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Comparative and functional analysis of microRNAs during zebrafish gonadal development

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Preface

This dissertation is submitted in fulfillment of the requirements for the degree of Philosophiae Doctor (PhD) at the Faculty of Biosciences and Aquaculture (FBA), Nord University. The presented original research was performed as part of the project FishmiR – Role of small non-coding RNA in fish development: comparative study in regulatory mechanisms, with financial support from the Norwegian Research Council (Grant # 213825).

The PhD project team consisted of the following members:

Christopher Presslauer, MSc, FBA, Nord University: PhD Student
Igor Babiak, Professor, FBA, Nord University: primary supervisor
Jorge M.O. Fernandes, Professor, FBA, Nord University: co-supervisor
Teshome T. Bizuayehu, Post-doc, FBA, Nord University: co-supervisor

Christopher Presslauer
Bodø, January 31, 2017

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Dedication

I would like to dedicate this dissertation to my late uncle, Peter Alan Brownrigg (1947-2016). Peter was a beloved member of the family who found joy in life from bringing happiness to others, and always encouraged me to pursue my passions in life.

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Paper III. **Presslauer C,** Bizuayehu TT, Fernandes JMO, Babiak I. Spatial expression patterns of gonadal miRNAs and function of maternal miR-92a-3p in zebrafish. Manuscript.

List of abbreviations

- 3’ UTR – 3’ untranslated region
- Ago – Argonaute
- eGFP – enhanced green fluorescent protein
- hpf – hours post fertilization
- ISH - *in situ* hybridization
- LNA – locked nucleic acid
- mRNA – messenger RNA
- miRNA – microRNA
- miRISC – miRNA-induced silencing complex
- MO – morpholino oligomer
- MZT – maternal to zygotic transition
- nt - nucleotide
- PGC – primordial germ cell
- piRNA – Piwi-interacting RNA
- pre-miRNA – precursor microRNA
- pri-miRNA – primary microRNA
- siRNA – small interfering RNA
- TSB – target site blocker
- VMO – vivo-morpholino oligomer
- WISH – whole mount *in situ* hybridization
- wpf – weeks post fertilization

Abstract

Zebrafish (*Danio rerio*) is a small tropical fish commonly used as a model organism in science due to several advantageous attributes, such as: small size, easy maintenance, short generation time, and easy reproduction in captivity. Historically, zebrafish were used in developmental biology, as their transparent embryos allowed for observation of the major stages of embryogenesis. More recently, assembly and annotation of the zebrafish genome expanded their use in molecular biology and genetics. microRNAs (miRNAs) are small non-coding RNAs which primarily function as post-transcriptional repressors of messenger RNAs (mRNAs). In metazoans, miRNAs have a variety of functions, including regulation of cell division, differentiation, migration, and apoptosis. However, most research on miRNAs has been performed on mammals. In addition, the function of miRNAs during teleost gonad development remains unknown. Therefore, characterization of the miRNA repertoire in the gonads of zebrafish would provide an important resource for reproductive biology. Unfortunately, developing gonads of zebrafish are too small to identify and sample from juvenile individuals.

To overcome this problem, *See-Thru-Gonad* line was established by crossing an established transparent line with a transgenic line modified to have fluorescently labeled germ cells. *See-Thru-Gonad* allowed observation of germline development *in vivo*, as well as identification and isolation of immature gonads. The advantages of the line were further demonstrated in a targeted gene knockdown experiment. Injection of a morpholino oligomer antisense to miR-92a-3p directly into the mature ovary significantly reduced miR-92a-3p expression in embryos, while significantly increasing the rate of developmental arrest during the first cleavage.

Small RNA sequencing was performed on zebrafish gonads at several stages of development, from undifferentiated stage to sexual maturation. In total, 520 mature miRNAs were identified in zebrafish gonads. miRNA expression was dynamic, with 111 miRNAs exhibiting significant changes in abundance over time, and 50 miRNAs showing

significant differences in abundance between testis and ovary. Among the 27 most abundant miRNAs throughout all gonadal development, 26 had a significant change in abundance over time and/or were differentially expressed between testis and ovary. The gonadal miRNA repertoire was heterogeneous, and all abundant miRNAs had multiple size and sequence variants (isomiRs). In addition, a miRNA arm switch was detected, with miR-202-3p the more abundant miRNA in earlier gonadal stages, and miR-202-5p more abundant in sexually mature gonads.

The spatial expression patterns, demonstrated for six miRNAs, were diverse, with some miRNAs specific to either germline (miR-430b-3p) or somatic cells (miR-202-3p), or with ubiquitous expression (miR-92a-3p). *In silico* target prediction was performed for miR-92a-3p, the most abundant zebrafish gonadal miRNA which was also maternally stocked in unfertilized eggs. The predicted targets included multiple transcripts involved in cell cycle regulation, such as *wee1 homolog 2* (*wee2*). Spatial and temporal expression patterns for both miR-92a-3p and *wee2* were determined in zebrafish embryos using *in situ* hybridization. They were co-expressed during embryonic cleavages, then *wee2* transcripts diminished during the blastula stage. Target validation assays were performed using a chimeric RNA containing the green fluorescent protein coding region fused with the *wee2* 3' untranslated region (3' UTR). The validation assays demonstrated miR-92a-3p was capable of interacting the *wee2* 3' UTR to suppress its translation.

In conclusion, the miRNA repertoire in zebrafish gonads is dynamic. The changing abundance patterns, isomiR heterogeneity, and diverse spatial expression patterns all suggest a specialized role of miRNAs in fine-tuning gonadal development.

Abstract (in Norwegian)

Komparativ og funksjonell analyse av microRNAer gjennom sebrafiskens gonadeutvikling

Sebrafisk (*Danio reiro*) er en tropisk fisk med mange egenskaper som gjør den godt egnet som modellorganisme i forskning. Den er liten, enkel å vedlikeholde, har kort generasjonstid og enkel reproduksjon i fangenskap. Historisk sett ble sebrafisk benyttet i utviklingsbiologi, der de transparente embryoene gjorde det mulig å observere de grunnleggende stadiene av embryogenesen. I nyere tid har sammensetningen og annoteringen av sebrafiskgenomet utvidet dens bruk til å også inkludere molekylærbiologi og genetikk. microRNAer (miRNAer) er små ikke-kodende RNAer som hovedsakelig fungerer som post transkriptoniske repressorer av budbringer RNA, eng. 'messenger RNA' (mRNAs). I metazoer har miRNAer en rekke funksjoner, inkludert regulering av celledeling, differensiering, migrasjon og apoptose. Størsteparten av den eksisterende forskningen på miRNA har blitt utført på pattedyr, og funksjonen til miRNAer i gonadeutviklingen hos teleoster er fremdeles helt ukjent. En karakterisering av miRNA repertoaret i gonadene til sebafisk vil av den grunn være en viktig ressurs for reproduksjonsbiologi. Gjennom utviklingen er uheldigvis gonadene til sebrafiskyngel for små til å kunne identifiseres og tas prøver av.

For å overvinne dette problemet ble *See-Thru-Gonad* avlslinjen etablert ved å krysse en etablert transparent avlslinje med en transgen avlslinje modifisert for fluorescensmerkede kjønnsceller. *See-Thru-Gonad* gjorde det mulig å observere kjønnsutviklingen *in vivo*, i tillegg til å kunne identifisere og isolere underutviklede gonader. Fordelen med denne avlslinjen ble videre demonstrert i et målrettet 'gene-knockdown' (eng.) eksperiment. Injisering av en 'morpholino oligomer antisense' (eng.) til miR-92a-3p direkte inn i ferdig utviklede eggstokker medførte en signifikant miR-92a-3p uttrykk i embryoer, samtidig som graden av utviklingsarrest økte signifikant gjennom den første celledelingen.

'Small RNA' (eng.) sekvensering ble utført på sebrafiskgonader gjennom flere stadier i utviklingen, fra det udelte cellestadiet til kjønnsmodning. Totalt ble 520 ferdig utviklede miRNAer identifisert i sebrafiskgonader. miRNA uttrykket var dynamisk, med 111 miRNA som utviste signifikante endringer i mengde over tid, og 50 miRNA som utviste signifikante forskjeller i mengde ved sammenligning av testikler og eggstokker. Blant de 27 vanligste miRNAene gjennom hele gonadeutviklingen, hadde 26 en signifikant forskjell i mengde over tid og/eller var differensielt uttrykt for testikler og eggstokker. Gonadenes miRNA repertoar var heterogent og alle av de mest tallrike miRNAer hadde flere størrelses og sekvensvarianter (isomiRs). I tillegg, ble det oppdaget et miRNA arm-skifte, med miR-202-3p som den mest tallrike miRNAen, i de tidligste gonadestadiet og miR-202-5p som den mest tallrike i kjønnsmodne gonader.

De romlige uttrykksmønstrene, demonstrert for seks miRNAer, var variable, med noen miRNAer som var spesifikke for enten kjønnslinjen (miR-92a-3p) eller somatiske celler (miR-202-3p), eller med et utbredt genuttrykk (miR-92a-3p). *In silico* mål-forutsigelse, eng. 'target prediction', ble utført for miR-92a-3p, den mest vanlige miRNAen i sebrafisk gonader, som også var maternalt lagret i ubefruktede egg. De forutsatte målene inkluderte flere transkripter som er involvert i regulering av cellesyklusen, som for eksempel *wee1 homolog 2* (*wee2*). Romlige and temporale uttrykksmønstre for både miR-92a-3p og *wee2* ble påvist i sebrafiskembryoer ved bruk av *in situ* hybridisering. Disse var begge uttrykt gjennom den embryoniske celledelingen og deretter forsvant *wee2* transkriptene i løpet av blastula stadiet. Mål-validerings analyse, eng. 'target validation assays', ble utført ved bruk av et kimærisk RNA som inneholdt et grønt fluorescerende protein koblet på *wee2 3'* utranslert område, eng. 'untranslated region' (3' UTR). Validering av analysen viste at miR-92a-3p var i stand til å interagere med *wee2 3'* UTR for å undertrykke dets translasjon.

For å konkludere, miRNA repertoaret i sebrafiskgonader er dynamisk. De variable mangfoldighetsmønstrene, 'abundance patterns' (eng.), isomiR heterogeniteten og de

mangfoldige romlige uttrykksmønstrene indikerer at miRNAer har en spesialisert rolle i fin-justeringen av gonadeutviklingen.

1. Introduction

1.1 Zebrafish in research

Zebrafish (*Danio rerio*) is a small freshwater fish native to the rivers and streams of tropical Asia, including Pakistan, India, Bangladesh, and Nepal (Talwar and Jhingran, 1991). While zebrafish are not an important species for food consumption, they belong to the family Cyprinidae, which is the largest family of teleosts with over 3000 described species (Nelson, 2006) and includes several of the most cultivated species in aquaculture, such as common carp (*Cyprinus carpio*), silver carp (*Hypophthalmichthys molitrix*), and grass carp (*Ctenopharyngodon idella*) (FAO, 2014). Zebrafish are also commercially valuable as an ornamental fish. Known for being active with a playful disposition, zebrafish are easy to maintain in household aquaria, an attribute which helped lead them to the forefront of science (Gerhard and Cheng, 2002).

The development of zebrafish as a model species for science is largely credited to Dr. George Streisinger and his colleagues at the University of Oregon, U.S.A., who pioneered zebrafish research through the 1970s and 1980s. This research group chose zebrafish as a model for several desirable traits including short generation time (3-4 months), weekly spawning intervals, rapid, external, and synchronous embryonic development, as well as their small size and ease of aquarium rearing (Streisinger et al., 1981). Notably, that lab produced the first reference zebrafish line (AB; ZFIN ID: ZBD-GENO-960809-7), which originated in the late 1970s from two zebrafish (ironically named A and B) purchased at different times from a pet store in Albany, Oregon, USA (Wilson et al., 2014). These fish were subsequently used to produce the first homozygous diploid zebrafish clones (Streisinger et al., 1981). As a result of their efforts, the University of Oregon established the "zebrafish model organism database" (http://www.zfin.org) and produced "The Zebrafish Book: a guide for the laboratory use of zebrafish (*Danio rerio*)", which remains a standard resource for zebrafish researchers today.

Another milestone in zebrafish research was the detailed staging of embryonic development (Kimmel et al., 1995). That study took advantage of the optical transparency of zebrafish embryos to produce high quality images of different stages of morphogenesis during the first three days after fertilization. To date, this study is the most cited zebrafish article with over 3500 citations, and is a foundation for research in zebrafish developmental biology (Kinth et al., 2013).

With the establishment of zebrafish as a model species for science, its use has expanded to a variety of disciplines. While the traditional focus on developmental biology is still relevant, in 2013, biochemistry/molecular biology surpassed developmental biology as the top subject area for zebrafish research (Kinth et al., 2013). Other popular fields such as cell biology, neurology, and genetics have also seen steady increases in research activity in recent years (Kinth et al., 2013). In addition, while zebrafish are still an excellent model for studying teleost development, sequencing of the zebrafish genome has highlighted their genetic similarity to mammals, with approximately 70 % of human protein-coding genes having an obvious zebrafish orthologue (Howe et al., 2013). One area of zebrafish research which is comparatively underdeveloped is reproductive biology. Despite the ease of breeding, the small size of immature zebrafish gonads makes them challenging to isolate. In addition, while there is evidence zebrafish follow a polygenic sex determination system, there are no clearly defined sex determining genetic markers (Liew et al., 2012).

1.2 Zebrafish reproduction

1.2.1 Primordial germ cells

Metazoan reproduction begins with the primordial germ cells (PGCs). These cells are specified during embryogenesis and give rise to all future gametes; therefore, they are the cells responsible for passing on hereditary information to the next generation. There are two known mechanisms of PGC specification in metazoans: epigenetic and

preformation (Extavour and Akam, 2003). The epigenetic model involves inductive signals from somatic cells in localized regions of the embryo, as in the mechanism described for mouse, *Mus musculus* (Tsang et al., 2001). In zebrafish and all known teleosts to date, PGCs are specified through preformation, which involves a population of cells inheriting maternally synthesized RNAs and proteins referred to as the germ plasm (Extavour and Akam, 2003). In zebrafish, the germ plasm components are stored in the oocytes during oogenesis (Raz, 2003). During the initial embryo cleavage, the germ plasm components condense within the cleavage furrow but remain outside the cells. At the 32-cell stage, the germ plasm localizes within four cells, which are then fated as PGCs. In subsequent cell divisions, the germ plasm remains packaged within the cell cytoplasm (Raz, 2003, Yoon et al., 1997). PGC specification occurs during the maternal to zygotic transition (MZT), at approximately the 1000-cell stage, when the germ plasm components become unpackaged and are observed throughout the cytoplasm (Yoon et al., 1997). After specification, the PGCs proliferate before undergoing an active migration towards the future site of the gonad (Yoon et al., 1997). To migrate, the PGCs are guided by the ligand stromal-cell-derived factor-1a (Sdf1a). The ligand is released by somatic cells at staging points in the embryo, and the signal is received by chemokine c-x-c motif receptor 4a (Cxcr4a), expressed within PGCs (Blaser et al., 2006, Knaut et al., 2003). During early somitogenesis, PGCs form two clusters on either side of the notochord, between the third and fifth somites (Yoon et al., 1997); it is in the location of the future gonad.

1.2.2 Sexual differentiation

In zebrafish, the process of sex differentiation begins shortly after hatching when the gonads undergo a form of juvenile hermaphroditism (Maack and Segner, 2003, Sun et al., 2013, Takahashi, 1977). For the first few days after hatch, PGCs proliferate and some of them differentiate into gonocytes. The gonocytes continue to proliferate mitotically, forming clusters, which are surrounded by a layer of granulosa precursor cells; the clustered gonocytes subsequently initiate meiosis. In the following days, the gonocytes

become isolated from the cluster and become enveloped individually by somatic cells (Lubzens et al., 2010, Selman et al., 1993, Sun et al., 2013, Takahashi, 1977). At around three weeks post fertilization (wpf), zebrafish gonads appear as immature ovaries, with some gonocytes having entered the primary growth stage of oogenesis. However, in the subsequent days, in approximately half of the juveniles, the gonads enter an altered state characterized by the progressive degeneration of the oocytes which had formed. In these transitioning gonads, somatic cells of the stroma increase in number before infiltrating the gonad to surround the gonocytes. The gonad then rapidly follows testicular morphogenesis. Zebrafish sexual differentiation is typically complete by approximately 30 days post fertilization, although this is highly variable and dependent on zebrafish line and environment (Maack and Segner, 2003, Maack et al., 2003, Takahashi, 1977).

Although all zebrafish gonads enter this initial ovary-like stage, both Takahashi (1977) and Sun et al., (2013) described two different variations of the early gonad. In gonads which presumably developed into ovaries, the majority of germ cells entered meiosis and became perinucleolar oocytes, whereas in other gonads there were noticeably fewer meiotic germ cells, and an increased number of somatic cells were observed. One of the prominent hypotheses regarding zebrafish sexual differentiation is that sex is determined by whether a threshold number of germ cells is met. If so, ovarian development continues; if the threshold is not met, the gonad eventually transitions into a testis. Evidence for this hypothesis comes from several studies correlating the number of germ cells with sex ratios. For example, zebrafish which have had their germ cells ablated during embryogenesis develop as sterile but phenotypically male fish (Siegfriend and Nusslein-Volhard, 2008, Slanchev et al., 2005). In the study by Slanchev et al., (2005), it was noted that in a small number of zebrafish the treatment failed and the fish were sexually mature, likely due to a small number of germ cells surviving the treatment and successfully colonizing the gonad. However, among these treated fish the sex ratio was overwhelmingly male (76 %) compared to an even split in control fish (51 %). In addition, a single transplanted PGC was sufficient

to develop the zebrafish gonad, but the fish all developed as males (Saito et al., 2008). In a more recent study, researchers estimated the number of PGCs present in the gonad of juvenile zebrafish and checked for correlation with the sex ratio (Tzung et al., 2015). At 14 days post fertilization (dpf), the PGC distribution was bimodal, with individuals exhibiting either a low (< 60) or high (> 65) PGC count. Families with lower PGC counts were disproportionately male-dominant, whereas families with higher PGC counts were disproportionately female-dominant. Therefore, a reduced number of germ cells in the juvenile gonad greatly increases the probability of ovarian degeneration and subsequent testis development (Fig. 1).

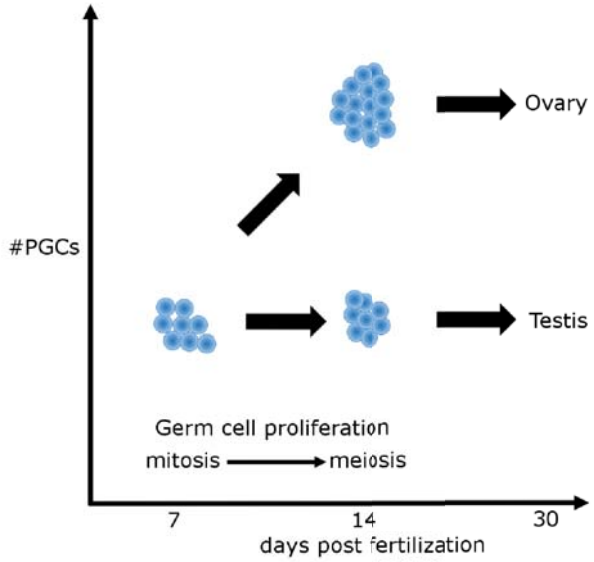

Figure 1. The correlation between the number of meiotic germ cells in the zebrafish gonad and sex differentiation. Modified from Tzung et al. (2015).

In zebrafish gonads, both *cytochrome P450, family 19, subfamily A, polypeptide 1a* (*cyp19a1a*) and *sex determining region Y box-9a* (*sox9a*) are expressed at 25 dpf within somatic cells (Siegfriend and Nusslein-Volhard, 2008). The gene *cyp19a1a* encodes the enzyme aromatase cytochrome P450, which is responsible for converting androgens to estrogens, and is essential to ovarian development (Jørgensen et al., 2008). By

comparison, *sox9a* is a transcription factor which acts as an upstream positive regulator of several genes involved in testis differentiation, including *anti-Müllerian hormone* (*amh*) (Rodríguez Marí et al., 2005). AMH negatively regulates *cyp19a1a* and inhibits primordial follicle assembly in the ovary (Rodríguez Marí et al., 2005, Nilsson et al., 2011). By 35 dpf, as zebrafish gonads enter sexual differentiation, both *cyp19a1a* and *sox9a* exhibit dimorphic expression (Siegfriend and Nusslein-Volhard, 2008); gonads which will develop as ovaries maintain high expression of *cyp19a1a* but have reduced *sox9a* expression, while the opposite is true for transitioning gonads. Germ cell-depleted gonads also exhibit high expression of *sox9a*, while *cyp19a1a* is not detectable (Siegfriend and Nusslein-Volhard, 2008). A possible mechanism by which germ cells promote ovarian development was hypothesized (Siegfriend and Nusslein-Volhard, 2008); meiotic germ cells recruit bi-potential gonadal-somatic cells and through their interactions reinforce expression of *cyp19a1a* as well as promote *forkhead box l2* (*foxl2*), a pro-ovary transcription factor which also represses *sox9a* (Georges et al., 2014). Somatic cells unable to recruit meiotic germ cells would have higher expression of *sox9a* and subsequently *amh,* leading to the repression of *cyp19a1a*. In the entire gonad, the number of meiotic germ cells would therefore influence the balance between *cyp19a1a* and ovary development, or *sox9a* and testis development.

1.2.3 Oogenesis

Zebrafish oogenesis is typically divided into five stages: primary growth, cortical alveolus stage, vitellogenesis, oocyte maturation, and fully mature egg (Lubzens et al., 2010, Selman et al., 1993). The primary growth stage is characterized by the onset and arrest of meiosis during prophase I, the recruitment of the follicle layer (theca and granulosa cell layers), assembly of organelles, and intense RNA transcription. Many RNA molecules with functional roles later during oogenesis, such as vitellogenin receptors, are formed during this time, as well as maternal mRNAs which are stored in the oocyte to regulate embryogenesis.

The appearance of the cortical alveoli marks the transition of the oocyte to the secondary growth stages (Lubzens et al., 2010, Selman et al., 1993). Cortical alveoli are membrane-limited vesicles which are stocked with proteins destined for the restructuring of the egg envelope during the cortical reaction after fertilization (Selman et al., 1993). Lectins are typically associated with cortical alveoli, and have functions in preventing polyspermy while facilitating the formation of the chorion (Luckenbach et al., 2008, Dong et al., 2004). During this stage, an oocyte forms the tripartite vitelline envelope, also known as the zona radiata. This envelope consists of three layers: the zona radiata externa, the zona radiata interna 1, and the zona radiata interna 2 (Selman et al., 1993). The zona externa consists of zona pellucida proteins. These proteins form primary components of the egg chorion, although other functions include regulation of nutrient uptake during oogenesis, and guidance of spermatozoa to the micropyle (Berois et al., 2011).

The vitellogenic stage marks a period of major oocyte growth, primarily due to the uptake and storage of the yolk precursor protein vitellogenin, as well as other lipids and vitamins (Lubzens et al., 2010, Selman et al., 1993). Vitellogenins are the precursors for lipoproteins and phosphoproteins which constitute a major source of nutrients during early development. Teleosts have at least three vitellogenin genes (*vtga, vtgb, vtgc*). They are primarily synthesized in the liver and transported to the ovary, where their transport is facilitated through the vitelline envelope pore canals and are received by vitellogenin-specific receptors which mediate endocytosis. By the end of vitellogenesis, the follicle cells become competent to respond to endogenous hormones, and begin the process of oocyte maturation (Selman et al., 1993).

Oocyte maturation includes the resumption of meiosis and ovulation. First, the germinal vesicle (the term for the oocyte nucleus arrested during meiotic prophase) migrates to the oocyte periphery where the nuclear envelope breaks down (Selman et al., 1993). The first meiotic division is completed and meiosis continues to metaphase II, where meiosis is arrested again until fertilization. During this time, the final growth of

the oocyte occurs due to hydration. Subsequently, the follicle cells release the oocyte from the ovary into the ovarian lumen.

1.2.4 Spermatogenesis

Teleost spermatogenesis can be divided into three general phases: the mitotic proliferative phase (or spermatogonial), the meiotic (or spermatocytary), and the spermiogenic (Schulz et al., 2010). In the zebrafish transforming gonad, concurrent to the degeneration of the initial primary oocytes, stromal cells encompass gonocytes and differentiate into Sertoli cells and spermatogonia cysts, respectively (Sun et al., 2013). Within the cysts, mitotic gonocytes differentiate into type A spermatogonia, and begin a period of proliferation (Schulz et al., 2010). While a number of type A spermatogonia is maintained for the renewal of the population, others continue through several mitotic divisions to produce type B spermatogonia. From the first mitotic divisions until spermiogenesis, incomplete cytokinesis results in cytoplasmic bridges between spermatogonia. These allow synchronized development of all germ cells within a cyst (Schulz et al., 2010).

The transition from a spermatogonium to a spermatocyte is characterized by the meiotic division (Rupik et al., 2011, Schulz et al., 2010). Primary spermatocytes (meiosis I) undergo a prolonged period of prophase I and are typically observed in the testis. The completion of meiosis I produces the haploid secondary spermatocytes. However, secondary spermatocytes are rarely observed in zebrafish, as they rapidly enter meiosis II (Rupik et al., 2011, Schulz et al., 2010). At the completion of meiosis II, the spermatocyte transforms to a spermatid and the process of spermiogenesis begins.

During spermiogenesis, spermatids undergo a series of complex morphological changes, eventually resulting in the mature spermatozoa. These include nuclear condensation, elimination of organelles and cytoplasm, flagellum formation, and a re-arrangement of cellular organelles (Schulz et al., 2010, Jamieson, 1991). Upon

completion, the intercellular bridges between spermatids, as well as between them and Sertoli cells, are broken. The cyst complex opens up and the spermatozoa are released into the tubular lumen.

1.3 microRNAs

microRNAs (miRNAs) are a family of small non-coding RNAs approximately 22 nucleotides long which regulate gene expression (Bartel and Chen, 2004). The first miRNA was discovered in 1993 during a study of a null-mutation in *Caenorhabditis elegans* which caused failure in temporal development during embryogenesis (Lee et al., 1993). The authors found lin-4 locus within the intronic region of an unrelated gene, and used transformational rescue to confirm it as the source of the phenotype. The authors were unable to find conventional start and stop codons within the sequence, but did find RNA transcripts with lengths of 22 and 61 nucleotides. Within the transcripts, there were sequences complementary to the 3' untranslated (3' UTR) region of the gene *lin-14*. Null-mutations for *lin-14* caused the opposite phenotype as those observed in lin-4 mutants, leading the authors to suggest that *lin-4* negatively regulates *lin-14* translation via antisense RNA-RNA interactions (Lee et al., 1993).

Other miRNAs were not discovered until 2000 when researchers identified let-7. Similar to lin-4, researchers found let-7 to be essential to temporal development in *C. elegans*, specifically in the L4 larva to adult transition, where it downregulated *lin-41* (Reinhart et al., 2000). However, while lin-4 function appeared to be specific to *C. elegans*, let-7 is highly conserved in bilaterian animal species including fly (*Drosophila melanogaster*), sea urchin (*Stronglyocentrotus purpuratus*), zebrafish, chicken (*Gallus gallus*), and human (*Homo sapiens*) (Pasquinelli et al., 2000). It was found that multiple miRNAs shared common sequence identity with let-7 at the 5' end, which was termed the seed region and theorized to be the site of RNA binding activity (Brennecke et al., 2005). These miRNAs were categorized as the let-7 family, and several shared redundant functions in regulating larval transitions in *C. elegans* (Abbott et al., 2005).

The discovery that miRNAs may be widespread in metazoans increased interest in their research. Since the early studies in *C. elegans*, miRNA research has become a major field of transcriptomics, accounting for 6,560 publications in 2013 (Casey et al., 2015). miRNAs have functions in cell division (Hwang and Mendell, 2006), differentiation (Ivey and Srivastava, 2010), migration (Staton et al., 2011), and apoptosis (Su et al., 2015). However, miRNA research is predominantly focused on mammals. In teleosts, which are the largest group of vertebrates (Nelson, 2006), only 1,367 mature miRNA have been described to date, compared to 13,460 mature miRNAs in mammals (miRBase v.21).

1.3.1 miRNA biogenesis

miRNA genes can be located in various regions of the genome, including introns and exons of protein-coding genes, as well as intergenic regions (Wahid et al., 2010). In many cases, miRNAs are clustered together with a shared promoter to form poly-cistronic primary transcripts (Wahid et al., 2010); for example, the miR-17-92 cluster is highly conserved in animals and produces a single primary transcript containing miR-17, 18a, 19a, 19b, 20, and 92a (Olive et al., 2013). However, miRNAs may also be mono-cistronic with their own promoter, such as miR-202 (Roush and Slack, 2008).

Transcription of miRNAs is generally performed by RNA polymerase II (Ha and Kim, 2014, Lee et al., 2004); although in human a small group of miRNAs associated with Alu repeats were shown to be transcribed by RNA polymerase III (Borchert et al., 2006). The resulting RNA transcripts are referred to as primary miRNAs (pri-miRNAs) and may be several thousand nucleotides long. In the case of poly-cistronic transcripts, each miRNA will form its own stem loop secondary structure (Wahid et al., 2010).

miRNA biogenesis typically follows the canonical pathway (Fig. 2), which requires two RNaseIII enzymes: Drosha and Dicer (Chong et al., 2010). First, the stem-loop of a pri-miRNA is cropped by the microprocessor complex which consists of Drosha and its RNA-

binding partner Dgcr8/Pasha, resulting in a short stem-loop precursor miRNA (pre-miRNA) approximately 70 nucleotides in length (Denli et al., 2004). The cropping typically occurs approximately 11 nucleotides from the basal junction, and 22 nucleotides from the apical junction. In addition, it tends to leave a 2 nucleotide long 3' overhang. Following processing, the pre-miRNA is exported from the nucleus to the cytoplasm. Typically this is performed by the protein Exportin 5 (Bohnsack et al., 2004). However, some miRNAs use alternative pathways, such as the miR-320 family, which uses Exportin 1 (Xie et al., 2013). In the cytoplasm, pre-miRNA are further processed by Dicer, which interacts with the double-stranded RNA binding domain protein (TAR RNA binding protein, TRBP) to cleave the pre-miRNA near its terminal loop, resulting in a small RNA duplex (Ha and Kim, 2014, Li and Rana, 2014). Typically, Dicer interacts with the 3' overhang left by Drosha and cleaves 21 to 25 nucleotides upstream (MacRae et al., 2006). However, there are also examples of Dicer interacting with the 5' end, and cleaving 22 nucleotides upstream (Park et al., 2011).

The miRNA duplex is loaded into a member of the Argonaute (Ago) protein family, which together form part of the miRNA-induced silencing complex (miRISC). Within the Ago protein, one strand of the duplex is removed (passenger strand) and the remaining strand (guide strand) is used by the miRISC complex to interact and suppress the translation of its target mRNAs (Ha and Kim, 2014, Li and Rana, 2014). Both the 5p and 3p miRNA arms (as defined by their location in the pre-miRNA relative to the 5' and 3' ends) may act as the guide strand. Preferential loading into the Ago protein is generally thought to be determined by the thermodynamic stability of the ends, with a preference for the arm with the lower thermodynamic stability at its 5' end (Khvorova et al., 2003).

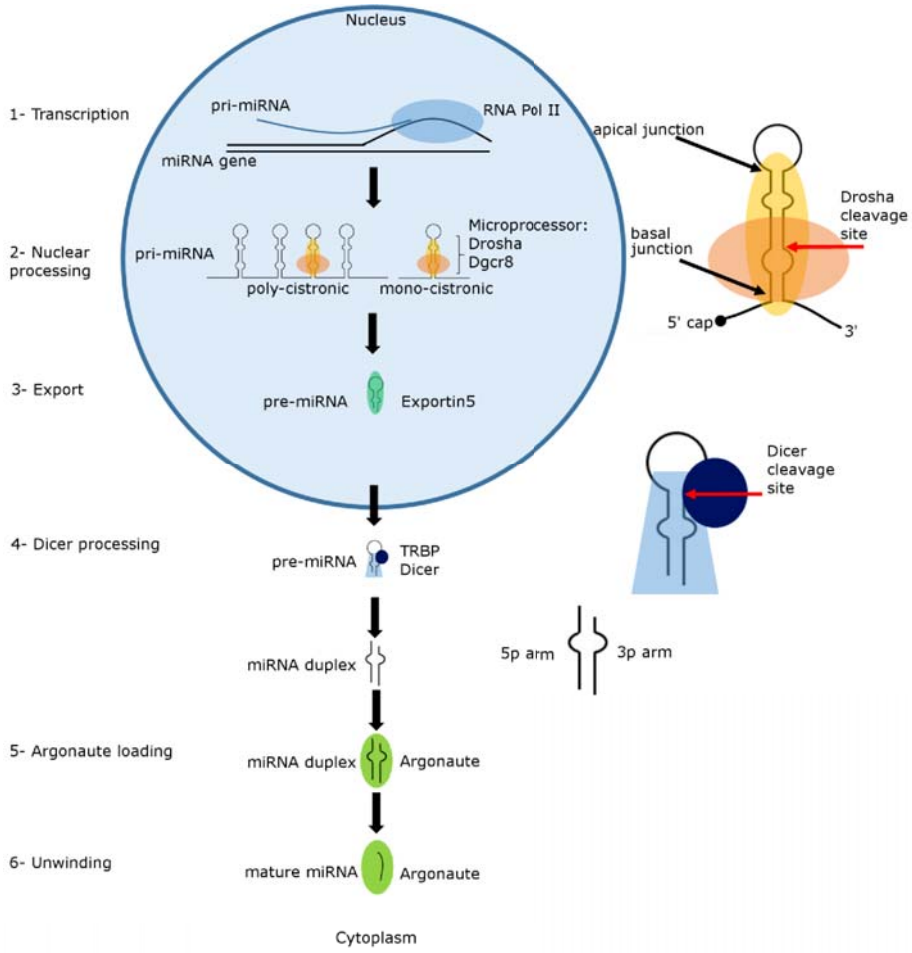

Figure 2. The major steps of the miRNA canonical biogenesis pathway. Modified from Ha et al. (2014).

