CHARACTERIZATION OF MICRORNA EXPRESSION PROFILES AND ROLE OF NODAL-RELATED GENES IN ZEBRAFISH OVARIAN FOLLICLES

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ABSTRACT

Zebrafish is a valuable model to study the biology of reproduction as the processes that regulate follicle development and oocyte maturation are conserved among vertebrates. In zebrafish, early vitellogenic (stage IIIa) ovarian follicles are maturationally incompetent while mid-late vitellogenic (stage IIIb) follicles are able to undergo oocyte maturation in response to maturation-inducing hormone signals. Signaling molecules derived from the ovary, such as microRNAs (miRNAs) and growth factors, are important in controlling ovarian function. To determine whether miRNAs may play a role in maturation competency acquisition, we characterized miRNA expression profiles in follicular cells isolated from stage IIIa and IIIb follicles. Bioinformatics analysis uncovered 214 known, 31 conserved novel and 44 novel miRNAs, of which 24 miRNAs were significantly regulated between stage IIIa and IIIb follicular cells. In addition, gene enrichment and pathway analyses of the predicted targets of the significantly regulated miRNAs supported the involvement of several key signaling pathways in regulating ovarian function. We then investigated the role of Nodal, a member of the transforming growth factor-β family, in regulating zebrafish ovarian function. We used real-time PCR to detect the zebrafish Nodal orthologs, nodal-related (ndr1) and ndr2 and found that they were expressed in ovarian follicles at all stages of development. We also detected the mRNAs for Nodal signaling components in follicular cells of vitellogenic follicles. Recombinant human Nodal activated Smad3, CREB, and ERK, and inhibited cell proliferation in ovarian follicular primary cell cultures. The mRNA levels of cyp17a1, hsd3b2 and paqr8 were increased in response to Nodal treatment. Subsequently, we used CRISPR/Cas9 technology to generate *ndr1* and *ndr2* null mutants, which caused severe defects in early development. To overcome this lethality *in vivo,* we developed a fluorescently-labeled, Doxycycline-inducible CRISPR-ON system that expresses single or multiplexed sgRNAs to knockout

ndr1, *ndr2*, and *ndr3.* Activation of the system induced gene editing in the designated genomic loci. Our findings suggest that miRNAs and Nodal play a role in zebrafish follicles. The CRISPR-ON system will facilitate further investigating the roles of miRNAs and Nodal in adult zebrafish *in vivo*.

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DEDICATION

I dedicate this work to my husband, **Ahmed Awad**. Thank you for being my best friend, my confidant and my pillar of strength since high school. Thank you for being there for me for almost 2 decades. You are an amazing father to our daughters and truly beautiful inside and out. Thank you.

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LIST OF ABBREVIATIONS

AGO	Argonaute protein
ALK	Activin receptor-like kinase
ALK7,	activin receptor-like kinase 7
AMH	Anti-Müllerian Hormone
BMP	Bone Morphogenetic Proteins
bp	base pair
cAMP	cyclic adenosine monophosphate
Cas9	CRISPR-associated endonuclease 9
CCK8	Cell Counting Kit 8
Co-Smad	Common Smad
CreER	estrogen receptor-controlled Cre
CRISPR	clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
cyc	cyclops
DGCR8	DiGeorge critical region 8
Dox	Doxycycline
dpf	days post fertilization
DSB	double-strand breaks
dsRNA	double-stranded RNA
E2	Estradiol
F0	Founder fish
F1	first generation
F2	second generation
fgf	fibroblast growth factors
FSH	follicle stimulating hormone
FSH	follicle stimulating hormone
Fshr	FSH receptors
GDFs	Growth and Differentiation Factors
GnRH	gonadotropin-releasing hormone
GnRH	Gonadotropin-releasing hormone

GO	Gene ontology
gsdf	gonadal somatic cell-derived factor
GV	germinal vesicle
GVBD	germinal vesicle break down
hCG	chorionic gonadotropin
hCG	human chorionic gonadotropin
HDR	homology-directed repair
HMA	Heteroduplex Mobility Assay
HPE	Holoprosencephaly
hpf	hours post fertilization
Hsd17b	17β-hydroxysteroid-dehydrogenase
Hsd3b	3β-hydroxysteroid-dehydrogenase
indel	insertion and/or deletion
indels	insertions or deletions
inhba	inhibin βA
I-Smads	Inhibitory Smads
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC	lens-specific *cryaa*:mCreulean (LC)
LH	luteinizing hormone
Lhcgr	LH receptors
LR	left–right
MIH	maturation inducing hormone
miRISC	RNA-induced silencing complex
miRLC	miRISC Loading Complex
miRNAs	MicroRNAs
MMEJ	microhomology-mediated end joining
MPF,	maturation promoting factor
mPRs	membrane progestin receptors
ncRNAs	non-coding RNAs
ndr	nodal-related
NES	nuclear export signal
NHEJ	non-homologous end joining

NLS	nuclear localizing signal
nt	nucleotides
P450Arom or Cyp19a	aromatase
P450c17	Cyp17a
P450scc	Cyp11a1
PAM	protospacer adjacent motif
pre-miRNAs	precursor-miRNAs
pre-miRNAs,	precursor miRNAs
pri-miRNA	primary miRNA
qPCR	Real-time PCR
R-Smads	Receptor Smads
SARA	Smad Anchor for Receptor Activation
sgRNA	single guide RNA
spaw	southpaw
sqt	squint
StAR	Steroidogenic acute regulatory protein
TALENs	transcription-activator-like effector nucleases
Tet-	tetracycline
TetA	TetActivator
TetRE	TetResponder
TGF-β	transforming growth factor-β
WT	Wild-type
xnr	*Xenopus* nodal-related
ZFNs	zinc finger nucleases

CHAPTER 1

LITERATURE REVIEW

I ZEBRAFISH AS A RESEARCH MODEL ORGANISM

The use of zebrafish (Danio rerio) in scientific research has been growing in popularity among researchers since the establishment of zebrafish as a research model organism over 30 years ago (Link and Megason, 2008). Zebrafish is the most studied of teleost fish, which encompasses the highest number of vertebrate species (Nelson, 2006). The popularity of the use of zebrafish in research stems primarily from the highly conserved genome of zebrafish with higher vertebrates. Specifically, the genome of zebrafish bears over 70% homology to that of human genes (Figure 1.1), of which 82% are orthologues to human disease-related genes (Howe et al., 2013).

In addition, there are several other traits of zebrafish that placed them in the spotlight. For example, the complete zebrafish genome is sequenced and is available publicly. This, in turn, facilitates conducting genome-editing and bioinformatics analyses. The conveniently available zebrafish genome has also been used as a reference genome for several fish species to compensate for the lack of their own genomes (Ma et al., 2012; Xu et al., 2013; Li et al., 2014). Moreover, zebrafish are characterized by having a relatively short generation time, with eggs developing into larvae in three days and reaching maturity within three-four months (Figure 1.2).

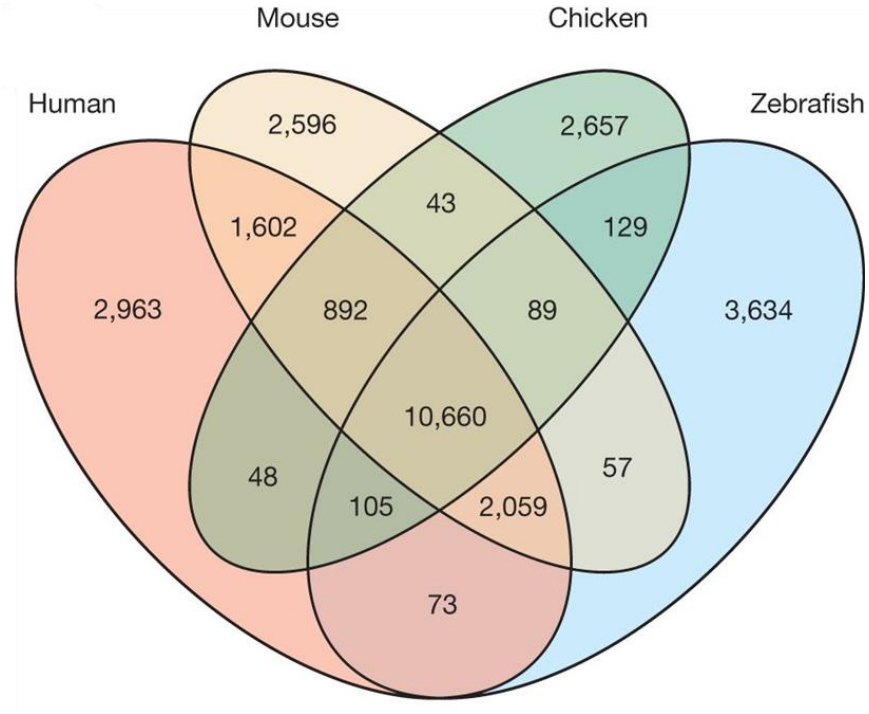


Figure 1.1. Gene homology between zebrafish and other organisms

Overlap between zebrafish genes orthologous to human, mouse and chicken genomes. Of the human genes, 71.4% have at least one zebrafish orthologue. Modified and reproduced with permission from Howe, et al., (2013)

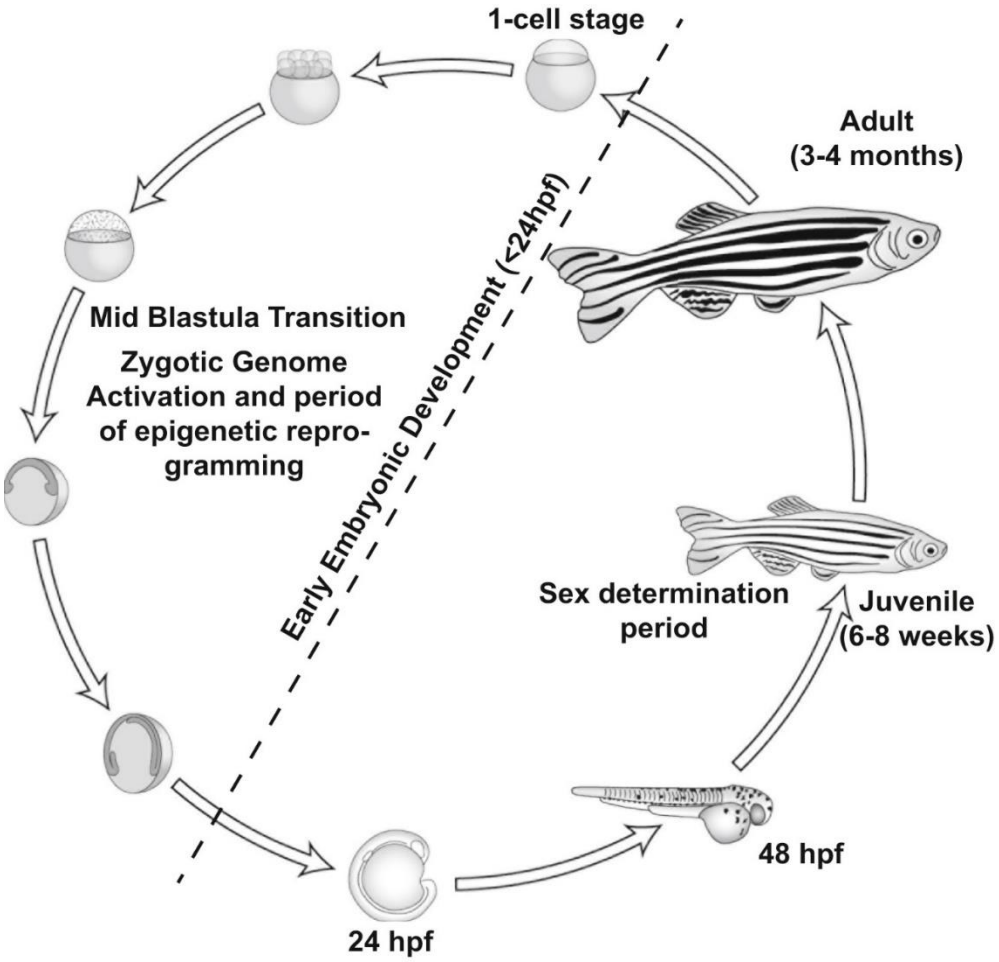


Figure 1.2. Zebrafish as a research model organism

Zebrafish is a great research model organism because of its short generation time and external development. Zebrafish enter the sex determination period at 6-8 weeks post fertilization and reach sexual maturity within 3-4 months after hatching. Zebrafish embryos are transparent and develop rapidly; thus, their use facilitates conducting embryological and early developmental studies. hpf, hours post fertilization. Reproduced with permission from Aluru (2017)

Importantly, zebrafish embryos are optically transparent and develop rapidly and externally to the mother. Therefore, the ability to observe internal structures in living embryos grants them with the necessary amenability to conduct embryological and early development research. The use of zebrafish is particularly useful in studying reproductive biology as the processes governing folliculogenesis and oogenesis are generally conserved among vertebrates. Additionally, zebrafish are known to be highly fecund with 200-300 eggs produced weekly by each mature female. Although wild species of zebrafish are considered annual breeders (Spence et al., 2008), lab-grown zebrafish are capable of spawning year-round under optimized laboratory conditions (Niimi and LaHam, 1974). The asynchronous ovaries of zebrafish encompass follicles at different developmental stages simultaneously within the same ovary. As such, zebrafish has been extensively used to study endocrine and paracrine pathways, and has proven to be a valuable model to study follicle development and oocyte maturation (Clelland and Peng, 2009).

II OVARIAN DEVELOPMENT IN ZEBRAFISH

II.1 Formation of the Ovary

The molecular regulation of zebrafish gonad differentiation is a complex process. The mature gonads originate from 'juvenile ovaries', which may remain as ovaries in females or transform into testes in males after the death of the oocytes (Slanchev et al., 2005). The duration in which the juvenile ovary phase persists ranges from a few days to more than a week in each individual (Wang et al., 2007). Juvenile ovaries consist of oogonia and primary oocytes (Maack and Segner, 2003), which can be first detected at 2.5-4 weeks post-fertilization (Liew and Orban, 2014). Once the juvenile ovary phase ends, the gonad either continues developing as an ovary or changes into testes (Liew and Orban, 2014). The ovary-to-testis transformation is initiated by an apoptotic event of the oocytes leading to a prompt deterioration in oocyte numbers and the ultimate exclusion from the gonad (Uchida et al., 2002). Although it is unclear how this process is activated, it has been shown that the number of primordial germ cells plays a role in this process as their reduction or complete elimination seems to be a factor in testis development (Dranow et al., 2013). In adult fish, germ cells are involved in maintaining ovarian function (Dranow et al., 2013). In addition, it was reported that tumor protein 53 is involved in activating oocyte apoptosis leading to a biased formation of testes rather than maintaining the ovaries (Rodriguez-Mari et al., 2010)

II.2 Follicle Development

Zebrafish follicles are composed of oocytes enclosed in two layers of somatic cells, inner granulosa and outer theca cell layers, separated by a thin basement membrane. Together, the granulosa and theca cell layers are called the follicular layer (Clelland and Peng, 2009). There are two stages that characterize folliculogenesis: the growth stage and maturation stage, which are influenced by the action of gonadotropins. The brain produces the gonadotropin-releasing hormone (GnRH), which stimulates the pituitary to secrete the follicle stimulating hormone (FSH) and the luteinizing hormone (LH). FSH acts through the FSH receptors (Fshr), which are specific to FSH binding and LH binds LH receptors (Lhcgr), which respond to both FSH and LH. Thus, gonadotropins control further ensuing hormones and factors that regulate follicle growth and oocyte maturation in the ovary (Figure 1.3).

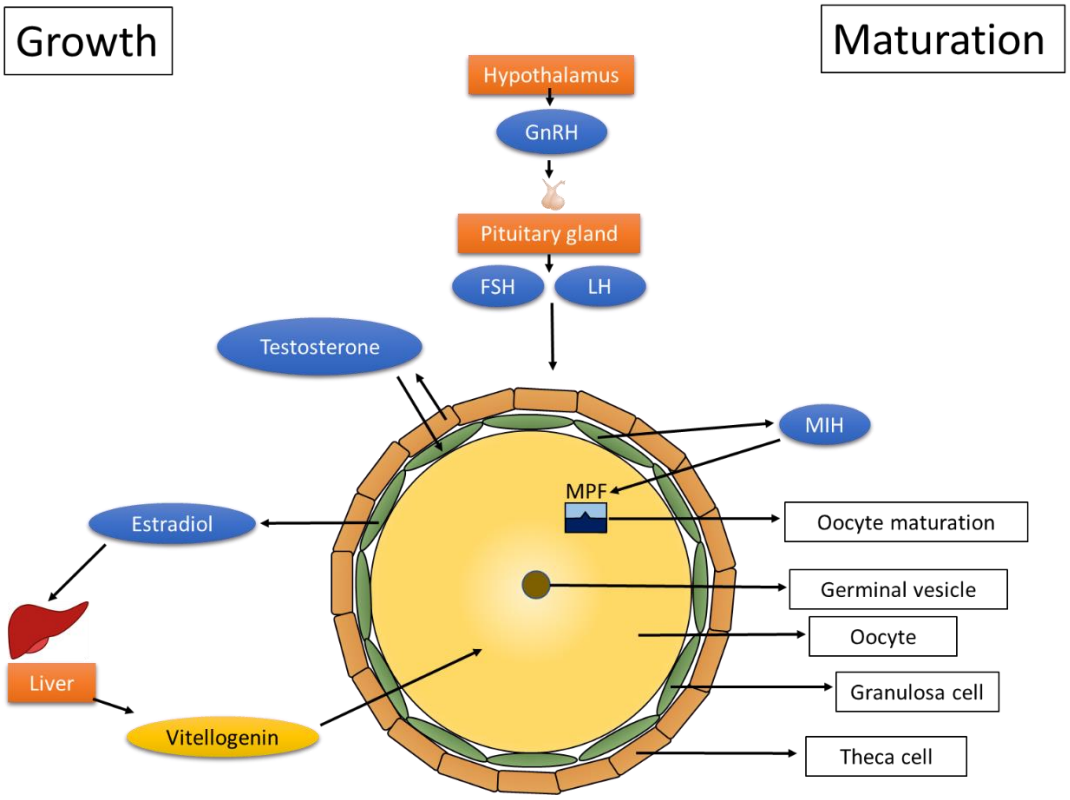


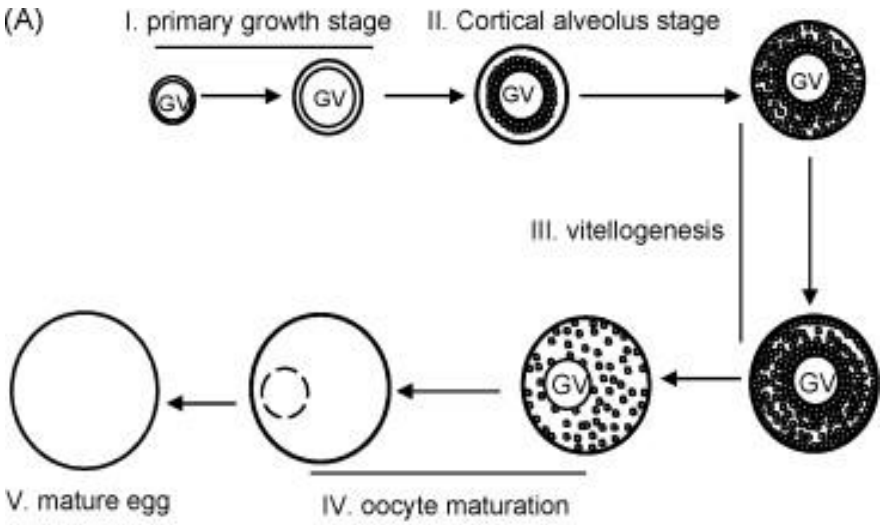
Figure 1.3. Control of follicle development during growth and maturation phases

The zebrafish follicle is composed of an oocyte surrounded by two thin cell layers, the granulosa and theca cells. During the growth phase, FSH is released from the pituitary to stimulate the aromatization of testosterone to estradiol in the granulosa cells. Estradiol travels to the liver and initiates the production of vitellogenin, which is amassed by the growing follicle. Once follicles are fully grown, the maturation of the oocytes begins. Secreted from the pituitary, LH induces the production of the maturation inducing hormone (MIH) in granulosa cells, which leads to the formation of the maturation promoting factor (MPF) and the subsequent maturation of the oocytes.

The stages of follicle development in zebrafish have been described based on their morphological and biochemical attributes, in the pioneering work of Selman et. al. (Selman et al., 1993). Generally, there are five main stages that were identified in the zebrafish ovary (Wang and Ge, 2004) (Figures 1.4A,1.4B). Primary formation and growth of follicles begin in the first stage (stage I) as oocytes expand in size and small follicles are formed. Meiosis in the oocytes of stage I follicles is arrested at prophase I. In the second stage (Stage II or cortical alveolus stage), the oocyte amasses cortical alveoli, which are yolk vesicles that contain zona pellucida proteins. The third stage, (Stage III or vitellogenesis), is marked by the onset of vitellogenin uptake, which leads to a significant increase in the size of the follicles.

The third stage is further subdivided into two sub-stages, stage III-a and stages III-b, based on the ability of the oocytes to respond to maturation hormones such as MIH or human chorionic gonadotropin (hCG; LH analog). As such, IIIa follicles are maturationally incompetent and fail to respond to maturation hormones whereas IIIb follicles have the capacity to undergo maturation when induced with maturation hormones (Wu et al., 2000; Clelland and Peng, 2009). In stage IV, maturation is initiated in the oocytes of the fully grown follicles when meiosis is resumed. This leads to signaling the breakdown of the nuclear membrane and the subsequent migration of the germinal vesicle from the center of the oocyte to its margin in a process termed germinal vesicle break down (GVBD) (Selman et al., 1993). In the last stage, stage V, the eggs are fully mature and their ooplasm becomes

relatively transparent. Following maturation, ovulation takes place as the follicular sheath ruptures, and the fertilizable eggs are released, ready for spawning (Selman et al., 1993; Clelland and Peng, 2009).


(A)
I. primary growth stage
II. Cortical alveolus stage
GV
GV
GV
GV
III. vitellogenesis
GV
GV
V. mature egg
IV. oocyte maturation

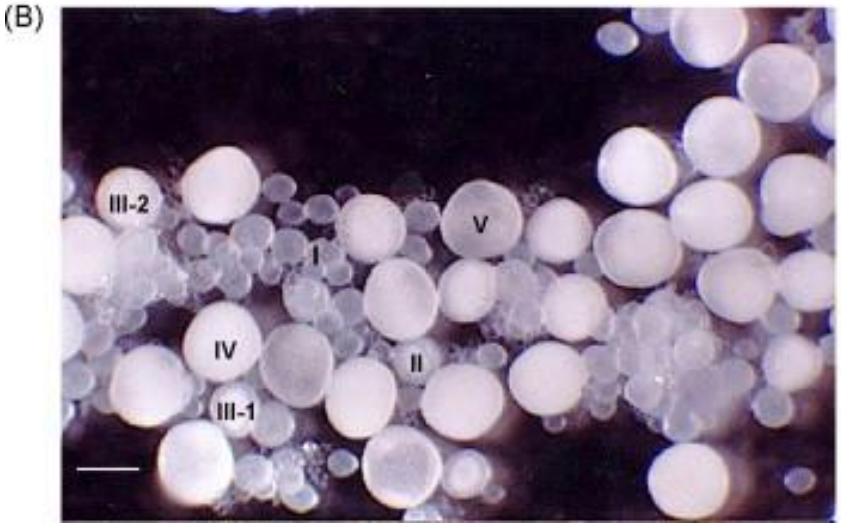

(B)
III-2
V
I
IV
II
III-1

Figure 1.4. Stages of follicle development in zebrafish.

(A) An illustration of the five stages of follicle development. Stage I (or primary growth) is the phase at which follicle formation begins and oocytes begin to enlarge. Stage II, cortical alveolus stage, is when cortical alveoli accumulate within the oocytes. Stage III follicles undergo vitellogenesis and increase significantly in size. At stage IV, oocyte maturation takes place and the germinal vesicle (GV) migrates from the center of the oocyte to the periphery as the nuclear membrane breaks down. Mature eggs are ovulated and ready for spawning in stage IV. (B) An image displaying the different zebrafish follicles at the five main developmental stages. Scale bar: 0.7 mm. Reproduced with permission from Clelland and Peng (2009)

II.3 Oocyte Maturation

The onset of early oocyte maturation involves the follicular cells acquiring the ability to synthesize MIH while the oocytes prepare to respond to MIH. Meiotic resumption and the accompanying nuclear and cytoplasmic events of maturing oocytes occur in three steps. First, LH is released from the pituitary and stimulates the production of MIH from the follicular cells (Nagahama and Yamashita, 2008). Second, MIH initiates the formation of the MPF complex through binding to receptors that are present on the surface of the oocyte. Finally, GVBD takes place resulting in the extrusion of the first polar body and maturation of the oocyte.

The MPF complex is composed of two proteins, Cdc2, and Cyclin B. In zebrafish oocytes, an inactive Cdc2 pre-exists in the oocytes and is detected prior to maturation, while Cyclin B is synthesized *de novo* (Nagahama and Yamashita, 2008). Once Cdc2 binds to Cyclin B, the MPF complex is formed, which allows the follicles to resume meiotic division, releasing the follicles from prophase I arrest (Nagahama and Yamashita, 2008; Clelland and Peng, 2009). The mature follicles then become fertilizable eggs and the MPF is inactivated through the degradation of Cyclin B. Meiosis stops once more at metaphase II in the activated eggs while they await fertilization.

In addition to GVBD and the accompanying nuclear events in the maturing oocytes of marine fish, cytoplasmic events for vitellogenin

processing, have been shown to take place turning opaque immature oocytes into transparent mature ones (Carnevali et al., 1999; LaFleur Jr et al., 2005). Similar ooplasm clearing takes place in zebrafish oocytes and is used as a visually distinctive sign of oocyte maturation (Clelland and Peng, 2009).

Prior to oocyte maturation, high cyclic adenosine monophosphate (cAMP) levels are maintained in the oocytes of developing follicles that lead to the inhibition of the MPF complex activity. The cAMP levels also prevent the meiotic resumption of prophase-metaphase; thus, the meiotic arrest of oocytes in growing follicles is maintained (Conti et al., 2002; Mehlmann et al., 2004). Both, the follicular cells and the oocyte, contribute to the synthesis of cAMP. The follicular cells deliver the cAMP they produced to the oocyte through gap junction communication (Downs and Eppig, 1984; Mehlmann et al., 2004; Ledent et al., 2005).

In order for the oocyte to resume meiosis, cAMP levels must decrease. (Hoffmann and Oris, 2006; Tokumoto et al., 2006). Upon the release of MIH in maturing oocytes, MIH binds membrane progestin receptors (mPRs) on the surface of the oocyte, which in turn causes the cAMP levels to decline to allow for the activation of the MPF (Tokumoto et al., 2004). Additionally, another set of events must take place for meiotic resumption to occur including the migration of the GV towards the animal pole followed by GV breakdown (GVBD), the clearing of the ooplasm and the hydration of the oocyte (Nagahama et al., 1995; Nagahama and Yamashita, 2008; Clelland

and Peng, 2009). Other factors such as ligand members of the transforming growth factor- β superfamily have been shown to regulate oocyte maturation.

II.4 Regulation of Follicle Growth and Oocyte Maturation

Follicle development and oocyte maturation are complicated processes that entail the coordinated action of several endocrine signals for the appropriate functional and morphological changes to take place within the follicle. With many factors at play, ovarian development of zebrafish begins 10 days after hatching and the fish become sexually mature by 3 months (Clelland and Peng, 2009).

In the ovaries of mature fish, steroidogenesis takes place in the follicular cells and is controlled by a sequence of enzymatic reactions (Figure 1.5). The production of Estradiol (E2), which acts in a conserved fashion to other vertebrates, is required for follicle growth to begin. In the theca cells, cholesterol is ultimately converted into testosterone through the action of several enzymes. This conversion process begins with the change of cholesterol to pregnenolone by Cyp11a1 (P450scc) (Nagahama et al., 1995), which is located in the mitochondria. To get catalyzed by Cyp11a1, cholesterol is transferred to mitochondria by the steroidogenic acute regulatory protein (StAR) (Ings and Van Der Kraak, 2006; Nagahama and Yamashita, 2008). Pregnenolone is then converted to progesterone through the action of 3β-hydroxysteroid-dehydrogenase (Hsd3b) (Hsu et al., 2006). In a 2-step process, progesterone is converted to androstenedione. This

conversion is catalyzed by Cyp17a (P450c17) through its 17α-hydroxylase activity that converts progesterone to 17α-hydroxyprogesterone, followed by its 17,20 lyase activity that converts 17α-hydroxyprogesterone to androstenedione. Finally, androstenedione is converted to testosterone through the action of 17β-hydroxysteroid-dehydrogenase (Hsd17b), which marks the last step in theca cells. Following the production of the testosterone, remaining steroidogenic events take place in granulosa cells. Testosterone gets secreted from the theca cells and diffuses into the granulosa cells where it is converted to estradiol-17β (E2) by aromatase (P450Arom or Cyp19a) (Clelland and Peng, 2009). E2 travels to the liver via the blood, diffuses into hepatocytes and initiates vitellogenin synthesis, which then accumulates in the oocytes of growing follicles (Okumura et al., 2002).

Once follicles are fully grown, oocyte maturation takes place. Prior to oocyte maturation, steroidogenic E2 production is shifted to MIH production (Nagahama and Yamashita, 2008).The conversion of 17α-hydroxyprogesterone to MIH is then catalyzed by Hsd20b. MIH is released from granulosa cells to act on the mPRs on the oocyte surface leading to oocyte maturation (Zhu et al., 2003).

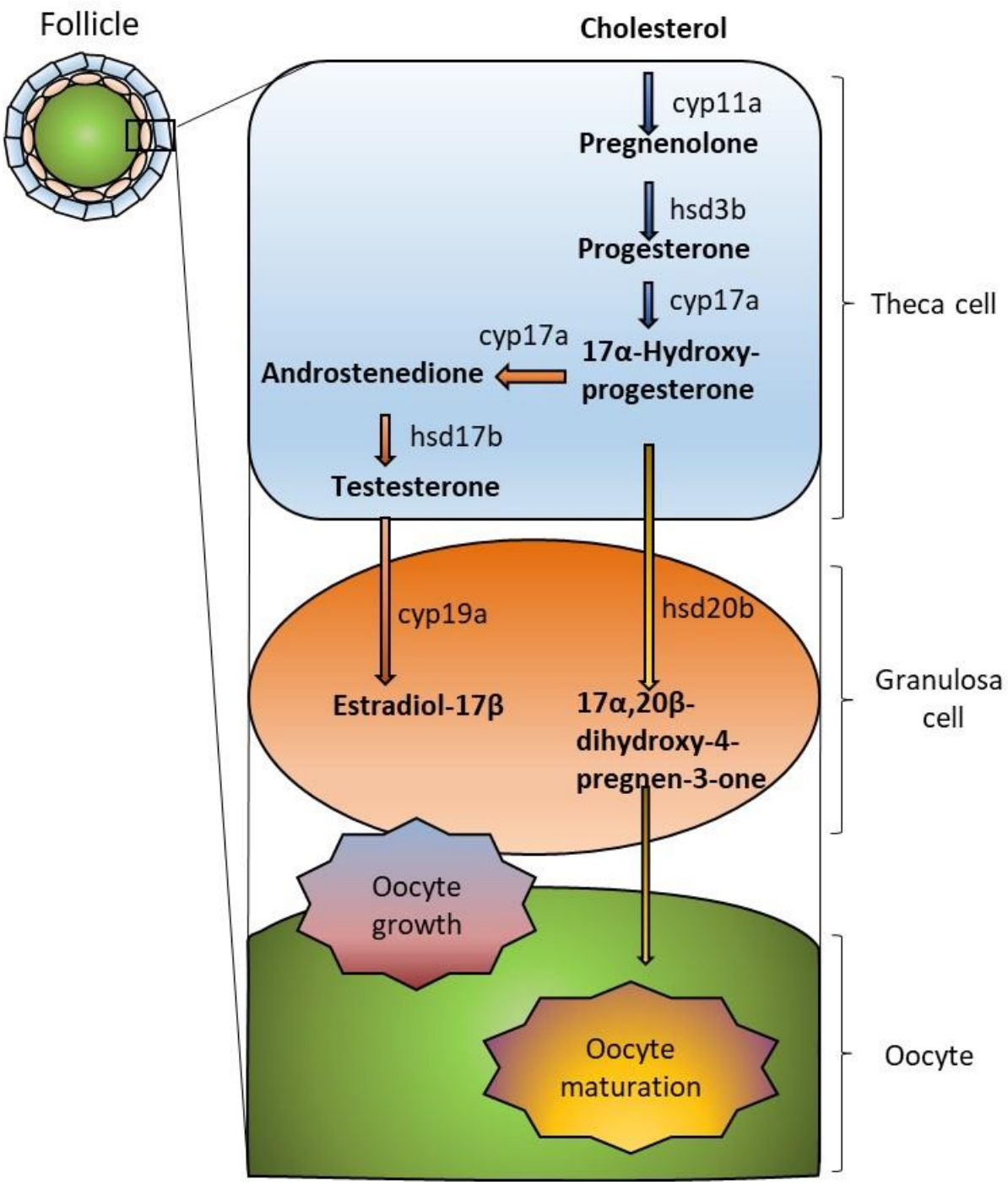

Follicle
Cholesterol
cyp11a
Pregnenolone
hsd3b
Progesterone
cyp17a
17α-Hydroxy-progesterone
cyp17a
Androstenedione
hsd17b
Testesterone
Theca cell
cyp19a
Estradiol-17β
hsd20b
17α,20β-dihydroxy-4-pregnen-3-one
Granulosa cell
Oocyte growth
Oocyte maturation
Oocyte

Figure 1.5. Steroidogenic pathways in fish follicles.

In theca cells, cholesterol is converted to 17α-hydroxy-progesterone and testosterone. In the granulosa cell, 17α-hydroxy-progesterone and testosterone are converted to 17,20β-dihydroxy-4-pregnen-3-one, one of the common forms of MIH, and estradiol, respectively. Estradiol leads to the growth of the follicle during the growth phase. Meanwhile, during the maturation phase, MIH binds to receptors on the oocyte surface, which results in oocyte maturation. The model is based on Clelland and Peng (2009)

III MICRORNAS

MicroRNAs (miRNAs) represent a very abundant class of small non-coding RNAs (ncRNAs) that regulate the expression of genes post-transcriptionally (O'Brien et al., 2018). Being single-stranded RNA molecules 18~26 nucleotides (nt) in length, miRNAs are involved in various regulatory pathways of gene expression. They regulate gene activity by mostly targeting the 3' UTR mRNA transcripts for cleavage or translational repression. miRNAs originate from larger precursor-miRNAs (pre-miRNAs) with a characteristic hairpin structure (Ha and Kim, 2014). miRNAs gained their importance from being implicated in numerous developmental, cellular and biological processes (Fu et al., 2013) as well as being associated with many diseases (Paul et al., 2018) making them the focus of numerous studies over the past 2 decades.

III.1 Discovery and Annotation

The discovery of miRNAs was made by the Ambros and Ruvkun groups, when they found the very first miRNA, *lin-4,* in *Caenorhabditis elegans* (*C. elegans*) (Lee et al., 1993). They later characterized it as a small noncoding RNA, rather than, the then more commonly-known protein-coding RNAs (Almeida et al., 2011). Since *lin-14* was being regulated through its 3' UTR and *lin-4* has a sequence that was complementary to the 3' UTR of *lin-14*, Ambros and Ruvkan proposed that *lin-4* regulated *lin-14* post-transcriptionally (Wightman et al., 1993). It was not until seven years later

when a second miRNA, *let-7*, was discovered, also in *C. elegans* (Reinhart et al., 2000). This discovery transformed the field of molecular biology, and because of the prevalence of bioinformatics techniques, the discovery of miRNAs in other organisms, as well as the prediction of other miRNAs along with their targets, were made relatively easy. Currently, 26 years later, there are 48860 mature microRNAs from 271 organisms in the miRBase release 22.1 (Kozomara et al., 2018).

The criteria for miRNA annotation and identification have been changing to accommodate the dynamic nature of the discoveries relating to the origin and regulation of miRNAs. Initially, miRNAs retained their gene name. (such as *lin-4*). Later on, miRNAs discovered from cloning or sequencing received numerical names (for example, *lin-4* homologs are called *mir-125* in other species) (Ha and Kim, 2014). Subsequently, more miRNA nomenclature guidelines were established. The miRNA name contains a prefix to determine the species in which the reported miRNA exists, such as “dre” for *Danio rerio*. This prefix is followed by either “mir”, which labels precursor hairpins or “miR” to indicate that it is a mature miRNA sequence. MicroRNAs are generally assigned sequential numerical identifiers based on the temporal order of their discovery. For example, miR-17 was discovered before miR-20 (Griffiths-Jones et al., 2006).

Since miRNAs can be derived from either the 5’ or the 3’ arms of their subsequent pre-miRNA, the less predominant miRNAs were given a “star

notion" symbolized by adding a "*" at the end of the miRNA name. More recently, this "star notion" has been disregarded in naming the less predominant miRNAs, but instead mature miRNAs that are generated from opposite arms of the same pre-miRNA are assigned a suffix that indicates the arm from which they were generated. For example, miR-17-5p (previously named miR-17) originates from the 5' arm while miR-17-3p (previously named miR-17*) is produced from the opposite 3' arm. Mature miRNAs that differ in 1-2 nucleotides are further assigned a lettered suffix such as dre-miR-430a and dre-miR-430b, while identical mature miRNAs that are produced from different loci are given a numbered suffix such as dme-miR-281-1 and dme-miR-281-2). Similarly, if identical mature miRNAs are driven from pre-miRNAs with different genomic locations, such pre-miRNAs are assigned numeric suffixes after the name of the miRNA (for example, dre-mir-199-1, dre-mir-199-2 and dre-mir-199-3) (Griffiths-Jones et al., 2006; Ha and Kim, 2014).

III.2 Biogenesis

miRNAs are present in both intergenic and intragenic locations. Intergenic miRNAs are mostly processed from the introns of their host genes while the transcription of intragenic miRNAs is regulated by their own promoters (de Rie et al., 2017). In the nucleus, miRNAs are transcribed by RNA polymerase II/III from their respective miRNA genes producing a primary miRNA (pri-miRNA) transcript, which is composed of a hairpin stem, a terminal loop, and two single-stranded flanking regions (Felekkis et al., 2010). The transcription of the pri-miRNA is followed by its cleavage into a pre-

miRNA by RNase III nuclease, Drosha, associated with its critical cofactor, the DiGeorge critical region 8 (DGCR8) protein. Together, Drosha and DGCR8 form a complex called the microprocessor (Denli et al., 2004) (Figure 1.6). As a part of the microprocessor, DGCR8 identifies the N6-methyladenylated GGAC motif in the pri-miRNA (Lee et al., 2003). Meanwhile, Drosha functions to define the terminus of the miRNA to be cleaved, determining its specificity in the process. Accordingly, Drosha cleaves the pri-miRNA approximately 22 bp away from the top junction linked to the terminal loop, and approximately 11 bp away from the basal junction between the single-stranded RNA and the double-stranded RNA (dsRNA) (Han et al., 2006). This cropping action on the pri-miRNA stem-loop produces a ~65 nt hairpin-shaped pre-miRNA with a 2 nt 3′ overhang (Lee et al., 2003).

Following the production of the pre-miRNA inside the nucleus, it is then exported into the cytoplasm by an exportin5/RanGTP complex (Denzler et al., 2016) and is cleaved by another RNase III, Dicer (Denli et al., 2004). The cleavage occurs close to the terminal loop, releasing a small mature RNA duplex that is ~ 22 nt in length (Zhang et al., 2004). The miRNA duplex that was formed from both arms of the same pre-miRNA then gets separated into two single strands, a predominantly-present guide strand and a passenger strand (Ha and Kim 2014). Either strand can be loaded into the RNA-induced silencing complex (miRISC), which is composed primarily of a member of the Argonaute (AGO) protein family (Yoda et al., 2010).

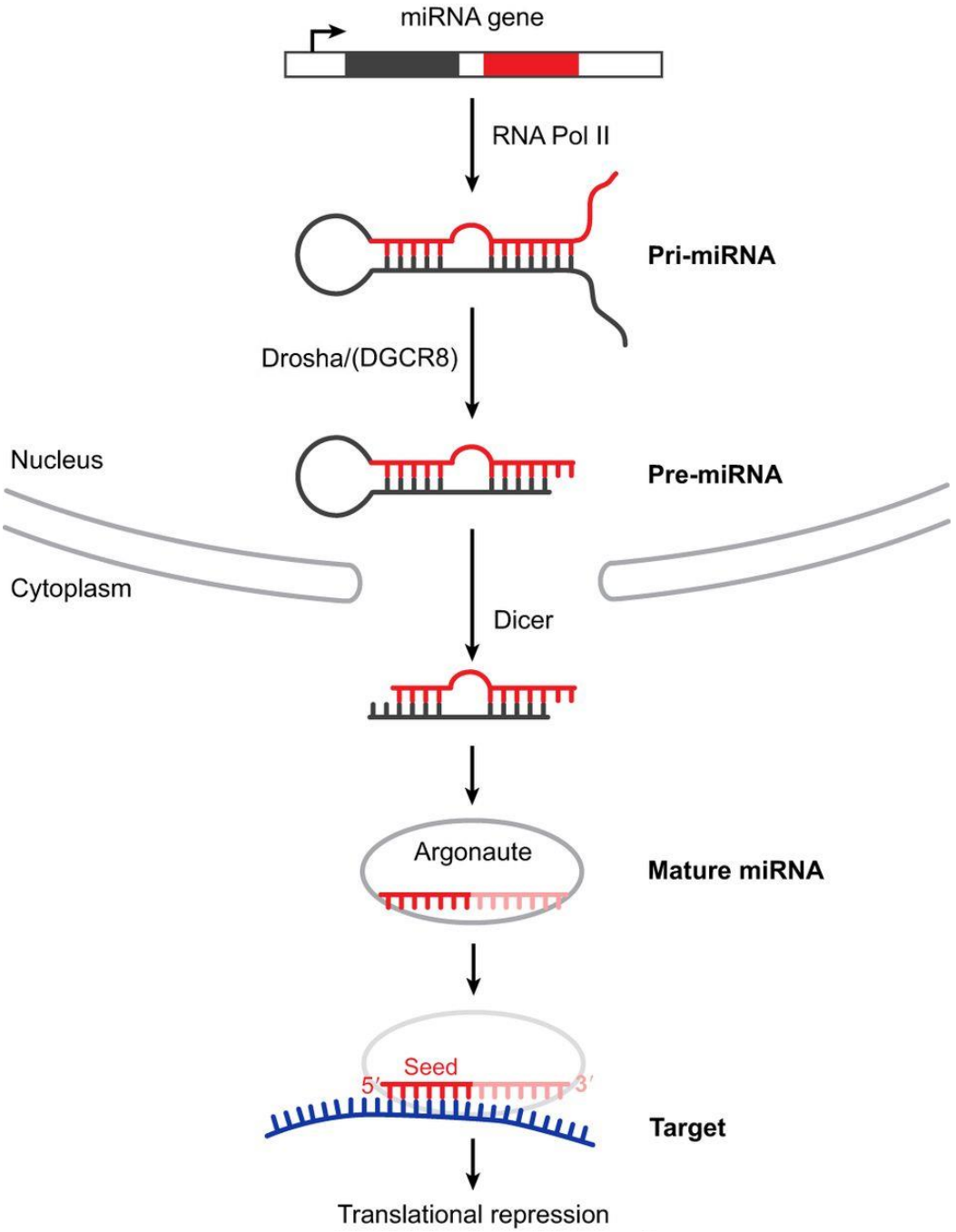

miRNA gene
RNA Pol II
Pri-miRNA
Drosha/(DGCR8)
Nucleus
Pre-miRNA
Cytoplasm
Dicer
Argonaute
Mature miRNA
Seed
5′
3′
Target
Translational repression

Figure 1.6. Canonical pathway of miRNA biogenesis.

The canonical pathway of miRNA biogenesis begins in the nucleus where the transcription of a primary miRNA (pri-miRNA), from the miRNA locus, takes place by polymerase II or III. The pri-miRNA is then cleaved by Drosha and DGCR8 to produce a hairpin precursor miRNA (pre-miRNA), which is exported outside the nucleus by Exportin 5. In the cytoplasm, Dicer cleaves the pre-miRNA hairpin generating a mature miRNA duplex, from which the mature strand binds Argonaute protein (AGO2) forming the RNA-induced silencing complex (RISC). RISC is guided to target mRNAs whose expression is silenced by translational repression. Modified and reproduced with permission from Alberti and Cochella (2017).

III.3 Mechanisms of miRNA-Mediated Gene Regulation

Based on the level of complementarity between the miRNA and the 3' UTR of target mRNA, either cleavage or translational inhibition takes place. The vital region of a miRNA for target recognition, also known as the "seed region", is located in the nucleotides 2-8 from the beginning of the 5' end of the mature miRNA sequence (Bhattacharyya et al., 2006). The seed region requires conserved Watson-Crick pairing to the target mRNA (Friedman et al., 2009). The presence of identical seed sequences in different miRNAs generally indicates that they originate from the same miRNA family as some miRNAs share a common evolutionary origin but diverge in the miRNA seed (Bartel, 2009). For example, there are 14 paralogous loci in the human genome that are grouped in the let-7 family. There are also 34 phylogenetically conserved miRNA families from *C.elegans* to humans, and 196 conserved mammalian miRNA families (Chiang et al., 2010). While some miRNA sisters have distinct roles, other miRNA sisters have been noted to have a redundant effect on their interaction with their target mRNAs (Ventura et al., 2008).

