRNAs could be responsible for declines in regenerative capacity (Chapter 3).

Stem cells and aging:

Aging is one of the most recognizable phenomena of biology and yet, to date, is one of the least understood (105). This is largely due to the fact that the aging process is an accumulation of a host of complex changes in an organism over time (106). There are characteristics of aging that are common across all species which result in similar phenotypes (105). Yet, there is no prescribed group of methodologies or ideal model organism that allows us to address all of the factors that contribute to aging. Decreases in maintenance of tissue homeostasis and repair of damaged tissue implicate stem cells as a key factor in the aging process (105-108). Best summarized by Ho et al., "as the regenerative prowess of a living organism is determined by the ability and potential of its stem cells to replace damaged tissue or worn out cells, a living organism is therefore as old as its stem cells" (104).

Understanding how adult stem cell populations are involved in regeneration is paramount to the advancement of the field. Stem cells are undifferentiated and can replace themselves indefinitely, producing daughter cells which have the potential to differentiate into various different mature cell types (105, 110). Embryonic stem cells have the ability to become any differentiated cell type in an organism. In contrast, adult stem cells are generally lineage specific and committed to a specialized cell type from their originating tissue (104, 111-113). Numerous reviews detail the many genetic and biochemical processes which contribute to stem cell aging (i.e. telomerase attrition, DNA damage, reactive oxygen species, and changes in chromatin dynamics and epigenetic regulation) (104-106, 110, 114). Regardless of the mechanism, aging of tissue-specific stem cell and progenitor cell populations is thought to be a contributor to the reduction of integrity and function in tissues and organs (104-108).

Role of miRNAs in tissue aging:

miRNAs are implicated in regulating numerous aspects of cellular senescence and organismal lifespans (4). Several reviews detail the role of miRNAs in the regulation of cellular senescence induced by stress, reactive oxygen species, DNA damage, modulation of tumor suppresser activation, replicative senescence and telomerase length (4, 5, 105, 115, 116). Studies have also identified functional miRNA-mRNA interactions that influence lifespan through regulation of the age-related insulin/IGF1, steroid and rapamycin (TOR) signaling

pathways (as reviewed in (4, 5)). For the purposes of this research, we are keenly interested in the role of miRNAs in tissue aging and how age-related changes in miRNAs can affect mechanisms that control the ability to maintain and repair tissue.

Only a few studies have been published to date on this subject which have documented changes in miRNA expression in older tissue compared to younger tissue (5). Age-related changes in miRNA expression were demonstrated, in mice and rats, to control the expression of key transcripts necessary for liver maintenance (117-119). miRNA expression profiles were also conducted and revealed several distinct mechanisms through which miRNAs mediate aging in brains of mice, chimpanzees, humans and macaques (120-123). Additionally, studies revealed that miRNAs are associated with aging of skeletal muscle through regulation of pathways that control the differentiation of myogenic precursor cells and satellite cell turnover (124, 125). Given the relative scarcity of research conducted on the role of miRNAs in tissue aging to date, we chose to use zebrafish as a model organism to examine if age-related changes in miRNAs could be responsible for the decline in tissue maintenance and repair capacity associated with age.

Zebrafish model for aging research:

Zebrafish have recently emerged as a vertebrate model for aging research (126-128). Zebrafish research benefits from the organism having a fully characterized genome and numerous mutant and transgenic lines available to query the development of age-related phenotypes in various tissues and organs (127). Additionally, there are a host of molecular techniques that allow the investigation of mechanisms *in vivo* (127). Zebrafish can live over 3 years, but generally begin to exhibit signs of gradual senescence similar to humans around 2 years (129). As zebrafish age they begin to demonstrate age-related changes in β-galactosidase activity in skin and oxidized protein accumulation in their muscles (128-130). Accumulation of lipofuscin and drusen-like lesion in the retinal pigment epithelium, which mimic human age-related macular degeneration, was documented with age (130). Aging also was shown to cause a decrease in heat shock responses (131, 132) and an increase in oxidative stress responses (133). Zebrafish also exhibited age-related changes in circadian rhythms (134) and cognitive performance (135). Of particular interest to us is that zebrafish

demonstrate age-related deficits in regenerative capacity (128, 136). Taken together, zebrafish provide us with the ideal model to investigate miRNA mediated effects on aging and the ability to repair tissue.

The latent capacity of key developmental signaling processes must be reawakened to regenerate lost structures in adults (137-139). Regeneration of adult tissues requires activation of new stem cell populations and reactivation of progenitor cells. Until recently, the lineage of the cell types involved in fin regeneration of zebrafish was largely unknown. A recent study investigated the lineage specificity of various cell types in a regenerating adult caudal fin. The results demonstrated that some cell types are derived from unique lineages, such as osteoblasts and dermal fibroblasts; whereas others, the artery and vein, are created from the same pool of progenitors (140). Additionally, four neuroectodermal cell types examined (melanocyte, irridophore, intraray glia and lateral line) were found to have independent organ-founding stem cells in the fin primordium (141). These findings supplemented the growing body of knowledge on vertebrate tissue regeneration and highlighted its usefulness for elucidating mechanisms involved in the dedifferentiation and fate specification of cells necessary to regenerate tissue.

Regeneration

The phenomenon of regeneration is awe-inspiring. It has captivated biologists and non-scientists alike. In 1768, Lazzaro Spallanzani reported on how decapitated snails regenerate their heads. This intrigued scientists, scholars and laymen, who clamored to witness this astonishing biological marvel (111). Still today, this remains one of the most compelling questions of evolutionary biology; why can some animals regenerate injured tissue while others cannot? (142). To explore this question, the field of regeneration research harnesses the innate ability of lower vertebrates, such as salamanders, newts and zebrafish, to better understand these evolutionary inconsistencies (142-144). Results from investigations of molecular pathways controlling regeneration in non-mammalian models fuel stem cell research and further our understanding of *de novo* post development of tissue in whole organisms.

Regeneration in zebrafish:

Zebrafish (*Danio rerio*) are a preeminent model organism for investigating tissue regeneration given the ability of zebrafish to regenerate numerous organs and cells including fin (143), heart (145, 146), liver (147), optic nerve (148), retina (149), spinal cord (150), sensory hair cells (151), melanocytes (152), maxillary barbel (153), and olfactory bulb (154). There are many advancements in zebrafish genetics and genomics which contribute to the powerfulness of this model (127). In this research we used the caudal fin regeneration model to study why vertebrates demonstrate a decline in wound healing and regenerative capacity with age.

Zebrafish caudal fin regeneration:

The adult caudal fin is a complex and organized structure. The fin has 16-18 lepidotrichia (fin rays) connected to the skeleton by soft tissue. The individual rays are defined on each side by hemirays that are comprised of bony segments encased by a single layer of scleroblasts (bone-secreting cells). Between the hemirays are blood vessels, nerves, mesenchymal cells, fibroblast and melanocytes. An epidermal cell layer covers the entire lepidotrichia. Upon injury or surgical amputation, regrowth occurs by the gradual addition of bone segments to the distal end of the fin (as reviewed in (143)).

The restoration of lost or damaged caudal fin structures is mediated by a network of signaling cascades that instruct cells to coordinately commence the various stages of regeneration which include: wound healing, blastema formation, outgrowth, and termination. The regenerative process through which teleosts restore lost caudal fin structures is termed epimorphic regeneration. This is characterized by the formation of a mass of undifferentiated proliferative mesenchymal cells, called a blastema (155). After surgical amputation of a fin, the lost structure is completely replaced in approximately three days in larval fish and two weeks in adult fish (156). In the adult model, following amputation, wound healing begins with the lateral migration of non-proliferating epithelial cells over the injury plane to form an apical epithelial wound cap (AEC) within the first 12 hours post amputation (hpa) (157). Canonical Wnt signaling is required for formation of the wound epithelium (158, 159). Fibroblast Growth Factor (FGF) signaling is also critical to the

formation of the wound epidermis (160). Once the AEC is formed, mesenchymal cells proximal to the amputation site dedifferentiate and proliferate as they migrate to the area under the AEC to form a blastema within 48 hpa. Wnt/β-catenin and FGF signaling continues to be necessary for blastema formation and were demonstrated to be negatively regulated at this stage by miR-203 and miR-133, respectively (159, 161). The Activin-βA ligand, TGF-β, is also involved in regulating the molecular events involved with both wound healing and blastema formation (162). More recently, IGF-signaling was found to be required between the wound epidermis and blastema for a successful regenerative response (163). The later regenerative events that control outgrowth also require Wnt/β-catenin and FGF signaling (159, 161). Furthermore, Activin-βA also has a continued role in patterning of the outgrowth (143). RA signaling is active in the formation of wound epidermis and blastema, along with patterning and bone deposition (164-168). Both *hedgehog* (shh) and several bone morphogenic proteins (bmp) control skeletal regeneration and patterning (169, 170). Therefore, since miRNAs act to temporally and spatially regulate gene expression, it is plausible that miRNAs mediate the expression of key regulatory transcripts that coordinately initiate and terminate signaling pathways necessary for regeneration (171). Studies have identified individual miRNA-mRNA interactions important for regeneration (172, 173). To obtain a complete picture of all the regulatory molecules involved in regeneration, we conducted high-throughput RNA sequencing on regenerating and non-regenerating tissue. To the best of our knowledge, our research provides the field with the first fully profiled expression analysis of transcripts and small RNAs involved in zebrafish tissue regeneration.

miRNA role in caudal fin tissue regeneration:

There have only been two studies that investigated the role of miRNAs and caudal fin regeneration (172, 173). Both discovered that miRNAs target key regeneration-signaling molecules, Wnt and FGF, which are necessary for wound healing, blastema formation and regenerative outgrowth. One study transiently knocked down *dicer* in adult caudal fin tissue and demonstrated that miRNA biogenesis is necessary for regeneration (172). Additionally, this study showed that miR-203 negatively regulates the Wnt signaling molecule, Lef1 (172). The second study showed that Mpsk1 kinase, which is a positive regulator of blastemal

proliferation, is targeted by miR-133. Both studies demonstrated that miRNAs mediate the expression of transcripts necessary for a successful regenerative response.

This research uses the zebrafish model to explore how misregulation of miRNAs could have negative consequences during embryonic development and aging. Although these processes are distinctly positioned on opposite ends of a lifespan, we were able to use common bioinformatics, genomic and molecular tools to uncover the role of miRNAs in eliciting:

1. RA-induced vertebrate axis defects during development (Chapter 2)
2. age-related declines in tissue regeneration capacity (Chapter 3)

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Chapter 2 - Retinoic acid-dependent regulation of miR-19 expression elicits vertebrate axis defects

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Abstract

Retinoic acid (RA) is involved in multifarious and complex functions necessary for proper vertebrate growth and development. RA signaling is reliant on strict enzymatic regulation of RA synthesis and tissue specific metabolism. Improper spatial and temporal expression of RA during development can result in vertebrate body axis defects. microRNAs (miRNAs) are also pivotal in orchestrating complex developmental processes. While mechanistic links between miRNAs in early embryonic patterning and axial development are established, the role of miRNAs in regulating metabolic enzymes responsible for RA abundance during axis formation has yet to be elucidated. Our experimental results uncovered an undescribed role of miR-19 family members in controlling RA metabolism through the regulation of CYP26A1 expression during vertebrate axis formation. We conducted global miRNA expression profiling and report that developmental RA exposure suppressed the expression of several miR-19 family members during early stages of zebrafish somitogenesis. A physiological reporter assay was used to confirm that cyp26a1 is a bona fide target of miR-19 *in vivo*. Transient knockdown of miR-19 expression phenocopied body axis defects observed following developmental RA exposure. Furthermore, cyp26a1 expression during somitogenesis was increased in RA exposed embryos and miR-19 morphants. In gain-of-function studies, exogenous miR-19 rescued the axis defects induced by RA exposure. Taken together, these results indicate that the teratogenic effects of RA exposure result, in part, from repression of miR-19 expression and the subsequent misregulation of cyp26a1. This highlights a previously unidentified role of miR-19 in facilitating normal vertebrate axis development via regulation of the RA signaling pathway.

Introduction

Vitamin A (retinol) is an essential nutrient necessary for normal vertebrate growth and development. Retinoic acid (RA) is an active byproduct of vitamin A synthesis and a critical signaling molecule in morphogenesis. In vertebrates, numerous studies have identified a diverse range of functions of RA signaling including early axial development and patterning, regional patterning of the central nervous system, regulation of neurogenesis and limb development and a variety of roles during organogenesis (as reviewed in (1-3)). Both excesses and deficiencies in RA levels during development result in teratogenic effects (1, 4). Vitamin A deficiency induces congenital malformations in the cardiac, respiratory, ocular and urogenital systems (5, 6) whereas; excessive amounts of vitamin A can cause abnormal morphological development of the central nervous system, skeletal system, liver and skin (4, 7). Even though the etiology of many of these developmental abnormalities has been known for over 50 years, the molecular mechanisms underlying RA-induced teratogenicity are not fully understood.

The pleiotropic functions of RA signaling require strict control over tissue distribution during development (3). Two important tissues for RA production during embryonic development in vertebrates include the presomatic mesoderm (PSM)or mesenchymal tissue that lines both sides of the neural tube (as reviewed in (8)). In addition, the somites, which are epithelial spheres of mesoderm generated in a rhythmic pattern from the PSM give rise to the vertebrae and skeletal muscles in an RA-dependent fashion (8-11). RA signaling is required to orchestrate the directional, periodic, and synchronous segmentation of the PSM into somites through control of somite positioning along the anterior-posterior (A-P) body axis in a "clock and wavefront" type model (10, 12-14). RA signaling also controls symmetric somite formation along the left-right (LR) body axis through the segmentation clock (9, 11, 15-20). Perturbations of RA signaling during somitogenesis desynchronize somite formation in quail, zebrafish, mouse, and chicken (11, 18, 19, 21) mimicking RA related defects of the human vertebral column (22-24). RA mediates its key role in vertebrate segmentation by antagonizing the traveling FGF/WNT signaling gradient that controls the mechanism for somite spacing (25). In addition to mesoderm segmentation, evidence suggests that the

RA/FGF mutually negative feedback loop regulates neurogenesis and growth and differentiation of the elongating embryonic axis (as reviewed in (1)).

Although tight control over RA synthesis is essential during vertebrate segmentation, regulation of RA catabolism is equally important. Cyp26 enzymes, members of the RA cytochrome P450 family, convert all-trans-RA to polar metabolites (4-hydroxy-RA, 4-oxo-RA, 18-hyrdroxy-RA and 5,8-epoxy-RA) that are subject to conjugation and elimination (1, 2, 26). In most vertebrates, the Cyp26 family of enzymes includes Cyp26a1, Cyp26b1 and Cyp26c1, each with its own distinct expression domains during development (as reviewed in (2, 27)). Targeted manipulation of Cyp26a1 and Cyp26b1 results in abnormalities that mimic the teratogenic effects of exogenous RA exposure (28, 29), suggesting that Cyp26 enzymes function as primary players in RA detoxification. This supports the critical role of these enzymes in regulating the spatiotemporal distribution of RA and preventing inappropriate RA signaling during development (30, 31).

In addition to somite formation, RA has a pivotal function in protecting the paraxial mesoderm from molecular signals directing LR patterning and, thus, maintaining metamery in the PSM (11, 18-20). Disruptions in RA signaling causes asymmetry in somite formation (18). In a seminal paper on the topic, the role of key RA synthesizing (retinal dehydrogenase 2, raldh2) and metabolizing enzymes (cyp26a1, cyp26b1, and cyp26c1) in the mechanism that controls lateralization of RA in response to the LR information cascade was investigated. The expression of these enzymes was evaluated after experimental manipulation of the LR signaling network and no disparate asymmetries in transcript expression patterns were observed (18). Therefore, it was speculated that regulation of RA signaling by the LR information cascade is likely to be post-transcriptional (18).

microRNAs (miRNAs) are critical post-transcriptional regulators of developmental timing in vertebrates (32-34). Mature miRNAs are ~22 nucleotide long endogenous non-coding RNAs that bind the 3' UTR of target mRNAs resulting in mRNA destabilization and/or translational repression (35-38). Somitogenesis requires precise temporal regulation and is governed in part by miRNAs (39-46). Maternal zygotic *dicer* zebrafish mutants that lack the dicer enzyme

necessary to generate mature miRNAs have severe defects in somitogenesis, gastrulation, and heart and brain development (40). These mutants exhibit truncated tails resultant from reduced axis extension (40). Similarly, inactivation of *Dicer* during mesoderm development in mice elicited reduction in somite size, a reduced A-P axis, and caudalization of somites (46). Numerous studies have revealed the importance of miRNAs in somite derived sclerotome and dermomyotome development in mice (47-49), chicken (50, 51), zebrafish (39, 52, 53), xenopus (54) and segment formation in *Drosophila* (55). Taken together, these findings suggest miRNAs importance as post-transcriptional regulators of somitogenesis.

Several reports describe a mechanistic link between miRNAs, early embryonic patterning and somitogenesis. For example, in developing zebrafish, misregulation of miR-92 resulted in aberrant LR patterning (56). miR-In300 targets a gene involved in Wnt signaling, repressing the zebrafish myf5 promoter activity during somite formation (43). Delta-like-1, a ligand of the Notch signaling pathway, an important regulator of cyclic gene expression that dictates rhythmic somite production, is targeted by numerous miRNAs during somite development (42). miRNAs also modulate the expression of Hox cluster genes that participate in the patterning of the A-P axis (41, 44, 57-59). RA directly also regulates the transcription of several Hox genes (60-62), further suggestive of the importance RA-mediated miRNA regulation of somitogenesis. For example, miR-196 influences axial patterning through putative regulation of Hox genes expression, and it influences pectoral appendage development, specifically through direct regulation of the RA receptor gene, *rarb.* (41). Additionally, high throughput sequencing revealed the presence of novel miRNAs in chicken somite tissue (45). Despite the critical involvement of RA signaling in somite formation and vertebrate symmetry, the role of RA-controlled miRNAs in these developmental processes has yet to be investigated.

In this study we investigated the role of miRNAs in orchestrating teratogenic axis defects elicited by developmental exposure to RA. Our unbiased global miRNA expression profiling revealed that RA exposure dysregulated miR-19 expression during the early stages of zebrafish somitogenesis. Empirical experiments confirmed the role of miR-19 family members in cyp26a1 regulation and disruption of the RA signaling gradient. These findings

demonstrate the necessity for miR-19 in post-transcriptional regulation of RA metabolism and define the role for miR-19 in facilitating somitogenesis and normal vertebrate development.

