Identification and characterization of hypoxia-responsive microRNAs and target genes potentially associated with regulation of reproductive functions in male marine medaka

by

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Submitted in partial fulfillment of the requirements for the degree of

BACHELOR OF SCIENCE (HONS) IN APPLIED BIOLOGY

from

City University of Hong Kong

March 2017

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Abstract

Aquatic hypoxia has become one of the most widespread and serious problems in the coastal oceans worldwide, posing enormous threats to the fish population. A recent study demonstrated that fish exposed to chronic hypoxia can cause transgenerational reproductive impairments in male fish. Genome-wide mRNA transcriptome and DNA methylome changes were observed that were shown by bioinformatics analysis to correlate with impairment in sperm motility and testes development in F0 and transgenerational group of F2 male fish. To follow up on this earlier study, this project aims to identify and characterize several hypoxia-responsive miRNAs in marine medaka testes that may have a role in regulating other target genes that control reproductive functions in male fish. Four miRNAs – miR-125b, miR-103b, miR-204-5p, and miR-451a – that showed differential expression in hypoxic medaka testes were selected for further investigations in this study. Bioinformatic analyses showed that miR-125-5b may potentially target PNMA1 mRNA, miR-103b may target COR1C mRNA and K2C8 mRNA, miR-204-5p may target PPM1D mRNA and miR-451a may target PIM1. PNMA1, PPM1D, PIM1, COR1C and K2C8 mRNAs were selected for further analysis because these 5 genes were shown to be co-regulated transgenerationally in a previous study, and may have a role in reproductive functions. The expression level of the four miRNAs and their target mRNAs were validated in testis of hypoxic marine medaka males using qRT-PCR assays. While none of the predicted miRNA-mRNA pairs showed a significant inverse correlation expression pattern, the expression of miR-204-6p and PIM1 (serine/threonine-protein kinase pim1) was significantly downregulated. Further multiple sequence alignment on PIM1 has found that it is conserved from fish to human and may have a role in the regulation of apoptosis and/or spermatogenesis.

Chapter 1
Introduction

1.1 Hypoxia

Anthropogenic- and eutrophication-induced hypoxia has been one of the most widespread and serious problems in coastal zones, posing enormous threats to the aquatic ecosystems worldwide. In 2008, over 400 dead zones have been reported, covering a total area of over 245,000 km^2 and the situation is likely to worsen in the coming years (Diaz & Rosenberg, 2008; Selman et al., 2008). Both *in vitro* and *in vivo* research have proven that hypoxia can lead to reproductive and developmental impairments in fishes by disrupting various hormones and genes that regulate reproductive processes (Wu, 2009). A recent study further revealed that hypoxia-induced reproductive impairments in fish could be more serious than we thought as the reproductive effects may be transgenerationally transmitted to future offsprings (Wang et al. 2016). Anthropogenic-induced hypoxia has become a serious threat to the global population of fish.

1.1.1 Occurrence of Aquatic Hypoxia

Aquatic hypoxia is defined as a concentration of dissolved oxygen (DO) below 2 mg O_2/L (Diaz & Rosenberg, 2008). Eutrophication-induced hypoxia is closely associated with human activities including agriculture and human waste discharge, urban runoffs, industrial effluent, and fossil fuel combustion (Selman et al., 2008), so its distribution is mainly centered at continental shelves where dense human population is found, such as the Baltic, Kattegat, Black sea, Gulf of Mexico and East China Sea (Diaz & Rosenberg, 2008).