There are five seed-match types with varying levels of efficacy that are preferably conserved in miRNAs (Figure 1.7) (Bartel, 2018). Seed sequences in 8mer seed type have perfect complementarity in position 2-8 nucleotides with an A nt in the preceding first position. miRNAs with 8mer seed types represent miRNAs with the highest efficacy levels. Intermediate efficacy levels were observed in 7mer-8mer seed sequences in miRNAs with perfect Watson-Crick complementarity in positions 2-8 without a preceding A nt. 7mer-A1 seed regions with conservation across positions 2-7 but with an A nt in position 1 have slightly lower levels of efficacy. The least efficient seed types were the 6mer and offset 6mer seed sequences, which contain complementary seed sequences across positions 2-7 or 3-8 in the seed sequence, respectively (Friedman et al., 2009).

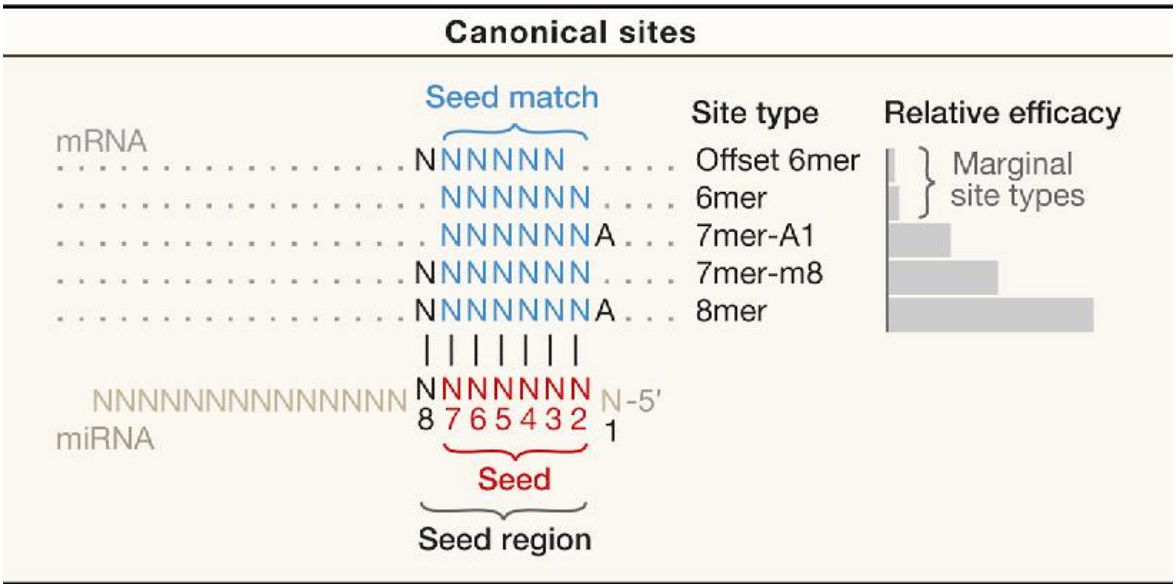


Figure 1.7. Canonical mRNA target seed match types.

Canonical seed sites possess 6-7 matched nucleotides between target mRNA and miRNA seed region (miRNA positions 2-8). Two sites, 7mer-A1 and 8mer, have an A in the first position. The graph on the right shows relative site efficacy in mammalian cells. The most effective sites are 7–8 nucleotide (nt) sites with perfect matching to the miRNA seed sequence in positions 2–7 (red). The level of efficacy declines as the matching between mRNA site and miRNA seed sequence decreases. The least efficient seed types are 6mer and offset 6mer seed sequences. Modified and reproduced with permission from Bartel (2018).

Following miRNA biogenesis, miRNAs associate with, Dicer and a member of the argonaute (AGO) protein family to form a miRISC Loading Complex (miRLC), which possesses precursor-processing and RISC-cleavage activities (Bose and Bhattacharyya, 2016). In humans, only one of the four AGO subclade proteins, AGO2, has cleaving activity (Huntzinger and Izaurralde, 2011). AGO2 in the miRISC complex performs an endonucleolytic cleavage of the target mRNA if perfect complementarity between the miRNA sequence and a corresponding region of the 3'UTR of the respective mRNA was found. Meanwhile, if there was partial complementarity between the miRNA and target mRNA, the latter is inactivated by deadenylation coupled to exonucleolytic degradation (Suarez and Sessa, 2009).

miRNAs are generally regarded as highly stable molecules as it has been reported that the half-lives of some miRNAs ranged from several hours to several days, as well as being resistant to degradation in unfavourable conditions such as freeze-thaw cycles, boiling, and high or low pH (Van Rooij et al., 2007; Chen et al., 2008; Mitchell et al., 2008). However, this stability has been suggested to be a specific feature for each miRNA in different tissues (Davis and Hata, 2009).

III.4 Role of microRNAs in Regulating Ovarian Development

The regulation of oogenesis and follicle development requires precise spatiotemporal control of gene expression, making miRNAs the perfect gene regulators for this task. The role of miRNAs in gene regulation in several

physiological processes including reproduction and endocrinology is well established (Bushati and Cohen, 2007; Toms et al., 2017; Hayder et al., 2018). Moreover, the detection of miRNAs in the ovaries of several fish species including zebrafish has been reported (Ma et al., 2012; Juanchich et al., 2013; Desvignes et al., 2014; Wong et al., 2017). Several studies described the role of miRNAs in mammalian ovarian functions. For example, miRNAs were found to suppress granulosa cell proliferation (Yan et al., 2012) and inhibited apoptosis in mouse granulosa (Carletti et al., 2010; Liu et al., 2014). Several miRNAs were reported to regulate gonadotropin or androgen production including the regulation of estradiol production (Xu et al., 2011; Yin et al., 2012), the regulation of the progesterone receptor leading to suppressing the expression of progesterone (Toms et al., 2015), as well as the regulation of LH/CG receptors (Troppmann et al., 2014). In zebrafish, the expression of two miRNAs,miR-17a and miR-430b, was regulated by hCG in follicular cells (Abramov et al., 2013) Recently, a critical role of miRNAs in regulating fish reproduction was described (Gay et al., 2018), indicating that miRNAs are vital for proper ovarian function. In addition, miRNAs have been shown to target signaling molecules such as signaling components of the Transforming growth factor-beta superfamily in regulating ovarian function (Imbar and Eisenberg, 2014)

IV THE TRANSFORMING GROWTH FACTOR- β (TGF-β) SIGNALING

The transforming growth factor-β (TGF-β) superfamily comprises a large group of growth and differentiation factors and secreted factors of over 30 members (Weiss and Attisano, 2013). Members of the TGF-β superfamily are evolutionarily conserved as many homologs with similar functions have been identified in several organisms such as frogs, fish, flies, and worms. Functionally, TGF-β members can be categorized into two groups, the TGFβ-like group, and the Bone Morphogenetic Proteins (BMP)-like group. The TGF-β -like group contains TGF-βs, activins, Nodal and some Growth and Differentiation Factors (GDFs) while the BMP-like group comprises BMPs, most GDFs and Anti-Müllerian Hormone (AMH) (Weiss and Attisano, 2013). Since the discovery of the first TGF-β polypeptide four decades ago (Moses et al., 2016), TGF-β members have been found to be produced by different tissues and cell types allowing them to be involved in regulating many cellular activities, including autophagy, cell survival, proliferation, differentiation, migration and apoptosis (Schuster and Krieglstein, 2002; Koesters et al., 2010; Bragado et al., 2013; Guo et al., 2014; Boswell et al., 2017; Zhang et al., 2017). It is through the critical roles that they play in different biological processes, TGF-β members have become at the center of a plethora of research studies. Particularly, several members of the TGF-βs play central roles in controlling reproductive processes such as oocyte maturation, germ

cell development, placental regulation and function, and hormone production by the hypothalamus-pituitary-gonadal axis and granulosa-lutein cells (Peng, 2003; Chang et al., 2016; Olguin-Alor et al., 2016; Spiller et al., 2017; Li et al., 2019).

IV.1 TGF-β Structure and Signaling Transduction

The production of the TGF-β family members starts with the synthesis of structurally similar long precursor molecules, which include an amino-terminal signal peptide, a large propeptide, and a highly conserved carboxyl-terminal polypeptide. Proteolytic cleavage of the carboxyl-terminal polypeptide then takes place leading to the formation of the mature, active ligand (Morikawa et al., 2016). Most TGF-β members form dimers between the mature ligands and are present as both homodimers and heterodimers (Morikawa et al., 2016). Another critical aspect of the structure of the carboxyl-terminal polypeptide is the number and location of the cysteine residues that it contains. Particularly, having seven or nine cysteines is what allows for the formation of functional disulfide-linked dimers (Daopin et al., 1992).

Active, dimerized members of the TGF-β family initiate signaling by binding a family of structurally-related transmembrane serine/threonine receptor kinases (Figure 1.8). In general, receptors of the TGF-β family can be split into two groups, type I and type II receptors (Yamashita et al., 1994). Evidently, a total of seven type I activin receptor-like kinase (ALK) and five

type II (TGF-βRII, ACVRIIA, ACVRIIB, BMPRII, and AMHRII) receptors were identified in mammals (Yamashita et al., 1994). Type I and type II receptors are present on the surface of the cell as homodimers or heterotetramers based on their proximity (Yamashita et al., 1994). Signaling is initiated by the TGF-β family members as they bind to their type II receptors. Subsequently, the receptor kinase domain of the TGF-β-bound type II receptor phosphorylates the glycine-serine repeat domain in the type I receptor (Weiss and Attisano, 2013).

Upon the activation of the type I receptors, signaling is driven to the nucleus through the Smad proteins. There are three types of Smads: Receptor Smads (R-Smads), Common Smads (Co-Smads), and Inhibitory Smads (I-Smads) (Chen et al., 2002). The five known R-Smads are Smad-1, 2, 3, 5 and 8. R-Smads are able to interact with type I receptors, join other Smads or interact with other signaling molecules (Hata and Chen, 2016). There is only one Co- Smad, Smad 4, which has the capacity to bind all R-Smads. Meanwhile, Smad7 can inhibit the signaling of all TGF-βs, while Smad6 specifically suppresses BMPs (Weiss and Attisano, 2013).

The ability of the TGF-β members to execute a diverse amount of unrelated biological tasks can be attributed, in part, to the interchangeability and non-specificity of receptor interactions with the different TGF-β ligands. In general, TGF-β signaling can produce a transcriptional response that is classified as BMP-like or TGF-β/activin-like. Typically, type II receptors are

not associated with a distinct TGF-β signaling response and oscillate between both groups. In contrast, type I receptors are more specialized as BMP-like signals interact with ALK-1, -2, -3, or -6, while TGF-β/activin-like signals activate ALK-4, -5, or ALK-7 type I receptors. Likewise, the activation of the R-Smads by type I receptors is also grouped into BMP-like pathways (Smad 1, 5 or 8) or TGF-β/activin pathways (Smad 2 or 3) (Morikawa et al., 2016).

The phosphorylation of R-Smads by type I receptors allows them to complex with Smad 4 (Hata and Chen, 2016). R-Smads usually form trimers, where a trimer comprises two R-Smads and the Co-Smad 4 forming a complex that is translocated to the nucleus. The cellular machinery involved in facilitating nuclear translocation depends on the Smad type that is entering the nucleus. To be transported into the nucleus, Smads are first detached from cytoplasmic anchors, then Smads either unveil their nuclear localizing signal (NLS) or block their nuclear export signal (NES) (Hata and Chen, 2016). After entering the nucleus, the trimeric R-Smad/Co-Smad complex modulates gene transcription through binding the DNA.

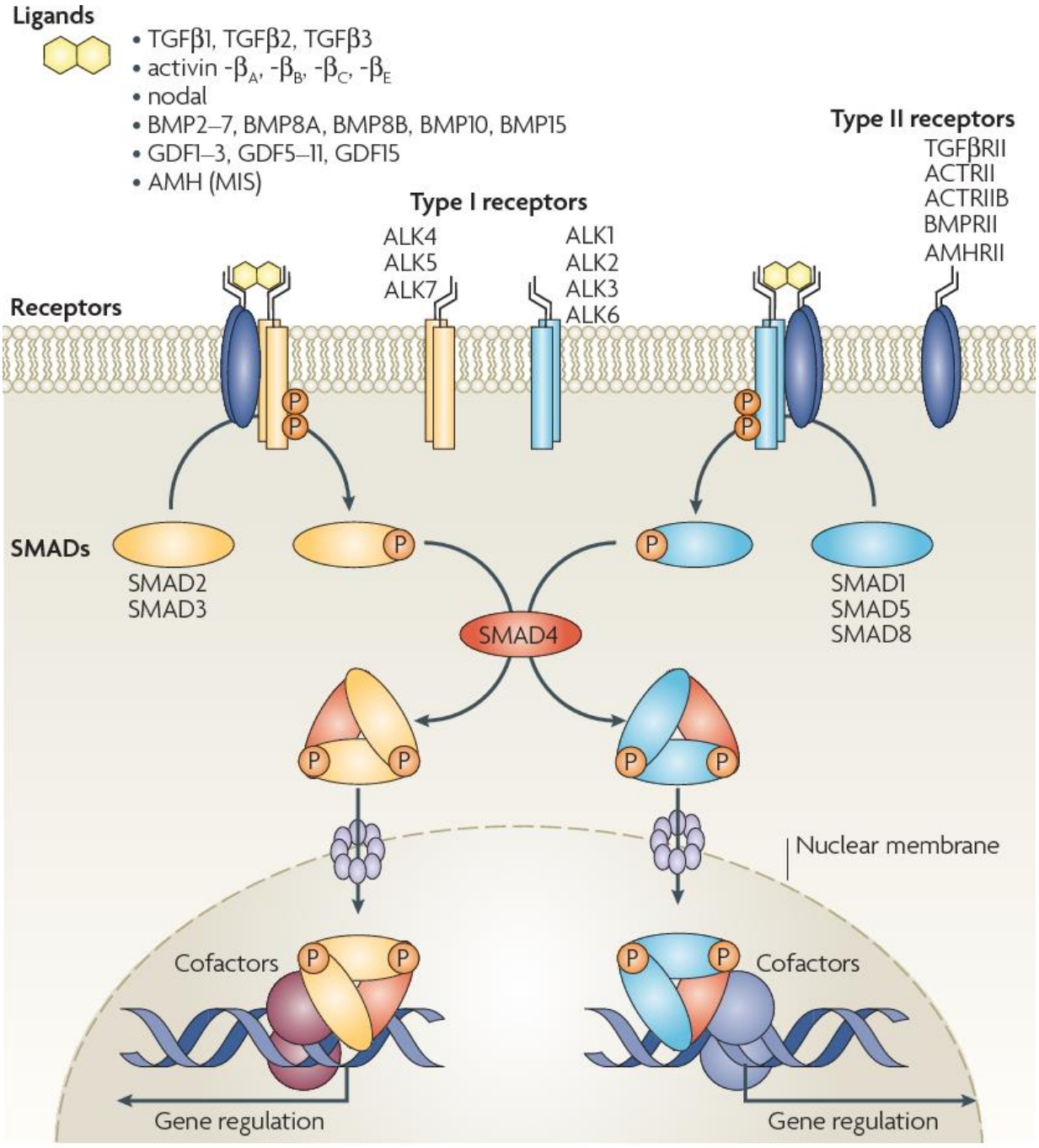

Ligands
• TGFβ1, TGFβ2, TGFβ3
• activin -β_A, -β_B, -β_C, -β_E
• nodal
• BMP2–7, BMP8A, BMP8B, BMP10, BMP15
• GDF1–3, GDF5–11, GDF15
• AMH (MIS)
Type II receptors
TGFβRII
ACTRII
ACTRIIB
BMPRII
AMHRII
Type I receptors
ALK4
ALK5
ALK7
ALK1
ALK2
ALK3
ALK6
Receptors
P
SMADs
SMAD2
SMAD3
SMAD4
SMAD1
SMAD5
SMAD8
Nuclear membrane
Cofactors
Cofactors
Gene regulation
Gene regulation

Figure 1.8. Overview of TGF-β superfamily signaling

Signaling of the TGF-β superfamily requires ligand-binding to specific receptors, which leads to the activation of specific Smads. There are two main groups of TGF-β ligands: 1) TGF-βs, activins and Nodal signal via type I receptors, ALK4, ALK5, and ALK7 to activate Smad2/3, and 2) BMPs, GDFs and AMH that interact with ALK1, ALK2, ALK3 and ALK6, which phosphorylate Smad1/5/8. These activated Smads complex with Smad4 and enter the nucleus to regulate gene transcription. ACTR, activin receptors; ALK, activin receptor-like kinase; AMH, Anti-Müllerian hormone; BMPs, bone morphogenetic proteins; GDFs, growth and differentiation factors; TGFβs, transforming growth factor-β. Reproduced with permission from Schmierer and Hill (2007).

The regulation of TGF-β signaling is controlled at several levels of the transduction pathway. The availability and the activity of TGF-β ligands offer the first form of TGF-β signaling control. Another regulation mechanism is present in the form of inhibitors that block the activity of the ligands or their receptors. For example, Follistatin inhibits the release of FSH as it inhibits the activity of activin via blocking the binding sites for activin type I and type II receptors (Nakamura et al., 1990). In contrast, Noggin is an inhibitory protein that binds BMPs; thus, the ligands are prevented from interacting with their receptors (Zimmerman et al., 1996). Some TGF-β members, such as inhibins, have the capacity to act on other members as antagonists so that ligand availability is negatively regulated (Namwanje and Brown, 2016).

The signaling transduction of the TGF-β family is strongly impacted by the structure of the active receptor complex as well as the co-receptor availability. For example, TGF-β type III receptor, Betaglycan, is essential for the binding of TGF-β2 to its type II receptor (Rotzer et al., 2001). Betaglycan also acts as a co-receptor of inhibin to reduce the amounts of inhibin that are available to interrupt activin signaling (Lewis et al., 2000). Furthermore, easy access to R-Smads regulates signaling as cofactors such as Smad Anchor for Receptor Activation (SARA) are needed to enable the interaction between R-Smads and their receptors (Tsukazaki et al., 1998). Conversely, I-Smads inhibit interactions with activin type I receptors and R-Smads (Goto et al., 2007).

IV.2 Role of TGF-β Superfamily in Regulating Ovarian Function

The regulation of follicle development and oocyte maturation by gonadotropins is well established in teleosts (Clelland and Peng, 2009). The roles of FSH and LH in follicle growth and oocyte maturation, respectively, have been shown to be regulated by members of the TGF-β superfamily (Kohli et al., 2003; Ge, 2005; Clelland et al., 2007). For example, the secretion of gonadotropin from the pituitaries of teleosts has been shown to be influenced by inhibin and activin (Garg et al., 1999). The expression of FSH in goldfish is stimulated by activinA and activinB. Conversely, activinA (also known as inhibinA) and activinB suppressed the expression of LH (Yam et al., 1999), whereas inhibin stimulated the secretion of LH from the pituitary of goldfish (Ge, 2000). Activin was also detected in the zebrafish brain (Garg et al., 1999). In the zebrafish pituitary, the expression of other TGF-β superfamily members including TGF-β1 (Kohli et al., 2003) and BMP15 (Clelland et al., 2006) was observed. TGF-β1 up-regulated the expression of *fshr*, which activates the production of E2 leading to the production of vitellogenin (Kohli et al., 2005).

The involvement of members of the TGF-β superfamily directly in ovarian tissue to control the regulatory processes that govern vitellogenesis and oocyte maturation in teleosts has been reported in multiple studies. For instance, it was implied that Bmp15 was involved in preventing precocious oocyte maturation in zebrafish (Clelland et al., 2007). Oocyte-specific Gdf9

has been shown to regulate early follicle development in mammals (Shimasaki et al., 2004). In zebrafish, the expression levels of *gdf9* were highest in stage 1 follicles and decreased in later stages. It was also found to be predominantly expressed in the zebrafish oocytes (Liu and Ge, 2007), implying a role for Gdf9 in zebrafish follicle development. In granulosa cells, *amh* is more highly expressed in late-stage I and stage II follicles (Rodríguez-Marí et al., 2005) whereas the expression of inhibin βA (*inhba*) increased in vitellogenic follicles (Wang and Ge, 2004). Similarly, the expression levels of TGF-β1 were detected in all follicle developmental stages; however, they declined in mature follicles (Kohli et al., 2003). The gonadal somatic cell-derived factor (gsdf), which is a granulosa specific paralog of Gdf9 and Bmp15 (Yan et al., 2017), also showed a preferential increase in expression in previtellogenic zebrafish follicles relative to later stages (Gautier et al., 2011). Recently, the TGF-β receptor signaling pathway, transmembrane receptor protein serine/threonine kinase signaling pathway, BMP signaling pathway were among the regulated pathways in zebrafish pre-vitellogenic follicles (Zhu et al., 2018).

Several members of the TGF-β superfamily also regulate oocyte maturation with either stimulatory or inhibitory effect on oocyte maturation. The role of activin in inducing oocyte maturation through controlling LH in zebrafish is widely accepted (Pang and Ge, 1999; Wu et al., 2000). The expression of activin and its associated signaling components in the zebrafish ovary is upregulated by hCG (Wu et al., 2000; Pang and Ge, 2002; Wang and

Ge, 2003; DiMuccio et al., 2005). On the other hand, the higher expression of TGF-β1 and Bmp15 in previtellogenic follicles suggested that the two TGF-β family members play a role in the growing follicles, possibly prohibiting the onset of premature oocyte maturation. Both TGF-β1 and Bmp15 were found to suppress LH-induced oocyte maturation (Kohli et al., 2003; Clelland et al., 2006). These results implied that TGF-β1 and Bmp15 are important to prevent the precocious maturation of zebrafish oocytes. Recently, Gsdf was shown to play an important role in zebrafish oocyte maturation. Mutant female *gsdf* zebrafish were shown to contain non-vitellogenic follicles, indicating a critical role for Gsdf for successful oocyte maturation to take place (Yan et al., 2017).

IV.3 Nodal

Nodal, a member of the TGF-β superfamily, was first identified in a 7.5 post-coitum mouse by transgenic screening as a recessive lethal mutation (termed 413.d) caused severe gastrulation defects (Conlon et al., 1991; Iannaccone et al., 1992). Mutating this then novel TGF-β-like gene produced embryonic defects and eventual lethality that mirrored 413.d mice (Zhou et al., 1993; Conlon et al., 1994). These discoveries highlighted the critical role that Nodal played during embryogenesis. Nodal has since been found to be involved in regulating processes in embryogenesis such as mesendoderm formation and left-right asymmetry patterning in vertebrates (Dougan et al., 2003; Schier, 2003).

The number of Nodal homologs reported varies in different organisms. For example, a single Nodal ligand was identified in human, mouse and chick (Schier, 2003; Fan and Dougan, 2007; Shen, 2007) whereas, in zebrafish and medaka, 3 *nodal-related* (*ndr1*, *ndr2* and *ndr3*) genes were identified (Fan and Dougan, 2007; Shen, 2007) and six *Xenopus nodal-related* (*xnr1*, *2,3, 4,5* and *6*) genes (Tadjuidje et al., 2016). Like their mammalian counterparts, zebrafish *ndr* orthologs are vital for proper embryogenesis to take place (Dougan et al., 2003; Schier, 2003; Shen, 2007).

Nodal is translated as a precursor proprotein that gets activated after post-translation modification by proteolytic cleavage. The mature form is then secreted via exocytosis (Beck et al., 2002; Blanchet et al., 2008). Like other TGF-β superfamily members, secreted mature Nodal ligand interacts with extracellular

membrane-bound type I and type II serine/threonine receptor complexes (Reissmann et al., 2001). The type I receptor complexes that have been reported to interact with Nodal are ALK4 and ALK7 (Reissmann et al., 2001), whereas type II receptors, ActRIIA and ActRIIB. Both ALK7 and ALK4 contain similar serine/threonine kinase domains; however, they differ in their extracellular domains as ALK7 contains a distinct extracellular domain that is unlike other ALKs. Thus, ALK7 was thought to act in a similar manner as TGF-β and activin receptors while being specific to other ligands. This view was demonstrated when ALK7 did not bind TGF-β, activin or BMP7 alone or with type II receptors (Reissmann et al., 2001). On the other hand, the binding of activin ligands with ALK7 was reported. Activin B and activin AB were reported to act through ALK7 in a pancreatic β cell line (Tsuchida et al., 1996) while activin B was shown to bind both ALK4 and ALK7 in gonadotrope cells (Bernard et al., 2006).

Furthermore, Nodal interacts with the co-receptor Cripto, which facilitates Nodal binding to ALK4 (Blanchet et al., 2008). Nodal binding to its receptors activates the internal signals, Smad 2 and Smad 3, which form a complex with Smad 4 and translocate to the nucleus to influence gene expression by interacting with transcription factors (Ross and Hill, 2008). Nodal signaling has been reported to target several downstream genes, which can be specific to species, tissues or functions. Notably, Nodal can target its own expression, creating a positive feedback loop. Interestingly, Nodal signaling also induces the production of its inhibitor, Lefty, which exerts its inhibitory role on Nodal through

binding Cripto or by binding Nodal itself (Meno et al., 1999; Chen and Shen, 2004), and thus an effective negative feedback response is created.

Although Nodal is mainly implicated in the regulation of embryonic processes, Nodal signaling has been implicated in the regulation of reproduction. For instance, Nodal was shown to signal through ALK7 to regulate follicular atresia of non-dominant follicles before ovulation and to promote apoptosis in ovarian granulosa cells (Wang et al., 2006). Nodal was also demonstrated to regulate proliferation in trophoblast cells (Nadeem et al., 2011).

V CRISPR/CAS9

The RNA-guided clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR-associated endonuclease 9 (Cas9) as a system for genome editing has revolutionized the process of generating model organisms. This technology has provided an efficient way to target genomic loci (Liu et al., 2019). In 2012, the Cas9-endonuclease from Streptococcus pyogenes or Streptococcus thermophilus gained wide recognition when its DNA cleaving ability in vitro was demonstrated in combination with CRISPR RNA (crRNA) (Doudna and Charpentier, 2014).

There are two components in the CRISPR/Cas9 system: a single guide RNA (sgRNA) that targets a specific sequence in the genome and a Cas9 protein with RNA-guided endonuclease activity (Zhang, 2015) (Figure 1.9). The sgRNA, which is a short synthetic RNA, is composed of 1) a user-defined ~20 nucleotide spacer that identifies the genomic target to be modified through base pairing; and 2) a scaffold sequence necessary for Cas9 binding. Hence, changing the target sequence present in the sgRNA warrants changing the genomic target that matches the new sequence. The sgRNA directs the Cas9-endonuclease to the genomic target site to induce a double-stranded break (DSB) (Jinek et al., 2012).

In addition to the guiding trait of the sgRNA, the Cas9 protein binds the DNA region sequence “NGG,” known as the protospacer adjacent motif (PAM) sequence. This PAM-Cas9 interaction leads to a sgRNA-target sequence interaction. If there is adequate matching between the target sequence and the sgRNA, the Cas9-endonuclease cleaves both sides of the DNA strand 3 nt upstream of the PAM sequence. This DSB then prompts the activation of the DNA repair mechanisms: non-homologous end-joining (NHEJ) or homology-directed repair (HDR) pathways (Figure 1.9).

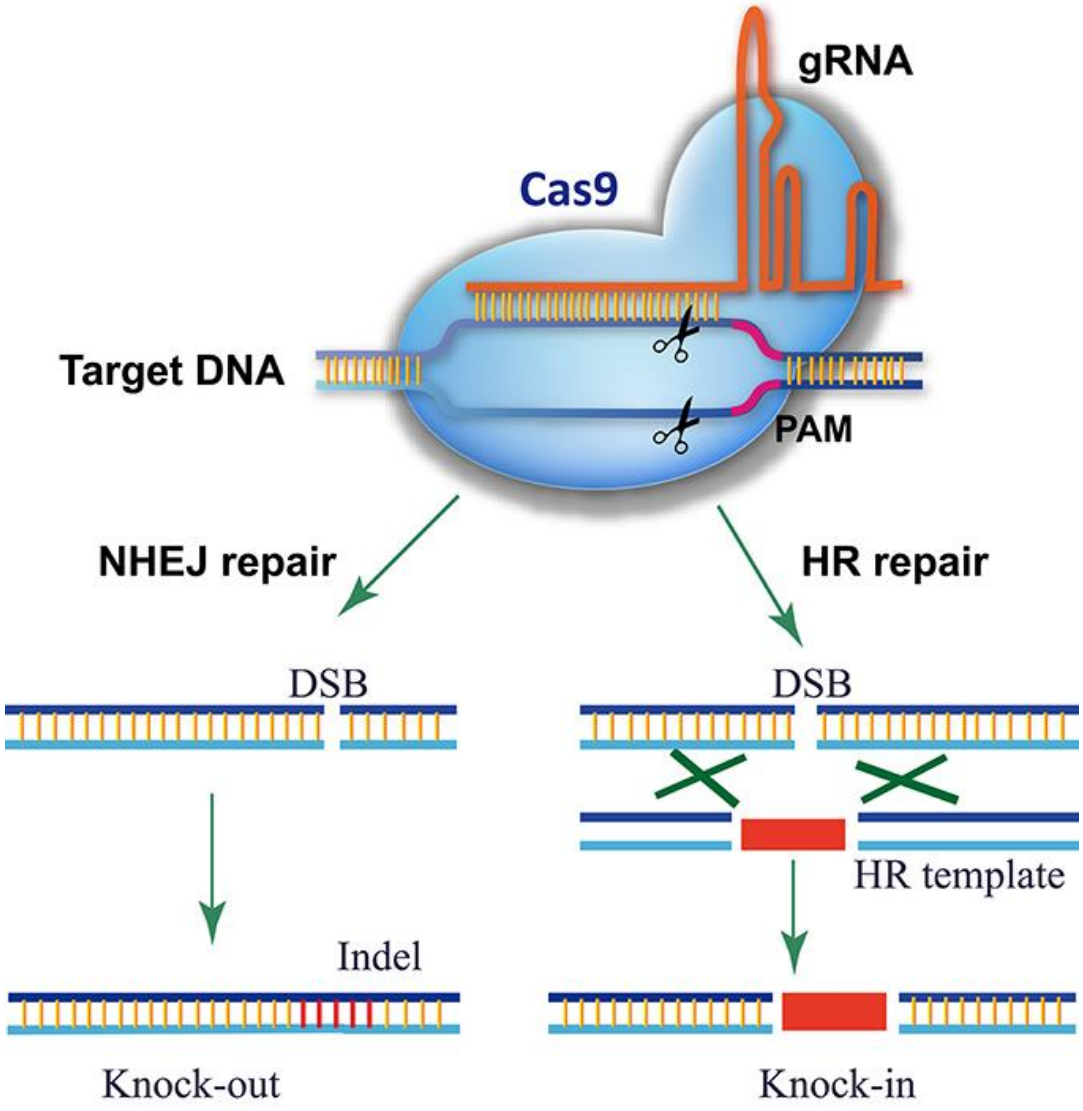

gRNA
Cas9
Target DNA
PAM
NHEJ repair
HR repair
DSB
DSB
HR template
Indel
Knock-out
Knock-in

Figure 1.9. Schematic of CRISPR/Cas9 genome-editing

Cas9-endonuclease is guided by an RNA molecule, single guide RNA (sgRNA) to cleave double-stranded genomic DNA at a specific site. The Cas9 protein introduces a double-stranded break (DSB) 3 nucleotides upstream of the photospacer adjacent motif (PAM) sequence. There are 2 main DNA repair pathways after a DSB takes place: Non-homologous end joining (NHEJ), which leads to the introduction of random insertions or deletions at the cut site and possible gene knockout, or homology-directed repair (HDR) pathways if a DNA template is provided leading to gene knock-in. Reproduced with permission from Ding et al., (2016)

Being error-prone, the NHEJ repair pathway generates fairly random insertions or deletions (indels) or base substitutions at the repair sites (Menon and Povirk, 2016; Pawelczak et al., 2017; Pannunzio et al., 2018). The random nature of these indels includes being heterogeneous in size and in DNA context. Therefore, Cas9-induced indels can inactivate a gene when a frame-shift indel is produced. However, when generating knockouts using this method, a mix of frame-shift and in-frame mutants are created. As a result, further screening is required to identify knockout clones/animals. In addition to generating germline mutations, indels and base substitutions lead to mosaic somatic mutations, which can cause extensive biallelic mutations and phenotypes in injected animals (Jao et al., 2013).

Prior to CRISPR/Cas9, other genome editing methods included zinc finger nucleases (ZFNs) or transcription-activator-like effector nucleases (TALENs). Both ZFNs and TALENS required creating a new nuclease pair for every genomic target (Zu et al., 2013; Bradford et al., 2017), which can be time-consuming and costly. Thus, the flexibility of the simple programmable CRISPR/Cas9 technology aided in popularizing it as an effective genome-editing method. Since its development, the efficacy of the CRISPR/Cas9 technology has been demonstrated in several model systems ranging from cultured cells to whole organisms (Doudna and Charpentier, 2014; Hsu et al., 2014; Zhang, 2015).

VI RATIONALE, HYPOTHESES, AND OBJECTIVES

Zebrafish as a research model has become a fast-growing system for basic and translational research. This stems from the conservation of many of its genes, with 70% gene homology to human genes, as well as the ease of replicating mutant phenotypes to support candidate gene predictions in humans (Bradford et al., 2017). Due to this high conservation of zebrafish with humans as well as the general conservation of the reproductive endocrine and paracrine signaling control in vertebrates, zebrafish will allow us to better understand human reproductive health. In addition, enhancing our knowledge about the regulation of fish reproduction can lead to desirable outcomes in aquaculture.

The role of miRNAs in many biological processes has been extensively studied since their discovery in 1993. miRNAs have been linked to regulating virtually all physiological and pathological processes including mammalian reproduction (Bushati and Cohen, 2007; Toms et al., 2017; Hayder et al., 2018), gonadotropin production and ovarian functions (Carletti et al., 2010; Xu et al., 2011; Yan et al., 2012; Yin et al., 2012; Liu et al., 2014; Troppmann et al., 2014; Toms et al., 2015). Recently, miRNAs were shown to be important regulators of fish reproduction (Gay et al., 2018). Our lab has previously detected miR-17a and miR-430b in zebrafish follicular cells and found that their expression levels are regulated by hCG (Abramov et al., 2013). Although these findings suggest that miRNAs may play a role in oocyte maturation, this role in regulating oocyte maturation is still mostly unclear. Therefore, we hypothesized that miRNAs are

expressed in the zebrafish ovary and may have regulated expression between stage IIIa and stage IIIb follicular cells.

Nodal, which is well known for its critical role during embryogenesis (Zinski et al., 2018), has been emerging as an important regulator of reproduction (Park and Dufort, 2013). Specifically, Nodal was found to act on rat granulosa cells (Wang et al., 2006). Much of what has been learnt about the role of Nodal in embryogenesis was derived from research done on the zebrafish Nodal homologs, ndr1, and ndr2 (Zinski et al., 2018). In healthy human follicles, the expression of Nodal and ALK7 were found in ovarian follicular cells (Wang et al., 2006). However, studying Nodal in adult zebrafish ovaries poses a challenge because of the early developmental lethality associated with knocking it out.

Based on these findings, we hypothesized that miRNAs are actively present at the time of the acquisition of maturation competency in stage IIIb follicles and that Nodal is an important regulator in the zebrafish ovarian follicles. The objectives of my Ph.D. studies were to 1) characterize miRNA expression profiles in IIIa and IIIb follicular cells; 2) investigate the roles of ndr1 and ndr2 in zebrafish follicular cells; 3) develop an in vivo model to study Nodal and miRNAs in the adult zebrafish ovary.

CHAPTER 2

IDENTIFICATION OF NOVEL MICRORNAS AND CHARACTERIZATION OF MICRORNA EXPRESSION PROFILES IN ZEBRAFISH OVARIAN FOLLICULAR CELLS

Identification of novel microRNAs and characterization of microRNA expression profiles in zebrafish ovarian follicular cells

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Author Contributions

YZ and XQ designed and performed the experiments. YZ analyzed the data and drafted the manuscript. CP supervised the study and was involved in experimental design, data analyses, and manuscript writing. All authors approved the submission of the manuscript.

ABSTRACT

MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression primarily at the post-transcriptional levels and thereby play important roles in regulating many physiological and developmental processes. Oocyte maturation in fish is induced by hormones produced from the hypothalamus, pituitary, and ovary. Gonadotropin-releasing hormone (GnRH) stimulates the secretion of luteinizing hormone (LH), which in turn, induces the secretion of maturation inducing hormone (MIH) from the ovary. It is documented that small early vitellogenic (or stage IIIa) follicles are unable to undergo oocyte maturation whereas oocytes in mid- to late vitellogenic (stage IIIb) follicles can be induced by LH and MIH to become mature. To determine whether miRNAs may be involved in the growth and acquisition of maturational competency of ovarian follicles, we determined the miRNA expression profiles in follicular cells collected from stage IIIa and IIIb follicles using next-generation sequencing. It was found that miRNAs are abundantly expressed in the follicular cells from both stages IIIa and IIIb follicles. Furthermore, bioinformatics analysis revealed the presence of 214 known, 31 conserved novel and 44 novel miRNAs in zebrafish vitellogenic ovarian follicular cells. Most mature miRNAs in follicular cells were found to be in the length of 22 nucleotides. Differential expression analysis revealed that 11 miRNAs were significantly up-regulated, and 13 miRNAs were significantly down-regulated in the stage IIIb follicular cells as compared with stage IIIa follicular cells. The expression of four of the significantly regulated miRNAs, dre-miR-22a-3p, dre-miR-16a, dre-miR-181a-3p, and dre-miR-29a, was validated by real-time PCR. Finally,

gene enrichment and pathway analyses of the predicted targets of the significantly regulated miRNAs supported the involvement of several key signaling pathways in regulating ovarian function, including oocyte maturation. Taken together, this study identifies novel zebrafish miRNAs and characterizes miRNA expression profiles in somatic cells within the zebrafish ovarian follicles. The differential expression of miRNAs between stage IIIa and IIIb follicular cells suggests that these miRNAs are important regulators of zebrafish ovarian follicle development and/or oocyte maturation.

INTRODUCTION

Follicle development and oocyte maturation in vertebrates are complex events that require the coordination of hormones originated from the hypothalamus-pituitary-gonadal axis. The hypothalamus produces gonadotropin-releasing hormone (GnRH), which stimulates the secretion of gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), from the pituitary gland. In fish, FSH plays a major role in promoting follicle development by inducing estradiol production from ovarian follicular cells while LH acts on follicular cells to induce the production of 17α, 20β-dihydroxy progesterone, known as the maturation-inducing hormone (MIH) (Ge, 2005; Clelland and Peng, 2009). MIH then binds to membrane progestin receptors (mPRs), particularly mPRα, expressed on the surface of oocytes (Hanna and Zhu, 2011; Thomas, 2017). This, in turn, activates the maturation promoting factor (MPF), leading to the release of the oocyte from its meiotic arrest and the maturation of the oocyte (Nagahama et al., 1995; Patino et al., 2001). In addition, many signaling molecules produced within the follicular cells and/or oocytes also act locally to regulate follicle development and oocyte maturation (Ge, 2005; Clelland and Peng, 2009).

Prior to engaging in the maturation process, zebrafish follicles develop through three stages. In stage I or the primary growth phase, oocytes begin to grow, and follicles start to form. Stage II is known as the cortical alveolus or previtellogenic stage in which cortical alveoli accumulate within the oocytes. Stage III is characterized by vitellogenesis (Selman et al., 1993). During this stage, follicles not only increase in size but also develop maturational competency. It has

been reported that small early vitellogenic follicles are unable to undergo maturation when treated with human chorionic gonadotropin (hCG, used as an analog of LH), or MIH. In contrast, larger follicles in mid to late vitellogenesis can be induced by these hormones and enter the maturation phase (Selman et al., 1993; Wu et al., 2000). Therefore, the small and mid-late vitellogenic follicles are described as stages III-1 (or IIIa) and III-2 (or IIIb), respectively (Wu et al., 2000; Gioacchini et al., 2012). Signaling molecules produced within the follicles, such as members of the transforming growth factor-β (TGF-β) superfamily, have been shown to regulate maturational competency (Pang and Ge, 2002; Clelland et al., 2007; Peng et al., 2009).

MicroRNAs (miRNAs) constitute an abundant class of the small, single-stranded, non-coding RNA of about 18~26 nucleotides (nt) in length (Ambros, 2004; Bartel, 2004; O'Brien et al., 2018). In general, miRNAs are first transcribed from intronic or intergenic DNA into primary miRNAs, processed into precursor miRNAs (pre-miRNAs), and then exported into the cytoplasm. The pre-miRNAs have a hairpin structure and further processed into mature miRNA duplexes, which unwind into single-stranded mature miRNAs. In most cases, mature miRNAs interact with the 3' untranslated region of target mRNAs to reduce their stability and to inhibit translation (Zhang et al., 2007; Winter et al., 2009; O'Brien et al., 2018). By regulating gene expression, miRNAs are involved in a plethora of developmental and physiological events, including reproduction (Bushati and Cohen, 2007; Toms et al., 2017; Hayder et al., 2018). miRNAs have been detected in the ovary of many species, including fish (Ma et al., 2012; Juanchich et

al., 2013; Desvignes et al., 2014; Wong et al., 2017). It has been reported that miRNAs regulate ovarian functions in mammals, such as granulosa cell proliferation (Yan et al., 2012) and apoptosis (Carletti et al., 2010; Liu et al., 2014), estradiol production (Xu et al., 2011; Yin et al., 2012), and the expression of progesterone (Toms et al., 2015) and LH/CG receptors (Troppmann et al., 2014). A recent study demonstrates that miRNAs are also important regulators of fish reproduction (Gay et al., 2018). However, the functions of miRNAs during fish follicle development and oocyte maturation are still largely unknown.

We have previously detected miR-17a and miR-430b in zebrafish follicular cells and found that their expression levels are regulated by hCG (Abramov et al., 2013), suggesting that miRNAs may play a role in oocyte maturation. To further investigate whether miRNAs are involved in follicle development and oocyte maturation, especially in the acquisition of maturational competency, we used next-generation RNA sequencing (RNA-seq) to compare the miRNA expression profiles in follicular cells between stage IIIa and IIIb follicles. We identified the significantly regulated miRNAs, predicted novel miRNAs and validated the expression of four of the significantly regulated miRNAs. Finally, through gene enrichment and pathway analyses of predicted target genes of the differentially expressed miRNAs, we identified key pathways that may be important during follicle development and oocyte maturation.

MATERIALS AND METHODS

Animals

Zebrafish were purchased from a local supplier and maintained in 10 L tanks of an AHAB System (Aquatic Habitats, FL) at 28 °C, under a 14-h light, 10-h dark cycle. The fish were fed twice a day with commercial tropical fish food. The study protocol was approved by the York University Animal Care Committee. All experiments were performed according to the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care.

Isolation of ovarian follicular cells

Female zebrafish were anesthetized with 3-aminobenzoic acid ethyl ester (Sigma–Aldrich Canada Inc., Oakville, ON) and decapitated. The ovaries were extracted and maintained in a 100-mm culture dish containing 60% Leibovitz L-15 medium without phenol red (Life Technologies, ThemoFisher Scientific, Burlington, ON). Stage IIIa (0.35-0.51mm) and IIIb (0.52-0.65mm) follicles were manually separated and collected according to their size. Follicular cell layers were collected mechanically using fine forceps.

RNA extraction and small RNA sequencing

Three samples were prepared from each of stage IIIa and IIIb follicular cells for a total of six samples. Each sample contained cells isolated from 120-150 follicles pooled from 3-4 fish. miRNA-enriched total RNA was extracted using miRNeasy mini kit (Qiagen, Germantown, MD) according to the manufacturer's

instructions. The sequencing of small RNAs was performed by the Génome Québec Innovation Centre at McGill University using the Illumina HiSeq 2500 Ultra-High-Throughput Sequencing platform.

Raw sequencing reads were processed using the ACGT101-miR program (LC Sciences, Houston, Texas, USA). Adaptor dimers, junk, low complexity, common RNA families and repeats were removed, and only unique sequences of 18-26 nt in length were retained. Unique reads were then mapped to zebrafish precursors obtained from miRBase 22.0. One mismatch within the sequence and length variation at both 3' and 5' ends was allowed when aligning reads to zebrafish precursors using BLAST search. Unique reads that were mapped to known precursors were considered known miRNAs. Unannotated reads that mapped to the opposite arm of known pre-miRNAs were considered known miRNAs but were designated either a p3 or p5 depending on whether they were mapped to the 3' or 5'end, respectively. The remaining sequences were mapped to other selected species precursors in miRBase 22.0 by BLAST search and were designated conserved novel miRNAs. Unmapped sequences were BLASTed against the zebrafish genome version CRCz11, and hairpin RNA structures containing sequences were predicted using RNAfold (Lorenz et al., 2011). These predicted miRNAs were considered novel. The following criteria were used to predict the secondary structure of pre-miRNAs: (1) the number of nt in one bulge in stem was ≤12; (2) the number of base pairs in the stem region of the predicted hairpin was ≥16; (3) cutoff of free energy (kCal/mol) was ≤-15; (4) the length of hairpin, up and down stems and terminal loop was ≥50; (5) the length of

hairpin loop was ≤20; (6) the number of nt in one bulge in the mature region was ≤8; (7) the number of biased errors in one bulge in the mature region was ≤4; (8) the number of biased bulges in mature region was ≤2; (9) the number of errors in mature region was ≤7; (10) the number of base pairs in the mature region of the predicted hairpin was ≥12; and (11) the percent of mature region in the stem-loop was ≥80. The results were further refined to only retain miRNAs that met the following criteria: (1) each miRNA should have at least one predicted pre-miRNA and such pre-miRNA should be able to form a hairpin structure, whose genomic coordinates should not overlap with known pre-miRNAs included in this analysis; (2) miRNAs with exact sequence and count matches but had different predicted pre-miRNAs were counted only once; and (3) the retained miRNAs should have at least 100 counts in all sample replicates of either IIIa or IIIb sample sets.