Materials and Methods

Fish care and husbandry: All zebrafish (*Danio rerio*) were reared according to Institutional Animal Care and Use Committee protocols at the Sinnhuber Aquatic Research Laboratory, Oregon State University. The Tropical 5D strain was used for the described experiments. Adults were raised on a recirculating water system (28±1℃) with a 14 h light/10 h dark schedule. Spawning and embryo collection procedures were followed as previously described (89).

Retinoic acid exposure: All-trans retinoic acid (RA) (EMD Chemicals, Cat No. 554720) was dissolved in DMSO. A range finding experiment was conducted by batch exposing embryos (50-75 embryos) to 1- 1000 nM RA in buffered embryo medium(90) (100 μL/embryo) in 20 mL glass vials with Teflon-lined lids (VWR International). Each vial was treated as a single replicate. Embryos were exposed to RA or embryo medium control at 6 hpf. The vials were covered with aluminum foil to prevent photodegradation and incubated at 28 ± 1 $^{\circ}$C. At 48 hpf, the embryos were assessed for mortality and morphological defects. The concentration that elicited no mortality and only a single morphological defect of interest, a posterior curved body axis, in 100% of the embryos was determined to be 5 nM (data not shown). This concentration was used for the remaining experiments. Depending on experiment, fish were humanely euthanized using MS-222 overdose (90) at various time points (12, 24, 36, 48 or 120 hpf). Sub-groups were fixed with 4% paraformaldehyde (JT Baker) for *in situ* hybridization or immunohistochemistry experiments or homogenized in QIAzol Lysis Reagent (Qiagen) for gene expression analyses.

miRNA microarray assay: Total RNA was isolated from pooled tissue that was harvested from 75 embryos batch exposed to either 5 nM RA or embryo medium at 12, 24, 36 and 48 hpf (n=2) using miRNEasy Kits (Qiagen). LC Sciences performed the microarray assay on miR

Zebrafish V12 Chips that contained probes for all the sequences present in the miRBase Sequence Database (V12) as described previously (91). The data were first background-subtracted and normalized by LOWESS (locally weighted regression (92)). T-tests were conducted for differential expression (P < 0.05). The array dataset is available through the U.S. National Center for Biotechnology Information Gene Expression Omnibus (93) series accession number GSExxxxx (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc_GSExxxx).

Real-time quantitative PCR: Total RNA was isolated from whole body homogenate of pooled embryos using miRNEasy Kits (Qiagen) and oligo(dT)-primed cDNA was synthesized using Superscript III (Invitrogen) (n=3). Quantitative RT-PCR (qRT-PCR) was performed with gene specific primers for cyp26a1 and β-actin (Eurofins MGW Operon) listed in Table 2-S1 using the DyNAmo SYBR Green qPCR kit (Finnzymes) on the Opticon 2 real-time detection system (MJ Research). All samples were normalized to β-actin. To quantify miRNA expression, the miRCURY LNA™ microRNA PCR System (Exiqon) was used. Primer sets for miR-19 (PN 204781) and U6 small nucleolar RNA (snoRNA) (control, PN 203907) were used to assess miR-19 expression on a Step One Plus Instrument (Applied Biosystems).

Whole mount in situ hybridization: *In situ* hybridization was performed as previously described (94) using a digoxygenin-labeled antisense RNA probe to cyp26a1. To synthesize the probe, the cyp26a1 transcript was cloned using RT-PCR gene specific primers (Table 2-S1, Eurofins MGW Operon) and cDNA prepared from RNA isolated as described above from whole zebrafish at 24 hpf. Digital images were captured using a Nikon Coolpix E500 digital camera mounted on a Nikon SMZ 1500 stereomicroscope.

Immunohistochemistry: Embryos were fixed at 24 hpf in 4% paraformaldehyde overnight at 4 °C. Rabbit α-zebrafish CYP26A1 (dilution: 1:1000, AnaSpec, Catalog No. 55733) primary antibody and Alexafluor® 555 goat α-rabbit (dilution: 1:1000,Molecular Probes) secondary antibody were used. Briefly, fixed embryos were washed with PBST followed by Milli-Q water (Millipore™) for 1 h. A 10 min collagenase (0.0001 g/mL PBST, Sigma, C9891) treatment was performed to permeabilize the embryos, followed by a 30 min rinse with PBST. The embryos were blocked with 10% Normal Goat Serum (Sigma, G6767) for 1 h at room temperature

prior to adding the primary antibody, in which the samples were incubated overnight at 4 °C. The following day all samples were rinsed in PBST for 1.5 h, incubated with secondary antibody for 1.5 h, and washed 2X for 15 min followed by 3X for 30 min with PBST. Embryos were imaged on an inverted Zeiss Axiovert 200 M epi-flourescence microscope using a Zeiss Axiocam HRm camera.

Microinjections: An antisense oligonucleotide morpholino (MO) designed against both the guide strand and *dicer* cleavage site of dre-miR-19 or a control 3' fluorescein-tagged MO (Gene Tools) (Table 2-S2) were injected into single-cell stage embryos. Approximately 2 nl of 1.5 mM morpholino in ultrapure water with 0.5% phenol red was microinjected into each embryo. At 24 hpf, the fish were screened for uniform incorporation of the MO by fluorescein visualization under ultraviolet light. A synthetic miR-19 or control Dharmacon miRIDIAN mimic was injected at 10 µM (Thermo Scientific, C-300488-03-0005).100 ng of the cyp26a1 3' UTR GFP reporter was co-injected with either the synthetic miR-19 or control mimic (10 µM) into single-cell stage embryos. Following microinjection of miR-19 MO or mimic, qRT-PCR was performed to examine miR-19 expression at 12 and 24 hpf.

GFP reporter assay: The zebrafish cyp26a1 3' UTR sequence was amplified by RT-PCR using adult zebrafish cDNA and gene-specific primers (Table 2-S1, Eurofins MGW Operon). The sequence was subcloned downstream of the GFP open reading frame (ORF) that was inserted into pCS2+ vector (95). Reporter injected animals were placed into 384 Well Polystyrene Microplates and imaged using an Image Xpress Micro (Molecular Devices). Measurements of pixel count/embryo were obtained from whole body images of 8 embryos using Metaexpress Software (Molecular Devices) at 24 hpf after 5 independent injection days (n = 40).

Statistical analysis: To analyze differences between treatment and control groups, a Student's *t*-test or 1-way ANOVA with a Dunnett's or Tukey's multiple-comparison *post hoc* tests were conducted. Data shown represents means ± SEM; values of $P \leq 0.05$ were considered statistically significant. Results were calculated using Prism 5.01 (Graph-Pad).

Results

RA exposure misregulates miRNA expression during zebrafish somitogenesis. Exposure to 5 nM RA from 6-24 hpf resulted in a single distinct posterior curved body axis defect in 100 % of the larvae at 48 hpf (Fig. 2-1B) in comparison to control (Fig. 2-1A). To determine whether misexpression of miRNAs may play a role in observed body axis defects, a miRNA microarray was performed with whole embryo homogenate collected at four time points spanning early zebrafish organogenesis (12, 24, 36 and 48 hpf). Numerous miRNAs were significantly differentially expressed in comparison to vehicle exposed controls (Fig. 2-S1). The greatest number of misexpressed miRNAs was observed at 12 hpf, which falls within the early stages of zebrafish somitogenesis (10-24 hpf). Three members of the zebrafish miR-19 family (miR-19a, miR-19c and miR-19d) were significantly decreased relative to the control (Table 2-1). In addition to the miR-19 family members, the expression of miR-22a was decreased 1.5 fold in RA exposed embryos (Table 2-1). Validation of the miRNA microarray results by qRT-PCR confirmed repression of miR-19 expression by RA at 12 hpf (Fig. 2-1C).

RA exposure disrupts cyp26a1 expression at the onset of somitogenesis. The posterior body axis curvature elicited by RA exposure is consistent with the asymmetrical patterning and irregular somite sizes that result during misregulation of somitogenesis (11, 18, 19). Bioinformatics analysis was conducted with the miRNA tools incorporated in Bioinformatics Resource Manager (http://www.sysbio.org/dataresources/brm.stm) (63) to uncover potential targets that are both related to RA signaling and somitogenesis, and targeted by misexpressed miRNA at 12 hpf. The 3' UTR of zebrafish cyp26a1, the primary enzyme responsible for converting active RA into its inactive polar metabolites, is predicted by EMBL-EBI microCosm (www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) to be targeted by miR-19a, c, and d, miRNAs that were significantly repressed by RA exposure at 12 hpf (Table 2-1). Of all miRNAs examined following transient developmental RA exposure, these miR-19 family members exhibited the largest magnitude decrease in expression at 12 hpf in comparison to control (Table 2-1). In contrast, a fourth miR-19 family member, miR-19b, was not predicted to target the cyp26a1 3'UTR nor was it significantly downregulated at 12 hpf. Consistent with the concept that miR-19 family members post-transcriptionally regulate

cyp26a1, there was a concomitant increase in cyp26a1 transcript expression relative to controls at 12 hpf in embryos exposed to RA (Fig. 2-2A). To investigate the spatial distribution of cyp26a1 transcript expression during the later stages of somitogenesis, whole mount in situ hybridization analysis was employed at 24 hpf and indicated that there was a significant expansion of cyp26a1 transcript expression in the tailbud region of RA exposed embryos compared to controls (Fig. 2-2B, C). Immunohistochemical analysis showed that RA induced CYP26A1 protein expression at 24 hpf in a pattern similar to that of the transcript (Fig. 2-2D, E).

Transient knockdown of miR-19 recapitulates axis defects induced by RA exposure. To determine whether decreased miR-19 was sufficient to produce the developmental axis defect shown in Fig. 2-1A, a single MO was designed to knock down miR-19a, miR-19-c, and miR-19-d during development (Fig. 2-3A, Table 2-S2). Embryos injected with the miR-19 MO exhibited the posterior curved body axis defect observed in embryos exposed to RA (Fig. 2-3B-E). Validation of miR-19 knockdown in miR-19 morpholino injected animals was confirmed via qRT-PCR analysis (Fig. 2-3F). Additionally, the expression of cyp26a1 transcript (Fig. 2-3G, H) and CYP26A1 protein (Fig. 2-3I, J) was increased in the tailbud region of the miR-19 MO injected embryos relative to controls at 24hpf.

miR-19 targets the 3' UTR of cyp26a1 in vivo. To confirm that cyp26a1 is targeted by miR-19 *in vivo,* a reporter was designed that contained the 3' UTR of cyp26a1 downstream of GFP (GFP-cyp26a1-3'UTR , Fig. 2-4A). The reporter was co-injected into the single-cell of embryos with either a miR-19 mimic or a control mimic and GFP expression was quantified at 24 hpf. Co-injection of the GFP-cyp26a1-3'UTR reporter and miR-19 mimic produced a notable decrease in GFP expression in comparison to animals co-injected with the reporter and control mimic (Fig. 2-4B). Quantification of the pixel count per embryo demonstrated a significant decrease in fluorescence in the GFP-cyp26a1 3' UTR reporter and miR-19 mimic co-injected animals in comparison to GFP-cyp26a1 3' UTR reporter and control mimic injected animals (Fig. 2-4C).

Exogenous miR-19 represses cyp26a1 and rescues RA induced axial defects. To determine whether rescue of miR-19 repression is sufficient to prevent axial defects induced by RA exposure, embryos were injected with an exogenous miR-19 mimic then exposed to RA from 6-24 hpf. Injection of synthetic miR-19 mimic significantly increased the level of miR-19 transcript present (Fig. 2-5A) and decreased cyp26a1 transcript expression (Fig. 2-5B) at 12 hpf. *In situ* hybridization revealed that miR 19-mimic embryos were nearly devoid of cyp26a1 transcript expression in the tailbud region during the later stages of somitogenesis (24 hpf) as compared to the control mimic injected embryos (Fig. 2-5C, D). Injection of exogenous miR-19 rescued the RA-induced curved body axis (Fig. 2-5E, F).

Discussion

Perturbations in developmental RA signaling result in teratogenic effects (as reviewed in (1, 3)) and miRNAs are known to absolve transcriptional programs from previous developmental stages (as reviewed in (64-66)). Therefore, we sought to determine whether dysregulation of miRNA signaling resulted in the hallmark curved body axis phenotype triggered by RA exposure. Studies suggest that the developmental role of RA is strongly related to the enzymatic regulation of its synthesis and tissue specific metabolism (as reviewed in (1)). Therefore, understanding how miRNAs might regulate enzymes responsible for RA abundance that, in turn, affect the expression of RA-responsive genes is critically important. During somitogenesis, CYP26A1 is necessary for maintaining homeostasis of RA activity in the PSM (as reviewed in (1, 3)). Studies have demonstrated the importance of CYP26A1 in vertebrate axis formation (28, 67). Our experimental results uncovered a role for several miR-19 family members in controlling metabolism of RA, specifically through the regulation of CYP26A1 expression during the early stages of zebrafish somitogenesis.

Our data indicate that developmental exposure to RA represses the expression of miR-19 family members (miR-19a, miR-19c, and miR-19d) during early somitogenesis (12 hpf), but not at a later stage investigated (24 hpf) (Table 2-1 and Supplemental Fig. 2-1). This is consistent with previous findings that RA is only necessary during the early stages of somitogenesis (somites 1-6 in mice) to assure proper and synchronous development of the

remaining somites (7, 68). While all three of these miR-19 family members are predicted to target the 3' UTR of cyp26a1, this had not been confirmed *in vivo* prior to our study. We showed that the 3' UTR of cyp26a1 is a bona fide target of miR-19 using *in vivo* reporter analysis (Fig. 2-4A-C). Co-expressed miRNAs are known to act cooperatively to regulate the 3' UTR of a common mRNA (69), supporting the concept that RA induced repression of miR-19 family members likely acts as a compensatory mechanism to increase the expression of one of the molecule's main detoxifying enzymes. Additionally, repression of miR-19 *in vivo* recapitulated the distinct posterior curved body axis morphology (Fig. 2-3C, E) and exogenous miR-19 rescued the classic body axis defects associated with RA exposure (Fig. 2-5F). These findings demonstrate a direct post-transcriptional miRNA interaction with an enzyme indispensable for RA metabolism, and its consequences on somitogenesis and, more broadly, the regulation of vertebrate axis formation.

The morphological defects exhibited by zebrafish developmentally exposed to RA are similar to those demonstrated by both transient and conditional knockdown of CYP26A1 and retinal dehydrogenase 2 (RALDH2) in diverse species (28, 70). CYP26A1-null mutants demonstrate morphological defects that are strikingly similar to those exhibited upon exposure to excess RA, including spina bifida and posterior truncation of the tail and lumbosacral region (28, 71). Similar to the morphological abnormalities we documented in embryonic zebrafish upon developmental exposure to RA, CYP26A1-null embryos also presented with abnormally curved notochords (28). Lack of CYP26A1 expression in the tailbud prevents the clearance of RA from the rostrally adjacent mesoderm (28). *Adu-Abed et al.* hypothesized that the resulting dearth of CYP26A1 creates a state of RA "endogenous" teratogenicity that recapitulates the effects of excess exogenous RA exposure (28). RALDH2-/- mutants that are deficient in endogenous RA also demonstrate shortened body axes and abnormally small somites that can be rescued by maternal administration of RA (70). In this study, exposure to RA suppressed miR-19 expression. Transient knockdown of miR-19 mimicked RA induced body axis defects and resulted in increased cyp26a1 expression (Fig. 2-3B-J). Although many miRNA knockouts fail to confer a gross phenotype (as reviewed in (72)), our knockdown of all the miR-19 seed family members predicted to target cyp26a1 resulted in an observable morphological defect in the whole animal. Elevated levels of miR-19 also reduced cyp26a1

expression (Fig. 2-5A-D). Additionally, in our rescue experiment, the body axis curvature caused by exogenous RA was rescued by artificially increased miR-19 levels and subsequent suppression of CYP26A1 expression. Hence, we postulate a negative feedback loop in which exogenous RA exposure during development downregulates miRNAs that negatively regulate CYP26A1, leading to increased CYP26A1 expression and decreases in endogenous RA expression. This builds on a recent paper that purposed a paradoxical mechanism for RA teratogenicity by demonstrating that adverse effects observed by RA exposure are a result of local RA deficiency (73). Thus, our findings place post-transcriptional regulation of cyp26a1 by a conserved class of miRNAs at the nexus of RA induced teratogenicity.

miRNA regulation of multiple genes in a shared pathway strengthens their impact (as reviewed in (72)). During somitogenesis, opposing RA and FGF pathways act antagonistically in the elongating body axis (25). Our microRNA array data also indicated that at 12 hpf, early developmental exposure caused a significant decrease in miR-22a (Table 2-1), the only miRNA predicted by EMBL-EBI microCosm (www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) to target the 3' UTR of fgf8, suggesting that FGF signaling may also be controlled by RA-sensitive miRNAs during early somitogenesis. RA reportedly attenuates FGF8 levels by transcriptional repression or mRNA decay (10). Additionally, FGF8 represses the onset of RALDH2 expression in the PSM and downregulation of FGF8 is necessary for normal axis formation (25, 74). Fgf8 transcript expression is non-existent in CYP26A1-deficient mice and is shown to have increased expression in response to decreased RA signaling molecules (25, 28). Changes in FGF8 expression in RA-deficient embryos result in aberrations in the determination wave-front that controls somite size, further demonstrating the mutual inhibitory role of RA and FGF signaling in axis development (1, 10, 19, 25, 68). Our data raises the possibility that RA-sensitive miRNA that target FGF signaling might also play a role in RA toxicity. Much remains to be learned about the post-transcriptional regulation of RA and FGF interactions during vertebrate segmentation.

Beyond cyp26a1, our findings have broader implications to altered temporal-spatial expression of genes transcriptionally regulated by RA or induced by FGF expression. These targets include members of the Hox gene family that regulate axis patterning, specification

and left-right symmetry (74). RA acts as a transcriptional activator for several Hox genes and Notch signaling (58, 60-62). Additionally, FGF signaling is functionally associated with Hox gene expression and axis patterning (as reviewed in (75)). Several studies have explored the role of miRNAs conserved within vertebrate Hox clusters in regulating Hox gene expression and subsequently impacting axial development and patterning (41, 44, 57, 59). Furthermore, specific Hox gene expression changes can be partially modulated by miR-196 regulation of the retinoic acid receptor gene, rarb (41). This supports our findings on the role of miRNAs in controlling RA abundance during morphogenesis and further confirms the necessity of miRNAs in the precise regulation of RA signaling during vertebrate axis development.