1.1.2 Hypoxia and Fish Reproduction

Given the large variations of oxygen level in the aquatic environment, fish have developed a wide range of intrinsic hypoxia tolerance and adaptive mechanisms. (Chapman & McKenzie, 2009).. In fact, the effect of hypoxia on fish is often sub-lethal (Pedersen, 1987) and the responses can be behavioral, physiological and biochemical (Wu, 2009). Studies have demonstrated that hypoxia can alter reproductive behaviors ranging from courtship, mate choice to reproductive efforts (Jones & Reynolds, 1999a; Jones & Reynolds, 1999b; Reynolds & Jones, 1999; Lissåker et al., 2003; Wang et al., 2008). There is extensive evidence demonstrating the negative effect of hypoxia on fish physiology, including retarded gonad development and gametogenesis, decreased number and quality of gametes, reduced

spawning, fertilization and hatching success, and impaired larvae survival (Wu et al., 2003; Shang & Wu, 2004; Thomas et al., 2006, 2007; Landry et al., 2007). It has been shown that hypoxia impairs reproductive processes by affecting hormones, neurotransmitters, and receptors along the hypothalamus-pituitary-gonad (HPG) axis and enzymes controlling steroidogenesis (Wu, 2009).

1.1.3 Hypoxia as Endocrine Disrupter in Fish

Wu (2003) has first reported hypoxia can disrupt endocrine systems and impair reproduction in carp. Numerous laboratory (Shang & Wu, 2004; Shang et al., 2006; Landry et al., 2007; Martinovic et al, 2009; Hala et al., 2012; Jiang et al., 2011; Yu et al., 2012) and field (Thomas et al., 2006, 2007; Thomas & Rahman, 2011) research have identified the same phenomenon in other fish species (zebrafish, Gulf killifish, fathead minnows, bovine corpus luteum and Atlantic croaker), as evidenced by the change in mRNA expression, the activity of steroidogenic enzymes and the synthesis of steroid hormones (testosterone and estrogen) under hypoxia. Hypoxia-induced endocrine disruption could bring about reproductive impairments, but the underlying molecular mechanisms remain largely unknown. In recent years, researchers have used cDNA microarray (Martinovic et al, 2009) and next-generation sequencing (NGS) (Lau et al., 2014; Lai et al., 2016; Wang et al, 2016) to study the gene expression profile under hypoxia or other methods (Shang et al., 2006; Jiang et al., 2011) to identify the genes and possible mechanism that are responsible for such effect.

1.2 Epigenetics

Epigenetics is the study of potentially heritable alternations in chromatin that changes gene activity and expression without changing the DNA sequence (Allis et al., 2015). There are several possible mechanisms of epigenetic changes including histone modification, DNA methylation, and non-coding RNA interference (Allis & Jenuwein 2016). Emerging evidence further indicated that these mechanisms could work together to produce non-genetic regulation of gene expression (Goldberg et al., 2007). Other than the traditional genetic pathway, hypoxia may exert its influence through epigenetic regulation. More importantly, since all epigenetic mechanisms share some degree of ability to bypass the reprogramming barrier and pass down the epigenetic tags to offspring, it challenges the conventional concept that acquired traits cannot be inherited to the offspring. In environmental toxicology, this means the environmental changes like toxic and stress may confer a more long-lasting effect

than we have previously thought. In other words, hypoxic exposure to a single generation could give rise to a transgenerational effect that is transmitted to several generations through epigenetic inheritance.

1.2.1 RNA Interference (RNAi) Mediated Gene Silencing in Animal

RNA interference (RNAi) is one of the several epigenetic mechanisms that can mediate gene expression without interrupting the DNA sequence. By definition, RNAi is the process by which small non-coding RNAs silence homologous gene transcripts. In the case of the animal, RNase III enzymes catalyze the processing of double-stranded RNAs (dsRNA) into small interfering RNAs (siRNAs) or microRNAs (miRNAs), couple with Piwi-interacting RNAs (piRNAs), all of these small RNAs then associate with a specific protein called Argonaute to inhibit endogenous targets expression by deadenylation and translational repression (Okamura & Lai, 2008). This extra layer of control on gene expression after transcription has proven to be essential to the cell differentiation and body development of multicellular organisms, with examples about the roles of miRNAs (Papaioannou & Nef, 2009; Kotaja, 2014; Wang & Xu, 2014) and piRNAs (Kuramochi-Miyagawa, 2004; Carmell et al., 2007; Wang & Reinke, 2008) on the spermatogenesis of animals. It would not be surprising to find the same phenomenon in the testis of marine medaka.