Differential expression analysis, target prediction and enrichment analyses

Sequencing counts were first normalized by the library size parameter of the corresponding sample. The differential expression of miRNAs based on the normalized sequencing counts was analyzed using Student's t-test ($p \leq 0.05$) and visualized using Heatmapper (Babicki et al., 2016). Potential targets of the significantly up- or down-regulated miRNAs were predicted by overlapping prediction data of two computational target prediction algorithms: TargetScan (Ulitsky et al., 2012) and miRanda 3.3a (Gabow et al., 2008). Gene ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling enrichment analyses were performed using the ClueGO tool kit (Bindea et al.,

2009). The GO terms and KEGG pathways that have a p value ≤0.05 were considered significant.

Real-time PCR

miRNA was extracted as described above. The extracted miRNA was polyadenylated and reversely transcribed into cDNA using the NCode miRNA First-Strand Synthesis Kit (Life Technologies) according to the manufacturer's instructions. Real-time PCR (qPCR) was performed using the NCode universal reverse primer along with a forward miRNA-specific primer (Table 2.1) and EvaGreen qPCR master mix, following the manufacturer's suggested protocol. Relative miRNA levels were determined using the $\Delta\Delta C_t$ method after normalization to the endogenous U6 levels.

Table 2.1. List of primers used in real-time PCR

miRNA	**Primer sequence (5' to 3')**
miR-29a	TAGCACCATTTGAAATCGGT
miR-22a-3p	AAGCTGCCAGCTGAAGAACTGT
miR-16a	TAGCAGCACGTAAATATTGGTG
miR-181-3p	ACCATCGACCGTTGATTGTACC
U6 Forward	CTTGCTTCGGCAGCACATATAC
U6 Reverse	AACGCTTCACGAATTTGCGTG

Statistical analysis

Student's t-test was used for comparison in miRNA levels between stage IIIa and stage IIIb using GraphPad Prism.

RESULTS

Characterization of miRNAs in zebrafish ovarian follicular cells

Six cDNA libraries were prepared from miRNAs isolated from three pools of stage IIIa and three pools of stage IIIb follicular cells (Figures 2.1A,2.1B). Subsequently, Illumina's TruSeq Massively Parallel Sequencing was used to determine the miRNA expression profiles of these samples. After removing low-quality reads, the numbers of mappable reads in stage IIIa and stage IIIb cells were 31601023 and 40205729, respectively. Mapping of the sequencing reads to the zebrafish miRNAs from miRBase 22.0 revealed the presence of 214 known miRNAs. These included 200 annotated miRNAs (Table S2.1), as well as 9 miRNAs derived from the 3' arm and 5 miRNAs derived from the 5' arm of known pre-miRNAs (Table S2.2). Unmapped reads were then compared to mature miRNA sequences from other species and their genomic locations in the zebrafish genome were determined, resulting in the identification of 31 conserved novel miRNAs (Table S2.3, S2.4.). The remaining unmapped reads were further mapped to the zebrafish genome and 44 novel miRNAs with predicted precursor miRNAs were identified in zebrafish ovarian follicular cells (Tables S2.5).

Compared to the other RNA populations (i.e. mRNAs, rRNAs, snoRNAs, tRNAs, snRNAs, and other miscellaneous RNAs), the number of miRNAs accounted for 66.16% and 70.40% of the sequencing reads in IIIa and IIIb follicular cells, respectively (Figure 2.1C). The majority of the mappable miRNA reads detected fell between 21-26 nt, with most miRNA reads being 22 nt in length

(Figure 2.1D). Furthermore, the top ten most abundant miRNAs detected were miR-202-5p, miR-143, miR-22a-3p, miR-92a-3p, miR-26a-5p, miR-181a-5p, miR-21, miR-30d, miR-27c-3p, and miR-27b-3p. They constituted 72.64% of the number of mapped miRNAs (Figure 2.1E) and were expressed in both stages IIIa and IIIb ovarian follicular cells.

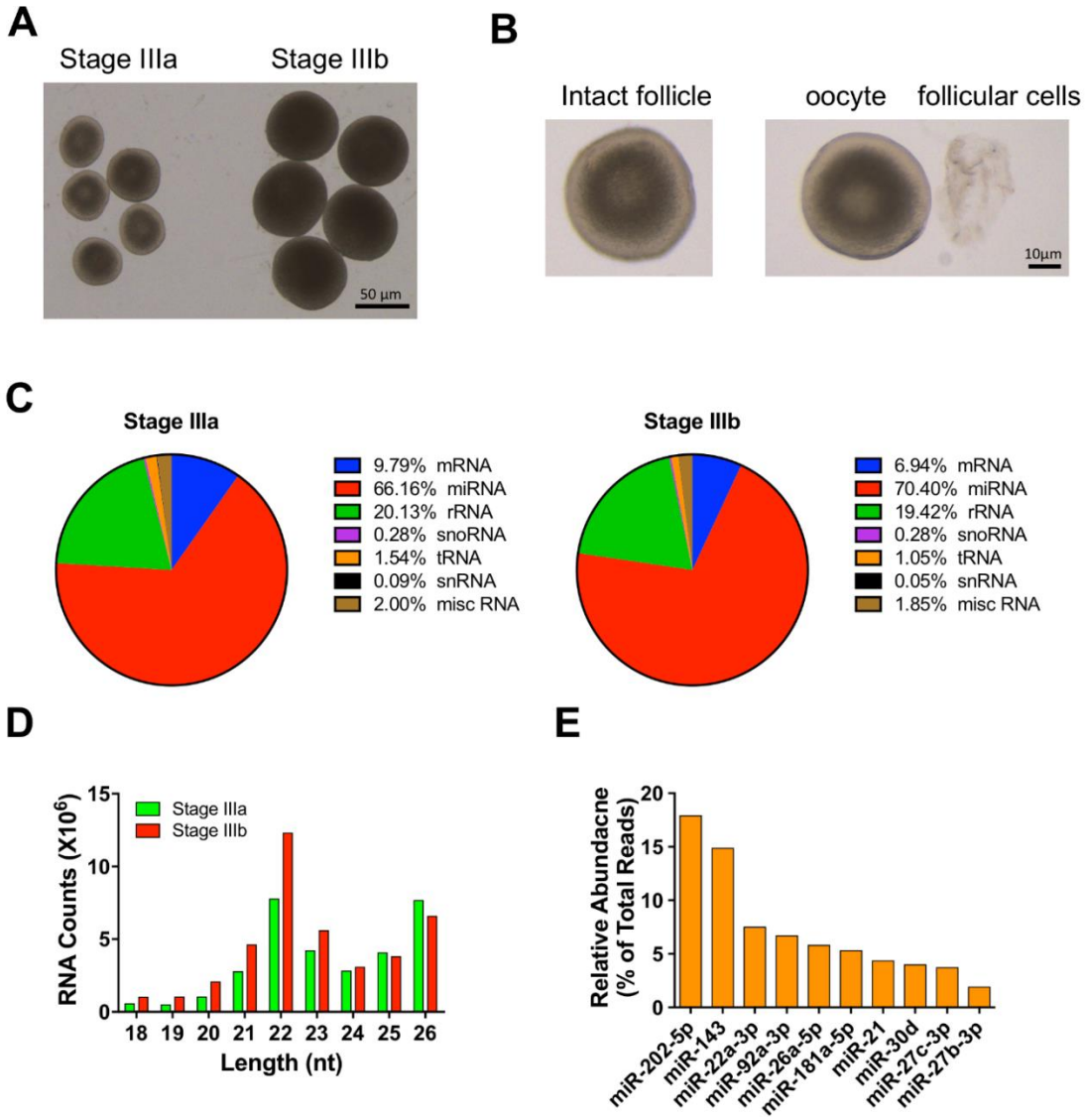


Figure 2.1. Overview of small RNA-seq data in follicular cells isolated from stage III follicles.

A) A picture of stage IIIa and stage IIIb vitellogenic follicles. B) A picture of a stage IIIa follicle before and after follicular cells isolation. C) Distribution of RNA populations in stage IIIa and IIIb follicular cells. D) Read length distribution of small RNA-seq dataset. E) Relative abundance of the 10 most highly expressed miRNAs.

Differential expression of miRNAs in stage IIIa and stage IIIb follicular cells

Out of the 289 miRNAs detected in the follicular cells, 24 were differentially expressed between stages IIIa and IIIb, of which 8 were significantly up-regulated and 8 were significantly down-regulated by more than 2 folds, respectively (Figure 2.2A). If no limit on the fold change was applied, 9 annotated and 2 conserved novel miRNAs were found to be up-regulated, while 12 annotated and 1 novel miRNA were down-regulated, in stage IIIb cells when compared with those in stage IIIa (Figures 2.2B, 2.2C).

Four of the differentially expressed miRNAs identified from the RNA-seq, dre-miR-22a-3p, dre-miR-16a, dre-miR-181a-3p, and dre-miR-29a, were validated using qPCR. New sets of samples were prepared from stage IIIa and IIIb follicles and qPCR was performed. While the trend between the results of sequencing (Figure 2.3A) and qPCR analyses (Figure 2.3B) was similar, the qPCR experiments showed a more significant and/or stronger changes in these miRNA levels between the two groups of samples.

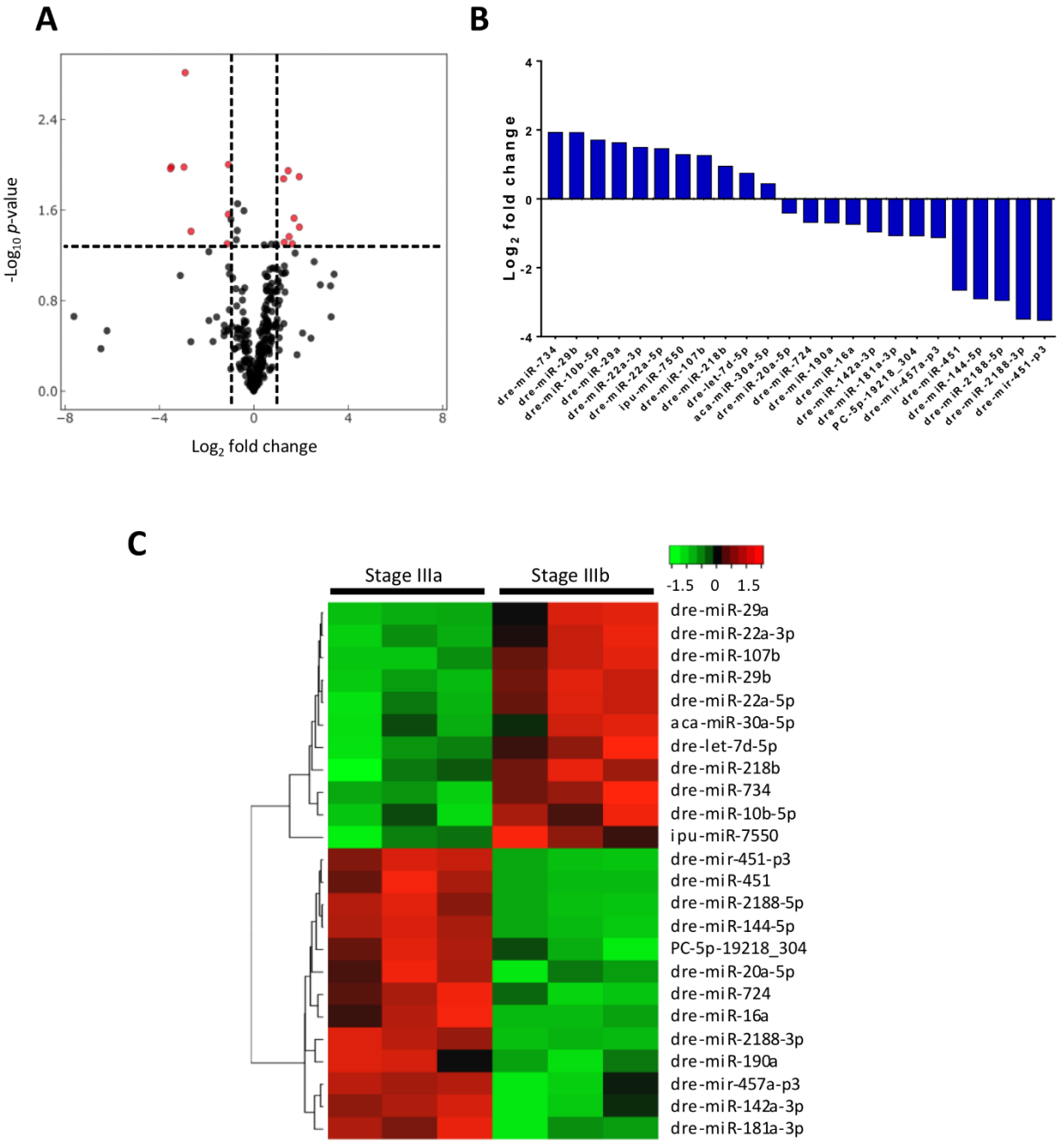

A
B
C
-Log10 p-value
Log2 fold change
Log2 fold change
dre-miR-734
dre-miR-29b
dre-miR-10b-5p
dre-miR-29a
dre-miR-22a-3p
dre-miR-22a-5p
ipu-miR-7550
dre-miR-107b
dre-miR-218b
dre-let-7d-5p
aca-miR-30a-5p
dre-miR-20a-5p
dre-miR-724
dre-miR-190a
dre-miR-16a
dre-miR-142a-3p
dre-miR-181a-3p
PC-5p-19218_304
dre-mir-457a-p3
dre-miR-451
dre-miR-144-5p
dre-miR-2188-5p
dre-miR-2188-3p
dre-mir-451-p3
Stage IIIa
Stage IIIb
-1.5 0 1.5
dre-miR-29a
dre-miR-22a-3p
dre-miR-107b
dre-miR-29b
dre-miR-22a-5p
aca-miR-30a-5p
dre-let-7d-5p
dre-miR-218b
dre-miR-734
dre-miR-10b-5p
ipu-miR-7550
dre-mir-451-p3
dre-miR-451
dre-miR-2188-5p
dre-miR-144-5p
PC-5p-19218_304
dre-miR-20a-5p
dre-miR-724
dre-miR-16a
dre-miR-2188-3p
dre-miR-190a
dre-mir-457a-p3
dre-miR-142a-3p
dre-miR-181a-3p

Figure 2.2. Differential expressions of 24 miRNAs in stage IIIa and IIIb follicular cells

A) Volcano plot of miRNAs expressed in stage IIIb vs stage IIIa follicular cells. Vertical lines indicate fold change < 1/2 and > 2 and the horizontal line represents the threshold of significant change ($p \leq 0.05$). B) Relative mean expression of the miRNAs (log2 fold change) in stage IIIb follicular cells compared to stage IIIa cells (P-value ≤0.05). C) Heatmap of differentially expressed miRNAs between stage IIIa and IIIb follicular cells.

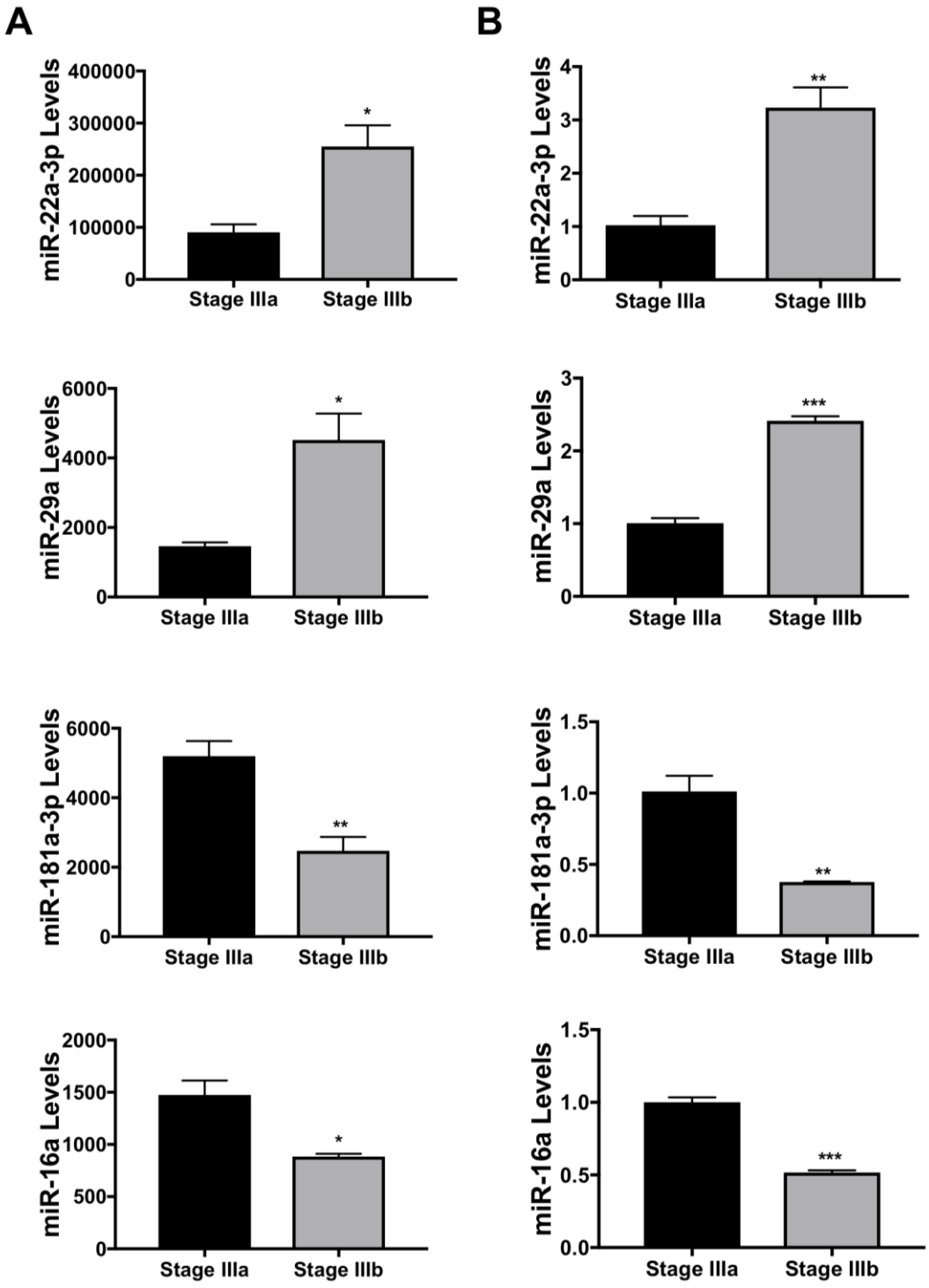

A
B
miR-22a-3p Levels
400000
300000
200000
100000
0
*
Stage IIIa
Stage IIIb
miR-22a-3p Levels
4
3
2
1
0
**
Stage IIIa
Stage IIIb
miR-29a Levels
6000
4000
2000
0
*
Stage IIIa
Stage IIIb
miR-29a Levels
3
2
1
0
***
Stage IIIa
Stage IIIb
miR-181a-3p Levels
6000
4000
2000
0
**
Stage IIIa
Stage IIIb
miR-181a-3p Levels
1.5
1.0
0.5
0.0
**
Stage IIIa
Stage IIIb
miR-16a Levels
2000
1500
1000
500
0
*
Stage IIIa
Stage IIIb
miR-16a Levels
1.5
1.0
0.5
0.0
***
Stage IIIa
Stage IIIb

Figure 2.3. Validation of several miRNAs differentially expressed between stage IIIa and IIIb follicular cells.

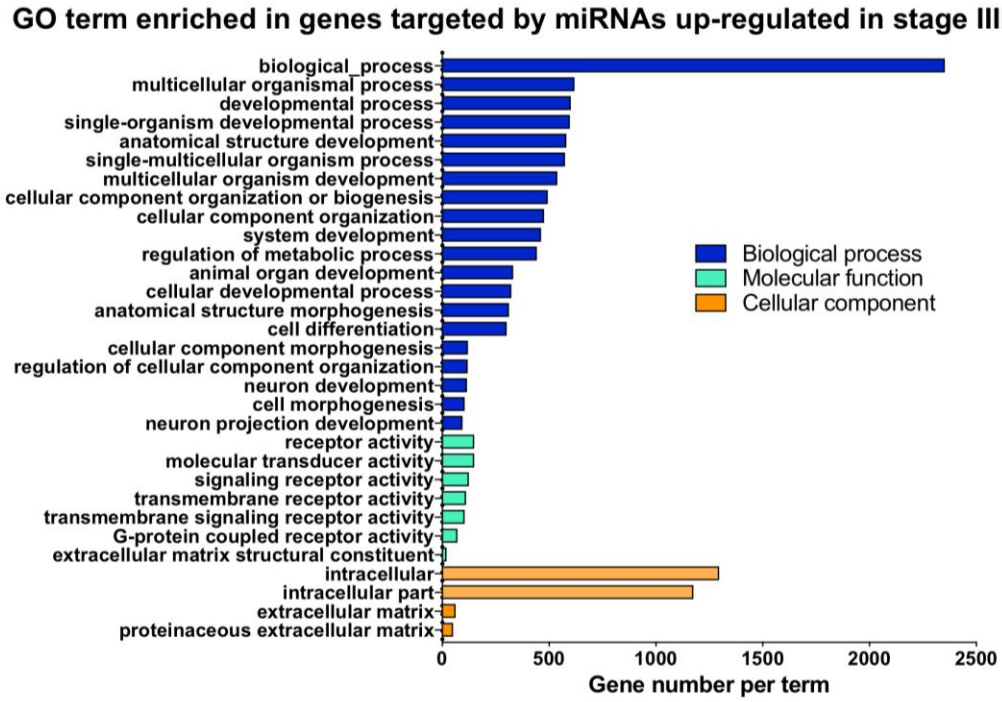
A) miRNA expression levels of dre-miR-22a-3p, dre-miR-29a, dre-miR-181a-3p and dre-miR-16a detected by the small RNA-seq. B) Relative expression levels of the same miRNAs measured by real-time PCR. Data represent mean + SEM (n=3 biological replicates). *P ≤ 0.05; **P < 0.01; ***P < 0.001 as analyzed by Student's t-test.

Prediction of target genes and pathway analyses

To gain some understanding of the biological functions and signaling pathways regulated by the miRNAs that showed significant changes between IIIa and IIIb follicular cells, we performed GO and KEGG enrichment analyses on the predicted targets of these miRNAs. Most of the biological processes regulated by the differentially expressed miRNAs were related to development (Figure 2.4). Interestingly, transmembrane receptor activity, signaling receptor activity, and G-protein coupled receptor activity were also amongst the most significantly enriched GO terms (Figure 2.5). KEGG pathway analysis revealed several key pathways that were enriched in the predicted targets of miRNAs that were differentially expressed between stage IIIa and IIIb cells. Specifically, the MAPK signaling, endocytosis, regulation of actin cytoskeleton, FoxO signaling, insulin signaling, AGE-RAGE signaling, TGF-β signaling, and p53 signaling pathways were significantly enriched in the predicted targets of miRNAs that were both up- and down-regulated in stage IIIb follicular cells. However, cell cycle, herpes simplex infection, salmonella infection, phosphatidylinositol signaling, ECM-receptor interaction, and base excision repair pathways were enriched only in the genes potentially regulated by miRNAs up-regulated in stage IIIb follicular cells (Figure 2.4A). On the other hand, focal adhesion, apoptosis, mTOR signaling, ErbB signaling, progesterone-mediated oocyte maturation, and VEGF signaling pathways were significantly enriched in the predicted targets of miRNAs down-regulated in stage IIIb. Examination of genes associated with these KEGG pathways (Table S2.6, S2.7) revealed that some of the genes were listed under

multiple pathways. For example, several genes associated with the MAPK pathway, were also related to FoxO, AGE-RAGE signaling pathway in diabetic complications, and salmonella infection pathway. For pathways that were enriched in the target genes of both up- and down-regulated miRNAs, there were specific genes that were found to be targeted only by the up- or down-regulated miRNAs. Notably, among the TGF-β pathway, ndr1 was targeted by miRNAs down-regulated in stage IIIb follicular cells while tgfb1a, tgfb1b, and tgfb2 were found only in the gene list targeted by miRNAs that were up-regulated in stage IIIb (Table S2.6). For the MAPK pathway, several fibroblast growth factors (fgf) ligands and receptors, namely fgf13a, fgf3, fgf4, and fgfr2, were only targeted by miRNAs down-regulated in stage IIIb cells (Table S2.7).

A


GO term enriched in genes targeted by miRNAs up-regulated in stage IIIb
biological_process
multicellular organismal process
developmental process
single-organism developmental process
anatomical structure development
single-multicellular organism process
multicellular organism development
cellular component organization or biogenesis
cellular component organization
system development
regulation of metabolic process
animal organ development
cellular developmental process
anatomical structure morphogenesis
cell differentiation
cellular component morphogenesis
regulation of cellular component organization
neuron development
cell morphogenesis
neuron projection development
receptor activity
molecular transducer activity
signaling receptor activity
transmembrane receptor activity
transmembrane signaling receptor activity
G-protein coupled receptor activity
extracellular matrix structural constituent
intracellular
intracellular part
extracellular matrix
proteinaceous extracellular matrix
Biological process
Molecular function
Cellular component
0
500
1000
1500
2000
2500
Gene number per term

B

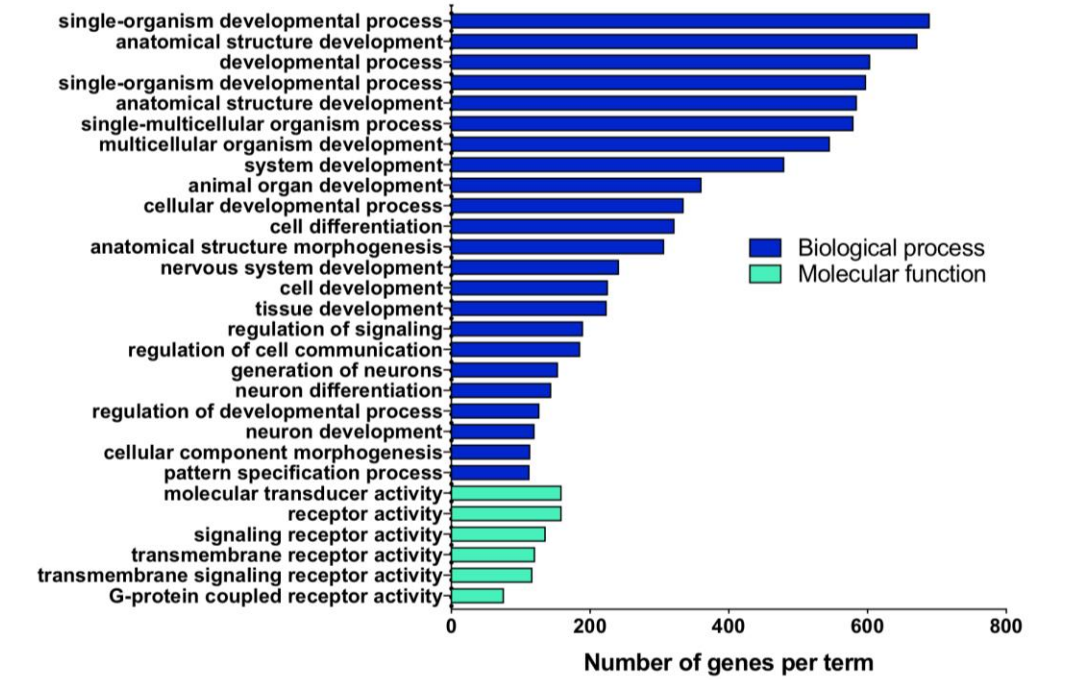
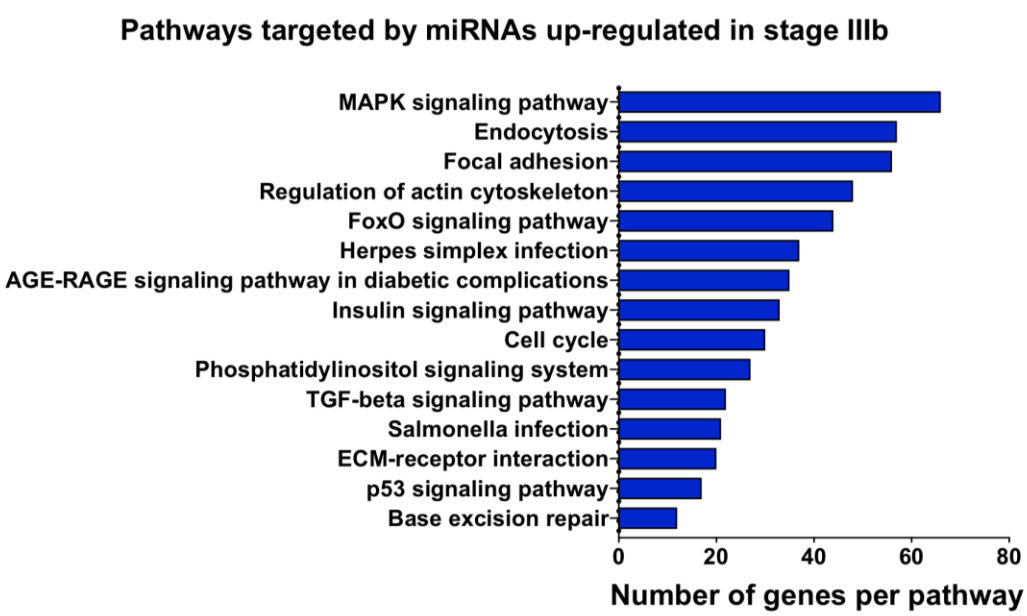

GO term enriched in genes targeted by miRNAs down-regulated in stage IIIb
single-organism developmental process
anatomical structure development
developmental process
single-organism developmental process
anatomical structure development
single-multicellular organism process
multicellular organism development
system development
animal organ development
cellular developmental process
cell differentiation
anatomical structure morphogenesis
nervous system development
cell development
tissue development
regulation of signaling
regulation of cell communication
generation of neurons
neuron differentiation
regulation of developmental process
neuron development
cellular component morphogenesis
pattern specification process
molecular transducer activity
receptor activity
signaling receptor activity
transmembrane receptor activity
transmembrane signaling receptor activity
G-protein coupled receptor activity
Biological process
Molecular function
0
200
400
600
800
Number of genes per term

Figure 2.4. Gene ontology (GO) analysis of the predicted targets of the miRNAs that were differentially expressed between stage IIIa and stage IIIb follicular cells.

GO enrichment analysis was performed using ClueGO on the predicted target genes of both up-regulated (A) and down-regulated (B) miRNAs. X-axis represents the number of target genes while the y-axis shows the terms related to biological processes, molecular function, or cellular components.

A


Pathways targeted by miRNAs up-regulated in stage IIIb
MAPK signaling pathway
Endocytosis
Focal adhesion
Regulation of actin cytoskeleton
FoxO signaling pathway
Herpes simplex infection
AGE-RAGE signaling pathway in diabetic complications
Insulin signaling pathway
Cell cycle
Phosphatidylinositol signaling system
TGF-beta signaling pathway
Salmonella infection
ECM-receptor interaction
p53 signaling pathway
Base excision repair
0
20
40
60
80
Number of genes per pathway

B

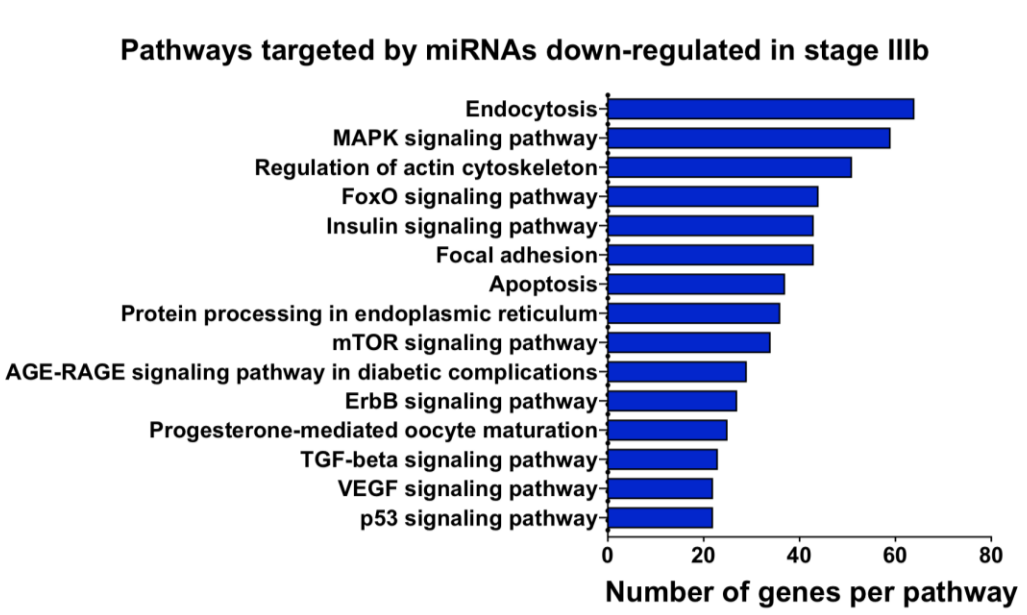

Pathways targeted by miRNAs down-regulated in stage IIIb
Endocytosis
MAPK signaling pathway
Regulation of actin cytoskeleton
FoxO signaling pathway
Insulin signaling pathway
Focal adhesion
Apoptosis
Protein processing in endoplasmic reticulum
mTOR signaling pathway
AGE-RAGE signaling pathway in diabetic complications
ErbB signaling pathway
Progesterone-mediated oocyte maturation
TGF-beta signaling pathway
VEGF signaling pathway
p53 signaling pathway
0
20
40
60
80
Number of genes per pathway

Figure 2.5. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the genes predicted to be targeted by up-regulated (A) and down-regulated (B) miRNAs in stage IIIb follicular cells. KEGG analysis was completed using ClueGO. The x-axis represents the number of target genes and the y-axis denotes the enriched pathway category.

To determine if the miRNAs differentially expressed between stage IIIa and IIIb follicular cells may play a role in oocyte maturation, we further analyzed three key genes known to play critical roles in oocyte maturation, *lhcgr* (encodes LH receptor), *paqr7b* (encodes mPRα), and *pgrmc1*(encodes progesterone receptor membrane component 1). Among them, pgrmc1 mRNA was the predicted target of miR-451 and miR-144-3p, both were down-regulated by more than 80% in stage IIIb cells. Although the difference in miR-144-3p levels between the two stages were not statistically significant (p=0.095), it was included in this analysis due to the magnitude of its down-regulation. On the other hand, lhcgr and paqr7b, were predicted to be targeted by miRNAs that were up- or down-regulated in stage IIIb cells. However, most of the up-regulated miRNAs were present at much lower levels (Table 2.2).

Table 2.2. Differentially expressed miRNAs that are predicted to target key genes in oocyte maturation

miRNA	**Mean count (IIIA)**	**Mean count (IIIB)**	**Target genes**
dre-miR-142a-3p	484	247	*lhcgr*
dre-miR-144-3p	732	84*	*lhcgr, pgrmc1, paqr7b*
dre-miR-16a	1474	883	*paqr7b*
dre-miR-20a-5p	2721	2035	*paqr7b*
dre-miR-451	17468	2925	*lhcgr, pgrmc1*
dre-miR-724	2270	1723	*lhcgr, paqr7b*
dre-miR-107b	53	127	*paqr7b*
dre-miR-218b	43	82	*lhcgr*
dre-miR-29a	1459	4517	*lhcgr*
dre-miR-29b	29	110	*lhcgr*
dre-miR-30a-5p	914	2215	*paqr7b*
dre-miR-734	485	1846	*paqr7b*

*, not statistically significant between IIIa and IIIb (p=0.095).

DISCUSSION

The role of miRNAs in fish reproduction is still largely unknown. Using RNA-seq analyses, we characterized miRNAs expressed in follicular cells isolated from ovarian follicles at the stage of vitellogenesis. This led to the prediction of 31 conserved novel and 44 novel miRNAs, thus enhancing our knowledge of miRNAs in zebrafish. In addition, we identified miRNAs that were differentially expressed between stage IIIa follicular cells, which are maturationally incompetent, and stage IIIb follicular cells, which can respond to hormonal signals that induce oocyte maturation. Analyses of predicted target genes of the differentially expressed miRNAs further support the involvement of several key pathways in regulating follicle growth and oocyte maturation and reveal new pathways that can be investigated in future studies.

The most abundant miRNA expressed in the follicular cells of vitellogenic follicles was miR-202-5p, constituting approximately 18% of the total miRNA counts. This miRNA has been shown to be predominantly expressed in the gonads of various vertebrates, including fish (Jia et al., 2015; Wong et al., 2017; Gay et al., 2018; Qiu et al., 2018). The high expression of this miRNA suggests that it is an important regulator of ovarian development and function in vertebrates. In zebrafish, miR-202-5p levels in stage II follicles (pre-vitellogenesis) were strongly up-regulated when compared to stage I (primary growth) follicles (Wong et al., 2017). Results from this study suggest that this high level of expression is maintained in stage III, during follicle growth and in preparation for oocyte

maturation. Thus, it is highly likely that miR-202-5p plays important roles throughout zebrafish follicle development and oocyte maturation. Indeed, a recent study conducted in medaka provides strong evidence to support the critical role of mir-202 in oogenesis/folliculogenesis. Knockout of *mir-202* resulted in the impairment of early follicle development and strongly reduced the number of eggs produced (Gay et al., 2018). In human, miR-202-5p levels in follicular fluid are positively correlated with the fertilization potential of oocytes (Machtinger et al., 2017), suggesting a role for miR-202-5p in the proper development of oocytes. Moreover, miR-202-5p has been shown to regulate PI3K (Liu et al., 2019) and TGF-β signaling, both of which play important roles in folliculogenesis and oocyte maturation (Kohli et al., 2005; Yao et al., 2014).

Two other most abundant miRNAs identified in our study were miR-143 and miR-22a-3p. These miRNAs have also been reported to be highly expressed in mammalian (Yao et al., 2009) and fish (Bizuayehu et al., 2012; Gu et al., 2014; Presslauer et al., 2017) ovaries. The role of these miRNAs in fish reproduction has not been reported. However, several studies in mammals suggest that they regulate follicle development. For example, miR-143 has been reported to inhibit the formation of mouse primordial follicles by down-regulating the expression of cell cycle genes and suppressing the proliferation of pre-granulosa cells (Zhang et al., 2013). miR-143 also targets *FSHR*, the gene encoding for the FSH receptor (Du et al., 2016), and inhibits granulosa cell proliferation and estradiol production (Zhang et al., 2017). The expression of miR-143 is inhibited by FSH (Zhang et al., 2017) and TGF–β (Du et al., 2016). hsa-miR-22-3p, the homolog of dre-miR-22a-

3p, was found to be significantly down-regulated in the plasma of patients with premature ovarian insufficiency (Dang et al., 2015), suggesting a potential role of this miRNA in maintaining proper follicle development. The function and regulation of miR-143 and miR-22a-3p during zebrafish follicle development will be investigated in the future.

In this study, we identified 24 miRNAs that were up- or down-regulated in stage IIIb follicular cells when compared to follicular cells from stage IIIa follicles. Since follicles at stage IIIa are maturationally incompetent, while stage IIIb are capable of responding to hormonal signals and undergo maturation (Clelland and Peng, 2009), the miRNAs that are differentially expressed between these two stages are likely involved in regulating maturational competency of these follicles. We, therefore, examined 3 genes that play critical roles in LH- and MIH-induced oocyte maturation to determine if they might be regulated by miRNAs differentially expressed in stage IIIa and IIIb cells. Interestingly, we found that two miRNAs predicted to target *pgrmc1*, whose knockout or inhibition impaired MIH-induced oocyte maturation (Aizen et al., 2018; Wu et al., 2018), were strongly down-regulated in stage IIIb cells. It has been reported that oocytes from follicles ranged from 0.55 to 0.65mm, which are similar to the stage IIIb follicles used in our study, had much higher pgrmc1 protein levels than oocytes at earlier vitellogenic stages (Aizen and Thomas, 2015). Since miRNAs can be secreted and exert paracrine/endocrine regulatory effects on other cells (O'Brien et al., 2018), it is possible that the decrease in these two miRNAs contributes to the higher pgrmc1 protein levels observed in oocytes at this stage. Several other differentially

expressed miRNAs are predicted to target genes encoding LH receptor and mPRα, which mediate the maturation-inducing effects of LH and MIH, respectively (Kwok et al., 2005; Chu et al., 2015; Aizen et al., 2018). While lhcgr and paqr7b are predicted targets of both up- and down-regulated miRNAs, it was noted that three of the up-regulated miRNAs predicted to target lhcgr or paqr7b had very low levels and therefore may not play a major role in regulating the expression of these genes. Future studies will investigate whether and how these miRNAs target the genes involved in LH and MIH signaling to regulate maturational competency in zebrafish.

Pathway analyses revealed that several key pathways known to be important in regulating oocyte maturation, are among the predicted targets of miRNAs down-regulated in stage IIIb. These include progesterone-mediated oocyte maturation, ErbB, mTOR, and VEGF signaling pathways. In fish, it is well documented that follicular cells produce 17α, 20β-dihydroxyprogesterone, which acts on membrane progestin receptor to induce oocyte maturation (Hanna and Zhu, 2011). The role of ErbB in oocyte maturation has also been reported in zebrafish (Aizen et al., 2018). A recent study showed that treatment with rapamycin, an mTOR inhibitor, prevented the development of mid and late vitellogenic follicles (Chen et al., 2018), suggesting that this pathway is important in promoting the growth of follicles from stage IIIa to IIIb. The mTOR pathway has been shown to promote follicle growth (Cheng et al., 2015) and may also be involved in oocyte maturation (Zhou et al., 2017) in mammals. VEGF was reported to enhance the effect of FSH on promoting granulosa cell proliferation

(Doyle et al., 2010) and to inhibit ovarian granulosa cell apoptosis (Kosaka et al., 2007), suggesting that it plays a role in promoting follicle growth in mammals. Whether and how the VEGF pathway regulates fish follicle development requires further investigation.

One of the most highly enriched pathways targeted by miRNAs differentially expressed between stages IIIa and IIIb follicular cells was the MAPK signaling pathway. This pathway has been well studied in vertebrates and shown to regulate follicle development and oocyte maturation (Zhang et al., 2009; Conti et al., 2012; Fan et al., 2012; Chaves et al., 2013; Alvarez-Mora et al., 2015). In fish, the MAPK pathway has been suggested to mediate the actions of various hormones and growth factors in the ovary, such as regulation of steroid production (Mendez et al., 2005; Qi et al., 2016) and activin/inhibin subunit expression (Chung and Ge, 2012), as well as oocyte maturation (Fitzgerald et al., 2015; Aizen et al., 2018). Interestingly, analyses of zebrafish ovarian transcriptomes revealed that the MAPK pathway was also strongly regulated when follicles transitioned from stage I to stage II (Zhu et al., 2018) and most strongly affected by β-diketone antibiotics, which have toxic effects in the reproductive system (Wang et al., 2017). In this study, we found that this pathway may be targeted by miRNAs either up- or down-regulated in stage IIIb cells. However, some target genes, such as several fgfs and their receptors, were only found in the gene list that may be targeted by miRNAs down-regulated in stage IIIb cells. Although the role of these fgfs in the fish ovary is unknown, several related FGF ligands have been reported to promote oocyte maturation in mammals (Cailliau et al., 2003; Zhang et al., 2010). Together,

these findings strongly suggest that the MAPK pathway plays a central role in ovarian follicle development and oocyte maturation and its activity is dynamically regulated by miRNAs.

Many studies have shown that ligands, receptors, and downstream signaling molecules of the TGF–β superfamily are expressed in the zebrafish ovary (Garg et al., 1999; Melino et al., 2000; Kohli et al., 2003; DiMuccio et al., 2005; Clelland et al., 2006) and that this pathway is involved in early follicle development, follicle growth, and oocyte maturation in zebrafish (Pang and Ge, 2002; Tan et al., 2009a; Tan et al., 2009b; Chung and Ge, 2012; Chen et al., 2017). Among the TGF-β family members, activin has potent effects on promoting maturational competency and oocyte maturation (Wu et al., 2000; Pang and Ge, 2002) while TGF-β1 inhibits oocyte maturation (Kohli et al., 2003; Kohli et al., 2005; Tan et al., 2009a). Several bone morphogenetic proteins (BMP), such as BMP-15 (Clelland et al., 2006; Clelland et al., 2007), BMP2b and BMP4 (Li and Ge, 2013), have been suggested to inhibit precocious oocyte maturation. Consistent with the inhibitory role of TGF-β1 on oocyte maturation, we found that tgfb1a, tgfb1b and tgfb2 were among the targets of miRNAs up-regulated in stage IIIb follicular cells. However, activin type I and type II receptors (ie. acvr1ba and acvr2aa) were predicted to be targeted by both up- and down-regulated miRNAs in stage IIIb follicular cells. Smad1 and Smad5, which mediate signaling by BMPs, were regulated by the miRNAs down- and up-regulated, respectively, in stage IIIb follicular cells. These findings suggest that activins and BMPs are tightly regulated by miRNAs during vitellogenesis. It is possible that different ligands signaling

through these receptors and/or Smads have differential effects on regulating follicle growth and/or oocyte maturation.