While the vast majority of miRNAs follow the canonical biogenesis pathway, there are several exceptions in which Drosha-mediated processing or Dicer processing are bypassed (Ha and Kim, 2014). One example of an alternative biogenesis pathway involves mirtrons, where miRNAs are generated through mRNA splicing. In this case, the lariat produced from splicing is refolded into a pre-miRNA structure (Ruby et al., 2007). In another example, miRNAs may be derived as a result of processing precursor structures of other non-coding RNAs, such as tRNA (Maute et al., 2013). Furthermore, some miRNAs, such as human miR-320a, are transcribed directly as pre-miRNAs, and

therefore do not require Drosha mediated cleavage (Kim et al., 2016, Xie et al., 2013). In this scenario, because the pre-miRNA was generated directly from transcription, the 5' end has a 7-methylguanosine cap, which is recognized for nuclear export by the PHAX-exportin 1 pathway (Xie et al., 2013). For these miRNAs, only the 3p arm is loaded onto Argonaute protein, possibly because the 5' cap will interfere with 5p arm loading (Ha and Kim, 2014).

miRNA biogenesis in which Dicer-mediated processing is bypassed has also been described (Cheloufi et al., 2010). Because the stem-region of pre-miR-451 is unusually short (17 nucleotides), it is thought to be too small to be recognized and processed by Dicer (Cheloufi et al., 2010). Instead, after Drosha mediated processing, the pre-miR-451 is loaded directly onto Ago2 protein (Cheloufi et al., 2010).

1.3.2 miRISC function

At present, the miRISC mechanisms of action are not fully understood, with evidence suggesting multiple modes of interaction. The core components of miRISC include a miRNA, an Ago protein, and an Argonaute-bound GW182 protein (Fabian and Sonenberg, 2012). Upon loading into the Ago protein, miRNAs serve as an antisense template to guide the miRISC complex to its target. Studies in which Ago proteins were artificially tethered to the mRNA 3' UTR demonstrated that the miRNA itself is not required for silencing activity (Pillai et al., 2004), whereas Ago and GW182 are essential for miRISC function (Schmitter et al., 2006, Eulalio et al., 2008).

The primary function of the miRISC complex is in post-transcriptional regulation of mRNA, either through mRNA degradation or translational repression (Huntzinger and Izaurralde, 2011, Morozova et al., 2012). mRNA degradation can occur either through endonucleolytic cleavage or directing the target mRNA to the 5' to 3' mRNA decay pathway. The Ago protein contains PAZ and PIWI domains (Höck and Meister, 2008, Hutvagner and Simard, 2008, Lingel et al., 2004). The PAZ domain contains a binding

pocket which anchors the two nucleotide overhang found at the 3' end of the miRNA guide strand. The PIWI domain is homologous to RNase H, and vertebrate Ago2 has retained this endonuclease activity. miRNAs with near or full complementarity to their target mRNA can enable Ago2 to directly cleave the mRNA, for example miR-196 and *Homeobox b8 (Hoxb8*) in mouse (Yekta et al., 2004). However, while full complementarity is common in plant miRNAs, it is relatively rare in animals, with most mRNAs entering the 5' to 3' mRNA decay pathway instead (Behm-Ansmant et al., 2006, Dalmay, 2013, Rehwinkel et al., 2005). For example, the widespread clearance of maternal mRNAs during zebrafish embryogenesis, mediated by miR-430, follows the 5' to 3' mRNA decay pathway (Giraldez et al., 2006). In this scenario, GW182 facilitates recruitment of enzymes, which de-adenylate the mRNA 3' UTR, followed by 5' cap removal, and eventually 5' to 3' degradation (Huntzinger and Izaurralde, 2011).

There are several possible mechanisms by which the miRISC complex acts to suppress mRNA translation. Translation initiation can be inhibited by competition for the 5' cap structure by the miRISC complex with the eIF4F complex, as well as by inhibition of the ribosomal 60S subunit associating with 40S. The miRISC complex may also act to inhibit translational elongation, either by blocking elongation or promoting ribosome "drop-off". An additional proposed mechanism suggests that translation is not inhibited, but instead the polypeptide chain is degraded co-translationally. The variety of mechanisms suggests a great degree of flexibility in which miRNAs can "fine tune" mRNA expression (Eulalio et al., 2008, Huntzinger and Izaurralde, 2011, Morozova et al., 2012).

1.3.3 miRNAs in teleost reproduction

The miR-430 family are the most studied miRNAs during fish embryogenesis because of their overwhelming representation around the maternal-to-zygotic transition. miR-430 are biologically active in clearance of maternal mRNAs (Giraldez et al., 2006). Several maternal mRNAs essential to proper germline development, including *nanos homolog 1* (*nos1*)*, tudor domain containing 7* (*tdrd7*)*,* and *hubble* (*hub*), are miR-430 targets (Kedde

et al., 2007, Mickoleit et al., 2011, Mishima et al., 2006). During PGC specification, the maternal factor *dead end* (*dnd*) encodes a protein which binds these target transcripts and prevents miR-430-mediated decay (Kedde et al., 2007). PGC migration is regulated by Sdf1a chemotaxis (section 1.2.1). During the migration, PGCs group at staging points within the embryo before proceeding (Blaser et al., 2006). As the PGCs progress, miR-430 facilitates clearance of *sdf1a* transcript from previously expressing somatic cells. In addition, miR-430 regulates expression of the decoy *chemokine receptor 7b* (*cxcr7b*), in order to prevent excessive depletion of Sdf1a ligand (Staton et al., 2011). PGCs in zebrafish embryos lacking both maternal and zygotic Dicer were frequently mislocalized, while addition of exogenous miR-430 was able to rescue PGC migration (Staton et al., 2011).

miRNA function during gonadal development is poorly understood. Zebrafish with germline mutations for *dicer* and *ago2* are able to reach sexual maturity and successfully reproduce (Giraldez et al., 2005, Cifuentes et al., 2010). In mammals, germline *Dicer1* mutations result in sterility; however, it should be noted that Dicer functions in the biogenesis of both miRNA and small interfering RNAs (siRNAs), with the latter possibly responsible for the observed phenotypes (Hayashi et al., 2008, Murchison et al., 2007, Suh et al., 2010). Another group of small non-coding RNAs called Piwi-interacting RNA (piRNA) are predominantly expressed within the animal germline (Grivna et al., 2006a, Grivna et al., 2006b, Vagin et al., 2006). piRNA are longer than miRNAs (approximately 30 nucleotides long) and interact with Piwi proteins, a distinct sub-class of Argonaute protein (Carmell et al., 2002). In zebrafish, Piwi proteins and piRNAs are essential to germ cell maintenance, differentiation, and meiosis (Houwing et al., 2008, Houwing et al., 2007)

miRNAs exhibit dynamic expression patterns throughout gonadal development. In teleosts, sexually dimorphic expression patterns have been reported in the gonads of Atlantic halibut (*Hippoglossus hippoglossus*), yellow catfish (*Pylodictus fulvidraco*), Nile tilapia (*Oreochromis niloticus*), zebrafish, and rainbow trout (*Oncorhynchus mykiss*)

(Bizuayehu et al., 2012a, Jing et al., 2014, Xiao et al., 2014, Vaz et al., 2015, Juanchich et al., 2016, Tao et al., 2016). miRNA expression profiles are also dynamic during teleost sex differentiation (Liu et al., 2015, Tao et al., 2016). In these studies, many of the gonadal enriched miRNAs are predicted to target steroidogenic enzymes or proteins involved in vertebrate sex differentiation. In zebrafish, miR-17-5p and miR-430b-3p were enriched in follicular cells during early vitellogenesis, and their expression could be manipulated using a luteinizing hormone analog (Abramov et al., 2013).

Abundant miRNAs with dynamic expression patterns suggest an important role in fine-tuning gonadal development in teleosts. However, to date, no miRNA has been functionally validated in teleost gonads. In addition, small RNA sequencing studies typically focus on sexually mature gonads (Juanchich et al., 2016, Vaz et al., 2015, Xiao et al., 2014), or sexual differentiation (Jing et al., 2014, Tao et al., 2016), with fewer examples of studies examining changing miRNA repertoire over time (Bizuayehu et al., 2012a, Juanchich et al., 2013). In zebrafish, the gonadal miRNA repertoire has only been described in sexually mature testis, ovary, and spermatozoa (Jia et al., 2015, Vaz et al., 2015).

Zebrafish are a major animal model with substantial genomic resources available. However, the miRNome has yet to be characterized. miRNAs are important regulatory elements and knowledge of their abundance during gonadal development would provide new information on their gonadal function. In addition, knowledge of spatiotemporal expression patterns of gonadal miRNAs will allow *in silico* targeting to be refined towards co-expressed targets. Gonadal miRNAs may then be functionally validated.

2. Objectives

The general objective of this dissertation was to establish zebrafish as a model species for teleost reproductive biology, and use it to determine the miRNA repertoire in the gonads of zebrafish. This includes determining spatial and temporal expression patterns of miRNAs, and examining their biological function in gonads. The specific objectives addressed in the manuscripts were as follows:

1. Despite zebrafish being a model organism, their small size makes examination of immature gonads challenging. To address this issue, a transparent and fluorescently labeled zebrafish line was established, and its benefits validated in a miRNA functional study (**paper I**).

2. Small RNA sequencing has identified miRNAs in the gonads of several teleost species. However, sequencing experiments typically focus on single time points. To date, no study has reported comprehensive small RNA sequencing throughout zebrafish gonadal development. To determine the dynamics of the miRNA repertoire, isolated zebrafish gonads were sequenced at five major stages of development, from an undifferentiated stage to maturation (**paper II**).

3. It is known that miRNAs are abundant in teleost gonads. However, their functions remain largely unknown. In order to validate miRNA function, spatial expression was determined for several gonad abundant miRNAs. For miR-92a-3p, co-expression with *wee2* was established, followed by target validation assays (**paper III**).

3. General Discussion

The general aim of this dissertation was to establish a zebrafish line for reproductive biology research, and to use the line to characterize miRNAs involved in gonadal development. The "*See-Thru-Gonad*" zebrafish line was created by crossing fish with mutations causing a lack of pigment and muscle transparency, with those containing the *vasa:vasa-eGFP* transgene, which labels the germline (**paper I**). *See-Thru-Gonad* zebrafish were useful for reproductive biology purposes, with the germline easily observable *in vivo* throughout the lifecycle. The line allowed accurate injections of a vivo-morpholino targeting miR-92a-3p directly to the ovary, which resulted in embryos arrested during cleavage. The *See-Thru-Gonad* line was used for isolation and small RNA sequencing of gonads from 3 wpf through sexual maturation (**paper II**). The comprehensive small RNA sequencing identified the most abundant miRNAs in gonads, as well as identified significant changes in miRNA abundance between developmental stages and between sexes. The temporal expression patterns of six miRNAs abundant in gonads were determined using *in situ* hybridization (ISH; **paper III**), which identified diverse expression patterns between the miRNAs. miR-92a-3p, which was the most consistently abundant miRNA in zebrafish gonads (**paper II**), was selected for functional analysis. The predicted target *wee1 homolog 2* (*wee2*) was validated, and targeted gene knockdown in embryos was performed (**paper III**). The information acquired on zebrafish gonadal miRNAs will be valuable to future studies in reproductive biology and better understanding of the biological functions of miRNAs in the gonad. Therefore, the aims of the dissertation have been fulfilled.

3.1 Zebrafish as a model for reproductive biology

In **paper I**, the use of the *See-Thru-Gonad* line allowed high resolution imaging of the germline *in vivo*. One of the major benefits of *See-Thru-Gonad* was the ability to observe PGC proliferation during embryogenesis and in the first two wpf. The process of zebrafish juvenile hermaphroditism has been well documented, and there is a consensus

that the number of PGCs correlates with differential *sox9* and *cyp19a1a* expression, which ultimately determines the fate of the gonad (Maack and Segner, 2003, Siegfriend and Nusslein-Volhard, 2008, Sun et al., 2013, Takahashi, 1977, Tong et al., 2010). However, details of the genetic mechanism which determines the rate of early PGC proliferation, and how exactly PGCs influence *sox9* and *cyp19a1a* expression remains unclear. Most evidence suggests zebrafish use a polygenic sex determination system, where allelic combinations within a sex determining loci ultimately determine sex (Liew et al., 2012, Liew and Orbán, 2014). However, the exact number of loci and how they interact are not yet known.

Using the *See-Thru-Gonad* line, we were able to observe individual and clustered PGCs during the crucial 7 to 14 dpf period, where PGC proliferation correlates with sexual differentiation (**paper I**). In **paper II**, small RNA sequencing procedures were modified for reduced total RNA input, allowing for sequencing of pooled gonads from three wpf individuals. However, using reduced total RNA inputs greatly decreased the amount of miRNA reads obtained, resulting in a lack of sequencing depth (**paper II**); only the most abundant miRNAs could be characterized. It is likely this protocol could be optimized further, either with more sensitive library preparation, or increasing the number of pooled gonads per library, in order to assess earlier stages of gonadal development. In addition, PGC-specific signaling allows for their potential isolation and individual sequencing. At present, single cell sequencing is restricted to longer RNAs (Fan et al., 2015, Sandberg, 2014); but small RNA sequencing procedures could soon be optimized for single cells (Faridani et al., 2016). The use of *See-Thru-Gonad* would allow for staging of 7 to 14 dpf gonads based on the size of the proliferating gonad, and subsequent transcriptome analysis. Discovering the exact mechanism of zebrafish sex determination would greatly benefit the field of reproductive biology.

Many aspects of reproductive biology are conserved in vertebrates. For example, *Doublesex and Mab-3 related transcription factor* (*Dmrt*) genes are ancient and highly conserved transcription factors, which function as sex determining genes in animals by

promoting testis-related genes and suppressing ovarian genes (Herpin and Schartl, 2011, Kopp, 2012). In medaka (*Oryzias latipes*), a duplicated copy of *dmrt1* gave rise to *dmrt1bY*, the male sex determining gene (Kondo et al., 2006, Nanda et al., 2002). In zebrafish, *dmrt1* is required for oocyte apoptosis in the transforming gonad, as well as initiation of testis lumen formation (Webster et al., 2017). Recent studies have identified miRNAs predicted to suppress *dmrt1* expression in the gonads of swamp eel (*Monopterus albus*), zebrafish, and Nile tilapia (Liu et al., 2015, Tao et al., 2016). In zebrafish, a luciferase assay confirmed miR-19a/b can interact with the zebrafish *dmrt1* 3' UTR (Liu et al., 2015). It is probable that many miRNA/mRNA target interactions are conserved in metazoans, and zebrafish may be a suitable model for primary research in reproductive biology.

3.2 Determining miRNA functions in teleost gonadal development

In **paper II**, dynamic miRNA expression was detected throughout gonadal development, which when compared to gonad morphology (**paper I**) and miRNA spatial expression (**paper III**), allows improved prediction of miRNA function. Currently, miRNA target prediction is based on miRNA seed complementarity with mRNA 3' UTRs (Friedman et al., 2009, John et al., 2004, Krek et al., 2005) and does not take into account miRNA/mRNA co-expression. Because of this, miRNA target prediction typically identifies thousands of potential mRNA targets, many of which likely have no biological relation. Comparing the miRNA repertoire with known biological pathways occurring at specific developmental stages should allow researchers to narrow the list of potential targets.

In **paper I**, reduced miR-92a-3p expression in zebrafish embryos correlated with embryonic arrest during cleavage, suggesting miR-92a-3p functioned as a regulator of the cell cycle. Using this knowledge, *in silico* target prediction for miR-92a-3p focused on potential targets within the cell cycle pathway (**paper III**), which reduced the list of potential targets from thousands to a manageable number (33). Among these, *wee2* was

selected for validation based on its expression as a maternal factor in zebrafish embryos (Aanes et al., 2011). ISH confirmed *wee2* and miR-92a-3p are spatially and temporally co-expressed during embryonic cleavage (**paper III**). Target validation studies then confirmed miR-92a-3p is capable of interacting with the *wee2* 3' UTR to suppress translation.

In animals, miR-202 has conserved expression within the gonads (Bannister et al., 2009, Li et al., 2011, Michalak and Malone, 2008, Otsuka et al., 2008, Ro et al., 2007a, Wainwright et al., 2013). In both Atlantic halibut and zebrafish, miR-202 expression is gonad-enriched (Bizuayehu et al., 2012a, Vaz et al., 2015). These findings, along with the spatial and temporal expression patterns between zebrafish arms (**papers II** and **III**) suggest miR-202 has a specialized and conserved role in animal gonadal development. Studies in mouse and chicken suggest miR-202 acts downstream of SOX9 to promote testis differentiation (Bannister et al., 2011, Bannister et al., 2009, Wainwright et al., 2013). However, in zebrafish (**paper II**) and rainbow trout (Juanchich et al., 2013), miR-202-5p was abundant during the vitellogenic stages of oogenesis. In zebrafish, miR-202-5p was significantly more abundant in ovaries at 9 wpf relative to testis (**paper II**), suggesting this development stage may correlate to function. At this stage, the zebrafish ovary enters the secondary growth stage, characterized by the uptake of vitamins and nutrients into the oocyte (Lubzens et al., 2010, Selman et al., 1993); this suggests that, unlike in birds and mammals, miR-202-5p may have a role in oocyte maturation processes in fish.

According to miRmap target prediction software (Vejnar and Zdobnov, 2012), one of the highest predicted targets for miR-202-5p is *solute carrier family 19 member 1 a* (*slc19a1*), which contains three predicted binding sites within its 3' UTR. In mammals and amphibians, *slc19a1* encodes for the predominant folate transporter in oocytes (Kooistra et al., 2013, Meredith et al., 2016, Subramanian et al., 2001). Folate is a vitamin essential as a 1-carbon source in multiple cellular pathways, including DNA synthesis and maintenance, as well as DNA, RNA, and protein methylation (Crider et al., 2012).

Epigenetic modifications of the germline are frequently observed, and function in germline specific gene regulation, as well as imprinting genes for embryonic development (Wu et al., 2011). In mouse, oocyte methylation coincides with the transition from primary to secondary growth stages (Tomizawa et al., 2011, Tomizawa et al., 2012). Therefore, *slc19a1* would be a potent candidate for miR-202-5p functional validation.

3.3 Conservation of abundant gonadal miRNAs

One of the highlights of the dissertation was the identification of miR-92a-3p as a dominant miRNA during gonadal development of zebrafish. miR-92a-3p is stocked as a maternal factor in oocytes, and is expressed in both germline and gonadal somatic cells (**papers II and III**). However, a comparison of miRNA repertoires in the mature gonads of other teleost species found little conservation of miR-92a-3p abundance (**paper II**). While miR-92a-3p gonadal abundance in zebrafish was corroborated by an additional sequencing study (Vaz et al., 2015), only olive flounder (*Paralichthys olivaceus*) shared relatively high gonadal abundance of miR-92a-3p (Gu et al., 2014). miR-92a is an ancient miRNA, which differentiated into the miR-17-92 cluster approximately 500 million years ago (Liu et al., 2015). In *D. melanogaster*, miR-92a remains highly expressed in oocytes, and is stocked as a maternal factor (Marco, 2014). Therefore, gonadal abundance of miR-92a-3p in zebrafish may be a remnant of its ancestral function.

The most highly conserved gonadal miRNAs in teleosts were miR-143-3p and miR-21-5p (**paper II**). In contrast to miR-92a-3p, no homologues of miR-143-3p or miR-21-5p have been described in *D. melanogaster*. However, both miRNAs are consistently among the most abundant miRNAs in the gonads of mammals (Khan et al., 2015, Li et al., 2011, Mishima et al., 2008). Notably, while miR-143-3p is abundant in the oocytes of frog (*Xenopus tropicalis*), miR-21-5p is absent (Armisen et al., 2009).

Novel miRNAs are constantly emerging in the metazoan genome, and are rarely lost in descendant lineages (Berezikov, 2011, Wheeler et al., 2009). There are multiple mechanisms through which miRNA can appear, including: whole-genome duplication, localized gene duplication, folding of intronic regions, by-products from processing of other RNA transcripts, transposable elements, and antisense miRNA transcripts (reviewed by Berezikov, 2011). Vertebrates have undergone at least two whole genome duplication events. All teleosts have undergone an additional duplication, with some groups, such as salmonids, having undergone a further independent whole genome duplication (Glasauer and Neuhauss, 2014). It would be interesting to determine how teleost whole genome duplication shaped miRNA evolution in teleost fish. In addition, teleosts are the largest group of vertebrates, accounting for approximately half of all described vertebrate species (Nelson, 2006). The evolutionary distance between phylogenetic groups can be quite large. For example, zebrafish and medaka diverged approximately 110 million years ago, greater than the estimated 96 million years separating human and mouse (Nei et al., 2001, Wittbrodt et al., 2002). The large evolutionary distances may explain the lack of conservation among additional gonadal miRNAs.

3.4 miRNA arm preference

In general, Ago proteins preferentially load one arm from the miRNA duplex. However, there are examples where miRNA arm preference varies between developmental stage and cell type (Griffiths-Jones et al., 2011, Ro et al., 2007b). Maturation of both miRNA arms greatly increases the repertoire of predicted mRNA targets from a single miRNA gene. In **paper II**, both miR-202-5p and -3p arms were abundant at different stages of gonadal development; miR-202-3p was more abundant at 3 and 12 wpf, while -5p was more abundant at 24 wpf. In **paper III**, the 5p and 3p arms were found to follow different spatial expression patterns; miR-202-5p was detected predominantly within the germline, whereas -3p was exclusively expressed within somatic cells.

The process which regulates arm switching is not yet understood. Under normal conditions, the relative thermodynamic stability at the termini of the miRNA duplex determines the probability of each arm being incorporated into Ago protein (Khvorova et al., 2003). However, under this mechanism alone, the 5p/3p ratio should be consistent, and would not explain spatial and temporal variations in arm preference. In insects, examples of variable miR-10 arm preference between species have been reported; the beetle *Tribolium castaneum* preferentially expresses miR-10-5p, whereas the *D. melanogaster* expresses miR-10-3p, despite the predicted miR-10 duplex sequence being identical in both species (Griffiths-Jones et al., 2011). In that study, the authors also reported that supplementing *D. melanogaster* cells with the *T. castaneum* pre-miR-10 construct resulted in 5p arm preference, suggesting regulation of arm preference may occur upstream of Dicer processing.

There is evidence in *D. melanogaster* that different AGO proteins have variations in arm preference; AGO1 associated RNAs were highly enriched in 5' uracil (Czech et al., 2008). It was subsequently demonstrated that *Dme*-miR-267a-5p, which contains a 5' terminal adenine is preferentially loaded into AGO2, and -3p, which has a 5' terminal uracil, is preferentially loaded into AGO1 (Okamura et al., 2009). The authors then demonstrated that swapping the 5' terminal adenine and uracil residues resulted in a switch, with AGO1 now preferentially loading the 5p arm. The preference of AGO2 to incorporate the -5p arm was dependent on the presence of internal mismatches at nucleotides 9 and 10. Modifications of nucleotide 9 in particular were sufficient to caused AGO2 to select the 3p arm instead. However, in human, no evidence was found in selective loading of miRNA for a specific Ago protein (Su et al., 2009).

It is currently unclear whether teleost Ago proteins exhibit preferential miRNA arm loading patterns. Transcripts for the miRNA associated Ago proteins 1,2 and 4 were detected with strong expression within the gonads of the swamp eel (Liu et al., 2015). The authors did not detect significant differences in the relative expression of the *ago*

genes between testis and ovary. However, it would be interesting to examine teleost *ago* gene expression throughout gonadal development, as well as between germline and somatic cell lineages. In mouse, there were significant differences in the fractional abundance of AGO proteins between the brain of embryos and adults (Juvvuna et al., 2012). Similar changes in teleosts may help to explain a shift in arm preference for miR-202.

3.5 isomiRs

Mature miRNAs are frequently produced with multiple length and sequence variants referred to as isomiRs. In teleost fish, they were first reported in Atlantic halibut and zebrafish (Bizuayehu et al., 2012b, Wei et al., 2012), although they have been widely detected in mammalian small RNA sequencing data (Landgraf et al., 2007). The use of synthetic spike-in RNA during sequencing provided strong evidence that isomiRs are not the result of sequencing error (Lee et al., 2010). Rather, it appears that miRNAs are heterogeneous, with isomiRs having a role in fine-tuning miRNA function (Neilsen et al., 2012).

In **paper II**, each of the 27 miRNAs contributing to the most abundant miRNAs in zebrafish gonads consisted of multiple isomiRs. These included 5' and 3' templated addition, 5' and 3' truncation, 3' untemplated additions, and sequence variants near the 3' terminal (represented by miR-10b in Fig. 3). While miRNA degradation cannot be ruled out, the non-random nature of isomiR frequency observed suggests functional relevance.

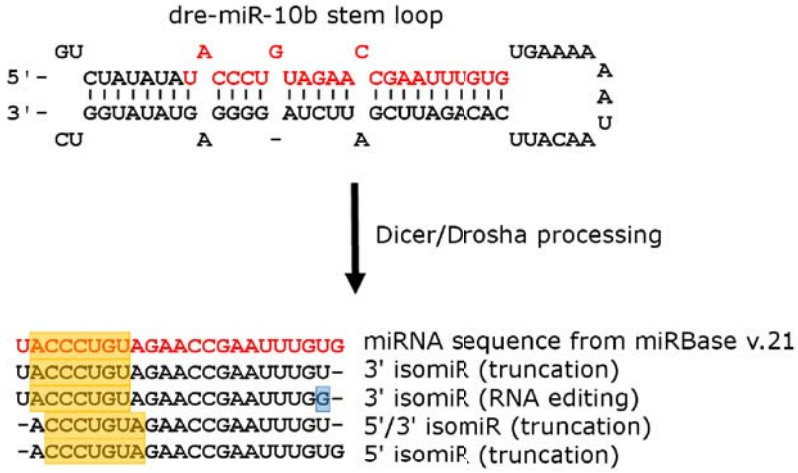

Figure 3. miR-10b isomiRs detected in zebrafish gonads. The miRNA seed sequence is highlighted in yellow.

IsomiRs with 3' modifications have been widely observed in plants and animals (Burroughs et al., 2010, Lee et al., 2010, Neilsen et al., 2012, Newman et al., 2011, Wyman et al., 2011). The most likely source of templated sequence variation is alternative Drosha- and Dicer-mediated processing, although multiple enzymes with functions in 3' trimming, nucleotide editing or addition have been identified (Fig. 4; reviewed by Neilsen et al. 2012). The relevance of 3' isomiRs appears to be variable. Adenylation of mammalian miR-122 improved its stability (Katoh et al., 2009). However, in *D. melanogaster*, adenylation encouraged degradation of maternal miRNAs (Lee et al., 2014). 3' isomiRs may also affect Ago protein preference. In human, a longer miR-182 isomiR associates exclusive with AGO1 and AGO3, whereas an isomiR missing the terminal 3' guanine preferentially associated with AGO2 (Burroughs et al., 2011). Because 3' terminal modifications would also affect the thermodynamic stability, 3' isomiRs may also affect miRNA arm preference. In **paper II**, longer miR-202-5p and -3p isomiRs generally correlated with 3p arm preference, whereas shorter -5p and -3p isomiRs correlated to 5p arm preference.

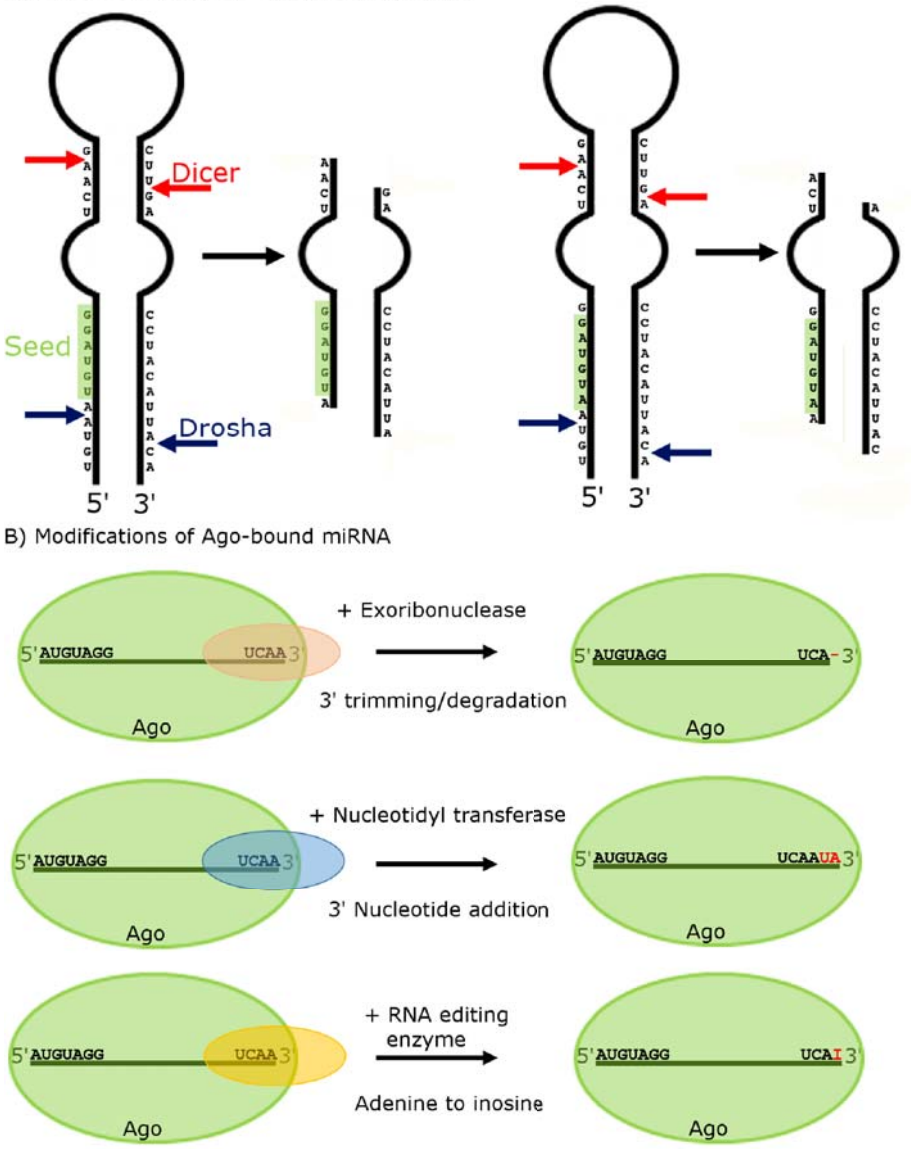

Figure 4. Frequent mechanisms of isomiR biogenesis. A) Canonical biogenesis of isomiRs by alternative Dicer and Drosha processing result in 5′ and 3′ modifications. 5′ modifications can affect the seed sequence (green). B) Frequent mechanisms of Ago-bound miRNA modification. Nucleotides in red identify editing. Modified from Neilsen et al. (2012).

In **paper II**, multiple 5' isomiRs were detected among the most abundant miRNAs. In particular, miR-10a-5p, miR-10b-5p, miR-10c-5p, miR-202-5p, and miR-202-3p had relatively large proportions of 5' isomiRs. Modifications to the 5' end of a mature miRNA are of particular evolutionary and functional importance. Because the miRNA seed sequence (nucleotides 2-8) is the predominant factor in miRISC guidance, a seed-shift can potentially change the repertoire of miRNA targets. In human, alternative mRNA targeting was validated for a miR-9 isomiR with a modified 5' end (Tan et al., 2014). In addition, there are examples of evolutionary 5' isomiR switching, such as in human miR-500a, where the dominant 5' isomiR differs from mouse miR-500a (Tan et al., 2014).

Notably, the dominant isomiR for zebrafish miR-202-3p in gonads differs from the annotated sequence of miRBase (http://www.miRbase.org), which is a frequently used database for miRNA annotation. miRNA target prediction algorithms, such as miRmap (Vejnar and Zdobnov, 2012) and Targetscan (Agarwal et al., 2015) use miRBase for input miRNA sequences. In the case of zebrafish miR-202-3p, miRBase predicted the dominant isomiR based on sequencing of zebrafish embryos, during which time miR-202-3p was poorly expressed (Chen et al., 2005). In the future, miRNA dominant form classification as well as target prediction will need to account for the heterogeneous nature of miRNAs.

3.6 Maternal miRNAs

In all animals, early embryonic development is driven by maternal factors, gene products stored in the eggs during oogenesis (Pelegri, 2003). The transition from maternal to zygotic regulation is a critical point in embryonic development, and involves the initiation of zygotic transcription in conjunction with the clearance of maternal mRNA and proteins (Tadros and Lipshitz, 2009). To date, much of the focus on miRNA during the zebrafish MZT has been on miR-430, which is a major maternal mRNA clearance factor (Giraldez et al., 2006). However, miR-430 targets approximately 10 percent of maternal mRNAs only (Giraldez et al., 2006), suggesting additional

degradation pathways. Recently, a zebrafish maternal degradation pathway was described, in which maternal mRNA were deadenylated by the CCR4-NOT complex based on enrichment of uncommon codons, such as leucine, within the open reading frame, and short 3' UTRs (Mishima and Tomari, 2016). The CCR4-NOT complex can be recruited by GW182 to the miRISC complex to perform miRNA-mediated deadenylation (Fabian et al., 2011). However, CCR4-NOT can also be recruited by RNA-binding proteins for targeted mRNA degradation (Bhandari et al., 2014); therefore, it is not clear if maternal miRNAs function in the described zebrafish maternal decay pathway. In zebrafish, maternal miR-34-5p regulates Notch signaling during neurogenesis (Soni et al., 2013). In addition, abundant miRNAs were detected in the unfertilized eggs of rainbow trout, which may be stocked as maternal factors (Juanchich et al., 2016, Ma et al., 2012). Our ongoing research has characterized cohorts of maternal miRNAs, which exhibit dynamic changes in abundance during the early stages of zebrafish embryogenesis (*FishmiR* project, unpublished data). Future work will be performed to functionally validate these maternal miRNAs.