1.2.2 Transgenerational Epigenetic Inheritance

Transgenerational epigenetic inheritance occurs when epigenetic marks are stably propagated from somatic cells to germ cells without direct exposure to the initial signal or environment that trigger the change, and maintained throughout the genome-wide epigenetic reprogramming during gametogenesis and embryogenesis (Allis et al., 2015). In the study of animal transgenerational epigenetic inheritance, biologists often concentrate on the paternal effect (inheritance through sperm) as the mother could transfer environmental cues to a fetus during pregnancy (which is considered as an intergenerational effect) (Bohacek & Mansuy, 2015). Research on mammalian germ cells have shown that most epigenetic markers would be altered or washed away during gametogenesis and after fertilization (Bohacek & Mansuy, 2015). Similar phenomenon with differences in mechanism and phasing is also observed in fish. In the spermatogenesis of zebrafish, even though protamines are not involved in chromatin packaging, the sperm chromatin still retains histone modifications like that in mammals (Wu et al., 2011). In the early embryogenesis of zebrafish, the paternal and maternal DNA methylation patterns are maintained and gradually discarded (Potok et al.,

2013). On the other hand, various types of mRNAs and ncRNAs, including miRNAs and tsRNAs, are present in mammalian sperms. Since RNAs can have a huge impact on gene expression and only those epigenetic markers that survive reprogramming can produce a transgenerational effect, RNA molecules probably are the key carriers of paternal germline inheritance in eukaryotes (Ostermeier et al., 2005; Lalancette et al., 2008). It is possible that paternal RNAs could associate with the DNA to induce epigenetic changes such as histone modification and/or DNA methylation (Allis et al., 2015; Chen et al., 2016).

1.2.3 RNA-Mediated Paternal Inheritance

Among the mechanisms of transgenerational epigenetic inheritance, RNA-based transmission could be the most plausible since identification of inherited DNA methylation in mammals is rare and more studies have demonstrated that spermatozoal RNAs can mediate transmission of acquired phenotypes (Allis et al., 2015; Chen et al., 2016). The most significant evidence come from experiments that use microinjection to transfer sperm RNAs from treated male mice into normal zygotes, which displayed altered phenotypes (Gapp et al., 2014; Rodgers et al., 2015). However, the mechanism of how sperm RNAs initiate the recapitulation of paternal acquired traits and produce long-lasting effect till adulthood remains largely unknown. Some possible theories include reshaping the embryonic development through transcriptional cascade, regulating transposable elements, remodeling chromatin structure, and RNA modifications (Chen et al., 2016). If hypoxia indeed impairs fish reproductive function through RNAi, such effect could be inherited to the offspring through the processes suggested above.

1.3 Hypoxia, Fish Reproduction, and RNAi

The idea that hypoxia impairs fish reproduction by affecting the steroidogenesis is quite established. However, with the discovery of RNAi, it was suggested that miRNAs may also play a certain role in the process. Several pieces of research have provided some initial suggestion towards such possibility. Experiments using steroid-producing human cell line H295R have identified some miRNAs that affect steroidogenesis genes under hypoxia (Nusrin et al., 2014; Kong et al., 2014). Studies in animals reveal the potential roles of miRNAs (Papaioannou & Nef, 2009; Kotaja, 2014; Wang & Xu, 2014) and piRNAs (Kuramochi-Miyagawa, 2004; Carmell et al., 2007; Wang & Reinke, 2008) on regulating spermatogenesis through the RNAi pathway. However, the most important cues came from

Lau (2014), who performed the miRNAs transcriptome analysis of brain, liver, and gonads from marine medaka exposed to hypoxia (Lau et al., 2014). Based on the result of the follow-up computational and bioinformatic analysis, researchers formally proposed that hypoxia could alter reproductive functions in marine medaka through miRNAs regulation (Tse et al., 2015, 2016; Lai et al., 2016). Wang et al. (2016) further demonstrated that the hypoxia effect on male fish reproductive functions is transgenerational and is associated with DNA methylation. Coupled with the studies on hypoxia-responsive miRNAs in mammals (Huang et al., 2009; Chen et al., 2013) and the transgenerational effect caused by environmental stresses (e.g. hypoxia) and toxicants (e.g. endocrine disrupters) (Anway et al., 2005; Skinner & Anway, 2005; Guerrero-Bosagna et al., 2014), our current understanding of the previously described topics suggested that RNAi-mediated gene silencing could be related to the hypoxia-induced reproduction malfunctioning in fish, leading to a potential transgenerational effect, and the underlying mechanism could involve spermatogenesis, steroidogenesis, HPG axis, and hypoxia-induced factors.