In conclusion, our study characterizes miRNA expression profiles in follicular cells of zebrafish vitellogenic follicles. We determined the abundance of miRNAs, predicted novel miRNAs, and identified miRNAs differentially expressed between stage IIIa and IIIb follicular cells. Comprehensive gene ontology and pathway enrichment analyses of the predicted targets of the significantly regulated miRNAs revealed several signaling pathways that may be crucial for follicle development and oocyte maturation. Further studies are required to determine how these pathways are regulated by miRNAs and how they are involved in ovarian functions.

CHAPTER 2

SUPPLEMENTARY DATA

Table S2.1. Annotated miRNAs detected in stage III zebrafish follicular cells

miRNA name	Mature sequence	IIIa-1	IIIa-2	IIIa-3	IIIb-1	IIIb-2	IIIb-3
dre-let-7a	TGAGGTAGTAGGTTGTATAGTT	13303	18627	19306	19137	28120	27789
dre-let-7b	TGAGGTAGTAGGTTGTGTGGTT	5386	5052	6029	4877	7140	11352
dre-let-7c-5p	TGAGGTAGTAGGTTGTATGGTT	1315	2151	1566	1667	2232	2761
dre-let-7d-5p	TGAGGTAGTTGGTTGTATGGTT	1880	2614	2791	3412	3827	4966
dre-let-7e	TGAGGTAGTAGATTGAATAGTT	27029	36608	46298	29061	36453	44397
dre-let-7f	TGAGGTAGTAGATTGTATAGTT	5214	6730	8746	5143	6723	6676
dre-let-7h	TGAGGTAGTAAGTTGTGTTGTT	2684	3769	4429	3755	5050	7834
dre-let-7i	TGAGGTAGTAGTTTGTGCTGTT	6874	6313	9310	4795	4639	8067
dre-let-7j	TGAGGTAGTTGTTTGTACAGTT	4526	5475	8552	7248	7534	13163
dre-miR-100-2-3p	CAAGCTCGTGTCTATAGGTATG	403	635	538	554	611	959
dre-miR-100-3p	CAAGCTTGTATCTATAGGTATC	85	218	139	129	125	220
dre-miR-100-5p	AACCCGTAGATCCGAACTTGTG	19144	29463	26489	22529	22839	28978
dre-miR-101a	TACAGTACTGTGATAACTGAAG	6835	17058	13989	16430	17197	21344
dre-miR-101b	TACAGTACTATGATAACTGAAG	571	1219	1276	897	912	941
dre-miR-103	AGCAGCATTGTACAGGGCTATGA	2538	3514	2952	3370	4353	4811
dre-miR-107a-3p	AGCAGCATTGTACAGGGCTATCA	2042	3433	3132	3670	4422	5711
dre-miR-107a-5p	AGCTTCTTTACAGTGTTGTCTTG	139	155	176	122	110	182
dre-miR-107b	AGCAGCATTGTACAGGGCTTT	46	47	66	103	132	145
dre-miR-10a-5p	TACCCTGTAGATCCGAATTTGT	307	604	354	640	935	523
dre-miR-10b-5p	TACCCTGTAGAACCGAATTTGTG	2244	6142	1508	10474	7970	13832
dre-miR-10c-5p	TACCCTGTAGATCCGGATTTGT	12050	18251	13049	21856	21957	38058
dre-miR-122	TGGAGTGTGACAATGGTGTTTG	0	112	78	165	784	72
dre-miR-125a	TCCCTGAGACCCTTAACCTGTG	15611	10124	12836	10935	17635	26192
dre-miR-125b-1-3p	ACGGGTTAGGTTCTTGGGAGCT	102	173	147	129	109	283
dre-miR-125b-2-3p	CGGGTTGGGTTCTCGGGAGCT	117	112	135	116	78	163
dre-miR-125b-3-3p	ACAGGTTAAGCTCTTGGGACCT	106	164	163	120	165	301
dre-miR-125b-5p	TCCCTGAGACCCTAACTTGTGA	3489	4107	4541	2985	4283	5682
dre-miR-125c-5p	TCCCTGAGACCCTAACTCGTGA	2963	2352	2741	2648	2595	7146
dre-miR-126a-3p	TCGTACCGTGAGTAATAATGC	12970	20702	12834	13022	16645	20916
dre-miR-126b-5p	CATTATTACTTTTGGTACGCG	11970	27377	22749	19955	25431	37437
dre-miR-128-3p	TCACAGTGAACCGGTCTCTTTT	1634	1999	3508	1989	3106	3457
dre-miR-128-5p	CGGGGCCGTGGCACTGTATGAGA	43	35	39	26	30	51
dre-miR-130a	CAGTGCAATGTTAAAAGGGCAT	1940	3080	3199	2817	4207	4680
dre-miR-130b	CAGTGCAATAATGAAAGGGCAT	1410	1655	1427	1384	1843	2509

dre-miR-130c-3p	CAGTGCAATATTAAAAGGGCAT	672	1047	956	1069	1196	1644
dre-miR-130c-5p	GCCCTTTTTCTGTTGTACTACT	19	33	60	38	41	92
dre-miR-132-3p	TAACAGTCTACAGCCATGGTCG	14	47	40	38	56	105
dre-miR-132-5p	ACCGTGGCATTAGATTGTTACT	32	85	55	52	104	156
dre-miR-133a-3p	TTTGGTCCCCTTCAACCAGCTG	72	93	113	32	117	161
dre-miR-135a	TATGGCTTTTTATTCCTATGTGA	44	67	53	42	44	31
dre-miR-1388-3p	ATCTCAGGTTCGTCAGCCCATG	2121	2498	1357	665	1030	1534
dre-miR-1388-5p	AGGACTGTCCAACCTGAGAATG	3462	8165	4046	2218	3262	4209
dre-miR-140-3p	TACCACAGGGTAGAACCACGGAC	4896	4087	4615	4739	5792	7729
dre-miR-140-5p	CAGTGGTTTTACCCTATGGTAG	199	252	345	304	386	456
dre-miR-141-3p	TAACACTGTCTGGTAACGATGC	1365	2073	1844	2235	2214	5028
dre-miR-142a-3p	TGTAGTGTTTCCTACTTTATGGA	442	480	530	174	217	351
dre-miR-142a-5p	CATAAAGTAGAAAGCACTACT	3440	8308	8443	3249	3769	6733
dre-miR-143	TGAGATGAAGCACTGTAGCTC	172661	454651	309002	271963	288373	550719
dre-miR-144-3p	TACAGTATAGATGATGTACT	326	1074	796	133	81	40
dre-miR-144-5p	GGATATCATCGTATACTGTAAGT	1765	2140	1723	374	251	126
dre-miR-145-3p	GGATTCCTGGAAATACTGTTCT	2211	6017	4114	2483	2723	4497
dre-miR-145-5p	GTCCAGTTTTCCCAGGAATCCC	5076	5972	3527	3068	4328	6053
dre-miR-146a	TGAGAACTGAATTCCATAGATGG	12123	16564	13483	10583	13655	21980
dre-miR-146b	TGAGAACTGAATTCCAAGGGTG	4072	2738	4557	1954	3083	3900
dre-miR-148	TCAGTGCATTACAGAACTTTGT	2782	2156	3403	4416	4903	3190
dre-miR-150	TCTCCCAATCCTTGTACCAGTG	1607	1601	1450	708	1337	2062
dre-miR-153b-3p	TTGCATAGTCACAAAAATGAGC	522	644	580	569	605	839
dre-miR-155	TTAATGCTAATCGTGATAGGGG	145	90	73	61	71	90
dre-miR-15a-5p	TAGCAGCACAGAATGGTTTGTG	538	659	747	411	482	658
dre-miR-15b-3p	CGAATCATGATGTGCTGTCACT	233	226	294	127	188	297
dre-miR-15b-5p	TAGCAGCACATCATGGTTTGTA	11285	12237	16715	7403	9454	13338
dre-miR-15c	AAGCAGCGCGTCATGGTTTTC	1321	1422	1887	841	1034	1449
dre-miR-16a	TAGCAGCACGTAAATATTGGTG	1231	1481	1711	855	857	939
dre-miR-16b	TAGCAGCACGTAAATATTGGAG	2651	2271	2609	1247	1726	2517
dre-miR-16c-3p	TCCAATATTGCTCGTGCTGCTGA	2050	1456	2080	1150	1703	2502
dre-miR-16c-5p	TAGCAGCATGTAAATATTGGAG	7462	7579	8561	5262	5982	8497
dre-miR-17a-5p	CAAAGTGCTTACAGTGCAGGTA	1521	1910	2028	1091	1498	1595
dre-miR-181a-3-3p	ACCATCGAGTGTTGAGTGTACC	39	41	63	21	28	63
dre-miR-181a-3p	ACCATCGACCGTTGATTGTACC	5123	4489	5983	1683	2960	2778
dre-miR-181a-5-3p	ACCATCGACCGTTGACTGTGCC	960	1211	722	1587	1005	1763
dre-miR-181a-5p	AACATTCAACGCTGTCGGTGAGT	127656	142728	127202	83158	126224	126979

dre-miR-181b-5p	AACATTCATTGCTGTCGGTGGG	11107	5938	9252	3256	4153	5151
dre-miR-181c-5p	CACATTCATTGCTGTCGGTGGG	1283	1023	986	561	565	1222
dre-miR-182-5p	TTTGGCAATGGTAGAACTCACA	280	118	146	133	272	153
dre-miR-184	TGGACGGAGAACTGATAAGGGC	91	35	96	24	81	29
dre-miR-187	TCGTGTCTTGTGTTGCAGCC	176	141	72	113	132	105
dre-miR-18a	TAAGGTGCATCTAGTGCAGATA	70	93	124	50	57	62
dre-miR-18c	TAAGGTGCATCTTGTGTAGTTA	54	82	141	96	129	181
dre-miR-190a	TGATATGTTTGATATATTAGGT	107	104	77	61	48	68
dre-miR-192	ATGACCTATGAATTGACAGCC	1552	12674	5806	4755	25759	2422
dre-miR-194a	TGTAACAGCAACTCCATGTGG	49	303	127	93	599	69
dre-miR-194b	TGTAACAGCCGCTCCATGTGGA	55	33	81	71	61	55
dre-miR-196b	TAGGTAGTTTCAAGTTGTTGGG	38	32	27	45	27	40
dre-miR-199-3p	TACAGTAGTCTGCACATTGGTT	2566	4904	3818	3465	4001	7036
dre-miR-199-5p	CCCAGTGTTCAGACTACCTGTTC	1392	1006	772	604	614	1319
dre-miR-19a-3p	TGTGCAAATCTATGCAAAACTGA	1744	3031	2764	1970	1980	1741
dre-miR-19b-3p	TGTGCAAATCCATGCAAAACTGA	6937	11623	11038	8679	10027	9113
dre-miR-19c-3p	TGTGCAAATCCATGCAAAACTCG	279	426	613	386	508	674
dre-miR-19d-3p	TGTGCAAACCCATGCAAAACTGA	974	1207	1359	823	936	1303
dre-miR-200a-5p	CATCTTACCGGACAGTGCTGGA	25	47	35	57	40	86
dre-miR-200b-3p	TAATACTGCCTGGTAATGATGA	332	402	465	368	311	639
dre-miR-200c-3p	TAATACTGCCTGGTAATGATGC	138	294	358	302	588	790
dre-miR-202-3p	AGAGGCATAGGGCATGGGAAAA	4832	3173	5324	3230	3800	3202
dre-miR-202-5p	TTCCTATGCATATACCTCTTTG	307969	349173	369309	299371	622606	514008
dre-miR-203a-3p	GTGAAATGTTTAGGACCACTTG	8	47	69	28	22	114
dre-miR-203b-3p	GTGAAATGTTCAGGACCACTTG	93	117	172	83	72	32
dre-miR-204-5p	TTCCCTTTGTCATCCTATGCCT	419	585	505	735	554	385
dre-miR-205-5p	TCCTTCATTCCACCGGAGTCTG	109	36	129	88	69	18
dre-miR-20a-3p	ACTGCAGTGTGAGCACTTGAAG	123	58	152	98	100	116
dre-miR-20a-5p	TAAAGTGCTTATAGTGCAGGTAG	2469	2991	2704	1803	2199	2104
dre-miR-20b-5p	CAAAGTGCTCACAGTGCAGGTAG	84	55	126	54	83	178
dre-miR-21	TAGCTTATCAGACTGGTGTTGGC	106157	104384	88178	83739	86097	134969
dre-miR-210-3p	CTGTGCGTGTGACAGCGGCTAA	1300	1674	2851	2513	3017	4310
dre-miR-210-5p	AGCCACTGACTAACGCACATTG	749	642	1055	902	1026	1802
dre-miR-212-5p	ACCTTGGCTCTAGACTGCTTACT	22	34	30	31	41	57
dre-miR-214	ACAGCAGGCACAGACAGGCAG	1801	2052	1440	1345	1176	2676
dre-miR-216b	TAATCTCTGCAGGCAACTGTGA	8	75	20	34	80	50
dre-miR-2184	AACAGTAAGAGTTTATGTGCT	3461	6496	3148	4813	4769	8116

dre-miR-2185-5p	CGGTGCAGGACTCCGCGGCTC	47	1860	1543	1013	10854	404
dre-miR-2188-3p	CTGTGTGAGGTTAGACCTATC	189	161	135	12	17	14
dre-miR-2188-5p	AAGGTCCAACCTCACATGTCC	2980	3657	2438	634	310	224
dre-miR-2189	TGATTGTTTGTATCAGCTGTGT	60	75	64	189	11	36
dre-miR-218a	TTGTGCTTGATCTAACCATGTG	2402	2010	2001	1982	2843	2745
dre-miR-218b	TTGTGCTTGATCTAACCATGCA	20	52	56	71	97	79
dre-miR-221-3p	AGCTACATTGTCTGCTGGGTTTC	6209	8724	5763	6208	6271	8633
dre-miR-221-5p	ACCTGGCATACAATGTAGATTTCTGT	1191	384	468	299	223	336
dre-miR-222a-3p	AGCTACATCTGGCTACTGGGTCTC	22906	11374	15058	7264	10838	15527
dre-miR-222a-5p	TGCTCAGTAGTCAGTGTAGATCC	5782	5867	5729	4935	5963	7887
dre-miR-222b	AGCTACATCTGAATACTGGGTCA	280	414	324	267	308	608
dre-miR-223	TGTCAGTTTGTCAAATACCCC	916	599	902	319	505	1082
dre-miR-22a-3p	AAGCTGCCAGCTGAAGAACTGT	63539	116201	91747	176927	273005	315083
dre-miR-22a-5p	AGTTCTTCACTGGCAAGCTTTA	164	438	305	662	956	871
dre-miR-22b-3p	AAGCTGCCAGTTGAAGAGCTGT	1168	1728	1737	1379	2214	2809
dre-miR-23a-3p	ATCACATTGCCAGGGATTTCCA	1071	1996	1587	1698	2854	2824
dre-miR-23b	ATCACATTGCCAGGGATTACCA	311	516	507	565	775	1121
dre-miR-24	TGGCTCAGTTCAGCAGGAACAG	193	381	323	315	609	738
dre-miR-24b-3p	TGGCTCAGTTCAGCAGGAACCG	36	295	145	142	295	364
dre-miR-24b-5p	GTGCCTACTGAGCTGATAACAGT	9	46	18	23	46	70
dre-miR-25-3p	CATTGCACTTGTCTCGGTCTGA	22263	25210	28884	24360	24855	32465
dre-miR-25-5p	AGGCGGAGACTTGGGCAGCTGCC	33	27	37	68	53	100
dre-miR-26a-2-3p	CCTATTCATGATTACTTGCACT	24	33	30	33	35	51
dre-miR-26a-3p	CCTATTCGGGATGACTTGGTTC	167	108	171	68	119	227
dre-miR-26a-5p	TTCAAGTAATCCAGGATAGGCT	76867	108707	109293	125177	174042	208507
dre-miR-27a-3p	TTCACAGTGGCTAAGTTCCGCT	1356	2421	1924	2880	3666	5908
dre-miR-27a-5p	AGGACTTAGCTCACTCTGTGAACA	176	487	240	328	656	857
dre-miR-27b-3p	TTCACAGTGGCTAAGTTCTGCA	12464	30438	34602	44449	58083	87483
dre-miR-27b-5p	AGAGCTTAGCTGATTGGTGAACA	199	277	330	296	452	990
dre-miR-27c-3p	TTCACAGTGGTTAAGTTCTGC	46616	68264	68276	77466	123418	132306
dre-miR-27d	TTCACAGTGGCTAAGTTCTTCA	23	111	164	171	199	375
dre-miR-27e	TTCACAGTGGCTAAGTTCAGTG	162	376	411	477	583	846
dre-miR-29a	TAGCACCATTTGAAATCGGTTA	1246	1523	1606	3001	5188	5361
dre-miR-29b	TAGCACCATTTGAAATCAGTGT	20	39	28	86	129	115
dre-miR-301a	CAGTGCAATAGTATTGTCAAAG	3791	3517	3896	2954	3667	4490
dre-miR-301c-3p	CAGTGCAATAGTATTGTCATAG	385	446	460	359	435	430
dre-miR-30a-5p	TGTAAACATTCCCGACTGGAAG	968	871	904	1366	2650	2628

dre-miR-30b	TGTAAACATCCTACACTCAGCT	5446	8841	8901	8641	13716	14721
dre-miR-30c-3p	CCGGGAGTGGGATGTTTGCGCT	304	330	254	278	314	602
dre-miR-30c-5p	TGTAAACATCCTACACTCTCAG	20139	14100	13096	10025	17122	19969
dre-miR-30d	TGTAAACATCCCCGACTGGAAG	82222	70075	77658	72479	120011	129711
dre-miR-30e-3p	CTTTCAGTCGGATGTTTGCAGC	3108	4549	4074	4348	5782	6772
dre-miR-30e-5p	TGTAAACATCCTTGACTGGAAG	45449	34362	32881	22213	36288	41116
dre-miR-31	TGGCAAGATGTTGGCATAGCTG	22	69	32	192	93	128
dre-miR-338	TCCAGCATCAGTGATTTTGTTG	819	1132	1444	1300	2133	2306
dre-miR-34a	TGGCAGTGTCTTAGCTGGTTGT	38	61	44	49	46	64
dre-miR-34b	TAGGCAGTGTTGTTAGCTGATTG	20	20	87	44	41	15
dre-miR-34c-3p	AATCACTAACCTCACTACCAGG	35	41	63	94	71	41
dre-miR-34c-5p	AGGCAGTGCAGTTAGTTGATTAC	60	34	68	74	58	31
dre-miR-363-3p	AATTGCACGGTATCCATCTGTA	277	275	470	371	408	651
dre-miR-365	TAATGCCCCTAAAAATCCTTAT	42	39	49	35	53	74
dre-miR-375	TTTGTTCGTTCGGCTCGCGTTA	75	82	263	82	96	59
dre-miR-429a	TAATACTGTCTGGTAATGCCGT	172	291	426	338	341	697
dre-miR-430a-3p	TAAGTGCTATTTGTTGGGGTAG	11	78	73	104	49	31
dre-miR-430b-3p	AAAGTGCTATCAAGTTGGGGTAG	209	772	825	1240	467	305
dre-miR-430c-3p	TAAGTGCTTCTCTTTGGGGTAG	39	102	100	144	61	44
dre-miR-451	AAACCGTTACCATTACTGAGTT	13294	24490	17619	3875	2473	2427
dre-miR-454a	TAGTGCAATATTGCTAATAGGG	1146	729	957	708	799	1371
dre-miR-454b	TAGTGCAATATTGCTTATAGGG	7197	4962	3983	2759	3610	5830
dre-miR-455-2-5p	GTATGTGCCCTTGGACTACATT	121	148	184	132	183	236
dre-miR-455-3p	ATGCAGTCCATGGGCATATACAC	307	316	293	157	325	375
dre-miR-455-5p	TATGTGCCCTTGGACTACATCG	4430	3963	4638	2523	4283	5572
dre-miR-456	CAGGCTGGTTAGATGGTTGTCA	2640	5088	3523	1829	3111	3872
dre-miR-457a	AAGCAGCACATCAATATTGGCA	1622	2535	2638	1795	2173	2360
dre-miR-457b-3p	TCCAGTATTGCTGTTCTGCTGT	20	49	60	28	22	93
dre-miR-457b-5p	AAGCAGCACATAAATACTGGAG	123	191	215	118	163	224
dre-miR-458-3p	ATAGCTCTTTGAATGGTACTGC	1183	3041	1238	1569	908	2966
dre-miR-459-5p	TCAGTAACAAGGATTCATCCTG	8	109	28	13	100	17
dre-miR-460-3p	CACAGCGCATACAATGTGGATG	36	126	98	70	97	174
dre-miR-462	TAACGGAACCCATAATGCAGCT	20678	13871	20204	11590	28022	27771
dre-miR-489	AGTGACATCATATGTACGGCTGC	134	122	142	117	123	187
dre-miR-7133-3p	TAGTTTGATTCACAGCACAAGA	16	53	58	44	37	34
dre-miR-7145	ACAATGGAAGCCAATGGTTACC	0	95	30	40	26	73
dre-miR-7148-5p	ATGGAAATACTCGCTGATACTG	11	16	32	28	32	41

dre-miR-722	TTTTTTGCAGAAACGTTTCAGATT	17	34	124	222	334	1142
dre-miR-724	TTAAAGGGAATTTGCGACTGTT	2408	2733	3171	2051	1501	1618
dre-miR-725-3p	TTCAGTCATTGTTTCTAGTAGT	85	120	204	176	173	242
dre-miR-731	AATGACACGTTTTCTCCCGGATCG	2778	1320	2095	1033	2559	2339
dre-miR-734	GTAAATGCTGCAGAATCGTACCG	534	659	261	1432	1651	2457
dre-miR-735-3p	CTCTCCCACCGCTAAACTTGAC	8	0	70	181	143	421
dre-miR-735-5p	GGCTGGTCCGAAGGCGGT	613	167	309	465	254	640
dre-miR-738	GCTACGGCCCGCGTCGGGACCTC	17	30	60	97	50	35
dre-miR-7a	TGGAAGACTAGTGATTTTGTTGT	105	90	90	100	144	101
dre-miR-7b	TGGAAGACTTGTGATTTTGTT	255	140	159	117	118	102
dre-miR-92a-2-5p	AGGTTGGGATCGGCCGCAATGCT	46	25	59	44	40	63
dre-miR-92a-3p	TATTGCACTTGTCCCGGCCTGT	249073	116078	154906	94195	148129	160783
dre-miR-92a-5p	AGGTTGGGATTGGTAGCAATGCT	77	67	99	113	74	95
dre-miR-92b-3p	TATTGCACTCGTCCCGGCCTCC	4247	3247	5222	2045	4268	4905
dre-miR-93	AAAAGTGCTGTTTGTGCAGGTA	1102	1486	1640	1133	1231	1319
dre-miR-9-4-3p	TAAAGCTAGAGAACCGAATGTA	16	80	47	47	73	13
dre-miR-9-5p	TCTTTGGTTATCTAGCTGTATGA	45	53	80	66	41	30
dre-miR-99	AACCCGTAGATCCGATCTTGTG	2941	2912	2555	2118	2841	3660

Table S2.2. Novel miRNAs derived from the 3' end of known miRNA precursors

miRNA name	Mature sequence	Pre-miRNA	Chromosome	Strand	Start	End	IIIa-1	IIIa-2	IIIa-3	IIIb-1	IIIb-2	IIIb-3
dre-miR-152-p5	AAGTTCTGTGATACACTCAGACT	CTGTTCACCTGGCTCAAGTTCTGTGATACACTCAGACTTTGAATCAGTGGTAGTCAGTGCATGACAGAACTTTGGCCCGG	chr3	+	24095957	24096036	927	843	985	1339	807	1105
dre-miR-222b-p5	ACTCAGTACTCGGTGTAGAGTC	GACGTGATCTGCAGACTCAGTACTCGGTGTAGAGTCTGTGTGATTCAGAAGAGCAGCTACATCTGAATACTGGGTCAGTGGAGACGTCACACGCTAC	chr21	-	44568170	44568266	33	76	43	32	25	37
dre-miR-27c-p5	AGGACTTAACCCACTTGTGAAC	GGTTGTGTGGTGTCAGGACTTAACCCACTTGTGAACAATGCATCGAACTTCAATGTTCACAGTGGTTAAGTTCTGCCGCCCCTAGACC	chr3	+	13406451	13406538	1083	754	746	836	1296	1753
dre-miR-29a-p5	TGACTGATTTCCTTTGGTGCT	TGAAGACCCTCATCTCTCTCTCTCTCTCCCCACCAAACGATGACTGATTTCCTTTGGTGCTTAGAGTCCCATCTGTCATC	chr4	-	11606758	11606883	54	87	74	258	603	400

		TAGCACCATTTGAAATCGGTTATAATGACTGGGGATCAATTCTTCA										
dre-miR-338-2-p5	AACAATATCCTGATGCTGAAT	GTGTGTGTGTGTGTTTCTGGTGCCTGCTGAGAACAATATCCTGATGCTGAATGAGTGTGTTGAAGGAAACTCCAGCATCAGTGATTTTGTTGCCAGAGGAGCACTTTGGGCATCCTGTGTATAT	chr12	+	17727528	17727651	72	96	78	77	70	170

Table S2.3. Novel miRNAs derived from the 3' end of known miRNA precursors

miRNA name	Mature sequence	Pre-miRNA	Chromosome	strand	start	end	IIIa-1	IIIa-2	IIIa-3	IIIb-1	IIIb-2	IIIb-3
dre-let-7j-p3	CTGTACAGACTACTGCCTTGCC	GGTTGAGGTAGTTGTTTGTACAGTTTTTAGGGTCTGTTATTCTGCCCTGTTAAGGAGCTAACTGTACAGACTACTGCCTTGCC	chr6	+	41454759	41454879	1503	1642	2816	1954	1697	3440
dre-miR-125a-1-p3	CAGGTGAGGTCCTCAGGAAC	GTATGTCTCTTTGTCCCTGAGACCCTTAACCTGTGAGGTCAAACTAGGTCACAGGTGAGGTCCTCAGGAACAGGGCTGCATGC	chr16	+	24898171	24898253	61	52	88	100	111	190
dre-miR-146a-p3	ATCTATGGGCTCAGTTCTTCT	GAGTTTGTTTTGAGCACTTTTCCCTGAGAACTGAATTCCATAGATGGTGTTCATGAAAAGTTCATCTATGGGCTCAGTTCTTCTGGCAATCTGTTTAATGTCTGCTACAAATTC	chr13	+	11822170	11822283	54	86	105	83	82	195
dre-miR-2185-1-p3	TGCACCAGCACTTTCTTGGCCAGACG	CAGGTTCTCTGGCTTGACGTCCCGGTGCAGGACTCCGCGGCTCTCGCAGTGTTTCAGAGCCGCGATCAGCTGCACCAGCACTTTCTTGGCCAGACGCT	chr17	+	39771488	39771585	8	53	48	69	152	10
dre-miR-451-p3	TTTAGTA	AGAGGCGGCG	chr5	-	42271971	42272030	588	879	807	114	51	32

	ATGGTAAGGGTTCT	AAACCGTTACCATTACTGAGTTTAGTAATGGTAAGGGTTCTGCTGCCTTT										
dre-miR-457a-p3	CCAGTATGGTTTGTGCTGCTCCCG	TGCCTGACAGAAGCAGCACATCAATATTGGCAGCTGCCCTCTCTCTGGGTTGCCAGTATGGTTTGTGCTGCTCCCGTCAGACA	chr7	+	26071426	26071508	74	69	73	20	27	52
dre-miR-733-p3	TCTGTGATCCCTGAAAAATCA	TGGCTTTTCCGTAGTGTCGCTGTTGCGTTGGTTTAGCTCAGTGGTTACTTCTGTGATCCCTGAAAAATCAACCTTTCATGGTCACCTCGGTGCCA	chr14	-	2406978	2407072	154	47	62	64	74	66
dre-miR-99-1-p3	CAAGCTCGATTCTATGGGTCTC	GCCACTTGTCATTAACCCGTAGATCCGATCTTGTGATAAGTTTGATGGCACAAGCTCGATTCTATGGGTCTCTGTCTCTGTGGT	chr15	+	29795583	29795666	27	40	41	42	29	62
dre-miR-99-2-p3	CAAGCTCGATTCTGTGGGTCT	CACTTGTCACAAACCCGTAGATCCGATCTTGTGGCGTAATCGGCAACCCAAGCTCGATTCTGTGGGTCTCTGTCACTGTG	chr10	-	38074312	38074391	258	545	452	376	512	577

Table S2.4. Conserved novel miRNAs identified in stage III zebrafish ovarian follicular cells

miRNA name	Mature sequence	Pre-miRNA	Chromosome	Strand	Start	End	IIIa-1	IIIa-2	IIIa-3	IIIb-1	IIIb-2	IIIb-3
aca-let-7a-2-3p	CTATACAGTCTATTGCCTTCCT	AGGTGAGGTAGTAGGTTGTATAGTTTGTGGGAGGGATTACATCCCATTTCAGGTGATAACTATACAGTCTATTGCCTTCCTTA	chr6	-	54506458	54506586	600	703	693	552	792	931
aca-miR-101-1-5p	TCAGTTATCACTGTGCTGATGC	GCTGCCCTGGCTCAGTTATCACTGTGCTGATGCTGTCTATACTAAAGGTACAGTACTGTGATAACTGAAGGATGGCAGC	chr6	+	31365432	31365535	63	137	131	109	162	210
aca-miR-30a-5p	TGTAAACATCCTCGACTGGAAG	TGTTGACGGTGAGCGACTGTAAACATCCTCGACTGGAAGCTGTGAAGCAGTAGATGAAGCTTTCAGTCGGATGTTTGCAGCTGCCAACTGCCAC	chr13	+	27795118	27795218	40	51	45	52	65	67
ccr-miR-22a-p5	AGTTCTTCTCTGGCAAACTTTA	AGTGAATATTGGCTGACCTACCGCAGTTCTTCTCTGGCAAACTTTATGACCCTGTCCTGACGCTAAAGCTGCCAGCTGAAGAACTGTTGTGGTCAGCTCT	chr21	-	39267220	39267317	57	165	100	139	236	282
ccr-miR-734-p5	TCCTTTGATATTGAGAGCT	ATCCTTTGATATTGAGAGCTGAACCGTTCTGCAGCATGGCTGCCTGTGGATCCTCTGGGGAAAGGTAAATGCTGCAGAATCGTACCGTTCTTGGTATCAC	chr1	+	34122874	34122973	9	32	18	89	100	231
ccr-miR-738-p5	CGCGTCGGGACCTCCGTC	GCTACGGCCCGCGTCGGGACCTCCGTCTCGGTGACCCCCTC	chr5	-	821403	821489	378	46	130	273	120	138

		TCTCGGGAGGGGGCCCGGGGCGGGCGGCCTCCCCGGCGCGGCGCCTC										
cin-miR-199-3p	ACAGTAGTCTGCACATTGGGTG	TTGATAGGACTTGATGTGGCGGTAAAGGATACACCTGCAGCAACTTTGCGGCACTTCGGCATACGACAGTAGTCTGCACATTGGGTGTCTAGCTGCTACATG	chr5	-	63709570	63709654	43	49	44	37	49	95
cpi-miR-218-3p	AACATGGTTCTGTCAAGCAC	TGGGGTTTTCCTTTGTGCTTGATCTAACCATGTGGTAGAACAATACAAATTGAACATGGTTCTGTCAAGCACCATGGAAGGCTGCAT	chr1	+	23866878	23866965	117	168	227	183	224	256
efu-miR-9277-p3	TCCCCGCGCAGGTTCGAATCCT	TCGTGGCCGAGTGGTTAAGGCGATGGACTTGAAATCCATTGGGGTCTCCCCGCGCAGGTTCGAATCCTGCCGACTACGCAGTTACGCTT	chr3	-	8975708	8975785	35	47	41	35	116	84
ipu-miR-223	GAGTATTTGACAGACTGTGG	GAGTATTTGACAGACTGTGGTTGACACTCGATCTAAAGGGGTGTCAGTTTGTCAAATACC	chr5	-	22234407	22234505	189	36	101	15	32	89
ipu-miR-29a-p3	CTAGCACCATTTGAAATCGGTC	ACTGATTTCCTCTGGTGTTCAGAGTGGACTGGGTTTTTCTAGCACCATTTGAAATCGGTCG	chr23	-	20348637	20348697	121	219	207	150	517	261
ipu-miR-30d	CTTTCAGTTGGATGTTTGCTGT	TGTAAACATCCCCGACTGGAAGCTGTGCTACGCGGAAAACGAGCTTTCAGTTGGATGTTTGCTGT	chr16	+	25341416	25341543	608	575	697	559	646	831
ipu-miR-7550	ATCCGG	ATCCGGCTCGAAGG	chr13	-	37556934	3755698	38	137	146	340	241	201

	CTCGAAGGACCA	ACCAATTTTGTAGAGGAGTTAGCTGGTTCTTAGGTGAATTT				8						
mdo-miR-29a-3p	TAGCACCATTTGAAATCGGTT	ATGACTGATTTCTTTTGGTGTTCAGAGTCAATATCATTTTCTAGCACCATTTGAAATCGGTTAT	chr23	-	32439964	32440052	95	74	85	108	195	213
ocu-let-7a-3p	CTATACAATCTACTGTCTTTCC	TGAGGTAGTAGGTTGTATAGTTTGGGGCTCCGCCCTGCTATGGGATAACTATACAATCTACTGTCTTTCC	chr4	-	18654155	18654261	464	688	719	421	788	866
ocu-let-7f-2-3p	CTATACAGTCTACTGTCTTTCC	TGTGGGATGAGGTAGTAGATTGTATAGTTTTAGGGTCATACCCCATCTTGGAGATAACTATACAGTCTACTGTCTTTCC	chr23	+	28536361	28536457	115	176	181	136	208	234
ocu-miR-130b-5p	ACTCTTTCCCTGTTGCACTACT	ACTCTTTCCCTGTTGCACTACTGTGGACCACTGGGAAGCAGTGCAATGATGAAAGGGCAT	chr5	+	13299328	13299422	529	541	692	506	677	1077
ocu-miR-24-5p	GTGCCTACTGAGCTGATATCAGT	GTGCCTACTGAGCTGATATCAGTTCTCGTTTCACACACTGGCTCAGTTCAGCAGGAACAG	chr8	-	30162492	30162633	60	69	68	53	96	170
oga-miR-100-p5	CGTAGATCCGAACTTGTGA	AACCCGTAGATCCGAACTTGTGATGATAGTCCACACAAGCTTGTGTCTATAGGTATG	chr15	+	29795594	29795654	16	28	22	24	35	45
oha-miR-26-3-3p	CCTATTCTTGATTACTTG	CAGCCTGGTTCAAGTAATCCAGGATAGGCTGTTACCAGGCAACACGGCCTATTCTTGATTACTTGTTTCAGGAGGCGGCTTCTTG	chr23	-	24675207	24675303	28	75	47	55	68	113
ola-let-7a-3p	CTATACAACTTACT	ACGTCCTTTGGGGTGAGGTAGTAGGTTG	chr25	+	2189964	2190061	479	529	758	304	558	725

	GTCTTTC	TATAGTTTTAGGGTCATTCCCATGCTGTCAGATGACTATACAACTTACTGTCTTTCCTGAAGCGGCT										
pol-let-7b-3p	CTATACAACCTACTGCCTTCC	TGAGGTAGTAGGTTGTGTGGTTTCAGGGTTGTGATTTTACCCCATCAGGAGCTAACTATACAACCTACTGCCTTCC	chr4	-	18653148	18653241	712	653	942	471	712	1326
pol-miR-21-3p	CGACAACAGTCTGAAGGCTGTC	TAGCTTATCAGACTGGTGTTGGCTGTTTAGATTGCAAGGCGACAACAGTCTGAAGGCTGTC	chr10	-	28198894	28199041	69	171	169	201	66	116
ssa-let-7h-3p	CTATACAACTTACTGCCTTCCT	TGAGGTAGTAAGTTGTGTTGTTGTTGGGGATCAGGATAGTGCGCCCCGTACGGGAGATAACTATACAACTTACTGCCTTCCT	chr23	+	28536665	28536778	153	232	252	172	255	360
ssa-miR-101b-5p	CAGTTATCATGGTACCGGTGCTGT	CAGTTATCATGGTACCGGTGCTGTGTCCCTGTCAAGTACAGTACTATGATAACTGAA	chr5	-	1837149	1837260	271	127	199	125	187	234
ssa-miR-130b-2-5p	TGCCCCTTTTATATTGTCCTACT	TGCCCCTTTTATATTGTCCTACTGGTGACCCTCTAATGAAAGCAGTGCAATGTTAAAAGGGCA	chr10	-	33377130	33377214	22	22	49	18	28	55
ssa-miR-148a-5p	AAGTTCTGTGATACACTTCGACT	AAGTTCTGTGATACACTTCGACTCTAATTGTTTGCAGTCAGTGCATTACAGAACTTTGT	chr9	-	1644683	1644761	1598	515	1151	1406	1929	723
ssa-miR-218-3p	ACATGGTTCCGTCAAGCACCAGG	TTGTGCTTGATCTAACCATGTGCCCCCGCCTACACTCGCCTCACATGGTTCCGTCAAGCACCAGG	chr14	-	24562800	24562887	203	101	183	96	195	275
tgu-miR-19a-3p	TGTGCAA	CTGTTAGTTTTGCAT	chr9	-	53436690	5343677	55	65	55	49	67	55

	ATCTATGCAAAGC	AGTTGCACTACAAGAAGAGAGTGGTTGTGCAAATCTATGCAAAGCTGATGGTGGC				1						
xla-miR-143-5p	GGTGCAGTGCTGCATCTCTGGT	GGTGCAGTGCTGCATCTCTGGTCAATTGTGAGTCTGAGATGAAGCACTGTAGCTC	chr14	+	38742313	38742411	646	445	458	249	284	613
xla-miR-214-5p	GCCTGTCTACACTTGCTGTGC	GCCTGTCTACACTTGCTGTGCAGAACATCCTCTCACCTGTACAGCAGGCACAGACAGGCAG	chr20	+	14877639	14877738	139	224	200	125	129	326

Table S2.5. Novel miRNAs identified in zebrafish stage III follicular cells

miRNA name	Mature sequence	Pre-miRNA	Chromosome	Strand	Start	End	IIIa-1	IIIa-2	IIIa-3	IIIb-1	IIIb-2	IIIb-3
PC-3p-11296_502	CACTTCAGTATCTGGTTGACTGGG	GACGGCCAGCCAGCCTACACGGTCCACCGGATACTGGATTCCAGGAGGGTCCAGAACTCACTTCAGTATCTGGTTGACTGGGAGGGCTACGGGCCAGA	chr11	+	16517471	16517568	30	38	31	45	47	22
PC-3p-11493_494	TATCATCTCATCTGTTTGGCCTGGA	TTCAGTACTCATCTGTATTTTTCAGGATAGTGAGCAGGCTTTTGATGGTGTTACTGCCCAATATCATCTCATCTGTTTGGCCTGGAACAACCAGCACAGGTATTATC	chr4	+	42736726	42736832	66	67	40	81	33	19
PC-3p-11648_488	AGGCGTACCTAACCGAACTGT	TTTTGGTGCCCTTTCAAGTGGTATGGTACGGTTTGGTTCGGTATGCCTTTTGACAGTGGAAACGGCTATAAAGGCGTACCTAACCGAACTGTACCGTACCACTCAGTGAAAGCGGGCCATAA	chr9	-	49939488	49939609	24	39	77	16	0	79
PC-3p-11987_476	CTGAAGTCATTATTATTAGGGC	AAATTCAAAGGAGGGCTAATAATTCTGGCTTCAAGTGTATATATATCCAAAGCTGAAGTCATTATTATTAGGGCTCCTGTGAAATG	chr11	+	5432908	5432993	0	44	3	4	61	132
PC-3p-13143_435	TACTGTATCACTCGGACATGGTGCCA	AAACTTTGGCACAACGAGATTGATGCGGTGCTTTGTCAGTGTTACTGTATCAC	chr4	+	58989176	58989249	30	35	34	71	37	21

		TCGGACATGGTGCCAGAGAGT										
PC-3p-13293_430	ATGACTCAAACTTGAGGACTT	TTAGTTCACCTTTCGAGTCTTCTGGTTCTTGAGTCGTTCGTTCATTTCGTGACAGAATGACTCAAACTTGAGGACTTGAGGGTTGAATAGA	chr4	+	32412985	32413075	16	36	46	30	36	88
PC-3p-140_40238	TGCGCATGCGTGAACTTTGTACC	ACAGTGCAAAGTTTAACATGCGCGCTGTGTGTGTCAGCTGTGTGCGCATGCGTGAACTTTGTACCGA	chr20	+	21349981	21350047	3582	2856	2599	2646	3683	8168
PC-3p-15383_376	ATAACTCAAACCCGAGGACTGG	ATAGGAGCCATGATGATTAGTTCACCTTTCAAGTCTTCTGGTTCTTGAGTTGTTCGTTCATAATGTGACAGAATAACTCAAACCCGAGGACTGGAGAAATGAACGGATCAATTCTTTTTCCGGCTCTAAA	chr9	+	5242718	5242847	82	118	96	56	114	203
PC-3p-17251_338	TATGAAAGTCAATGGTTACACT	AAGATATTTTGAAGAAGGCTGCAATCCTGTAACCATTGACTTCCATAGTATTTGTTTTTCCTATACTATGAAAGTCAATGGTTACACTTTTTTAGCTTTCTTCAGAATATGAT	chr21	-	11975430	11975542	0	11	5	26	87	60
PC-3p-17252_338	TAATGATGTACAGAACGGAGCGCCT	CACGCTCTTCTTTCTACGTCATTAAACCGCGGAGAAGCACCACATTGTCGCAAGTTTAATGATGTACAGAACGGAACGCCTC	chr20	-	3884678	3884759	65	22	20	34	19	9
PC-3p-1759_2936	TCAGCA	GATTGCACAAGGG	chr1	+	10987358	1098745	26	292	114	139	311	150

	CTCGGACAGCCTCTT	TGAAGAGGCTGTCCGAGTGCTGATATTACATTAATGTCACGACTATCAGCACTCGGACAGCCTCTTCACCCTTGTGCATTA				1						
PC-3p-18741_312	TAAAGGACTAATCATTTTGACT	GCGGTACAGTTGAAGACAGAATTATTAGCCCTCCTATATATATATATATATATATAAAGGACTAATCATTTTGACTTCAACTGTATTAA	chr24	-	2066730	2066820	0	9	98	35	7	12
PC-3p-20924_279	TACTGGAGATCTCCGCTAGGTGGA	TGGCCGCCTGGCGGAGCTGTTGTTCCAGTCGATCCTGTCTGGCTGTAAGTTGCTCAGAGATGGAATTCTGTTTACTGGAGATCTCCGCTAGGTGGAGC	chr20	+	17052844	17052941	43	29	45	36	34	38
PC-3p-2101_2469	TACTCCTTGGATGTTGACTGGA	TTTGAATGTTTCTAGTCACATTTGGGCGAGTTCTTTGTTCATCTTGAATTCATGGTACTCCTTGGATGTTGACTGGAAGTATTCTGG	chr17	+	53416825	53416911	17	96	92	48	39	7
PC-3p-29434_200	CACTAGAGCAACTTCAGGACTAAACT	GTGCTTTCTCTGTCACGGTTTTAAAAGTCTAAGACTCAGTCTAGTCCAGAACTGAATTGGCTGATTTCCTGGAGGAACACTAGAGCAACTTCAGGACTAAACTGAGGCTTAAATTAAACCTATCAGGAGGCAGGTT	chr4	+	75797756	75797891	88	21	0	15	0	2
PC-3p-30868_191	TACTCTGAGGCTTA	TTTGGTCATGCCCAAGACGTTTTGAG	chr15	+	5508394	5508476	74	25	5	29	0	2

	TTCTTGTGACCT	TTTTGGAGCAAAATTTTCAACTTTTACTCTGAGGCTTATTCTTGTGACCTAGCCCCT										
PC-3p-3862_1376	TGAGATTTCTGACTTGTTGAATTTGT	CTTTAAAATGTTGAATAGAGGAAGAAACATTTCAAAAGGTTCATTTTAATGCTGTTTGAGAAATGCATTTTAGTGTTTCTTGAGATTTCTGACTTGTTGAATTTTTGTTAAACTG	chr7	-	29424424	29424538	9	40	139	26	50	248
PC-3p-4095_1308	ACAACTCAAGAACCAGAAGACT	GCGTTTAGGGCCAGAAAAAAAATTTATCCGTTAATCTCTCGAGTCCTCAGGTTTGAGTCATTCTGTCACATGATGAACGAACAACTCAAGAACCAGAAGACTCGAAAGGTGAACTAAATATCATGGTTCCTATCGACT	chr5	+	6778475	6778612	28	32	80	61	36	67
PC-3p-4240_1269	TAAGATTGTTTTGCTTACACC	GTTAATGAAGAGGCATCATATCAAGCAAAATAATCTGCCAATGGGGTCAGCAAAATAATCTTGTTTTCCGATTTGCGATAAGATTGTTTTGCTTACACCATTGGCAGATTATTTTGCTTGATTTAAGGTAAACTCACTTCATTTTG	chr9	+	5252530	5252675	33	91	55	81	10	118
PC-3p-47249_125	CTTCATTCTAGAACTGTGCCCGTGA	AAGGAAAAATCATGGTTACATTCGGTATGGGGTATCGGAGACAATGCTTCATTCTAGAACTGTGCCCGTGAAATTTCG	chr12	-	18790977	18791056	44	89	48	66	12	18