In zebrafish, quantitative real-time polymerase chain reaction (RT-qPCR; **paper I**) and ISH (**paper III**) confirmed miR-92a-3p transcripts are present in 1-cell stage zebrafish embryos, while small RNA sequencing detected them in high abundance in unfertilized eggs (**paper II**); these findings suggest miR-92a-3p is stocked as a maternal factor. In addition, targeted gene knockdown of miR-92a-3p in mature zebrafish ovaries did not have an obvious effect on gonadal morphology, but had a significant effect on the cleavage stage of embryogenesis (**paper I**). miR-92a-3p was confirmed to interact with the 3' UTR of *wee2*, a maternal cell cycle regulator with functions in the G2/M checkpoint (**paper III**). These findings demonstrate miR-92a-3p is a maternal factor and is one of the first maternal miRNAs to be characterized in zebrafish.

In unfertilized zebrafish eggs, miR-92a-3p transcripts were abundantly stocked and were predominantly isomiRs with one (17%) or two (47%) additional templated adenine residues when compared to the dominant variant in all other developmental stages

(**paper II**). Adenylation of maternal miRNAs is conserved in *D. melanogaster*, sea urchin (*S. purpuratus*), and mouse (Lee et al., 2014). The adenylation in *D. melanogaster* is performed by the protein Wispy, and may contribute to the clearance of maternal miRNAs during the MZT. However, to date, no teleost homologue of Wispy has been described. Because the miR-92a-3p adenine residues were templated, maternal miRNAs in teleosts may be modified primarily through alternative Dicer and Drosha processing mechanisms. However, additional maternal miRNAs will need to be identified to confirm this hypothesis. In zebrafish eggs, miRNAs contribute a very small proportion of total RNA (Lee et al., 2014). In **paper II**, only nine miRNAs were sequenced with sufficient reads to analyze isomiR frequencies in unfertilized eggs; among these miRNAs, miR-92a-3p was the only one with a noticeable increase in 3' adenine residues compared to other developmental stages. Increased depth of small RNA sequencing in unfertilized eggs and a comparison to isomiRs in pre-MZT zebrafish may help to understand the role, if any, additional adenine residues have in maternal miRNA function.

4. Conclusions

In the present study, a transparent zebrafish line with a fluorescently labeled germline was established and used to examine the gonadal miRNA repertoire.

- The *See-Thru-Gonad* line allowed high resolution visualization of germ cells *in vivo*.
- Fluorescent labeling of the germline allowed isolation and small RNA sequencing of zebrafish gonads from undifferentiated stage to maturation.
- Targeted gene knockdown of miR-92a-3p using a *vivo*-morpholino resulted in a vertical transmission of the effect to embryos.
- miRNAs were differentially expressed at each developmental stage examined, as well as between testis and ovary.
- Multiple isomiRs were detected among the most abundant gonadal miRNAs, including examples of 5' truncation and templated addition, which affected the miRNA seed sequence.
- *In situ* hybridization demonstrated diverse spatial expression patterns of miRNAs in zebrafish gonads.
- Target validation studies provided evidence of a maternal miRNA with functions during zebrafish embryogenesis.

5. Future Perspectives

The current study revealed the dynamic nature of the miRNA repertoire in zebrafish gonads. However, while research on the role of miRNAs in reproduction is growing, it is still primarily based on next-generation sequencing and *in silico* target prediction. An increase in functional validation will have a profound effect on our knowledge of miRNAs, as their place within biological pathways can be confirmed. The research presented in this dissertation will serve as an important platform from which studies in miRNA functional validation can be performed.

The processing mechanisms and functions of isomiRs should be addressed. It will be important to distinguish between isomiRs which occur as a result of degradation or processing, and isomiRs which have functional roles, such as preferential association with specific Ago proteins, miRNA arm selection, and mRNA targeting. At present, miRNA *in silico* target prediction does not account for 5' isomiRs, and as a result, may miss important miRNA/mRNA interactions.

The vertical transmission of the genetic knockdown effect, observed in **paper I**, where targeted knockdown of miR-92a-3p in mature ovaries affected embryogenesis, should be further explored. The zebrafish ovarian cycle is 2-5 days, depending on temperature (Hisaoka and Firlit, 1962). Spawning occurred 3 days after injection, meaning a full ovarian cycle may have occurred between treatment and the observed phenotype. Determining whether the phenotype was a result of miR-92a-3p knockdown at a specific stage of oogenesis, or as a result of reduced maternal miR-92a-3p stocking, will be crucial to characterizing miR-92a-3p in zebrafish gonads.

The possibility for vertical transmission of the effect of genetic knockdown has important implications for research on maternal miRNAs. Embryonic arrest in zebrafish maternal *dicer1* mutants demonstrated the importance of miRNA function during embryogenesis (Giraldez et al., 2005). However, knockdown of maternal miRNAs is

challenging, as miRNAs may function during and immediately after fertilization. If maternal stocking of a specific miRNA can be prevented, its functional role could be characterized during the early stages of embryogenesis.

miRNAs are suspected to function in epigenetic pathways (Sato et al., 2011). However, their role in sex differentiation and gonadal development of fish has yet to be described. Identifying and validating miRNA targets with predicted epigenetic functions, such as miR-202-5p and *slc19a1a*, would improve knowledge in this field.

One of the strengths of the dissertation was the comprehensive sequencing of small RNAs with biological replicates at several stages of gonadal development. However, as a result of the number of libraries required, the relative sequencing depth of individual libraries was poor. Therefore, only the most abundant miRNAs could be examined. Improved sequencing depth could uncover novel miRNAs with functions in gonadal development.

6. References

Aanes, H., Winata, C.L., Lin, C.H., Chen, J.P., Srinivasan, K.G., Lee, S.G.P., et al. (2011). Zebrafish mRNA sequencing deciphers novelties in transcriptome dynamics during maternal to zygotic transition. *Genome Res,* 21**:** 1328-1338. DOI 10.1101/gr.116012.110

Abbott, A.L., Alvarez-Saavedra, E., Miska, E.A., Lau, N.C., Bartel, D.P., Horvitz, H.R., et al. (2005). The let-7 microRNA family members mir-48, mir-84, and mir-241 function together to regulate developmental timing in *Caenorhabditis elegans*. *Dev Cell,* 9**:** 403-414. DOI 10.1016/j.devcel.2005.07.009

Abramov, R., Fu, G., Zhang, Y. & Peng, C. (2013). Expression and regulation of miR-17a and miR-430b in zebrafish ovarian follicles. *Gen Comp Endocrinol,* 188**:** 309-315. DOI 10.1016/j.ygcen.2013.02.012

Agarwal, V., Bell, G.W., Nam, J.-W. & Bartel, D.P. (2015). Predicting effective microRNA target sites in mammalian mRNAs. *eLife,* 4**:** e05005. DOI 10.7554/eLife.05005

Armisen, J., Gilchrist, M.J., Wilczynska, A., Standart, N. & Miska, E.A. (2009). Abundant and dynamically expressed miRNAs, piRNAs, and other small RNAs in the vertebrate Xenopus tropicalis. *Genome Res,* 19**:** 1766-1775. DOI 10.1101/gr.093054.109

Bannister, S.C., Smith, C.A., Roeszler, K.N., Doran, T.J., Sinclair, A.H. & Tizard, M.L.V. (2011). Manipulation of estrogen synthesis alters MIR202* expression in embryonic chicken gonads. *Biol Reprod,* 85**:** 22-30. DOI 10.1095/biolreprod.110.088476

Bannister, S.C., Tizard, M.L.V., Doran, T.J., Sinclair, A.H. & Smith, C.A. (2009). Sexually dimorphic microRNA expression during chicken embryonic gonadal development. *Biol Reprod,* 81**:** 165-176. DOI 10.1095/biolreprod.108.074005

Bartel, D.P. & Chen, C.Z. (2004). Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. *Nat Rev Genet,* 5. DOI 10.1038/nrg1328

Behm-Ansmant, I., Rehwinkel, J., Doerks, T., Stark, A., Bork, P. & Izaurralde, E. (2006). mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. *Genes Dev,* 20**:** 1885-1898. DOI 10.1101/gad.1424106

Berezikov, E. (2011). Evolution of microRNA diversity and regulation in animals. *Nat Rev Genet,* 12**:** 846-860.

Berois, N., Arezo, M.J. & Papa, N.G. (2011). Gamete interactions in teleost fish: the egg envelope. Basic studies and perspectives as environmental biomonitor. *Biol Res,* 44**:** 119-124. DOI 10.4067/s0716-97602011000200002

Bhandari, D., Raisch, T., Weichenrieder, O., Jonas, S. & Izaurralde, E. (2014). Structural basis for the Nanos-mediated recruitment of the CCR4–NOT complex and translational repression. *Genes Dev,* 28**:** 888-901. DOI 10.1101/gad.237289.113

Bizuayehu, T.T., Babiak, J., Norberg, B., Fernandes, J.M.O., Johansen, S.D. & Babiak, I. (2012a). Sex-Biased miRNA expression in Atlantic halibut (*Hippoglossus hippoglossus*) brain and gonads. *Sex Dev,* 6**:** 257-266. DOI 10.1159/000341378

Bizuayehu, T.T., Lanes, C.F., Furmanek, T., Karlsen, B.O., Fernandes, J.M., Johansen, S.D., et al. (2012b). Differential expression patterns of conserved miRNAs and isomiRs during Atlantic halibut development. *BMC Genomics,* 13**:** 11. DOI 10.1186/1471-2164-13-11

Blaser, H., Reichman-Fried, M., Castanon, I., Dumstrei, K., Marlow, F.L., Kawakami, K., et al. (2006). Migration of zebrafish primordial germ cells: a role for myosin contraction and cytoplasmic flow. *Dev Cell,* 11**:** 613-627. DOI 10.1016/j.devcel.2006.09.023

Bohnsack, M.T., Czaplinski, K. & Görlich, D. (2004). Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *RNA,* 10**:** 185-191. DOI 10.1261/rna.5167604

Borchert, G.M., Lanier, W. & Davidson, B.L. (2006). RNA polymerase III transcribes human miRNAs. *Nat Struct Mol Biol,* 13**:** 1097-1101. DOI 10.1038/nsmb1167

Brennecke, J., Stark, A., Russell, R.B. & Cohen, S.M. (2005). Principles of microRNA–target recognition. *PLoS Biol,* 3**:** e85. DOI 10.1371/journal.pbio.0030085

Burroughs, A.M., Ando, Y., De Hoon, M.J.L., Tomaru, Y., Nishibu, T., Ukekawa, R., et al. (2010). A comprehensive survey of 3′ animal miRNA modification events and a possible role for 3′ adenylation in modulating miRNA targeting effectiveness. *Genome Res,* 20**:** 1398-1410. DOI 10.1101/gr.106054.110

Burroughs, A.M., Ando, Y., De Hoon, M.L., Tomaru, Y., Suzuki, H., Hayashizaki, Y., et al. (2011). Deep-sequencing of human Argonaute-associated small RNAs provides insight into miRNA sorting and reveals Argonaute association with RNA fragments of diverse origin. *RNA Biol,* 8**:** 158-177. DOI 10.4161/rna.8.1.14300

Carmell, M.A., Xuan, Z., Zhang, M.Q. & Hannon, G.J. (2002). The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. *Genes Dev,* 16**:** 2733-2742. DOI 10.1101/gad.1026102

Casey, M., Kerin, M., Brown, J. & Kj, S. (2015). Evolution of a research field-a micro (RNA) example. *PeerJ,* 3. DOI 10.7717/peerj.829

Cheloufi, S., Dos Santos, C.O., Chong, M.M.W. & Hannon, G.J. (2010). A dicer-independent miRNA biogenesis pathway that requires Ago catalysis. *Nature,* 465**:** 584-589. DOI 10.1038/nature09092

Chen, P.Y., Manninga, H., Slanchev, K., Chien, M., Russo, J.J., Ju, J., et al. (2005). The developmental miRNA profiles of zebrafish as determined by small RNA cloning. *Genes Dev,* 19**:** 1288-1293. DOI 10.1101/gad.1310605

Chong, M.M.W., Zhang, G., Cheloufi, S., Neubert, T.A., Hannon, G.J. & Littman, D.R. (2010). Canonical and alternate functions of the microRNA biogenesis machinery. *Genes Dev,* 24**:** 1951-1960. DOI 10.1101/gad.1953310

Cifuentes, D., Xue, H., Taylor, D.W., Patnode, H., Mishima, Y., Cheloufi, S., et al. (2010). A novel miRNA processing pathway independent of Dicer requires Argonaute2 catalytic activity. *Science,* 328**:** 1694-1698. DOI 10.1126/science.1190809

Crider, K.S., Yang, T.P., Berry, R.J. & Bailey, L.B. (2012). Folate and DNA methylation: a review of molecular mechanisms and the evidence for folate's role. *Adv Nutr,* 3**:** 21-38. DOI 10.3945/an.111.000992

Czech, B., Malone, C.D., Zhou, R., Stark, A., Schlingeheyde, C., Dus, M., et al. (2008). An endogenous small interfering RNA pathway in Drosophila. *Nature,* 453**:** 798-802. DOI 10.1038/nature07007

Dalmay, T. (2013). Mechanism of miRNA-mediated repression of mRNA translation. *Essays Biochem,* 54**:** 29-38. DOI 10.1042/bse0540029

Denli, A.M., Tops, B.B.J., Plasterk, R.H.A., Ketting, R.F. & Hannon, G.J. (2004). Processing of primary microRNAs by the Microprocessor complex. *Nature,* 432**:** 231-235. DOI 10.1038/nature03049

Dong, C.-H., Yang, S.-T., Yang, Z.-A., Zhang, L. & Gui, J.-F. (2004). A C-type lectin associated and translocated with cortical granules during oocyte maturation and egg fertilization in fish. *Dev Biol,* 265**:** 341-354. DOI 10.1016/j.ydbio.2003.08.028

Eulalio, A., Huntzinger, E. & Izaurralde, E. (2008). GW182 interaction with Argonaute is essential for miRNA-mediated translational repression and mRNA decay. *Nat Struct Mol Biol,* 15**:** 346-353. DOI 10.1038/nsmb.1405

Extavour, C.G. & Akam, M. (2003). Mechanisms of germ cell specification across the metazoans: epigenesis and preformation. *Development,* 130**:** 5869-5884. DOI 10.1242/dev.00804

Fabian, M.R., Cieplak, M.K., Frank, F., Morita, M., Green, J., Srikumar, T., et al. (2011). miRNA-mediated deadenylation is orchestrated by GW182 through two conserved motifs that interact with CCR4–NOT. *Nat Struct Mol Biol,* 18**:** 1211-1217. DOI 10.1038/nsmb.2149

Fabian, M.R. & Sonenberg, N. (2012). The mechanics of miRNA-mediated gene silencing: a look under the hood of miRISC. *Nat Struct Mol Biol,* 19**:** 586-593.

Fan, X., Zhang, X., Wu, X., Guo, H., Hu, Y., Tang, F., et al. (2015). Single-cell RNA-seq transcriptome analysis of linear and circular RNAs in mouse preimplantation embryos. *Genome Biol,* 16**:** 148. DOI 10.1186/s13059-015-0706-1

FAO (2014). *FAO Yearbook. Fishery and Aquaculture Statistics*: Food and Agricultural Organization of the United Nations. ISBN 9789250092683

Faridani, O.R., Abdullayev, I., Hagemann-Jensen, M., Schell, J.P., Lanner, F. & Sandberg, R. (2016). Single-cell sequencing of the small-RNA transcriptome. *Nat Biotech,* advance online publication. DOI 10.1038/nbt.3701

Friedman, R.C., Farh, K.K.-H., Burge, C.B. & Bartel, D.P. (2009). Most mammalian mRNAs are conserved targets of microRNAs. *Genome Research,* 19**:** 92-105. DOI 10.1101/gr.082701.108

Georges, A., L'hote, D., Todeschini, A.L., Auguste, A., Legois, B., Zider, A., et al. (2014). The transcription factor FOXL2 mobilizes estrogen signaling to maintain the identity of ovarian granulosa cells. *eLife,* 3**:** e04207. DOI 10.7554/eLife.04207

Gerhard, G.S. & Cheng, K.C. (2002). A call to fins! Zebrafish as a gerontological model. *Aging Cell,* 1**:** 104-111. DOI 10.1046/j.1474-9728.2002.00012.x

Giraldez, A.J., Cinalli, R.M., Glasner, M.E., Enright, A.J., Thomson, J.M., Baskerville, S., et al. (2005). MicroRNAs regulate brain morphogenesis in zebrafish. *Science,* 308**:** 833. DOI 10.1126/science.1109020

Giraldez, A.J., Mishima, Y., Rihel, J., Grocok, R.J., Van Dongen, S., Inoue, K., et al. (2006). Zebrafish miR-430 promotes deadenylation and clearance of maternal mRNAs. *Science,* 312**:** 75-79. DOI 10.1126/science.1122689

Glasauer, S.M.K. & Neuhauss, S.C.F. (2014). Whole-genome duplication in teleost fishes and its evolutionary consequences. *Mol Genet Genomics,* 289**:** 1045-1060. DOI 10.1007/s00438-014-0889-2

Griffiths-Jones, S., Hui, J.H.L., Marco, A. & Ronshaugen, M. (2011). MicroRNA evolution by arm switching. *EMBO Reports,* 12**:** 172-177. DOI 10.1038/embor.2010.191

Grivna, S.T., Beyret, E., Wang, Z. & Lin, H. (2006a). A novel class of small RNAs in mouse spermatogenic cells. *Genes Dev,* 20**:** 1709-1714. DOI 10.1101/gad.1434406

Grivna, S.T., Pyhtila, B. & Lin, H. (2006b). MIWI associates with translational machinery and PIWI-interacting RNAs (piRNAs) in regulating spermatogenesis. *Proc Natl Acad Sci USA,* 103**:** 13415-13420. DOI 10.1073/pnas.0605506103

Gu, Y., Zhang, L. & Chen, X. (2014). Differential expression analysis of *Paralichthys olivaceus* microRNAs in adult ovary and testis by deep sequencing. *Gen Comp Endocrinol,* 204**:** 181-184. DOI 10.1016/j.ygcen.2014.05.019

Ha, M. & Kim, V.N. (2014). Regulation of microRNA biogenesis. *Nat Rev Mol Cell Biol,* 15**:** 509-524. DOI 10.1038/nrm3838

Hayashi, K., Chuva De Sousa Lopes, S.M., Kaneda, M., Tang, F., Hajkova, P., Lao, K., et al. (2008). MicroRNA biogenesis is required for mouse primordial germ cell development and spermatogenesis. *PLoS ONE,* 3**:** e1738. DOI 10.1371/journal.pone.0001738

Herpin, A. & Schartl, M. (2011). Dmrt1 genes at the crossroads: a widespread and central class of sexual development factors in fish. *FEBS Journal,* 278**:** 1010-1019. DOI 10.1111/j.1742-4658.2011.08030.x

Hisaoka, K.K. & Firlit, C.F. (1962). Ovarian cycle and egg production in the Zebrafish, *Brachydanio rerio*. *Copeia,* 1962**:** 788-792. DOI 10.2307/1440680

Höck, J. & Meister, G. (2008). The Argonaute protein family. *Genome Biol,* 9**:** 210-210. DOI 10.1186/gb-2008-9-2-210

Houwing, S., Berezikov, E. & Ketting, R.F. (2008). Zili is required for germ cell differentiation and meiosis in zebrafish. *EMBO J,* 27**:** 2702-2711. DOI 10.1038/emboj.2008.204

Houwing, S., Kamminga, L.M., Berezikov, E., Cronembold, D., Girard, A., Van Den Elst, H., et al. (2007). A role for Piwi and piRNAs in germ cell maintenance and transposon silencing in zebrafish. *Cell,* 129**:** 69-82. DOI 10.1016/j.cell.2007.03.026

Howe, K., Clark, M.D., Torroja, C.F., Torrance, J., Berthelot, C., Muffato, M., et al. (2013). The zebrafish reference genome sequence and its relationship to the human genome. *Nature,* 496**:** 498-503. DOI 10.1038/nature12111

Huntzinger, E. & Izaurralde, E. (2011). Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nat Rev Genet,* 12**:** 99-110. DOI 10.1038/nrg2936

Hutvagner, G. & Simard, M.J. (2008). Argonaute proteins: key players in RNA silencing. *Nat Rev Mol Cell Biol,* 9**:** 22-32. DOI 10.1038/nrm2321

Hwang, H.W. & Mendell, J.T. (2006). MicroRNAs in cell proliferation, cell death, and tumorigenesis. *Br J Cancer,* 94**:** 776-780. DOI 10.1038/sj.bjc.6603023

Ivey, K.N. & Srivastava, D. (2010). MicroRNAs as regulators of differentiation and cell fate decisions. *Cell Stem Cell,* 7**:** 36-41. DOI 10.1016/j.stem.2010.06.012

Jamieson, B.G.M. (1991). *Fish evolution and systematics: evidence from spermatozoa,* Cambridge: Cambridge University Press

Jia, K.-T., Zhang, J., Jia, P., Zeng, L., Jin, Y., Yuan, Y., et al. (2015). Identification of microRNAs in zebrafish spermatozoa. *Zebrafish,* 12**:** 387-397. DOI 10.1089/zeb.2015.1115

Jing, J., Wu, J., Liu, W., Xiong, S., Ma, W., Zhang, J., et al. (2014). Sex-biased miRNAs in gonad and their potential roles for testis development in yellow catfish. *PLoS ONE,* 9**:** e107946. DOI 10.1371/journal.pone.0107946

John, B., Enright, A.J., Aravin, A., Tuschl, T., Sander, C. & Marks, D.S. (2004). Human MicroRNA Targets. *PLoS Biology,* 2**:** e363. DOI 10.1371/journal.pbio.0020363

Jørgensen, A., Morthorst, J.E., Andersen, O., Rasmussen, L.J. & Bjerregaard, P. (2008). Expression profiles for six zebrafish genes during gonadal sex differentiation. *Reprod Biol Endocrinol,* 6**:** 25-25. DOI 10.1186/1477-7827-6-25

Juanchich, A., Bardou, P., Rué, O., Gabillard, J.-C., Gaspin, C., Bobe, J., et al. (2016). Characterization of an extensive rainbow trout miRNA transcriptome by next generation sequencing. *BMC Genomics,* 17**:** 164. DOI 10.1186/s12864-016-2505-9

Juanchich, A., Le Cam, A., Montfort, J., Guiguen, Y. & Bobe, J. (2013). Identification of differentially expressed miRNAs and their potential targets during fish ovarian development. *Biol Reprod,* 88**:** 128, 1-11. DOI 10.1095/biolreprod.112.105361

Juvvuna, P.K., Khandelia, P., Lee, L.M. & Makeyev, E.V. (2012). Argonaute identity defines the length of mature mammalian microRNAs. *Nucleic Acids Res,* 40**:** 6808-6820. DOI 10.1093/nar/gks293

Katoh, T., Sakaguchi, Y., Miyauchi, K., Suzuki, T., Kashiwabara, S.-I., Baba, T., et al. (2009). Selective stabilization of mammalian microRNAs by 3' adenylation mediated by the cytoplasmic poly(A) polymerase GLD-2. *Genes Dev,* 23**:** 433-438. DOI 10.1101/gad.1761509

Kedde, M., Strasser, M.J., Boldajipour, B., Oude Vrielink, J.a.F., Slanchev, K., Le Sage, C., et al. (2007). RNA-binding protein Dnd1 inhibits microRNA access to target mRNA. *Cell,* 131**:** 1273-1286. DOI 10.1016/j.cell.2007.11.034

Khan, H.A., Zhao, Y., Wang, L., Li, Q., Du, Y.-A., Dan, Y., et al. (2015). Identification of miRNAs during mouse postnatal ovarian development and superovulation. *J Ovarian Res,* 8**:** 44. DOI 10.1186/s13048-015-0170-2

Khvorova, A., Reynolds, A. & Jayasena, S.D. (2003). Functional siRNAs and miRNAs exhibit strand bias. *Cell,* 115**:** 209-216. DOI 10.1016/S0092-8674(03)00801-8

Kim, Y.-K., Kim, B. & Kim, V.N. (2016). Re-evaluation of the roles of DROSHA, Exportin 5, and DICER in microRNA biogenesis. *Proc Natl Acad Sci USA,* 113**:** E1881-E1889. DOI 10.1073/pnas.1602532113

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B. & Schilling, T.F. (1995). Stages of embryonic development of the zebrafish. *Dev Dynam,* 203**:** 253-310. DOI 10.1002/aja.1002030302

Kinth, P., Mahesh, G. & Panwar, Y. (2013). Mapping of zebrafish research: a global outlook. *Zebrafish,* 10**:** 510-517. DOI 10.1089/zeb.2012.0854

Knaut, H., Werz, C., Geisler, R., The Tubingen Screen, C. & Nusslein-Volhard, C. (2003). A zebrafish homologue of the chemokine receptor Cxcr4 is a germ-cell guidance receptor. *Nature,* 421**:** 279-282. DOI 10.1038/nature01338

Kondo, M., Hornung, U., Nanda, I., Imai, S., Sasaki, T., Shimizu, A., et al. (2006). Genomic organization of the sex-determining and adjacent regions of the sex chromosomes of medaka. *Genome Res,* 16**:** 815-826. DOI 10.1101/gr.5016106

Kooistra, M., Trasler, J.M. & Baltz, J.M. (2013). Folate transport in mouse cumulus-oocyte complexes and preimplantation embryos. *Biol Reprod,* 89**:** 63, 1-9. DOI 10.1095/biolreprod.113.111146

Kopp, A. (2012). Dmrt genes in the development and evolution of sexual dimorphism. *Trends Genet,* 28**:** 175-184. DOI 10.1016/j.tig.2012.02.002

Krek, A., Grun, D., Poy, M.N., Wolf, R., Rosenberg, L., Epstein, E.J., et al. (2005). Combinatorial microRNA target predictions. *Nat Genet,* 37**:** 495-500. DOI 10.1038/ng1536

Landgraf, P., Rusu, M., Sheridan, R., Sewer, A., Iovino, N., Aravin, A., et al. (2007). A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell,* 129**:** 1401-1414. DOI 10.1016/j.cell.2007.04.040

Lee, L.W., Zhang, S., Etheridge, A., Ma, L., Martin, D., Galas, D., et al. (2010). Complexity of the microRNA repertoire revealed by next-generation sequencing. *RNA,* 16**:** 2170-2180. DOI 10.1261/rna.2225110

Lee, M., Choi, Y., Kim, K., Jin, H., Lim, J., Nguyen, T.A., et al. (2014). Adenylation of maternally inherited microRNAs by Wispy. *Mol Cell,* 56**:** 696-707. DOI 10.1016/j.molcel.2014.10.011

Lee, R.C., Feinbaum, R.L. & Ambros, V. (1993). The C. elegans Heterochronic Gene *lin-4* Encodes Small RNAs with Antisense Complementarity to *lin-14*. *Cell,* 75**:** 843-854. DOI 10.1016/0092-8674(93)90529-Y

Lee, Y., Kim, M., Han, J., Yeom, K.-H., Lee, S., Baek, S.H., et al. (2004). microRNA genes are transcribed by RNA polymerase II. *EMBO J,* 23**:** 4051-4060. DOI 10.1038/sj.emboj.7600385

Li, M., Liu, Y., Wang, T., Guan, J., Luo, Z., Chen, H., et al. (2011). Repertoire of porcine microRNAs in adult ovary and testis by deep sequencing. *Int J Biol Sci,* 7**:** 1045-1055.

Li, Z. & Rana, T.M. (2014). Therapeutic targeting of microRNAs: current status and future challenges. *Nat Rev Drug Discov,* 13**:** 622-638. DOI 10.1038/nrd4359

Liew, W.C., Bartfai, R., Lim, Z., Sreenivasan, R., Siegfried, K.R. & Orban, L. (2012). Polygenic sex determination system in zebrafish. *PLoS ONE,* 7**:** e34397. DOI 10.1371/journal.pone.0034397

Liew, W.C. & Orbán, L. (2014). Zebrafish sex: a complicated affair. *Brief Funct Genomics,* 13**:** 172-187. DOI 10.1093/bfgp/elt041

Lingel, A., Simon, B., Izaurralde, E. & Sattler, M. (2004). Nucleic acid 3'-end recognition by the Argonaute2 PAZ domain. *Nat Struct Mol Biol,* 11**:** 576-577. DOI 10.1038/nsmb777

Liu, J., Luo, M., Sheng, Y., Hong, Q., Cheng, H. & Zhou, R. (2015). Dynamic evolution and biogenesis of small RNAs during sex reversal. *Sci Rep,* 5**:** 9999. DOI 10.1038/srep09999

Lubzens, E., Young, G., Bobe, J. & Cerda, J. (2010). Oogenesis in teleosts: How fish eggs are formed. *Gen Comp Endocrinol,* 165**:** 367-389. DOI 10.1016/j.ygcen.2009.05.022

Luckenbach, J.A., Iliev, D.B., Goetz, F.W. & Swanson, P. (2008). Identification of differentially expressed ovarian genes during primary and early secondary oocyte growth in coho salmon (*Oncorhynchus kisutch*). *Reprod Biol Endocrinol,* 6. DOI 10.1186/1477-7827-6-2

Ma, H., Hostuttler, M., Wei, H., Rexroad, C.E., Iii & Yao, J. (2012). Characterization of the rainbow trout egg microRNA transcriptome. *PLoS ONE,* 7**:** e39649. DOI 10.1371/journal.pone.0039649

Maack, G. & Segner, H. (2003). Morphological development of the gonads in zebrafish. *J Fish Biol,* 62**:** 895-906. DOI 10.1046/j.1095-8649.2003.00074.x

Maack, G., Segner, H. & Tyler, C. (2003). Ontogeny of sexual differentiation in different strains of zebrafish (*Danio rerio*). *Fish Physiol Biochem,* 28**:** 125-128. DOI 10.1023/b:fish.0000030497.59378.88

Macrae, I.J., Zhou, K., Li, F., Repic, A., Brooks, A.N., Cande, W.Z., et al. (2006). Structural basis for double-stranded RNA processing by Dicer. *Science,* 311**:** 195-198. DOI 10.1126/science.1121638

Marco, A. (2014). Sex-biased expression of microRNAs in *Drosophila melanogaster*. *Open Biol,* 4. DOI 10.1098/rsob.140024

Maute, R.L., Schneider, C., Sumazin, P., Holmes, A., Califano, A., Basso, K., et al. (2013). tRNA-derived microRNA modulates proliferation and the DNA damage response and is down-regulated in B cell lymphoma. *Proc Natl Acad Sci USA,* 110**:** 1404-1409. DOI 10.1073/pnas.1206761110

Meredith, M., Macneil, A.H., Trasler, J.M. & Baltz, J.M. (2016). Growing mouse oocytes transiently activate folate transport via folate receptors as they approach full size. *Biol Reprod,* 94**:** 125, 1-12. DOI 10.1095/biolreprod.115.137687

Michalak, P. & Malone, J.H. (2008). Testis-derived microRNA profiles of African clawed frogs (*Xenopus*) and their sterile hybrids. *Genomics,* 91**:** 158-164. DOI 10.1016/j.ygeno.2007.10.013

Mickoleit, M., Banisch, T.U. & Raz, E. (2011). Regulation of *hub* mRNA stability and translation by miR430 and the *dead end* protein promotes preferential expression in zebrafish primordial germ cells. *Dev Dynam,* 240**:** 695-703. DOI 10.1002/dvdy.22571

Mishima, T., Takizawa, T., Luo, S.-S., Ishibashi, O., Kawahigashi, Y., Mizuguchi, Y., et al. (2008). MicroRNA (miRNA) cloning analysis reveals sex differences in miRNA expression profiles between adult mouse testis and ovary. *Reproduction,* 136**:** 811-822. DOI 10.1530/rep-08-0349

Mishima, Y., Giraldez, A.J., Takeda, Y., Fujiwara, T., Sakamoto, H., Schier, A.F., et al. (2006). Differential regulation of germline mRNAs in soma and germ cells by zebrafish miR-430. *Curr Biol,* 16**:** 2135-2142. DOI 10.1016/j.cub.2006.08.086

Mishima, Y. & Tomari, Y. (2016). Codon usage and 3' UTR length determine maternal mRNA stability in zebrafish. *Mol Cell,* 61**:** 874-885. DOI 10.1016/j.molcel.2016.02.027

Morozova, N., Zinovyev, A., Nonne, N., Pritchard, L.-L., Gorban, A.N. & Harel-Bellan, A. (2012). Kinetic signatures of microRNA modes of action. *RNA,* 18**:** 1635-1655. DOI 10.1261/rna.032284.112

Murchison, E.P., Stein, P., Xuan, Z., Pan, H., Zhang, M.Q., Schultz, R.M., et al. (2007). Critical roles for Dicer in the female germline. *Genes Dev,* 21**:** 682-693. DOI 10.1101/gad.1521307

Nanda, I., Kondo, M., Hornung, U., Asakawa, S., Winkler, C., Shimizu, A., et al. (2002). A duplicated copy of DMRT1 in the sex-determining region of the Y chromosome of the medaka, *Oryzias latipes*. *Proc Natl Acad Sci USA,* 99**:** 11778-11783. DOI 10.1073/pnas.182314699

Nei, M., Xu, P. & Glazko, G. (2001). Estimation of divergence times from multiprotein sequences for a few mammalian species and several distantly related organisms. *Proc Natl Acad Sci USA,* 98**:** 2497-2502. DOI 10.1073/pnas.051611498

Neilsen, C.T., Goodall, G.J. & Bracken, C.P. (2012). IsomiRs – the overlooked repertoire in the dynamic microRNAome. *Trends Genet,* 28**:** 544-549. DOI 10.1016/j.tig.2012.07.005

Nelson, J.S. (2006). *Fishes of the world,* New York: John Wiley and Sons, Inc.0-471-25031-7

Newman, M.A., Mani, V. & Hammond, S.M. (2011). Deep sequencing of microRNA precursors reveals extensive 3′ end modification. *RNA,* 17**:** 1795-1803. DOI 10.1261/rna.2713611

Nilsson, E.E., Schindler, R., Savenkova, M.I. & Skinner, M.K. (2011). Inhibitory actions of Anti-Mullerian Hormone (AMH) on ovarian primordial follicle assembly. *PLoS One,* 6**:** e20087. DOI 10.1371/journal.pone.0020087

Okamura, K., Liu, N. & Lai, E.C. (2009). Distinct mechanisms for microRNA strand selection by Drosophila Argonautes. *Mol Cell,* 36**:** 431-444. DOI 10.1016/j.molcel.2009.09.027

Olive, V., Li, Q. & He, L. (2013). mir-17-92, a polycistronic oncomir with pleiotropic functions. *Immunol Rev,* 253**:** 158-166. DOI 10.1111/imr.12054

Otsuka, M., Zheng, M., Hayashi, M., Lee, J.-D., Yoshino, O., Lin, S., et al. (2008). Impaired microRNA processing causes corpus luteum insufficiency and infertility in mice. *J Clin Invest,* 118**:** 1944-1954. DOI 10.1172/JCI33680

Park, J.-E., Heo, I., Tian, Y., Simanshu, D.K., Chang, H., Jee, D., et al. (2011). Dicer recognizes the 5′ end of RNA for efficient and accurate processing. *Nature,* 475**:** 201-205. DOI 10.1038/nature10198

Pasquinelli, A.E., Reinhart, B.J., Slack, F., Martindale, M.Q., Kuroda, M.I., Maller, B., et al. (2000). Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature,* 408**:** 86-89.