In addition to the role of miR-19 in RA metabolism, our finding that miR-19 family members regulate vertebrate axis formation provides compelling insight into their evolutionary significance. The miR-19 family has no known homologs in invertebrates (76, 77). The introduction of miRNA families correlates with drastic increases in morphological complexity (77-79). This is attributed to miRNAs role in stabilizing gene expression, and, in turn, rendering phenotypic traits influenced by miRNA-targets more likely to evolve (77, 80). It is hypothesized that the continuous addition of novel miRNAs to metazoan genomes is influential in the canalization, or evolved robustness, of a trait and contributes to the production of distinct developmental outcomes (77, 80). This in conjunction with our experimental data suggests the evolution and necessity for miR-19 in vertebrate identity. Taken together, the findings presented stress the evolutionary significance of miR-19 in somitogenesis, a developmental phenomenon common to all vertebrates.

Beyond its importance as miRNA family acquired by vertebrates, miR-19 is a member of the evolutionarily conserved miR-17-92 cluster (as reviewed in (81)). In zebrafish, the miR-19 family members are located in three conserved clusters miR-17-92, miR-106a-363, and miR-106b-2 (Table 2-S3). The high conservation of these clusters underscores their functional relevance, while the unique sequences of the mature miRNAs direct their interactions with target mRNAs and ultimately govern their function (82). The miR-17-92 cluster encodes the miRNA genes miR-17, miR-18a, miR-19a, miR-20a, miR-19b and miR-92a1 in humans, mice, zebrafish, and over 25 other known vertebrate species (miRBase). To date, miR-19c and miR-

19d miRNAs have only been identified in lamprey and medaka, respectively, in addition to zebrafish (miRBase). This is likely due to the genome duplication events leading to teleost fish (80, 83, 84). The emergence of miR-19 in the miR-17-92 cluster and its paralogs supports the speculation that tandem duplication events play a pivotal evolutionary role in the emergence of novel miRNA (76, 80). Additionally, the origin of novel miRNAs in pre-established clusters eliminates the necessity for the evolution of separate promoters for the new miRNAs (80).

An elegant study demonstrated an essential function of miR-17-92 for normal development in mice (85). The results also demonstrated a functional cooperation between miR-17-92 and miR-106b-25 clusters. Additionally, it was speculated that among cluster members, it was the loss of miR-19 family members and miR-18 that were responsible for the observed developmental phenotypes in mice (85). The teratogenic effects we observed due to developmental RA exposure were notably accompanied by a decrease in the expression of three miR-19 family members, miR-19a, miR-19c, miR-19d, each located in one of the three miR-17-92 paralog clusters. This also suggests a plausible functional synergy between the clusters dictating the observed developmental axis defects.

The only miR-19 family member that was not misregulated upon developmental RA exposure in this study was miR-19b, which is located in the miR-17-92 cluster with miR-19a (Table 2-1 and S2). Previous research highlighted differences in miR-19a and miR-19b expression, suggesting their functional divergences even though they evolved from the same cluster (86). Structural analysis of the miR-17-92 cluster in humans suggests that efficient enzymatic (Drosha) processing of miR-19b is sterically hindered (87). A similar structural limitation in the zebrafish miR-17-92 cluster may explain why miR-19b expression was not affected by RA exposure in stark contrast to its homolog, miR-19a.

In support of our findings implicating miR-17-92 cluster members in vertebrate axis formation, a germline deletion of the miR-17-92 cluster was found to elicit skeletal abnormalities in humans (88). This was the first report of a miRNA mutation causing a hereditary condition responsible for developmental defects in humans. While the cluster

regulates TGF-β and sonic hedgehog signaling, two pathways influential in skeletal development, the authors state that other targets of the miR-17-92 cluster that control skeletal growth and patterning likely exist (88). Our data expand on these findings to describe a mechanism through which specific miR-17-92 cluster members controls the proper development of somites, precursors to skeletal formation.

Together, this study highlights a role for miR-19 in facilitating normal vertebrate development by serving as a RA-sensitive switch to promote CYP26A1 mediated RA turnover during somitogenesis. Our findings suggest a previously undescribed miRNA-driven compensatory mechanism initiated to increase the expression of CYP26A1 during a period in which spatial maintenance of endogenous RA abundance is critical. Furthermore, these data underscore the evolutionary significance and plausible relevance of miR-19 as a vertebrate innovation.

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Acknowledgments

Figure 2-1 Developmental RA exposure results in axial defects and suppression of miR-19 expression.
Embryos were exposed to 5 nM RA from 6 – 24 hpf. Representative images of (A) control and (B) RA exposed animals at 48 hpf. (C) miR-19 expression levels were measured by qRT-PCR at 12 hpf in pools of 25 embryos exposed to 5 nM RA. (Values represent fold-change compared to unexposed control embryos, mean ± SEM, n = 3, *P < 0.05, independent samples *t* test.)
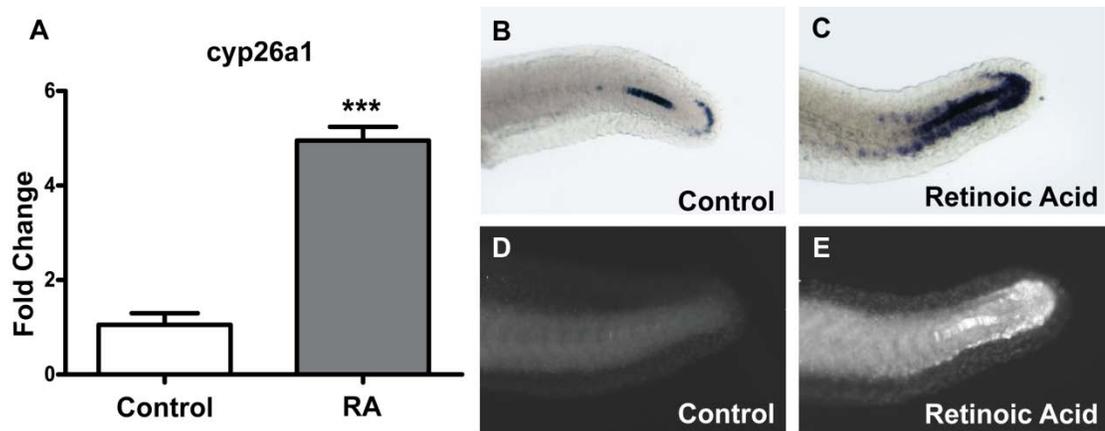
Figure 2-2. Transient developmental exposure to RA disrupts cyp26a1 expression during somitogenesis.
 (A) Embryos were exposed to 5 nM RA from 6-12 hpf and RNA was collected at 12 hpf. cyp26a1 expression levels at 12 hpf in embryos exposed to 5 nM RA. (The data represent fold-change relative to control exposed embryos, mean ± SEM, n = 3, ***P < 0.001, independent samples *t* test.)   Representative images at 24 hpf showing measurements of cyp26a1 transcript detected by *in situ* hybridization and protein levels detected by IHC in (B,D) controls as compared to (C,E)RA exposed embryos.

A

B

CT MO

C

miR-19 MO

D

CT MO

E

miR-19 MO

F

miR-19

G

CT MO

H

miR-19 MO

I

CT MO

J

miR-19 MO
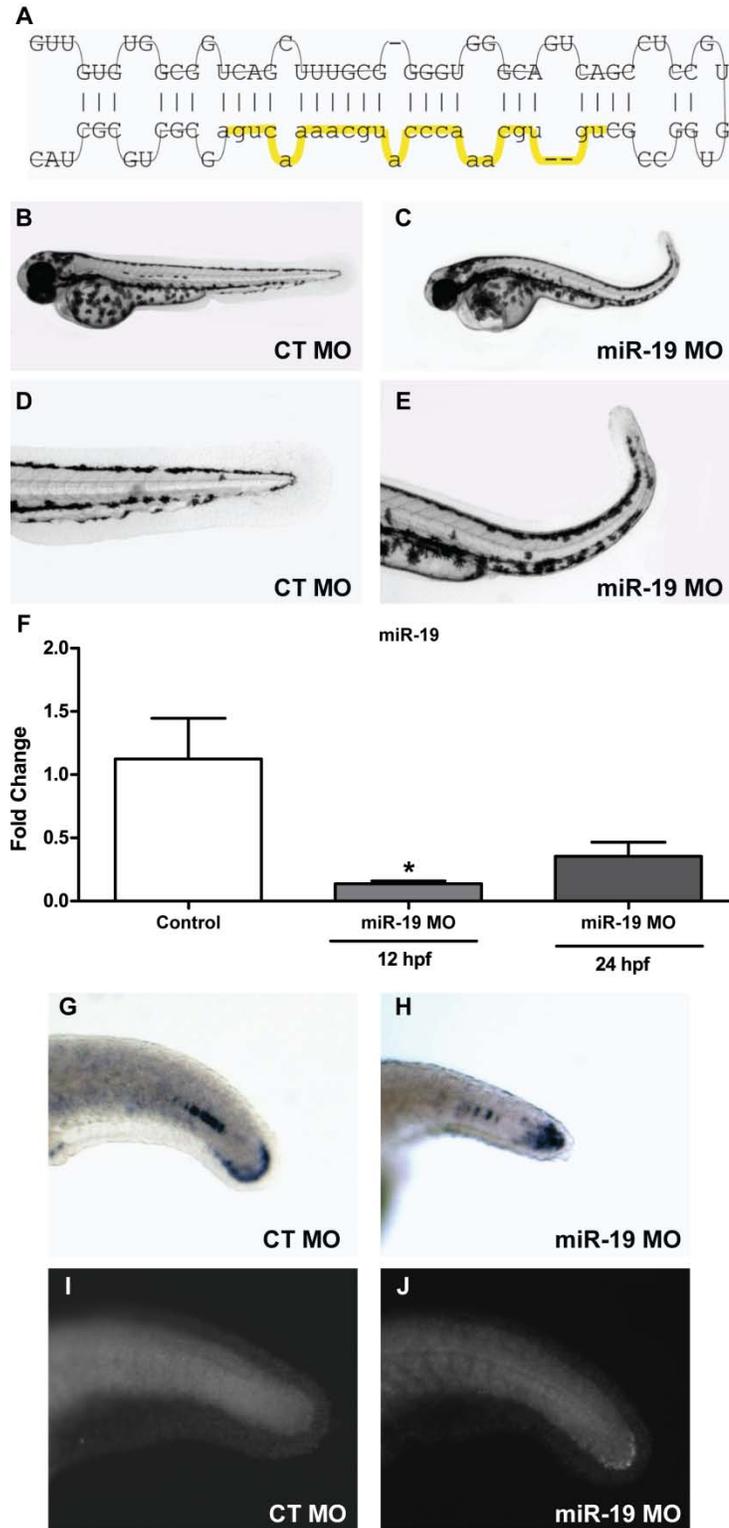
Figure 2-3. miR-19 disrupts axial patterning and development through post transcriptional regulation of cyp26a1.

(A) Schematic of the miR-19d MO target site on the pre-miR-19d sequence that was injected into single-cell staged embryos. Representative images of the axis defects elicited by miR-19d MO (C,E) in 48 hpf embryos in comparison to (B,D) control (CT) MO injected embryos (F) Effective knockdown of miR-19 transcript levels confirmed in miR-19d MO injected embryos at 12 hpf and 24 hpf via qRT-PCR. (Values reflect fold-change relative to control MO injected embryos, mean ± SEM, n = 3,*P < 0.05, One-way ANOVA with Dunnett's Multiple Comparison Test.) Representative images of cyp26a1 transcript and protein expression at 24 hpf in (G,I) controls relative to (H,J) miR-19 morphants as demonstrated by *in situ* hybridization and IHC, respectively.
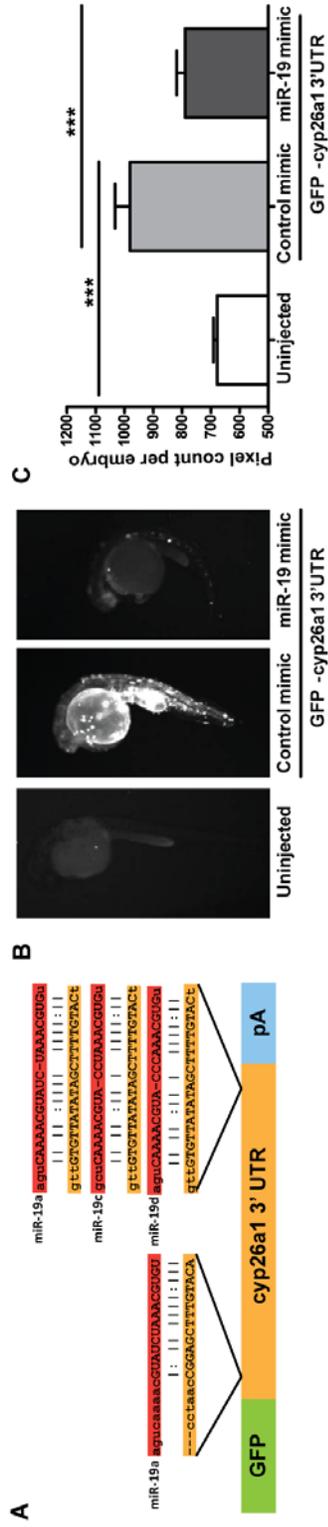
Figure 2-4. Physiological reporter assay confirms that cyp26a1 is a bona fide target of miR-19 *in vivo*.

(A) Simplified schematic of cyp26a1 3' UTR reporter assay along with predicted miR-19 a, c and d MREs. Single-cell stage embryos were co-injected with the GFP-cyp26a1 3' UTR reporter and either miR-19 mimic or control (CT) mimic. (B) Representative images at 24 hpf and (C) the quantification of pixel count per embryo was measured. (Significance represents co-injected GFP-cyp26a1 3' UTR reporter and miR-19 mimic in comparison to reporter and control mimic or reporter and mimic coinjected animals in comparison to uninjected controls, mean ± SEM, n = 40, ***P < 0.001, One-way ANOVA with Tukey's Multiple Comparison Test.)
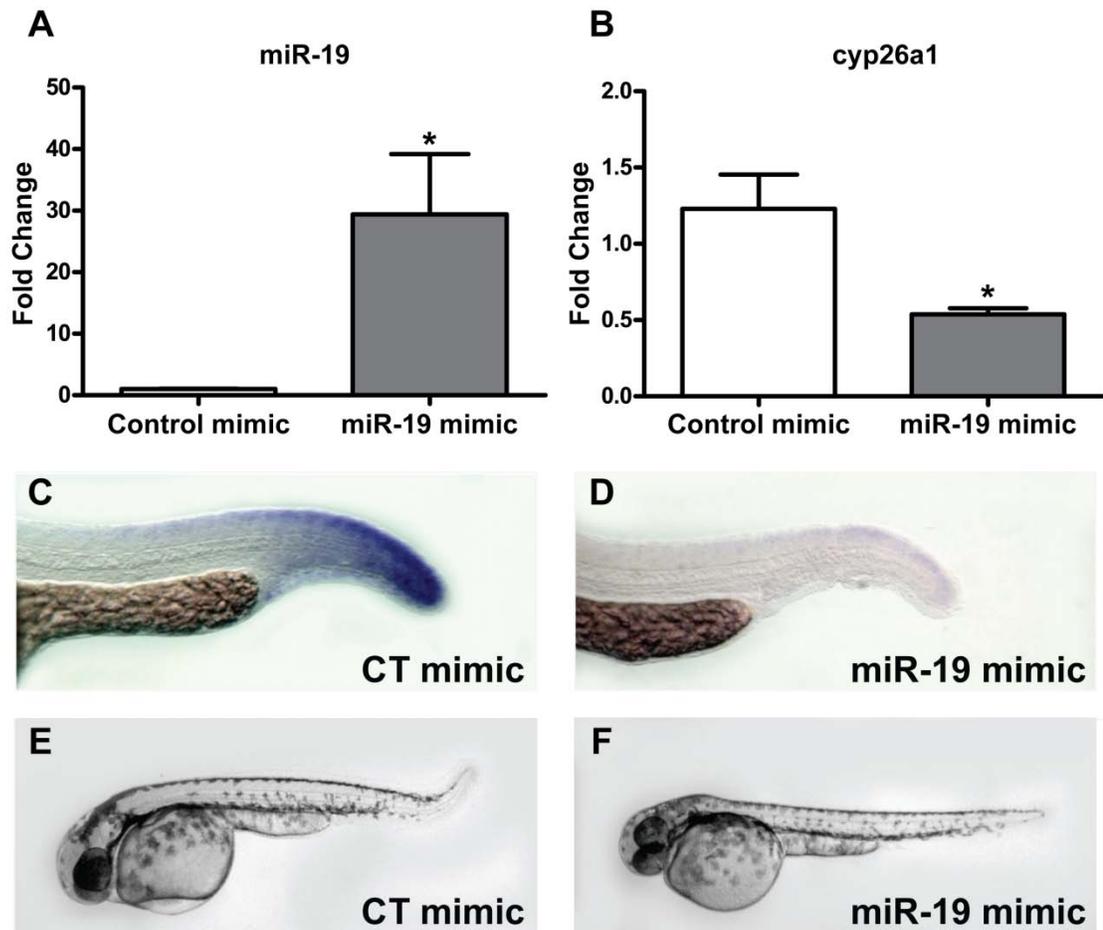
Figure 2-5. Exogenous miR-19 reduces cpy26a1 expression and rescues the body axis defects elicited by RA exposure.

(A) miR-19 transcript expression levels at 12 hpf in pools of embryos injected with miR-19 or control mimics. (Values represent fold-change compared to control mimic injected embryos, mean ± SEM, n = 3,*P < 0.05, independent samples *t* test.) (B) cyp26a1 expression levels measured by qRT-PCR in 12 hpf embryos injected with exogenous miR-19 mimic. (The data represents fold-change relative to control exposed embryos, mean ± SEM, n = 3,*P < 0.05, independent samples *t* test.) Representative images of cyp26a1 expression in the tail bud as measured by *in situ* hybridization in (C) control mimic or (D) miR-19 mimic injected embryos. Embryos injected with (E) control mimic or (F) miR-19 mimic were exposed to RA from 6-24 hpf. Representative images showing that miR-19 mimic rescues RA induced body axis defects.