1.4 Objectives of This Study

The objectives of this study are: (1) Examine sperm motility to look for impairment of reproductive function in male medaka exposed to prolonged hypoxia for 50 days; (2) Perform qRT-PCR of selected miRNAs and mRNAs in normoxic and hypoxic fish testes to validate their expression pattern; and (3) Perform multiple sequence alignment to characterize the function of candidate genes. It is envisaged that through characterizing the expression of miRNAs and their targeted genes, additional clues on how epigenetics, particularly, miRNAs, might contribute to the hypoxia-induced transgenerational effect of male fish reproduction impairment.

Chapter 2
Materials and Methods

2.1 Medaka Maintenance and Hypoxia Exposure

Marine medaka *O. melastigma* were maintained and kept in two continuous flow systems (28 ± 2 °C and 14h light/10h dark cycle). The fish were fed with hormone-free TetraMin Tropical Fish Food Flakes (Tetra., Blacksburg, VA) and Otohime β1 (Nisshin Co, Japan) three times per day. In each system, marine medaka (four months old) were reared in four replicate net cages (each contained 10 males and 10 females) under normoxia (4.5 ± 0.5 mg L^{-1}) and hypoxia (0.7 ± 0.2 mg L^{-1}) for 50 days. The oxygen level was achieved by pumping nitrogen into water and monitoring DO continuously using a DO meter.

2.2 Sperm Motility Test

After normoxic and hypoxic exposure, a total of four sexually mature males were randomly sampled from each group. The testis was dissected from each fish and gently pressed to extrude semen into 200 μl of artificial seawater for immediate analysis of sperm motility using the CRISMAS image analysis system (Image House, Copenhagen). 40 μl of sample was loaded on the chip to record the swimming patterns of sperms, in which 10 different fields of the sample were captured using a color CCD camera (Axioplan 2 imaging, ZEISS, Germany). The mean curvilinear velocity (VCL), mean straight line velocity (VSL) and mean angular path velocity (VAP) of normoxic and hypoxic groups were calculated.

2.3 Total RNA Extraction and Quantification

Total RNA of testis was prepared as in the previous study (Wang et al., 2016). The samples were stored at -80 °C and the total RNA was extracted using TRIzol reagent (Invitrogen, Inc., Carlsbad, CA). Each sample was first mixed with 0.2 ml TRIzol reagent and ground using a tissue grinder with a disposable pestle. The homogenate was stored for 5 minutes at room temperature after adding another 0.8 ml of TRIzol reagent. 0.2 ml of chloroform was added, and the tube was shaken vigorously by hand for 15 seconds. The sample was stored for 3 minutes at room temperature and centrifuged at 12,000 rpm for 15 minutes at 4 °C. The

aqueous phase was transferred to a clear tube. 0.5 ml of ice-cold isopropanol was added and the sample was mixed by turning up and down. It was stored at -80 °C for 2 hours and centrifuged at 12,000 rpm for 10 minutes at 4 °C. All supernatant was removed from the tube, leaving the white RNA pellet. The pellet was washed with 1 ml of ice-cold 75% ethanol, and the solution was centrifuged at 12,000 rpm for 10 minutes at 4 °C. All ethanol was discarded from the tube, and the RNA pellet was air-dried for 5-10 minutes and resuspended in 20 µl RNase-free water by tapping the tube several times. The RNA concentration was then measured using NanoDrop 1000 photospectrometer.