Pelegri, F. (2003). Maternal factors in zebrafish development. *Dev Dyn,* 228**:** 535-554. DOI 10.1002/dvdy.10390

Pillai, R.S., Artus, C.G. & Filipowicz, W. (2004). Tethering of human Ago proteins to mRNA mimics the miRNA-mediated repression of protein synthesis. *RNA,* 10**:** 1518-1525. DOI 10.1261/rna.7131604

Raz, E. (2003). Primordial germ-cell development: the zebrafish perspective. *Nat Rev Genet,* 4**:** 690-700. DOI 10.1038/nrg1154

Rehwinkel, J., Behm-Ansmant, I., Gatfield, D. & Izaurralde, E. (2005). A crucial role for GW182 and the DCP1:DCP2 decapping complex in miRNA-mediated gene silencing. *RNA,* 11**:** 1640-1647. DOI 10.1261/rna.2191905

Reinhart, B.J., Slack, F.J., Basson, M., Pasquinelli, A.E., Bettinger, J.C., Rougvie, A.E., et al. (2000). The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature,* 403**:** 901-906.

Ro, S., Park, C., Sanders, K.M., Mccarrey, J.R. & Yan, W. (2007a). Cloning and expression profiling of testis-expressed microRNAs. *Dev Biol,* 311**:** 592-602. DOI 10.1016/j.ydbio.2007.09.009

Ro, S., Park, C., Young, D., Sanders, K.M. & Yan, W. (2007b). Tissue-dependent paired expression of miRNAs. *Nucleic Acids Res,* 35**:** 5944-5953. DOI 10.1093/nar/gkm641

Rodríguez Marí, A., Yan, Y.L., Bremiller, R.A., Wilson, C., Canestro, C. & Postlethwait, J.H. (2005). Characterization and expression pattern of zebrafish Anti-Mmullerian hormone (Amh) relative to sox9a, sox9b, and cyp19a1a, during gonad development. *Gene Expr Patterns,* 5**:** 655-667. DOI 10.1016/j.modgep.2005.02.008

Roush, S. & Slack, F.J. (2008). The let-7 family of microRNAs. *Trends Cell Biol,* 18**:** 505-516. DOI 10.1016/j.tcb.2008.07.007

Ruby, J.G., Jan, C.H. & Bartel, D.P. (2007). Intronic microRNA precursors that bypass Drosha processing. *Nature,* 448**:** 83-86. DOI 10.1038/nature05983

Rupik, W., Huszno, J. & Klag, J. (2011). Cellular organisation of the mature testes and stages of spermiogenesis in *Danio rerio* (Cyprinidae: Teleostei) - Structural and ultrastructural studies. *Micron,* 42**:** 833-839. DOI 10.1016/j.micron.2011.05.006

Saito, T., Goto-Kazeto, R., Arai, K. & Yamaha, E. (2008). Xenogenesis in teleost fish through generation of germ-line chimeras by single primordial germ cell tansplantation. *Biol Reprod,* 78**:** 159-166. DOI 10.1095/biolreprod.107.060038

Sandberg, R. (2014). Entering the era of single-cell transcriptomics in biology and medicine. *Nat Meth,* 11**:** 22-24. DOI 10.1038/nmeth.2764

Sato, F., Tsuchiya, S., Meltzer, S.J. & Shimizu, K. (2011). MicroRNAs and epigenetics. *FEBS Journal,* 278**:** 1598-1609. DOI 10.1111/j.1742-4658.2011.08089.x

Schmitter, D., Filkowski, J., Sewer, A., Pillai, R.S., Oakeley, E.J., Zavolan, M., et al. (2006). Effects of Dicer and Argonaute down-regulation on mRNA levels in human HEK293 cells. *Nucleic Acids Res,* 34**:** 4801-4815. DOI 10.1093/nar/gkl646

Schulz, R.W., Renato De Franca, L., Lareyre, J.-J., Legac, F., Chiarini-Garcia, H., Nobrega, R.H., et al. (2010). Spermatogenesis in fish. *Gen Comp Endocrinol,* 165**:** 390-311. DOI 10.1016/j.ygcen.2009.02.013

Selman, K., Wallace, R.A., Sarka, A. & Qi, X. (1993). Stages of oocyte development in the zebrafish, *Brachydanio rerio*. *J Morphol,* 218**:** 203-224. DOI 10.1002/jmor.1052180209

Siegfriend, K.R. & Nusslein-Volhard, C. (2008). Germ line control of female sex determination in zebrafish. *Dev Biol,* 324**:** 277-287. DOI 10.1016/j.ydbio.2008.09.025

Slanchev, K., Stebler, J., De La Cueva-Méndez, G. & Raz, E. (2005). Development without germ cells: The role of the germ line in zebrafish sex differentiation. *Proc Natl Acad Sci USA,* 102**:** 4074-4079. DOI 10.1073/pnas.0407475102

Soni, K., Choudhary, A., Patowary, A., Singh, A.R., Bhatia, S., Sivasubbu, S., et al. (2013). miR-34 is maternally inherited in Drosophila melanogaster and Danio rerio. *Nucleic Acids Res,* 41**:** 4470-4480. DOI 10.1093/nar/gkt139

Staton, A.A., Knaut, H. & Giraldez, A.J. (2011). miRNA regulation of Sdf1 chemokine signaling provides genetic robustness to germ cell migration. *Nat Genet,* 43**:** 204-211. DOI 10.1038/ng.758

Streisinger, G., Walker, C., Dower, N., Knauber, D. & Singer, F. (1981). Production of clones of homozygous diploid zebra fish (*Brachydanio rerio*). *Nature,* 291**:** 293-296.

Su, H., Trombly, M.I., Chen, J. & Wang, X. (2009). Essential and overlapping functions for mammalian Argonautes in microRNA silencing. *Genes Dev,* 23**:** 304-317. DOI 10.1101/gad.1749809

Su, Z., Yang, Z., Xu, Y., Chen, Y. & Yu, Q. (2015). MicroRNAs in apoptosis, autophagy and necroptosis. *Oncotarget,* 6**:** 8474-8490. DOI 10.18632/oncotarget.3523

Subramanian, V.S., Marchant, J.S., Parker, I. & Said, H.M. (2001). Intracellular trafficking/membrane targeting of human reduced folate carrier expressed in *Xenopus* oocytes. *Am J Physiol Gastrointest Liver Physiol,* 281**:** G1477-G1486.

Suh, N., Baehner, L., Moltzahn, F., Melton, C., Shenoy, A., Chen, J., et al. (2010). MicroRNA function is globally suppressed in mouse oocytes and early embryos. *Curr Biol,* 20**:** 271-277. DOI 10.1016/j.cub.2009.12.044

Sun, D., Zhang, Y., Wang, C., Hua, X., Zhang, X.A. & Yan, J. (2013). Sox9-related signaling controls zebrafish juvenile ovary-testis transformation. *Cell Death Dis,* 4**:** e930. DOI 10.1038/cddis.2013.456

Tadros, W. & Lipshitz, H.D. (2009). The maternal-to-zygotic transition: a play in two acts. *Development,* 136**:** 3033-3042. DOI 10.1242/dev.033183

Takahashi, H. (1977). Juvenile hermaphroditism in the zebrafish, *Brachydanio rerio*. *Bull Fac Fish Hokkaido Univ,* 28**:** 57-65.

Talwar, P.K. & Jhingran, A.G. (1991). *Inland fishes of India and adjacent countries,* Rotterdam: CRC Press

Tan, G.C., Chan, E., Molnar, A., Sarkar, R., Alexieva, D., Isa, I.M., et al. (2014). 5′ isomiR variation is of functional and evolutionary importance. *Nucleic Acids Res,* 42**:** 9424-9435. DOI 10.1093/nar/gku656

Tao, W., Sun, L., Shi, H., Cheng, Y., Jiang, D., Fu, B., et al. (2016). Integrated analysis of miRNA and mRNA expression profiles in tilapia gonads at an early stage of sex differentiation. *BMC Genom,* 17**:** 1-13. DOI 10.1186/s12864-016-2636-z

Tomizawa, S.-I., Kobayashi, H., Watanabe, T., Andrews, S., Hata, K., Kelsey, G., et al. (2011). Dynamic stage-specific changes in imprinted differentially methylated regions during early mammalian development and prevalence of non-CpG methylation in oocytes. *Development,* 138**:** 811-820. DOI 10.1242/dev.061416

Tomizawa, S.-I., Nowacka-Woszuk, J. & Kelsey, G. (2012). DNA methylation establishment during oocyte growth: mechanisms and significance. *Int J Dev Biol,* 56**:** 867-875. DOI 10.1387/ijdb.120152gk

Tong, S.-K., Hsu, H.-J. & Chung, B.-C. (2010). Zebrafish monosex population reveals female dominance in sex determination and earliest events of gonad differentiation. *Dev Biol,* 344**:** 849-856. DOI 10.1016/j.ydbio.2010.05.515

Tsang, T.E., Khoo, P.L., Jamieson, R.V., Zhou, S.X., Ang, S.L., Behringer, R., et al. (2001). The allocation and differentiation of mouse primordial germ cells. *Int J Dev Biol,* 45**:** 549-555.

Tzung, K.-W., Goto, R., Saju, Jolly m., Sreenivasan, R., Saito, T., Arai, K., et al. (2015). Early depletion of primordial germ cells in zebrafish promotes testis formation. *Stem Cell Reports,* 4**:** 61-73. DOI 10.1016/j.stemcr.2014.10.011

Vagin, V.V., Sigova, A., Li, C., Seitz, H., Gvozdev, V. & Zamore, P.D. (2006). A distinct small RNA pathway silences selfish genetic elements in the germline. *Science,* 313. DOI 10.1126/science.1129333

Vaz, C., Wee, C.W., Lee, G.P.S., Ingham, P.W., Tanavde, V. & Mathavan, S. (2015). Deep sequencing of small RNA facilitates tissue and sex associated microRNA discovery in zebrafish. *BMC Genom,* 16**:** 1-16. DOI 10.1186/s12864-015-2135-7

Vejnar, C.E. & Zdobnov, E.M. (2012). miRmap: Comprehensive prediction of microRNA target repression strength. *Nucleic Acids Res,* 40**:** 11673-83. DOI 10.1093/nar/gks901

Wahid, F., Shehzad, A., Khan, T. & Kim, Y.Y. (2010). microRNAs: Synthesis, mechanism, function, and recent clinical trials. *Biochim Biophys Acta,* 1803**:** 1231-1243. DOI 10.1016/j.bbamcr.2010.06.013

Wainwright, E.N., Jorgensen, J.S., Kim, Y., Truong, V., Bagheri-Fam, S., Davidson, T., et al. (2013). SOX9 regulates microRNA miR-202-5p/3p expression during mouse testis differentiation. *Biol Reprod,* 89**:** 34, 1-12. DOI 10.1095/biolreprod.113.110155

Webster, K.A., Schach, U., Ordaz, A., Steinfeld, J.S., Draper, B.W. & Siegfried, K.R. (2017). Dmrt1 is necessary for male sexual development in zebrafish. *Dev Biol,* 422**:** 33-46. DOI 10.1016/j.ydbio.2016.12.008

Wei, C., Salichos, L., Wittgrove, C.M., Rokas, A. & Patton, J.G. (2012). Transcriptome-wide analysis of small RNA expression in early zebrafish development. *RNA,* 18**:** 915-929. DOI 10.1261/rna.029090.111

Wheeler, B.M., Heimberg, A.M., Moy, V.N., Sperling, E.A., Holstein, T.W., Heber, S., et al. (2009). The deep evolution of metazoan microRNAs. *Evol Dev,* 11**:** 50-68. DOI 10.1111/j.1525-142X.2008.00302.x

Wilson, C.A., High, S.K., Mccluskey, B.M., Amores, A., Yan, Y.-L., Titus, T.A., et al. (2014). Wild sex in zebrafish: loss of the natural sex determinant in domesticated strains. *Genetics,* 198**:** 1291-1308. DOI 10.1534/genetics.114.169284

Wittbrodt, J., Shima, A. & Schartl, M. (2002). Medaka - a model organism from the far east. *Nat Rev Genet,* 3**:** 53-64. DOI 10.1038/nrg704

Wu, S.-F., Zhang, H., Hammoud, S.S., Potok, M., Nix, D.A., Jones, D.A., et al. (2011). Chapter 18 - DNA Methylation Profiling in Zebrafish. IN H. William Detrich, M.W. & Leonard, I.Z. (Eds.) *Methods in Cell Biology*. Academic Press.

Wyman, S.K., Knouf, E.C., Parkin, R.K., Fritz, B.R., Lin, D.W., Dennis, L.M., et al. (2011). Post-transcriptional generation of miRNA variants by multiple nucleotidyl transferases contributes to miRNA transcriptome complexity. *Genome Res,* 21**:** 1450-1461. DOI 10.1101/gr.118059.110

Xiao, J., Zhong, H., Zhou, Y., Yu, F., Gao, Y., Luo, Y., et al. (2014). Identification and characterization of microRNAs in ovary and testis of Nile tilapia (*Oreochromis niloticus*) by using solexa sequencing technology. *PLoS ONE,* 9**:** e86821. DOI 10.1371/journal.pone.0086821

Xie, M., Li, M., Vilborg, A., Lee, N., Shu, M.-D., Yartseva, V., et al. (2013). Mammalian 5'-capped microRNA precursors that generate a single microRNA. *Cell,* 155**:** 1568-1580. DOI 10.1016/j.cell.2013.11.027

Yekta, S., Shih, I.-H. & Bartel, D.P. (2004). MicroRNA-directed cleavage of *HOXB8* mRNA. *Science,* 304**:** 594-596. DOI 10.1126/science.1097434

Yoon, C., Kawakami, K. & Hopkins, N. (1997). Zebrafish *vasa* homologue RNA is localized to the cleavage planes of 2- and 4-cell stage embryos and is expressed in the primordial germ cells. *Development,* 124**:** 3157-3165.

Paper II

SCIENTIFIC REPORTS

OPEN

Dynamics of miRNA transcriptome during gonadal development of zebrafish

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Studies in non-teleost vertebrates have found microRNAs (miRNAs) to be essential for proper gonadal development. However, comparatively little is known about their role during gonadal development in teleost fishes. So far in zebrafish, a model teleost, transcript profiling throughout gonadal development has not been established because of a tiny size of an organ in juvenile stages and its poor distinguishability from surrounding tissues. We performed small RNA sequencing on isolated gonads of *See-Thru-Gonad* line, from the undifferentiated state at 3 weeks post fertilization (wpf) to fully mature adults at 24 wpf. We identified 520 gonadal mature miRNAs; 111 of them had significant changes in abundance over time, while 50 miRNAs were either testis- or ovary-enriched significantly in at least one developmental stage. We characterized patterns of miRNA abundance over time including isomiR variants. We identified putative germline versus gonadal somatic miRNAs through differential small RNA sequencing of isolated gametes versus the whole gonads. This report is the most comprehensive analysis of the miRNA repertoire in zebrafish gonads during the sexual development to date and provides an important database from which functional studies can be performed.

MicroRNAs (miRNAs) are a highly conserved class of small regulatory RNAs, approximately 22 nucleotides (nt) long, which have a primary function in repressing the post-transcriptional expression of target messenger RNAs (mRNAs)[1]. The functions of miRNAs are diverse, including regulation of cell division[2], differentiation[3], migration[4], and apoptosis[5]. However, most knowledge on miRNA in vertebrates is restricted to mammals. As of 2014, 11,717 mature miRNAs have been identified in mammals, compared to only 1,044 in teleost fishes[6], despite teleosts being distinctively the most speciose group of all vertebrates with over 23,500 described species[7].

The function of miRNAs during gonadal development is currently a subject of debate. In the more common canonical miRNA biogenesis pathway, miRNAs are transcribed as long primary transcripts (pri-miRNA)[8]. Within the nucleus, pri-miRNAs are recognized by the microprocessor Drosha/DGCR8 complex, which cleaves the miRNA hairpin to produce the precursor miRNA (pre-miRNA)[8]. After export to the cytoplasm, the pre-miRNA is processed by the enzyme Dicer to produce an RNA duplex, which is loaded into an Argonaute (Ago) protein[8]. In mouse (*Mus musculus*), germline mutations for either Dicer or Ago resulted in oocytes which were unable to complete meiosis and were sterile[9–11]. Similarly, ablation of *Dicer1* in gonadal somatic cells resulted in widespread suppression of miRNA expression, leading to abnormal gonad morphology and sterility[12,13]. By comparison, knockout of Dicer in male mice affected germ cell and spermatogonia proliferation and differentiation, but Ago2 deficient testis developed normally[14]. In another study on mouse, *Dgcr8* deficient oocytes were able to develop normally[15]. The authors found evidence that since Dicer also processes small interfering RNAs (siRNAs)[8], it is likely their loss which explained the previous Dicer mutant phenotypes in oocytes, whereas miRNA activity is suppressed during oocyte maturation[15]. However, the authors also reported diminished brood size in *Dgcr8* knockout mice, suggesting a role for maternal miRNAs[15].

Studies in zebrafish have found miRNAs to be essential for proper development. In *dicer1* mutants, developmental arrest was observed at approximately 10 days, after maternal Dicer1 activity has ceased[16], while maternal dicer mutant embryos had severe morphological deformities early during embryogenesis[17]. However, the role of miRNAs during gonadal development is unclear. Zebrafish with germline *dicer* and *ago2* mutations are able to reach sexual maturity and produce offspring[17,18]. Instead, both Zili, and Ziwi, two proteins from the Piwi subclass of Argonaute proteins which interact with Piwi-interacting RNAs (piRNAs), are required for germline meiosis

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and survival[19,20]. The primary functions of teleost gonadal miRNAs may therefore be related to gonadal-somatic cell development or interactions between somatic and germ cells. Establishing miRNA repertoires between germline and gonadal-somatic cells may help to address this question.

In recent years, several studies have reported differential expression of miRNAs during gonadal development of commercially important teleost species. The first study was performed in Atlantic halibut (*Hippoglossus hippoglossus*), where sexually dimorphic miRNA expression was detected between immature testis and ovaries, as well as between sexually mature and immature testis[21]. Further studies in rainbow trout, (*Oncorhynchus mykiss*) have identified miRNAs in several developmental stages of testes, ovary, and unfertilized egg[22,23]. In yellow catfish (*Pylodictis fulvidraco*), the miRNA repertoire of immature gonads was determined[24], and in Nile tilapia (*Oreochromis niloticus*), gonads during sexual differentiation[25] as well as at sexual maturity have been sequenced[26]. While these studies provide important information about the miRNA repertoire during specific stages of development, the findings lack context without a more complete overview of the gonadal miRNA repertoire. As of yet, no study is reported to characterize miRNAs in both testis and ovary throughout gonadal development in a teleost. A more complete overview of miRNA abundance would be beneficial to understand the role of miRNAs in teleost reproduction.

Zebrafish (*Danio rerio*) is a model teleost species for developmental biology. However, due to its small size, sequencing of small transcriptome from zebrafish gonads has thus far been restricted to sexually mature organs[27] and spermatozoa[28] only. To overcome the challenge of distinguishing zebrafish gonads during juvenile stages of development, we have recently established *See-Thru-Gonad* line[29]; this line is a hybrid of the zf45Tg[30] and *nacre transparent* (−/−)[31,32] lines. In *See-Thru-Gonad* fish, the germline is visualized *in vivo* under epifluorescent light during its lifetime[29].

The objective of the present study was to explore the miRNA repertoire during gonadal development of the zebrafish. We isolated gonads throughout gonadal development of *See-Thru-Gonad* line, from undifferentiated gonads at 3 weeks post fertilization (wpf) to fully mature gonads at 24 wpf. We also sampled mature oocytes and spermatozoa. With the exception of 3 wpf gonads and sperm, which were pooled for sequencing, we performed small RNA sequencing on individual testes and ovaries throughout gonadal development assuring true biological replications. For this purpose, we developed RNA low-input protocol for library construction and tested its reliability for scarce sample amounts. For the first time, we characterized miRNA dynamics during zebrafish gonadal development.

Results

Sequence statistics. Zebrafish gonads from nine stages of development, as well as gametes (Fig. 1) were sampled and used to prepare a total of 47 small RNA libraries. Sequencing of these libraries generated over 227 million reads (Supplementary Dataset S1). After trimming, over 191 million good quality reads remained, of which over 155 million reads were mapped to the zebrafish genome. In all libraries, Piwi-interacting RNA (piRNA) were the most abundant RNA fraction, constituting from 31% (24 wpf testis) to 79% (unfertilized eggs) of all annotated small RNAs (Supplementary Fig. S1). Ovary and testis at 24 wpf had a comparatively large proportion of transfer RNA (tRNA, 24% and 31%, respectively). Large intergenic non-coding RNAs (lincRNAs) composed from 9% (24 wpf ovary) to 29% (spermatozoa) of small RNA reads. The proportion of miRNA greatly varied between libraries, ranging from 2 to 21% in unfertilized eggs and 24 wpf ovary, respectively.

The majority of small RNA reads in each library was between 26 and 31 nucleotides (nt) long (Supplementary Fig. S1). Libraries from undifferentiated 3 wpf gonads and gametes had a relatively large proportion of reads smaller than 20 nt. Libraries from gonads at 6 and 9 wpf generally showed few reads outside the 26 to 31 nt range. In contrast, libraries from 12 and 24 wpf gonads had strong peaks at 22 and 34 nt.

Low input RNA protocol. Total number of miRNA reads obtained from reduced RNA inputs were reduced to 73% and 45% in testis, and 48% and 14% in ovary, for libraries produced from 100 and 25 ng total RNA, respectively (Fig. 2a, Supplementary Dataset S2). Despite a reduction in the total number of miRNA reads, the frequency of mapped miRNAs in all RNA input levels was stable, as well as highly and positively correlated between input sizes (Fig. 2b). In 5 cases, individual miRNAs were not detected in the ovary library prepared from 25 ng total RNA input, while they were detected when using larger RNA inputs (Fig. 2b).

Dynamics of miRNA abundance during gonadal development. In total, 520 mature miRNAs were identified in zebrafish gonads (Supplementary Dataset S3). The samples were clustered according to sex (ovary versus testis) and developmental advancement (Fig. 3a). Samples from 6 and 9 wpf gonads were closely clustered within the respective sex, while 12 and 24 wpf gonad samples were clustered separately. The undifferentiated 3 wpf gonad sample was most similar to 6 and 9 wpf testis. A single ovary sample at 12 wpf was separated from the rest of the 12 wpf ovaries. When comparing gametes with 24 wpf gonads, the samples were tightly clustered as testis, ovary, unfertilized egg, and spermatozoa (Fig. 3b).

Twenty-seven miRNAs contributed to the ten most abundant miRNAs in each gonadal sampling point (Fig. 4a,b). Five miRNAs were abundant only in purified spermatozoa or unfertilized eggs, but not in whole gonads (Fig. 4c). miR-92a-3p was the most frequently abundant miRNA, which was among the top five most abundant miRNAs in every gonadal stage as well as in gametes, while miR-92b-3p was highly abundant in gonads from 3 to 12 wpf, and in spermatozoa. In addition, miR-10b-5p, miR-125a-5p, miR-143–3p, miR-181a-5p, and miR-21–5p were among the most abundant miRNAs in at least six of nine gonadal stages (Fig. 4b). Several miRNAs, namely let-7a-1–5p, let-7c-5p, let-7d-5p, miR-181a-5p, miR-222a-3p, miR-430b-3p, and miR-462-5p were typically more abundant in earlier stages of gonadal development, whereas miR-100-5p, miR-10a-5p, miR-10b-5p, miR-10c-5p, miR-202-5p, and miR-30d-5p were abundant during later stages of development. miR-22a-3p and

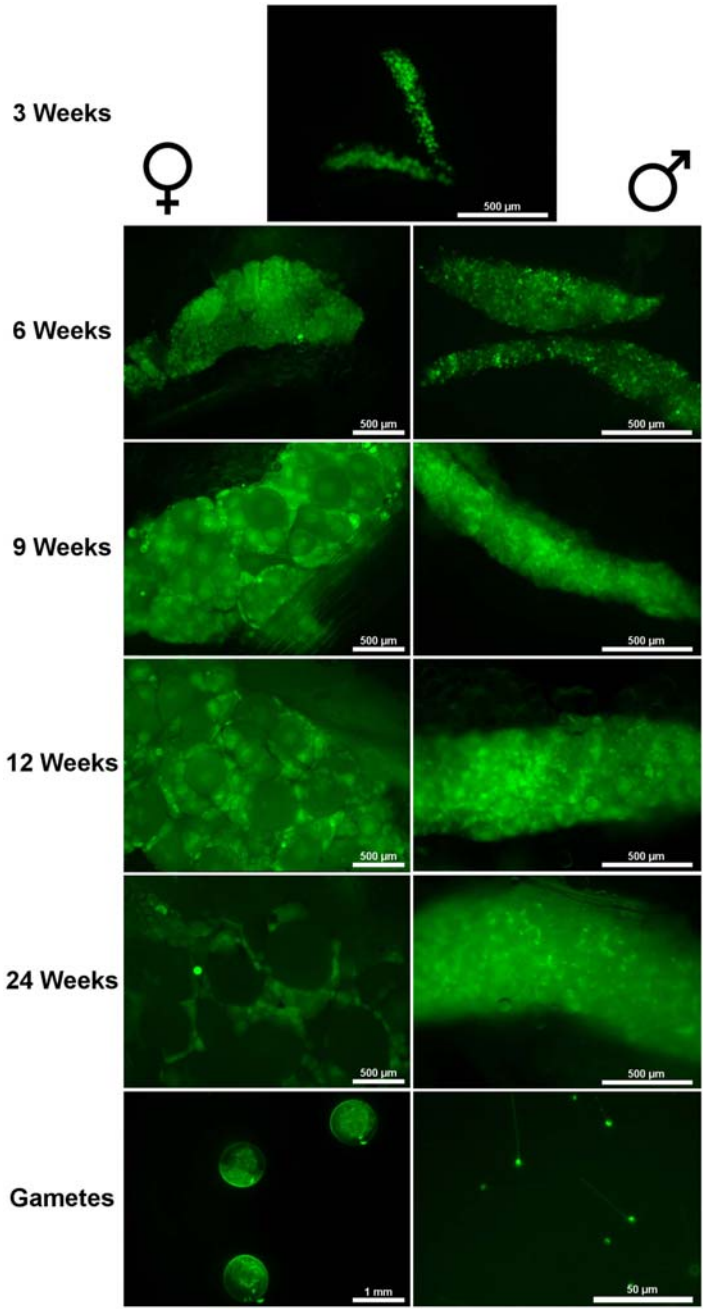

Figure 1. Representation of zebrafish gonads expressing vasa:vasa-eGFP transgene at each sequenced gonadal stage.

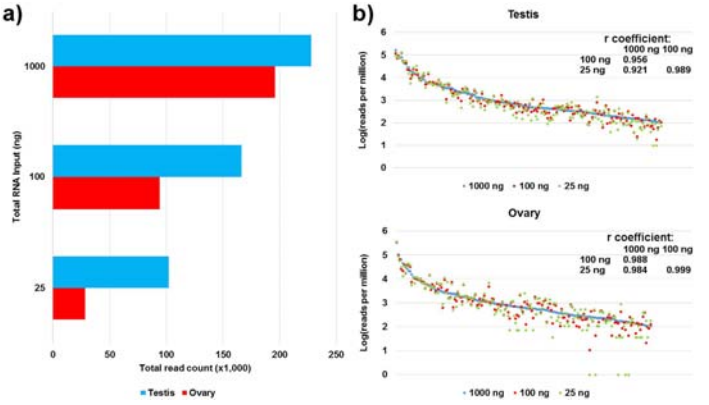

Figure 2. Efficiency of low-input RNA library preparation. (**a**) Total number of miRNA reads obtained from libraries prepared using different total RNA inputs. (**b**) Normalized miRNA reads from total RNA low-input libraries plotted against the data derived from the standard 1000 ng total RNA input library. Pearson's product-moment correlation coefficient *r* is given for miRNAs with a threshold abundance >100 reads per million.

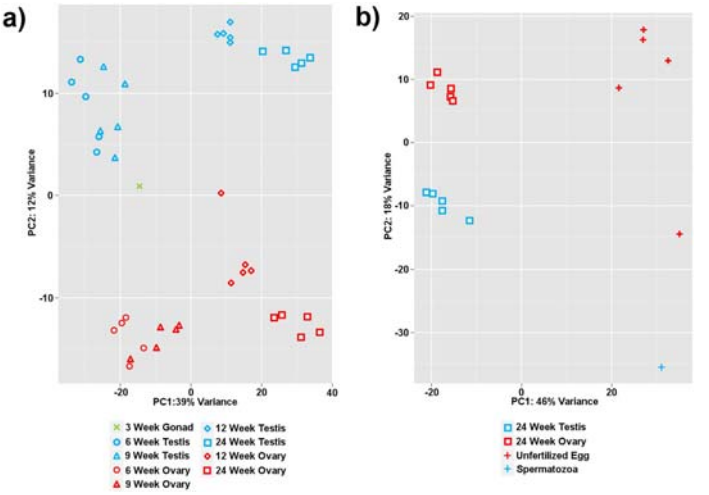

Figure 3. Principal component analysis of miRNAs from zebrafish gonads and gametes. (**a**) Clustering of miRNAs according to gonadal sex and developmental advancement. (**b**) Clustering of miRNAs in gametes versus respective gonad samples derived from sexually mature 24-week-old zebrafish. Blue and red colors denote male and female sex samples, respectively, while green color marks sexually undifferentiated 3-week-old juvenile gonad.

miR-430b-3p were only abundant in the ovary, whereas miR-125c-5p and miR-462-5p were more frequently abundant in the testis (6 to 12 wpf) compared to the ovary (only 6 wpf).

Differential expression analysis of the dynamics of miRNA over the course of gonadal development revealed significant changes in miRNA abundance over time (Supplementary Dataset S4). Of the 520 mature miRNA identified in zebrafish gonads, 111 had at least one significant change in abundance over time. Abundance of 62 miRNAs in the testis and 65 miRNA in the ovary was significantly different from undifferentiated gonads in at least one sampling point. Additionally, 73 and 70 miRNAs had a significant change in abundance over time within the testis and ovary, respectively. The dynamics of expression of the 27 most abundant miRNAs (Fig. 4) had several distinct patterns (Fig. 5). miR-222a-3p was the only miRNA with no significant change in abundance over time, while miR-10a-5p, miR-10c-5p, miR-125a-5p, miR26a-5p, and miR-92b-3p showed no significant change in abundance from 3 to 12 wpf. Notably, all miRNAs from the miR-10 family showed a significant increase in

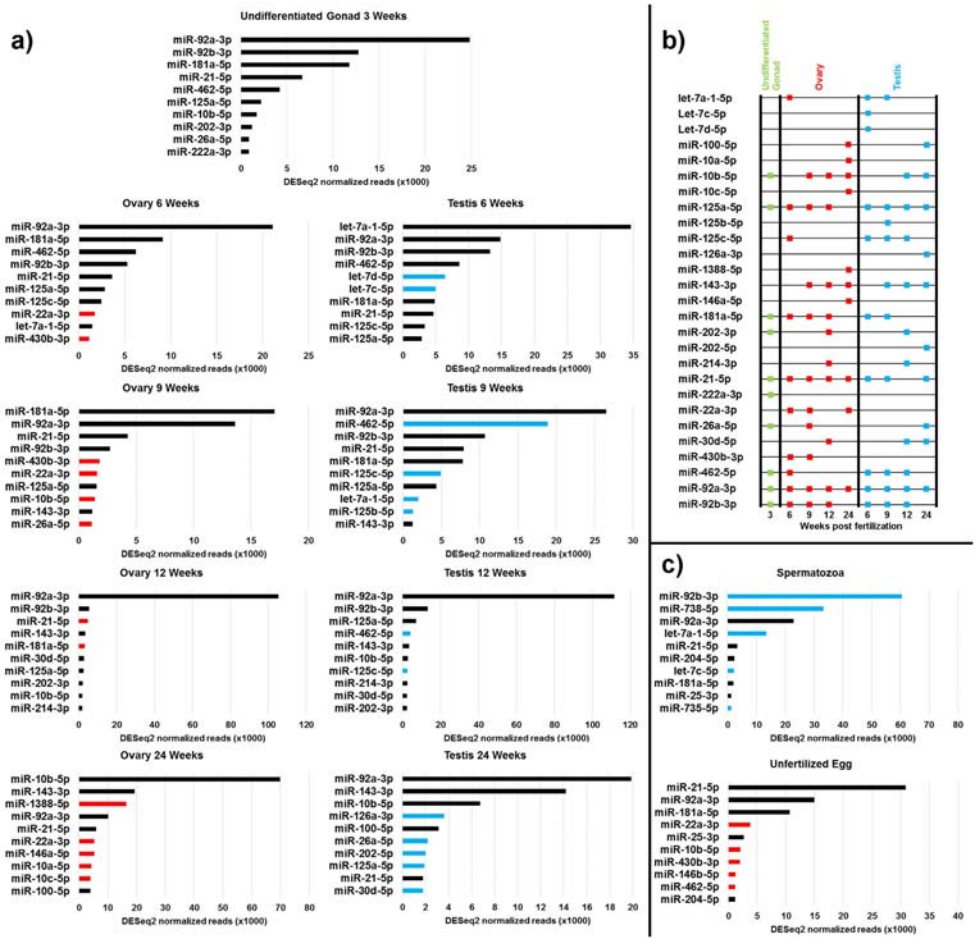

Figure 4. The most abundant miRNAs in zebrafish gonads and gametes. (**a**) The 10 most abundant miRNAs at each sampled stage of gonadal development in both testis and ovary. (**b**) The frequency of presence of a miRNA among the 10 most abundant miRNAs throughout the stages of gonadal development. (**c**) The 10 most abundant miRNAs in the spermatozoa and unfertilized eggs. Black bars represent miRNAs commonly abundant in both sexes at a given sampling time point, while red and blue bars mark miRNAs abundant in either ovary or testis samples, respectively.

abundance in ovaries at 24 wpf, while miRNAs from the let-7 family showed a significant increase at 6 wpf only. By comparison, miR-125c-5p, miR-430b-3p, and miR-462-5p all showed increase in abundance at 9 wpf. The abundance of several miRNAs, namely miR-100-5p, miR-126a-3p, miR-143-3p, and miR-202-5p was gradually increasing in time. In contrast, abundance of miR-181a-5p steadily decreased over time.