Table 2-1. miRNA microarray results at 12hpf.

miRNA Microarray Data: 12 hpf		
miRNA	**p-value**	**Log2**
dre-miR-22a	2.52E-02	-1.98
dre-miR-19c	2.95E-02	-2.40
dre-miR-21	3.05E-02	-0.52
dre-miR-19a	4.64E-02	-1.71
dre-miR-19d	4.78E-02	-2.74
dre-miR-145	3.13E-02	-0.55
dre-miR-143	4.50E-02	0.55
dre-miR-218a	6.06E-03	0.55
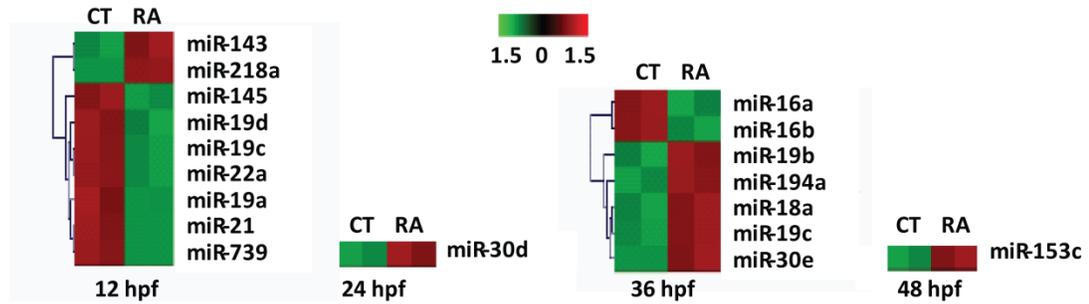
Figure 2-S1. Heatmap of miRNA microarray results.
Results of miRNA microarray analysis performed on samples obtained at 12, 24, 36 or 48 hpf from embryos batch exposed to 5 nM RA from 6 – 24 hpf (n=2). Heatmap representing bi-directional hierarchical clustering of significant miRNA genes (The data represents fold-change relative to control exposed embryos, n = 2, P < 0.05, 5% FDR, *t* test.) miR-739 was removed from the miRBase database due to its prediction as a fragment of rRNA.

Table 2-S1. Cyp26a1 and β-actin primer sequences used for experiments.

Gene	Experiment	Forward (5'-3')	Reverse (5'-3')
b-actin	qRT-PCR	AAGCAGGAGTACGATGAGTC	TGGAGTCCTCAGATGCATTG
cyp26a1	qRT-PCR	CAGCAGGAGGTGAAGAGCGCC	TCCACCAGTTCTTGCTCGTCCG
cyp26a1	*In Situ* Hybridization	CCGTTTTACTCTTTCTCGCC	CTGCACCACTTCTGTGTTCA
cyp26a1 (3' UTR)	Reporter Assay	TAGCCTAACCGGAGCTTTGTAC	CTCACTCAGTCCTACTGAAATTTG

Table 2-S2. Morpholino sequences.
Sequences for the miR-19d (miRNA Accession number) and control morpholino.

miRNA	Accession	MO sequence (5'-3')	MO type
dre-miR-19d	MIMAT0001785	TCAGTTTTGCATGGGTTTGCACA	Guide Dicer
control MO	-	CCTCTTACCTCAGTTACAATTTATA	-

Table 2-S3. Zebrafish miR-19 family members.
Mature sequences (seed sequence shown in red) and genomic cluster context information for members of zebrafish miR-19 family.

	Sequence	Cluster	Additional cluster members
dre-miR-19a	UGUGCAAAUCUAUGCAAAACUGA	**miR-17-92**	miR-17a-1, miR-18a, miR-20a, **miR-19b**, miR-92a-1
dre-miR-19b	UGUGCAAAUCCAUGCAAAACUGA	**miR-17-92**	miR-17a-1, miR-18a, **miR-19a**, miR-20a, miR-92a-1
dre-miR-19c	UGUGCAAAUCCAUGCAAAACUCG	**miR-106a-363**	miR-18c, miR-20b, miR-363
dre-miR-19d	UGUGCAAACCCAUGCAAAACUGA	**miR-106b-2**	miR-93, miR-25

Chapter 3 – Regulatory role of miRNAs in age-related declines in tissue regeneration capacity

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Abstract

Aging is coupled with a striking decline in the ability to maintain and repair damaged tissue. microRNAs (miRNAs), small non-coding RNAs that post-transcriptionally repress gene expression, are implicated in the regulation of aging in various tissues. While links between miRNAs and aging are established, the mechanistic role of miRNAs in declining regenerative capacity with age has yet to be elucidated. To determine whether age-related changes in miRNAs trigger deficits in tissue restoration, we performed RNA sequencing on regenerating and non-regenerating zebrafish caudal fin tissue from three developmental cohorts (juveniles, adults and aged). This is the first unbiased look at small and messenger RNAs in tandem, and provides a comprehensive picture of regulatory molecules responsible for age-related declines in regenerative capacity. We identified 13 transcripts that had significant but inverse expression in our aged population, which have a compromised regenerative response, as compared to juveniles and adults that regenerate successfully. Cbx7, chromobox protein homolog 7, was significantly decreased in aged regenerating tissue and increased in younger regenerating tissue. While the role of cbx7 in aging has been defined, this is the first report of its role in tissue regeneration. Subsequently, a non-biased bioinformatics approach was used to discover mRNAs expressed during regeneration which are targets of the significantly expressed miRNAs in regenerating tissue. Members of the miR-21 and miR-181a family were the most abundant miRNAs in regenerating and non-regenerating tissue across age cohorts and both were predicted to target the 3' UTR of cbx7. qRT-PCR confirmed that miR-21 and miR-181a* were significantly increased in regenerating tissue compared to non-regenerating tissue and miR-21 expression was attenuated in aged tissue upon regeneration. A physiological reporter assay was used to demonstrate that cbx7 is a *bona fide* target of miR-21 and miR-181a*, and microinjection of exogenous miR-21 reduced cbx7 expression *in vivo.* Transient knockdown of miR-21 inhibited tissue regeneration in larval zebrafish, suggesting a role for miRNA mediated regulation of cbx7 in tissue regeneration. Taken together, these results identify a novel, age-dependent regenerative function of cbx7 and underscore the importance of miR-21 as a master regulator of vertebrate regenerative responses.

Introduction

One of the hallmarks of aging is a compromised ability to maintain tissue homeostasis and repair damaged tissue. A decline in regenerative capacity is common to most animals and is often attributed to a reduced ability to properly reactivate quiescent progenitor populations required to restore tissue. Organismal lifespans are defined by accumulated molecular, cellular, and tissue damage balanced against the ability to reawaken latent developmental signaling cues necessary to facilitate regeneration (1).

Research over the past decade has implicated the role of microRNAs (miRNAs) in development and diseases (2, 3). Recent work has begun to uncover the functional role miRNAs have in aging and cellular senescence (as reviewed in (1, 4, 5)). Additionally, research demonstrated that miRNAs regulate somatic stem cells required for tissue homeostasis and repair (as reviewed in (6)). miRNAs are small, ~21-24 nucleotide, non-coding RNAs involved in post-transcriptional regulation of gene expression (2). In animals, interaction between miRNAs and target mRNAs often occurs through base pairing of nucleotides 2 – 8 of miRNAs to complementary target sequences in the 3' untranslated region (UTR) of mRNAs, resulting in mRNA degradation or translational repression (2). miRNAs have important functions in cell fate decisions which dictate differentiation, pluripotency, and proliferation (6-8). This underscores their importance in the complex processes of aging and regeneration. To date, functional roles for miRNAs were demonstrated in regenerating heart (9), hair cells (10), skin (11), pancreas (12), skeletal muscle (13), limbs (14), fins (15), oligodendrocytes (16), retina (17) and liver (18).

It is difficult to study regeneration in mammalian models because, like humans, mammals fail to respond to most tissue injury with a regenerative response. Aging and regeneration research present similar challenges in choosing the most suitable model that will best translate to humans. Aging is uniquely challenging in that subtle changes over many months or years of a mammal's life preclude study of a discrete mechanism. To tackle this problem, a vertebrate model with a short lifespan has proven to be ideal (19). How and why evolution led to the loss of regenerative capacity in mammals is still unclear, but we can begin to

understand the molecular events that drive regeneration by using model vertebrates, such as amphibians and teleosts, which have maintained the remarkable capacity to regenerate tissues and organs (20).

We used zebrafish as a model vertebrate organism to investigate mechanisms for the age-dependent decrease in wound healing capacity, and to better understand why mammals cannot regenerate most tissues. Numerous studies have highlighted advantages of zebrafish as a model of aging (as reviewed in (19, 21, 22)), and regeneration (as reviewed in (20, 23-25)). Additionally, zebrafish can regenerate certain tissues, such as the caudal fin, throughout their entire lifespan (this research). However, this regenerative capacity often becomes compromised with age, in turn, making zebrafish an ideal model for studying age related declines in tissue regeneration. The zebrafish caudal fin regeneration paradigm was used for this study, in which zebrafish restore lost caudal fin tissue through a process termed epimorphic tissue regeneration. This is characterized by wound healing commencing with the lateral migration of epithelial cells to form an apical epithelial wound cap (23-26). Following wound healing, a mass of undifferentiated highly proliferative mesenchymal cells, called a blastema, is formed (23-26). In the following stages, various signaling cascades are activated to mediate outgrowth, patterning and termination (20, 25). We focused this study on the events involved in blastema formation. This pivotal step in epimorphic tissue regeneration in zebrafish involves dedifferentiation and reactivation of progenitor populations (15), and presents a powerful opportunity to further understanding of the intricacies involved in reprogramming genes to fuel *de novo* post development of tissue in whole organisms.

Both 78-base pair (bp) and 40-bp RNA Sequencing (RNA-Seq) analysis were conducted on regenerating and non-regenerating caudal fin tissue in juvenile (4 weeks), adult (4 month) and aged (2+ years) zebrafish, providing the first fully-defined repertoire of both transcript and small RNAs involved in tissue regeneration. We identified transcripts that had significant but inverse expression in our aged population, which have a compromised regenerative response, as compared to juveniles and adults that regenerate successfully. We identified putative mRNA targets of miRNAs with significant differential expression in regenerating tissue as compared to non-regenerating tissue. Cbx7, a chromobox protein family member

and Polycomb (PC) ortholog, was one of the most significantly differentially expressed transcripts we discovered that had an aberrant expression pattern in aged tissue. Cbx7 was predicted to be targeted by miR-21 which had significantly attenuated expression in aged tissue in comparison to younger tissue. Empirical experiments confirmed the role of miR-21 in post-transcriptional regulation of cbx7 and transient knockdown of miR-21 impaired regeneration. While the role of cbx7 has been reported in aging (27) and in the regulation of self-renewal and differentiation in embryonic stem cells (28, 29), this is the first report of its role in tissue regeneration in adult animals. Taken together, our data highlight a novel age-dependent role for cbx7 in regulating vertebrate tissue regeneration via the master regulator actions of pro-regenerative miR-21.

Materials and Methods

Fish care and husbandry: All zebrafish (*Danio rerio*) were reared according to Institutional Animal Care and Use Committee protocols at the Sinnhuber Aquatic Research Laboratory, Oregon State University. The wild-type AB strain was used for the described sample collection for Illumina® RNA-Sequencing. The wild-type Tropical 5D strain was used for all experiments with embryonic zebrafish. Adults were raised on a recirculating water system (28 ± 1 $^{\circ}$C) with a 14 h light/10 h dark schedule. Spawning and embryo collection procedures were followed as previously described (30).

Sample collection for Illumina® mRNA and small RNA sequencing:

Animal handling, surgical fin amputations, and sample collection: To fully define the mRNA and small RNA expression profiles in regenerating zebrafish caudal fins, three aged cohorts were used: juveniles (4 weeks), adults (4 months), and aged zebrafish (2+ years). In preparation for the surgical caudal fin amputations, the fish were first anesthetized in a 150 mg/L MS222 (3-amino benzoic acidethylester) solution. Once anesthetized, fish were placed on a either a sterile glass microscope slide (juveniles) or sterile glass petri dish (adults and aged) and visualized under an Olympus SV61 stereoscope. The caudal fins of the fish were then partially amputated using a sterile razor blade directly anterior to the caudal fin bifurcation. Amputated tissue was collected using forceps and placed directly into an RNase,

DNase free sterile microtube containing 500 µL of RNA*later®* (Invitrogen). This fin tissue collected for RNA isolation was considered the control, or non-regenerating fin tissue (0 days post-amputation, 0 dpa, (Figure 3-1). Caudal fin tissue collected from 10 animals was combined for each biological replicate in the adult and aged cohorts. Since the juvenile fish were significantly smaller than the other two cohorts, caudal fin tissue from 100 animals was combined for each juvenile biological replicate group to obtain the necessary amount of RNA to perform RNA-Sequencing analysis. Three biological replicates were collected for each age group. Immediately post-amputation, the fish were placed in a tank of anesthetic-free fish water for recovery. Once recovered, all of the fish of an individual biological replicate were placed together in a tank and housed as previously described. The next day, the same fish were reamputated ~ 0.25 cm below the original amputation plane to harvest the regenerating tissue (1 dpa) (Figure 3-1).

Sample preparation and processing: Prior to RNA isolation, the RNA*later®* was removed and 0.5 nM zirconium oxide beads (Next Advance, #ZrOB05) and 700 uL of QiaZol Lysis reagent (Qiagen) were added to each sample. Tissue was homogenized in a Bullet Blender (Next Advance) and stored at-80 ^{o}C until RNA isolation. Total RNA was isolated using miRNEasy Kits (Qiagen). A Nanodrop- 1000 Spectrophotometer and Agilent Bioanalyzer were used to measure the quantity and quality of RNA yielded. All samples had A260/A280 ≥ 1.8 and A260/A230 ≥ 1.8 and RNA Integrity Numbers (RIN) ≥ 9. Six µg of total RNA (100 ng/uL) for each of the 18 samples (3 biological replicates per time point and age group) were provided to the Genomics Core of Lerner Research Institute (Cleveland, Ohio) for Illumina® RNA-Sequencing library preparation and processing. Three µg of total RNA from each sample was prepared for 78-bp RNA-Sequencing using the Illumina® TruSeq RNA sample preparation kit. The remaining 3 µg was prepared for 40-bp RNA-Sequencing using the Illumina® TruSeq small RNA sample preparation kit. For both the 78-bp and 40-bp RNA sequencing reads, six lanes of an 8 lane flow cell were used for the collected samples and one for sequencing controls. Three lanes were used for the control (0 dpa) samples and three lanes were used for the regenerating (1 dpa) samples. Each lane contained 3 bar-coded samples (juvenile, adult and aged) that were sequenced using single-end reads, either 78-bp or 40-bp in length, on an Illumina® Genome Analyzer IIx. The libraries were sequenced and the number of

sequencing reads obtained per lane are provided in Table 3-S1. Both the 78-bp and 40-bp RNA sequencing analysis datasets are available through the U.S. National Center for Biotechnology Information Gene Expression Omnibus (31) series accession number GSExxxxx (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc_GSExxxx).

Illumina® 78-bp data analysis: Sequece.txt files for each of the 18 individual samples were mapped to the zebrafish Ensembl reference genome (Zv9.64) using Tophat v1.3.3 with Bowtie 2 (32, 33). To customize the Tophat pipeline for analysis of zebrafish genome data, the following specific parameters were changed: minimum intron distance, 50; maximum intron distance, 1000; inner distance between mate set, 165. The remainder of the Tophat parameters were run at default settings. To assemble the transcripts to the zebrafish reference annotation (Ensembl Zv9.64.gtf), Tophat output Bam hit files were processed using Cufflinks v1.3.0 (34-36) with a maximum intron length of 10,000. This provided GTF output files containing Fragment per Kilobase Million Fragments Mapped (FPKM) values for the aligned transcripts. All the GTF output files from Cufflinks were used as input in Cuffmerge (v1.3.0) along with the zebrafish reference annotation (Ensembl Zv9.64) and reference genomic DNA sequences (Zv9.64.fa) to merge novel isoforms and known isoforms to maximize the assembly quality. This product of Cuffmerge, a merged GTF annotation file, was used in Cuffdiff (v1.3.0) to evaluate the significant changes in transcript expression in the control (0 dpa) versus regenerating (1 dpa) samples. In Cuffdiff, the minimum alignment count parameter was changed to 10 for these analyses. Upper quartile normalization was performed. Additionally, bias correction was run in Cuffdiff using the zebrafish reference genome (Ensembl Zv9.64.gtf). In Cuffdiff, the biological replicates for each time point (CON, 0 dpa; REG 1 dpa) and age group (G, A, J) were pooled for statistical comparison with $p < 0.05$ and a false discovery rate (FDR) of 5%. The differential expression profiles created were further filtered to remove transcripts that were too complex or shallowly sequenced (LOWDATA), had few alignments (NOTEST), too many fragments in loci (HIDATA), or an exception that prevented testing in Cuffdiff (FAILED) (37).

Illumina® 40-bp Data Analysis: The 18 raw sequence files from the 40-bp Illumina® were processed through the CASHX pipeline (38). This pipeline parsed small RNA sequences from

the 3′ adapter, reduced the data to a unique read set, counted the number of reads per unique set, and aligned sequences to the zebrafish Ensembl reference genome (Zv9.64). The parsing process identified all small RNA sequences ranging in size from 18-30 nucleotides. Parsed and aligned sequences were inserted into a MySQL database for organization and flexibility. This setup provided methods to combine sequence information from multiple runs and further reduced the amount of storage needed for data processing. After the data were processed into the database, scripts provided with the CASHX pipeline were updated to accommodate analysis of genomes, such as *Danio rerio*, with non-standard naming of chromosomes and scaffolds. These tools were used in combination with downloaded files from miRBase (v.18) (www.mirBase.org) containing the mature miRNA sequence information (mature.fa) and genome coordinate information (dre.gff) (39, 40). These files were combined and used to analyze miRNA loci and miRNA foldbacks for read counts. These scripts counted reads for these miRNA features that had perfect hits to the genome and were found within a 4 nucleotide flank of the miRNA feature. The database was then updated with miRNA annotation information. Biological replicates were generated for each sequencing library and replicate counts were pooled to determine differential expression profiles for statistical comparisons with $p < 0.05$ for each annotation feature.