2.4 Quantitative Real Time PCR (qRT-PCR) of Hypoxia-Responsive miRNAs

TaqMan® MicroRNA Assay (Applied Biosystems) was used to confirm the expression patterns of the 4 selected miRNAs in testis of normoxic and hypoxic marine medaka, which were found to be hypoxia-responsive previously by small RNA sequencing (Tse et al., 2016). Each 15 µl reverse transcription reaction contained 1 µl of total RNA (10 ng/µl), 3 µl of stem-loop RT primer (5X), 0.15 µl dNTP Mix (100 mM), 1 µl of MultiScribe Reverse Transcriptase (50U/µl), 0.19 µl of MultiScribe Reverse Transcriptase (20U/µl), and 8.16 µl of RNase-free water (TaqMan® MicroRNA Reverse Transcription Kit, Applied Biosystems). The RT reactions were incubated as follows: 30 minutes at 16 °C, 30 minutes at 42 °C and 5 minutes at 85 °C. qRT-PCR of miRNA was performed using StepOnePlus Real-Time PCR system (Applied Biosystems). Each 10 µl PCR contained 0.5 µl of RT product, 0.50 µl of TaqMan® Small RNA Assay (20X), 5 µl of TaqMan® Universal PCR Master Mix II (2X) without UNG (Applied Biosystems), and 4 µl of RNase-free water. The reactions were loaded in the MicroAmp Fast Optical 96-well Reaction Plate (Applied Biosystems), centrifuged at 3,000 rpm for 2 minutes and incubated as follows:

Step	**Cycles**	**Temperature**	**Time**
Hot-start activation	1	95°C	10 minutes
Denaturation	40	95°C	15 seconds
Annealing/Extension		60°C	60 seconds

Reactions were run in triplicates and included a no-template control for each gene. Expression levels were normalized against 18S rRNA and calculated using the comparative Ct method. Student's *t*-test was used to test for differences between groups with statistical significance set at $*P < 0.05$. Each group included 10 replicates (n = 10). All targets sequences and assay ID are listed in table 2.1.

Table 2.1. Hypoxia-responsive miRNAs in testis of marine medaka and the assay ID

miRNA	miRNA Sequence (5'-3')	Assay ID
miR-125b-5p	UCCCUGAGACCCUAACUUGUGA	000449
miR-103b	UCAUAGCCCUGUACAAUGCUGCU	121115_mat
miR-204-5p	UUCCCUUUGUCAUCCUAUGCCU	000508
miR-451a	AAACCGUUACCAUUACUGAGUU	001141

2.5 Quantitative Real Time PCR (qRT-PCR) of Hypoxia-Responsive mRNAs

qRT-PCR was performed to confirm the expression patterns of the 5 selected mRNAs from the testis of marine medaka, which were found to be hypoxia-responsive using transcriptome sequencing (Wang et al., 2016). Genomic DNA removal and reverse transcription was conducted using RNase-free DNase kit (Promega, Cat# M6101) and QuantiTect reverse transcription kit (Qiagen) as previously described (Wang et al., 2016), while some samples were prepared using PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa, Cat# RR047A). Each 10 µl genomic DNA elimination reaction contained 1 µg total RNA, 2 µl of gDNA Eraser Buffer (5X), 1 µl of gDNA Eraser, and an appropriate volume of RNase-free water to reach a final volume of 10 µl. The reaction was incubated as follows: 2 minutes at 42 °C and 5 seconds at 85 °C. Each 20 µl reverse transcription reaction contained 10 µl of samples from the previous step, 4 µl of PrimeScript Buffer 2 (5X), 1 µl of PrimeScript RT Enzyme Mix I, 1 µl of RT Primer Mix, and 4 µl of RNase-free water. The RT reaction was then incubated as follows: 15 minutes at 37 °C and 5 seconds at 85 °C. The PCR product can be stored at -20 °C.

qRT-PCR of mRNA was performed using either 7500 Real-Time PCR system (Applied Biosystems) or StepOnePlus Real-Time PCR system (Applied Biosystems). Each 10 µl PCR contained 2 µl of template DNA, 5 µl GoTaq® qPCR Master Mix (2X) (Promega), 0.2 µl of forward and reverse primer of the target gene respectively (10 µM), and 2.6 µl of nuclease-free water. The reactions were loaded in the MicroAmp Fast Optical 96-well Reaction Plate (Applied Biosystems), centrifuged at 3,000 rpm for 2 minutes and incubated as follows:

Step	**Cycles**	**Temperature**	**Time**
Hot-start activation	1	95°C	2 minutes
Denaturation	40	95°C	15 seconds
Annealing/Extension		60°C	60 seconds
Meth curve	1	95°C	15 seconds
		60°C	60 seconds
		95°C	15 seconds

Reactions were run in triplicate and included a no-template control for each gene. Expression levels were normalized against 18S rRNA and calculated using the comparative Ct method. Student's *t*-test was used to test for differences between groups with statistical significance set at $*P < 0.05$. Each group included 22 replicates (n = 22). The primer sequences of the genes were designed by Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and listed in table 2.2.

Table 2.2. Forward and reverse primer sequences used for qRT-PCR

Genes	**Forward primer sequence (5' → 3')**	**Reverse primer sequence (5' → 3')**
PIM1	GTCAAAAAGGGGCGTTTCCC	ACAGTAAAAGACGGAGCGCA
COR1C	TTTAAGGAACCTTTGCCCCGA	GTTTGGCAGGACTCAGACCC
K2C8	CCTTCTCCCGCAGCACTATC	TGGTCGGACTCAACGACAAG
PNMA1	GAGCCAGCCAGGAGATTGTT	CATACAGACAGCTAGGCCCG
PPM1D	AGCACCAGCAGTGTTCAACT	AAACGCTCCAATGATCCGGT
18S (Internal control)	GACAAATCGCTCCACCAACT	CCTGCGGCTTAATTTGACCC

Chapter 3
Results

3.1 Screening of Transgenerational Hypoxia-Responsive miRNA-mRNA Pairs

In order to understand the regulatory role of miRNAs on reproductive functions of marine medaka testis under hypoxia, 5 miRNA-mRNA pairs that fulfill the following requirements are selected to be the focus of this study: (1) The miRNAs and their target mRNAs are hypoxia-responsive; (2) The mRNAs could be targeted by miRNA and regulated through RNAi; (3) The expression of miRNA and mRNA has an inverse correlation; (4) The mRNAs are co-regulated transgenerationally; and (5) The mRNAs are associated with reproductive function.

The workflow of screening miRNA-mRNA pairs is explained in Figure 3.1. In the previous study (Tse et al., 2016), small RNA sequencing identified 14 hypoxia-responsive miRNAs in marine medaka testis and miRanda algorithm predicted 3257 genes to be the mRNA target of those hypoxia-responsive miRNAs. In another study (Wang et al., 2016), transcriptome sequencing identified 2411 genes in marine medaka testis are differentially expressed under hypoxia. The target mRNAs were then overlaid with the hypoxia-responsive mRNAs to identify pairs with a direct inverse correlation between the expression of miRNAs and genes, i.e. when the miRNA is upregulated in response to hypoxia, its predicted mRNA target should be downregulated, and vice versa (Tse et al., 2016). To further explore the role of miRNAs on transgenerational effect, 24 genes that are co-regulated transgenerationally, i.e. genes that are co-upregulated or co-downregulated in F0H, F2T, and F2H groups, were selected from 1833 differentially expressed genes (Figure 3.2). In the last step of screening, transgenerationally co-regulated genes that could be targeted by hypoxic-responsive miRNAs but not associate with reproductive functions were filtered out (see section 3.2). Ultimately, 5 miRNA-mRNA pairs that are associated with reproductive functions and have a transgenerational inverse correlation in expression level were selected.