In total, 27 miRNAs were significantly more abundant in testis than in ovary in at least one developmental stage, whereas 23 miRNAs were more abundant in the ovary compared to the testis (Supplementary Dataset S5). Of the testis-enriched miRNAs, the let-7 family was well represented at 6 wpf (Fig. 6a), while the miR-125 family was abundant at 6, 9, and 24 wpf (Fig. 6a,b,d). miR-462-5p and miR-26a-5p were abundant at 9 and 24 wpf, respectively (Fig. 6b,d). In ovaries, miR-430b-3p was highly enriched compared to testes from 6 to 12 wpf (Fig. 6a,b,c). At 24 wpf, miR-10a-5p, miR-10b-5p, miR-146a-5p, miR-22a-3p, and miR-1388-5p were abundant and ovary-enriched (Fig. 6d).

Comparison of miRNA abundance between gametes and sexually mature gonad. Comparing miRNA reads in gametes to the sexually mature gonads at 24 wpf, when both gametes and gonads were isolated, identified seven miRNAs with significantly increased abundance (Fig. 7, Supplementary Dataset S5).

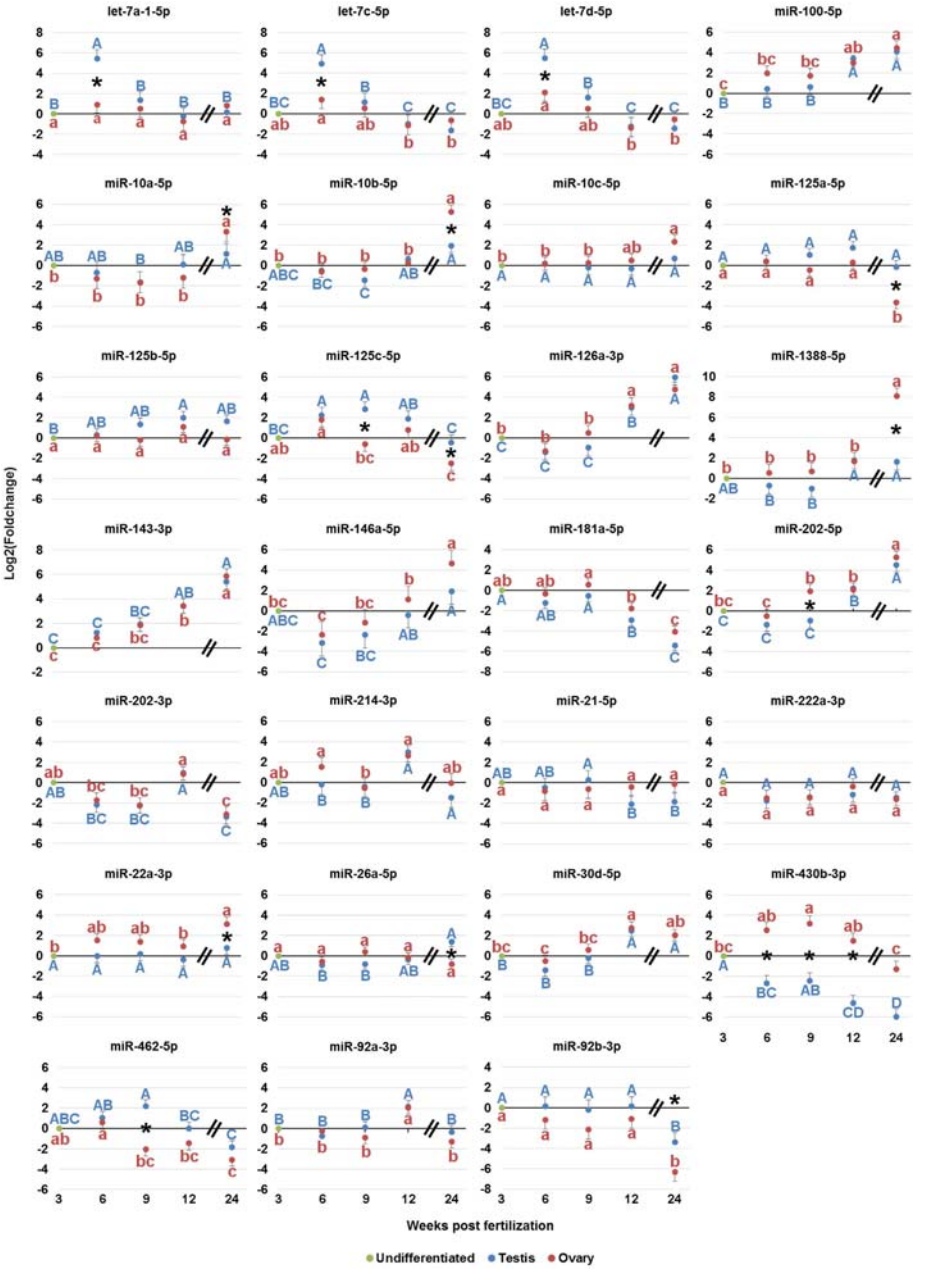

Figure 5. Dynamics of miRNA expression in zebrafish gonads over time. Each data point represents the average log-transformed fold change for the reads at a given time point (n = 5 for each testis and ovary sampling point) relative to undifferentiated gonads (pooled sample) at 3 weeks post fertilization. Within each sex, values marked with different letters (blue upper case for testis, red lower case for ovary) are significantly different from each other. Reads were normalized using DESeq2 and statistical significance was determined by an adjusted p-value ≤ 0.01 and a log2 fold change $\geq |2|$. Significant differences between sexes at a given time point are marked with asterisks. The double break at X-axis highlights the discontinuous sampling interval between 12 and 24 weeks post fertilization. Vertical bars represent standard deviation.

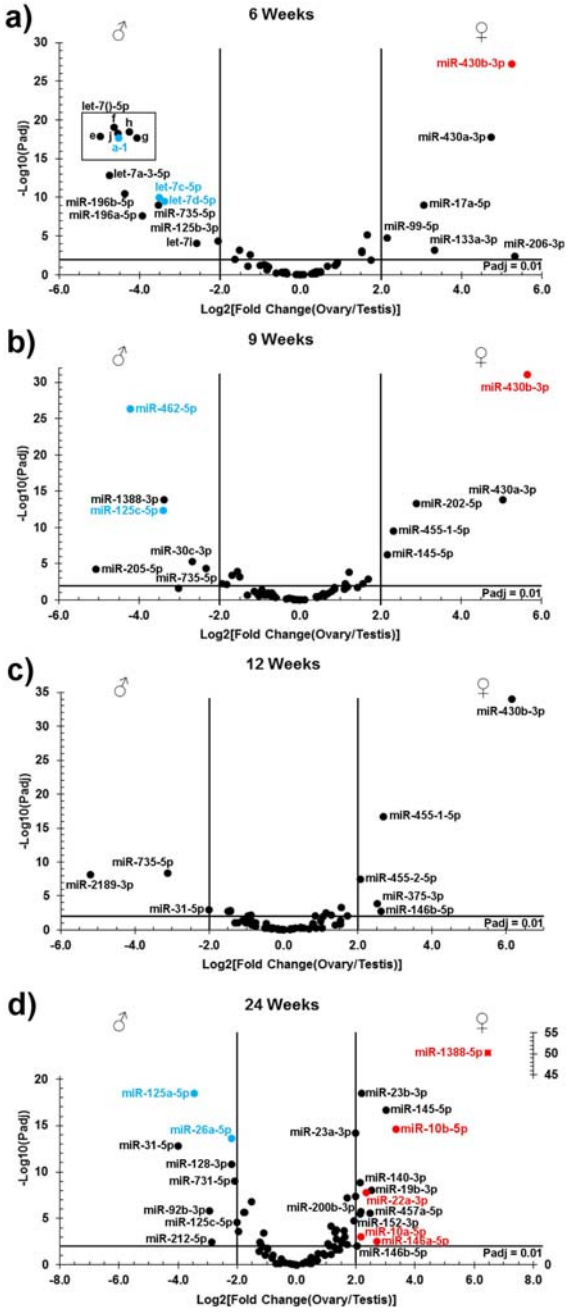

Figure 6. Differences in miRNA abundance between testes and ovaries. The plots show adjusted *p*-value (y-axis) against the log2-transformed fold change (ovary/testis) for miRNAs with a normalized read count ≥ 100 at 6 (panel a), 9 (**b**), 12 (**c**), and 24 weeks post fertilization (**d**). Named miRNAs appearing with a log2 fold change value $\geq |2|$ and an adjusted p-value ≤ 0.01 were considered as differentially expressed. Blue and red labels identify miRNA present among the 10 most abundant miRNA at the given time point for testis and ovary, respectively. The square data point in panel D (miR-1388-5p) is plotted against the secondary y-axis.

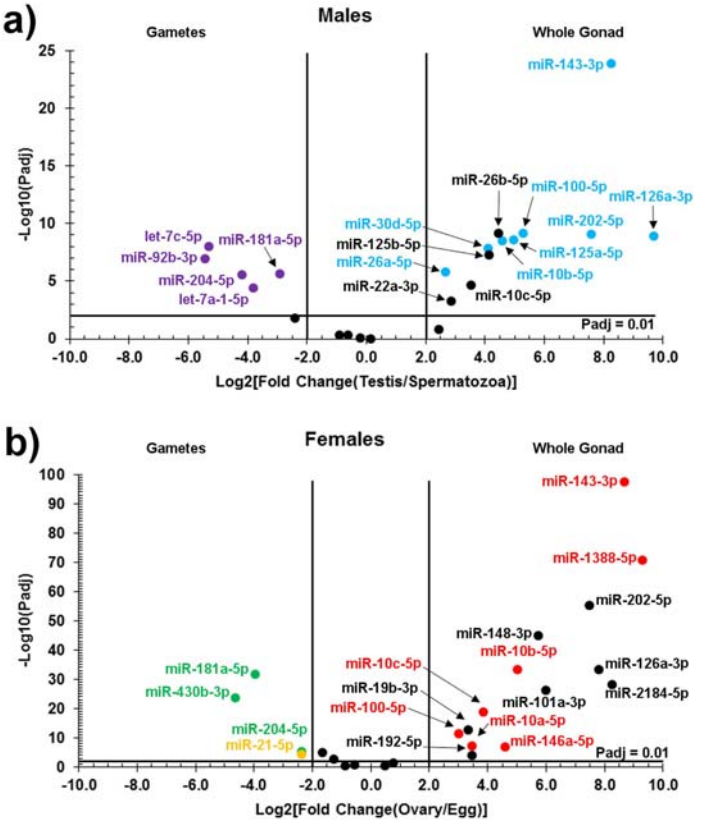

Figure 7. Differences in miRNA abundance between mature gonads and gametes. The adjusted *p*-value (y-axis) is plotted against the log- transformed fold change (gonad/gamete) for mature male (panel a) and female (**b**) zebrafish at 24 weeks post fertilization. Only miRNAs with a normalized read count $\geq$1000 are plotted. Named miRNAs appearing with a log2 fold change value $\geq |2|$ and an adjusted *p*-value ≤ 0.01 were considered differentially expressed. Colored labels identify miRNA among the 10 most abundant in their respective sample: blue for testis, red for ovary, purple for spermatozoa, green for unfertilized eggs. Yellow coloring identifies miRNA among the 10 most abundant in both ovaries and unfertilized eggs.

miR-181a-5p and miR-204-5p were significantly more abundant in both spermatozoa and oocytes. let-7a-1-5p, let-7c-5p and miR-92b-3p were more abundant in spermatozoa only, whereas miR-430b-3p and miR-21-5p were more abundant in oocytes only. miR-21-5p was the only miRNA which was among the most abundant miRNA in both unfertilized eggs and the ovary (Fig. 4a,c). By comparison, 20 miRNAs were significantly more abundant in gonads compared to gametes. The miR-10 family was well represented; miR-125a-5p and miR-125b-5p were more abundant in the testis than spermatozoa, miR-10a-5p was more abundant in the ovary than unfertilized eggs, while miR-10b-5p, miR-10c-5p and miR-100-5p were more abundant in both testes and ovaries compared to gametes.

Presence of isomiRs during zebrafish gonadal development. Multiple variations in miRNA sequences (isomiRs) were observed throughout gonadal development (Supplementary Dataset S6). Of the 27 most abundant miRNAs from Fig. 4, all had at least one isomiR variant accounting for at least 5% of the miRNA reads in at least one developmental stage. Nucleotide additions were the most frequent variant, present in 23 out of the 27 miRNA examined (Supplementary Dataset S6). Of these, 13 miRNA had variants only containing templated additions, 4 miRNAs had only untemplated variants, and 6 miRNAs had both templated and untemplated variants. Nucleotide substitutions at 3′ end were observed in 18 of 27 miRNA (Supplementary Dataset S6). The most frequent substitutions were either uracil or adenine to guanine, found in 11 and 6 miRNAs, respectively (Fig. 8a). The U-to-G substitutions were frequently detected at 6 wpf in both testis and ovary, as well as 9 wpf in ovary, but not in 12 and 24 wpf gonads. The A-to-G substitutions were most frequent at

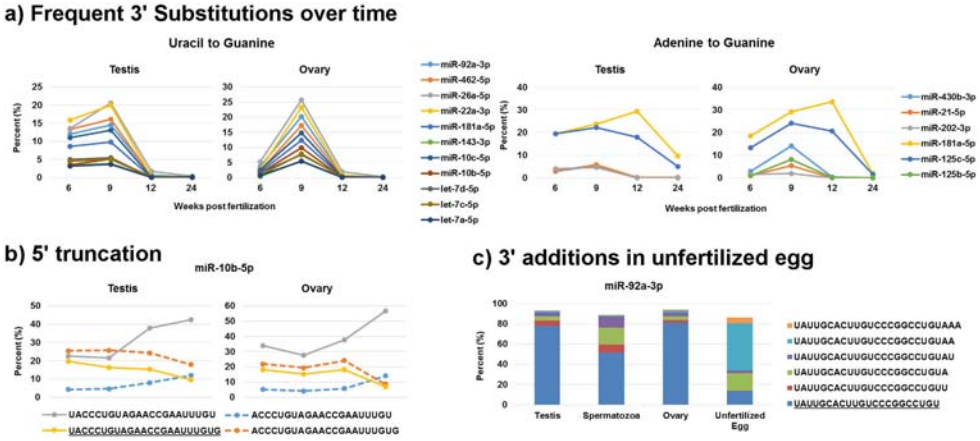

Figure 8. General features of isomiRs in zebrafish gonads. (**a**) Dynamics in the relative composition of frequently occurring nucleotide substitutions among the most abundantly expressed miRNAs in zebrafish gonads. (**b**) Representation of 5′ truncations observed in miR-10b-5p. Dotted lines represent isomiRs with 5′ truncation compared to the reference variant. (**c**) Abundant 3′ additions of templated adenine and untemplated uracil in miR-92a-3 in gametes versus mature whole gonads. The reference variants for miR-10b-5p (**b**) and miR-92a-3p (**c**) are underlined. Only isomiRs accounting for ≥5% of the total miRNA reads are shown.

9 and 12 wpf. Modifications at 5′ end were detected in 8 miRNAs; miR-100-5p, miR-10a-5p, miR-10b-5p, miR-10c-5p, and miR-126a-5p were truncated, while miR-202-5p, miR-202-3p, and miR-214-3p had templated additions (Supplementary Dataset S6). In the case of miR-10b-5p, one of the 5′ truncated variants accounted for up to 26% of all reads, and was the dominant variant in the testis at 6 and 9 wpf (Fig. 8b). miR-202-3p had 10 variants with at least 5% representation, and the 5′ templated addition variants accounted for the majority of the reads in all developmental stages (Supplementary Dataset S6). In gametes, miR-92a-3p had several variants with 3′ templated adenine and untemplated uracil additions (Fig. 8c). In unfertilized eggs, isomiRs with an additional 1 to 3 adenine residues accounted for a combined 69% of all reads, compared to 7% in 24 wpf ovaries.

GO term enrichment analysis. Gene ontogeny (GO) term enrichment analysis of the 27 most abundant gonadal miRNAs identified 16 significantly enriched biological process GO terms (Supplementary Dataset S7). The most enriched GO term was segment polarity determination (GO:0007367), which contained target mRNA for the miR-10 and miR-125 families, as well as miR-181a-5p miR-21-5p, miR-222a-3p, and miR-430b-3p. The second most enriched GO term was negative regulation of substrate adhesion-dependent cell spreading (GO:1900025), and contained target mRNA for miR-181a-3p, miR-202-5p, miR-25-3p, miR-30d-5p, miR-430b-3p, miR-92a-3p, and miR-92b-3p.

Discussion

A comparison of the zebrafish miRNA repertoire in sexually mature gonads with sequencing results in olive flounder (*Paralichthys olivaceus*)[33], marine medaka (*Oryzias melastigma*)[34], Nile tilapia[26], rainbow trout[22], and another zebrafish study[27], identified several miRNAs with varying levels of gonadal conservation, while highlighting the dynamic nature of miRNA expression (Supplementary Fig. S2). Among the most frequently abundant miRNAs between species were let-7a-5p, miR-143-3p, miR-181a-5p, miR-202-5p, and miR-21-5p (Supplementary Fig. S2B). The most frequently abundant was miR-143-3p, which was among the 10 most abundant miRNAs in both testis and ovary in all five species examined, followed by miR-21-3p, which was abundant in all except Nile tilapia. Relative abundance of let-7a-5p, miR-181a-5p, and miR-202-5p varied between different species as well as the two zebrafish studies. We detected high abundance of let-7a-5p in testis at 6 wpf (Fig. 4), but not in sexually mature gonads, in contrast to the other four fish species, and the other zebrafish study[27]. miR-202-5p had high abundance in rainbow trout, marine medaka, and the other zebrafish study, but we detected comparatively lower abundance (Fig. 4), similar to Nile tilapia. miR-181a-5p was highly abundant in Nile tilapia and olive flounder, as well as 12 wpf zebrafish ovaries (Fig. 4), but not highly abundant at 24 wpf ovary or in the related zebrafish study.

We report relatively high precision in small RNA sequencing between biological replicates, allowing us to detect significant differences in abundance between the gonadal stages sampled (Fig. 5, Supplementary Dataset S4). However, we also observed changes in the miRNA repertoire between 12- and 24-week-old gonads, both of which were sexually mature, but differed in gonadal morphology (Fig. 1). These findings suggest small RNA sequencing at a single developmental point may be inadequate to reliably determine the miRNA transcriptome of a gonad.

Since zebrafish germline *dicer* and *ago2* mutants are fertile[17,18], and germline miRNA activity in mice is suppressed during oocyte maturation[15], there are questions over the essentiality of miRNAs in gonadal development. By comparison, piRNA and Piwi proteins are essential to zebrafish gonadal development[19,20]. Zebrafish *ziwi* mutants have normal gonadal development at 2 wpf, before suffering severe germ cell depletion by 3 wpf as a result of apoptosis[20]. In *zili* mutants, a nonsense mutation resulted in complete germline ablation after 7 wpf, while a missense mutant resulted in germ cell development but caused defects during meiosis I in oocytes and ended in sterility[19]. Between 2 and 3 wpf, wildtype zebrafish germ cells are proliferating and entering the early stages of meiosis prior to sex determination[29], while the number of germ cells produced influences the eventual sex of the animal[35,36]. In our study, we observed that the miRNAs abundant in 3 wpf gonads generally maintained steady levels of expression throughout the gonadal development, and had putative functions in repressing stem cell differentiation, notably: miR-10b-5p, miR-222a-3p, and miR-26a-3p (Figs 4 and 5). miR-10b-5p belongs to the evolutionarily conserved miR-10/100 family, which are associated with suppressing *homeobox* (*hox*) genes[37]; *hox* genes are highly conserved transcription factors which guide stem cell differentiation[38]. In mice, miR-10b-5p was enriched in germ cells, and suggested to function in repressing germ cell differentiation[39]. In chicken (*Gallus gallus*), miR-222-3p is germline enriched and represses *de novo methyltransferase* (*DNMT3B*)[40]. In the olive flounder, miR-26a-5p targets *empty spiracles homeobox 2* (*emx2*)[41]; in mice, *Emx2* is required to trigger germ cell differentiation[42]. These findings suggest there may be a relationship between miRNAs functioning to repress early gonadal cell differentiation, while piRNAs are required for germ cell differentiation, an initiation of meiosis, and to prevent germ cell apoptosis.

The role of gonadal miRNAs may also be more essential to gonadal somatic cell development rather than to the germline. In this study, while miRNAs made up over 20 percent of all small RNA reads in gonads at 24 wpf, they made up less than 3 percent of small RNA reads in isolated gametes (Supplementary Fig. S1). In addition, we found far more miRNAs with significantly higher abundance in whole 24 wpf gonads compared to isolated gametes (Fig. 7). While germline *dicer* and *ago2* mutations effectively prevented mature miRNAs transcription within the zebrafish germline[17,18], they would not have prevented miRNA expression in gonadal somatic cells. In mice, Dicer loss in Sertoli cells led to infertility[13], with evidence suggesting the altered proteome was a result of miRNA depletion[43]. While it is possible that miRNAs are more abundant within the germline in earlier stages of gametogenesis than in mature gametes, the differences we observed were striking and should be investigated further. *In situ* hybridization experiments identifying spatial expression patterns of miRNAs would assist in determining whether they have primary functions in the germline, gonadal somatic cells, or both.

In contrast to the miRNAs abundant at 3 wpf, many of the miRNAs abundant later in gonadal development had putative functions in promoting cell differentiation. We found significantly increased abundance of let-7 miRNAs in testis at 6 wpf (Fig. 5). At this developmental stage, the gonad is already differentiated (Fig. 1) and typically consists of spermatogonia and spermatocytes with some spermatids[29]. The let-7 miRNA family is highly conserved and typically associated with promoting progenitor cell differentiation and proliferation, often through interactions with *lin41/trim71* and *lin28*[44]. Lin28 interacts with pre-let-7 to prevent its maturation, which results in cells maintaining an undifferentiated state. Trim71 promotes Lin28 repression of let-7 and antagonizes Argonaute 2 (Ago2), resulting in protection of let-7-targeted mRNA from repression[44–46]. miRNAs from the let-7 family target *trim71*, meaning increased abundance of let-7 causes a positive feedback loop of let-7 maturation and function, thereby encouraging progenitor cell differentiation[44–46]. In mouse, Trim71 is present within the male germline, and let-7a promotes spermatogonia differentiation[47]. However, in fruit fly (*Drosophila melanogaster*), let-7 promotes germline differentiation through regulating gonadal-somatic cell behaviour[48]. The specific timing of let-7 abundance observed in zebrafish testis suggests a conserved function in promoting testis differentiation, although future studies will be needed to determine whether teleost let-7 functions primarily through the germline or gonadal somatic cells.

Notable miRNAs exhibiting a pattern of increasing abundance throughout the gonadal development included miR-100-5p, miR-126a-3p, and miR-143-3p (Fig. 5), all of which also have putative functions in promoting cell differentiation. In human, miR-100-5p promotes stem cell differentiation and impairs the self-renewing ability of breast cancer stem cells[49], while miR-126-3p was suggested to function in the differentiation and morphology of PGCs[39,50]. Several studies have shown mammalian miR-143-3p to function in suppressing cell proliferation[51,52], regulating cell apoptosis[53], and regulating differentiation of cultured adipocyte cells[54].

miR-92a-3p was the most dominant gonadal miRNA in our study (Fig. 4). While miR-92a-3p abundance at 12 wpf was overwhelming, our observations were similar to studies in the mature gonads of zebrafish and yellow catfish (*Pelteobagrus fulvidraco*)[24,27]. The miR-17/92 cluster is well conserved and has been linked to regulating the cell cycle, proliferation, and apoptosis[55]. In a previous study, we demonstrated that the knockdown of miR-92a-3p in zebrafish gonads resulted in significantly reduced miR-92a-3p expression in 1-cell stage embryos, which also had a significantly higher rate of embryonic developmental arrest[29]. This led us to hypothesize miR-92a-3p had a function as a maternal miRNA in zebrafish embryogenesis. Here, we found that an exceptionally large proportion of miR-92a-3p transcripts in unfertilized egg, and to a lesser extent spermatozoa, had templated 3′ nucleotide additions (Fig. 8c). Maternal miRNAs are highly adenylated in oocytes and early embryos of fruit fly, sea urchin (*Strongylocentrotus purpuratus*), and mouse[56], with the adenylation functioning to regulate miRNA stability[57–59]. Our findings further suggest miR-92a-3p is stored as a maternal transcript in unfertilized eggs.

At present, relatively little is known about how maternal miRNAs function in zebrafish embryos. The most studied embryonic miRNA in zebrafish is miR-430-3p, which is transcribed before the maternal to zygotic transition and functions as part of the zygotic decay pathway to destabilize maternal mRNAs[60]. However, zebrafish miR-430-3p only targets a relatively small number (~10%) of maternal mRNAs[60], and many maternal mRNAs are instead regulated through a maternal decay pathway[61,62]. Recently, studies of the maternal decay pathway in zebrafish identified the presence of uncommon codons, such a leucine, and short 3′UTRs as important

determinants in maternal mRNA stability[63]. In the future, it would be interesting to examine if there is a correlation between predicted targets of maternal miRNAs and these attributes.

Several miRNAs were consistently more abundant in testis than ovary, including miR-125a-5p, miR-125b-5p, miR-125c-5p, and miR-462-5p, whereas miR-22a-3p and miR-430b-3p were more abundant in the ovary (Fig. 6). Our findings were similar to deep sequencing results in mature zebrafish gonads, which also reported higher abundance of 125a-5p, miR-125b-5p, miR-125c-5p, and miR-462-5p in the testis, although that study did not find higher abundance of miR-22a-3p in the ovaries[27]. In mammals, miR-22 was shown to regulate estrogen receptor α [64], which acts as a transcription factor to regulate reproductive development[65]. Neither our study, nor the study by Vaz *et al.*[27] found miR-430b-3p to be abundant in mature ovaries. However, we did find miR-430b-3p to be among the most abundant miRNA in ovulated oocytes (Fig. 4c). A previous study on zebrafish ovarian development found miR-430b-3p to be differentially expressed in oocyte and follicular cells[66]. In follicular cells, miR-430b-3p expression was highest in early vitellogenic stages before being significantly reduced during late vitellogenesis and maturation, whereas its expression in oocytes was highest during late vitellogenesis and was still strongly expressed in mature oocytes. Among the predicted targets of miR-430b-3p are *inhibin beta Aa* (*inhbaa*), a subunit of Activin, *activin-A receptor type II-like* (*acvrl1*), as well as *smad3a* and *smad3b*[67]. Activin, which is essential to zebrafish follicle development and ovary maturation[68,69], is an extracellular signaling ligand which activates the intracellular Smad signaling pathway upon binding with its type-II receptor[70]. Luteinizing hormone, which up-regulates activin A production in ovarian follicles to induce maturation[71], also inhibits miR-430b-3p expression within the follicles[66], suggesting miR-430b-3p may act within follicle cells to suppress ovary maturation.

The testis-enriched miRNAs, found in our study, share similar putative functions as regulators of cell proliferation and apoptosis, including miR-125a-5p and miR-125b-5p[72–74]. Additionally, in mouse it was shown that Dicer, which is required for miRNA biogenesis, is essential for Sertoli cell survival and subsequent germ cell development[13]. Sertoli cells with a Dicer-knockout mutation lacked several miRNAs, including miR-125a-3p, while having an overabundance of SOD-1, a protein linked to apoptotic cell death[43]. In zebrafish embryos, miR-462–5p was also shown to regulate cell proliferation and apoptosis[75].

In the present study, we observed a shift in the dominant miR-202 arm from 3p to 5p between 12 and 24 wpf (Figs 3 and 4). Our findings are in agreement with other studies on teleost, which found miR-202-5p to be the dominant arm in the mature gonads of zebrafish[27] and tilapia[26], while miR-202-3p was the dominant arm in the immature gonads of Atlantic halibut[76]. Arm switching in miRNA has been documented in mammals, where the dominant arm of several miRNA varied between tissues and greatly increases the number of regulatory targets of a pre-miRNA[77]. For miR-202, the observed arm-switch may be related to different arm preference between gonadal-somatic and germline cells. In mouse, both 5p and 3p arms were expressed in embryonic gonadal-somatic cells but not cells of the germline[78]. However, in frog (*Xenopus tropicalis*), miR-202-5p was detected in oocytes during oogenesis[79].

Currently, the function of miR-202 in teleosts is unknown, and as such, it is difficult to explain the shift in arm preference between developmental stages. In chicken and mouse, miR-202 is enriched in testis and pri-miR-202 was suggested to be a direct transcriptional target of SOX9/SDF1 and involved in promoting testis differentiation[78,80,81]. However, in zebrafish, neither miR-202-5p nor miR-202-3p were found to be enriched in testis compared to ovary, a finding which is supported by another recent zebrafish study[27]. Similarly, in rainbow trout, both 5p and 3p arms were present in ovaries during late vitellogenesis[23], where target prediction suggested miR-202-5p targets several miRNAs essential to oogenesis, such as *transforming growth factor β receptor II* (*tgfbr2*)[23]. In mouse, *Tgfbr2* is required to maintain primordial follicle quiescence, and its suppression leads to the initiation of ovarian primordial follicle development[82]. In zebrafish ovaries, the follicle layer is formed by 9 wpf[83], which coincides with the significant increase in miR-202-5p abundance observed in our study. miR-202-3p in human has been shown to function in suppressing cell proliferation[84,85]. In the present study, we found miR-202-3p to be among the most abundant gonadal miRNA at 3 and 12 wpf (Fig. 4). High abundance at 3 wpf may suggest miR-202-3p functions in maintaining the state of the undifferentiated gonad, while high abundance at 12 wpf, when zebrafish gonads are reaching sexual maturity, may be related to the meiotic arrest of gametes.

Among the isomiRs detected in zebrafish gonads, 3′ substitutions of either uracil or adenine to guanine were frequently found at 6 and 9 wpf (Fig. 8a). Adenine-to-inosine RNA editing has previously been documented as a widespread post-transcriptional modification of miRNA precursors[86,87]. As inosine resembles guanosine, it would appear in sequencing as an A to G substitution and explain our observations. To our knowledge, no mechanisms for uracil to guanine or inosine substitutions have been described and false positives in RNA editing are a known bias of RNA sequencing[88]. We attempted to validate isomiRs using Sanger sequencing but it was unsuccessful (data not presented), likely due to amplification of the dominant variant over lesser represented isomiRs. Regardless, the frequency of uracil-to-guanine substitutions was striking, particularly in ovary at 9 wpf, where in the case of miR-26a-5p it exceeded 25% of the miRNAs' reads (Fig. 5). In mouse, isomiRs with guanine at the 3′ terminus preferentially localize to the nucleus of neurons[89]. The authors suggested the 3′ terminal guanine may have higher stability in the nucleus, or the 3′ guanine may mediate active transport to the nucleus through interaction with Argonaute proteins. It was also suggested that altering the sub-cellular localization of a miRNA could result in distinct functions at particular developmental stages.

Trimming variants at the 5′ and 3′ ends of miRNAs have been previously reported[90,91]. The 5′ end change can affect the seed sequence and alter the function of a miRNA[90]. In our study, we detected several miRNAs with either templated additions or truncations at the 5′ end, notably in the miR-10 family and in both miR-202-5p and 3p arms (Fig. 8b, Supplementary Dataset S6). While we were unable to validate these isomiRs, a 5′ trimming variant of miR-202-5p with a similar relative proportion of reads was also detected in the testis and ovary of marine medaka[34].

The zebrafish used in the experiment are the hybrid *See-Thru-Gonad* line[29]. To date, there have been no studies comparing the miRNA repertoire between zebrafish lines, and we therefore cannot rule out mutations or the

eGFP transgene affecting gonadal miRNA expression. However, a previous study which used the *nacre transparent* (−/−), zf45Tg, and TAB reference line found no morphological differences in gonadal development between them[92].

In summary, this study reports the miRNA repertoire in zebrafish gonads throughout the development from undifferentiated gonads to sexual maturation. We have demonstrated the dynamic changes in miRNA abundance at key stages of gonadal development. Our data show various trends in miRNA abundance, with several miRNAs consistently among the most abundant miRNAs in all gonadal stages, whereas others show specific time or sex dependent expression. The reported miRNA abundance patterns also often correlated to the putative miRNA functions. This is the first report on the dynamics of small RNA transcriptome in both testis and ovary in a teleost, and therefore provides the most complete overview of changes in miRNA abundance in gonads over time. The results from this study will serve as an important platform from which future studies can begin to validate the functional roles of gonadal miRNAs.