		GA										
PC-3p-5289_1028	TAACGATACAGCAGACGAACTCGGGC	ACGGCCCGATTCCGGTGCATGGTTACGGCAGAGTCGGCTCGCTTCTGCCGCCGCATCTGGCCCGAGTAACGATACAGCAGACGGACTCGGGCAGG	chr1	-	2851730	2851824	68	63	49	72	9	4
PC-3p-5463_997	TTAAGTTGGTACAACATGAAGT	TTAGGTTAACTTAATTGTTTTATGTTTAATCAACTTAAATTTGGAAGTTAACTTGATTTAAGTTGGGACAACATGAAGTGATTGTGTGTAACCCTG	chr5	-	69778926	69779021	0	384	204	0	0	0
PC-3p-5780_949	TGCAGAGGATGGATTGGATGACAGCT	CCAGTGATTCGTTCAGCTGTTTGGACTATTCGCTGTAGTCTACGGAATTCGGTTTTGGCAGTGGATGCAGAGGATGGATTGGATGACAGCTGAGTAGAATTGTAAG	chr20	+	20657123	20657228	110	58	47	35	52	19
PC-3p-6360_864	TTGTATGTAAGTGCAGGCTGTGGAG	TTGACGCTCCACAGCTTGTATGGCATCCACAGCAAAAGTGAATGACAGAAAAATCTGTCTGGAGCTGGAGCCTGATTGTATGTAAGTGCAGGCTGTGGAGGGTAAT	chr4	+	48683186	48683291	110	91	70	100	50	26
PC-3p-7308_758	CAAAGTGGGTCGGAGAGCTGTT	GACCAGTTCCCCGTCCTGTTGGTGTTGTAATTTTAGTCTTAACAAAGTGGGTCGGAGAGCTGTTCT	chr19	+	4916274	4916339	36	62	46	64	83	201
PC-3p-77075_73	TTCTGGTAAGTTTC	AACGTTAAATAATGGCTGTGAAATAAC	chr15	+	8498666	8498825	180	24	20	0	0	0

	TGTAATTCAACC	AGAAATTAACTGTAAAATGACATTAAGTTACAGAAATTTCCTGACATTTAAATTTCTGGTAAGTTTCTGTAATTCAACCTCTGTTTTTTTAAAGTAAATTTCTGTAAATTAATTGCCATTATTTTAAGTTTTT										
PC-3p-8524_654	CAAAAGTGTACCGAACCAAACC	TACGGTATGGTACGGTTCGGTTTGGTACGCTTTTATGGCCGTTTCCACTGTCAAAAGTGTACCGAACCAAACCGTACTGTACCACT	chr11	+	12797257	12797342	28	53	59	25	30	51
PC-3p-875_5810	TAAGATTGTTTTGCTTGCACTG	GCAAATGTGCCAGCAGAGTAAAAGCAGAATAACCTAGTTTTTGGGTTAAAATAATAAGATTGTTTTGCTTACACTGCTGGCAGATTATT	chr6	+	59694110	59694198	0	630	305	142	629	5
PC-5p-1458_3508	TGACTTTAGAGAATTTGAT	CAGTAACAATTTAAAGAAATAATTGACTTTAGAGAATTTGATTGGATTTAAGGGGAAAAAAATGTTTTGTCATCTTTCTCAAAGTCAGCTATGTCATTTGTTAGTA	chr21	+	29340397	29340502	38	190	111	212	66	23
PC-5p-14585_395	TTCGAGAATCTGTCCACCACAGTGAG	GTGCCGCCTTCGAGAATCTGTCCACCACAGTGAGGACTACTGAATTGCCACTGGATGGGGGTAACCCAGTGACAAAGTCCATCGCTATGTGTGATCAGGGTCGCGAAGGGACGG	chr13	+	19849938	19850051	50	28	33	32	32	25
PC-5p-14802_390	TTAGTGT	AGATTTAGTGTGA	chr4	+	28761860	2876193	88	40	24	25	14	12

	GAGAAAGTGACTGTAGAAG	GAAAGTGACTGTAGAAGCTTAAATGCTGGAGCCACTTGCGGAGGCATGCTCGCGCTAAAGCT				4						
PC-5p-15041_384	TCTGTTGCAACCCTGATCAAAC	CATTTCTGTTGCAACCCTGACCAAACACACCTGTTTGTAATTATCAAGTGCTGCTTTAGGTACTATTAATTGGTTCAGGTGTGTTCGATCAGGGTTGGGACTGAAT	chr19	-	30527377	30527482	0	267	0	0	0	0
PC-5p-15213_380	AATCCTGTAACCGTTGACTTCC	TTATTCAGTTTAACACAAAAGAAGATACTTTGAAGAAAGCTGAAATCCTGTAACCGTTGACTTCCACAGTCAGAAAAGCAAATGGAAGTCAATAGTTACAGGTTTCCTGCTTTTTTCAAAATATCAACTGTTTTGTTCAGCAGAA	chr23	+	19640008	19640152	46	56	62	16	22	183
PC-5p-15460_374	TACGAGAGTCAGGAGTTGGCCACT	CAGGAACCGCTGCAATGCTTTACGAGAGTCAGGAGTTGGCCACTCGGCAACAGCCCTTACCTTAGAGGGGTCAGCTTTGATCTCACC	chr13	+	28896514	28896600	129	26	54	105	19	15
PC-5p-15979_363	TCTGAATCAAAGACTGTAGAGTACTT	TCTGAATCAAAGACTGTAGAGTACTTAAAGGGACTTTATGTTTGCTTCAGTAAGTACTTTCGCTTTTTGTTTCTCA	chr4	+	62592390	62592465	65	58	8	64	15	6
PC-5p-19218_304	TGAACAATGACAGAAGAGCCT	TACAAGAGGGCCAGTGAACAATGACAGAAGAGCCTTTACCAACTTCCCTAAGATGGGGAACCTCG	chr4	+	19587679	19587780	36	51	44	30	21	12

		TTCCTCCTCCTGTCATCTTCATGGCAAAAATCTTACT										
PC-5p-19602_298	TGCAAGTAATCATGAATAGGCC	CCACCTAGTGCAAGTAATCATGAATAGGCCTCCAGGACAGACAGCCTATCCTGGATTACTTGAACAAGGGAA	chr2	-	21375529	21375600	112	25	45	0	0	49
PC-5p-22971_255	TAGCATTGAAGTTCCCCATCTGAAGG	CTTTGTAGCATTGAAGTTCCCCATCTGAAGGGGAGCGCTCCAGTTACTAAAGTAACCCTCGCTCCCCGAGGGGGGGAACGAAAATGCTATATTC	chr4	+	74307054	74307147	49	29	25	16	7	5
PC-5p-2332_2235	ATCATTTTTGTGACTATGCAACT	GCAGTCACATCTGTCTGTGCTCATTTTTGTGACTATGCAACTGGAACCAGACTACTAGCTGCAAACCACAAAAATGACACAGACAGCTGTGCAAT	chr6	-	13981004	13981098	101	98	69	49	76	120
PC-5p-4490_1200	TTTGGTAGAATACGAGGATTTGCTT	TTCTTTGGTAGAATACGAGGATTTGCTTGCTGTTGAACAAGATGCGCTGTTAATTCCTCCTGTTTTTTCAGAATT	chr24	+	15011291	15011365	113	68	58	27	84	38
PC-5p-49914_118	TTGGGCAGCTGCAGATCT	TTTTTTTAATGATTTTGGGCAGCTGCAGATCTTTCATAGTCCTCTCTGCAGTAGAGCTGCTATGGATTCAGCTGCCCTACAGTAAAGTAG	chr2	+	8657373	8657462	0	15	24	40	38	22
PC-5p-6571_838	TGTCAAGGTCCGGTACCCCTGTAGC	TGTCAAGGTCCGGTACCCCTGTAGCGTGGTTGTTATCAGTCCCTTTGTCCCTT	chr2	-	14092389	14092462	41	80	30	32	59	22

		GCAGGGGTACCG TGACCTGCT										
PC-5p-768_6590	TATTACA TACAACT GTTCCTG ACTGT	TCGGATATTACATA CAACTGTTCCTGA CTGTTCCAGCTGT TTCAGTTCTTACTG GTAATTGACATTTG TGTTGAATATTTAT	chr14	-	13319229	1331931 0	503	259	317	1392	496	384
PC-5p-8253_677	TGTTGTA GGATGT AGATGTA TAAGTC	TGTTGTAGGATGT AGATGTATAAGTC AGATAGAAATGCA TTAGGTTTTGCATA TAAATATATGTGCT TAACACATTTGTTT TAGTTTGTTATTTG CCTCTTACATG	chr4	+	67495807	6749591 2	273	83	78	118	30	33

Table S2.6. Enriched pathways of predicted targets of miRNAs up-regulated in stage IIIb follicular cells

GOTerm	Number of Genes	% Associated Genes	Term PValue	Term PValue Corrected with Bonferroni step down	Associated Genes Found	miRNAs
MAPK signaling pathway	66	20.2454	6.26E-10	6.89E-08	[arrb2a, atf7b, bdnf, cacna2d1a, cacng3b, cacng4b, cacng7a, cacng8b, casp3b, cdc25b, crk, crkl, dusp1, egf, elk4, fgf8a, fgfr1a, flnb, fosaa, fosab, gadd45ga, map2k2a, map2k5, map3k14, mapk11, mapk12b, mapk8a, mapk8b, mapk9, mapkapk2a, max, mef2cb, mknk1, nf1b, nfatc3a, nlk1, nr4a1, nras, pdgfab, pdgfbb, ppm1aa, ppm1bb, ppp3r1b, ppp5c, rac1a, rac1l, rac3b, rap1aa, rasa1a, rasgrp4, rps6ka5, rras2, si:ch211-153j24.3, si:ch211-79m20.1, si:dkey-253i9.4, si:zfos-2326c3.2, tab2, taok1a, tgfb1a, tgfb1b, tgfb2, tgfbr1a, tnfrsf1a, tp53, traf6, zgc:171775]	[dre-miR-107b, dre-miR-10b-3p, dre-miR-22a-3p, dre-miR-22a-5p, dre-miR-29a, dre-miR-29b, dre-miR-734]
Endocytosis	57	16.332378	1.86E-05	1.98E-03	[amph, ap2b1, arf2b, arfgap3, arpc4l, arpc5la, arrb2a, asap1a, asap2a, bin1b, chmp7, clint1a, clta, cltca, cyth3b, dnm1b, egf, ehd1a, epn3a, erbb3a, gbf1, git2b, grk1a, il2rb, kitb, mdm2, nedd4l, pard6gb, pip5k1ab, pip5k1bb, pld1a, pld2, psd3l, rab11fip4b, sb:cb649, sh3gl1b, sh3gl3b, sh3glb1a, sh3glb1b, sh3glb2b, si:ch211-168b3.1, smap1, smurf2, snx3, snx5, tfr1a, tgfb1a, tgfb1b, tgfb2, tgfbr1a, traf6, vps37b, vps37c, wasb, wipf1b, wipf2a, zgc:114173]	[dre-miR-107b, dre-miR-10b-3p, dre-miR-218b, dre-miR-22a-3p, dre-miR-22a-5p, dre-miR-29a, dre-miR-29b, dre-miR-734, ipu-miR-7550]
Focal adhesion	56	22.310757	2.89E-10	3.24E-08	[LOC101885790, actn1, ccnd2a, chad, col1a1b, col1a2, col2a1a, col2a1b, col4a1, col4a5, col4a6, col6a1, col6a2, col9a1b, col9a3, crk, crkl, egf, flnb, fn1a,	[dre-miR-107b, dre-miR-29a, dre-miR-29b, dre-miR-10b-3p,

					fyna, itga10, itga11a, itga5, lamc1, mapk8a, mapk8b, mapk9, mylk2, mylk4a, pak4, pak6b, parvaa, parvg, pdgfab, pdgfbb, pik3ca, pik3r1, pik3r2, ppp1cab, ptenb, pxna, rac1a, rac1l, rac3b, rap1aa, rapgef1a, rock1, rock2a, shc1, si:ch211-79m20.1, tln2a, tnc, vav3b, vegfaa, vegfc]	dre-miR-22a-5p, dre-miR-734, dre-miR-22a-3p, ipu-miR-7550]
Regulation of actin cytoskeleton	48	17.204302	2.15E-05	2.26E-03	[LOC101885790, actn1, araf, arhgef4, arhgef6, arpc4l, arpc5la, cfl1, crk, crkl, egf, ezrb, fgf8a, fgfr1a, fn1a, itga10, itga11a, itga5, map2k2a, msna, mylk2, mylk4a, nras, pak4, pak6b, pdgfab, pdgfbb, pfn2, pik3ca, pik3r1, pik3r2, pikfyve, pip4k2aa, pip5k1ab, pip5k1bb, ppp1cab, pxna, rac1a, rac1l, rac3b, rock1, rock2a, rras2, si:ch211-203d1.3, si:ch211-79m20.1, tmsb, vav3b, wasb]	[dre-miR-107b, dre-miR-10b-3p, dre-miR-218b, dre-miR-22a-5p, dre-miR-29a, dre-miR-22a-3p, dre-miR-734, dre-miR-29b, ipu-miR-7550]
FoxO signaling pathway	44	25	5.40E-10	5.99E-08	[araf, cat, ccnd2a, ccng2, egf, ep300a, ep300b, fbxo25, fbxo32, foxo1a, foxo3a, g6pca.1, gabarapa, gadd45ga, il7r, irs1, map2k2a, mapk11, mapk12b, mapk8a, mapk8b, mapk9, mdm2, nlk1, nras, pik3ca, pik3r1, pik3r2, prkab1a, prkag2a, prkag2b, ptenb, rag1, rag2, sgk1, sgk2b, skp2, stat3, tgfb1a, tgfb1b, tgfb2, tgfbr1a, usp7, zgc:171775]	[dre-miR-29a, dre-miR-107b, dre-miR-22a-5p, ipu-miR-7550, dre-miR-10b-3p, dre-miR-29b, dre-miR-734, dre-miR-22a-3p]
Herpes simplex infection	37	19.892473	8.09E-06	8.73E-04	[LOC791723, arntl1b, casp3b, clocka, clockb, crfb2, csnk2a2a, csnk2a4, cul1a, ep300a, ep300b, fb06f03, fosaa, fosab, il10rb, il15, irf9, jak1, jak2b, mapk8a, mapk8b, mapk9, mavs, per1a, per1b, per2, per3, ppp1cab, si:ch211-153j24.3, skp2, srpk1b, stat1a, tab2, tnfrsf1a, tp53, traf6, usp7]	[dre-miR-29a, dre-miR-107b, dre-miR-22a-5p, ipu-miR-7550, dre-miR-10b-3p, dre-miR-29b, dre-miR-734, dre-miR-22a-3p]
AGE-RAGE signaling pathway in diabetic	35	25.362318	2.17E-08	2.36E-06	[LOC100004321, casp3b, col1a1b, col1a2, col4a1, col4a5, col4a6, fn1a,	[dre-miR-29a, dre-miR-10b-3p,

complications					foxo1a, jak2b, mapk11, mapk12b, mapk8a, mapk8b, mapk9, nras, pik3ca, pik3r1, pik3r2, plcd4a, plcg1, plcg2, prkcda, rac1a, rac1l, stat1a, stat3, stat5b, tgfb1a, tgfb1b, tgfb2, tgfbr1a, vegfaa, vegfc, zgc:171775]	dre-miR-22a-5p, dre-miR-29b, dre-miR-29a, dre-miR-107b, dre-miR-734]
Insulin signaling pathway	33	18.435755	1.18E-04	0.012031555	[araf, crk, crkl, eif4ea, fbp1a, fbp2, flot1a, flot1b, foxo1a, g6pca.1, gys1, irs1, map2k2a, mapk8a, mapk8b, mapk9, mknk1, nras, phka2, phkg1b, phkg2, pik3ca, pik3r1, pik3r2, ppp1cab, ppp1r3db, prkab1a, prkag2a, prkag2b, ptprfa, rapgef1a, shc1, si:ch211-79e4.4]	[dre-miR-10b-3p, dre-miR-22a-5p, dre-miR-29b, dre-miR-29a, dre-miR-22a-3p, dre-miR-107b, dre-miR-734, ipu-miR-7550]
Cell cycle	30	21.73913	8.36E-06	8.95E-04	[bub1bb, ccnd2a, ccne1, cdc14b, cdc16, cdc25b, cdc27, cdk6, cul1a, ep300a, ep300b, espl1, gadd45ga, mcm3, mdm2, pcna, rad21b, rb1, skp2, stag1b, stag2a, stag2b, tfdp1a, tfdp2, tgfb1a, tgfb1b, tgfb2, tp53, ywhabl, zbtb17]	[dre-miR-29a, dre-miR-107b, dre-miR-29b, dre-miR-22a-5p, dre-miR-734, dre-miR-10b-3p]
Phosphatidylinositol signaling system	27	19.014084	3.37E-04	0.033660897	[cds1, dgkaa, inpp5kb, ip6k1, ippk, itpk1a, itpka, itpkb, itpr1a, itpr1b, mtmr3, mtmr6, ocrl, pi4k2a, pik3ca, pik3r1, pik3r2, pikfyve, pip4k2aa, pip5k1ab, pip5k1bb, plcd4a, plcg1, plcg2, ppip5k1a, ptenb, synj1]	[dre-miR-29a, dre-miR-734, dre-miR-107b, dre-miR-10b-3p, ipu-miR-7550, dre-miR-22a-5p, dre-miR-29b, dre-miR-22a-3p]
TGF-beta signaling pathway	22	21.359222	1.93E-04	0.019531448	[acvr1ba, acvr2aa, bmpr1aa, bmpr1ab, chd, cul1a, ep300a, ep300b, id4, nog2, nog3, rock1, smad1, smad5, smad7, smurf2, tfdp1a, tgfb1a, tgfb1b, tgfb2, tgfbr1a, zgc:56064]	[dre-miR-10b-3p, dre-miR-29b, dre-miR-29a, dre-miR-22a-5p, dre-miR-734, dre-miR-107b]

Salmonella infection	21	20.79208	3.66E-04	0.035829694	[arpc4l, arpc5la, flnb, fosaa, fosab, mapk11, mapk12b, mapk8a, mapk8b, mapk9, pfn2, rac1a, rac1l, rhogc, rhogd, rock1, rock2a, si:ch211-153j24.3, tjp1b, wasb, zgc:171775]	[dre-miR-10b-3p, dre-miR-218b, dre-miR-29a, dre-miR-22a-3p, dre-miR-22a-5p, ipu-miR-7550, dre-miR-734, dre-miR-29b]
ECM-receptor interaction	20	24.691359	3.49E-05	3.63E-03	[chad, col1a1b, col1a2, col2a1a, col2a1b, col4a1, col4a5, col4a6, col6a1, col6a2, col9a1b, col9a3, fn1a, hspg2, itga10, itga11a, itga5, lamc1, sdc4, tnc]	[dre-miR-218b, dre-miR-107b, dre-miR-29b, dre-miR-29a, dre-miR-22a-5p, dre-miR-10b-3p]
p53 signaling pathway	17	22.972973	3.39E-04	0.033517685	[LOC100004321, casp3b, ccnd2a, ccne1, ccng2, cd82a, cdk6, ei24, gadd45ga, gorab, gtse1, igfbp3, mdm2, pmaip1, ptenb, sesn3, tp53]	[dre-miR-10b-3p, dre-miR-22a-5p, dre-miR-29a, dre-miR-107b, dre-miR-22a-3p, dre-miR-734, dre-miR-29b, ipu-miR-7550]
Base excision repair	12	32.432434	7.77E-05	0.008005749	[apex1, apex2, lig1, lig3, parp3, pcna, polb, pold3, poll, tdg.1, tdg.2, zgc:110269]	[dre-miR-29a, dre-miR-22a-3p, dre-miR-734, dre-miR-29b]

Table S2.7. Enriched pathways of predicted targets of miRNAs down-regulated in stage IIIb follicular cells

Pathways	Number of Genes	% Associated Genes	Term PValue	Term PValue Corrected with Bonferroni step down	Associated Genes Found	miRNAs
Endocytosis	64	18.338108	8.27E-08	8.85E-06	[adrb2a, ap2m1a, arap3, arf6b, arpc4, arpc4l, arrb2a, asap1a, capza1b, cav3, cdc42, clint1a, cxcr4a, cyth1b, dnajc6, dnm1a, dnm1b, ehd1a, epn1, fam21c, fgfr2, gbf1, git1, grk1a, hrasa, hsp70l, il2rb, mdm2, nedd4l, pard6gb, pld2, psd2, psd3l, rab11a, rab11bb, rab11fip4b, rnf41, sh3gl1b, sh3glb1a, sh3glb1b, sh3glb2a, sh3glb2b, si:ch211-204c21.1, smap1, smurf2, snx3, snx5, snx6, spg21, stam2, stambpa, tfr1a, tgfbr2, unm_sa1614, vps25, vps26b, vps35, vps37c, vps45, waslb, wipf1b, wipf2a, zgc:114173, zgc:91909]	[dre-miR-142a-3p, dre-miR-144-5p, dre-miR-16a, dre-miR-190a, dre-miR-20a-5p, dre-miR-2188-3p, dre-miR-2188-5p, dre-miR-451, dre-mir-451-p3, dre-miR-724, PC-5p-19218_304]
MAPK signaling pathway	59	18.09816	4.59E-07	4.87E-05	[arrb2a, bdnf, cacng6a, casp3a, casp3b, cdc25b, cdc42, chuk, crk, crkl, daxx, fgf13a, fgf3, fgf4, fgf8a, fgfr1a, fgfr2, gadd45ab, grb2b, hrasa, hsp70l, jund, map2k1, map2k2a, map3k14, map3k3, map3k5, mapk1, mapk8b, mapkapk2a, mef2cb, mknk1, nfatc3a, nlk1, nr4a1, nras, pdgfab, ppm1aa, ppm1na, ppp3cb, ppp3r1b, ppp5c, prkacab, prkacba, rac1a, rac1l, rac3b, raf1a, rap1aa, rasgrp4, rps6ka3b, rps6ka5, si:dkey-253i9.4, si:zfos-2326c3.2, sos1, tab2, tgfbr2, tp53, zgc:55558]	[dre-miR-142a-3p, dre-miR-144-5p, dre-miR-16a, dre-miR-181a-3p, dre-miR-190a, dre-miR-20a-5p, dre-miR-2188-3p, dre-miR-2188-5p, dre-miR-451, dre-mir-451-p3, dre-miR-724]
Regulation of actin cytoskeleton	51	18.27957	2.65E-06	2.73E-04	[actn1, araf, arhgef4, arhgef6, arpc4, arpc4l, cdc42, chrm3a, crk, crkl, f2, fgf13a, fgf3, fgf4, fgf8a, fgfr1a, fgfr2, git1, hrasa, ins, itga10, itgb4, map2k1, map2k2a, mapk1, msna, mylk3, mylk4b, nckap1, nras, pdgfab, pik3r2, pik3r3a, pik3r3b, pik3r5, pikfyve, pip4k2aa, ptk2ab, pxna, rac1a,	[dre-miR-142a-3p, dre-miR-144-5p, dre-miR-16a, dre-miR-181a-3p, dre-miR-190a, dre-miR-20a-5p, dre-miR-2188-3p,

					rac1l, rac3b, raf1a, scinla, scinlb, si:ch211-175f11.5, sos1, spata13, wasf2, waslb, zgc:55558]	dre-miR-2188-5p, dre-miR-451, dre-mir-451-p3, dre-miR-724]
FoxO signaling pathway	44	25	5.83E-10	6.35E-08	[araf, cat, ccnb1, ccnb2, ccnd1, cdkn1a, cdkn1bb, chuk, fbxo25, foxo1a, foxo3a, gabarapa, gabarapb, gadd45ab, grb2b, homer3, hrasa, il7r, ins, map2k1, map2k2a, mapk1, mapk8b, mdm2, nlk1, nras, pdpk1b, pik3r2, pik3r3a, pik3r3b, pik3r5, plk2b, plk3, prkab1a, prkag1, prkag2a, ptenb, raf1a, rag1, rbl2, sos1, tgfbr2, usp7, zgc:55558]	[dre-miR-142a-3p, dre-miR-144-5p, dre-miR-16a, dre-miR-181a-3p, dre-miR-190a, dre-miR-20a-5p, dre-miR-2188-3p, dre-miR-2188-5p, dre-miR-451, dre-mir-451-p3, dre-miR-724]
Focal adhesion	43	17.131474	8.97E-05	0.008614785	[actn1, birc2, cav3, ccnd1, cdc42, chad, col1a1b, crk, crkl, grb2b, hrasa, itga10, itgb4, lama4, map2k1, mapk1, mapk8b, mylk3, mylk4b, parvaa, pdgfab, pdpk1b, pgfb, pik3r2, pik3r3a, pik3r3b, pik3r5, ptenb, ptk2ab, pxna, rac1a, rac1l, rac3b, raf1a, rap1aa, shc1, sos1, thbs1b, tln1, tln2a, vegfaa, vegfc, vwf]	[dre-miR-142a-3p, dre-miR-144-5p, dre-miR-16a, dre-miR-181a-3p, dre-miR-190a, dre-miR-20a-5p, dre-miR-2188-3p, dre-miR-2188-5p, dre-miR-451, dre-mir-451-p3, dre-miR-724]
Insulin signaling pathway	43	24.022346	3.45E-09	3.72E-07	[araf, calm3a, crk, crkl, eif4e1c, eif4ea, fasn, fbp1a, flot1a, flot1b, flot2b, foxo1a, grb2b, gys1, hrasa, ins, map2k1, map2k2a, mapk1, mapk8b, mknk1, nras, pdpk1b, phkg1a, pik3r2, pik3r3a, pik3r3b, pik3r5, ppp1r3ab, ppp1r3da, ppp1r3db, prkab1a, prkacab, prkacba, prkag1, prkag2a, ptprfa, pygb, raf1a, rps6kb1a, shc1, sos1, zgc:55558]	[dre-miR-142a-3p, dre-miR-144-5p, dre-miR-16a, dre-miR-190a, dre-miR-20a-5p, dre-miR-2188-3p, dre-miR-2188-5p, dre-miR-451, dre-mir-451-p3, dre-

						miR-724, PC-5p-19218_304]
Apoptosis	37	20.786516	2.52E-06	2.62E-04	[LOC100004321, baxa, birc2, casp2, casp3a, casp3b, casp7, chuk, cycsb, dab2ipb, daxx, eif2ak3, gadd45ab, hrasa, lmna, lmnb2, lmnl3, map2k1, map2k2a, map3k14, map3k5, mapk1, mapk8b, nras, parp1, parp2, pdpk1b, pik3r2, pik3r3a, pik3r3b, pik3r5, raf1a, spna2, tp53, tuba8l4, zgc:55558, zgc:63831]	[dre-miR-142a-3p, dre-miR-144-5p, dre-miR-16a, dre-miR-190a, dre-miR-20a-5p, dre-miR-2188-3p, dre-miR-2188-5p, dre-miR-451, dre-mir-451-p3, dre-miR-724, PC-5p-19218_304]
Protein processing in endoplasmic reticulum	36	19.78022	1.25E-05	0.001245155	[LOC100004321, amfr, atf6, bag1, baxa, calr3a, cul1b, dnajb12a, dnajb1b, dnajc5gb, eif2ak3, ganab, herpud1, hsp70l, hyou1, map3k5, mapk8b, mbtps2, nfe2l2a, rnf185, sar1ab, sec23a, sec23b, sec24b, sel1l, stt3a, stt3b, syvn1, ube2d1a, ube2j1, ubxn6, wfs1b, yod1, zgc:100906, zgc:175088, zgc:77086]	[dre-miR-142a-3p, dre-miR-144-5p, dre-miR-16a, dre-miR-190a, dre-miR-20a-5p, dre-miR-2188-3p, dre-miR-2188-5p, dre-miR-451, dre-mir-451-p3, dre-miR-724, PC-5p-19218_304]
mTOR signaling pathway	34	17.616581	2.30E-04	0.021825993	[akt1s1, atp6v1h, chuk, depdc5, deptor, eif4e1c, eif4ea, fnip1, fzd3a, fzd3b, grb2b, hrasa, ins, map2k1, map2k2a, mapk1, mlst8, nras, pdpk1b, pik3r2, pik3r3a, pik3r3b, pik3r5, ptenb, raf1a, rps6ka3b, rps6kb1a, rragca, rragd, sos1, ulk2, wnt8b, wnt9b, zgc:55558]	[dre-miR-142a-3p, dre-miR-144-5p, dre-miR-16a, dre-miR-190a, dre-miR-20a-5p, dre-miR-2188-3p, dre-miR-2188-5p, dre-miR-451, dre-mir-451-p3, dre-miR-724, PC-5p-19218_304]

AGE-RAGE signaling pathway in diabetic complications	29	21.014492	2.20E-05	0.002179249	[LOC100004321, baxa, casp3a, casp3b, ccnd1, cdc42, cdkn1bb, col1a1b, cybb, foxo1a, hrasa, mapk1, mapk8b, nras, pik3r2, pik3r3a, pik3r3b, pik3r5, plcd1a, plcd4a, plcg2, rac1a, rac1l, stat1a, stat1b, tgfbr2, vegfaa, vegfc, zgc:55558]	[dre-miR-142a-3p, dre-miR-144-5p, dre-miR-16a, dre-miR-190a, dre-miR-20a-5p, dre-miR-2188-3p, dre-miR-2188-5p, dre-mir-451-p3, dre-miR-724, PC-5p-19218_304]
ErbB signaling pathway	27	23.275862	5.50E-06	5.61E-04	[abl2, araf, camk2d1, camk2g2, cdkn1a, cdkn1bb, crk, crkl, grb2b, hrasa, map2k1, map2k2a, mapk1, mapk8b, nck1b, nras, pik3r2, pik3r3a, pik3r3b, pik3r5, plcg2, ptk2ab, raf1a, rps6kb1a, shc1, sos1, zgc:55558]	[dre-miR-142a-3p, dre-miR-144-5p, dre-miR-16a, dre-miR-190a, dre-miR-20a-5p, dre-miR-2188-3p, dre-miR-2188-5p, dre-miR-451, dre-mir-451-p3, dre-miR-724, PC-5p-19218_304]
Progesterone-mediated oocyte maturation	25	21.73913	5.44E-05	0.005280876	[adcy7, araf, ccnb1, ccnb2, cdc25b, cdc27, cdk1, gnai2b, gnai3, ins, mad1l1, map2k1, mapk1, mapk8b, pik3r2, pik3r3a, pik3r3b, pik3r5, prkacab, prkacba, raf1a, rps6ka3b, si:ch211-132f19.7, si:dkey-206f10.1, zgc:55558]	[dre-miR-142a-3p, dre-miR-144-5p, dre-miR-16a, dre-miR-190a, dre-miR-20a-5p, dre-miR-2188-3p, dre-miR-2188-5p, dre-miR-451, dre-mir-451-p3, dre-miR-724, PC-5p-19218_304]
TGF-beta signaling	23	22.330097	5.42E-05	0.005311541	[acvr1ba, acvr2aa, bambia, bmp6, bmpr1ab, chd, cul1b, fsta, gdf6a, id2b, id4,	[dre-miR-142a-3p, dre-miR-144-5p,

pathway					lft1, mapk1, ndr1, nog2, rps6kb1a, smad1, smad7, smurf2, sp1, tfdp1a, tgfbr2, thbs1b]	dre-miR-16a, dre-miR-181a-3p, dre-miR-190a, dre-miR-20a-5p, dre-miR-2188-3p, dre-miR-2188-5p, dre-miR-451, dre-mir-451-p3, dre-miR-724]
p53 signaling pathway	22	29.72973	5.12E-07	5.37E-05	[LOC100004321, baxa, casp3a, casp3b, ccnb1, ccnb2, ccnd1, ccne1, cd82a, cdk1, cdk6, cdkn1a, cycsb, gadd45ab, gtse1, igfbp3, mdm2, perp, ptenb, rprma, thbs1b, tp53]	[dre-miR-142a-3p, dre-miR-144-5p, dre-miR-16a, dre-miR-181a-3p, dre-miR-20a-5p, dre-miR-2188-3p, dre-miR-2188-5p, dre-miR-451, dre-mir-451-p3, dre-miR-724, PC-5p-19218_304]
VEGF signaling pathway	22	25.287355	9.88E-06	9.98E-04	[cdc42, hrasa, map2k1, map2k2a, mapk1, mapkapk2a, nras, pik3r2, pik3r3a, pik3r3b, pik3r5, plcg2, ppp3cb, ppp3r1b, ptk2ab, pxna, rac1a, rac1l, rac3b, raf1a, vegfaa, zgc:55558]	[dre-miR-142a-3p, dre-miR-144-5p, dre-miR-16a, dre-miR-190a, dre-miR-20a-5p, dre-miR-2188-3p, dre-miR-2188-5p, dre-miR-451, dre-mir-451-p3, dre-miR-724, PC-5p-19218_304]

CHAPTER 3

EXPRESSION AND FUNCTION OF NODAL-RELATED GENES IN ZEBRAFISH OVARY

Expression and Function of Nodal-Related Genes in Zebrafish Ovary

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Conception and design: Y. Zayed and C. Peng
Development of methodology: Y. Zayed
Acquisition of data: Y. Zayed generated data for all figures except Figure 3.1B. X. Qi generated Figure 3.1B.
Analysis and interpretation of data: Y. Zayed and C. Peng
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Study supervision: C. Peng

ABSTRACT

Nodal, a secretory protein of the transforming growth factor-β (TGF-β) superfamily, plays critical roles during embryo development. Several members of the TGF-β superfamily are known to be involved in follicle development and oocyte maturation in zebrafish; however, the role of Nodal in these processes is not known. The objective of this study was to investigate if Nodal regulates ovarian functions. Using real-time PCR, we detected nodal orthologs in zebrafish, nodal-related (ndr1) and ndr2 mRNAs in ovarian follicles at all stages of the development. We found that mRNAs for ndr1 and ndr2 and the type I receptors for Nodal, acvr1b, and acvr1c, were significantly increased in late vitellogenic follicular cells when compared to early vitellogenic follicular cells. In primary cultures of ovarian follicular cells, treatment with recombinant human Nodal resulted in the activation of Smad3, CREB and transient activation of ERK. In addition, Nodal inhibited follicular cell proliferation and increased the mRNA levels of the steroidogenic enzymes hsd3b2 and cyp17a1, as well as membrane progestin receptor, paqr8. Conversely, knockdown of *ndr1* and *ndr2* using small interfering RNAs had the opposite effects on the expression of these enzymes and on paqr8. Taken together, these findings suggest that Nodal regulates steroidogenesis in zebrafish.

INTRODUCTION

Zebrafish follicles are composed of an oocyte surrounded by two thin layers of somatic follicular cells, namely the theca and granulosa cell layers. Follicle development in zebrafish can be divided into two stages, a relatively prolonged growth phase as follicles develop and grow and a relatively shorter maturation phase of the oocytes in the fully-grown follicle (Nagahama et al., 1995). Follicle development is mainly regulated by two gonadotropins, the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH), which control follicle growth and oocyte maturation, respectively. FSH acts through binding FSH receptor (Fshr) in the follicular cells to promote estradiol production, which in turn, stimulates vitellogenin production and uptake by the oocytes, resulting in the increase in follicle size (Clelland and Peng 2009; Nagahama and Yamashita 2008). Vitellogenic follicles are categorized into two sub-groups, stage IIIa, which are maturationally incompetent, and IIIb follicles, which can respond to hormonal signals that induce oocyte maturation (Clelland and Peng, 2009). When the vitellogenic follicles are fully grown, LH binds to LH receptor (Lhcgr) in the follicular cells to stimulate the production and the conversion of 17α-hydroxyprogesterone (17α-HP) to 17α, 20β-dihydroxy-4-pregnen-3-one (DHP), known to be the most common maturation-inducing hormone (MIH) in teleosts (Nagahama et al., 1995; Senthilkumaran et al., 2002; Tanaka et al., 2002; Clelland and Peng, 2009). MIH then binds to membrane progestin receptors (mPRs) on the oocyte surface (Hanna and Zhu, 2011; Thomas, 2017) and triggers the activation of the maturation promoter factor (MPF), which then releases the oocyte from its meiotic arrest leading to its maturation (Nagahama et al., 1995; Patino

et al., 2001). Other signaling molecules in the follicular cells and/or the oocyte are also involved in modulating follicle development and oocyte maturation (Ge, 2005; Clelland and Peng, 2009).

The role of the transforming growth factor-β (TGF-β) members in the regulation of oocyte maturation in zebrafish has been reported. For example, activin has been shown to stimulate oocyte maturation (Wu et al., 2000; Pang and Ge, 2002) while TGF-β1 and BMP-15 exert inhibitory effects on oocyte maturation (Kohli et al., 2005; Clelland et al., 2006; Clelland et al., 2007). Signaling components for the TGF-β superfamily, such as type I and type II receptors and Smad proteins have been detected in zebrafish ovaries (Pang and Ge, 2002; Wang and Ge, 2003; DiMuccio et al., 2005; Kohli et al., 2005). Knockdown of Smad2 has been shown to inhibit basal, activin-induced and hCG-induced oocyte maturation, suggesting that endogenous TGF-β superfamily signaling is crucial for oocyte maturation in zebrafish (Tan et al., 2009).

Nodal is an important morphogen that belongs to the TGF-β1 superfamily of growth and differentiation factors. Although Nodal is well known for its critical role during embryogenesis (Zinski et al., 2018), it has been emerging as an important regulator of reproduction (Park and Dufort, 2013) and has been reported to act on rat granulosa cells (Wang et al., 2006). Nodal is known to signal through type II receptors (ActRIIA/B) and type I receptors, activin receptor-like 4 (ALK4) and ALK7, as well as Smad2/3 (Hill, 2018). While Nodal and its homologs, including the zebrafish homologs, Nodal related1 (ndr1) and ndr2, are known as key regulators of

vertebrate embryogenesis (Zinski et al., 2018) , the expression of Nodal and ALK7 were reported in the theca and granulosa cells of healthy human follicles, respectively (Wang et al., 2006). We have recently reported that ndr1 is among the predicted targets of microRNAs (miRNAs) that are down-regulated in late vitellogenesis as fully-grown follicles prepare to enter the maturation phase (Zayed et al., 2019). To further elucidate the roles of ndr1 and ndr2 in regulating steroidogenesis and oocyte maturation, we attempted to discern the expression patterns, molecular functions, and pathways of Nodal signaling in ovarian follicular cells. In this study, we show a potential role for Nodal in steroidogenesis in zebrafish ovarian follicles.

MATERIALS AND METHODS

Animals

Zebrafish were maintained at 28°C, under a 14 hour-light, 10-hour dark photoperiod in acrylic tanks in a high-density rack system designed especially for zebrafish (Aquaneering Inc., San Diego, USA). Fish were fed twice daily, once with brine shrimp and another with a commercial pelleted diet (Zeigler adult zebrafish diet, Zeigler Bros Inc). The use of zebrafish in this study was approved by York University's Animal Care Committee.

Zebrafish ovarian follicle isolation

Gravid female zebrafish were anesthetized using Ethyl 3-aminobenzoate methanesulfonate (E10521; Sigma Aldrich), decapitated, ovaries removed, and follicles collected. Follicles were separated into 6 main groups according to size and morphological characteristics (Selman et al., 1993): primary growth (stage I; 0.07–0.14 mm diameter), cortical alveolus (or stage II; 0.15–0.34 mm diameter), early-vitellogenic (or stage IIIa; 0.35–0.52 mm in diameter), late-vitellogenic (or stage IIIb; 0.52–0.65 mm in diameter), full-grown immature (or stage IV; 0.66–0.75 mm in diameter) follicles and mature follicles (>0.75 mm in diameter). Some experiments required the isolation of the follicular cells from early and late vitellogenic follicles, which was performed manually using fine forceps.

RNA extraction, Reverse Transcription, and Real-Time PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen) and reverse transcribed into cDNA as described previously (Qi et al., 2016). Subsequently, qRT-PCR was performed using EvaGreen qRT-PCR Master Mix (ABM) according to the manufacturer's instructions. The levels of mRNA were normalized to the zebrafish elongation factor-1-alpha (ef1α). Gene levels were measured using zebrafish specific primers (Table 3.1) Relative gene expression levels were calculated using the $2^{-\Delta\Delta}C_t$ method.

Western blot

Cell lysates were prepared from zebrafish ovarian follicular cells in lysis buffer containing proteinase and phosphatase inhibitors. Samples were subjected to SDS-PAGE using 12% gels and blotted onto Polyvinylidene Difluoride membrane (Millipore). Membranes were then probed overnight at 4°C with primary antibodies (Table 3.2). Membranes were incubated with HRP-conjugated secondary antibody (anti-rabbit for Smad2/3 and ERK1/2 or anti-mouse for CREB and β-actin) for one hour then visualized using Clarity™ Western ECL Blotting Substrate.

Table 3.1. Primers used in real-time PCR

Gene	Orientation	Sequence
ef1α	Forward	AGGACATCCGTCGTGGTAA
	Reverse	AGCCTTGGGGTTGTCTTCA
ndr1	Forward	CACAAGAGCGTTCATCATCCT
	Reverse	TGGGAAACACCAGGAATCAT
ndr2	Forward	GGTCGGGAGATTCATAGCAG
	Reverse	CGTCCTCGCTTGACTTCTTT
Fshr	Forward	TTTTCAACGACCACTGTAAGG
	Reverse	GCACATTAGAAACCTGGGAAC
lhcgr	Forward	GACGGTTCTGTCAACTCGG
	Reverse	CAGGTCTGGATAGTGGAAGTC
paqr7b	Forward	GTGGTGGAAGAAGAATGGC
	Reverse	AAAAGAAGAGGACTTGGTAGC
paqrb	Forward	CATCTTGTCTGGTTATCGTCC
	Reverse	GCCACACTGAAACTGAGGTAG
acvr2a	Forward	TTGAGGGGAAACTTGAATGC
	Reverse	TTTCAACCGTTCCTGACACA
acvr2b	Forward	TGAGTGAGTGCGTGTGAGTG
	Reverse	CTTCTTGACGAGCTGGATGG
acvr1b	Forward	GTCGGTTTGTGGTTGTGTTG
	Reverse	GAGGTTGATGCTGTTGCAGA
acvr1c	Forward	TGAAGATTTCCAGCTGCCTTA
	Reverse	TGGCATCTCAACTGTGCTATG
hsd20b	Forward	GGAGAACAGGCTGAGGTGAC
	Reverse	CTGGACACATTGACCACTCG
hsd3b1	Forward	GAGGAGAGCTTGTAAAGGAGCA
	Reverse	TAGCTGAATTTAAGGCGAGAGG
hsd3b2	Forward	GAGAAAAGCTGGTCCGACTGT
	Reverse	CTACCTCAATGCTGCTGGTGTA
hsd3b7	Forward	GGTTGGGTGATGAGAGGAGTT
	Reverse	AGTTGTAGATGGGCTTCAGGAC
cyp17a1	Forward	ACAGGGGGAATCTACCTTATC
	Reverse	CCGAGACAAACACGCACC

Table 3.2. Antibody table

Name of Antibody	Manufacturer and Catalog Number	Dilution Used
Phospho-Smad2 (Ser465/467)	Cell Signaling, 3108S	1:500
Phospho-Smad3 (Ser423/425)	Cell Signaling, 9520S	1:500
Smad2/3 Antibody	Cell Signaling, 3102S	1:500
CREB (86B10) mouse mAb	Cell Signaling, 9104S	1:1000
Phospho-CREB (Ser133) (1B6) mouse mAb	Cell Signaling, 9196S	1:1000
p44/42 MAPK (Erk1/2) Antibody #9102	Cell Signaling, 9102S	1:1000
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Cell Signaling, 9101S	1:1000
β-Actin Antibody (ACTBD11B7)	Santa Cruz Biotechnology, sc-81178	1:1000

Primary cell culture, treatment and transfection

Primary ovarian follicular cells were cultured as previously described (Qi et al., 2016). Briefly, follicles of female zebrafish were isolated and washed with Medium 199. Follicles were then cultured in Medium 199 supplemented with 10% fetal bovine serum (FBS) at 28°C in 5% CO2 for 6 days for the follicle cells to proliferate. Cultured cells were sub-cultured in 12-well plates at a density of 2 X 10^5 per well for 24 hours then treated with recombinant human Nodal, recombinant human TGF-β or transfected with siRNAs oligomers. Cells were transiently transfected with 100 nM siRNAs that target ndr1 (CGCAAACCCGAUUAACUCA) or ndr2 (CGGAGAGAUGAUUCUGAGA) or the non-targeting Negative Control using the NanoJuice™ transfection kit (Novagen) according to the instructions provided in manufacturer's protocol. Cells were collected and processed either after 24 hours following transfection for gene analyses, or 5-120 minutes after treatment for protein analyses.