Bioinformatics: Unsupervised, bidirectional, hierarchical clustering was performed on the transcript differential FPKM outputs from RNA-Seq by applying a Euclidean distance metric and centroid linkage cluster which grouped the age cohorts and transcript expression patterns by similarity. To form hypotheses about putative miRNA-mRNA interactions that could be further tested functionally *in vivo,* the Bioinformatics Resource Manager (BRM) v2.3 (http://www.sysbio.org/dataresources/brm.stm, Pacific Northwest National Laboratory) was used (41). To identify miRNA and putative target transcripts, significant transcript and miRNA lists were uploaded to BRM. Since zebrafish miRNA target prediction tools are limited, the BRM XSpecies Identifier retrieval tool was used to identify orthologous genes in humans based on provided Ensembl gene IDs for the significantly expressed zebrafish transcripts and miRNAs. The lists containing the significantly expressed zebrafish miRNA and human orthologs were uploaded to BRM. The miRNA Target query was used to identify predicted target transcripts through three databases: TargetScan (www.targetscan.org), microCosm

(www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5) and miRNA.org (www.microrna.org). This output was then merged in BRM with the significantly expressed transcripts identified from the 78-bp RNA Sequencing analysis and filtered to contain only transcripts predicted to be targets of abundant miRNA, as determined by CASHX, and significantly expressed in the caudal fin.

Real-time quantitative PCR: Total RNA isolated from the control and regenerating caudal fin tissue for RNA-Sequencing was used to confirm the transcript and miRNA expression changes predicted from the Cuffdiff and CASHX data analyses, respectively. Additionally, whole body homogenate from embryonic morpholino and mimic injected fish was collected at 24 and 48 hpf from pooled embryos (25 per biological replicate) harvested using miRNEasy Kits (Qiagen), n=3. All samples were oligo(dT)-primed and cDNA was synthesized using Superscript III (Invitrogen) (n=3). Quantitative RT-PCR (qRT-PCR) was performed with gene specific primers for cbx7 and β-actin (Eurofins MGW Operon) listed in Table 3-S2 using the Power SYBR® Green Master Mix (Applied Biosystems) on a Step One Plus Instrument (Applied Biosystems). All samples were normalized to β-actin. To quantify miRNA expression, the miRCURY LNA™ Universal cDNA Synthesis Kit and SYBR Green Master Mix (Exiqon) were used. Exiqon stock primer sets for miR-181a (PN 204566-01), miR-181a* (PN 204110-01), U6 small nucleolar RNA (snoRNA) (control, PN 203907), and custom primer sets for miR-21, miR-93, miR-140*, miR-200c and miR-726 (PN 206999) were used to assess miRNA expression as previously described (42).

Microinjections: Antisense oligonucleotide morpholinos (MO) were used to knock down cbx7 and miR-21 levels in developing embryos (Gene Tools) (Table 3-S3). The cbx7 MO (i1e2) was a splice blocking morpholino designed against the intron (i) 1, exon (e) 2 boundary involved in pre-mRNA splicing (43). The miR-21 MO targeted the guide strand and *dicer* cleavage site. All morpholinos, including a standard control MO, were injected into single-cell stage embryos. Approximately 2 nl cbx7 MO (0.75 mM) or miR-21 MO (1.5 mM) MO in ultrapure water with 0.5% phenol red were microinjected into each embryo. At 6 hpf, the fish were screened for uniform incorporation of the MO by fluorescein visualization under ultraviolet light. A synthetic miR-21(Thermo Scientific, C-300495-03-0005), miR-181a*(Thermo

Scientific, C-300568-05-0005) or control Dharmacon miRIDIAN mimic was injected at 10 µM (Thermo Scientific, CN-001000-01-05). The cbx7 3' UTR GFP reporter (200 ng/µL) was co-injected with either the synthetic miR-21, miR-181a* or control mimic (25 µM) into single-cell stage embryos. Depending on experiment, fish were humanely euthanized using MS-222 overdose (44) at various time points (24, 48 or 120 hpf). Sub-groups were fixed with 4% paraformaldehyde (JT Baker) for immunohistochemistry experiments or homogenized in QIAzol Lysis Reagent (Qiagen) for gene expression analyses.

GFP reporter assay: The zebrafish cbx7 3'-UTR sequence was amplified by PCR using zebrafish cDNA and gene-specific primers (Table 3-S2, Eurofins MGW Operon). The sequence was subcloned downstream of the GFP open reading frame (ORF) that was inserted into pCS2+ vector (45) using a Cold Fusion Cloning Kit (System Biosciences). Reporter injected animals were placed into 96-Well Half Area Polystyrene Microplates (Greiner Bio-One) and imaged using an Image Xpress Micro (Molecular Devices). Measurements of average GFP Intensity/well were obtained from each well (n = 4) containing 12 animals/well using Metaexpress Software (Molecular Devices) at 24 hpf.

Larval zebrafish regeneration assay: Larval zebrafish caudal fin surgical amputations were performed on 2 dpf morpholino-injected fish. The morphants were placed into individual wells of a 96-well plate and screened for regenerative outgrowth capability at 3 dpa (46-49). Images of the entire plate were obtained using an Image Express High Content Imager (Molecular Devices) and images of individual fish were captured using a Nikon Coolpix E500 digital camera mounted on a Nikon SMZ 1500 stereomicroscope.

Immunohistochemsitry: Regenerating (1 dpa) and control (0 dpa) fin tissue samples collected from adult and aged zebrafish and cbx7, miR-21 and control morpholino injected embryos collected at 48 and 120 hpf were fixed in 4% paraformaldehyde overnight at 4 °C. Rabbit α-human CBX7 (dilution: 1:50, Santa Cruz Biotechnology, sc-70232) primary antibody, mouse monoclonal acetylated alpha tubulin primary (1:4000, Sigma, T6793), and Alexafluor® 555 or 594 goat α-rabbit or goat α- mouse (dilution: 1:500, LifeTechnologies) secondary antibodies were used. Briefly, fixed embryos were washed with PBST followed by a 1 h wash in

UltraPure water (LifeTechnologies). A 30 or 60 min collagenase (0.0001 g/mL PBST, Sigma, C9891) treatment was then performed to permeabilize the embryos or fin tissue, respectively, followed by a 30 min rinse with PBST. The embryos were blocked with 10% Normal Goat Serum (Sigma, G6767) for minimum of 1 h at room temperature prior to adding the primary antibody, in which the samples were incubated for 72 hours at 4 °C. Samples were then rinsed in multiple PBST washes for 1.5 h, incubated with secondary antibody for 2 h, and washed 2X for 15 min followed by 3X for 30 min with PBST. Embryos were imaged on an inverted Zeiss Axiovert 200 M epi-flourescence microscope using a Zeiss Axiocam HRM camera.

Hair cell staining: To investigate the effects of cbx7 and miR-21 knockdown on hair cell expression, Yo-Pro-1 (LifeTechnologies, Y3603) was used to selectively stain the hair cell nuclei. Cbx7, miR-21 and control morphants (120 hpf) were batch treated (20 per biological replicate) in 20 mL glass vials with 2 μM Yo-Pro-1 in embryo medium for 1 h (n=3). After removal from the stain, the larvae were washed 3 successive times with embryo media. The larvae were anesthetized with MS-222 prior to imaging on an inverted Zeiss Axiovert 200 M epi-flourescence microscope using a Zeiss Axiocam HRM camera.

Statistical analysis: To analyze differences between treatment and control groups or regenerating and non-regenerating tissue, a 2-tailed, paired Student's *t*-test, 1-way ANOVA with a Dunnet's multiple-comparison *post hoc* test, or 2-way ANOVA with a Bonferroni multiple comparison *post hoc* test were conducted, depending on the experiment. Data shown represent means ± SE; values of $P \leq 0.05$ were considered statistically significant. Results were calculated using Prism 5.01 (Graph-Pad).

Results

Regenerative outgrowths in aged fish are morphologically compromised. Juvenile, adult and aged zebrafish were amputated and representative images captured at 0, 1, 7 and 14 dpa. Aged zebrafish exhibited compromised morphology in regenerative outgrowths by 14 dpa compared to juveniles and adults (Figure 3-2).

Global transcriptome profiling predicts conserved and unique regenerative responses across age cohorts. To fully define the transcriptional repertoire of a regenerative response, RNA sequencing was performed on regenerating (1 dpa) and non-regenerating tissue (control, 0 dpa) in juvenile, adult and aged cohorts (Figure 3-2). Approximately 12 million reads per sample were mapped to the Ensembl zebrafish genome assembly (Zv9). Greater than 90% (>11 million reads) could be mapped to the genome (Table 3-S1). Statistical assessment of mRNA expression in regenerating versus non-regenerating tissue identified 9,012, 8,904, and 6,069 transcripts significantly differentially expressed relative to their 0 dpa (control) in the aged, adult and juvenile tissue, respectively ($p < 0.05$, 5% FDR). Approximately 30% of the differentially expressed transcripts were common to the aged and adult zebrafish, and 50% were common to aged and juveniles (Figure 3-3A). Approximately 18% of the total number of significantly expressed transcripts from all age cohorts, which had either increased or decreased changes, were common in aged, adult and juvenile tissue.

Inverse expression profiles in aged versus juvenile and adult regenerating tissue highlight transcripts that may be responsible for compromised regenerative response. To investigate the decline in regenerative capacity observed with age, additional filtering was conducted on the 2,694 transcripts that were significantly expressed in regenerating tissue across all three age cohorts. Of these, 2,645 of were commonly increased (1,170) or decreased (1,475) in all age groups (Figure 3-3B). Since both juveniles and adults could successfully regenerate caudal fins, we focused on the significant transcripts that had inverse expression in both juveniles and adults versus aged zebrafish. Thirteen transcripts were inversely expressed in juveniles and adults compared to the aged cohort. Seven transcripts had increased expression in regenerating fin tissue in both juveniles and adults and decreased expression in aged zebrafish. Six transcripts had decreased expression in the regenerating tissue of juvenile and adults and increased expression in the aged tissue (Figure 3-3C, Table 3-3-1). Cbx7 (2 of 2), referred to as cbx7 henceforth, was the most abundant (largest FPKM values) with increased expression in regenerating tissue in juveniles and adults and, conversely, decreased expression in aged tissue (Table 3-S4). CBX7 is known to play an important role in cellular aging and in mediating self-renewal and differentiation (27-29). Therefore, we chose

to experimentally evaluate the age-dependent role of cbx7 in caudal fin regeneration and its potential regulation by miRNAs.

Regeneration induces significant changes in miR-21 and miR-181a expression. The results from 40-bp RNA-Seq analysis showed that at 1 dpa, there was a total of 79 miRNAs expressed in either control or regenerating tissue at any lifestage. These results were filtered to represent only the 53 miRNAs that greater than 100 counts in at least one of the biological replicates. miR-21, a miRNA implicated for its role in aging (50-55) and regeneration (56-64), was the only significantly increased miRNA identified in regenerating tissue compared to control in all three age groups (Figure 3-4A-C). miR-21 expression in regenerating fin tissue was significantly lower in the geriatric cohort relative to the adult and juvenile cohorts (Table 3-2). miR-181a had a significant decrease in expression in regenerating tissue in all the age cohorts (Figure 3-4A-C).

Cbx7 is putatively targeted by miRNAs expressed during regeneration. Bioinformatics analysis was performed to identify significant changes in cbx7 transcript expression that may be subject to post-transcriptional regulation by miRNAs. miR-21 is predicted to have two mRNA recognition elements (MREs) located in the 3' UTR of cbx7 (Figure 3-5A). The multiple recognition elements within a 3' UTR can have a multiplicative regulatory effect (65). miR-181a was also significantly increased in regenerating tissue. The expression profiles of miRNAs predicted to target the 3' UTR of cbx7, miR-21, miR-140*, miR-200c, miR-93, miR-181a* and miR-726, along with miR-181a were examined. miR-21 demonstrated significantly increased expression in regenerating tissue in comparison to control in all three age groups (Figure 3-5B). There was no significant difference in the homeostatic level of miR-21 expressed in non-regenerating tissue across the age groups. Yet, miR-21 was significantly attenuated in the aged regenerating tissue in comparison to adult and juvenile regenerating tissue. miR-726 was only detected in aged tissue and showed a significant decrease in expression during regeneration, suggesting a putative age-dependent role for miR-726. miR-181a* expression in regenerating fin tissue samples indicated that it was significantly increased in both the adult and aged regenerating fin tissue.

Transient knockdown of miR-21 impairs regeneration. Since miR-21 was one of the most abundant and significantly increased miRNA in regenerating tissue across all cohorts and attenuated in aged regenerating tissue versus younger regenerating tissue (Figure 3-4A-C, 5B,C, Table 3-1), we tested whether the miR-21 is required for regeneration. A miR-21 antisense oligonucleotide morpholino (Figure 3-6A) was injected into single-cell stage embryos and knockdown was confirmed at 24 and 48 hpf via qRT-PCR (Figure 3-6B). Loss of miR-21 function did not result in developmental defects (Figure 3-6C, E), but caused a failed regenerative response in 75% percent of the miR-21 morphants (Figure 3-6D, F), confirming its necessity for caudal fin regeneration.

miR-21 and miR-181a* target the 3' UTR of cbx7a in vivo. To determine whether cbx7 is post-transcriptionally regulated by miR-21 and miR-181a*, a reporter was designed in which the cbx7 3' UTR was inserted downstream of GFP (GFP-cbx7a-3'UTR) (Figure 3-7A). The GFP-cbx7-3'UTR reporter was co-injected into single cell-stage embryos with either a miR-21 mimic, miR-181a* mimic or control mimic. Co-injection of the GFP-cbx7-3' UTR reporter and miR-21 or miR-181a* mimic significantly decreased the GFP expression as compared to embryos co-injected with the reporter and control mimic (Figure 3-7B, C). To further confirm whether miR-21 and miR-181a* can functionally regulate cbx7 expression *in vivo*, cbx7 transcript expression was quantified in embryos injected with exogenous miR-21 and miR-181a* mimic. The injection of miR-21 mimic significantly decreased cbx7 transcript expression (Figure 3-7D).

Transient knockdown of cbx7 confirms relationship with predicted targets. To further investigate the relationship between cbx7 and the miRNAs predicted to target its 3' UTR, a splice-blocking cbx7 morpholino targeting exon 2 was used to transiently knockdown its expression. Validation of knockdown was confirmed via RT-PCR amplification of tissue collected from whole body homogenate of cbx7 morphants and size-verified by gel electrophoresis, demonstrating the deletion of exon 2 (44 bp) (Figure 3-8A). Expression of miR-21, miR-140*, miR-200c, miR-93, miR-181a, miR-181a* and miR-726 was examined in morphants injected with the cbx7 morpholino. miR-726, present in aged tissue, was not detected in the embryos. At 48 hpf, all the miRNAs predicted to target the 3' UTR of cbx7,

including miR-181a, were significantly decreased, except miR-21(Figure 3-8B). Cbx7 morphants regenerated after caudal fin amputation by 3 dpa (data not shown).

Aged tissue exhibits decreases in CBX7 expression and neuronal integrity*.* Prior to this experiment the expression pattern of CBX7 in zebrafish had yet to be described. Therefore, we investigated CBX7 protein expression in caudal fin tissue via immunohistochemistry. CBX7 was localized to the neuromasts in the non-regenerating (control, 0 dpa) and regenerating tissue (1 dpa) in both adult (Figure 3-9A, C) and aged fish (Figure 3-9B, D). Acetylated alpha tubulin antibody was used to label all the neuronal cell bodies and axons in non-regenerating and regenerating adult (Figure 3-9E, G, I) and aged fin tissue (Figure 3-9F, H, J). Aged tissue exhibits a decrement in neuronal processes pre- (0 dpa) and post-amputation (1 dpa). Aged tissue has diminished nerve innervation under the apicial epithelial wound cap compared to adult tissue at 1 dpa (Figure 3-9I, J).

Aged tissue is devoid of several transcripts important in neuromast hair cell formation, neurogenesis, and regeneration. Given the localization of CBX7 to the neuromasts, we evaluated the 78-bp sequencing data for the expression of key transcripts involved in regeneration, neuromast formation and neurogenesis based on the literature. Expression of Sox2, Jagged1b and DeltaD, known regulators of hair cell formation, neurogenesis, and regeneration (66-76), were significantly decreased in regenerating adult and juvenile tissue as compared to control (Table 3-3). In contrast, expression of these transcripts was not detected in aged regenerating or non-regenerating tissue.

Neuromast hair cells and neurons have aberrant patterning in cbx7 and miR-21 morphants. We investigated the potential role of cbx7 and miR-21 in neurogenesis and hair cell development in cbx7 (Figure 3-10E-H) and miR-21 (Figure 3-10I-L) morphants compared to control (Figure 3-10A-D). Hair cell staining showed that both cbx7 and miR-21 morphants had reduced number of neuromast hair cells in the along the posterior lateral line in the trunk and tail, demonstrating a role for cbx7 and miR-21 in hair cell formation. miR-21 also had less hair cells in the anterior lateral line of the head. Transient knockdown of miR-21 considerably reduced the number of neuronal processes in the caudal fin of morphants in comparison to

cbx7 and control morphants at 48 and 120 hpf. In addition to regeneration, this highlights the pleiotropic role of miR-21.

Discussion

Aging is one of the most discernible phenomena of biology, with many questions remaining about its mechanisms (77). One of the hallmarks is a declining capacity to maintain tissue homeostasis and repair damaged tissue in response to injury, insult or disease. Zebrafish provide an attractive model for investigating the molecular signaling pathways involved in both regeneration (25, 26) and aging (19, 22). Here, we have reported the first full repertoire of transcripts and small RNAs expressed during caudal fin regeneration from three developmentally distinct age cohorts. Our data describe a previously unknown role for cbx7 in tissue regeneration and corroborate its known function in cellular senescence (27). We demonstrate that miR-21 regulates cbx7, and miR-21 loss-of-function inhibits regeneration, neurogenesis and neuromast hair cell formation. Hence, our findings build on recent studies that demonstrate miRNAs as master regulators of cbx7 and pluripotency related genes (29) which, in turn, actuate progenitor and stem cell populations necessary for embryonic tissue formation and adult tissue regeneration.