Methods

Fish. All experiments involving zebrafish were performed according to the Norwegian Regulation on Animal Experimentation (The Norwegian Animal Protection Act, No. 73 of 20 December 1974). All methods were approved by the National Animal Research Authority (Utvalg for forsøk med dyr, forsøksdyrutvalget, Norway) General License for Fish Maintenance and Breeding (Godkjenning av avdeling for forsøksdyr) no. 17.

The zebrafish used in the experiment were the *See-Thru-Gonad* line, previously established by crossing the *nacre transparent* (−/−)[31,32] and zf45Tg lines[30]. The resulting hybrid line is transparent with a fluorescently labeled germline throughout development[29].

Zebrafish were housed in a recirculating system (Pentair, Apopka FL, USA) and maintained according to standard procedures[93]. The system temperature was maintained at 28.5 ± 1.0 °C with a 12 h light and 12 h dark photoperiod. Adult zebrafish were housed in 10 L tanks at a density of 40 fish/tank with an approximate 1:1 sex ratio. When spawning, the fish were given a daily diet of newly hatched *Artemia* nauplii (Pentair) and SDS 400 zebrafish specific diet (Special Diet Services, Essex, United Kingdom). Juvenile zebrafish were produced using standard breeding techniques[93]. Post hatch, zebrafish larvae were kept in 3 L tanks with fine mesh baffles and restricted water flow. Feeding began at 2 days post hatch using SDS 100 zebrafish specific diet. From 2 to 4 weeks post fertilization (wpf) the juvenile fish were fed newly hatched *Artemia* nauplii and SDS 200 diet. After 4 wpf, the juveniles were fed SDS 300 diet alongside *Artemia* nauplii until the end of the experiment.

Sampling. Gonads from zebrafish were sampled at 5 time points: 3, 6, 9, 12, and 24 weeks post fertilization (Fig. 1). For each time point, five individuals of each sex were euthanized using 200 mg/L tricaine methanesulphonate (MS-222; Sigma Aldrich, Oslo, Norway) buffered with equal parts sodium bicarbonate, followed by partial decapitation once opercular movement had ceased. Zebrafish were submerged in a Petri dish of ice-cold nuclease-free phosphate-buffered saline (PBS; Sigma Aldrich) where they were measured for total length using an AxioZoom V.16 microscope (Carl Zeiss Microscopy GmbH, Göttingen, Germany) equipped with an AxioCam MRm monochrome camera (Zeiss) and Zen Pro imaging software. Images were taken using either bright field or epifluorescent light with an enhanced green fluorescent protein filter (excitation: 488 nm, emission: 509 nm). Epifluorescent light was used to identify the gonad, which was subsequently removed and examined to determine the sex. The gonads were transferred to RNA Later (Sigma Aldrich) and kept on ice until RNA extraction.

In addition to the whole gonads, sampling of spermatozoa and unfertilized oocytes was performed. For spermatozoa, 24 wpf adult male zebrafish were immobilized by immersion in chilled Hanks' balanced salt solution (Sigma Aldrich) following standard protocol for zebrafish cold-water immobilization[94]. The immobilized fish were patted dry with tissue paper before gently stroking the side of the fish. Sperm was collected from the genital pore using a 10 μL pipette with gentle suction. Spermatozoa from 15 fish was pooled in 200 μL ice-cold Hanks' balanced salt solution before proceeding. After pooling, the suspension was centrifuged at 2500 g for 10 min, resulting in a spermatozoa pellet. The supernatant was discarded and the pellet was re-suspended in 200 μL of chilled Hanks solution. A 10 μL sub-sample was checked for spermatozoa mobility and presence of non-sperm cell contaminants using the AxioZoom V.16 microscope. The remaining spermatozoa were pelleted a second time as previously described and the supernatant was discarded. The pellet was immediately taken for RNA extraction. Unfertilized eggs were collected from five females using a similar method, except the fish were first immobilized in chilled PBS. After the fish were patted dry, unfertilized eggs were collected into a plastic spoon and maintained as individual batches for each female separately. After collection, the unfertilized eggs were transferred to a Petri dish and washed three times in ice-cold PBS before being taken immediately for RNA extraction.

RNA extraction. Total RNA was extracted using QIAzol Lysis Reagent (Qiagen, Hilden, Germany) following manufacturer's instructions. Following ethanol precipitation, the extracted RNA was purified using the RNeasy MinElute Cleanup Kit (Qiagen). During column purification, genomic DNA was removed using a DNase I treatment (Qiagen). RNA integrity was assessed and quantified using the Agilent 2200 Tapestation instrument (Agilent, Santa Clara, USA) using High Sensitivity RNA screen tape (Agilent).

Small RNA library preparation, low-input RNA protocol optimization, and sequencing. Small RNA libraries were produced using TruSeq Small RNA Library Preparation kit (Illumina, San Diego, USA) following the manufacturer's protocol with modifications to down-scale input RNA. All whole gonad and unfertilized egg libraries were produced from individual fish, whereas the spermatozoa library input was pooled from 15 individuals. Because total RNA yields from immature zebrafish gonads are not sufficient for the recommended 1000 ng input, the following modifications were made: when using 100 or 25 ng total RNA inputs, both the 5′ and 3′ ligation adapters were diluted 2x before use, and the number of amplification cycles used was 15, compared to

13 when using 1000 ng total RNA input. Adapter-ligated constructs were selected with a size of 145 to 170 nt. To test the efficacy and reliability of low-input RNA library preparation, RNA from a 24 wpf ovary and testis were extracted and libraries were prepared using inputs of 1000 ng, 100 ng, and 25 ng.

Adult 24 wpf gonads were sequenced using the recommended minimum input of 1000 ng total RNA. Ovary libraries from 6 to 12 wpf were prepared using 100 ng of total RNA. For testis libraries, 9 and 12 wpf libraries were prepared using inputs of 100 ng total RNA. Inputs of 25 ng total RNA were used for the 3 wpf undifferentiated gonads and 6 wpf testis samples. For unfertilized eggs and spermatozoa libraries, inputs of 1000 and 100 ng total RNA were used, respectively. Sequencing was performed using the MiSeq Reagent Kit v3 (150 cycles with single reads; Illumina) on a MiSeq.

Data analysis. Adapter sequences and low quality (Phred quality score < 20) sequences were removed using cutadapt[95]. Trimmed good quality sequences were mapped to known zebrafish non-coding RNAs from Ensembl, GRCz10 (http://www.ensembl.org). miRDeep2[96] was used to identify mature zebrafish miRNAs with single mismatch using miRBase version 21 (http://www.mirbase.org). The expression levels of mature miRNAs and differential expression were assessed using DESeq2 algorithm[97]. To be considered differentially expressed, miRNAs from comparable developmental stages required a minimum of 100 DESeq2 normalized reads in one stage, a Log2 (Fold change) value $\geq |2|$, as well as an adjusted p-value ≤ 0.01. The normalization factor was estimated by taking the median of the ratio of a sample read count divided by the geometric mean across all samples[97].

Zebrafish 3′UTRs and miRNAs were downloaded from ensembl 87[98] and miRBase 21[99] respectively. miRNA targets were predicted using miRanda[100] with a minimum total alignment score of 155 and maximum free energy -20 kcal/mol. GO term enrichment analysis was performed for the 27 most abundant miRNAs (Fig. 4) using the empirical sampling approach[101].

References

1. Bartel, D. P. & Chen, C. Z. Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. *Nat. Rev. Genet.* **5,** doi: 10.1038/nrg1328 (2004).
2. Hwang, H. W. & Mendell, J. T. MicroRNAs in cell proliferation, cell death, and tumorigenesis. *Br. J. Cancer* **94,** 776–780, doi: 10.1038/sj.bjc.6603023 (2006).
3. Ivey, K. N. & Srivastava, D. MicroRNAs as regulators of differentiation and cell fate decisions. *Cell Stem Cell* **7,** 36–41, doi: 10.1016/j.stem.2010.06.012 (2010).
4. Staton, A. A., Knaut, H. & Giraldez, A. J. miRNA regulation of Sdf1 chemokine signaling provides genetic robustness to germ cell migration. *Nat. Genet.* **43,** 204–211, doi: 10.1038/ng.758 (2011).
5. Su, Z., Yang, Z., Xu, Y., Chen, Y. & Yu, Q. MicroRNAs in apoptosis, autophagy and necroptosis. *Oncotarget* **6,** 8474–8490 (2015).
6. Bizuayehu, T. T. & Babiak, I. MicroRNA in Teleost Fish. *Genome Biol. Evol.* **6,** 1911–1937, doi: 10.1093/gbe/evu151 (2014).
7. Volff, J. N. Genome evolution and biodiversity in teleost fish. *Heredity* **94,** 280–294 (2004).
8. Ha, M. & Kim, V. N. Regulation of microRNA biogenesis. *Nat. Rev. Mol. Cell. Biol.* **15,** 509–524, doi: 10.1038/nrm3838 (2014).
9. Kaneda, M., Tang, F., O'Carroll, D., Lao, K. & Surani, M. A. Essential role for Argonaute2 protein in mouse oogenesis. *Epigenetics Chromatin* **2,** 9, doi: 10.1186/1756-8935-2-9 (2009).
10. Murchison, E. P. *et al.* Critical roles for Dicer in the female germline. *Genes Dev.* **21,** 682–693, doi: 10.1101/gad.1521307 (2007).
11. Tang, F. *et al.* Maternal microRNAs are essential for mouse zygotic development. *Genes Dev.* **21,** 644–648, doi: 10.1101/gad.418707 (2007).
12. Nagaraja, A. K. *et al.* Deletion of dicer in somatic cells of the female reproductive tract causes sterility. *Mol. Endocrinol.* **22,** 2336–2352, doi: 10.1210/me.2008-0142 (2008).
13. Papaioannou, M. D. *et al.* Sertoli cell Dicer is essential for spermatogenesis in mice. *Dev. Biol.* **326,** 250–259, doi: 10.1016/j.ydbio.2008.11.011 (2009).
14. Hayashi, K. *et al.* MicroRNA biogenesis is required for mouse primordial germ cell development and spermatogenesis. *PLoS ONE* **3,** e1738, doi: 10.1371/journal.pone.0001738 (2008).
15. Suh, N. *et al.* MicroRNA function is globally suppressed in mouse oocytes and early embryos. *Curr. Biol.* **20,** 271–277, doi: 10.1016/j.cub.2009.12.044 (2010).
16. Wienholds, E., Koudijs, M. J., van Eeden, F. J. M., Cuppen, E. & Plasterk, R. H. A. The microRNA-producing enzyme Dicer1 is essential for zebrafish development. *Nat. Genet.* **35,** 217–218, doi: 10.1038/ng1251 (2003).
17. Giraldez, A. J. *et al.* MicroRNAs regulate brain morphogenesis in zebrafish. *Science* **308,** 833, doi: 10.1126/science.1109020 (2005).
18. Cifuentes, D. *et al.* A novel miRNA processing pathway independent of Dicer requires Argonaute2 catalytic activity. *Science* **328,** 1694–1698, doi: 10.1126/science.1190809 (2010).
19. Houwing, S., Berezikov, E. & Ketting, R. F. Zili is required for germ cell differentiation and meiosis in zebrafish. *EMBO J.* **27,** 2702–2711, doi: 10.1038/emboj.2008.204 (2008).
20. Houwing, S. *et al.* A role for Piwi and piRNAs in germ cell maintenance and transposon silencing in zebrafish. *Cell* **129,** 69–82, doi: 10.1016/j.cell.2007.03.026 (2007).
21. Bizuayehu, T. T. *et al.* Differential expression patterns of conserved miRNAs and isomiRs during Atlantic halibut development. *BMC Genomics* **13,** 1–14, doi: 10.1186/1471-2164-13-11 (2012).
22. Juanchich, A. *et al.* Characterization of an extensive rainbow trout miRNA transcriptome by next generation sequencing. *BMC Genomics* **17,** 164, doi: 10.1186/s12864-016-2505-9 (2016).
23. Juanchich, A., Le Cam, A., Montfort, J., Guiguen, Y. & Bobe, J. Identification of differentially expressed miRNAs and their potential targets during fish ovarian development. *Biol. Reprod.* **88,** 128, 121–111, doi: 10.1095/biolreprod.112.105361 (2013).
24. Jing, J. *et al.* Sex-biased miRNAs in gonad and their potential roles for testis development in yellow catfish. *PLoS ONE* **9,** e107946, doi: 10.1371/journal.pone.0107946 (2014).
25. Tao, W. *et al.* Integrated analysis of miRNA and mRNA expression profiles in tilapia gonads at an early stage of sex differentiation. *BMC Genomics* **17,** 1–13, doi: 10.1186/s12864-016-2636-z (2016).
26. Xiao, J. *et al.* Identification and characterization of microRNAs in ovary and testis of Nile tilapia (*Oreochromis niloticus*) by using solexa sequencing technology. *PLoS ONE* **9,** e86821, doi: 10.1371/journal.pone.0086821 (2014).
27. Vaz, C. *et al.* Deep sequencing of small RNA facilitates tissue and sex associated microRNA discovery in zebrafish. *BMC Genomics* **16,** 1–16, doi: 10.1186/s12864-015-2135-7 (2015).
28. Jia, K.-T. *et al.* Identification of miRNAs in zebrafish spermatozoa. *Zebrafish* **12,** 387–397, doi: 10.1089/zeb.2015.1115 (2015).
29. Presslauer, C., Bizuayehu, T. T., Razmi, K., Fernandes, J. M. O. & Babiak, I. *See-Thru-Gonad* zebrafish line: developmental and functional validation. *Reproduction* **152,** 507–517, doi: 10.1530/REP-16-0328 (2016).
30. Krøvel, A. V. & Olsen, L. C. Expression of a vas::EGFP transgene in primordial germ cells of the zebrafish. *Mech. Dev.* **116,** 141–150, doi: 10.1016/S0925-4773(02)00154-5 (2002).

31. Krauss, J., Astrinides, P., Frohnhöfer, H. G., Walderich, B. & Nüsslein-Volhard, C. *transparent*, a gene affecting stripe formation in Zebrafish, encodes the mitochondrial protein Mpv17 that is required for iridophore survival. *Biol. Open* (2013).
32. Lister, J. A., Robertson, C. P., Lepage, T., Johnson, S. L. & Raible, D. W. *nacre* encodes a zebrafish microphthalmia-related protein that regulates neural-crest-derived pigment cell fate. *Development* **126,** 3757–3767 (1999).
33. Gu, Y., Zhang, L. & Chen, X. Differential expression analysis of *Paralichthys olivaceus* microRNAs in adult ovary and testis by deep sequencing. *Gen. Comp. Endocrinol.* **204,** 181–184, doi: 10.1016/j.ygcen.2014.05.019 (2014).
34. Lau, K. *et al.* Identification and expression profiling of microRNAs in the brain, liver and gonads of marine medaka (*Oryzias melastigma*) and in response to hypoxia. *PLoS ONE* **9,** e110698, doi: 10.1371/journal.pone.0110698 (2014).
35. Siegfried, K. R. & Nüsslein-Volhard, C. Germ line control of female sex determination in zebrafish. *Dev. Biol.* **324,** 277–287, doi: 10.1016/j.ydbio.2008.09.025 (2008).
36. Tzung, K.-W. *et al.* Early depletion of primordial germ cells inzebrafish promotes testis formation. *Stem Cell Rep*. **4,** 61–73, doi: 10.1016/j.stemcr.2014.10.011 (2015).
37. Tehler, D., Høyland-Kroghsbo, N. M. & Lund, A. H. The miR-10 microRNA precursor family. *RNA Biol.* **8,** 728–734, doi: 10.4161/rna.8.5.16324 (2011).
38. Seifert, A., Werheid, D. F., Knapp, S. M. & Tobiasch, E. Role of Hox genes in stem cell differentiation. *World J. Stem Cells* **7,** 583–595, doi: 10.4252/wjsc.v7.i3.583 (2015).
39. Bhin, J. *et al.* PGC-enriched miRNAs control germ cell development. *Mol. Cells* **38,** 895–903, doi:10.14348/molcells.2015.0146 (2015).
40. Rengaraj, D., Lee, B. R., Lee, S. I., Seo, H. W. & Han, J. Y. Expression patterns and miRNA regulation of DNA methyltransferases in chicken primordial germ cells. *PLoS ONE* **6,** e19524, doi: 10.1371/journal.pone.0019524 (2011).
41. Yin, C. *et al.* Identification and expression of the target gene *emx2* of miR-26a and miR-26b in *Paralichthys olivaceus*. *Gene* **570,** 205–212, doi: 10.1016/j.gene.2015.06.030 (2015).
42. Tian-Zhong, M. *et al.* Critical role of Emx2 in the pluripotency – differentiation transition in male gonocytes via regulation of FGF9/NODAL pathway. *Reproduction* **151,** 673–681, doi: 10.1530/rep-16-0022 (2016).
43. Papaioannou, M. D. *et al.* Loss of dicer in Sertoli cells has a major impact on the testicular proteome of mice. *Mol. Cell. Proteomics* **10,** doi: 10.1074/mcp.M900587-MCP200 (2011).
44. Roush, S. & Slack, F. J. The let-7 family of microRNAs. *Trends Cell Biol.* **18,** 505–516, doi: 10.1016/j.tcb.2008.07.007 (2008).
45. Ecsedi, M. & Großhans, H. LIN-41/TRIM71: emancipation of a miRNA target. *Genes Dev.* **27,** 581–589, doi: 10.1101/gad.207266.112 (2013).
46. Lin, Y. *et al.* Human TRIM71 and its nematode homologue are targets of let-7 microRNA and its zebrafish orthologue is essential for development. *Mol. Biol. Evol.* **24,** 2525–2534 (2007).
47. Rybak, A. *et al.* The let-7 target gene mouse lin-41 is a stem cell specific E3 ubiquitin ligase for the miRNA pathway protein Ago2. *Nat. Cell Biol.* **11,** 1411–1420, doi: doi: 10.1038/ncb1987 (2009).
48. Fagegaltier, D. *et al.* A genome-wide survey of sexually dimorphic expression of drosophila miRNAs identifies the steroid hormone-induced miRNA let-7 as a regulator of sexual identity. *Genetics* **198,** 647–668 (2014).
49. Petrelli, A. *et al.* By promoting cell differentiation, miR-100 sensitizes basal-like breast cancer stem cells to hormonal therapy. *Oncotarget* **6,** 2315–2330 (2015).
50. Harris, T. A., Yamakuchi, M., Ferlito, M., Mendell, J. T. & Lowenstein, C. J. MicroRNA-126 regulates endothelial expression of vascular cell adhesion molecule 1. *Proc. Natl. Acad. Sci. USA* **105,** 1516–1521, doi: 10.1073/pnas.0707493105 (2008).
51. Gammell, P. MicroRNAs: recently discovered key regulators of proliferation and apoptosis in animal cells. *Cytotechnology* **53,** 55–63, doi: 10.1007/s10616-007-9049-4 (2007).
52. Sirotkin, A. V., Laukova, M., Ovcharenko, D., Brenaut, P. & Mlyncek, M. Identification of microRNAs controlling human ovarian cell proliferation and apoptosis. *J. Cell. Physiol.* **223,** 49–56, doi: 10.1002/jcp.21999 (2010).
53. Ugras, S. *et al.* Small RNA sequencing and functional characterization reveals MicroRNA-143 tumor suppressor activity in Liposarcoma. *Cancer Res.* **71,** 5659–5669, doi: 10.1158/0008-5472.can-11-0890 (2011).
54. Esau, C. *et al.* MicroRNA-143 regulates adipocyte differentiation. *J. Biol. Chem.* **279,** 52361–52365, doi: 10.1074/jbc.C400438200 (2004).
55. Mogilyansky, E. & Rigoutsos, I. The miR-17/92 cluster: a comprehensive update on its genomics, genetics, functions and increasingly important and numerous roles in health and disease. *Cell Death Differ*. **20,** 1603–1614, doi: 10.1038/cdd.2013.125 (2013).
56. Lee, M. *et al.* Adenylation of maternally inherited microRNAs by Wispy. *Mol. Cell* **56,** 696–707, doi: 10.1016/j.molcel.2014.10.011 (2014).
57. Burns, D., D'Ambrogio, A., Nottrott, S. & Richter, J. D. CPEB and two poly(A) polymerases control miR-122 stability and p53 mRNA translation. *Nature* **473,** 105–108, doi: 10.1038/nature09908 (2011).
58. D'Ambrogio, A., Gu, W., Udagawa, T., Mello, C. C. & Richter, J. D. Specific miRNA stabilization by Gld2-catalyzed monoadenylation. *Cell Rep*. **2,** 1537–1545, doi: 10.1016/j.celrep.2012.10.023 (2012).
59. Katoh, T. *et al.* Selective stabilization of mammalian microRNAs by 3′ adenylation mediated by the cytoplasmic poly(A) polymerase GLD-2. *Genes Dev.* **23,** 433–438, doi: 10.1101/gad.1761509 (2009).
60. Giraldez, A. J. *et al.* Zebrafish miR-430 promotes deadenylation and clearance of maternal mRNAs. *Science* **312,** 75–79, doi: 10.1126/science.1122689 (2006).
61. Mathavan, S. *et al.* Transcriptome analysis of zebrafish embryogenesis using microarrays. *PLoS Genet.* **1,** e29, doi: 10.1371/journal.pgen.0010029 (2005).
62. Tadros, W. & Lipshitz, H. D. The maternal-to-zygotic transition: a play in two acts. *Development* **136,** 3033–3042, doi: 10.1242/dev.033183 (2009).
63. Mishima, Y. & Tomari, Y. Codon usage and 3′ UTR length determine maternal mRNA stability in zebrafish. *Mol. Cell* **61,** 874–885, doi: 10.1016/j.molcel.2016.02.027.
64. Pandey, D. P. & Picard, D. miR-22 inhibits estrogen signaling by directly targeting the estrogen receptor α mRNA. *Mol. Cell. Biol.* **29,** 3783–3790, doi: 10.1128/mcb.01875-08 (2009).
65. Lee, H.-R., Kim, T.-H. & Choi, K.-C. Functions and physiological roles of two types of estrogen receptors, ERα and ERβ, identified by estrogen receptor knockout mouse. *Lab. Anim. Res.* **28,** 71–76 (2012).
66. Abramov, R., Fu, G., Zhang, Y. & Peng, C. Expression and regulation of miR-17a and miR-430b in zebrafish ovarian follicles. *Gen. Comp. Endocrinol.* **188,** 309–315, doi: 10.1016/j.ygcen.2013.02.012 (2013).
67. Vejnar, C. E. & Zdobnov, E. M. miRmap: Comprehensive prediction of microRNA target repression strength. *Nucleic Acids Res*. **40,** 11673–11683, doi: 10.1093/nar/gks901 (2012).
68. Tan, Q., Balofsky, A., Weisz, K. & Peng, C. Role of activin, transforming growth factor-β and bone morphogenetic protein 15 in regulating zebrafish oocyte maturation. *Comp. Biochem. Physiol., Part A: Mol. Integr. Physiol*. **153,** 18–23, doi: 10.1016/j.cbpa.2008.09.016 (2009).
69. Wang, Y. & Ge, W. Spatial expression patterns of activin and its signaling system in the zebrafish ovarian follicle: evidence for paracrine action of activin on the oocytes. *Biol. Reprod.* **69,** 1998–2006, doi: 10.1095/biolreprod.103.020826 (2003).
70. Pangas, S. A. & Woodruff, T. K. Activin signal transduction pathways. *Trends Endocrinol. Metab.* **11,** 309–314, doi: 10.1016/S1043-2760(00)00294-0 (2000).

71. Pang, Y. & Ge, W. Gonadotropin regulation of activin βA and activin type IIA receptor expression in the ovarian follicle cells of the zebrafish, Danio rerio. *Mol. Cell. Endocrinol.* **188,** 195–205, doi: 10.1016/S0303-7207(01)00719-5 (2002).
72. Haemmig, S. *et al.* miR-125b controls apoptosis and temozolomide resistance by targeting TNFAIP3 and NKIRAS2 in glioblastomas. *Cell Death Dis.* **5,** e1279, doi: 10.1038/cddis.2014.245 (2014).
73. Lee, Y. S., Kim, H. K., Chung, S., Kim, K.-S. & Dutta, A. Depletion of human micro-RNA miR-125b reveals that it is critical for the proliferation of differentiated cells but not for the down-regulation of putative targets during differentiation. *J. Biol. Chem.* **280,** 16635–16641, doi: 10.1074/jbc.M412247200 (2005).
74. Tiwari, A., Shivananda, S., Gopinath, K. S. & Kumar, A. microRNA-125a reduces proliferation and invasion of oral squamous cell carcinoma cells by targeting estrogen-related receptor alpha: implications for cancer therapeutics. *J. Biol. Chem.* doi: 10.1074/jbc.M114.584136 (2014).
75. Huang, C.-X. *et al.* The zebrafish miR-462/miR-731 cluster is induced under hypoxic stress via hypoxia-inducible factor 1α and functions in cellular adaptations. *FASEB J.* **29,** 4901–4913, doi: 10.1096/fj.14-267104 (2015).
76. Bizuayehu, T. T. *et al.* Sex-Biased miRNA expression in Atlantic halibut (*Hippoglossus hippoglossus*) brain and gonads. *Sex. Dev.* **6,** 257–266 (2012).
77. Ro, S., Park, C., Young, D., Sanders, K. M. & Yan, W. Tissue-dependent paired expression of miRNAs. *Nucleic Acids Res.* **35,** 5944–5953, doi: 10.1093/nar/gkm641 (2007).
78. Wainwright, E. N. *et al.* SOX9 regulates microRNA miR-202-5p/3p expression during mouse testis differentiation. *Biol. Reprod.* **89,** 34, 31–12, doi: 10.1095/biolreprod.113.110155 (2013).
79. Armisen, J., Gilchrist, M. J., Wilczynska, A., Standart, N. & Miska, E. A. Abundant and dynamically expressed miRNAs, piRNAs, and other small RNAs in the vertebrate Xenopus tropicalis. *Genome Res.* **19,** 1766–1775, doi: 10.1101/gr.093054.109 (2009).
80. Bannister, S. C. *et al.* Manipulation of estrogen synthesis alters miR202* expression in embryonic chicken gonads. *Biol. Reprod.* **85,** 22–30, doi: 10.1095/biolreprod.110.088476 (2011).
81. Bannister, S. C., Tizard, M. L. V., Doran, T. J., Sinclair, A. H. & Smith, C. A. Sexually dimorphic microRNA expression during chicken embryonic gonadal development. *Biol. Reprod.* **81,** 165–176, doi: 10.1095/biolreprod.108.074005 (2009).
82. Yang, S. *et al.* Expression patterns and regulatory functions of microRNAs during the initiation of primordial follicle development in the neonatal mouse ovary. *Biol. Reprod.* **89,** 126, 121–111, doi: 10.1095/biolreprod.113.107730 (2013).
83. Selman, K., Wallace, R. A., Sarka, A. & Qi, X. Stages of oocyte development in the zebrafish, Brachydanio rerio. *J. Morphol.* **218,** 203–224, doi: 10.1002/jmor.1052180209 (1993).
84. Wang, Q. *et al.* MicroRNA-202-3p inhibits cell Proliferation by targeting ADP-Ribosylation factor-like 5A in human colorectal carcinoma. *Clin. Cancer Res.* **20,** 1146–1157, doi: 10.1158/1078-0432.ccr-13-1023 (2014).
85. Zhang, Y. *et al.* miR-202 suppresses cell proliferation in human hepatocellular carcinoma by downregulating LRP6 post-transcriptionally. *FEBS Lett.* **588,** 1913–1920, doi: 10.1016/j.febslet.2014.03.030 (2014).
86. Kawahara, Y. *et al.* Frequency and fate of microRNA editing in human brain. *Nucleic Acids Res.* **36,** 5270–5280, doi: 10.1093/nar/gkn479 (2008).
87. Kawahara, Y. *et al.* Redirection of silencing targets by adenosine-to-inosine editing of miRNAs. *Science* **315,** 1137–1140 (2007).
88. Peng, Z. *et al.* Comprehensive analysis of RNA-Seq data reveals extensive RNA editing in a human transcriptome. *Nat. Biotech.* **30,** 253–260, doi: 10.1038/nbt.2122 (2012).
89. Khudayberdiev, S. A., Zampa, F., Rajman, M. & Schratt, G. A comprehensive characterization of the nuclear microRNA repertoire of post-mitotic neurons. *Front. Mol. Neurosci.* **6,** 43, doi: 10.3389/fnmol.2013.00043 (2013).
90. Llorens, F. *et al.* A highly expressed miR-101 isomiR is a functional silencing small RNA. *BMC Genom.* **14,** 1–14, doi: 10.1186/1471-2164-14-104 (2013).
91. Tan, G. C. *et al.* 5′ isomiR variation is of functional and evolutionary importance. *Nucleic Acids Res.* **42,** 9424–9435, doi: 10.1093/nar/gku656 (2014).
92. Presslauer, C. *et al.* Induced autoimmunity against gonadal proteins affects gonadal development in juvenile zebrafish. *PLoS ONE* **9,** e114209, doi: 10.1371/journal.pone.0114209 (2014).
93. Westerfield, M. *The zebrafish book. A guide for the laboratory use of zebrafish* (*Danio rerio*). 4th edn, (University of Oregon Press, 2000).
94. Kinkel, M. D., Eames, S. C., Philipson, L. H. & Prince, V. E. Intraperitoneal injection into adult zebrafish. *JoVE.* **e2126,** doi: 10.3791/2126 (2010).
95. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet* **17,** 10–12, doi: 10.14806/ej.17.1.200 (2011).
96. Friedlander, M. R., Mackowiak, S. D., Li, N., Chen, W. & Rajewsky, N. miRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. *Nucleic Acids Res.* **40,** doi: 10.1093/nar/gkr688 (2012).
97. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15,** 550, doi: 10.1186/s13059-014-0550-8 (2014).
98. Kinsella, R. J. *et al.* Ensembl BioMarts: a hub for data retrieval across taxonomic space. *Database* **2011,** bar030, doi: 10.1093/database/bar030 (2011).
99. Kozomara, A. & Griffiths-Jones, S. miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res.* **42,** D68–D73, doi: 10.1093/nar/gkt1181 (2014).
100. Miranda, K. C. *et al.* A pattern-based method for the identification of microRNA binding sites and their corresponding heteroduplexes. *Cell* **126,** 1203–1217, doi: 10.1016/j.cell.2006.07.031 (2006).
101. Bleazard, T., Lamb, J. A. & Griffiths-Jones, S. Bias in microRNA functional enrichment analysis. *Bioinformatics* **31,** 1592–1598, doi: 10.1093/bioinformatics/btv023 (2015).

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Author Contributions

I.B. and J.F. conceived the experiment. C.P. and M.K. conducted the sequencing, and C.P. performed sampling and imaging. T.B., C.P. and I.B. analysed the data. C.P. and I.B. drafted the manuscript. All authors reviewed the manuscript.

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Paper III

Spatial expression patterns of gonadal miRNAs and function of maternal miR-92a-3p in zebrafish

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Abstract

Small RNA sequencing experiments in zebrafish have identified abundant miRNAs in gonads throughout development. However, their biological functions remain largely unknown. In the present study, we first determined the spatial expression patterns of let-7a-5p, miR-202-5p, miR-202-3p, miR-430b-3p, miR-462-5p, and miR-92a-3p in 10 week old zebrafish gonads. Based on previous studies showing miR-92a-3p as a highly abundant miRNA during gonadal development, and with possible functions as a maternal miRNA, we selected it for functional analysis. After performing *in silico* target prediction, we identified *wee2* as a potential target of maternal miR-92a-3p during the early stages of zebrafish embryogenesis. Co-expression of both miR-92a-3p and *wee2* during embryo cleavage was confirmed using *in situ* hybridization. Next, we performed several target validation assays, as well as targeted knockdown of miR-92a-3p in zebrafish embryos. Our results suggest miR-92a-3p is maternally expressed, and capable of interacting with the predicted *wee2* 3'UTR binding site. In addition, the predicted binding site was strongly suppressed by endogenous miRNAs. Knockdown of miR-92a-3p resulted in delayed embryonic development over the first 24 hours, providing further evidence for its predicted role in regulating the cell cycle.

Keywords: embryonic development, microRNA, miR-92a-3p, zebrafish, *wee2*

Introduction

MicroRNAs (miRNAs) belong to a family of small non-coding RNAs with important roles as post-transcriptional repressors of protein-coding genes (Bartel, 2009). After transcription, miRNAs are processed into a small RNA duplex, which is loaded into a member of the Argonaute (Ago) protein family (Ha and Kim, 2014, Li and Rana, 2014). Ago binds the miRNA guide strand and releases the passenger strand (Ha and Kim, 2014, Li and Rana, 2014). Together, they form essential components of the miRNA-induced silence complex (miRISC), which targets messenger RNAs (mRNA) for suppression. The mature miRNA guide strand is approximately 22 nucleotides long, and acts as the target recognition component for the miRISC complex mainly based on base-pair complementarity between nucleotides 2-8 of the miRNA (the seed region) and the 3' untranslated region (3' UTR) of targeted mRNA (Ha and Kim, 2014). Base pair interaction causes the Ago protein to induce translation repression, mRNA deadenylation, or mRNA decay (Ha and Kim, 2014, Huntzinger and Izaurralde, 2011).

Currently, miRNA target prediction is based primarily on computational algorithms which perform alignments between miRNA seed sequences and complementary regions of mRNA 3'UTRs (Friedman et al., 2009, John et al., 2004, Krek et al., 2005). However, these methods often generate many false positive predictions (Ekimler and Sahin, 2014) and often do not take into account whether miRNA and their predicted targets are co-expressed (Ritchie et al., 2009). It is therefore important that miRNA/mRNA target interactions are verified experimentally.