Cell growth and Cell Counting Kit-8 (CCK8) assays

Primary follicular cells were seeded in 24-well plates at the density of at 6X10^4 cells per well. Cells were then treated with recombinant human Nodal (250ng/ml) or TGF-β1 (10ng/ml) for 24 and 48 hours and cell numbers were determined by manual cell counting. For CCK-8 assays, cells were seeded in 96-well plates (1X10^4 cells per well), treated with Nodal or TGF-β1 for 24 and 48 hours, and subjected to CCK-8 reagent as per the manufacturer's instructions.

Statistical analysis

Data presented are mean + SEM. Sample numbers are indicated in figure legends. All experiments were performed with at least three biological replicates. Student's t-test was used for comparison between two groups. $p<0.05$ was considered significant.

RESULTS

Expression pattern of ndr1 and ndr2 in zebrafish follicles

To determine whether Nodal is expressed during follicle development in zebrafish, follicles were separated into five stages, as described by Selman et. al (Selman et al., 1993), and the stage III follicles were further subdivided into IIIa (early vitellogenic) and IIIb (mid- to late vitellogenic), where IIIa follicles are unresponsive to maturation hormones while IIIb follicles are maturationally competent and are capable of undergoing oocyte maturation upon induction with maturation hormones (Figure 3.1A). Total RNA was extracted from the different developmental stages and the expression patterns of two zebrafish Nodal orthologous, *ndr1* and *ndr2*, were determined using real-time PCR. Overall, ndr1 showed a steady decrease in stages I-IV and increased again at stage V, while ndr2 levels increased in stage II then steadily decreased throughout the rest of folliculogenic stages (Figure 3.1B).

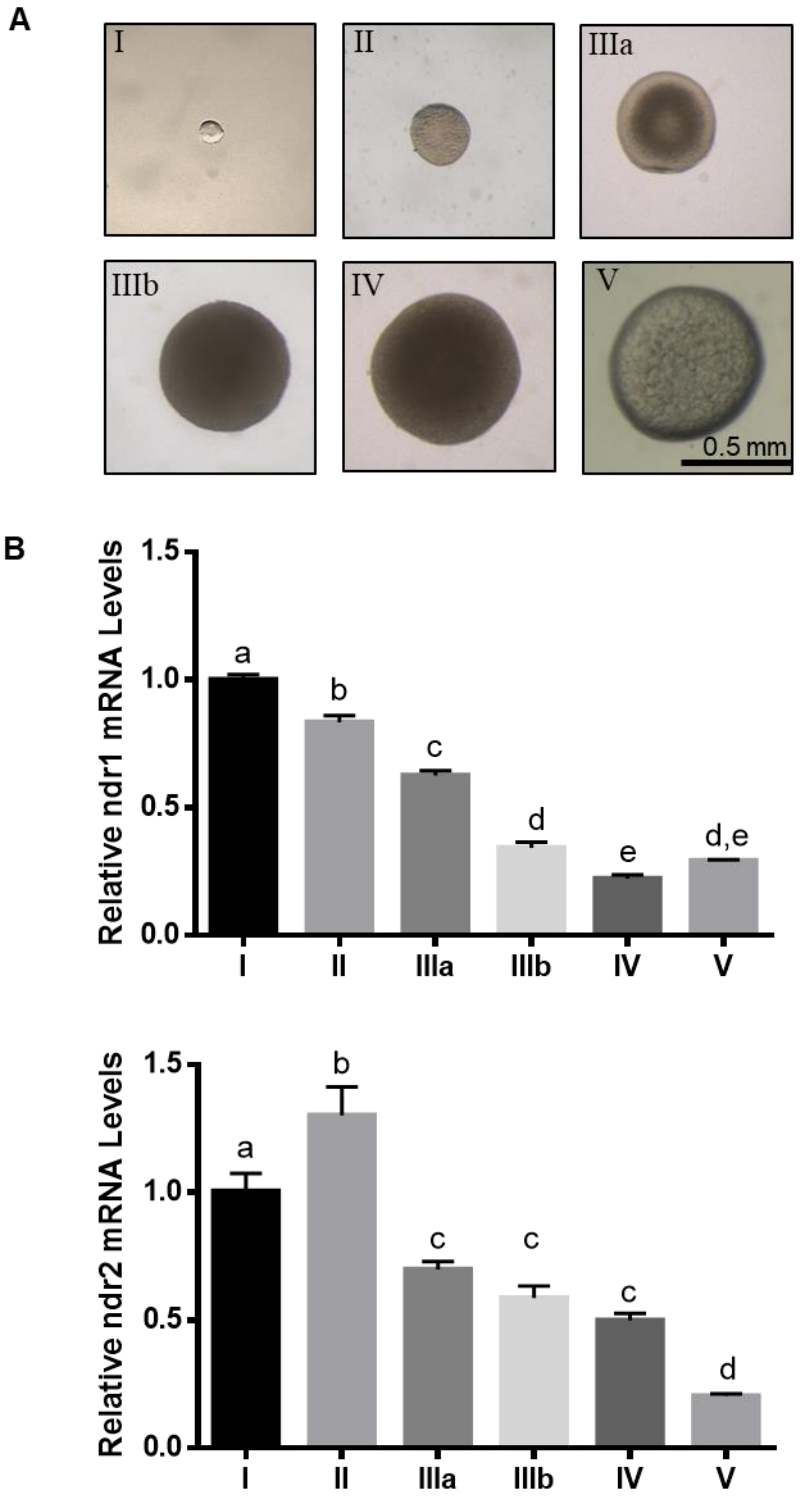

A
I
II
IIIa
IIIb
IV
V
0.5 mm
B
Relative ndr1 mRNA Levels
1.5
1.0
0.5
0.0
a
b
c
d
e
d,e
I
II
IIIa
IIIb
IV
V
Relative ndr2 mRNA Levels
1.5
1.0
0.5
0.0
a
b
c
c
c
d
I
II
IIIa
IIIb
IV
V

Figure 3.1. Ndr1 and ndr2 mRNA levels in different stages of follicle development.

A) A picture of the different follicular stages in the zebrafish ovary. B) Relative mRNA expression of ndr1 and ndr2 in the different developmental stages of zebrafish follicles measured by real-time PCR. Data represent mean + SEM (n=3). Different letters above bars indicate statistical significance ($P < 0.05$ versus control).

Nodal signaling components in follicular compartments

To determine whether nodal signaling components are present in the different compartments of vitellogenic follicles, follicular cells were separated from the oocytes of stages IIIa and IIIb, total RNA was extracted, and real-time PCR was performed. Both ndr1 and ndr2 mRNA levels were upregulated in IIIb follicular cells but downregulated in IIIb oocytes. Similarly, acvr1b and acvr1c showed an increase in mRNA expression levels in stage IIIb follicular cells but a mild decrease in the oocytes. However, acvr2a and acvr2b mRNA levels decreased significantly in the IIIb follicular cells. Meanwhile, acvr2a levels did not change while acvr2b levels increased in IIIb oocytes (Figure 3.2).

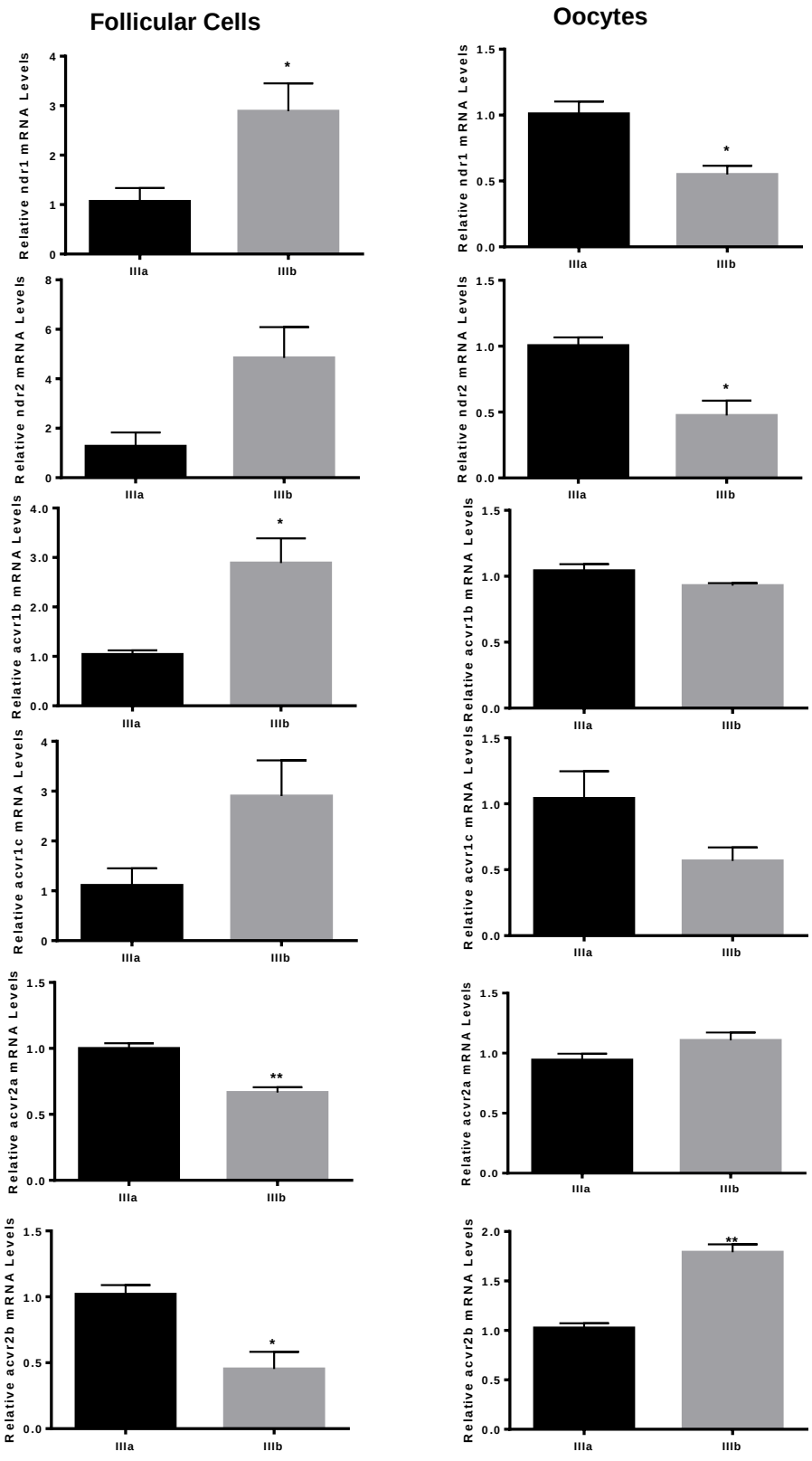

Follicular Cells
Oocytes
Relative ndr1 mRNA Levels
Relative ndr2 mRNA Levels
Relative acvr1b mRNA Levels
Relative acvr1c mRNA Levels
Relative acvr2a mRNA Levels
Relative acvr2b mRNA Levels
IIIa
IIIb
*
**

Figure 3.2. mRNA levels of ndrs and their signaling components in follicular cells and oocytes of early and late vitellogenic follicles.

Real-time PCR was used to measure mRNA expression levels of ndr1, ndr2, acvr1b, acvr1c, acvr2a and acvr2b from manually-isolated follicular cells and their denuded oocytes. Data represent mean + SEM (n=3 biological replicates). *P < 0.05; **P < 0.01 versus control, as analyzed by Student's t-test.

Effect of Nodal and TGF-β1 on Smad2/3, CREB and ERK phosphorylation in zebrafish ovarian cells

Since recombinant zebrafish nodal proteins are not available, recombinant human Nodal was used in this study. We first tested if recombinant human Nodal was active in zebrafish. Nodal signaling is known to induce its own expression in a positive feedback loop (Krebs et al., 2003). Therefore, we measured the mRNA levels of ndr1 and ndr2 in follicular cells after incubation with recombinant human Nodal for 24 hours. We observed a significant increase in both ndr1 and ndr2 mRNA levels after the treatment (Figure 3.3A).

Nodal and TGF-β1 have been reported to activate Smad2/3 (Wrighton et al., 2009) and ERK (van Boxtel et al., 2018) pathways. We have recently shown that CREB is involved in steroidogenesis regulation in zebrafish primary ovarian cell cultures (Qi et al., 2016). Therefore, we first tested if they activate Smad2, Smad3, and ERK. As shown in Figure 3.3B, a time-dependent incubation showed that recombinant human Nodal increased phospho (p-) Smad3, but not p-Smad2 levels while recombinant human TGF-β1 induced the phosphorylation of Smad2 but not p-Smad3. Although both Nodal and TGF-β1 increased the phosphorylation levels of CREB in a time-dependent manner, the induction levels of CREB by recombinant human Nodal generated more intense bands compared to the ones caused by recombinant human TGF-β1. The induced phosphorylation levels went down after 30 minutes of incubation (Figure 3.3B). To test whether Nodal signals through ERK pathway in ovarian follicular cells, we used Western blotting and found that

recombinant human Nodal and recombinant human TGF-β1 induced transient phosphorylation of ERK at 5 minutes, which turned into an inhibition after incubating the cells with either recombinant human Nodal or recombinant human TGF-β1 after 60 minutes (Figure 3.3C).

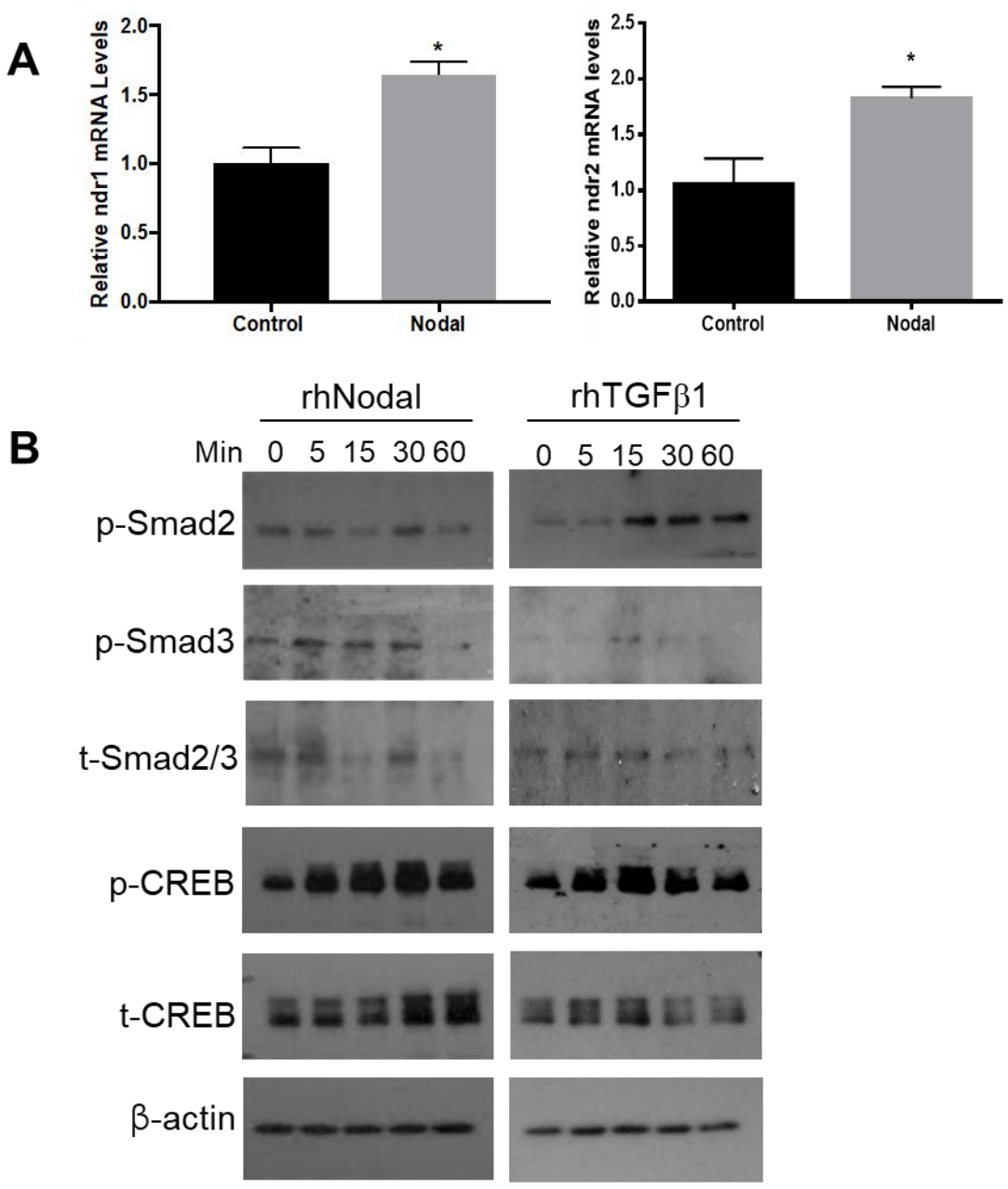

A
Relative ndr1 mRNA Levels
2.0
1.5
1.0
0.5
0.0
*
Control
Nodal
Relative ndr2 mRNA levels
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
Nodal
B
rhNodal
rhTGFβ1
Min 0 5 15 30 60
0 5 15 30 60
p-Smad2
p-Smad3
t-Smad2/3
p-CREB
t-CREB
β-actin
C
5 min
15 min
30 min
60 min
120 min
C N β1
p-ERK
t-ERK
β-actin

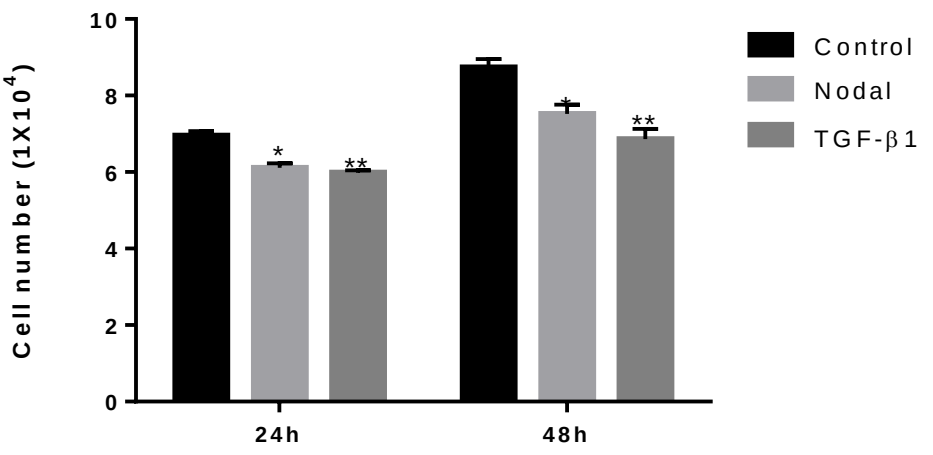
Figure 3.3. Nodal signaling pathway is active in cultured primary ovarian follicular cells

A) Relative expression levels of ndr1 and ndr2 mRNAs as measured by real-time PCR in primary ovarian follicular cells after treatment with 250 ng/ml of recombinant human Nodal for 24 hours. B) Follicular cells were treated with 250ng/ml recombinant human Nodal or 10 ng/ml recombinant human TGFB for 5-60 minutes. Phospho-Smad3, (p-Smad3), pSmad2, total (t)-Smad2/3, p-CREB and t-CREB. C) Treatment of follicular cells with 250ng/ml recombinant human Nodal or 10 ng/ml recombinant human TGFB for 5-120 minutes and p-ERK and t-ERK were detected by Western blotting. Data represent mean + SEM, with each experiment having a minimum N=3 biological replicates. Student's t-test was used. * denotes $P < 0.05$.

Nodal inhibits follicular cell proliferation:

Nodal was reported to regulate proliferation in human trophoblast cells (Nadeem et al., 2011) and ERK was reported to regulate proliferation in mouse granulosa cells (Fan et al., 2009). To assess the effect of Nodal on cell proliferation, primary follicular cells were treated and counted after treatment with recombinant human Nodal. We found that cell growth was significantly inhibited after treatment with Nodal for 24 and 48 hours (Figure 3.4A). To further confirm the inhibitory effects of Nodal on proliferation, CCK8 assays were performed. Treatment with recombinant human Nodal suppressed proliferation of ovarian follicular cells at 24 and 48 hours (Figure 3.4B). Similarly, recombinant human TGF-β1 inhibited cell proliferation as shown by CCK8 and manual cell counting (Figures 3.4A,3.4B).

A


Cell number (1X10^4)
10
8
6
4
2
0
*
**
*
**
24h
48h
Control
Nodal
TGF-β1

B

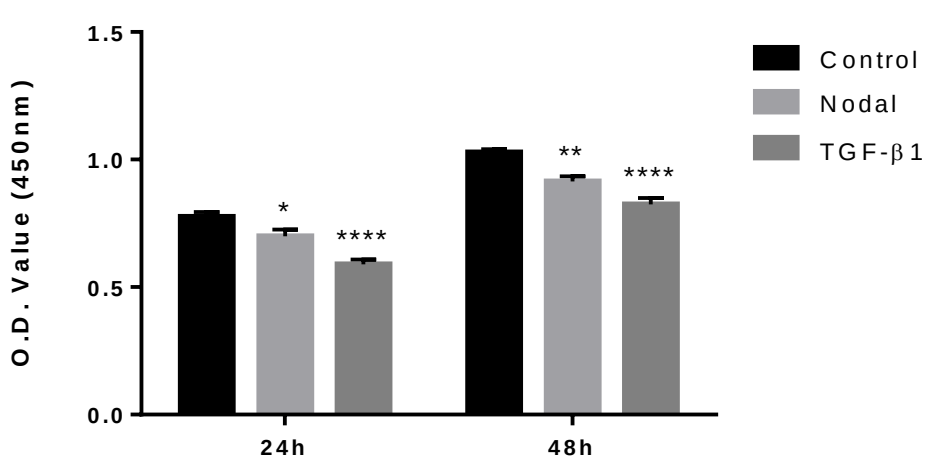

O.D. Value (450nm)
1.5
1.0
0.5
0.0
*
****
**
****
24h
48h
Control
Nodal
TGF-β1

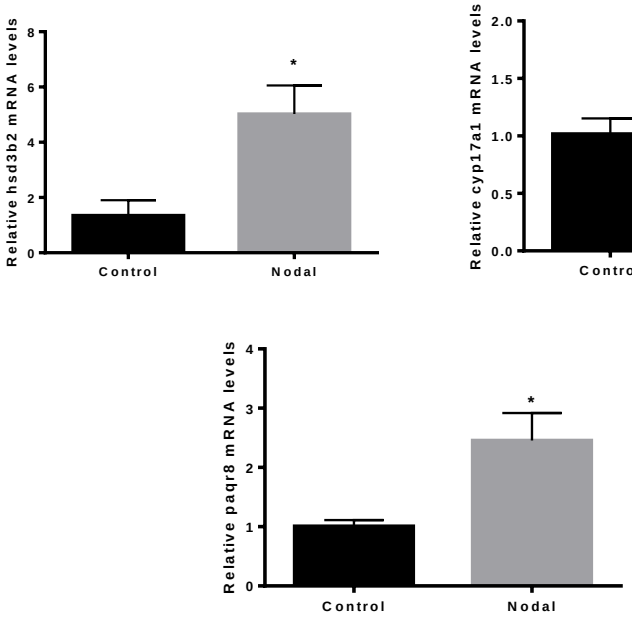
Figure 3.4. Nodal and TGF-β1 inhibit the proliferation of cultured primary ovarian follicular cells.

A) Cell proliferation of cultured primary follicular cells was measured using manual cell count after 24 and 48 hours of 250 ng/ml recombinant human Nodal and 10 ng/ml recombinant human TGF-β1 treatments. B) CCK8 assay of follicular cells treated with 250 ng/ml recombinant human Nodal and 10 ng/ml recombinant human TGF-β1 after 24 and 48 hours. Data represent mean + SEM (A, n=3; B, n=8 biological replicates). *P < 0.05; **P < 0.01; ****P < 0.0001 versus control as analyzed by ANOVA test.

Nodal regulates gene expression in follicular cells

To further investigate the role of Nodal in ovarian follicular cells, we determined the effects of Nodal on the expression of steroidogenic enzymes, as well as gonadotropin and MIH receptors. Follicular cells were first treated with recombinant human Nodal for 24 hours and mRNA levels of lhcgr, fshr, paqr7b, paqr8, hsd3b1, hsd3b2, hsd3b7, hsd20b, and cyp17a1 were determined by real-time PCR. Treatment of ovarian follicular cells with recombinant human Nodal for 24 hours led to a significant increase in the expression of hsd3b2, cyp17a1 as well as paqr8 (Figure 3.5A) while the expression of paqr7b, lhcgr, fshr, hsd20b, hsd3b1, and hsd3b7 mRNA levels were not affected (Figure 3.5B). To further confirm the effects of Nodal, we used two siRNAs to silence *ndr1* and *ndr2*, respectively. Transfection of siNdr1 siRNA resulted in a decrease in ndr1 mRNA levels. Unexpectedly, silencing of *ndr1* also reduced ndr2 mRNA levels. Similarly, siNdr2 siRNA decreased both ndr1 and ndr2 mRNA levels. Knockdown of *ndr1* and *ndr2* led to a significant decrease in the mRNA levels of hsd3b2, cyp17a1 and paqr8 (Figure 3.6) but did not affect lhcgr, fshr, hsd3b1, hsd3b7, hsd20b or paqr7b (Figure 3.7).

A


Relative hsd3b2 mRNA levels
8
6
4
2
0
*
Control
Nodal
Relative cyp17a1 mRNA levels
2.0
1.5
1.0
0.5
0.0
**
Control
Nodal
Relative paqr8 mRNA levels
4
3
2
1
0
*
Control
Nodal

B

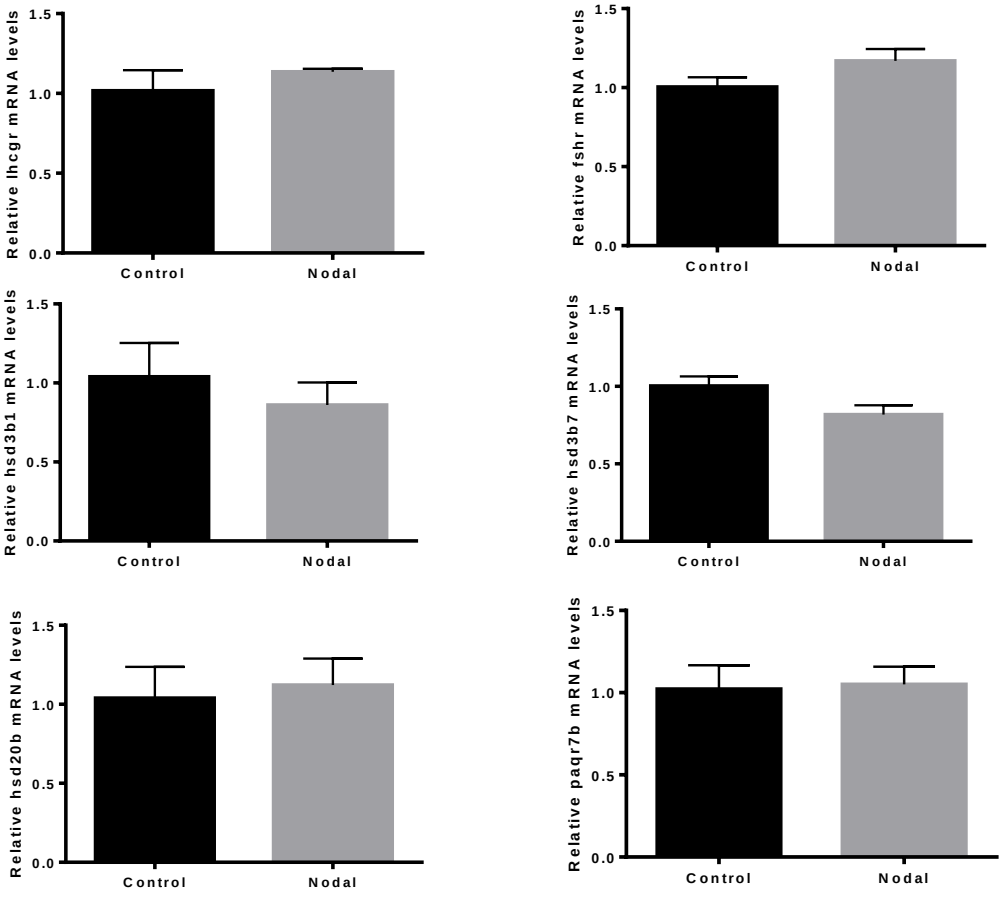
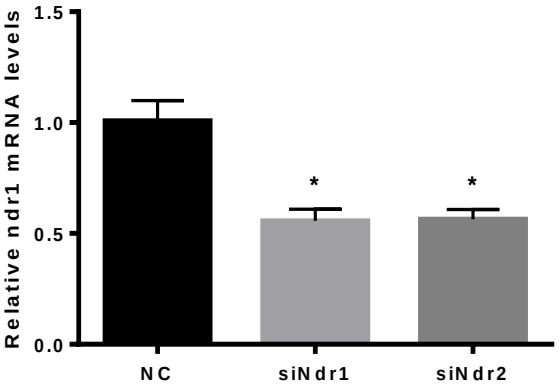
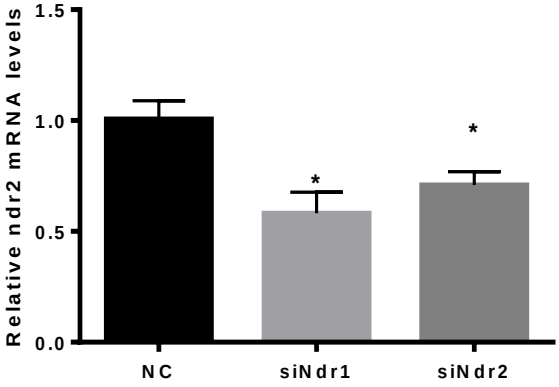
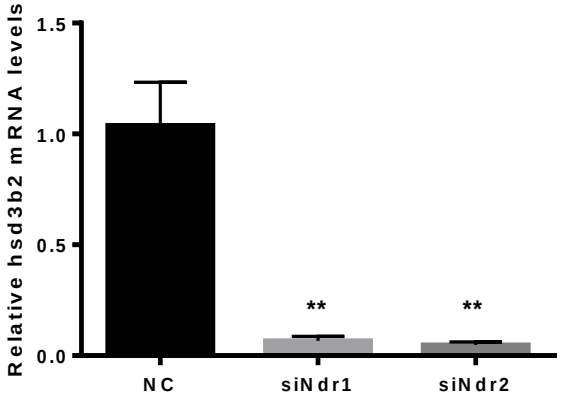

Relative lhcgr mRNA levels
1.5
1.0
0.5
0.0
Control
Nodal
Relative fshr mRNA levels
1.5
1.0
0.5
0.0
Control
Nodal
Relative hsd3b1 mRNA levels
1.5
1.0
0.5
0.0
Control
Nodal
Relative hsd3b7 mRNA levels
1.5
1.0
0.5
0.0
Control
Nodal
Relative hsd20b mRNA levels
1.5
1.0
0.5
0.0
Control
Nodal
Relative paqr7b mRNA levels
1.5
1.0
0.5
0.0
Control
Nodal

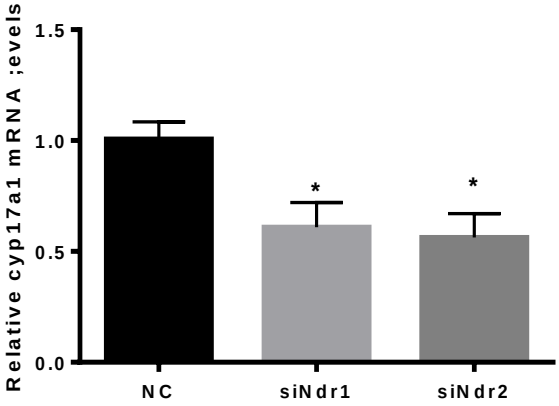
Figure 3.5. Effects of recombinant human Nodal on the gene expression in primary ovarian follicular cells

A) Ovarian primary follicular cells were treated with 250 ng/ml recombinant human Nodal for 24 hours and the mRNA levels of hsd3b2, cyp17a1, and paqr8, and B) lhcgr, fshr, hsd3b1, hsd3b7, 20bhsd, and paqr7b. Statistical analysis was performed using student's t-test. Data, mean + SEM (n=3 biological replicates). *P < 0.05; **P < 0.01 versus control.


Relative ndr1 mRNA levels
1.5
1.0
0.5
0.0
*
*
NC
siNdr1
siNdr2


Relative ndr2 mRNA levels
1.5
1.0
0.5
0.0
*
*
NC
siNdr1
siNdr2


Relative hsd3b2 mRNA levels
1.5
1.0
0.5
0.0
**
**
NC
siNdr1
siNdr2


Relative cyp17a1 mRNA ;evels
1.5
1.0
0.5
0.0
*
*
NC
siNdr1
siNdr2

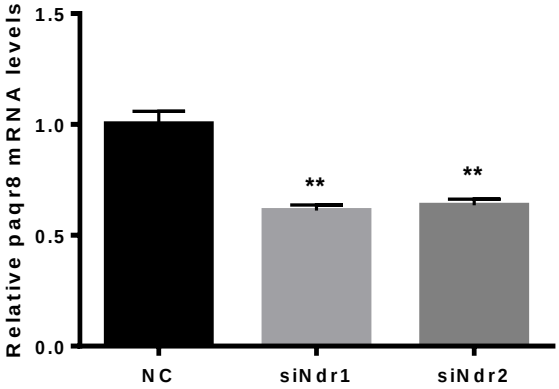
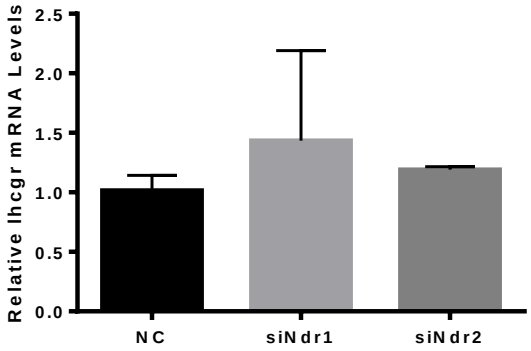
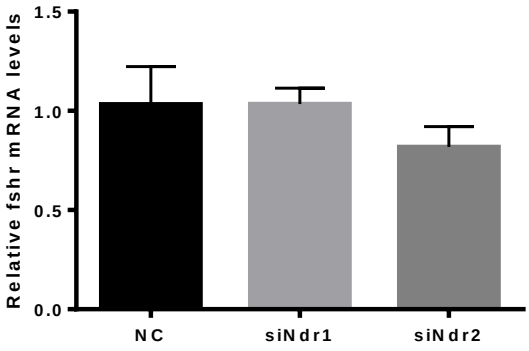
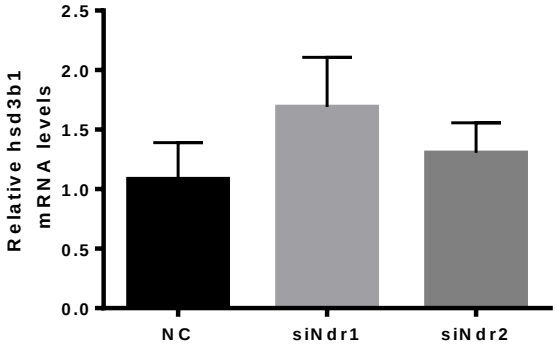

Relative paqr8 mRNA levels
1.5
1.0
0.5
0.0
**
**
NC
siNdr1
siNdr2

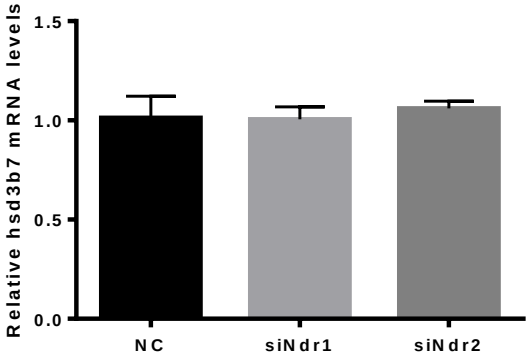
Figure 3.6. Effect of siNdr1 and siNdr2 siRNAs on the mRNA levels of ndr1, ndr2, hsd3b2, cyp17a1, and paqr8 in primary ovarian follicular cells

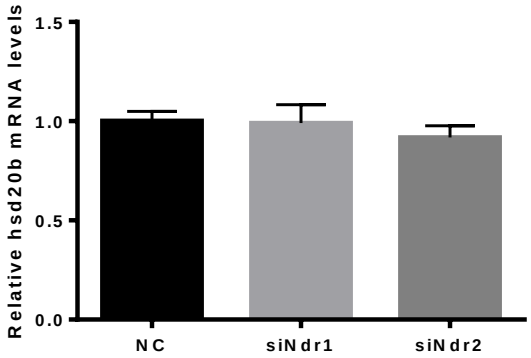
Ovarian primary follicular cells were transfected with 100 nM siNdr1 or siNdr2 siRNAs for 24 hours and the expression levels of ndr1, ndr2, hsd3b2, cyp17a1, and paqr8 mRNA were measured by real-time PCR. Data, mean + SEM (n=3 biological replicates) versus control. * denotes $P < 0.05$; **$P < 0.01$ as analyzed by ANOVA test.


Relative lhcgr mRNA Levels
2.5
2.0
1.5
1.0
0.5
0.0
NC
siNdr1
siNdr2


Relative fshr mRNA levels
1.5
1.0
0.5
0.0
NC
siNdr1
siNdr2


Relative hsd3b1 mRNA levels
2.5
2.0
1.5
1.0
0.5
0.0
NC
siNdr1
siNdr2


Relative hsd3b7 mRNA levels
1.5
1.0
0.5
0.0
NC
siNdr1
siNdr2


Relative hsd20b mRNA levels
1.5
1.0
0.5
0.0
NC
siNdr1
siNdr2

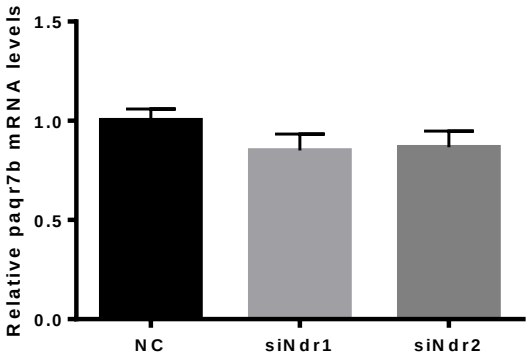

Relative paqr7b mRNA levels
1.5
1.0
0.5
0.0
NC
siNdr1
siNdr2

Figure 3.7. Effect of siNdrs on lhcgr, fshr, hsd3b1, hsd3b7, hsd20b and paqr7b in primary ovarian follicular cells.

Expression levels of lhcgr, fshr, hsd3b1, hsd3b7, hsd20b, and paqr7b mRNA were measured by real-time PCR in ovarian primary follicular cells after transfection with 100 uM siNdr1 or siNdr2 for 24 hours. Data represent mean + SEM (n=3 biological replicates). ANOVA test was used for statistical analysis.

DISCUSSION

This study describes the potential role of Nodal in ovarian follicular cells. Zebrafish Nodal homologs, ndr1 and ndr2, and Nodal signaling components, acvr1b, acvr1c, acvr2a, and acvr2b were detected in zebrafish ovarian follicular cells and oocytes. We further showed that recombinant human Nodal is active in zebrafish cells and that Nodal activates Smad3, and CREB pathways but transiently activates and then inhibits ERK pathways. While recombinant human Nodal had inhibitory effects on the proliferation of follicular cells, it stimulated the mRNA levels of the steroidogenic enzymes, hsd3b2 and cyp17a1, and paqr8, which encodes mPRβ. Taken together, these findings point to a potentially critical role for Nodal in regulating steroidogenesis in the follicular cells.

There are 3 homologs of Nodal in zebrafish, ndr1, ndr2 and ndr3 (Schier, 2003). In this study, we focused on ndr1 and ndr2 since the mRNA expression levels of ndr3 in the follicular cells were very low (data not shown). We detected ndr1 and ndr2 mRNAs in all stages of follicle development, suggesting that they play a role in regulating the development of follicles. We further examined the mRNA expression patterns of ndr1 and ndr2 in the follicular cells or the denuded oocytes of vitellogenic follicles and found that their levels were upregulated in stage IIIb isolated follicular cells while being down-regulated in IIIb oocytes. The higher mRNA expression of ndr1 and ndr2 in early follicle development suggests a potential role for Nodal signaling in regulating early stage of follicle development. The mRNA expression levels of both ndr1 and ndr2 are downregulated in stage IIIb compared to IIIa. Given

their relatively bigger size, we observed that denuded oocytes yield a much higher quantity of extracted total mRNA than the follicular cells of the same follicles. The higher mRNA quantity in oocytes may, in turn, mask the mRNA yield of the follicular cells. For this reason, it is plausible that the mRNA expression patterns of ndr1 and ndr2 showed similar trends in stage IIIb oocytes or follicles compared to their IIIa counterparts. On the other hand, the upregulated mRNA expression of ndr1 and ndr2 in IIIb follicular cells implied that the two Nodal homologs suggests that ndr1 and ndr2 may play a role in the transition of stage IIIa to IIIb.

Nodal is known to signal through type II receptors serine/threonine kinase receptors, ActRIIA, ActRIIB, and type I receptors, ALK4 and ALK7 (Hill, 2018). Several signaling components of Nodal signaling have been reported to act in concert with other TGF-β1 ligands in zebrafish. For example, ActRIIA, ActRIIB, and ALK4 were reported to promote oocyte maturational competence as part of activin signaling (Wang and Ge, 2003). In this study, we examined the expression patterns of Nodal signaling components in follicular cells and oocytes in vitellogenic follicles. When we compared the expression of these signaling components in IIIb follicular cells to their IIIa counterparts, we observed an increase in acvr1b and acvr1c matching the expression patterns of ndr1 and ndr2 while acvr2a and acvr2b were down-regulated in IIIb. In contrast, this expression pattern was reversed in IIIb oocytes as acvr1b and acvr1c were down-regulated and acvr2a and acvr2b were up-regulated. Despite the lack of more information regarding the regulation of these transcripts, the dynamic nature of their expression implies that they are involved in

regulating follicle development and oocyte maturation. The expression of acvr2a and acvr2b was found predominantly in fully grown oocytes rather than in follicular cells (Tse and Ge, 2010). It is probable that ActRIIA and ActRIIB are mainly needed in the oocyte rather than in the follicular cells of maturing follicles. One possible explanation is their regulation by gonadotropins as treatment of zebrafish follicles with human chorionic gonadotropin induced dose- and time-dependent increases in ActRIIA and ActRIIB mRNA (DiMuccio et al., 2005). More studies will be conducted in the future to discern the possible underlying mechanisms of the potential regulation of Nodal signaling components by gonadotropins.

Human Nodal is present in cells as homodimeric proproteins, which are cleaved into mature ligands extracellularly (Shen, 2007). Alignment of the mature peptides of Nodal to the mature regions of ndr1 and ndr2 revealed an identity of 57% and 67%, respectively. Thus, it was essential to determine whether recombinant human Nodal is functional in zebrafish. Treatment of primary ovarian follicular cells with recombinant human Nodal showed that there was an upregulation in the mRNA levels of ndr1 and ndr2. Nodal has been reported to induce its own expression (Zinski et al., 2018). The observed upregulation of ndr1 and ndr2 upon recombinant human Nodal treatment suggests that Nodal is indeed active in zebrafish cells and confirms that Nodal can induce its own expression.

Nodal and TGF-β1 are known to activate Smad2/3. We have previously shown that TGF-β1 inhibits oocyte maturation (Kohli et al., 2003) and activated Smad2/3 signaling (Tan et al., 2009). Thus, we compared potential signaling activated by

recombinant human Nodal or recombinant human TGF-β1 in primary cultures of ovarian follicular cells. We found that recombinant human Nodal activated Smad3 signaling while recombinant human TGF-β1 induced Smad2 signaling in follicular cells. While both recombinant human Nodal and TGF-β1 activated CREB, the activation signal was much stronger with recombinant human Nodal than with recombinant human TGF-β1. We further demonstrated that recombinant human Nodal and recombinant human TGF-β1 transiently activated then inhibited ERK pathway. Both ERK and CREB have been reported to be involved in the regulation of steroidogenesis (Manna et al., 2002; Duarte et al., 2014). Nodal signaling has been shown to act in concert with ERK signaling during embryogenesis (Poulain et al., 2006) and human Nodal has been shown to induce ERK phosphorylation in BeWo cells (Quail et al., 2014). ERK is also known to modulate proliferation cessation in granulosa cells (Karlsson et al., 2010). Therefore, Nodal signaling may play a vital role in regulating physiological and molecular processes in ovarian follicular cells. These findings also suggest that both Nodal and TGF-β1 act on zebrafish ovarian follicular cells and activate overlapping and different signaling pathways. The role of Smad2/3, ERK, and CREB in ovarian follicular cells will be studied in the future.

Using primary cultures of zebrafish follicular cells, we found that recombinant human Nodal inhibited the proliferation of the primary follicular cells. These results are in accord with a previous report of Nodal inhibiting proliferation in human trophoblast cells (Nadeem et al., 2011). In addition, using recombinant human Nodal treatment and siRNA-mediated gene silencing approaches, we demonstrated that

Nodal induces hsd3b2, cyp17a1, mRNA levels. Hsd3b2, which is evolutionarily similar to human HSD3b1, is involved in progesterone production (Lin et al., 2015). Cyp17a1 plays a role in the production of 17alpha-hydroxyprogesterone, as well as precursors for androgens (Wang and Ge, 2004). Therefore, the upregulation of these genes suggests that Nodal may induce the production of either progesterone, MIH or androgens. Future studies will measure the enzyme activity and the level of progesterone, testosterone, and MIH to confirm that Nodal regulates their production. In addition, mRNA levels of paqr8, which encodes mPRβ, were also upregulated by Nodal treatment. It was shown that mPRβ is expressed in cumulus cells and inhibition of its function impairs cumulus expansion, a process required for oocyte maturation in mammals (Qiu et al., 2008). The role of mPRβ in zebrafish follicular cells requires further investigation.