Results from our small RNA-Seq analysis demonstrated that members of the miR-21 and miR-181 family were the most abundant miRNAs present in zebrafish fin tissue and exhibited significant differential expression in regenerating tissue compared to non-regenerating tissue in all age cohorts (Figure 3-4A-C, Figure 3-5A, B, Table 3-2). Although miR-21 was significantly increased in regenerating tissue in comparison to non-regenerating tissue within each age groups, aged regenerates had significantly attenuated miR-21 expression in comparison to tissue from younger regeneration-competent fish (Figure 3-5B). Other models have shown that miR-21 is affiliated with both aging and regeneration. Circulating miR-21 was recently found to be a new biomarker for age-related inflammation (50) and to function in age-related matrix protein metabolism changes in the kidney (51), nitric oxide induced colon tissue senescence (52), aging of colon cancer stem-like cells (53), cardiac aging (54), and immortalization of mouse embryonic fibroblasts (55). miR-21 was demonstrated to have a role in regeneration of the limb (56), liver (57-62), and nerves (63, 64), along with stem cell-

dependent tissue repair (78, 79). miR-21 was also found to be involved in wound healing, the first event necessary to initiate a regenerative response (80-84). Our results support these findings demonstrating a critical role for miR-21 in regeneration and suggest that an attenuated expression change in miR-21 levels with age may be responsible, in part, for regenerative decline.

miR-21 was predicted to target cbx7, one of the 13 transcripts that had a significant inverse expression pattern in aged fish compared to juveniles and adults. The 3' UTR of cbx7 is predicted to have two miR-21 MREs (Figure 3-5A). Multiple conserved sites within an mRNA 3' UTR strengthens the regulatory association between a miRNA and its target (65). CBX7 is a polycomb protein that controls transcription of the INK4a/Arf locus and repression of tumor suppressors, p16^{INK4a} and Arf, which target the Rb and p53 pathways (27). CBX7-mediated suppression extends the lifespan of various types of human cells (27). p16^{INK4a} impairs regeneration in pancreatic islet cells (85) and reduces neural progenitor function, self-renewal potential and neurogenesis (86, 87). Increased p16^{INK4a} was also associated with stem cell aging, a process intimately linked to decline in tissue maintenance and repair (88).

Beyond its role in senescence, CBX7 was recently implicated as a master switch that acts in conjunction with Polycomb repressive complexes (PRCs) and Polycomb group (PcG) proteins to control embryonic stem cells (ESCs) decision to differentiate or self renew (28, 29). These proteins regulate chromatin status to orchestrate either the expression of pluripotent genes and repression of lineage specific genes or visa-versa (89). In accordance with the first discovered role of CBX7 in controlling cellular lifespan (27), our deep RNA sequencing results from regenerating caudal fin demonstrate that aged zebrafish, with compromised regeneration, have aberrant cbx7 expression upon regeneration compared to juveniles and adults that can mount a successful regenerative response (Figure 3-2, 3-3C, Table 3-1). Epimorphic tissue regeneration involves the formation of dedifferentiated progenitor cells which ultimately differentiate into skeletal structure, blood vessels, nerves, melanocytes and fibroblasts (25). Given the role of CBX7 in regulating ESC fate decisions (28, 29) along with the previously described mechanisms for PcG proteins in influencing lineage commitment (as

reviewed in (90)), we postulate that CBX7 has an equally significant, and potentially similar function in regulating progenitor cell populations involved in tissue regeneration.

Recently, research demonstrated post-transcriptional regulation of cbx7 by two miRNA families, miR-125 and miR-181, in ESCs (29). To investigate predicted miRNA-cbx7 interactions involved in tissue regeneration, we quantitatively validated the expression profile of all miRNAs predicted to bind the 3' UTR of cbx7 in regenerating tissue from the three age groups. Using this approach, we confirmed that both miR-21 and miR-181a*, but not miR-181a, were significantly increased in regenerating tissue in all cohorts (Figure 3-5B). Given that miR-181a* is transcribed and processed by Dicer in the same pre-miRNA as miR-181a, we speculated that miR-181a* could also have a functional regulatory role in regeneration. Interestingly, miR-21 induction was blunted in aged regenerating tissue relative to tissue collected from regeneration-competent juvenile and adult cohorts (Figure 3-5B). Additionally, we found that miR-726, which also has two predicted binding sites within the cbx7 3' UTR, was only detected in aged fish and significantly decreased in regenerating tissue. This suggests that, in addition to miR-21 and miR-181a*, miR-726 may also have an age-dependent role in mediating cbx7 expression during tissue regeneration.

To explore whether miRNAs could be regulating cbx7 expression in zebrafish, we used a physiological reporter assay and confirmed that cbx7 is a *bona fide* target of both miR-21 and miR-181a* *in vivo* (Figure 3-8B, C). Injection of an exogenous miR-21 mimic caused a significant decrease in expression in cbx7 by 48 hpf (Figure 3-8D). We did not observe a significant decrease of cbx7 upon miR-181a* mimic microinjection. This could be attributed to miR-181a* having only one predicted MRE in the cbx7 3' UTR, as opposed to miR-21 which has two. The expression pattern of all miRNAs predicted to target cbx7 was quantitatively assessed after transient knockdown of cbx7. The majority of miRNAs were significantly decreased at 48 hpf (Figure 3-8B), possibly as a result of a regulatory feedback loop. Interestingly, cbx7 knockdown did not induce a change in miR-21 expression, nor did it impair larval fish from regenerating following injury (data not shown). Transient knockdown of miR-21, however, did block regeneration in 75% of larvae (Figure 3-6E, F). It is plausible that, in the larval model, cbx7 is not necessary for regeneration, further supporting the

concept of age-related changes in regeneration signaling pathways. Or, cbx7 could be acting in concert with other miR-21 targets to facilitate regeneration. Further study in both the larval and adult fish will need to confirm these hypotheses.

We found that CBX7 expression was localized to the neuromasts in both the adult and aged caudal fin tissue (Figure 3-9A-D). In adult zebrafish, new neuromast cells populate regenerative outgrowths following surgical amputation (91). In fish, neuromasts are individual mechanosensory organs that are arranged in a symmetric pattern along both the body and the caudal fin. These innervated structures comprise the lateral line, a primary sensing tissue in the fish. Each neuromast contains 15-20 hair cells surrounded by supporting and mantle cells (92). Numerous models including axolotl salamanders, frog and various fish species can regenerate sensory hair cells (30, 91, 93-102), which are structurally and genetically equivalent to mammalian inner ear hair cells (92). Research in these models seeks to identify regenerative therapies for inner ear hair cells (103, 104). Similar to the decline in tissue regeneration capacity, hair cell loss is strongly correlated with age in mammals (105, 106). Molecular mechanisms necessary for regeneration are conserved across many biological systems (e.g. heart, retina, skeletal muscle, fin, liver, and hair cells)(25). Additionally, regeneration requires the reactivation of fundamental developmental signaling pathways in order to restore damaged or missing structures (107). Therefore, we investigated whether miR-21 or cbx7 were required for neuromast hair cell development. Knockdown of cbx7 and miR-21 caused a reduced number and aberrant patterning of neuromast hair cells (Figure 3-10E, F, I, J). These results implicate a functional role for miR-21 and cbx7 in tissues and cell types most affected by aging.

To explore this relationship further, we evaluated the 78-bp RNA-Seq transcript expression profile to determine whether key developmental regulatory genes known to be associated with both regeneration and neuromast formation had aberrant expression patterns with age. Sox2 and the Notch signaling pathway ligands Jagged1b (Jag1b) and DeltaD, were all significantly downregulated in regenerating tissue from adult and juveniles. Aged regenerating and non-regenerating tissue were completely devoid of these transcripts (Table 3-3). Sox2 is a transcription factor and pluripotency gene that belongs to the B1 subfamily of

Sox proteins (108). The decrease in sox2 expression observed in regenerating tissue from adults and juveniles recapitulated the sox2 expression profile previously shown in regenerating zebrafish caudal fins and xenopus limbs (66). Transient knockdown of sox2 impaired caudal fin regeneration and sox2 expression was restricted to the neural tube (66). These results suggest that sox2 is necessary for epimorphic fin regeneration and likely associated with nerve innervation (66). This was further supported by studies that demonstrated expression in regenerating newt tissues and the importance of Sox2 in providing an adequate nerve supply for regenerating tail and spinal cord tissue in xenopus (67, 68). Several additional studies demonstrate the dependence of regeneration on an intact nerve supply (14, 104, 109, 110)). Additionally, Sox2 deficiency impaired neurogenesis in the adult mouse brain (69). Similarly observed a decrement in the neuronal processes in aged regenerating tissue (Figure 3-9F, H, J). And, concomitantly, showed that miR-21 knockdown impairs neurogenesis (Figure 3-10K, L) and tissue regeneration (Figure 3-6E-F).

Our findings suggest an association between miRNA mediated cbx7 and sox2 expression in axon formation and regeneration. This is supported by evidence of a Polycomb autoregulatory mechanism that regulates sox2 and cbx7 expression (28). Sox2, oct4 and nanog are all predicted to bind the promoter regions of cbx7 in ESCs (29). During differentiation, miRNAs repress cbx7, which leads to transcriptional activation of lineage-specific genes and cbx2, cbx4 and cbx8 which, in turn, associate with PRC1 to form new repressor complexes that downregulate the expression of cbx7 and pluripotency related genes, such as sox2 (28, 29). Our RNA-Seq data from aged regenerating tissue showed a decrease in cbx7, which may result in repressed expression of pluripotency genes (Figure 3-3C, Table 3-1). Regenerating tissue of younger fish, in contrast, had significantly increased cbx7 expression, which would have resulted in activated pluripotency genes (Figure 3-3C, Table 3-1). Hence, it is possible that, in addition to miR-21 mediation of cbx7 expression, the age-related absence of sox2 is limiting the transcription of cbx7 during regeneration. Further study is needed to determine the potential relationship between cbx7 and sox2 during regeneration.

The Notch ligands Jag1b and DeltaD were absent in aged regenerating tissue. Reactivation of Notch signaling is important for regeneration in the inner ear (70), lateral line(101), liver (111), heart (71), fin (71), spinal cord (112), and axons (113). Additionally, sox2, jag1, and deltaD are known to have important roles in the formation and differentiation of hair cells and supporting cells (72-74, 76, 114). Sox2 mutant mice do not express Jag1 (73). Sox2-depleted zebrafish embryos cannot regenerate lateral line hair cells following laser ablation (75). Functional Sox2 and Jag1 deficiency causes loss of hair cells, similar to cbx7 and miR-21 morphants (Figure 3-10E, F, I, J) (73, 114-117). Jag1 was recently demonstrated to be required for maintenance of sox2 expression (118). Furthermore, sox2, jag1b, and deltaD were shown to be expressed in the neuromasts of embryonic zebrafish (119, 120). Similar to the expression pattern of sox2, our data show that jag1b and deltaD are significantly decreased in regenerating fin tissue in adults and juveniles, and absent in regenerating tissue from aged fish (Table 3-3). In contrast, aged regenerating tissue did have a significant increase in jag1a, which was not present in adults and juveniles in our data. Concomitant with our localization of CBX7 to neuromasts (Figure 3-9), aberrant hair cell expression upon cbx7 and miR-21 knockdown (Figure 3-10E, F, I, J) and the known expression patterns and function of sox2, jag1 and deltaD support miR-21 being an upstream regulator of an age-related cascade of events that likely mediates the expression of cbx7, sox2 and Notch ligands, severely limiting the capacity of progenitor cells involved with regeneration and formation of neuromast hair cells.

Congruent with our work, a recent study found miR-21 was overexpressed in regenerating limb blastemas in salamanders and demonstrated that Jag1 is a target of miR-21 during regeneration (56). To the best of our knowledge, miR-21 is not yet predicted to target Jag1 in zebrafish. Yet, our findings support the importance for miR-21 in regeneration and suggest that in zebrafish, miR-21 has a functional role in regeneration through regulation of cbx7, which acts as a permissive switch to stimulate the activity of developmentally repressed genes such as Sox2 and Notch ligands in progenitor cells.

In summation, we demonstrated that transient knockdown of miR-21 impaired regeneration in larval zebrafish, and that cbx7 is a *bona fide* target of miR-21 *in vivo*. We observed an age

related decrease in cbx7 in regenerating tissue that exhibits a compromised ability to regenerate. In ESCs, miRNA mediated Cbx7 expression is a critical regulator of pluripotency related genes (28, 29). Our data implicates, for the first time, the importance of miRNA mediated regulation of Cbx7 in adult somatic cells and outlines how dysregulation of pluripotency related genes affects tissue regeneration, neuromast hair cell development and neurogenesis. This research underscores the pleiotropic effects that miR-21 has on development and tissue repair, and, more broadly, highlights how the use of tandem small RNA and mRNA global profiling in tissue regeneration can uncover conserved pathways critical to the development and reconstruction of complex body plans.

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Acknowledgements

Figure 3-1. Caudal fin amputation and tissue collection experimental design.
Simplified schematic of zebrafish caudal fin amputation and collection of the non-regenerating (control) tissue at 0 dpa followed by subsequent amputation and collection of the blastema formed at 1 dpa.
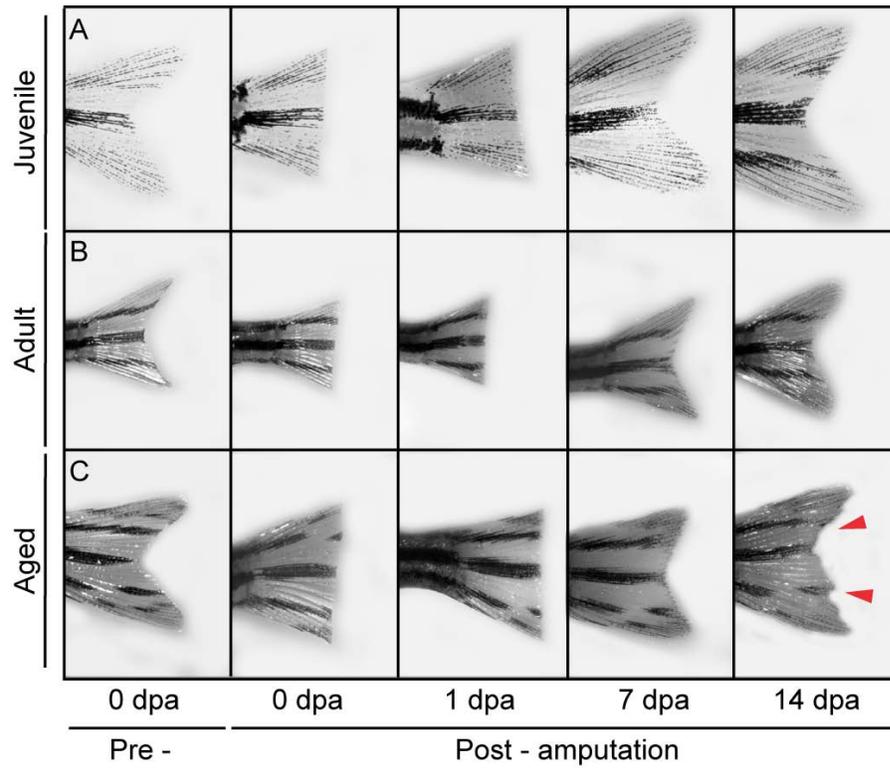
Figure 3-2. Zebrafish exhibit declines in regenerative capacity with age.
Representative images of caudal fin outgrowths prior to amputation and immediately post-amputation (0 dpa), 1 dpa, 7 dpa and 14 dpa in (A) juvenile, (B) adult and (C) aged zebrafish. Red arrows indicate areas of compromised regenerative outgrowth.
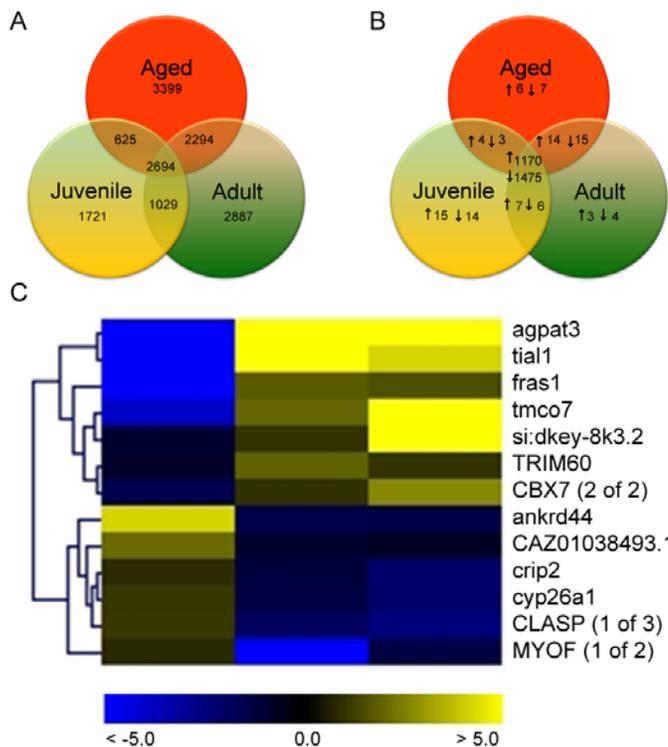
Figure 3-3. Global expression of transcripts in regenerating caudal fin tissue.
78-bp RNA-Seq analysis was conducted on caudal fin tissue samples of non-regenerating (control, 0 dpa) and regenerating tissue (1 dpa) collected from juvenile (4 weeks), adult (4 months) and aged (> 2 years) fish (n =3). Venn diagrams depicting (A) the overall number of statistically significant transcripts and (B) the number of statistically significant transcripts that were commonly expressed in regenerating tissue in comparison to control in all three age cohorts ($p < 0.05$, 5% FDR). (C) A heatmap of the expression pattern of the transcripts with significant and inverse differential expression profiles in aged tissue in comparisons to tissue from juveniles and adults. (The data represent unsupervised bidirectional hierarchical clustering performed on fold change (Log2) values obtained from transcript differential FPKM values in regenerating tissue in comparison to control in juveniles, adults and aged fish, $p < 0.05$, 5% FDR.)