The function of miRNAs during teleost gonad development is still poorly understood (Bizuayehu and Babiak, 2014). Several studies have profiled miRNA spatial and temporal expression patterns in teleost gonads (Bizuayehu et al., 2012, Jia et al., 2015, Jing et al., 2014, Juanchich et al., 2013, Tao et al., 2016, Vaz et al., 2015, Xiao et al., 2014); but no study has functionally validated a miRNA essential to teleost gonadal development. Previously, we described the miRNA repertoire in zebrafish gonads, which identified several miRNA with notable trends in abundance (Presslauer et al., 2017). Both let-7a-5p and miR-462-5p were significantly more abundant in testis than ovary at 6 and 9 weeks post fertilization, respectively, whereas miR-430b-3p was only detected within ovaries throughout gonadal development (Presslauer et al., 2017). In addition, an arm-switch was detected for miR-202; the 3p arm was more abundant in gonads during early development, whereas the 5p arm was more abundant after sexual maturation (Presslauer et al., 2017). Determining whether these miRNAs are predominantly germline or gonadal-somatic expressed would assist in predicting their biological functions.

miR-92a-3p is among the most abundant miRNAs in zebrafish gonads (Presslauer et al., 2017, Vaz et al., 2015). Notably, miR-92a-3p is also abundant in unfertilized eggs, where we previously reported the majority of reads were trimming variants, with one to three additional templated adenine residues, when compared to the dominant isomiR in whole gonads (Presslauer et al., 2017). Adenylation of maternal miRNAs in oocytes has been reported in fruit fly (*Drosophila melanogaster*), sea urchin (*Stronglyocentrotus purpuratus*), and mouse (*Mus musculus*) (Lee et al., 2014), suggesting miR-92a-3p may also be stored in zebrafish oocytes with a maternal function in embryos. In addition, targeted knockdown of miR-92a-3p in mature zebrafish ovaries resulted in a significant reduction of miR-92a-3p in 1-cell embryos, as well as a significant increase in the proportion of embryos with arrested development at the 1-cell stage (Presslauer et al., 2016). We hypothesized that zebrafish miR-92a-3p has a function as a maternal factor essential for early development of embryos.

In the present study, we aim to determine the spatial expression patterns of several miRNAs abundant in zebrafish gonads: let-7a-5p, miR-202-5p, miR-202-3p, miR-430b-3p, miR-462-5p, and miR-92a-3p. In addition, we identify a predicted target for miR-92a-3p with possible maternal functions. We demonstrate co-expression of both the miRNA and its target, then perform target validation assays using zebrafish embryos. Confirming miRNA/target interactions will assist in identifying essential roles for maternal miRNAs.

Methods

Fish

The zebrafish used in the experiment were from the inbred AB line, which was originally obtained from The Norwegian Zebrafish Platform (zebrafish.no), Norwegian University of Life Sciences (Oslo, Norway), before becoming established at Nord University (Bodø, Norway). The zebrafish were housed in an Aquatic Habitats recirculating system (Pentair, Apopka FL, USA) following standard zebrafish husbandry procedures (Westerfield, 2000). Zebrafish nutrition consisted of a daily mix of newly hatched *Artemia* nauplii (Pentair) and SDS zebrafish specific diet (Special Diet Services, Essex, United Kingdom) following the manufacturers recommended feeding regime.

All experimental procedures described in the present study were performed in accordance with the Norwegian Regulation on Animal Experimentation (The Norwegian Animal Protection Act, No. 73 of 20 December 1974) and were approved by the National Animal Research Authority (Utvalg for forsøk med dyr, forsøksdyrutvalget, Norway) General License for Fish Maintenance and Breeding (Godkjenning av avdeling for forsøksdyr) no. 17.

Fish Sampling

Total RNA was extracted from the ovaries of three mature zebrafish. The fish were first euthanized with 200 mg/L of MS-222 Tricaine; Sigma Aldrich, Oslo, Norway) buffered with equal parts sodium bicarbonate ($NaHCO_3$; Sigma Aldrich). After opercular movement had ceased, the fish were decapitated and the gonads were immediately removed, and total RNA was extracted using QIAzol Lysis Reagent (Qiagen, Hilden, Germany) following the manufacturer's instructions. RNA integrity was assessed using 1% (w/v) agarose gel electrophoresis and was quantified using a NanoDrop ND-1000 (Thermo Fisher Scientific, Saven & Werner AS, Kristiansand, Norway). cDNA synthesis was performed using the QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's protocol.

Ten week old zebrafish were sampled for *in situ* hybridization (ISH). The fish were first euthanized as described for RNA extraction before opening the abdominal cavity and placing the fish directly into pre-chilled Bouin's solution (4 °C; Sigma Aldrich). The samples were fixated overnight at 4°C before being dehydrated in a gradient series of ethanol washes (from 25 to 100 %) and embedded in paraffin wax. The samples were systematically sectioned (6 µm thickness) using a rotary microtome (Microm HM355S,

MICROM International GmbH, Germany) and were mounted on Polysine® slides (Thermo Fisher Scientific). Slides were stored at 4 °C for a maximum of one week before use.

Zebrafish embryos at the 1-cell, 2-cell, 256-cell, and 25-somite stages were collected after allowing natural spawning and fixed for whole mount *in situ* hybridization (WISH). The embryos were placed directly into pre-chilled 4% w/v paraformaldehyde (PFA; pH 7.0) and fixated overnight at 4 °C. The following day the embryos were repeatedly washed with a chilled solution of 0.1 % Tween-20 in phosphate buffered saline solution (PBST) before immediately proceeding with the WISH procedure.

in situ hybridization of zebrafish gonads

Locked nucleic acid (LNA) oligonucleotides antisense for the mature forms of let-7a, miR-202-5p, miR-202-3p, miR-430b-3p, miR-462-5p, and miR-92a-3p were designed and produced by Exiqon A/S (Vedbaek, Denmark; Table 1). The LNA probes were double digoxigenin labeled at the 5' and 3' ends.

The ISH procedure was performed in accordance to the miRCURY LNA™ microRNA ISH Optimization Kit (Exiqon) with some modifications. Melting of paraffin was performed for 1 h at 60 °C the day prior to the ISH experiment. Section permeability was improved using 12 µg/mL Proteinase-K (Roche) for 10 min at room temperature. LNA probe hybridization was performed with a probe concentration of 40 nM in microRNA ISH buffer (Exiqon). For each probe, hybridization occurred at 30 °C below the RNA Tm °C (Table 1) for 1 h.

miR-92a-3p target prediction

Targets were predicted for zebrafish miR-92a-3p (miRBase accession: MIMAT0001808) using both TargetScanFish (release 6.2; http://www.targetscan.org/fish_62/) and miRmap (http://mirmap.ezlab.org/) (Agarwal et al., 2015, Vejnar and Zdobnov, 2012). Potential targets were filtered using the KEGG pathway database to select those involved in the zebrafish cell cycle pathway (http://www.genome.jp/kegg/pathway.html).

Whole mount _in situ_ hybridization of zebrafish embryos

A cRNA probe for zebrafish *wee1 homolog 2* (*wee2;* Accession: NM_001037222) was developed by first amplifying a partial cDNA sequence which encompassed the majority of the 3' untranslated region (3' UTR). The primers used were:

TGGATTTCCTGGATACTAACAC and AGTTAAAACGCACCAATCGT. The fragment was amplified from the previously described ovarian cDNA using 30 PCR cycles with an annealing temperature of 53 °C and an extension time of 30 s. After PCR, a fragment of the expected 233 nucleotides was excised from a 1 % w/v agarose gel and purified using the QIAquick gel extraction kit according to the manufacturer's protocol (Qiagen). The cDNA fragment was inserted into the pCR4-TOPO® vector and transformed into OneShot® chemically competent *E. coli* cells. After propagation, the vector was purified using the QIAprep® Miniprep kit (Qiagen) and sequenced in both directions using M13 primers with the Big Dye® Terminator 3.1 (Applied Biosystems) sequencing template preparation method. The sequencing reactions were analyzed at the DNA Sekvenseringslab, University of Tromsø, Norway. The confirmed zebrafish *wee2* cDNA fragment was amplified from the pCR4-TOPO® vector into linear fragments using the M13 primers. The extracted PCR product was then used as a template for digoxigenin labeled cRNA probe transcription with T3 (anti-sense) and T7 (sense) RNA polymerase (Roche, Mannheim, Germany). The cRNA probes were purified using LiCl/ethanol precipitation and re-suspended in nuclease-free water before storage at -80 °C.

WISH for miR-92a-3p and *wee2* was performed essentially as previously described (Hall et al., 2003). Embryo permeability was improved using 2.5 µg/mL Proteinase-K (Roche) for 10 min at room temperature. miR-92a-3p probe hybridization was performed with a probe concentration of 40 nM for 1 h at 57 °C, whereas *wee2* probe hybridization was performed with a concentration of 1.0 µg/µL for 48 h at 70 °C. Bound probe was conjugated to alkaline-phosphatase labelled anti-DIG antibody (Roche) overnight at a 1:1000 dilution. The colour reaction was performed at room temperature for 1 and 10 h for miR-92a-3p and *wee2* respectively.

miR-92a-3p target validation

Zebrafish miR-92a-3p target validation was performed through a series of microinjection experiments. First, the predicted target *wee2* 3'UTR fragment previously amplified for ISH was excised from the pCR4-TOPO® vector using EcoRI digestion (Thermo Fisher Scientific). After gel purification, the fragment was ligated into a pGEM-T-Easy vector (Promega) containing the *hrgfp* coding region (Yoshizaki et al., 2005). To ensure proper insert orientation, several colonies were propagated and sequenced as previously described. Plasmids containing both the sense and antisense *wee2* 3'UTR were linearized using NdeI (Thermo Fisher Scientific) and *in vitro* transcription was performed using the Message Machine T7 Kit (Thermo Fisher Scientific). The transcribed RNAs were purified using LiCl precipitation and re-suspended in 20 µL nuclease-free water before being aliquoted and stored at -80 °C. The NanoDrop ND-1000 calculated

the stock concentration of *gfp-wee2* and *gfp-wee2-antisense* at 3200 ng/µL and 1000 ng/µL, respectively.

To determine the optimal concentration of chimeric RNA for injection, to obtain strong GFP signal, the *gfp-wee2* and *gfp-wee2-antisense* chimeric mRNA were diluted in freshly made and chilled embryo medium (Westerfield, 2000) before injecting into naturally spawned 1-cell zebrafish embryos approximately 30 to 60 min post fertilization. *gfp-wee2* injection concentrations were 3200, 1600, 800, and 200 ng/µL compared to 1000 and 200 ng/µL for the *gfp-wee2-antisense* control. For each injection, a total of 1 nL of each treatment was injected into the yolk directly below the blastodisc using an IM-300 microinjector (Narishige, London, UK). After injection, embryos were transferred to a fresh solution of embryo medium for incubation at 28.5 °C. All observations were performed using an AxioZoom V.16 microscope (Carl Zeiss, Göttingen, Germany) and Zen Pro (2012; Carl Zeiss) imaging software. Embryo staging was performed according to established literature (Kimmel et al., 1995).

After optimization of *gfp-wee2* injections, the chimeric RNA was co-injected with a miR-92a-3p target site blocker to observe effects on the GFP signal presence and intensity. The miR-92a-3p target site blocker assays were performed using a miRCURY LNA Power Target Site Blocker (TSB) specific for the miR-92a-3p binding site within the zebrafish *wee2* 3'UTR. The TSB (5'-ATTATTGCACCCAGTGCC-3'; the miR-92a-3p target site is underlined) was designed and produced by Exiqon A/S, with an additional scrambled TSB to act as a negative control (5'-TAACACGTGTATACGCCCA-3'). Upon receipt, the lyophilized TSBs were re-suspended in nuclease free water at a stock concentration of 50 µM and aliquoted for further use. The TSB assays were optimized for a 1 nL injection volume consisting of 320 ng/µL *gfp-wee2* and 1.0 µM TSB in freshly prepared embryo medium. Microinjections and general observations were made as previously described. In addition, the GFP mean intensity was measured using the ZEN Measurement Module (Zeiss). For each measurement, contours were traced to encompass the head, brain, and eye region of the embryo.

The effect of miR-92a-3p mimic on *gfp-wee2* expression was also measured. For this trial, a *mir*Vana® miRNA mimic for zebrafish miR-92a-3p was designed and produced by Thermo Fisher Scientific. The lyophilized mimic was re-suspended in nuclease-free water to a stock concentration of 100 µM and aliquoted for further use. The effect of miR-92a-3p mimic on *gfp-wee2* expression was observed by a series of 1.0 nL injections consisting of 5.0 µM mimic and 800, 1600, or 3200 ng/µL *gfp-wee2*. Observations and measurements were made as previously described.

miR-92a-3p knockdown

miR-92a-3p knockdown was performed using a vivo-morpholino (VMO) complementary to the dre-miR-92a-3p guide strand (5'-TACAGGCCGGGACAAGTGCAATACC-3') designed and produced by Genetools LLC (Philomath, OR, USA) with an additional 5-base mismatch VMO (5'-TAGACGCCGCGACAACTCCAATACC-3'; underlined letters indicate the mismatches) as a negative control. Microinjections and embryo staging were performed as previously described. Treatments consisted of 5.0 µM VMO and were rescued with an equal concentration of miR-92a-3p mimic. Embryos were examined for phenotypes at 1 and 2 days post fertilization.

Statistical analyses

GFP mean intensity values were tested for equal variance using an F-test before evaluating the effect of treatment using a two-tailed T-test. Percentage data (frequency of GFP expressing embryos and frequency of embryos with delayed development) were arcsin square root transformed. An evaluation of treatment on the percentage of GFP expressing embryos was tested for equal variance before using a pairwise two-tailed T-test. Evaluation of the miR-92a-3p VMO, a mismatch control VMO, miR-92a-3p mimic, and rescue treatments were performed using ANOVA. Multiple comparison analysis was performed using Tukey-Kramer test. Effects were considered significant at *p*-value of 0.05 or less (Zar, 1999).

Results

in situ hybridization of miRNAs expressed in zebrafish gonads

The 10 week old zebrafish testis predominantly consisted of spermatogonia, spermatocytes, and spermatids (Fig. 1 A). ISH for let-7a-5p resulted in weak signal within the spermatogonia, spermatocytes, and some clusters of spermatids, while stronger signal was observed in cells at the margin of spermatocyte clusters; these were presumably gonadal somatic Sertoli and Leydig cells. The miR-202-5p and miR-202-3p arms had contrasting spatial expression patterns. miR-202-5p was strongly expressed in cells of the germline, with strong signal visible in spermatogonia, spermatocytes, and spermatids. It was not conclusive if gonadal somatic cells expressed miR-202-5p or not. However, regions at the periphery of spermatocyte clusters had noticeably reduced signal, suggesting miR-202-5p may not be expressed in gonadal somatic cells. By comparison, miR-202-3p transcripts had an expression pattern typical of gonadal somatic cells. No signal was detected in any cells of the germline, while strong signal was observed in cells around the periphery of germline clusters. Both miR-462-5p and miR-92a-3p were expressed in both germline and gonadal somatic cell.

Zebrafish ovaries consisted primarily of stage Ib and II oocytes, with some stage Ia located at the periphery (Fig. 1 B). Similar to testis, ISH of miR-202-5p and miR-202-3p resulted in contrasting expression patterns between the arms, with miR-202-5p localized exclusively to cells of the germline (stage Ib and II oocytes), while miR-202-3p was only detected in the follicle cells surrounding the oocytes. Strong signal from miR-430b-3p transcripts were detected in stage Ia and Ib oocytes, while comparatively weak signal was detected in stage II oocytes. Notably, miR-430b-3p signal was exclusively detected within the nucleus. This was in contrast to miR-202-5p, miR-462-5p, and miR-92a-3p, which were predominantly expressed within the oocyte cytoplasm with comparatively weaker signal detected within the nucleus. miR-462-5p transcripts were detected within stage Ia, Ib, and II oocytes, but not in somatic cells, which was in contrast to testis. Similar to testis, miR-92a-3p was found in both cells of germline and somatic origins.

miR-92a-3p target prediction

Prediction of miR-92a-3p targets identified 25 protein families within the zebrafish cell cycle pathway with possible target transcripts (Supplementary Dataset S1). Notably, multiple components involved in DNA biosynthesis, such as the mini-chromosome-maintenance complex and origin recognition complex contained predicted targets. In addition, multiple predicted targets are involved in the cyclin dependent kinase (Cdk1)

pathway at the G2 / M checkpoint (Fig. 2). From the predicted Cdk1 pathway targets, *wee1 homolog 2* (*wee2*) was selected for target validation assays.

Whole mount *in situ* hybridization of zebrafish embryos

Mature miR-92a-3p transcripts were strongly expressed in zebrafish embryos at all stages examined (Fig. 3 A). At the 1-, 2-, and 256-cell stages, the transcripts were detected ubiquitously within the cells. At the 25 somite stage, miR-92a-3p transcripts were predominantly localized to the eyes and brain, but signal was detected throughout the body. Expression within the somites was comparatively weaker than the brain but consistent. Staining was noticeably reduced in the notochord.

Transcripts of *wee2* were strongly expressed during early cleavage but the signal appeared weaker at the blastula stage (Fig. 3 B). At the 25-somite stage, *wee2* transcripts were detected only in the brain and ventral portion of the somites.

Chimeric mRNA injections

The translation efficiency of chimeric *gfp*-3'UTR mRNA into GFP protein varied between the 3'UTR sequences (Fig. 4). Injections of 200 pg or 800 pg *gfp-wee2* resulted in no visible GFP signal (Fig. 4 A), similar to non-injected control embryos (Fig. 4 B). Injections of 1600 pg mRNA produced slightly elevated GFP signal within the yolk, while GFP signal within the body was barely detectable. An increased concentration of 3200 pg *gfp-wee2* resulted in consistent and strong expression of GFP throughout the embryo (Fig. 4 A). Notably, throughout the experiment, embryos with severe deformities produced intense GFP signal from reduced mRNA concentrations (Fig. 4 C). By comparison, embryos which received injections of chimeric mRNA containing the antisense sequence for the zebrafish *wee2* 3'UTR had a strong and ubiquitous GFP signal, which was easily visible from concentrations of 200 pg mRNA onwards, and highly intense at 1000 pg (Fig. 4 D).

miR-92a-3p target site blocker and *gfp-wee2* co-injections

Groups of embryos treated with *gfp-wee2* and either a scrambled TSB control or a miR-92a-3p TSB had significantly different proportions of GFP expressing embryos, as well as significantly different relative intensities of GFP signal (Fig. 5). While both treatments produced GFP signal in 25-somite embryos, the signal generally appeared more intense in those which received the miR-92a-3p TSB (Fig. 5 A). Across three trials, a significantly higher proportion of GFP expressing embryos were observed in groups which received the miR-92a-3p TSB, compared to those receiving the scrambled TSB

(Fig. 5 B). Among the GFP expressing embryos, mean GFP intensity was measured in the head and brain region, based on the strong expression of miR-92a-3p in this region (Fig. 3 A). Significantly increased GFP signal was detected in embryos which received the miR-92a-3p TSB, compared to the control TSB treatment group (Fig. 5 C). In addition, GFP mean intensity for the control TSB treatment group was consistently low, with barely detectable GFP values. By comparison, GFP mean intensities for the miR-92a-3p TSB group were highly variable.

miR-92a-3p mimic and GFP-wee2 co-injections

Embryos injected with *gfp-wee2* mRNA and miR-92a-3p mimic produced variable proportions of GFP expressing embryos depending on *gfp-wee2* concentration (Fig. 6). Generally, embryos treated with co-injections of *gfp-wee2* mRNA and miR-92a-3p mimic expressed noticeably less GFP than those receiving *gfp-wee2* mRNA only, although it was highly variable and often overlapping. A comparison of miR-92a-3p mimic injections with variable *gfp-wee2* mRNA concentrations resulted in no difference in the number of GFP expressing embryos at 3200 pg mRNA (Fig. 6 B). However, only 8 % of embryos receiving 1600 pg *gfp-wee2* mRNA and 5.0 µM miR-92a-3p mimic were GFP expressing compared to 76 % in embryos receiving *gfp-wee2* mRNA only (Fig. 5 B). Injections of 800 pg *gfp-wee2* mRNA resulted in few GFP-expressing embryos regardless of the presence of miR-92a-3p mimic. GFP signal intensities in the head region of embryos receiving 3200 pg *gfp-wee2* mRNA were highly variable between the treatment groups (Fig. 6 C).

miR-92a-3p knockdown and rescue

Injections of miR-92a-3p VMO frequently resulted in delayed embryonic development (Fig. 7 A). By 1 day post fertilization, embryos from the control VMO, miR-92a-3p mimic, and rescue treatment groups typically reached the 25-somite stage, whereas embryos receiving the miR-92a-3p VMO often displayed retarded development, with some embryos only reaching the 1-somite stage. Follow-up observations on the same group of embryos was performed at two days post fertilization, at which time embryos from the control VMO treatment group had reached the long pec stage of development (Fig. 7 B). Rescue and miR-92a-3p mimic embryos generally displayed slightly delayed development, and appeared closer to the high pec stage of development. Notably, the rescue and miR-92a-3p mimic treatments often displayed reduced pigmentation when compared to the VMO only treated embryos. All of the miR-92a-3p VMO treated embryos, which at 1 day post fertilization had severe delays in development (n = 21), had recovered and were now at the high pec stage of development, and exhibited a normal pigmentation pattern. The number of embryos in each treatment group which failed to reach the 20-somite stage by 1 day post fertilization were quantified (Fig. 7 C).

Across three trials, over 50 % of the miR-92a-3p VMO treated embryos failed to reach the 20-somite stage, while other treatments resulted in a significantly smaller proportion of embryos with delayed development.

Discussion

Diverse spatial expression patterns of gonadal miRNAs

Among the six miRNAs examined we observed multiple patterns of transcript localization (Fig. 1). These observations demonstrate the importance of understanding miRNA spatial expression when predicting biological function. Our current findings suggest that miR-202-5p is germline expressed, similar to another recent study in zebrafish gonads (Jia et al., 2015). However, while Jia et al., (2015) were only able to detect miR-202-5p in the testis, we reported strong expression in both testis and ovary, a finding corroborated by small RNA sequencing studies in zebrafish gonads (Presslauer et al., 2017, Vaz et al., 2015). Notably, our study reported miR-202-3p is localized to gonadal somatic cells (Fig. 1). This may explain the arm-switch previously reported during gonadal development of zebrafish (Presslauer et al., 2017). During gametogenesis, proliferation and differentiation of the germline would result in increased miR-202-5p expression, whereas gonadal-somatic cells would make up a comparatively smaller proportion of the gonad over time.

We detected strong let-7a-5p signal within the gonadal somatic cells of testis, with noticeably weaker signal found within the germline, particularly spermatocytes (Fig. 1 A). Small RNA sequencing of zebrafish testis detected the highest let-7a-5p abundance at 6 weeks post fertilization, after sexual differentiation (Presslauer et al., 2017). Let-7a-5p was also abundant in isolated spermatozoa, suggesting a function in the germline (Presslauer et al., 2017). Because the testis were still immature, it was not possible in the present study to detect if let-7a-5p signal was present in spermatozoa. However, the study by Jia et al., (2015) also reported strong let-7a-5p expression within spermatozoa. These findings suggest let-7a-5p functions in both germline and gonadal somatic cells, although it is possible its biological functions vary at different stages of gametogenesis.

We detected variations in the sub-cellular localization of miRNAs, observable particularly in the pre-vitellogenic Ia and Ib oocytes (Fig. 1 B). While the functions of miRNA are typically described as occurring in the cytoplasm, there is a growing number of studies which demonstrate the transport and localization of mature miRNA within the nucleus (Jeffries et al., 2011, Liao et al., 2010, Park et al., 2010, Roberts, 2014, Zeno et al., 2009), including miR-92a-3p (Khudayberdiev et al., 2013). In the present study, miR-202-5p, miR-462-5p, and miR-92a-3p all appeared predominantly expressed within the oocyte cytoplasm, with varying levels of transcript also detected within the nucleus (Fig. 1 B). However, miR-430b-3p was exclusively localized to the nucleus, with no detectable level of transcript within the cytoplasm (Fig. 1 B). While we cannot rule out the nuclear signals observed may be a result of the ISH probe binding to the pri- or pre-miRNA form,

the lack of any cytoplasmic signal for miR-430b-3p suggests this miRNA is nuclear enriched. While the nuclear function of miRNA is still poorly understood, sequencing of RNAs isolated by Argonaute protein crosslinking immunoprecipitation identified a substantial number of miRNA interaction sites within gene introns (12%) (Chi et al., 2009). In addition, nuclear miRNA can regulate biogenesis of other non-coding RNAs (Tang et al., 2012, Zisoulis et al., 2012).

miR-92a-3p is expressed prior to the maternal zygotic transition

In the present study, we demonstrate miR-92a-3p transcripts are localized within the cell prior to the first cleavage (Fig. 3). In the study by Ning et al., (2013), the authors reported relatively weak detection of miR-92a-3p in zebrafish embryos at 24 hours post fertilization (hpf), before it greatly increased in expression at 48 and 72 hpf. In addition, the study by Li et al., (2011) suggested miR-92a-3p was first expressed during the blastula stage. The relatively weak ISH staining observed by Ning et al., (2013) at 24 hpf may be explained by variations between ISH protocols. We previously were able to amplify mature miR-92a-3p from zebrafish embryos immediately after fertilization (Presslauer et al., 2016), and reported abundant miR-92a-3p transcripts in unfertilized zebrafish eggs (Presslauer et al., 2017), similar to a study profiling the rainbow trout (*Oncorhynchus mykiss*) egg transcriptome (Ma et al., 2012). This evidence strongly suggests that miR-92a-3p is a maternally inherited miRNA with functions in the zebrafish embryo prior to the maternal/zygotic transition.

***wee2* is a predicted target of miR-92a-3p**

Target prediction for miR-92a-3p identified multiple targets within the zebrafish cell cycle pathway (Fig. 2). miR-92a-3p is a member of the conserved miR-17/92 cluster, which are characterized for their role in regulating mammalian cell cycle, proliferation, and apoptosis (Manni et al., 2009, Mogilyansky and Rigoutsos, 2013, Zhou et al., 2015). Among the predicted cell cycle targets was *wee2* (Fig. 2). In zebrafish, *wee2* transcripts are abundant during the early developmental stages embryos (Aanes et al., 2011). In non-human primates, WEE2 is a conserved oocyte-specific meiosis inhibitor, which maintains the oocyte at the second stage of meiotic arrest (Hanna et al., 2010). In *Xenopus*, Wee2 was identified as a Cdk inhibitory kinase capable of stopping cell cycle progression (Leise and Mueller, 2002, Leise and Mueller, 2004). In addition, the *Xenopus* study demonstrated that microinjection of *wee2* mRNA into embryos resulted in developmental arrest during the cleavage stage (Leise and Mueller, 2002). Our previous finding, in which targeted gene knockdown of zebrafish miR-92a-3p resulted in developmental arrest during embryo cleavage, led us to hypothesize that suppression of *wee2* is an essential function of maternal miR-92a-3p.

In the present study, we isolated *wee2* cDNA from mature zebrafish ovaries and used ISH to demonstrate its expression during early zebrafish embryogenesis (Fig. 3 B). *wee2* was strongly expressed during the cleavage stage, before declining in the blastula stage, and was barely detectable during somitogenesis. Our findings are corroborated by transcriptome analysis of zebrafish embryos, which also reported strong expression of *wee2* at the 1-cell stage, following by a decline in expression, particularly following the maternal/zygotic transition (Aanes et al., 2011). The co-expression of *wee2* and miR-92a-3p suggest there is a strong potential for interaction between the miRNA and its potential target.

gfp-wee2 translation was suppressed by endogenous factors

Injections of 1600 pg of *gfp-wee2* mRNA were required for consistent, but faint, GFP signal to be detected (Fig. 4 A). This was in contrast with a reporter using the *wee2* 3' UTR antisense sequence, in which 200 pg produced strong and consistent GFP signal (Fig. 4 D), and previous studies using GFP reporter assays which used 250 pg (Yoshizaki et al., 2005). Observations that embryos with severe deformities would produce intense GFP signal from reduced *gfp-wee2* concentrations provided further evidence that endogenous factors were acting on the *wee2* 3'UTR during zebrafish embryogenesis. The predicted *wee2* target site for miR-92a-3p is shared with miR-25-3p and miR-92b-3p, while other miRNAs predicted to target the *wee2* 3'UTR include: miR-30, miR-96, miR-101, miR-137, miR-145, miR-148, and miR-156 (Agarwal et al., 2015). Among these miRNAs, miR-92a-3p is by far the most abundant during zebrafish embryogenesis (Vaz et al., 2015). However, zebrafish unfertilized egg and embryo also contain a relatively high abundance of miR-25-3p (Presslauer et al., 2017, Vaz et al., 2015). Therefore, there may be multiple factors in addition to miR-92a-3p which act during zebrafish embryogenesis to suppress *wee2* translation.

miR-92a-3p suppresses _gfp-wee2_ translation in early zebrafish development

In order to test our prediction that endogenous miR-92a-3p was capable of suppressing *gfp-wee2* translation, we co-injected a target site blocker for the miR-92a-3p binding site with a reduced concentration of the *gfp-wee2* reporter (Fig. 5). Our findings of a significantly increased proportion of GFP-expressing embryos, with significantly increased GFP intensity among those embryos, suggests the miR-92a-3p binding site is the primary binding site for endogenous miRNAs suppressing the *gfp-wee2* reporter. Next, we co-injected a miR-92a-3p mimic to confirm miR-92a-3p is capable of interacting with its predicted binding site (Fig. 6). We observed a decrease in the proportion of GFP-expressing cells, and a decrease in GFP intensity among those

embryos. Our evidence suggests that miR-92a-3p is functional in the down-regulation of maternal *wee2* during the early development of zebrafish.

miR-92a-3p knockdown slowed embryonic development

In the present study, micro-injection of miR-92a-3p VMO into zebrafish embryos resulted in a significant proportion of embryos displaying slowed development at 1 day post fertilization (Fig. 7). Embryos which received a scrambled control VMO, the miR-92a-3p mimic, or a rescue treatment (miR-92a-3p VMO and mimic) had reached the 25-somite stage. By comparison, a significant number of embryos receiving the miR-92a-3p VMO failed to reach the 20-somite stage. Interestingly, the phenotype was not lethal, and the embryos recovered by 2 days post fertilization. The observed phenotype was much weaker than in the previously described study, where miR-92a-3p knockdown in mature ovaries resulted in irreversible developmental arrest during embryo cleavage (Presslauer et al., 2016). Because maternal miR-92a-3p is naturally abundant in zebrafish embryos, it may have been able to complete its primary maternal functions prior to the actions of the morpholino oligomer. Future studies will be needed to determine the exact timing of the essential activities of maternal miR-92a-3p.

To date, two additional studies have performed targeted gene knockdown of miR-92a-3p in zebrafish embryos (Li et al., 2011, Ning et al., 2013). By comparison, the study by Li et al., (2011) reported defects in left/right patterning and endoderm specification, while Ning et al., (2013) reported loss of miR-92a-3p resulted in poor proliferation and differentiation of chondrogenic cell progenitors. The variation in phenotypes observed may in part be explained by differences in the knockdown injection. In the present study, we injected approximately 0.05 ng of VMO, compared to injections of up to 3 ng (Li et al., 2011) and 4 ng (Ning et al., 2013) of regular morpholino oligomer (MO). The strongest knockdown effects were observed by Li et al., (2011), which also used MO cocktails containing up to 3 MOs targeting multiple regions of the pre-miRNA, as well as miR-92b-3p.

Conclusion

The spatial expression patterns of the six gonadal miRNAs was diverse, both between cell lineage and intracellular localization; this information may greatly affect predicted biological function. miR-92a-3p is strongly expressed in both gonadal somatic and germline cells, and maternal miR-92a-3p was detected in zebrafish embryos. miR-92a-3p transcripts were co-expressed with their predicted target *wee2* during the 1-, and 2-cell stages of embryogenesis. Microinjection experiments provided evidence that endogenous miRNAs interact with the *wee2* 3'UTR, and synthetic miR-92a-3p is capable

of suppressing *gfp-wee2* translation. Finally, knockdown of miR-92a-3p in zebrafish embryos resulted in slowed development during the first day of embryogenesis, further suggesting a role for miR-92a-3p in regulating the cell cycle.

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Author contributions

IB and JF conceived the experiment. CP and TB performed the experimental procedures and analyzed the data. CP and IB drafted the manuscript. All authors reviewed the manuscript

Competing financial interests

The authors declare no competing financial interests.