We found that siNdr1 and siNdr2 reduced both ndr1 and ndr2 mRNA levels. This is surprising as a BLAST search of both siRNAs did not find any non-specific targeting. It may be possible that ndr1 can induce ndr2 expression and vice versa. This possibility will be tested in the future using *ndr1* or *ndr2* knockout lines described in Chapter 4. We observed that both ndr1 and ndr2, as well as the type I receptors, were upregulated in stage IIIb follicular cells. These results, together with the role of Nodal in enhancing hsd3b, cyp17a, and paqr8 expression, suggest that Nodal signaling is involved in regulating the processes that govern oocyte maturation. Future experiments will be performed to study the effect of Nodal on oocyte maturation.

In conclusion, this study provides direct evidence that Nodal signaling is active in zebrafish follicular cells and suggests that it may regulate steroidogenesis and oocyte maturation. Our findings also suggest that CREB, Smad3, and ERK pathways may mediate the actions of Nodal in ovarian follicular cells. More studies are required to fully understand the functions, as well as mechanisms of actions, of Nodal in ovarian follicle development and oocyte maturation.

CHAPTER 4

DEVELOPMENT OF A MULTIPLEXED INDUCIBLE CRISPR-ON SYSTEM FOR CONTROLLABLE GENOME EDITING OF NODAL-RELATED GENES

Development of a multiplexed inducible CRISPR-ON system for controllable genome editing of Nodal-related genes

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Keywords: Nodal, nodal-related, CRISPR/Cas9, TetON,

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Development of methodology: Y. Zayed and R. Kwong
Acquisition of data: Y. Zayed
Analysis and interpretation of data: Y. Zayed and C. Peng
Manuscript drafting and revision: Y.Zayed and C. Peng
Study supervision: C. Peng

ABSTRACT

Nodal morphogens are members of the transforming growth factor-β (TGF-β) superfamily that have been extensively studied in early development. In zebrafish, there are three Nodal homologs, ndr1, ndr2, and ndr3, which have been shown to regulate embryogenesis. However, little is known about the physiological role of Nodal in adult organisms. In order to examine the role of ndr1 and ndr2 in ovarian development *in vivo*, we used the CRISPR/Cas9 technology to develop *ndr1* and *ndr2* knockout lines. We found that the deletion of these genes caused severe defects and lethality in early development. Homozygous fish of both mutant lines displayed fully penetrant cyclopic phenotypes. Therefore, we developed a fluorescently-labeled, CRISPR-ON system that expresses a Dox-inducible Cas9-nuclease and a multiplexed sgRNA system to knockout *ndr1*, *ndr2*, and *ndr3*, either alone or in combinations. Upon Dox activation, this system successfully induced gene editing in the designated genomic loci. This system will allow for studying the functions of ndr1, ndr2, and ndr3 in adult zebrafish.

INTRODUCTION

Nodal morphogens are members of the transforming growth factor-β (TGF-β) superfamily that play conserved crucial roles in early vertebrate development. In humans and mouse, there is one copy of the Nodal gene (Zhou et al., 1993) whereas three Nodal homologs are present in zebrafish, namely, Nodal-related 1 (ndr1), ndr2 and ndr3, which are also known as squint (sqt), cyclops (cyc) and southpaw (spaw), respectively (Schier 2003). Nodal signaling has been shown to be vital for the specification of mesoderm and endoderm and in controlling left-right (LR) axis formation (Hill 2018). Although a single Nodal gene in humans and mice control these functions, the zebrafish genes seem to have specialized functions as ndr1 and ndr2 control the formation of the germ layers while ndr3 largely modulates LR axis patterning (Fan and Dougan 2007).

Holoprosencephaly (HPE) has been linked to mutations in the Nodal gene in humans (Roessler et al., 2009). In zebrafish, ndr2 is essential for the patterning of the ventral midline of the central nervous system (Dougan et al., 2003). Similar to developmental defects associated with human Nodal deficit, zebrafish *ndr2* mutants also display cyclopia and deformation in the forebrain (Tian et al., 2003; Lim et al., 2013; Turner et al., 2018). On the other hand, the role of ndr1 alone in embryogenesis is controversial. Mutations in *ndr1* produced no phenotype in any *ndr1* homozygous mutants (Dougan et al., 2003). Meanwhile, a cyclopic phenotype was observed in some homozygous mutants while other homozygous mutant embryos developed normally (Aquilina-Beck et al., 2007). More recently, transcription

activator-like effector nucleases (TALENs) and zinc-finger nucleases (ZFNs) were used to generate mutant ndr1 lines, which displayed a cyclopic phenotype with penetrance rate that ranged from 0 to 24.1% (Lim et al., 2013). A non-coding RNA generated by the ndr1 transcript was suggested to play a role in dorsal specification (Lim et al., 2012). However, this claim was not supported by a recent study (Goudarzi et al., 2019). Nonetheless, embryos with homozygous mutations in both *ndr1* and *ndr2* displayed more severe defects than either mutation alone (Dougan et al., 2003). While the penetrance of human HPE is displayed in heterozygous states, the same phenotype has not been reported in heterozygous fish for either *ndr1*, *ndr2*, or *ndr3*.

Increasing evidence suggests that Nodal plays a role in regulating reproduction (Park and Dufort 2013). In human trophoblasts, Nodal acts through a type I receptor, activin receptor-like kinase 7 (ALK7), to inhibit proliferation, migration, and invasion, and to induce apoptosis (Nadeem et al., 2011; Munir et al., 2004; Nadeem et al., 2013). Nodal has also been reported to induce apoptosis in rat granulosa cells (Wang et al., 2006). Conditional knockout of Nodal in mouse uterus impaired placental development and reduced pregnancy rate (Park et al., 2012). In chapter 3, we found that ndr1 and ndr2 were expressed in zebrafish follicular cells and oocytes and their levels were regulated during follicle development. We also found that Nodal regulated the expression of, hsd3b2, cyp17a1, and paqr8 in primary cultures of ovarian follicular cells (Zayed et al., Chapter 3). These findings suggest that Nodal regulates ovarian physiology in zebrafish.

Several approaches have been developed to temporally manipulate gene expression in zebrafish. The use of heat shock *hsp70l* promoter is one of the common methods to acquire temporal control on gene expression. However, it was reported that its expression control was lost at permissive temperatures (Hans et al., 2011). Another system is estrogen receptor-controlled Cre (CreER) (Hans et al., 2009), but its inducing reagent, Tamoxifen, can bind to estrogen receptor and may interfere with estrogen functions (Jo et al., 2018). Moreover, ecdysone receptor-Gal4 chimeric protein to control Gal4 activity in the Gal4/UAS system was reported, yet, it maintains sporadic expression (Esengil et al., 2007). The tetracycline (Tet)-inducible Tet-ON system has also been used in zebrafish. Although leaky expression has been reported in one of two transgenic lines (Huang et al., 2005), this inducible system possesses the ability to reverse its expression upon drug removal and therefore allows temporal control of transgene expression (Wehner, Jahn, and Weidinger 2015).

Recently, a breakthrough in genome-editing technologies with Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and the CRISPR-associated protein 9 (Cas9) has provided a powerful and efficient gene targeting (Jinek et al., 2012). CRISPR/Cas9 technology utilizes a synthetic single-guide RNA (sgRNA) that can be designed to direct the Cas9 nuclease to a specific region in the DNA. The Cas9 protein, in turn, causes double-strand breaks (DSB) in the genome, which induces insertion and/or deletion (indel) mutations in the targeted site (Wang et

al., 2013). Particularly, the use of CRISPR technology has been established in zebrafish (Chang et al., 2013; Jao, Wente, and Chen 2013; Hwang et al., 2013).

In Chapter 3, we detected active Nodal signaling in zebrafish follicular cells and found that Nodal regulated the expression of genes involved in steroidogenesis and oocyte maturation. These findings suggest that nodal signaling plays a role in regulating zebrafish ovarian functions. In order to study the functions of *ndr* genes *in vivo,* we used CRISPR/Cas9 technologies to delete *ndr1* or *ndr2* and observed strong embryonic defects and lethality during early development. Therefore, we aimed to generate an inducible system to knockout *ndr* genes during the adult stage. We introduced Cas9 nuclease into a Tet-inducible vector that also expresses the yellow YPet fluorescent protein (Wehner, Jahn, and Weidinger 2015) to allow rapid screening using fluorescent signals and a sgRNA scaffold that targets either *ndr1*, *ndr2* or *ndr3* individually, or multiplexed in pairs or all three simultaneously. We show here that the inducible CRISPR-ON system caused modification to the intended genomic loci of the *ndr* genes upon activation.

MATERIALS AND METHODS

Animal care

Wild-type TL/AB zebrafish (Danio rerio) were maintained on 14 h (light) to 10 h (dark) cycle in a temperature-controlled (28°C) multi-tank re-circulating water system (Aquaneering, San Diego, CA, USA). Adult fish were fed twice a day with live brine shrimp (Brine Shrimp Direct, Ogden, UT, USA) and high-protein diet (Zeigler®, USA). Larvae were fed once daily with rotifer 5-14 at days post fertilization (dpf) and then with live brine shrimp twice daily at 14-60 dpf. All animal works were approved by the York University Animal Care Committee. All experiments were performed according to the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care.

Cas9 mediated mutagenesis

sgRNAs were designed using CHOPCHOP (Montague et al., 2014) for each candidate: ndr1 (GCATCTCAGGTCTGAGTTGG), ndr2 (GGAACGTCAGATTCAGGCAG) and ndr3 (GGGTTGTAAGTCTCATCCAG) and synthesized as described by (Gagnon et al., 2014). Briefly, gene-specific oligonucleotides containing the T7 (*TAATACGACTCACTATA*) promoter sequence, the sgRNA sequence and a complementary region were annealed to a constant oligonucleotide (AAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTA ACTTGCTATTTCTAGCTCTAAAAC) encoding the reverse-complement of the

tracrRNA tail. Cas9 mRNA and sgRNA were transcribed *in vitro* from linear DNA templates using the mMESSAGE mMACHINE Ultra kit (Ambion). TL/AB wild-type one-cell stage embryos were co-injected with a ~1 nL mixture of gRNAs combined with Cas9 mRNA. Some injected embryos were used for mutation screens while the remaining injected embryos were raised to adulthood, crossed to wild-type fish and rate of passing mutant allele to the next generation was screened. Founder fish (F0) with mutations were selected, heterozygous first generation (F1) fish with same heteroduplex band pattern in mutant allele were crossed and mutation in the homozygous second generation (F2) fish were confirmed by Sanger sequencing of the amplified mutant allele.

Heteroduplex mobility assay

Genomic DNA from injected and un-injected sibling embryos was extracted with 50 mM NaOH and then neutralized with 1 M Tris·HCl. DNA amplification was done using 2X Taq Frogga mix (FroggaBio) and gene-specific primers to screen amplicons of targeted region for difference in amplified band pattern (Table 4.1). Samples were separated on a nondenaturing 10% polyacrylamide gel (10% acrylamide (29:1), 1× TBE, 0.05% ammonium persulfate, 0.05% TEMED) and run in 1× TBE buffer. Gels were stained with ethidium bromide (0.5 µg/ml) before imaging.

Table 4.1. List of primers

Primer Name	Sequence
ndr1-genoF	TTGACATGTCCTCCATATCAGC
ndr1-genoR	TCTGGTGCAACCAGTACTTGAG
ndr2-genoF	CAGATGTGCTCAACAATGACAA
ndr2-genoR	AGTGCAGAAGCAGATTTGTGAC
ndr3-genoF	TTGATATGTGGGTGGATTTTGA
ndr3-genoR	ACTTCCACTACTGAGAAACCCG
Cas9-genoF1	GTGGAGCAGCACAAGCATTA
Cas9-genoR1	GTCCAGCACCTCCTTAGTGG
sgRNA-genoF1	TCACCACCTCCCAAAAACAT
sgRNA-genoR1	CGGTGCCACTTTTTCAAGTT

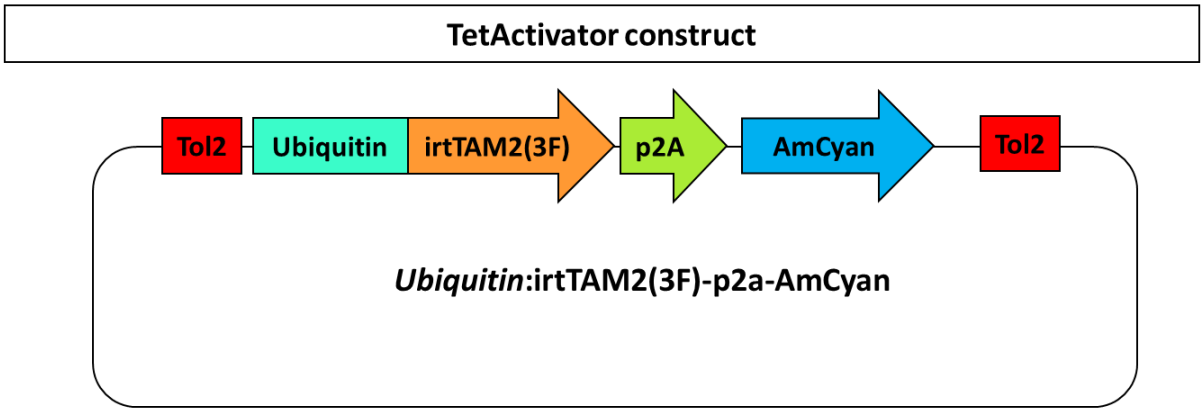
Plasmid construction

To employ inducible CRISPR mutagenesis, we used a tetracycline (Tet) inducible system, which is composed of two components: TetActivator (TetA) and TetResponder (TetRE) (Wehner, Jahn, and Weidinger 2015). The TetA construct produces the rtTA protein that binds to the promoter of the TetRE construct to induce gene expression. A DNA fragment composed of the zebrafish codon-optimized Cas9 and T2A cleaving peptide sequence was amplified from pME-Cas9-T2A-GFP (Ablain et al., 2015) into *TetRE*:polylinker-YPet to generate*TetRE*:Cas9-T2A-YPet (Figure 4.1).

Zebrafish zygotes were co-injected with both TetA (*ubiquitin*:irtTAM2(3F)-p2a-AmCyan) and *TetRE*:Cas9-T2A-YPet constructs along with Tol2 mRNA. Embryos were screened for AmCyan fluorescence and positive embryos were reared to adulthood. Positive colonies were selected and sequenced to assure the accuracy of the gene synthesis process.

Following the protocol provided by Yin, Maddison, and Li (2015), sgRNAs targeting *ndr1*, *ndr2*, or *ndr3* were subcloned into *pU6a*:sgRNA#1, *pU6a*:sgRNA#2, *pU6b*:sgRNA#3, respectively (Figure 4.2A).which were then cloned into lens-specific *cryaa*:mCreulean (LC) fluorescence-expressing pGGDestTol2LC (Yin, Maddison, and Li 2015). Then the three sgRNA cassettes from these vectors were cloned individually in pGGDestTol2LC-1sgRNA or in pairs in pGGDestTol2LC-2sgRNA or all three in pGGDestTol2LC-3sgRNA; thus, generating seven sgRNA cassettes that target *ndr1*, *ndr2* or *ndr3* individually (Figure 4.2B), in pairs (Figure 4.2C) or combined (Figure 4.2D). constructs that express *ndr*-targeting sgRNAs either alone or in combination. Positive colonies were selected and sequenced to assure the accuracy of the gene synthesis process.

A


TetActivator construct
Tol2
Ubiquitin
irtTAM2(3F)
p2A
AmCyan
Tol2
Ubiquitin:irtTAM2(3F)-p2a-AmCyan

B

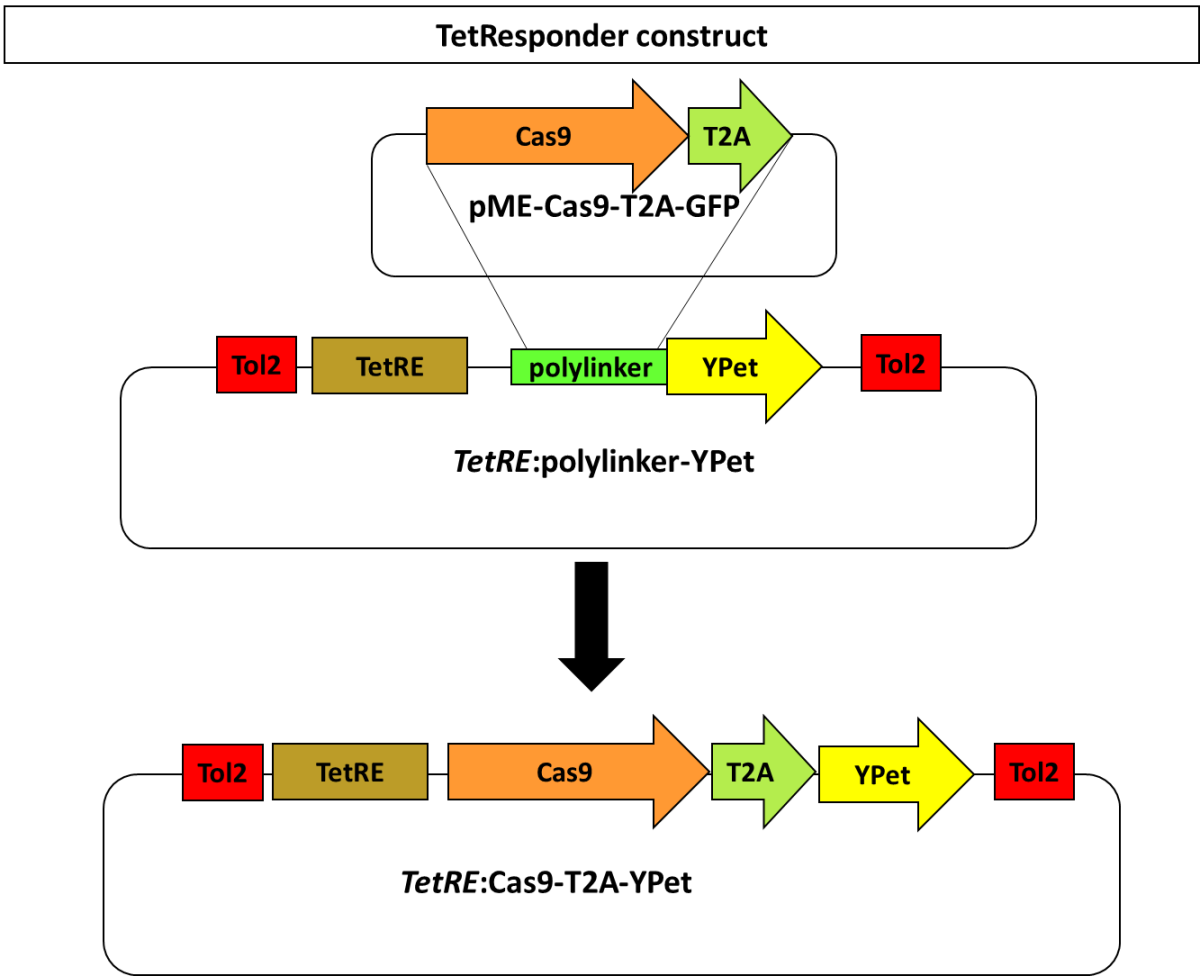
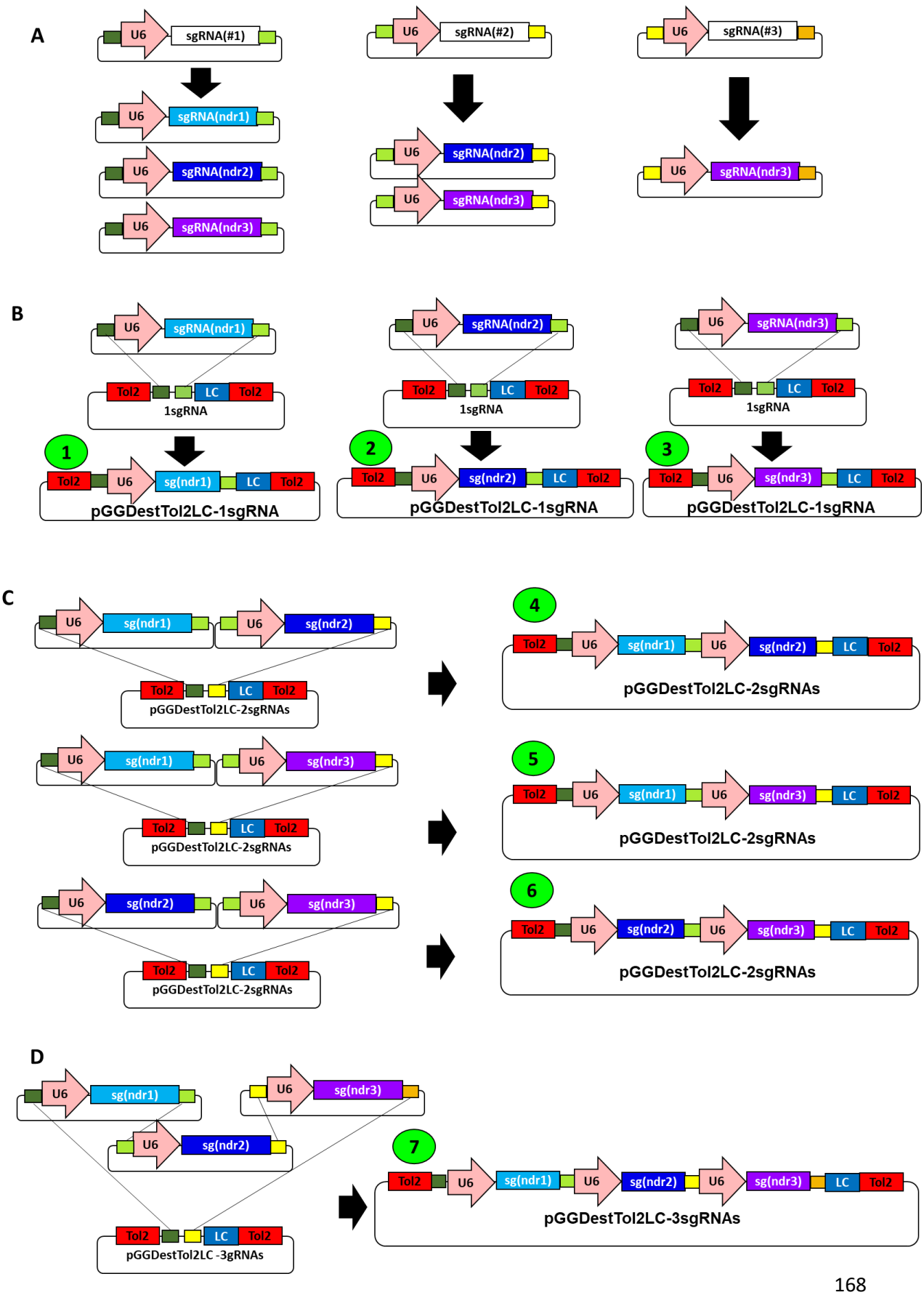

TetResponder construct
Cas9
T2A
pME-Cas9-T2A-GFP
Tol2
TetRE
polylinker
YPet
Tol2
TetRE:polylinker-YPet
Tol2
TetRE
Cas9
T2A
YPet
Tol2
TetRE:Cas9-T2A-YPet

Figure 4.1. Schematic of a TetON system expressing Cas9 endonuclease for zebrafish.

A) TetActivator irtTAM2(3F) construct under the control of a ubiquitin promoter. A P2A self-cleaving peptide separates the TetActivator from AmCyan that encodes fluorescence protein. The cassette is flanked by Tol2 transposon inverted repeats. B) A zebrafish-codon-optimized Cas9-endonuclease and a T2A self-cleaving peptide were cloned from pME-Cas9-T2A-GFP construct (Ablain et al., 2015) into the polylinker chain of the TetResponder construct, *TetRE*:polylinker-YPet (Wehner, Jahn, and Weidinger 2015). The resulting *TetRE*:Cas9-T2A-YPet cassette contains a YPet yellow fluorescence protein and is flanked by Tol2 transposon inverted repeats.


A
U6
sgRNA(#1)
sgRNA(ndr1)
sgRNA(ndr2)
sgRNA(ndr3)
sgRNA(#2)
sgRNA(#3)
B
Tol2
LC
1sgRNA
1
2
3
sg(ndr1)
sg(ndr2)
sg(ndr3)
pGGDestTol2LC-1sgRNA
C
pGGDestTol2LC-2sgRNAs
4
5
6
D
pGGDestTol2LC -3gRNAs
7
pGGDestTol2LC-3sgRNAs

Fi*gure 4.2. Generation of *ndr1*, *ndr2* and *ndr3* sgRNA *constructs.

A) Custom-designed sgRNAs targeting *ndr1*, *ndr2*, or *ndr3* were sub-cloned into sgRNA scaffold-containing *U6*:sgRNA vectors accommodating 1-3 sgRNAs. sgRNA cassettes were ligated into lens-specific mCreulean (LC) blue fluorescence pGGDestTol2LC to generate cassettes that contain 1 sgRNA (B), 2 sgRNAs (C) or 3 sgRNA (D). Green bubbles denote *ndr1*, *ndr2* or *ndr3* plasmid number. Final pGGDestTol2LC cassettes are flanked by Tol2 inverted repeats.

Transgenesis

To generate transgenic fish that carries the tet-on system, a total volume of ~1nl of the mixture of *in vitro*-transcribed Tol2 mRNA (25 pg) and either a sgRNA (20pg) plasmid or a mixture of TetActivator and TetResponder plasmids (15 pg each) were injected into the yolk of zygotes. Injected embryos were then incubated for 1-2 days and screened for fluorescence and positive larvae were raised.

Development of CRISPR-ON fish with the expression of TetA; TetRE, and single or multiplexed sgRNA

Zygotes were co-injected with the TetA and TetRE constructs generating double transgenic TetON fish (Figure 4.3). To establish fish that expressed the custom-designed *ndr1*, *ndr2* and/or *ndr3* sgRNAs, zygotes were injected with one of the seven sgRNA-expressing pGGDestTol2LC vectors along with Tol2 mRNA (Figure 4.3). Embryos were screened for LC at 48 hpf (hours post fertilization) and positive embryos were reared. One adult fish that transmitted the transgene to the offspring was selected from each of the seven lines for further crossings. The presence of the indicated sgRNA was confirmed by DNA sequencing in adult fish before crossing with other fish. Individual sgRNA-expressing lines were established and were then crossed to the TetON double transgenic fish, generating triple-transgenic CRISPR-ON fish.

Doxycycline Treatment

Doxycycline Hyclate (Sigma-Aldrich) was dissolved in 50% ethanol and kept at a stock concentration of 10 mg/mL Final Dox concentration of 10 µg/mL was added to egg water after embryo collection for 6, 24 and 48 hours.

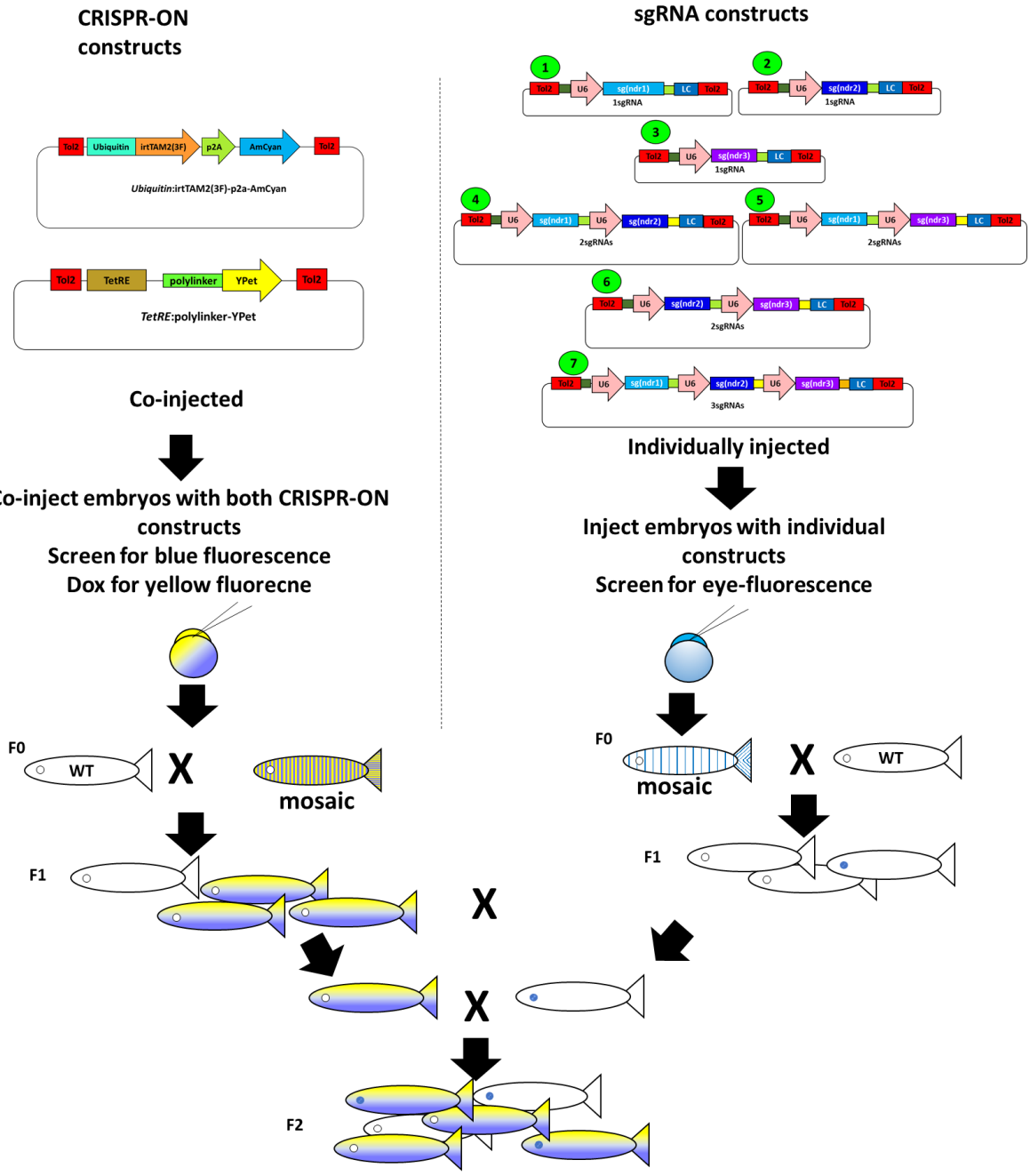

CRISPR-ON constructs
Tol2
Ubiquitin
irtTAM2(3F)
p2A
AmCyan
Tol2
Ubiquitin:irtTAM2(3F)-p2a-AmCyan
Tol2
TetRE
polylinker
YPet
Tol2
TetRE:polylinker-YPet
Co-injected
Co-inject embryos with both CRISPR-ON constructs
Screen for blue fluorescence
Dox for yellow fluorecne
F0
WT
X
mosaic
F1
sgRNA constructs
1
Tol2
U6
sg(ndr1)
LC
Tol2
1sgRNA
2
Tol2
U6
sg(ndr2)
LC
Tol2
1sgRNA
3
Tol2
U6
sg(ndr3)
LC
Tol2
1sgRNA
4
Tol2
U6
sg(ndr1)
U6
sg(ndr2)
LC
Tol2
2sgRNAs
5
Tol2
U6
sg(ndr1)
U6
sg(ndr3)
LC
Tol2
2sgRNAs
6
Tol2
U6
sg(ndr2)
U6
sg(ndr3)
LC
Tol2
2sgRNAs
7
Tol2
U6
sg(ndr1)
U6
sg(ndr2)
U6
sg(ndr3)
LC
Tol2
3sgRNAs
Individually injected
Inject embryos with individual constructs
Screen for eye-fluorescence
F0
mosaic
X
WT
F1
X
X
F2

Figure 4.3. A schematic outlining the workflow of generating triple-transgenic multiplexed CRISPR-ON fish.

Zygotes were either co-injected with *ubiquitin*:irtTAM2(3F)-p2a-AmCyan (TetA) (Wehner, Jahn, and Weidinger 2015) and*TetRE*:Cas9-YPet (TetRE; top two left vectors) or with one of the seven mCreulean fluorescence-labelled eye lens-specific *U6*:sgRNA vectors (Yin, Maddison, and Li 2015) that carry sgRNAs targeting *ndr1*,*ndr2*, *ndr3* individually, in pairs, or all three combined (top right constructs, green bubbles denote plasmid number). Injected founder (F0) zygotes were screened for positive fluorescence, positive fish were crossed to wild-type fish and transgenic fish with high germline transmittance were selected for further crossings. First generation (F1) F1 TetA and TetRE double transgenic fish are then crossed with F1 sgRNA transgenic fish to generate triple-transgenic fish which have full body blue and yellow fluorescent as well as blue lens-specific fluorescence.

RESULTS

Validation of single guide RNAs

To study the effect of the loss of the Nodal signals in zebrafish using the CRISPR/Cas9 system, we designed three sgRNAs that target each of *ndr1*, *ndr2* or *ndr3* (Figure 4.4A). To determine the efficiency of the *ndr1*, *ndr2,* and *ndr3* targeting sgRNAs, zebrafish zygotes were injected with *in vitro*-transcribed Cas 9 mRNA and *ndr1*, *ndr2*, or *ndr3* sgRNA. To assess whether the sgRNA induced indel mutations at the intended locations, Heteroduplex Mobility Assay (HMA) was performed on DNA extracted from the injected embryos 24 hours hpf and PCR was performed using primers that flanked the sgRNA targeting site. HMA showed high mutagenesis rates at the *ndr1*, *ndr2* or *ndr3* targets (85–100%) in seven randomly selected *ndr*-targeted embryos. Heteroduplex bands were found in all embryos injected with the *ndr1*, *ndr2* and *ndr3* sgRNA and Cas9 mRNA indicating that our custom-designed sgRNAs were efficiently guiding the Cas9 nuclease to the cutting site and as a result, indel mutations were being formed at the intended genomic locations (Figure 4.4B).

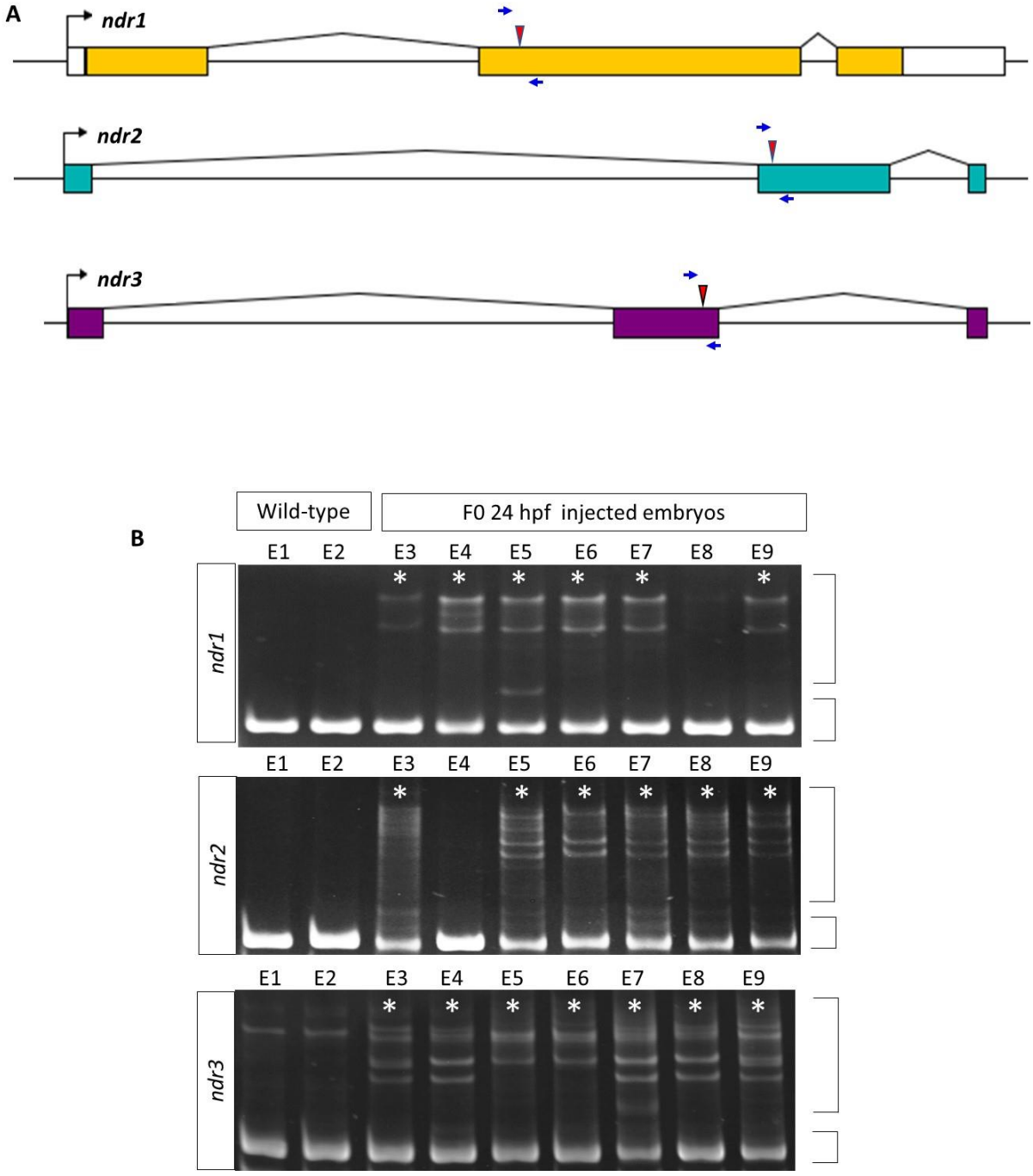

A
ndr1
ndr2
ndr3
B
Wild-type
F0 24 hpf injected embryos
E1
E2
E3
E4
E5
E6
E7
E8
E9
ndr1
ndr2
ndr3

Figure 4.4. Efficiency of mutations generated by CRISPR/Cas9 targeting of *ndr1*, *ndr2*, or *ndr3*.

A) Structure of *ndr1*, *ndr2*, and *ndr3* genes. Location of the single guide RNAs (sgRNAs) is denoted by red arrowheads. Blue arrows indicate location of genotyping PCR primers. B) Heteroduplex mobility assay (HMA) using DNA extracted from 24 hours post fertilization (hpf) embryos that were injected with *in vitro*-transcribed mRNA of CRISPR Cas9 and sgRNA targeting *ndr1*, *ndr2*, or *ndr3* (E3-E9) compared to uninjected wild-type embryos (E1-E2). PCR amplicons were run on polyacrylamide gels and stained with ethidium bromide. *denotes mutant mosaic embryos. Large square brackets indicate heteroduplex bands while small brackets point to homoduplex bands.

Establishing *ndr1*, *ndr2* or *ndr3* mutant zebrafish lines

To generate *ndr1*, *ndr2* or *ndr3* mutant zebrafish lines, embryos were injected with the validated sgRNAs and then reared to adulthood. Adult mosaic CRISPR/Cas9 mutant F0 were screened for mutations in DNA extracted from their fins using HMA. Several potential F0 fish were found to display heteroduplex band in the amplicons of the targeted regions, indicating the presence of mutations at the chosen cutting sites in *ndr1*, *ndr2* or *ndr3* (Figure 4.5A). Potential CRISPR/Cas9 F0 mutants were then crossed with wild-type fish and 24 hpf embryos from their F1 offspring were randomly selected for HMA screening (Figure 4.5B). Although the rate of passing mutations to the offspring was variable, several fish had high transmittance rate. The CRISPR/Cas9 F1 offspring of the fish with the highest germline transmittance of mutations were then reared and adult F1 fins were used for genotyping using HMA. One male and one female F1 fish that displayed similar heteroduplex patterns from each of *ndr1* or *ndr2* were crossed to each other to generate homozygous fish that potentially have the same biallelic mutations (Figure 4.5C). The F1 *ndr3* mutants are currently being reared to be genotyped and crossed in the future.

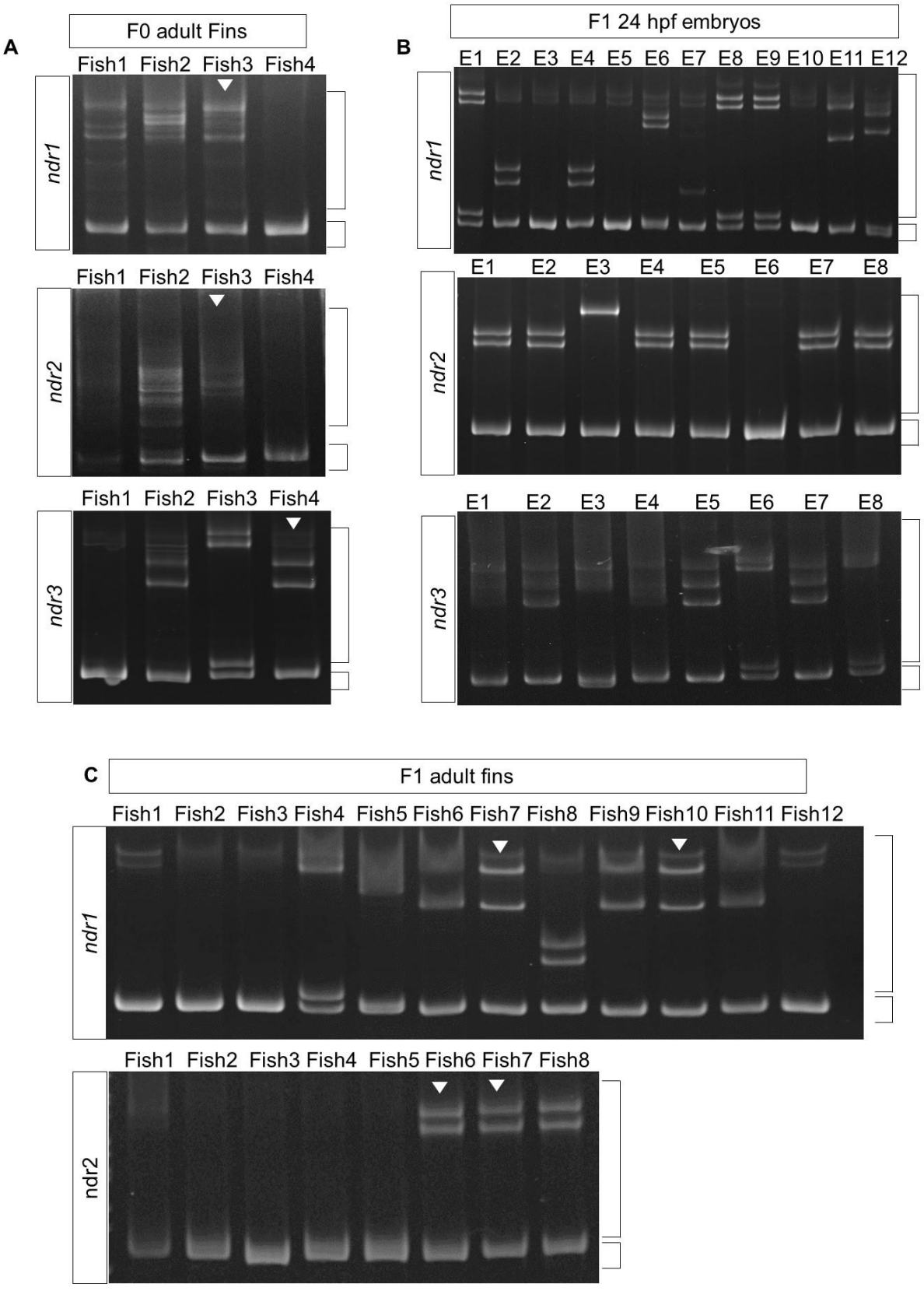

A
F0 adult Fins
Fish1 Fish2 Fish3 Fish4
ndr1
Fish1 Fish2 Fish3 Fish4
ndr2
Fish1 Fish2 Fish3 Fish4
ndr3
B
F1 24 hpf embryos
E1 E2 E3 E4 E5 E6 E7 E8 E9 E10 E11 E12
ndr1
E1 E2 E3 E4 E5 E6 E7 E8
ndr2
E1 E2 E3 E4 E5 E6 E7 E8
ndr3
C
F1 adult fins
Fish1 Fish2 Fish3 Fish4 Fish5 Fish6 Fish7 Fish8 Fish9 Fish10 Fish11 Fish12
ndr1
Fish1 Fish2 Fish3 Fish4 Fish5 Fish6 Fish7 Fish8
ndr2

Figure 4.5. Establishing *ndr1*, *ndr2* or *ndr3* mutant lines.