Figure 3-4. Regeneration induces significant changes in miR-21 and miR-181a expression.
Bar charts of CASHX output representing counts of the number of miRNA that aligned to mature miRNA loci in aged (G), adult (A), and juvenile (J) regenerating (REG) and non-regenerating (CON) tissue. miRNA with less than 100 counts in any of the biological replicates were excluded from this chart. (Significance values reflect counts in regenerating tissue relative to non-regenerating, mean ± SEM, n = 3,*P < 0.05,***P < 0.001, Two-way ANOVA with Bonferroni Multiple Comparison Test.)
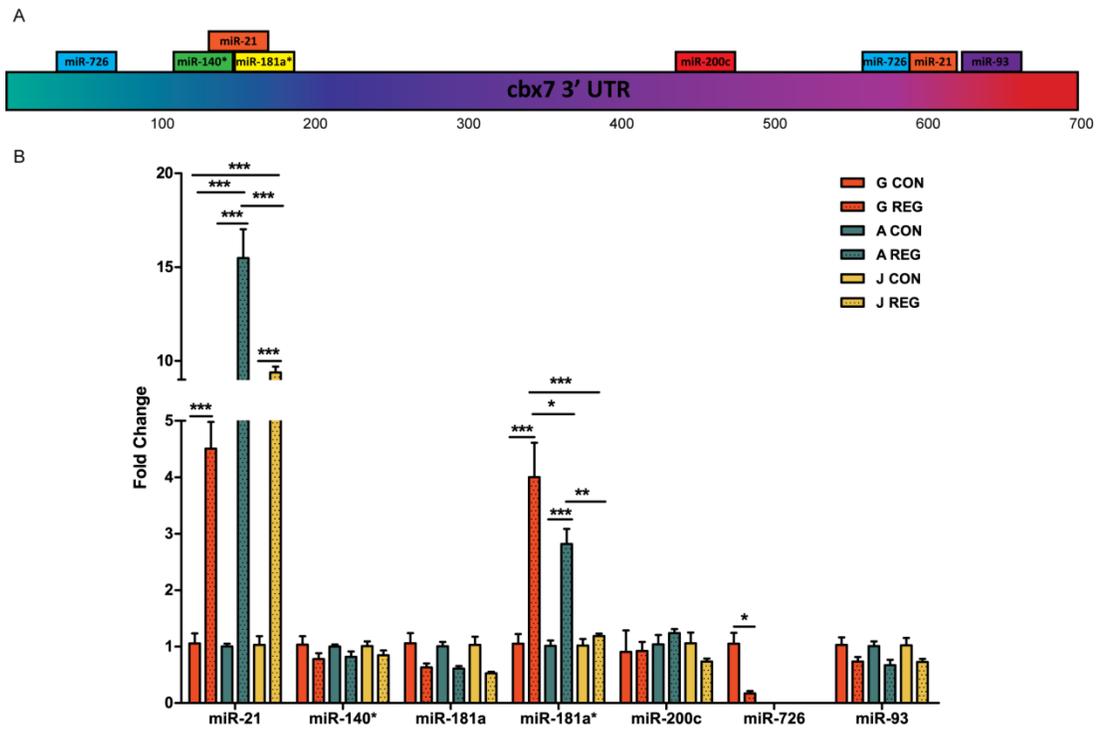
Figure 3-5. Significantly expressed miRNAs are predicted to target cbx7.
(A) Simplified schematic of MREs of the miRNAs predicted to target the 3' UTR of cbx7. (B) Expression levels, represented by fold-change, of miRNAs predicted to target cbx7 in regenerating and non-regenerating tissue from aged, adult and juvenile zebrafish. (The fold-change data is relative to non-regenerating tissue, mean ± SEM, n = 3, *P < 0.05, ** P < 0.01,***P < 0.001, Two-way ANOVA with Bonferroni Multiple Comparison Test.)
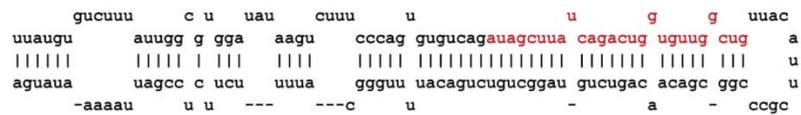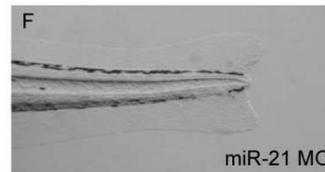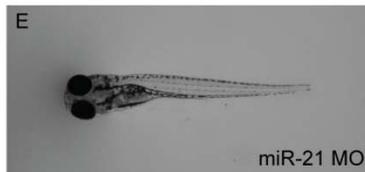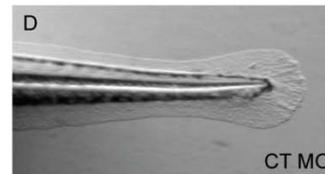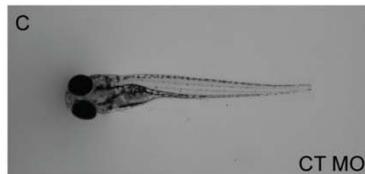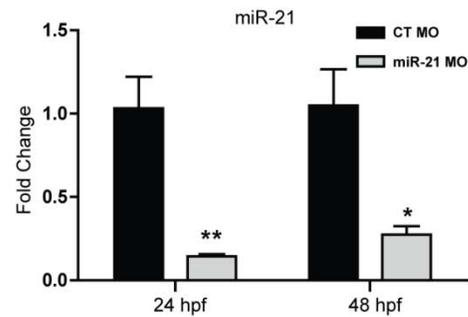
Figure 3-6. miR-21 is necessary for caudal fin regeneration.
(A) The miR-21 morpholino, designed to target the guide-dicer site (red) of the pre-miR-21 sequence, was injected into single-cell stage embryos. (B) Effective knockdown of miR-21 was confirmed in miR-21 morphants at 24 and 48 hpf via qRT-PCR. (Values reflect fold-change relative to control MO injected embryos, mean ± SEM, n = 3,*P < 0.05, **P <0.01, One-way ANOVA with Dunnett's Multiple Comparison Test.) miR-21 morphants demonstrated an aberrant regenerative caudal fin outgrowths in comparison to control morpholino injected and amputated larvae at 3 dpa. Representative whole body images and caudal fins of (C,D) control and (E,F) miR-21 morphants at 3 dpa (120 hpf).
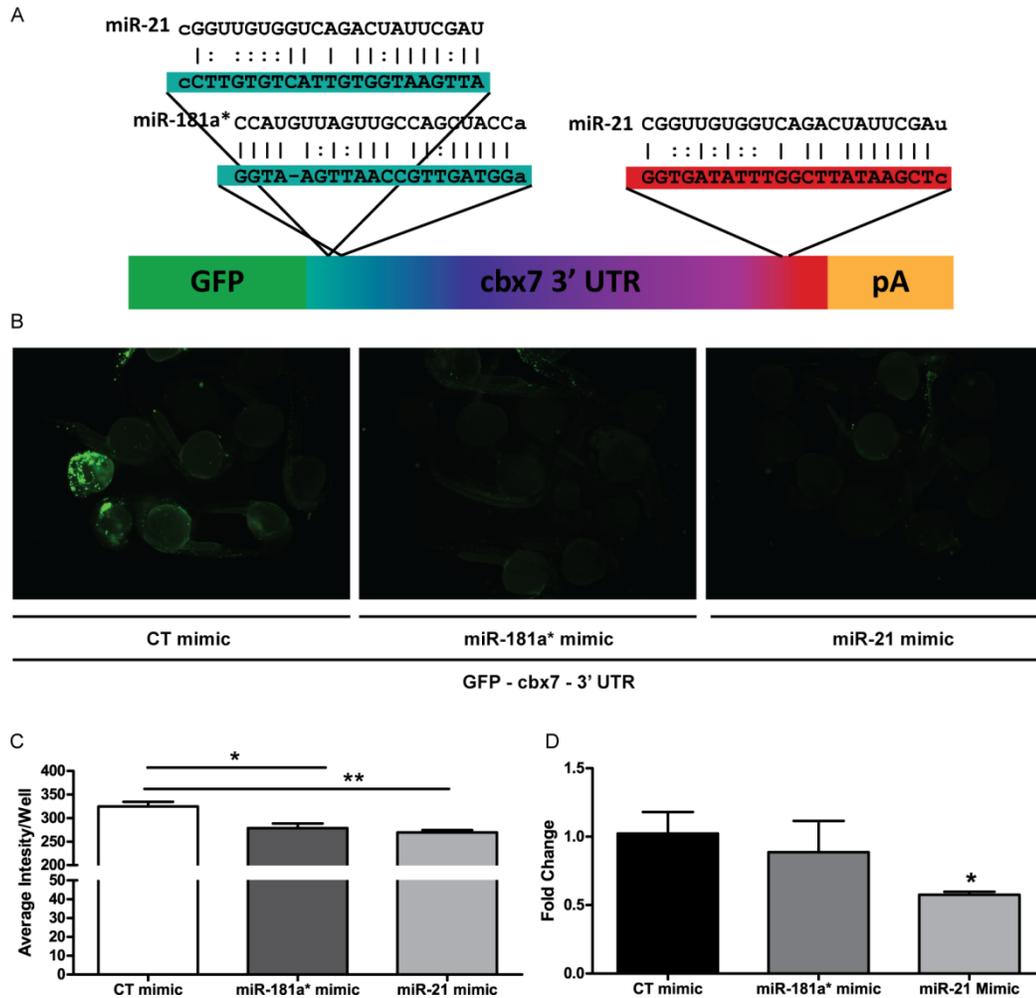
Figure 3-7. Cbx7a is a *bona fide* target of miR-21 and miR-181a* *in vivo*.
A GFP-cbx7a 3' UTR reporter (A) was co-injected with miR-21, miR-181a* or control morpholinos into single-cell embryos. (B) Representative images of fluorescence expression in embryos (n=12/well) and (C) the quantification of fluorescence intensity per well (n = 4) was measured. (Significance represents co-injected GFP-cbx7a 3' UTR reporter and miR-21 or miR-181a* mimic in comparison to control mimic, mean ± SEM, n = 4, *P < 0.05, ** P < 0.01, One-way ANOVA with Tukey's Multiple Comparison Test.) (D) Injection of miR-21 mimic decreased the expression of cbx7. (Values represent fold-change compared to control mimic injected embryos, mean ± SEM, n = 3,*P < 0.05, One-way ANOVA with Dunnett's Multiple Comparison Test.)
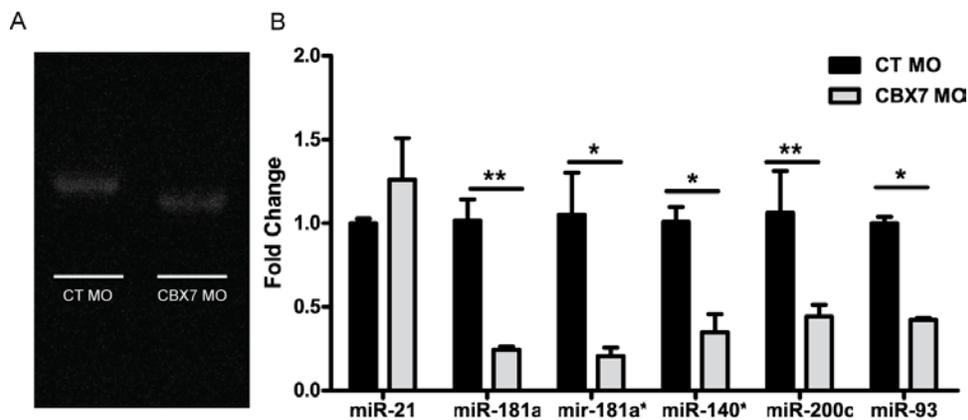
Figure 3-8. Cbx7 loss-of-function confirms relationship with target miRNA.
Cbx7 splice blocking morpholino targeting exon 2 (44bp) or control morpholino were injected into single stage animals. (A) Gel electrophoresis was used to confirm knockdown of PCR amplified products of pooled tissue from cbx7 and control morphants at 48 hpf. (C) Expression levels, represented by fold-change, of miRNAs predicted to target cbx7 in cbx7 morphants at 48 hpf (The fold-change data is relative to control morphants, mean ± SEM, n = 3, *P < 0.05,**P < 0.01, Two-way ANOVA with Bonferroni Multiple Comparison Test.)
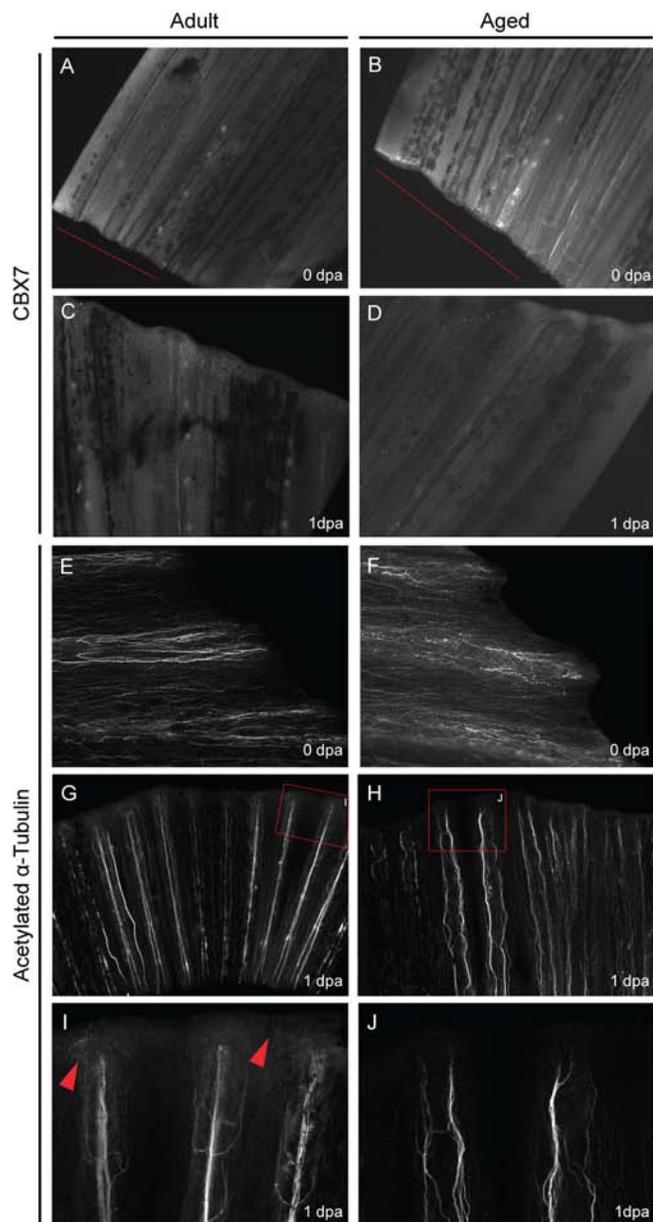
Figure 3-9. CBX7 expression and neuronal integrity of adult and aged fin tissue.
Representative images of CBX7 protein expression in neuromasts of non-regenerating (control) and regenerating tissue from (A,C) adult and (B,D) aged zebrafish (Red line represents amputated edge of fin). Acetylated alpha tubulin antibody labeled the neuronal cell bodies and axons in non-regenerating and regenerating (E,G,I) adult and (F,H,J) aged fin tissue. Magnified images of the regeneration front show neuronal processes extending under the apical epithelial wound cap of (I) adult tissue at 1 dpa and lack of innervation in the (J) aged tissue.
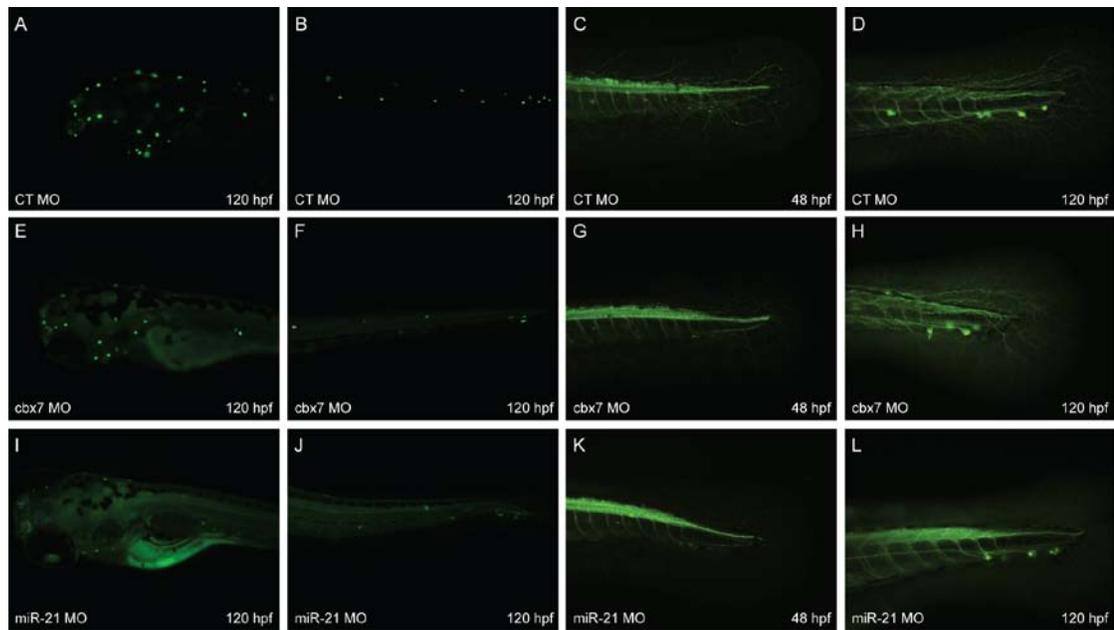
Figure 3-10. Transient knockdown of cbx7 and miR-21 impacts neuromast hair cell formation and neurogenesis.
Staining of the hair cells of the anterior lateral line and posterior lateral line in (A,B) control, (E,F) cbx7 and (I,J) miR-21 morphants at 120 hpf. Acetylated alpha tubulin antibody expression in the neuronal process of (C,D) control, (G,H) cbx7 and (K,L) miR-21 morphants at 48 and 120 hpf

Table 3-1. Fold change values for significant and inversely expressed transcripts in adults and juveniles versus aged tissue from 78-bp RNA-Seq differential expression analysis. Log2 for transcripts with significant and inverse expression profiles in caudal fin tissue from juveniles and adults in comparison to aged zebrafish (p < 0.05, 5% FDR).