References

Aanes, H., Winata, C.L., Lin, C.H., Chen, J.P., Srinivasan, K.G., Lee, S.G.P., et al. (2011). Zebrafish mRNA sequencing deciphers novelties in transcriptome dynamics during maternal to zygotic transition. *Genome Res,* 21**:** 1328-1338. DOI 10.1101/gr.116012.110

Agarwal, V., Bell, G.W., Nam, J.-W. & Bartel, D.P. (2015). Predicting effective microRNA target sites in mammalian mRNAs. *eLife,* 4**:** e05005. DOI 10.7554/eLife.05005

Bartel, D.P. (2009). MicroRNA target recognition and regulatory functions. *Cell,* 136**:** 215-233. DOI 10.1016/j.cell.2009.01.002

Bizuayehu, T.T. & Babiak, I. (2014). MicroRNA in teleost fish. *Genome Biol Evol,* 6**:** 1911-1937. DOI 10.1093/gbe/evu151

Bizuayehu, T.T., Lanes, C.F., Furmanek, T., Karlsen, B.O., Fernandes, J.M., Johansen, S.D., et al. (2012). Differential expression patterns of conserved miRNAs and isomiRs during Atlantic halibut development. *BMC Genomics,* 13. DOI 10.1186/1471-2164-13-11

Chi, S.W., Zang, J.B., Mele, A. & Darnell, R.B. (2009). Argonaute HITS-CLIP decodes microRNA-mRNA interaction maps. *Nature,* 460**:** 479-486. DOI 10.1038/nature08170

Ekimler, S. & Sahin, K. (2014). Computational methods for microRNA target prediction. *Genes,* 5**:** 671-683. DOI 10.3390/genes5030671

Friedman, R.C., Farh, K.K.-H., Burge, C.B. & Bartel, D.P. (2009). Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res,* 19**:** 92-105. DOI 10.1101/gr.082701.108

Ha, M. & Kim, V.N. (2014). Regulation of microRNA biogenesis. *Nat Rev Mol Cell Biol,* 15**:** 509-524. DOI 10.1038/nrm3838

Hall, T.E., Cole, N.J. & Johnston, I.A. (2003). Temperature and the expression of seven muscle-specific protein genes during embryogenesis in the Atlantic cod (Gadus morhua) L. *J Exp Biol,* 206**:** 3187-3200. DOI 10.1242/jeb.00535

Hanna, C.B., Yao, S., Patta, M.C., Jensen, J.T. & Wu, X. (2010). WEE2 is an oocyte-specific meiosis inhibitor in rhesus macaque monkeys. *Biol Reprod,* 82**:** 1190-1197. DOI 10.1095/biolreprod.109.081984

Huntzinger, E. & Izaurralde, E. (2011). Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nat Rev Genet,* 12**:** 99-110. DOI 10.1038/nrg2936

Jeffries, C.D., Fried, H.M. & Perkins, D.O. (2011). Nuclear and cytoplasmic localization of neural stem cell microRNAs. *RNA,* 17**:** 675-686. DOI 10.1261/rna.2006511

Jia, K.-T., Zhang, J., Jia, P., Zeng, L., Jin, Y., Yuan, Y., et al. (2015). Identification of miRNAs in zebrafish spermatozoa. *Zebrafish,* 12**:** 387-397. DOI 10.1089/zeb.2015.1115

Jing, J., Wu, J., Liu, W., Xiong, S., Ma, W., Zhang, J., et al. (2014). Sex-biased miRNAs in gonad and their potential roles for testis development in yellow catfish. *PLoS ONE,* 9**:** e107946. DOI 10.1371/journal.pone.0107946

John, B., Enright, A.J., Aravin, A., Tuschl, T., Sander, C. & Marks, D.S. (2004). Human MicroRNA Targets. *PLoS Biol,* 2**:** e363. DOI 10.1371/journal.pbio.0020363

Juanchich, A., Le Cam, A., Montfort, J., Guiguen, Y. & Bobe, J. (2013). Identification of differentially expressed miRNAs and their potential targets during fish ovarian development. *Biol Reprod,* 88**:** 128, 1-11. DOI 10.1095/biolreprod.112.105361

Khudayberdiev, S., Zampa, F., Rajman, M. & Schratt, G. (2013). A comprehensive characterization of the nuclear microRNA repertoire of post-mitotic neurons. *Front Mol Neurosci,* 6. DOI 10.3389/fnmol.2013.00043

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B. & Schilling, T.F. (1995). Stages of embryonic development of the zebrafish. *Dev Dynam,* 203**:** 253-310. DOI 10.1002/aja.1002030302

Krek, A., Grun, D., Poy, M.N., Wolf, R., Rosenberg, L., Epstein, E.J., et al. (2005). Combinatorial microRNA target predictions. *Nat Genet,* 37**:** 495-500. DOI 10.1038/ng1536

Lee, M., Choi, Y., Kim, K., Jin, H., Lim, J., Nguyen, T.A., et al. (2014). Adenylation of maternally inherited microRNAs by Wispy. *Mol Cell,* 56**:** 696-707. DOI 10.1016/j.molcel.2014.10.011

Leise, W.F. & Mueller, P.R. (2002). Multiple Cdk1 Inhibitory Kinases Regulate the Cell Cycle during Development. *Dev Biol,* 249**:** 156-173. DOI 10.1006/dbio.2002.0743

Leise, W.F. & Mueller, P.R. (2004). Inhibition of the cell cycle is required for convergent extension of the paraxial mesoderm during *Xenopus* neurulation. *Development,* 131**:** 1703-1715. DOI 10.1242/dev.01054

Li, N., Wei, C., Olena, A.F. & Patton, J.G. (2011). Regulation of endoderm formation and left-right asymmetry by miR-92 during early zebrafish development. *Development,* 138**:** 1817-1826. DOI 10.1242/dev.056697

Li, Z. & Rana, T.M. (2014). Therapeutic targeting of microRNAs: current status and future challenges. *Nat Rev Drug Discov,* 13**:** 622-638. DOI 10.1038/nrd4359

Liao, J.-Y., Ma, L.-M., Guo, Y.-H., Zhang, Y.-C., Zhou, H., Shao, P., et al. (2010). Deep sequencing of human nuclear and cytoplasmic small RNAs reveals an unexpectedly complex subcellular distribution of miRNAs and tRNA 3′ trailers. *PLoS ONE,* 5**:** e10563. DOI 10.1371/journal.pone.0010563

Ma, H., Hostuttler, M., Wei, H., Rexroad, C.E., Iii & Yao, J. (2012). Characterization of the rainbow trout egg microRNA transcriptome. *PLOS ONE,* 7**:** e39649. DOI 10.1371/journal.pone.0039649

Manni, I., Artuso, S., Careccia, S., Rizzo, M.G., Baserga, R., Piaggio, G., et al. (2009). The microRNA miR-92 increases proliferation of myeloid cells and by targeting p63 modulates the abundance of its isoforms. *FASEB J,* 23**:** 3957-3966. DOI 10.1096/fj.09-131847

Mogilyansky, E. & Rigoutsos, I. (2013). The miR-17/92 cluster: a comprehensive update on its genomics, genetics, functions and increasingly important and numerous roles in health and disease. *Cell Death Differ,* 20**:** 1603-1614. DOI 10.1038/cdd.2013.125

Ning, G., Liu, X., Dai, M., Meng, A. & Wang, Q. (2013). MicroRNA-92a upholds Bmp signaling by targeting noggin3 during pharyngeal cartilage formation. *Dev Cell,* 24**:** 283-295. DOI 10.1016/j.devcel.2012.12.016

Park, C.W., Zeng, Y., Zhang, X., Subramanian, S. & Steer, C.J. (2010). Mature microRNAs identified in highly purified nuclei from HCT116 colon cancer cells. *RNA Biol,* 7**:** 606-614. DOI 10.4161/rna.7.5.13215

669 Presslauer, C., Bizuayehu, T.T., Kopp, M., Fernandes, J.M.O. & Babiak, I. (2017).
670 Dynamics of miRNA repertoire during gonadal development of zebrafish (*Danio*
671 *rerio*). *Sci Rep,* 7**:** 43850. DOI 10.1038/srep43850
672 Presslauer, C., Bizuayehu, T.T., Razmi, K., Fernandes, J.M.O. & Babiak, I. (2016). See-
673 Thru-Gonad zebrafish line: developmental and functional validation.
674 *Reproduction,* 152**:** 507-517. DOI 10.1530/rep-16-0328
675 Ritchie, W., Flamant, S. & Rasko, J.E.J. (2009). Predicting microRNA targets and
676 functions: traps for the unwary. *Nat Meth,* 6**:** 397-398. DOI
677 http://www.nature.com/nmeth/journal/v6/n6/suppinfo/nmeth0609-397_S1.html
678 Roberts, T.C. (2014). The microRNA biology of the mammalian nucleus. *Mol Ther*
679 *Nucleic Acids,* 3**:** e188. DOI 10.1038/mtna.2014.40
680 Tang, R., Li, L., Zhu, D., Hou, D., Cao, T., Gu, H., et al. (2012). Mouse miRNA-709
681 directly regulates miRNA-15a/16-1 biogenesis at the posttranscriptional level in
682 the nucleus: evidence for a microRNA hierarchy system. *Cell Res,* 22**:** 504-515.
683 DOI 10.1038/cr.2011.137
684 Tao, W., Sun, L., Shi, H., Cheng, Y., Jiang, D., Fu, B., et al. (2016). Integrated analysis
685 of miRNA and mRNA expression profiles in tilapia gonads at an early stage of
686 sex differentiation. *BMC Genom,* 17**:** 1-13. DOI 10.1186/s12864-016-2636-z
687 Vaz, C., Wee, C.W., Lee, G.P.S., Ingham, P.W., Tanavde, V. & Mathavan, S. (2015).
688 Deep sequencing of small RNA facilitates tissue and sex associated microRNA
689 discovery in zebrafish. *BMC Genom,* 16**:** 1-16. DOI 10.1186/s12864-015-2135-7
690 Vejnar, C.E. & Zdobnov, E.M. (2012). miRmap: Comprehensive prediction of
691 microRNA target repression strength. *Nucleic Acids Res,* 40**:** 11673-11683. DOI
692 10.1093/nar/gks901
693 Westerfield, M. (2000). *The zebrafish book. A guide for the laboratory use of zebrafish*
694 *(Danio rerio). 4th ed.,* Eugene: University of Oregon Press
695 Xiao, J., Zhong, H., Zhou, Y., Yu, F., Gao, Y., Luo, Y., et al. (2014). Identification and
696 characterization of microRNAs in ovary and testis of nile tilapia (*Oreochromis*
697 *niloticus*) by using solexa sequencing technology. *PLoS ONE,* 9**:** e86821. DOI
698 10.1371/journal.pone.0086821
699 Yoshizaki, G., Tago, Y., Takeuchi, Y., Sawatari, E., Kobayashi, T. & Takeuchi, T.
700 (2005). Green fluorescent protein labeling of primordial germ cells using a
701 nontransgenic method and its application for germ cell transplantation in
702 Salmonidae. *Biol Reprod,* 73**:** 88-93. DOI 10.1095/biolreprod.104.034249
703 Zar, J.H. (1999). *Biostatistical Analysis, 4th edition,* New Jersey: Prentice
704 Hall.9780130815422
705 Zeno, F.-P., Karsten, K., Hauke, S., Reiner, B., Breunig, H.G., Aisada, U., et al. (2009).
706 Trafficking of Mature miRNA-122 into the Nucleus of Live Liver Cells. *Curr*
707 *Pharm Biotechnol,* 10**:** 569-578. DOI 10.2174/138920109789069332
708 Zhou, C., Shen, L., Mao, L., Wang, B., Li, Y. & Yu, H. (2015). miR-92a is upregulated
709 in cervical cancer and promotes cell proliferation and invasion by targeting
710 FBXW7. *Biochem Biophys Res Commun,* 458**:** 63-69. DOI
711 10.1016/j.bbrc.2015.01.066
712 Zisoulis, D.G., Kai, Z.S., Chang, R.K. & Pasquinelli, A.E. (2012). Autoregulation of
713 microRNA biogenesis by let-7 and Argonaute. *Nature,* 486**:** 541-544. DOI
714 10.1038/nature11134

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Tables

Table 1. LNA detection probe sequences

Target miRNA	Probe sequence	RNA Tm °C
dre-let-7a-5p	AACTATACAACCTACTACCTCA	84
dre-miR-202-5p	CAAAGAGGTATATGCATAGGAA	75
dre-miR-202-3p	TTTTCCCATGCCCTATGCCTCT	91
dre-miR-430b-3p	CTACCCCAACTTGATAGCACTTT	86
dre-miR-462-5p	AGCTGCATTATGGGTTCCGTTA	90
dre-miR-92a-3p	ACAGGCCGGGACAAGTGCAATA	87

Figures

Figure 1. *in situ* hybridization of miRNAs in 10 week old zebrafish testis (A) and ovary (B) compared with hematoxylin and eosin (HE) stained sections. Sg = spermatogonia, Sc = spermatocytes, St = spermatids, Fc = follicle cells, Ia = primary growth pre-follicle stage, Ib = primary growth follicle stage, II = cortical alveolus stage. Scalebars represent 100 µm.

Figure 2. Predicted miR-92a-3p targets within the Cdk1 regulatory pathway. Figure was modified from the KEGG pathway for the zebrafish cell cycle. Filled boxes indicate proteins with transcripts predicted as targets for miR-92a-3p, whereas empty boxes indicate non-targets.

Figure 3. Whole mount *in situ* hybridization of miR-92a-3p (A) and *wee2* (B) at the 1-cell, 2-cell, 256-cell, and 25-somite stages. The scalebar represents 500 µm.

Figure 4. GFP-3'UTR chimeric RNA injections into zebrafish embryos. A) Representative images of zebrafish embryos injected with 200, 800, 1600, or 3200 pg of *gfp-wee2* mRNA. 200 and 800 pg injections resulted in some GFP signal detected within the yolk, but when compared to non-injected embryos appeared to be a natural reflection of epifluorescent light. GFP signal was detected within the body of embryos injected with 1600 or 3200 pg *gfp-wee2* mRNA. B) A non-injected control embryo. C) A representative image for *gfp-wee2* injected embryos with deformities. D) Representative images of zebrafish embryos injected with 200 or 1000 pg *gfp-wee2-antisense* mRNA. All observations were made at 24 hpf at the 25-somite stage. The scalebar represents 500 µm.

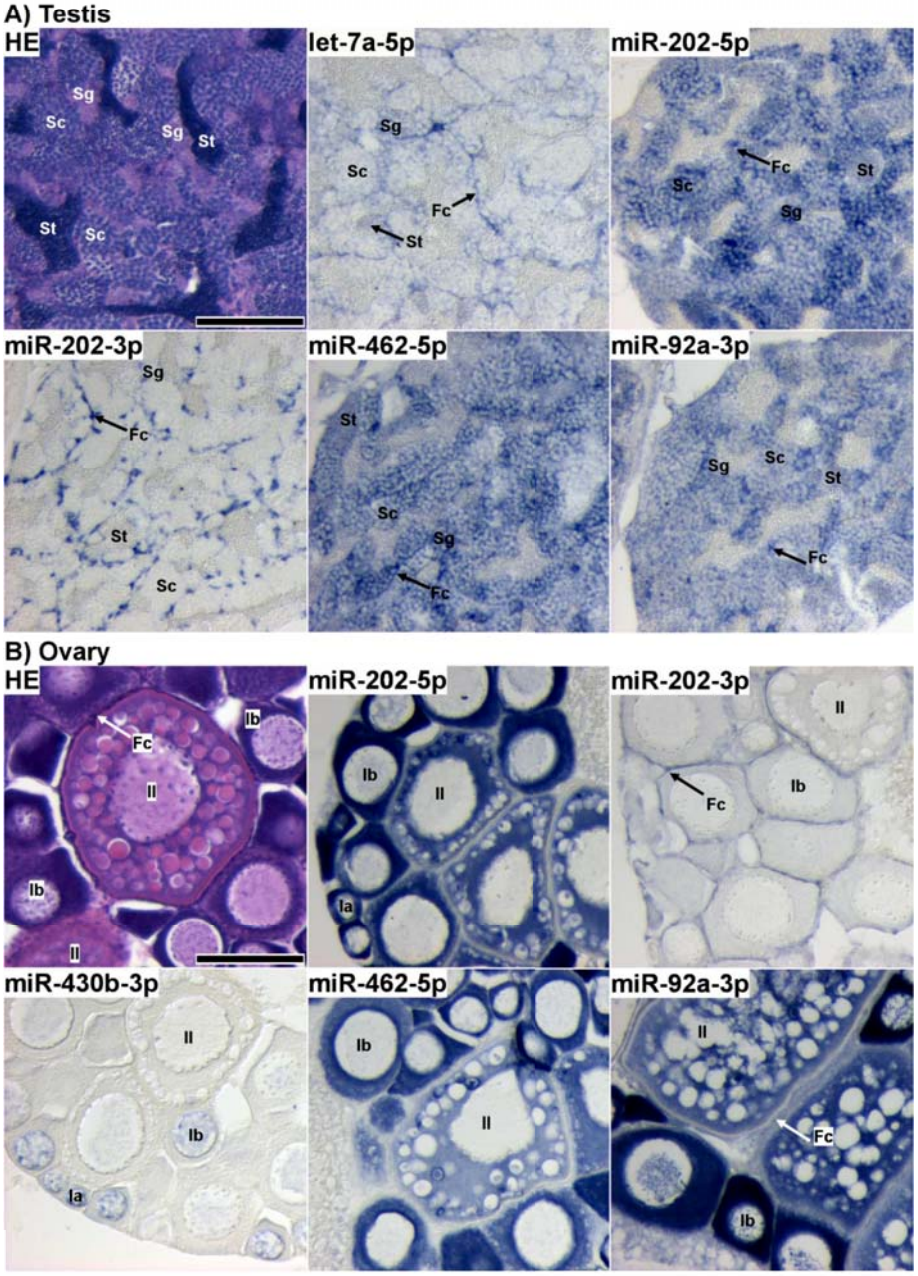
Figure 5. Zebrafish miR-92a-3p target protector assays. Microinjections of *gfp-wee2* chimeric RNA with a target site blocker (TSB) specific for the miR-92a-3p predicted binding site were made to determine miRNA/mRNA interaction. A) Representative embryos from the non-injected control, *gfp-wee2* and control TSB, or *gfp-wee2* with miR-92a-3p TSB treatment groups. White contours identify the area of the zebrafish head in which the GFP mean intensity (MI) value was measured. The scalebar represents 500 µm. B) The relative proportion of embryos with detectable GFP signal between the control TSB and miR-92a-3p TSB treatments. C) Box plots display the distribution of GFP intensity values from all embryos in the two treatment groups. Whiskers represent the upper and lower limits of GFP intensity. Boxes identify the first and third quartiles. The horizontal line identifies the median value. Asterisks mark significant p-values (< 0.05).

Figure 6. Zebrafish miR-92a-3p target validation assays. Microinjections of *gfp-wee2* chimeric RNA with synthetic miR-92a-3p mimic to assess miRNA/mRNA interactions. A) Representative embryos injected with *gfp-wee2* mRNA (3200 pg) or a co-injection of both *gfp-wee2* and miR-92a-3p mimic (5.0 μM). White contours identify the area of the zebrafish head in which the GFP mean intensity (MI) value was measured. The scalebar represents 500 μm. B) The relative proportion of embryos with detectable GFP signal between the control TSB and miR-92a-3p TSB treatments depending on the *gfp-wee2* concentration. C) Box plots display the distribution of GFP intensity values between the two treatments (*gfp-wee2* injection of 1600 pg). Whiskers represent the upper and lower limits of GFP intensity. Boxes identify the first and third quartiles. The horizontal line identifies the median value.

Figure 7. miR-92a-3p knockdown and rescue in zebrafish embryos. A) Representative images display typical development of zebrafish embryos at 1 day post fertilization after miR-92a-3p knockdown treatment. Non-injected control embryos display the wildtype 25-somite phenotype. Similar phenotypes were observed in embryos which received a scrambled MO, a miR-92a-3p MO and miR-92a-3p mimic co-injection, and a miR-92a-3p mimic only. Various levels of developmental delay were observed in embryos receiving miR-92a-3p MO only. B) Follow up observations at 2 days post fertilization. C) Relative proportion of embryos observed at 1 day post fertilization for each treatment group with delayed development, as defined by not having reached the 20-somite stage. Lettering identifies significant differences between treatment groups ($p < 0.05$).

781 **Fig. 1**

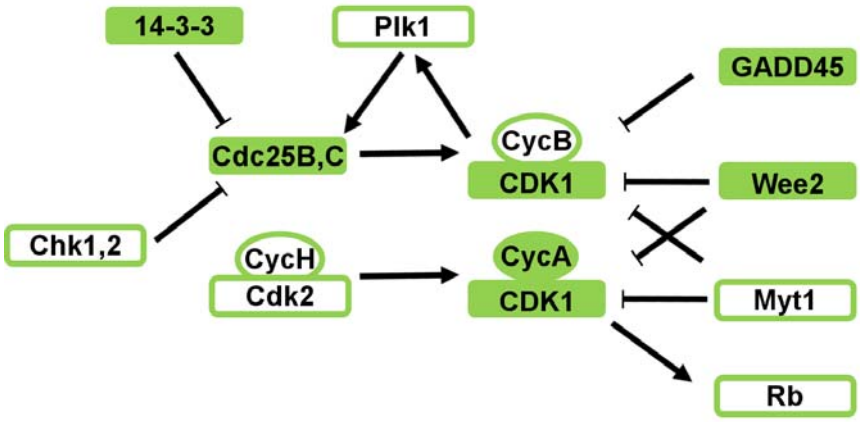
782



783

784 **Fig. 2**

785



786 **G2 / M Checkpoint**

Fig. 3

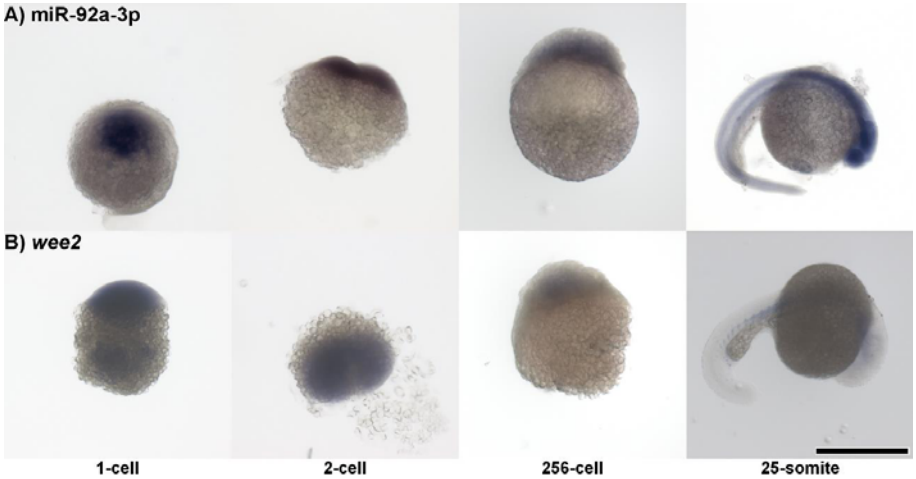


Fig. 4

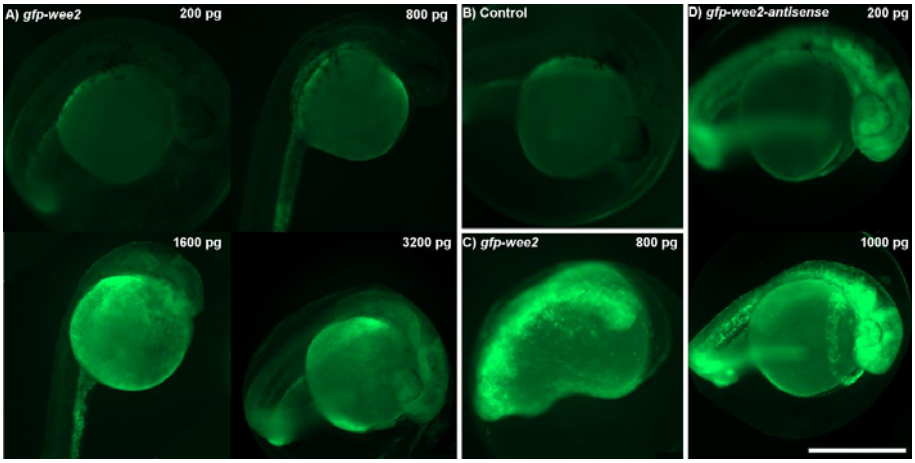

A) gfp-wee2
200 pg
800 pg
1600 pg
3200 pg
B) Control
C) gfp-wee2
800 pg
D) gfp-wee2-antisense
200 pg
1000 pg

Fig. 5

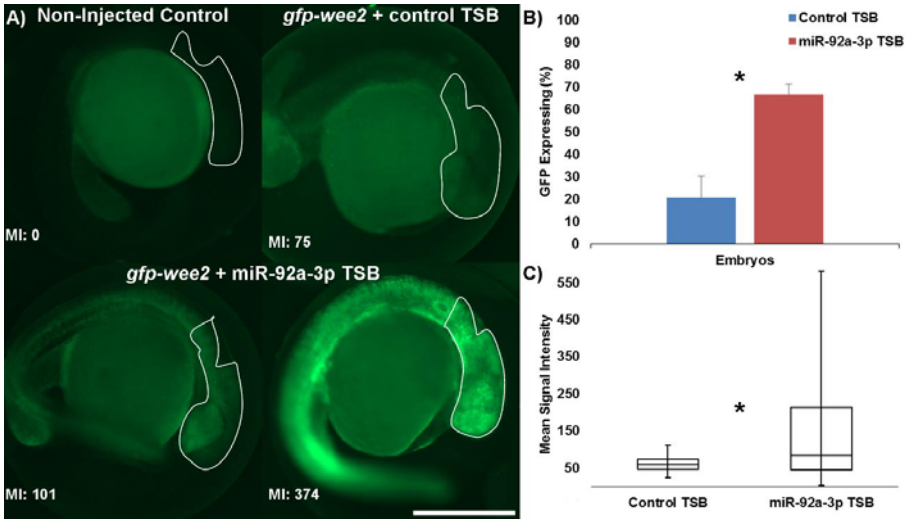
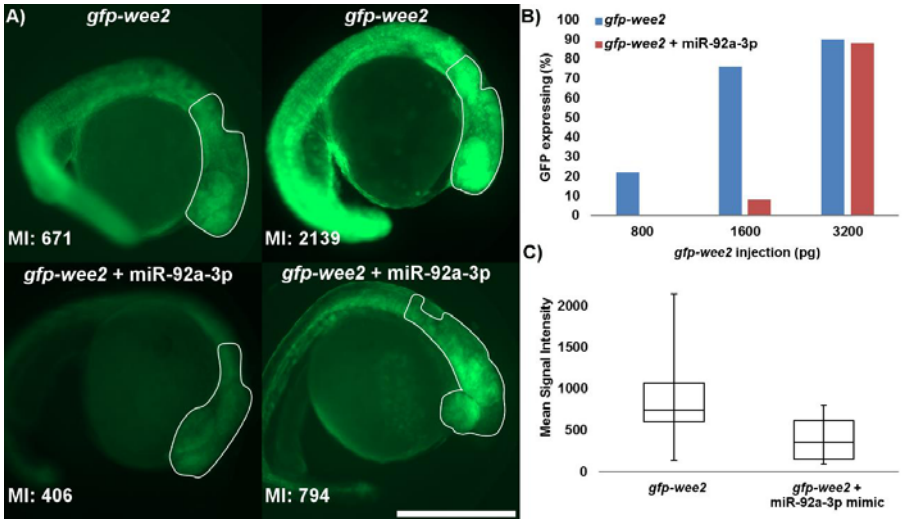
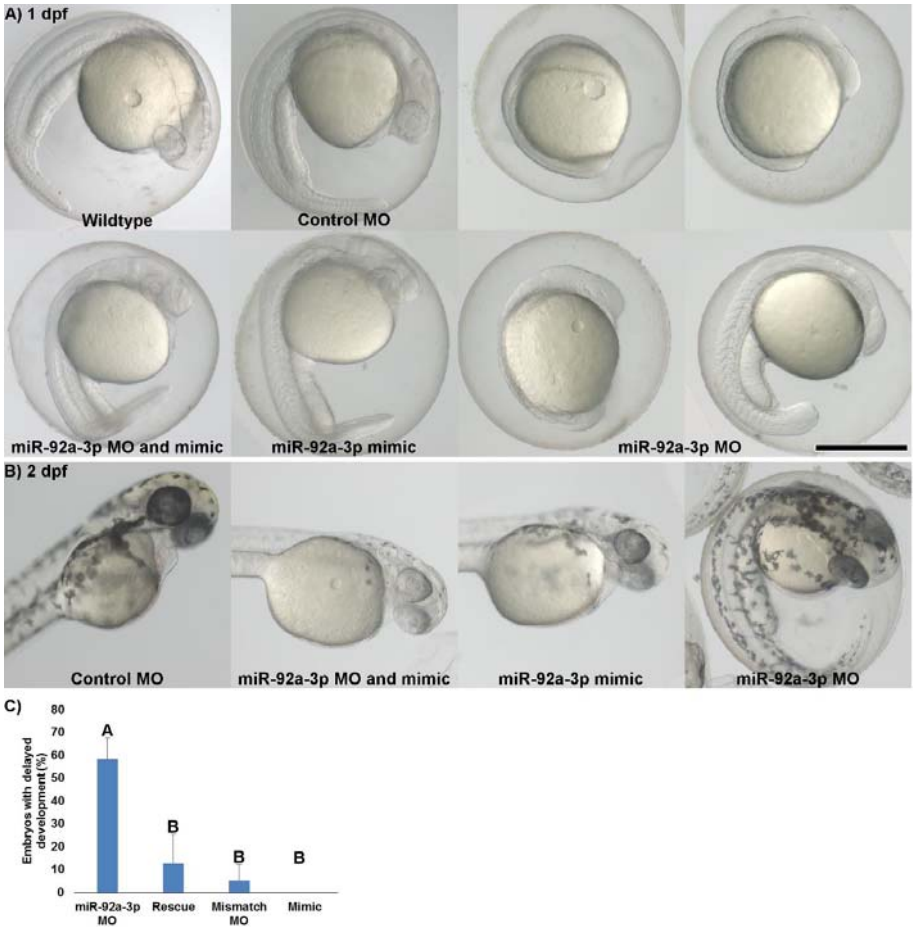

A) Non-Injected Control
gfp-wee2 + control TSB
MI: 0
MI: 75
gfp-wee2 + miR-92a-3p TSB
MI: 101
MI: 374
B)
Control TSB
miR-92a-3p TSB
GFP Expressing (%)
100
90
80
70
60
50
40
30
20
10
0
*
Embryos
C)
Mean Signal Intensity
550
450
350
250
150
50
*
Control TSB
miR-92a-3p TSB

Fig. 6


A)
gfp-wee2
gfp-wee2
MI: 671
MI: 2139
gfp-wee2 + miR-92a-3p
gfp-wee2 + miR-92a-3p
MI: 406
MI: 794
B)
gfp-wee2
gfp-wee2 + miR-92a-3p
GFP expressing (%)
100
90
80
70
60
50
40
30
20
10
0
800
1600
3200
gfp-wee2 injection (pg)
C)
Mean Signal Intensity
2000
1500
1000
500
0
gfp-wee2
gfp-wee2 +
miR-92a-3p mimic

804 **Fig. 7**

805



806

Supplementary Files

Supplementary Dataset S1. Predicted cell cycle targets for miR-92a-3p (miRBase accession: MIMAT0001808) using both TargetScanFish (release 6.2) and miRmap, and compared to the zebrafish cell cycle KEGG pathway.

Supplementary Dataset S1

Spatial expression patterns of gonadal miRNA and target validation of miR-92a-3p in the embryos of zebrafish

Christopher Presslauer, Teshome Tilahun Bizuayehu, Jorge M.O. Fernandes, Igor Babiak

Predicted cell cycle targets for miR-92a-3p (miRBase accession: MIMAT0001808) using both TargetScanFish (release 6.2) and miRmap, and compared to the zebrafish cell cycle KEGG pathway.

Protein family	Gene name	ENSEMBL ID	Score	
			TargetScan	miRmap
14-3-3	*ywhae1*	ENSDARG00000006399.1	-0.2	83.52
14-3-3	*ywhae2*	ENSDARG00000017014.1	-0.13	n/a
Abl	*abl1*	ENSDARG00000035187.1	-0.13	n/a
APC/C	*anapc13*	ENSDARG00000078435.1	n/a	33.55
Cdc14	*cdc14ab*	ENSDART00000105681.1	n/a	7.56
CDC25	*cdc25b*	ENSDARG00000010792.1	-0.1	41.49
CDK1	*cdk1*	ENSDARG00000087554.1	-0.11	15.61
CDK4,6	*cdk6*	ENSDART00000102888	n/a	5.71
CycA	*ccna1*	ENSDARG00000043236.1	-0.36	79.28
CycB	*ccnb1*	ENSDART00000063357.1	n/a	67.18
CycE	*ccne2*	ENSDART00000169412.1	n/a	23.84
E2F4	*e2f4*	ENSDARG00000029358.1	-0.42	n/a
Esp1	*espl1*	ENSDART00000156645.1	n/a	41.72
GADD45	*gadd45aa*	ENSDART00000063996.1	n/a	15.7
GADD45	*gadd45ba*	ENSDARG00000027744.1	-0.09	18.22
GSK3β	*gsk3b*	ENSDARG00000052468.1	-0.12	n/a
Mad1	*mad1l1*	ENSDARG00000033852.1	-0.02	68.86
MCM	*mcm3*	ENSDARG00000024204.1	-0.12	23.91
MCM	*mcm6*	ENSDARG00000057683.1	-0.11	78.87
MCM	*mcm7*	ENSDARG00000035761.1	-0.14	50
Orc	*orc1*	ENSDARG00000039217.1	-0.13	28.68
Orc	*orc3*	ENSDART00000047857.1	n/a	53.13
Orc	*orc4*	ENSDARG00000029014.1	-0.27	n/a
p18/Ink4c	*cdkn2c*	ENSDART00000133742.1	n/a	12.3
p21/Cip1	*cdkn1a*	ENSDART00000136722.1	n/a	38.74
p27,57/Kipl1,2	*cdkn1bb*	ENSDARG00000054271.1	-0.18	70.56
p27,57/Kipl1,2	*cdkn1c*	ENSDARG00000010878.1	-0.18	80.9
SCF	*cul1a*	ENSDARG00000019239.1	-0.13	67.81
SKP2	*skp2*	ENSDART00000101751.1	n/a	17.81
Stag1,2	*Stag2a*	ENSDART00000137655.1	n/a	4.82
Stag1,2	*Stag2b*	ENSDART00000161852.1	n/a	18.72
TGFβ	*tgfb2*	ENSDARG00000027087.1	-0.14	n/a
TGFβ	*tgfb3*	ENSDARG00000019367.1	-0.15	n/a
Wee	*wee2*	ENSDARG00000012718.1	-0.32	69.1

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This dissertation examines the role of microRNAs (miRNAs) during development of the gonads in zebrafish. miRNAs are small non-coding RNA, which fine-tune expression of protein-coding genes by preventing their translation. They are abundant in plants and animals, and there are many similarities between miRNAs in human and fish. Zebrafish is a popular ornamental fish, frequently used as a model animal in science. At present, relatively little is known about the role of miRNAs during fish reproduction. In this dissertation, a special zebrafish line was created allowing *in vivo* visualization of germ cells. Further, this line was used to characterize the most abundant miRNAs at various stages of gonadal development, from undifferentiated gonads to sexual maturation. Regulatory functions of selected miRNAs were investigated. miRNAs stocked in unfertilized eggs are likely maternal factors which function during embryonic development of the offspring. Collectively, the results indicate that miRNA repertoire in fish gonads is dynamic during the gonad development, with spatial, temporal, and gender-related specificity. The research provides an important resource on the miRNAs involved in fish gonad development.

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