A) Images of ethidium bromide-stained polyacrylamide gels showing separation of homoduplex and heteroduplex PCR amplicons in DNA extracted from the fins of F0 founder mutant fish that have been reared after being injected with CRISPR/Cas9 and ndr1, ndr2 or ndr3 gRNA at the 1 cell stage. Each of the fish was further screened for transmitting the mutation through the germline by crossing with wild-type fish and one fish was chosen for further analysis (arrowheads). B) Screening of 24h embryos that were produced from crossing the selected fish in (A) to wild-type fish. C) Screening of CRISPR/Cas9 mutations in the fins of reared embryos of fish selected in (A). Male and female fish harboring similar heteroduplex patterns (arrowheads) were crossed to each other to produce mutant homozygous fish. Large and small square brackets indicate heteroduplex and homoduplex bands, respectively.

ndr1 and *ndr2* mutants display cyclopia and body curvature

To determine whether CRISPR *ndr1* and *ndr2* mutants showed a phenotype at early developmental stages, the F1 mutant fish were crossed to each other and morphology of F2 24 and 48 hpf embryos were observed. While both *ndr1* and *ndr2* F2 embryos displayed cyclopia, *ndr1* mutants exhibited a spectrum of levels of eye fusion to the formation of 1 deformed eye without pigmentation within the same clutch (Figures 4.6 A-E,4.6 A'-E'). In addition, the body axis of cyclopic embryos was shortened and was either moderately or severely curved dorsally (Figures 4.6 F-H,4.6F'-H'). Quantification of the phenotypes of either fused or 1 eye with a moderately or severely curved body ranged from 5.78% to 7.8% of total embryos in the same clutch with a total of 26.88% of the embryos showing an abnormal phenotype (Figure 4.6 I). On the other hand, F2 of *ndr2* 24 and 48 hpf mutant embryos showed a single phenotype of fused eyes and a body axis with severe ventral curvature (Figures 4.7 A-D,4.7A'-D') that comprised 22.36% of embryos in the same clutch (Figure 4.7E). The penetrance of *ndr1* and *ndr2* phenotype was consistent upon repeated spawning of the same parental *ndr1* and *ndr2* heterozygous pairs.

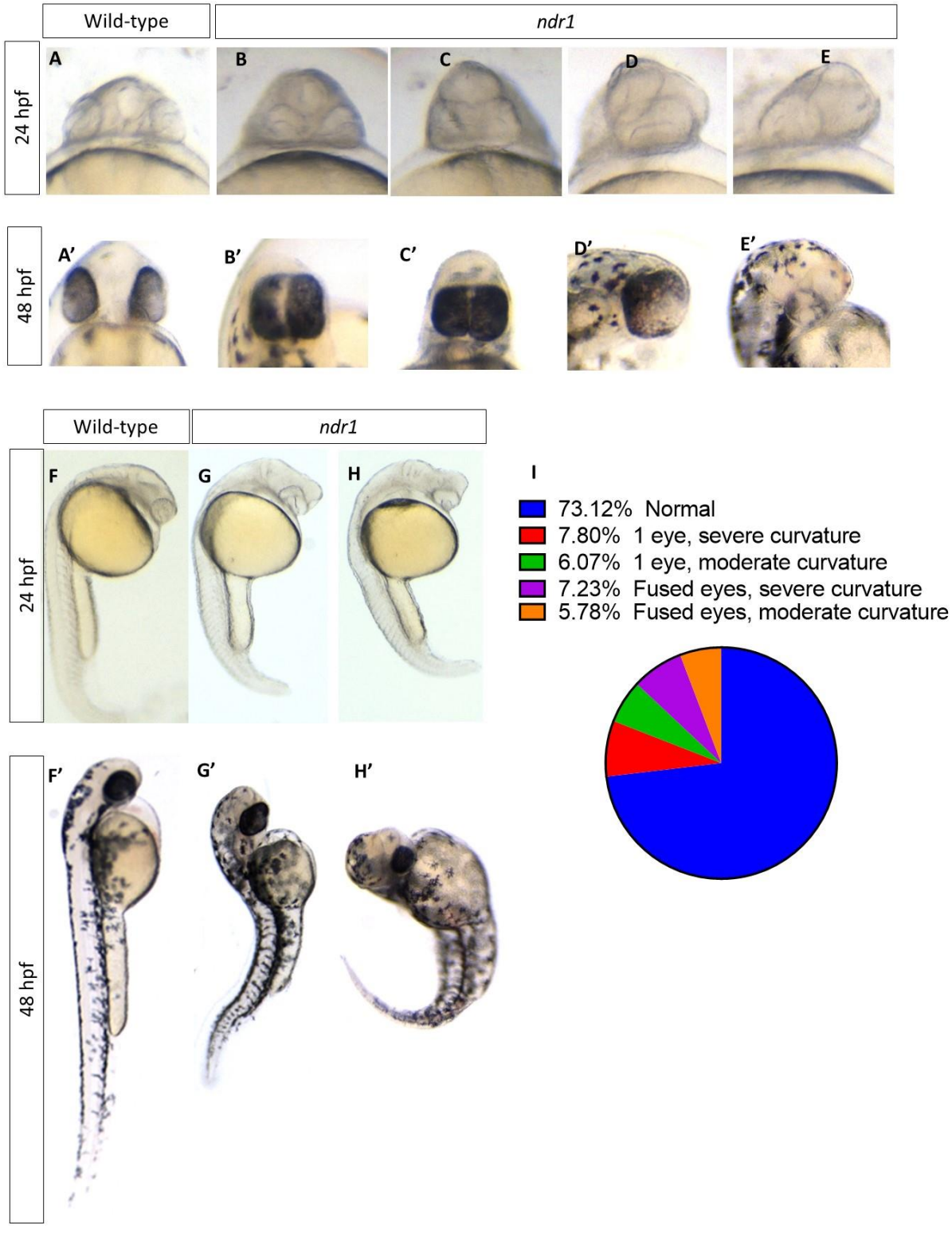

Wild-type
ndr1
24 hpf
A
B
C
D
E
48 hpf
A'
B'
C'
D'
E'
Wild-type
ndr1
24 hpf
F
G
H
I
73.12% Normal
7.80% 1 eye, severe curvature
6.07% 1 eye, moderate curvature
7.23% Fused eyes, severe curvature
5.78% Fused eyes, moderate curvature
48 hpf
F'
G'
H'

Figure 4.6. CRISPR/Cas9-targeted *ndr1* mutants show cyclopia with different levels of eye fusion, and shortened and curved body axis.

A-E) Frontal view of live F2 *ndr1* embryos showing cyclopia and other defects ranging from eye fusion to the formation of 1 deformed eye without pigmentation at 24 hours post fertilization (hpf). Wild-type fish was shown as the control. A'-E') Wild-type and mutant fish as in A-E at at 48 hpf. F-H) Lateral views of *ndr1* mutants exhibiting a shortened body axis with moderate or severe dorsal curvature at 24hpf (G-H) and 48hpf (G'-H'). I) Quantification of cyclopic phenotype in combination with body axis defect type at 48 hpf.

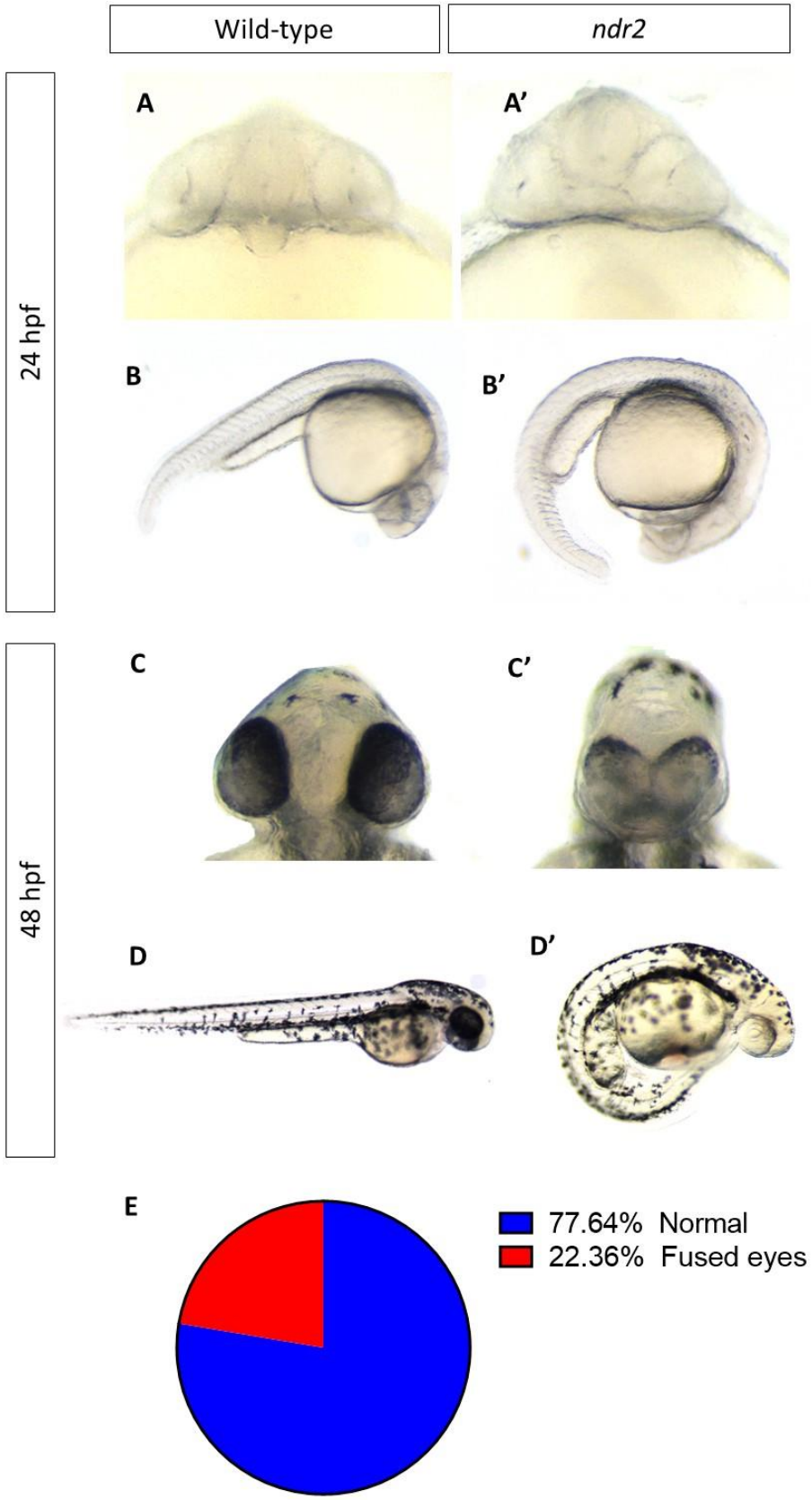
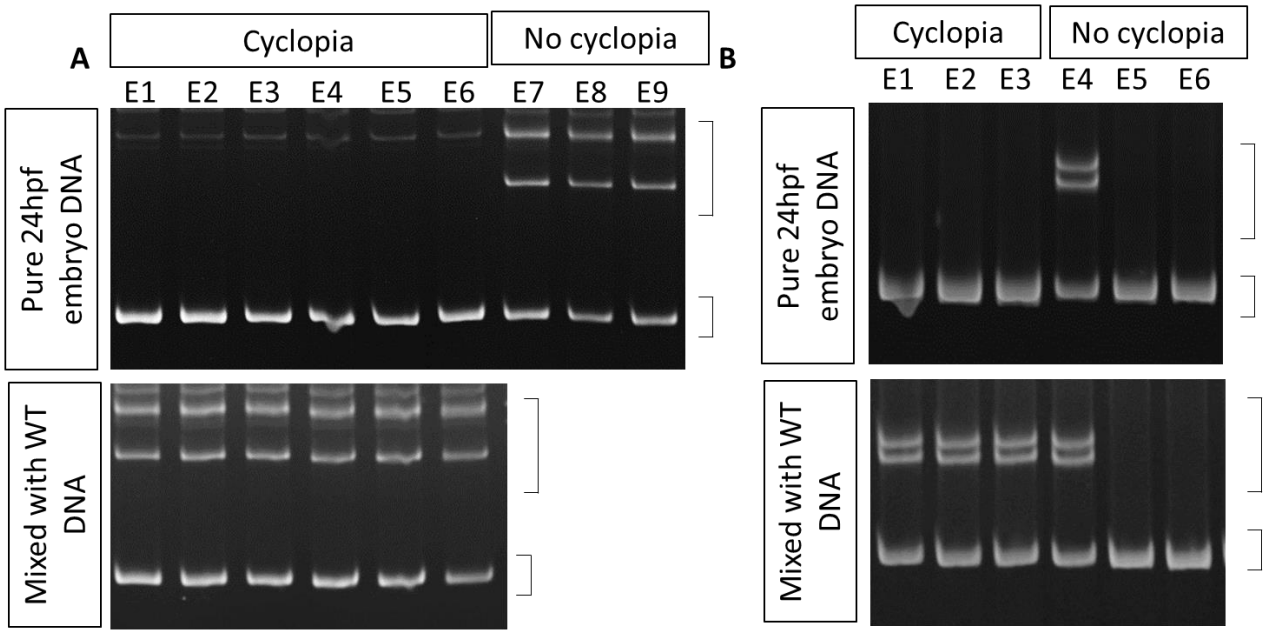

Wild-type
ndr2
24 hpf
48 hpf
A
A'
B
B'
C
C'
D
D'
E
77.64% Normal
22.36% Fused eyes

Figure 4.7. CRISPR/Cas9-targeted *ndr2* mutants show cyclopia and severely deformed body axis.

A-A') Frontal views of 24 hours post fertilization (hpf) Second generation (F2) *ndr2* mutants showing fused eyes and the corresponding phenotype in lateral views (B-B') compared to wild-type. C-C') Frontal view of F2 *ndr2* mutants at 48 hpf compared to wild-type and the corresponding lateral views (D-D') showing severe malformation in body axis in mutants. E) Quantification of eye fusion phenotype in *ndr2* mutants at 48 hpf.

Confirmation of mutations of *ndr1* and *ndr2* mutants

To determine whether the cyclopic *ndr1* and *ndr2* embryos were malformed because of the CRISPR/Cas9-induced mutations, 24 hpf embryos with cyclopia were selected from the F2 *ndr1* and *ndr2* mutant clutches for HMA. When pure cyclopic embryo DNA was used as a template for PCR, all deformed *ndr1* and *ndr2* mutants produced homoduplex bands only. On the other hand, embryos with no cyclopia produced homoduplex bands with or without heteroduplex bands (Figure 4.8A-B). Upon mixing the DNA of cyclopic mutants with wild-type DNA, HMA revealed both homoduplex and heteroduplex bands. Meanwhile, mixing wild-type DNA with the DNA of embryos with no cyclopia produced single homoduplex bands only (Figure 4.8A-B). Finally, DNA sequencing of cyclopic mutants confirmed that they were homozygous mutants with a 7 base pair (bp) deletion in the *ndr1* gene and 4 base pair deletion in the *ndr2* gene (Figure 4.8 C).



C

CCGCATCTCAGGTCTGAGTTGGAGGTGGATATTTATCACGCATCTACACC WT *ndr1*
CCGCATCTCAGGTCTGAG----------TGGATATTTATCACGCATCTACACC -7bp

TGTCGGAACGTCAGATTCAGGCAGCGGAGCTGCGGATCCGCGTC WT *ndr2*
TGTCGGAACGTCAGATT--------CAGCGGAGCTGCGGATCCGCGTC -4bp

Figure 4.8. Confirmation of mutations in *ndr1* and *ndr2*.

A) Heteroduplex mobility assay (HMA) of six F2 *ndr1* mutant embryos with variable levels of deformation (E1 to E6) and three embryos that showed no defects (E7 to E9). DNA was extracted from the embryos at 24hpf and HMA was performed without (top panel) or with (bottom panel) DNA extracted from wild-type (WT) embryos. The DNA of all deformed F2 mutant embryos produced homoduplex bands when amplified alone and generated heteroduplex bands upon mixing with wild-type DNA confirming the presence of a homozygous mutation. Meanwhile, embryos with no defects showed heteroduplex bands indicating that they have heterozygous mutations. B) HMA assay of three *ndr2* mutant embryos with deformed phenotype and three of their wild-type- looking siblings. DNA extracted from 24hpf embryos was amplified alone (top panel) or mixed with wild-type DNA (bottom panel). Formation of previously not present heteroduplex bands after the addition of wild-type DNA confirms the homozygous mutation while the presence of heteroduplex bands before or after wild-type DNA addition denotes a heterozygous mutation. Big square brackets indicate heteroduplex band formation and small square brackets indicate homoduplex band formation. C) Sequence information of regions targeted in *ndr1* and *ndr2* by their respective sgRNAs (marked in green; PAM sequence in red). *ndr1* and *ndr2* mutants contained 7bp and 4bp deletions, respectively.

Generation of CRISPR-ON system with single or multiplexed sgRNA to knockout *ndr1*, *ndr2* and/or *ndr3*

Generation of double-transgenic Tet-On zebrafish expressing Cas9-nuclease

Because of the deleterious effect of Nodal loss in early development, *in vivo* studies beyond embryonic stages are limited. To study the roles of ndr1, ndr2 and ndr3 in adult physiological processes, especially in regulating ovarian functions, we aimed to develop an inducible CRISPR/as9 system to knockout *ndr1*, *ndr2*, and *ndr3*, either alone or in combinations. The *TetRE*:Cas9-T2A-YPet (Figure 4.1) was combined with a zebrafish specific sgRNA multiplexing system that expresses up to three *ndr*-targeting sgRNAs simultaneously (Figure 4.2), producing the CRISPR-ON system (Figure 4.3).The transmittance of mutation through the germline was tested by crossing the *ubiquitin*:irtTAM2(3F)-p2a-AmCyan; *TetRE*:Cas9-T2A-YPet with wild-type fish and embryos were treated with 10uM Doxycycline (Dox) up to 48 hours. Embryos that showed both blue and yellow fluorescence, which indicate the expression of the TetA and the Cas9-expressing TetRE constructs were selected. We found that the fluorescence signal can be seen at 6 hours, although not very strong and some positive embryos can be missed (Figure 4.9A). Strong fluorescence signals can be observed at 24 hpf (Figure 4.9B) and 48 hpf (Figure 4.9C). We also observed that in one of the double transgenic lines, in over ten thousand embryos, green fluorescence was accompanied with yellow fluorescence that only appears upon adding Dox. Therefore, this line was chosen for further crossings.

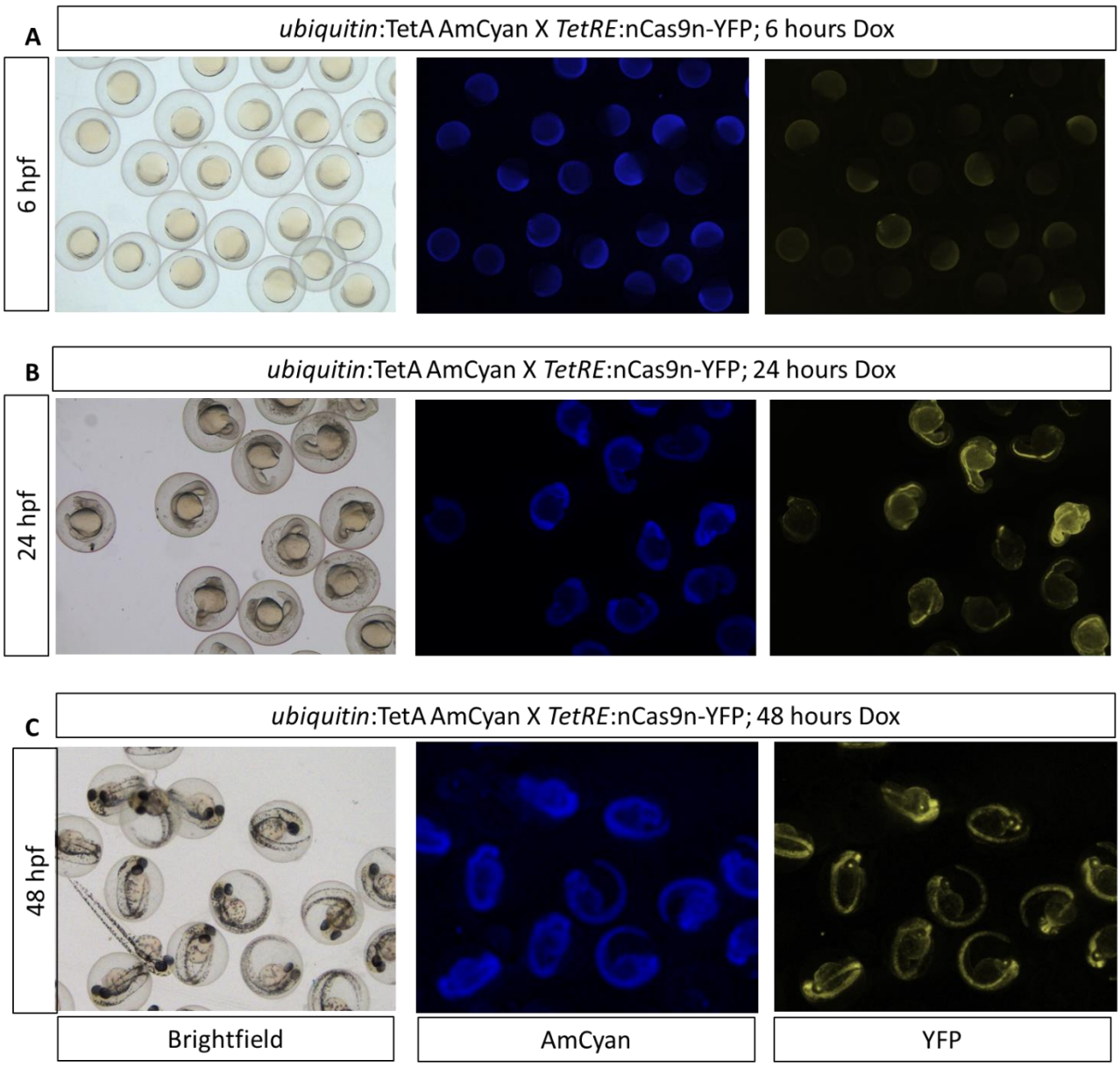

A
ubiquitin:TetA AmCyan X *TetRE*:nCas9n-YFP; 6 hours Dox
6 hpf
B
ubiquitin:TetA AmCyan X *TetRE*:nCas9n-YFP; 24 hours Dox
24 hpf
C
ubiquitin:TetA AmCyan X *TetRE*:nCas9n-YFP; 48 hours Dox
48 hpf
Brightfield
AmCyan
YFP

Figure 4.9. Characterization of inducible double transgenic Tet-ON fish.

A) Embryos were screened after being co-injected with TetActivator (*ubiquitin*:TetA AmCyan) and TetResponder (*TetRE*:Cas9-YFP) at the zygotic stage. Positive embryos carrying both TetActivator and TetResponder were identified by the presence of blue fluorescence and induction of yellow fluorescence following Doxycycline (Dox) treatment for 6 hours (A), 24 hours (B) and 48 hours (C). All embryos that contain the TetActivator transgene also carry the TetResponder transgene.

Generation and characterization of triple transgenic fish containing Tet-ON-Cas9 and sgRNA transgenes

To establish fish lines that express the CRISPR-ON system and the multiplexed sgRNAs, double transgenic *ubiquitin*:irtTAM2(3F)-p2a-AmCyan; *TetRE*:Cas9-T2A-YPet fish were crossed with *U6*:sgRNA(*ndr*)- *cryaa*:mCerulean and adult putative triple transgenic fish were first screened for the presence of *ubiquitin*:irtTAM2(3F)-p2a-AmCyan by blue fluorescence. Positive adults were then screened by PCR and Sanger sequencing against the Cas9 nuclease and against the gene specific region of the sgRNA scaffold. Interestingly, we noticed that uninduced *ubiquitin*:irtTAM2(3F)-p2a-AmCyan; *TetRE*:Cas9-T2A-YPet larvae do not display blue lens fluorescence (Figure 4.10A) whereas when they express the *U6*:sgRNA(*ndr*)- *cryaa*:mCerulean, they gain blue fluorescence in the lens (Figure 4.10B).

To determine whether the three transgenic components were compatible with each other, putative triple-transgenic embryos that contained the TetA transgene were induced by Dox for 24 hours and then tested for the presence of Cas9, sgRNA and gene-specific modification. HMA confirmed that when both Cas9 and sgRNA were present, heteroduplex bands were apparent in either *ndr1*, *ndr2* or *ndr3* triple-transgenic fish. However, a similar phenotype to the one observed with the ndr1 or *ndr2* CRISPR knock-out fish was not observed. While triple-transgenic fish expressing multiplexed sgRNA expression were established, their characterization needs to be performed (Figure 4.10 C-E).

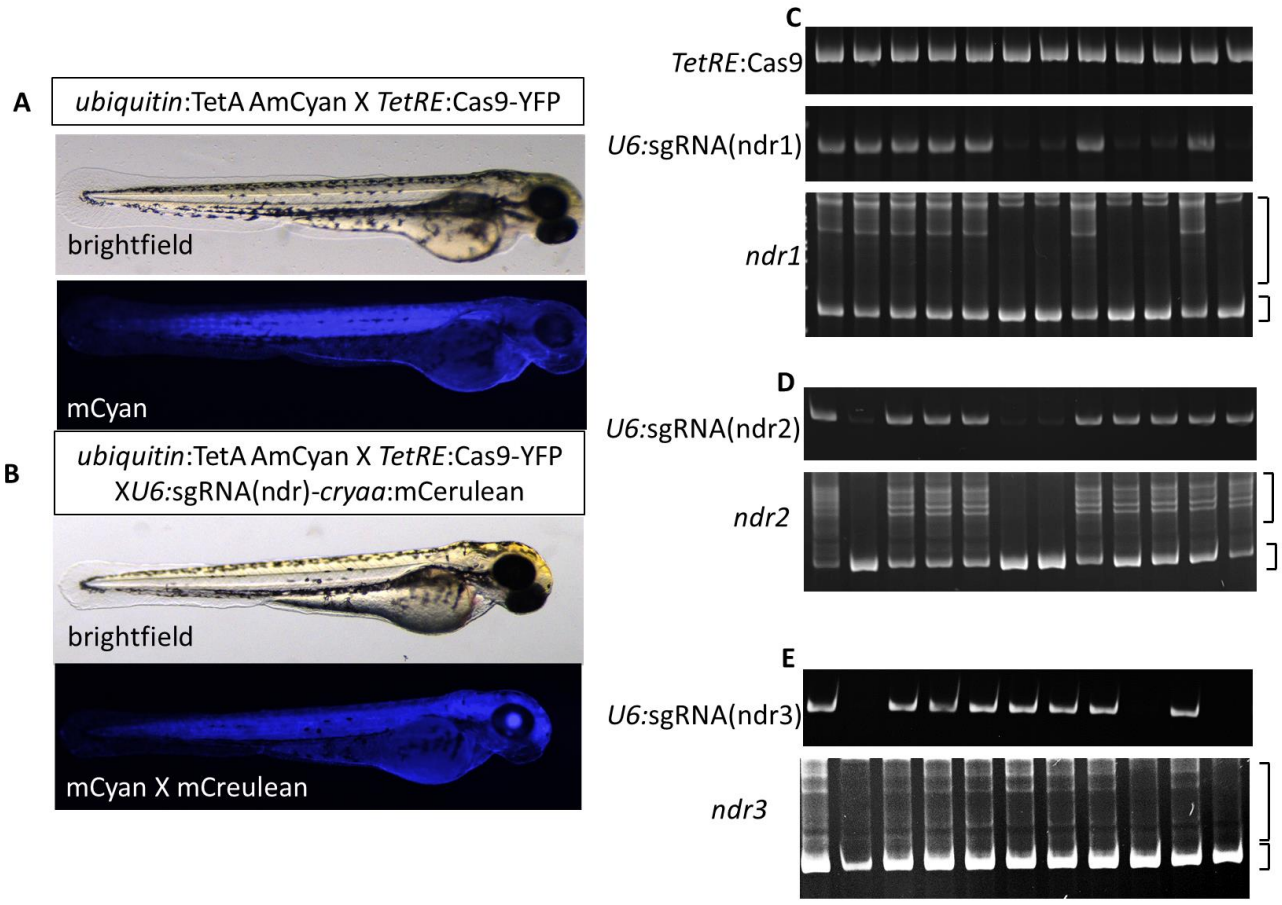

A
ubiquitin:TetA AmCyan X TetRE:Cas9-YFP
brightfield
mCyan
B
ubiquitin:TetA AmCyan X TetRE:Cas9-YFP
XU6:sgRNA(ndr)-cryaa:mCerulean
brightfield
mCyan X mCreulean
C
TetRE:Cas9
U6:sgRNA(ndr1)
ndr1
D
U6:sgRNA(ndr2)
ndr2
E
U6:sgRNA(ndr3)
ndr3

Figure 4.10. Identification of triple transgenic fish harboring inducible CRISPR-ON system as well as sgRNA expression targeting *ndr1*, *ndr2* or *ndr3*.

A) Screening of 48 hours post fertilization (hpf) larvae that were positive for TetActivator (*ubiquitin*:TetA AmCyan) and TetResponder (*TetRE*:Cas9-YFP) showed no retina fluorescence in the absence of *U6*:sgRNA(ndr)-mCreulean, whereas larvae positive for *U6*:sgRNA(ndr)-mCreulean showed the lens-specific fluorescence at 48 hpf, hence fish transmitting the transgene in the germline were identified (B). C-E) Validation of targeted genomic modification of triple transgenic fish that displayed yellow fluorescence upon Dox induction. The presence of the *TetRE*:Cas9-YFP was confirmed by PCR (top panel in C used as an example for *ndr1* triple transgenic fish). Heteroduplex bands were formed in randomly chosen 24 hpf Dox-induced embryos indicating the induction of genomic modification, in targeted region of *ndr1*, *ndr2* and *ndr3* C-E, bottom panels) only in the presence of sgRNA whose presence was confirmed by PCR of *U6*:sgRNA(ndr1)-mCreulean (C, middle panel), *U6*:sgRNA(ndr2)-mCreulean (and *U6*:sgRNA(ndr3)-mCreulean (D and E top panels, respectively). Big square brackets indicate heteroduplex band formation and small square brackets denote homoduplex bands.

DISCUSSION

Using the CRISPR/Cas9 technology, we created two mutant knockout lines, *ndr1* and *ndr2,* to determine whether loss of ndr1 and ndr2 cause deleterious effects in zebrafish early development. Contrary to previous reports (Dougan et al., 2003; Aquilina-Beck et al., 2007), we found that the phenotype of *ndr1* was fully penetrant, causing embryonic cyclopia and body axis defects. On the other hand, our *ndr2* mutants displayed a phenotype similar to what was found in earlier studies (Tian et al., 2003; Lim et al., 2013; Turner et al., 2018). We also developed a fluorescently-labeled, Tetracycline-inducible multiplexed system to knockout *ndr1*, *ndr2* or *ndr3,* individually or in combination. While further characterization is required, this system will be very useful for investigating the functions of the *ndr* genes during development and in adulthood.

In this study, we found that zygotes injected with the *in vitro*-transcribed Cas9 mRNA of and sgRNA targeting *ndr1*, *ndr2*, or *ndr3* generally displayed a high frequency of mutations in 24 hpf embryos and in the adult F0 fish. These results indicate that the sgRNAs we designed efficiently induce indel mutations. Further sequencing analyses of one line of *ndr1* and one line of *ndr2* mutants revealed a 7bp and 4 bp deletion at the designated genomic loci of *ndr1* and *ndr2*, respectively. However, the rate of transmittance of mutations to the offspring through the germline was variable, as anticipated because of the mosaic nature of mutagenesis in F0 fish. Therefore, it is essential to acquire F0 fish with a high transmittance rate to the offspring to facilitate further screening in the F1 generation.

Several lines of evidence support that the F1 *ndr1* and *ndr2* mutants are homozygous. First, their DNA produced heteroduplex bands only when wild-type DNA was added to their own DNA as indicated by HMA. Second, the offspring from F1 *ndr1* mutants displayed cyclopia in 26.88% of the embryos in the same clutch, which is very close to the expected 25% homozygous mutant population. This rate of phenotype penetrance is significantly higher than previous studies, which reported that *ndr1* loss caused cyclopia in some homozygous embryos while other homozygous mutants were not affected (Aquilina-Beck et al., 2007; Lim et al., 2013; Goudarzi et al., 2019). HMA further confirmed that all embryos with cyclopia were indeed homozygous for the mutation. Finally, sequencing of the DNA from homozygous embryos revealed that the induced indel mutation of those mutants was a 7 bp deletion. Similarly, almost a quarter of the F2 *ndr2* mutant population displayed cyclopia and were found to be homozygous mutants with a 4 bp deletion in the *ndr2* gene. These findings suggest that both ndr1 and ndr2 are individually essential for proper early development to occur.

The phenotype observed in our *ndr2* mutants is consistent with other reports; however, there are discrepancies in the *ndr1* phenotypes between this study and previous reports. There is either no phenotype (Dougan et al., 2003), partially penetrant phenotype (Aquilina-Beck et al., 2007; Goudarzi et al., 2019) or a range of phenotypes using TALENS and ZFNs (Lim et al., 2013). A non-coding RNA has been found in the *ndr1* gene and suggested to play a role in dorsal specification (Lim et al., 2012). However, this was not supported by a recent study (Goudarzi et al., 2019). To

rule out potential off-target effects, we are generating a second CRISPR *ndr1* knockout line utilizing a sgRNA that targets *ndr1* in a different locus to further assess the strong penetrance of the *ndr1* phenotype we observed. It may also be possible that knocking out *ndr1* reduces *ndr2* levels and vice versa, as we have observed with knockdown experiments of *ndr1* or *ndr2* in zebrafish ovarian primary cell cultures. The levels of ndr1 and ndr2 mRNAs in our knockout lines will be measured to examine whether knocking out either *ndr* gene affects the other. Rescue experiments are also needed to confirm the role of ndr1 in early embryogenesis.

The ubiquitin:irtTAM2(3F)-p2a-AmCyan and TetRE:polylinker-YPet plasmids were developed to introduce an inducible expression of any gene of interest in zebrafish (Wehner, Jahn, and Weidinger 2015). In this study, we used these vectors to generate fish that express a Dox-inducible zebrafish codon-optimized Cas9-nuclease. We also used the multiplexed *U6*:sgRNA vectors (Yin, Maddison, and Li 2015) to create seven different fish lines that express mCreulean fluorescence-labeled sgRNA constructs that target *ndr1*,*ndr2* and *ndr3*, individually, in pairs, or all 3 combined. These fish were then crossed to generate a fluorescently labeled, triple-transgenic multiplexed CRISPR-ON system. The fluorescent TetA transgene with self-reporting AmCyan allows for the first screening step in this triple-transgenic system. We found that the presence of the TetA transgene is always accompanied with the TetRE transgene, which, enhances the efficiency of the system as it significantly increases the probability of finding double transgenic fish without having to prematurely activate the system. In addition, the absence of retina-

specific fluorescence in *TetA*; *TetRE* transgenic fish solely indicates that the fish do not carry the sgRNA transgene, whereas *TetA*; *TetRE* that display lens fluorescence express the designated sgRNA cassette. This, in turn, simplifies the genotyping process required to identify triple-transgenic fish. In these transgenic lines, heteroduplex bands were detected after Dox induction. This result indicates that the three transgenic components, TetA, TetRE, and sgRNA constructs, are properly expressed and cause genomic modification to the designated genomic loci upon activation. Establishing a CRISPR-ON multiplexed system in zebrafish will facilitate studies of genes, such as *ndr1*, *ndr2,* and *ndr3*, whose loss impair early development. This system is the first multiplexed Dox-inducible CRISPR-ON system in zebrafish that would allow for conducting temporally controlled loss of function studies.

It is surprising that after Dox treatment to the embryos carrying the CRISPR-ON and the *ndr1* or *ndr2* sgRNA, we did not observe the cyclopic phenotype. However, it is possible that these embryos may still have the ndr1 and ndr2 transcripts, either from maternal source or expressed early in embryonic development. Ndr1 and ndr2 mRNAs have been detected in embryos at the four-cell stage (Gore et al., 2005). Although we started the Dox induction at the one-cell stage, strong fluorescent signals were not observed until 24h after. Future studies are required to determine the temporal expression levels of ndrs in control and Dox-induced embryos.

In summary, the data shown here describes phenotypes observed in *ndr1* and *ndr2* mutations using CRISPR/Cas9 mutagenesis. While *ndr2* phenotype was consistent with previous reports, we found a more significantly penetrant phenotype in *ndr1* homozygous mutants than previously reported. We have also created an inducible CRISPR-ON, multiplexed triple-transgenic system in zebrafish that will allow studying the functions of *ndr* genes *in vivo*.

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

SUMMARY

The objective of this dissertation was to study some of the different factors that are at play at the onset of maturational competence in the zebrafish ovary. Specifically, we have 1) characterized miRNA expression profiles in maturationally competent follicles; 2) investigated the role of Nodal-related (*ndr*) genes in zebrafish ovarian follicular cells and established knockout fish lines, as well as an inducible CRISPR-ON system in zebrafish to study the roles of *ndr* genes in female reproduction.

Aim 1: To characterize miRNA expression profiles in vitellogenic follicles

To determine whether miRNAs may be involved in the acquisition of maturational competency of ovarian follicles, in Chapter 2, we used next-generation sequencing to characterize the miRNA expression profiles in follicular cells collected from stage IIIa, which are maturationally incompetent, and IIIb follicles, which can respond to hormonal signals that induce oocyte maturation (Clelland and Peng, 2009). Our data revealed that miRNAs are abundantly expressed in the follicular cells from both stages IIIa and IIIb follicles. To further examine the sequencing data, we utilized bioinformatics analyses to further characterize the miRNA profiles in stages IIIa and IIIb follicular cells. It was revealed that 214 known, 31 conserved novel miRNAs and 44 novel miRNAs were present in our follicular cell samples. The majority of mature miRNAs were found to be in the length of 22 nucleotides.

Upon examining the differential expression of the miRNAs, we discovered that 11 miRNAs were significantly up-regulated, and 13 miRNAs were significantly down-regulated in the stage IIIb follicular cells when compared to stage IIIa follicular cells. In addition. We validated the expression of four of the significantly regulated miRNAs, dre-miR-22a-3p, dre-miR-16a, dre-miR-181a-3p, and dre-miR-29a, by real-time PCR. We further conducted gene enrichment analyses on the predicted targets of the significantly regulated miRNAs. It was revealed from our enrichment analyses that some key signaling pathways endorsed potential roles of the differentially expressed miRNAs in regulating ovarian functions including oocyte maturation. Overall, the differential expression of miRNAs between stage IIIa and IIIb follicular cells suggests that these miRNAs are important regulators of zebrafish ovarian follicle development and/or oocyte maturation.

In the future, we would like to expand our understanding of the role of miRNAs in vitellogenic follicles by studying some of the most abundant and most regulated miRNAs we found in this study. Our data revealed that two of the most abundant miRNAs identified in our study were miR-143 and miR-22a-3p. Although there are several lines of evidence, which suggest that these miRNAs are involved in regulating follicle development, their role in fish reproduction has not been reported. Specifically, miR-22a-3p is among the most abundant, most regulated and is one of the miRNAs whose expression we validated, making it an excellent candidate to pursue further. To examine the role of these miRNAs in the acquisition of maturational competence in zebrafish follicles, miRNA mimic and inhibitor oligos will

be transfected in primary ovarian cell cultures to study their effect on steroidogenesis. The oligos will also be microinjected into immature vitellogenic oocytes to observe whether these miRNAs influence oocyte maturation *in vitro*. Moreover, reporter assays will be used to identify the possible genes targeted by these miRNAs and such interactions will be further confirmed with real-time PCR and western blotting in follicular cells and vitellogenic oocytes. Functional rescue experiments will be used to confirm such interactions.

To examine the functions of miR-143 and miR-22a-3p in vivo, we will use the zebrafish CRISPR-ON system that was discussed in Chapter 4. Transgenic fish lines expressing a single guide (sgRNA) will be generated and will then be crossed with our established double-transgenic lines that express the Tet-ON, Doxycycline (Dox)-inducible Cas9-endonuclease. The resulting triple transgenic lines that harbor the two Tet-ON transgenes along with the sgRNA transgene will be selected and loss of function studies in the ovary will be conducted.

Our analysis identified 24 miRNAs that were up- or down-regulated in the maturationally competent stage IIIb follicular cells as compared with follicular cells from stage IIIa follicles. Therefore, the miRNAs that are differentially expressed between these two stages are possibly involved in regulating the maturational competence of these follicles. Upon examining genes that play critical roles in LH- and MIH-induced oocyte maturation, we found 3 of those genes, namely pgrmc1, paqr7b, and lhcgr, among the predicted targets of miRNAs that are differentially expressed in stage IIIa and IIIb follicular cells. These possible miRNA-mRNA

interactions between these genes and miRNAs reported in Table 2.2 will be examined using miRNA mimics and inhibitors, along with reporter assays, real-time PCR, and western blotting, as well as functional rescue experiments in vitellogenic oocytes and follicular cells. In addition, we have found that ndr1 as well as nodal/activin receptors were among the targets of the downregulated miRNAs in stage IIIb. This will be studied in the future.

Aim 2: To determine the role of Nodal in zebrafish follicles and to generate an *in vivo* model to study nodal-related genes.

Members of the TGF-β superfamily are known to be involved in follicle development and oocyte maturation in zebrafish. Nodal, a member of the TGF-β superfamily, plays critical roles during embryo development but its role in regulating ovarian functions in zebrafish is not known. To determine whether nodal is involved in regulating ovarian functions, we first measured the endogenous mRNA levels of ndr1 and ndr2, in all stages of ovarian follicle development as well as in the follicular cells and oocytes of IIIa and IIIb follicles. We found that the expression of ndr1 and ndr2 was regulated in different follicular stages. The expression patterns of ndr1 and ndr2 were also contrasted in follicular cells and oocytes of vitellogenic follicles.

In addition, we found that the ndr1 and ndr2, as well as other Nodal signaling components, acvr1b, and acvr1c, were significantly increased in stage IIIb when compared to IIIa follicular cells. This increase prompted further investigation of the role of Nodal in follicular cells. Treatment with recombinant human Nodal resulted

in the activation of Smad3 and CREB as well as a transient activation followed by inhibition of ERK providing evidence that Nodal is functional in the zebrafish ovary. The role of Smad2/3, CREB, and ERK in ovarian follicular cells will be investigated. Additionally, recombinant human Nodal inhibited follicular cell proliferation and increased the mRNA levels of the steroidogenic enzymes hsd3b2 and cyp17a1 as well as the membrane progestin receptor, paqr8. Conversely, knockdown of *ndr1* and *ndr2* using small interfering RNAs had the opposite effect on the expression of these enzymes, as well as on paqr8. These results suggest that Nodal is involved in regulating steroidogenesis. The protein levels of hsd3b, cyp17a1 and paqr8 will also be measured in the future.

Future studies will also investigate the role of Nodal in regulating oocyte maturation ovarian follicles using oocyte maturation assays. The regulation of paqr7b, paqr8, pgrmc1 in denuded oocytes by Nodal will also be investigated. The potential role of gonadotropins in regulating Nodal signaling components in follicular cells and oocytes will be explored.

Since cyp17a1 is involved in regulating the production of 17alpha-hydroxyprogesterone, as well as precursors for 17beta-estradiol and androgens (Wang and Ge, 2004), the upregulation of cyp17a1 by Nodal may lead to stimulating progesterone, MIH or estradiol production. To determine whether Nodal regulates any of these hormones, their levels will be measured in vitellogenic follicles and in primary ovarian follicular cells co-cultured with recombinant human Nodal.

In Chapter 4, using CRISPR/Cas9 technology, we created *ndr1* and *ndr2* single knockout (KO) lines that displayed cyclopia and early development lethality. Since the *ndr1* phenotype is controversial, a second *ndr1* KO line is being developed to exclude the possibility of an off-targeting effect. Likewise, an *ndr3* KO line is underway. Potential founder of the *ndr3* and the second *ndr1*mutant fish with germline transmittance have been identified and their F1 progeny are currently being raised. Upon establishing the second *ndr1* mutant line, its F2 offspring will be tested for showing a similar phenotype and penetrance rate to the first *ndr1* discussed in Chapter 4. The already established *ndr1* and *ndr2* lines will be further used to test the possibility that ndr1 can induce the expression of ndr2 and vice versa, which we observed with siNdr1 and siNdr2 in Chapter 3. The effect of Nodal loss in heterozygous fish has not been explored by studying double or triple heterozygous *ndr* mutant lines. Thus, *ndr1*, *ndr2,* and *ndr3* KO lines will be used to study the possibility of gene redundancy among the 3 *ndr* genes at the heterozygous level. Finally, we have established Doxycycline-inducible CRISPR-ON lines zebrafish with a single or multiplexed expression of single guide RNAs that target *ndr1*, *ndr2,* and *ndr3* transgenes. These lines will be induced and the effect of targeting the expression of *ndr1*, *ndr2* and *ndr3* will be studied upon induction with Doxycycline in adult fish ovaries.

CONCLUSION

We have discovered that miRNAs are abundantly present and are regulated in zebrafish vitellogenic ovarian follicular cells. We have identified known and novel miRNAs and elucidated the processes and pathways that are potentially targeted by the most regulated miRNAs in vitellogenic follicular cells. In addition, we provide evidence that Nodal may play a role in regulating ovarian steroidogenesis. Lastly, we have generated Dox-inducible CRISPR-ON lines that may bypass the early developmental lethality observed in *ndr1* and *ndr2* knockout lines. Hence, these inducible CRISPR-ON lines will facilitate the study of the roles of ndrs as well as miRNAs in adult zebrafish ovary.

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APPENDIX

ADDITIONAL PUBLICATIONS

Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation

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Frontier Endocrinol, 2018; 9: 402.

I contributed to this paper by writing about circulation, secretion and uptake of microRNAs and miRNAs in biological fluids.

I was also involved in the final revision of the manuscript.