	Age Cohort		
Transcript	**Aged**	**Adult**	**Juvenile**
agpat3	-1.79769e+308	1.79769e+308	1.79769e+308
tial1	-1.79769e+308	1.79769e+308	4.22414
tmco7	-3.90464	2.01474	1.79769e+308
TRIM60	-0.807662	1.92168	1.02129
fras1	-1.79769e+308	1.81466	1.57671
si:dkey-8k3.2	-0.917333	1.03233	1.79769e+308
CBX7 (2 of 2)	-1.55552	1.00438	2.69832
CABZ01038493.1	2.11188	-0.905684	-0.778159
crip2	0.896862	-1.20917	-2.1666
cyp26a1	1.11555	-1.29666	-2.10108
ankrd44	4.1798	-1.57574	-1.47502
CLASP1 (1 of 3)	1.13417	-1.92109	-2.43288
MYOF (1 of 2)	0.847512	-1.79769e+308	-1.318

Table 3-2. Fold change values for miRNAs from 40-bp RNA-Seq differential expression analysis.
miRNAs significantly differentially expressed in aged, adult or juvenile regenerating caudal fin tissue in comparison to control (Significance values reflect counts in regenerating tissue relative to non-regenerating, mean ± SEM, n = 3,*P < 0.05, ***P < 0.001, Two-way ANOVA with Bonferroni Multiple Comparison Test)

	Age Cohort					
miRNA	**Aged**		**Adult**		**Juvenile**	
let-7a	-2.42	***	-1.85	***	-1.62	
let-7g	-2.18		-1.80	***	-1.54	
miR-10b	-3.60	*	-2.24	***	-1.77	
miR-181a	-2.69	***	-2.55	***	-2.56	***
miR-21	3.04	***	9.51	***	7.20	***

Table 3-3. Fold change values for Sox2 and Notch ligands from 78-bp RNA-Seq differential expression analysis.
Log2 for deltaD (dld), jagged1a (jag1a), jagged1b (jag1b) and sox2 in regenerating compared to non-regenerating tissue (p < 0.05, 5% FDR).

Age Cohort						
miRNA	Aged		Adult		Juvenile	
dld			-2.84578	***	-2.14833	***
jag1a	3.57431	***				
jag1b			-1.35052	***	-0.63411	***
sox2			-1.03724	*	-1.10368	**

Table 3-S1. Sequence and read alignment data from 78-bp Illumina Sequencing.
Illumina® flow cell lane location for 78- bp RNA-Seq, total number of processed sequences, parsed sequences, sequences passing Q-threshold and number of aligned reads for each of the 18 individual samples. The bioinformatics IDs indicate the age cohort (G = aged; A = Adult; J = Juvenile), CON for control or non-regenerating tissue (0 dpa) and REG for regenerating tissue (1 dpa), and numbers denote the biological replicate.

Sample	Lane	Total_Processed_Seqs	Total_Parsed_Seqs	Total_Seqs_passing_Q-Threshold	Aligned_Read_SAMSTAT
G-CON-1	1	12,010,526	11,876,890	11,876,890	12,095,368
G-CON-2	2	11,462,101	11,285,766	11,285,766	10,958,271
G-CON-3	3	10,279,681	10,194,492	10,194,492	9,482,057
G-REG-1	5	14,942,673	14,926,147	14,926,147	13,994,913
G-REG-2	6	15,481,541	15,461,928	15,461,928	14,696,840
G-REG-3	7	12,505,648	12,492,314	12,492,314	11,780,744
A-CON-1	3	9,590,565	9,510,890	9,510,890	8,559,533
A-CON-2	1	11,759,748	11,630,260	11,630,260	10,767,689
A-CON-3	2	13,767,681	13,554,668	13,554,668	12,602,451
A-REG-1	7	12,383,753	12,370,522	12,370,522	11,310,669
A-REG-2	5	12,842,225	12,827,624	12,827,624	12,488,480
A-REG-3	6	11,423,817	11,409,502	11,409,502	10,425,682
J-CON-1	2	11,239,639	11,066,457	11,066,457	10,284,081
J-CON-2	3	8,057,565	7,989,441	7,989,441	7,141,018
J-CON-3	1	12,848,896	12,706,963	12,706,963	11,606,685
J-REG-1	6	11,083,992	11,069,884	11,069,884	10,031,527
J-REG-2	7	11,446,240	11,433,860	11,433,860	10,456,310
J-REG-3	5	8,739,084	8,729,403	8,729,403	7,967,022

Table 3-S2. Cbx7 and β-actin primers used for experiments.

Gene	Experiment	Forward (5'-3')	Reverse (5'-3')
b-actin	PCR	AAGCAGGAGTACGATGAGTC	TGGAGTCCTCAGATGCATTG
cbx7	PCR	AGTCTACGCGGGACATCTCGAC	CGTTTTGGTCCACTTCGGTGCTC
cbx7 (3' UTR)	Reporter Assay	AGTCAGGACCAAGTGAACGA	TCACCCACAACTTTATTTTTCCAT

Table 3-S3. Morpholino sequences.
Sequences for the cbx7, miR-21, and control morpholinos.

Name	Ensembl Transcript ID or Mature miRNA Accession No.	MO Sequence (5' to 3')	MO Type	MO Target
cbx7 MO	ENSDART00000055428	CACATTTCCCTGAAACGAGAGCGAA	splice blocking	i1e2
miR-21 MO	MI0001908	CAGCCAACACCAGTCTGATAAGCTA	miRNA	Guide-Dicer
Control MO	-	CCTCTTACCTCAGTTACAATTTATA	-	-

Table 3-S4. FPKM values for significant and inversely expressed transcripts in adults and juveniles versus aged tissue from 78-bp RNA-Seq differential expression analysis.

Ensembl transcript IDs, loci, FPKM values, fold change (Log2), p and q values (5% FDR) for transcripts with transcripts with significantly inverse expression profiles in juveniles and adults in comparison to aged obtained from 78-bp RNA-Seq analysis.

Transcript Name	Ensembl ID	Aged				Adult				Juvenile			
		FPKM value 0 dpa	FPKM value 1 dpa	Log2 (Fold Change)	p	FPKM value 0 dpa	FPKM value 1 dpa	Log2 (Fold Change)	p	FPKM value 0 dpa	FPKM value 1 dpa	Log2 (Fold Change)	p
agpat3	ENSDART00000115081	1.32937	0	-1.79769e+308	0.00026953	0	1.94922	1.79769e+308	1.46E-06	0	1.61943	1.79769e+308	9.25E-06
tial1	ENSDART00000135321	3.61061	0	-1.79769e+308	0.00012397	0	4.35719	1.79769e+308	1.10E-06	0.230232	4.30287	4.22414	0.00358033
tmco7	ENSDART00000042496	1.29979	0.0867879	-3.90464	0.0120007	0.645331	2.60783	2.01474	0.00175086	0	1.92793	1.79769e+308	1.08E-06
TRIM60	ENSDART00000085792	4.79631	2.74016	-0.807662	0.00541217	0.361193	1.36843	1.92168	0.00190633	1.43638	2.91546	1.02129	0.00646962
fras1	ENSDART00000097856	1.50877	0	-1.79769e+308	2.50E-08	0.538818	1.89544	1.81466	1.53E-05	1.07982	3.22098	1.57671	7.91E-06
si:dkey-8k3.2	ENSDART00000143550	4.71306	2.49551	-0.917333	0.00392114	1.91953	3.92607	1.03233	0.00032936	0	1.85412	1.79769e+308	7.51E-15
CBX7 (2 of 2)	ENSDART00000055428	17.7261	6.03055	-1.55552	3.01E-07	6.02922	12.0951	1.00438	0.0012463	4.16863	27.0563	2.69832	0
CABZ01038493.1	ENSDART00000130740	0.451526	1.95174	2.11188	0.0110555	8.38252	4.47442	-0.905684	0.00287592	9.26578	5.40297	-0.778159	0.00975927
crip2	ENSDART00000103980	4.64102	8.64163	0.896862	0.00356915	27.4297	11.8638	-1.20917	2.04E-06	69.3624	15.4494	-2.1666	0
cyp26a1	ENSDART00000041728	14.3089	31.0042	1.11555	4.60E-05	50.4744	20.5465	-1.29666	3.08E-07	64.3686	15.0033	-2.10108	2.22E-16
ankrd44	ENSDART00000037182	0.0911717	1.65236	4.1798	0.00115647	2.56242	0.859615	-1.57574	0.00031169	2.89686	1.04208	-1.47502	0.00083117
CLASP1 (1 of 3)	ENSDART00000102445	3.84497	8.43942	1.13417	7.99E-05	3.83476	1.01258	-1.92109	5.77E-06	3.76382	0.697041	-2.43288	1.42E-06
MYOF (1 of 2)	ENSDART00000026000	4.05885	7.30347	0.847512	0.00908081	1.14973	0	-1.79769e+308	7.13E-05	2.81687	1.12982	-1.318	0.00565796

Chapter 4 – Conclusion

In summation, the research presented in this thesis demonstrated the pivotal role of miRNAs during two distinct life stages, embryonic development and aging. Misregulation of miRNA expression was shown to be responsible for eliciting adverse developmental effects and the decline of tissue regenerative capacity with age. This is not surprising since many commonalities exist between development and regeneration. In essence, regeneration can be thought of as redevelopment (1). Regeneration requires the reactivation of fundamental developmental signaling pathways in order to restore damaged or missing structures (1). Both development and regeneration require precise control over spatiotemporal gene expression necessary to initiate and terminate molecular signaling cascades that guide cells to either create or recreate new tissue architecture (1). Accordingly, miRNAs are perhaps best known for their role in fine-tuning the spatiotemporal expression of genes (2). Hence, our data corroborated the evidence that misregulation of miRNA expression, whether induced by toxicant exposure or organism aging, can result in undesirable, and often irreversible, biological consequences.

First, to investigate the regulatory function of miRNAs in complex biological processes, RA was used as a model toxicant to explore the role of miRNAs in eliciting teratogenicity in the larval zebrafish model (Chapter 2). An initial toxicity assay demonstrated that 5 nM RA resulted in a distinct posterior curved body axis in larval zebrafish. Therefore, we sought to determine whether miRNAs were responsible for disrupting RA signaling, thereby leading to RA-induced body axis defects. Results from an unbiased global miRNA profiling analysis revealed that developmental RA exposure suppressed the expression of three miR-19 family members during the early stages of zebrafish somitogenesis. This is consistent with previous studies that showed RA is only necessary during the early stages of somitogenesis (somites 1-6 in mice) to assure proper and synchronous development of the remaining somites (3, 4). All three of these miR-19 family members are predicted to target the 3' UTR of cyp26a1, the main enzyme responsible for converting RA into its inactive polar metabolites (5). Through the use of a physiological reporter assay, we confirmed for the first time that the 3' UTR of cyp26a1 is a *bona fide* target of miR-19. Co-expressed miRNAs are known to act

cooperatively to regulate the 3' UTR of a common mRNA (6), supporting the concept that RA-induced repression of miR-19 family members likely acts as a compensatory mechanism to increase the expression of one of the molecule's main metabolizing enzymes, CYP26A1. Additionally, antisense repression of miR-19 *in vivo* recapitulated the distinct posterior curved body axis morphology and co-injection of exogenous miR-19 rescued the classic body axis defects associated with RA exposure. Together, this study highlights a role for miR-19 in facilitating normal vertebrate development by serving as a RA-sensitive switch to promote CYP26A1 mediated RA turnover during somitogenesis.

These findings also provide novel insight into the evolutionary significance of the miR-19 family as a vertebrate innovation. miR-19 has no known homologs in invertebrates (7, 8). The introduction of miRNA families correlates with drastic increases in morphological complexity (8-10). In addition, the data presented are consistent with the results of a seminal paper implicating the miR-17-92 cluster, in which miR-19 is a member, in vertebrate axis formation. This was the first report of a miRNA mutation causing a hereditary condition responsible for developmental defects in humans (11).This, in conjunction with our empirical results demonstrating the critical role of miR-19 family members in vertebrate axis formation, suggests the evolution and necessity for miR-19 in conferring proper formation and patterning of somites, a developmental phenomenon common to all vertebrates.

Our results suggest a novel miRNA-driven compensatory mechanism initiated to increase the expression of RA's main detoxifying enzyme, CYP26A1, during a period in which spatial maintenance of endogenous RA abundance is critical. Thus, this research places miR-19 at the nexus of RA induced teratogenicity and contributes to a more robust understanding of the regulation of RA metabolizing enzymes by describing the role of miRNAs in refining RA signaling during development. These results raise the interesting possibility that regulation of CYP26A1 abundance by miRNAs might also mediate hindbrain and forebrain development, neurogenesis and heart formation which are all regulated, in part, by RA signaling (5). To our knowledge, miRNAs have yet to be incorporated into existing bioinformatics models of the segmentation clock (12, 13). Our results deem this necessary to obtain a complete picture of the mechanisms that dictate somite formation and beg the question as to whether other

major signaling pathways that control axis formation, such as FGF, are similarly post-transcriptionally regulated by miRNAs.

Next, to investigate the potential role of miRNAs in the declining ability to repair tissue with age, we conducted 40-bp and 78-bp RNA sequencing on regenerating and non-regenerating caudal fin tissue from aged, adult and juvenile zebrafish (Chapter 3). This data provides the field with the first full repertoire of both transcripts and small RNAs involved in caudal fin tissue regeneration. Additionally, an unbiased approach was used to explore the differences in expression patterns of small RNAs and mRNAs in aged zebrafish, which have a compromised regenerative response, in tandem with expression patterns in juveniles and adults, which regenerate successfully. Only 13 transcripts exhibited a significant, but inversed, expression pattern in aged regenerating tissue compared to younger tissue. Cbx7 was the most abundant inversely expressed transcript that had decreased expression in geriatrics and increased expression in adults and juveniles. While this research supports the first identified role for cbx7 as a regulator of aging (14) and its recently discovered role in mediating self-renewal and differentiation (15, 16), our findings present the first documented evidence for a role of cbx7 in tissue regeneration in adult animals.

Subsequently, a non-biased bioinformatics approach was used to identify transcripts significantly expressed in regenerating tissue based on the 78-bp RNA-Seq data predicted to be targeted by significantly expressed miRNAs for the 40-bp RNA-Seq analysis. Members of the miR-21 and miR-181a family were the most abundant miRNAs in regenerating and non-regenerating tissue and were both predicted to target the 3' UTR of cbx7. qRT-PCR experiments demonstrated that miR-21 and miR-181a* were both significantly increased in regenerating tissue compared to non-regenerating tissue. A physiological reporter assay was used to confirm that cbx7 is a *bona fide* target of miR-21 and miR-181a*. Microinjection of exogenous miR-21 reduced cbx7 *in vivo,* also supporting the relevance of a functional regulatory interaction between miR-21 and cbx7. Furthermore, transient knockdown of miR-21 inhibited tissue regeneration in larval zebrafish, suggesting a role for miRNA mediated regulation of cbx7 in tissue regeneration. Taken together, these results highlight a novel age-

dependent role for cbx7 in regulating vertebrate tissue regeneration and underscore the importance of miR-21 as a master regulator of regenerative responses.

Data from the RNA-Seq analysis and our localization of CBX7 to the neuromasts also suggests that the mechanisms dictating regeneration in the caudal fin may be conserved in neuromast hair cell formation and neurogenesis, since the same molecular signaling pathways involved in tissue and structure formation are often required for the restoration of damaged structures (1). Additionally, many pathways (e.g., WNT and FGF signaling) are conserved across regeneration platforms as demonstrated in the fin, heart, retina, skeletal muscle, liver and hair cells (as reviewed in (17)). Sox2 and two Notch ligands, Jagged1 (jag1b) and DeltaD (dld), were all significantly decreased during regeneration in adults and juveniles and completely absent in aged tissue. Sox2 was demonstrated to be important for tissue regeneration (18, 19), cell fate specification (20-22), nerve innervation (18, 23), and neuromast and hair cell formation (20, 24-26). Reactivation of Notch signaling is critical for regeneration in the inner ear (27), lateral line (28), liver (29), heart (30), fin (30), spinal cord (31), and axons (32). Additionally, both Jag1b and DeltaD ligands were demonstrated to be involved with neuromast hair cell formation (33-38). Our data demonstrates that in addition to inhibiting regeneration, transient knockdown of miR-21 causes aberrant neuromast hair cell formation and impaired neurogenesis. Sox2 is an important pluripotency related gene whose expression was demonstrated to be mediated by Cbx7 expression (15, 16). Additionally, sox2, jag1, and deltaD interact concurrently to control the formation of developing hair cells (as reviewed in (38)). Taken together, our findings suggest the importance for miR-21 as a master regulator that acts as a permissive switch to regulate expression of cbx7 and likely influence other important developmental regulatory genes such as Sox2 and Notch ligands, which are necessary for both regeneration and development. Furthermore, this research underscores the critical role miRNAs play in conserved regenerative responses and developmental signaling pathways.

Regeneration research has rapidly re-emerged in the context of regenerative medicine (39). One main goal of *in vivo* regeneration research is to understand the molecular signaling processes which coordinately act to restore tissue in organisms that can regenerate. The

hope, of course, is to use our knowledge of these pathways to understand why other species cannot. Seifert et al. suggest that these traditional types of evolutionary comparisons, such as the presence or absence of regenerative capacity across species, may not broaden the lens enough to allow for a complete picture of regeneration. In many species, regeneration is coupled to developmental stage and regenerative capable organisms only have the ability to restore damaged tissue in larval and juvenile stages (39). Perhaps the more appropriate question for uncovering pathways that facilitate regeneration is: "What are the changes that occur throughout a lifetime of an individual to constrain regeneration?" (39). Our research provides answers to this question and suggests a putative role for miRNAs in mediating age-related changes in regenerative capacity. Furthermore, given the evolutionary role of miRNAs to confer the robustness of phenotypes (40) and that the decline of regenerative capacity with age is a conserved phenotype in most vertebrates (41-44), we posit that evolution and devolution of specific miRNAs that reinforce age-dependent transcriptional programs necessary for regeneration are responsible for the decline in regenerative capacity with age.

In regards to development, this research was the first to identify a role for miRNAs in eliciting RA-induced teratogenicity through the regulation of RA metabolizing enzymes. These findings are significant because although the etiology of RA-induced developmental defects has been known for over fifty years, underlying molecular mechanisms of RA signaling are not fully understood (45, 46). Through our aging research, we were the first to demonstrate an age related role for cbx7 in tissue regeneration in adult animals. Additionally, our findings show the pleiotropic role of miR-21 in the development and re-development or various types of cells and tissues. Collectively, this thesis highlights the role of miRNAs in two complex processes distinctly positioned on opposite ends of organismal lifespan and, more broadly, demonstrates the powerfulness of using tandem miRNA and mRNA global profiling to uncover conserved regulatory mechanisms involved in the development and reconstruction of complex body plans.

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