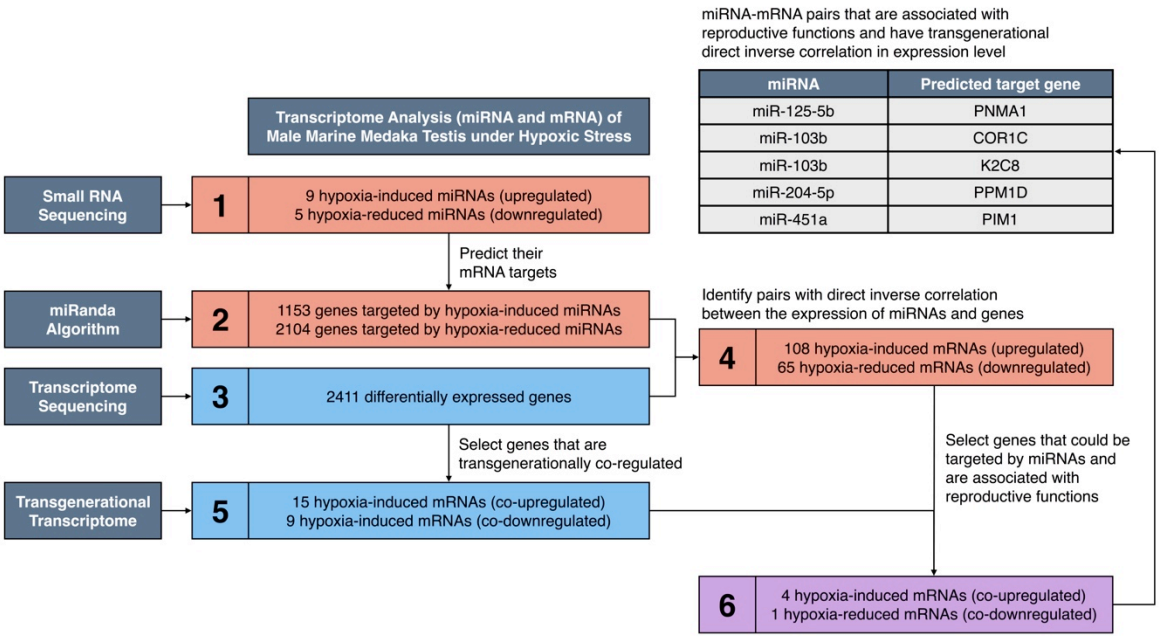

Figure 3.1. The workflow of screening for transgenerational hypoxia-responsive miRNA-mRNA pairs. (1) Small RNA sequencing of male marine medaka testis identified 9 upregulated and 5 downregulated miRNAs under hypoxic stress. (2) miRanda algorithm analysis predicted hypoxia-induced and hypoxia-reduced miRNAs target 1153 and 2104 genes, respectively. (3) Transcriptome sequencing of male marine medaka testis identified 2411 differentially expressed genes under hypoxic stress. (4) Overlaying the results of small RNA sequencing and transcriptome data identified 108 upregulated and 65 downregulated genes that have an inverse correlation with the expression of selected miRNAs and genes. (5) Analysis of the transgenerational transcriptome identified 15 co-upregulated and 9 co-downregulated genes in F0H, F2T, and F2H groups. (6) Overlaying the results of 4 and 5 identified genes that are transgenerationally co-regulated and targeted by miRNAs, coupled with the literature review on the functions of those genes, 4 co-upregulated and 1 co-regulated genes that are associated with reproductive functions were selected. The table in the top-right corner lists 5 miRNA-mRNA pairs that are potentially associated with reproductive functions and have a transgenerational direct inverse correlation in expression level.

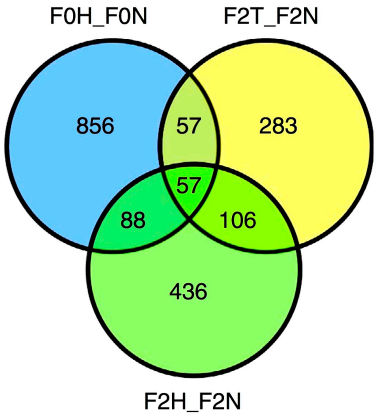

Figure 3.2. Venn diagram of the genes that are co-upregulated or co-downregulated transgenerationally among F0H_F0N, F2T_F2N and F2H_F2N. In the 57 differentially expressed genes overlapped among three groups, 15 genes were co-upregulated and 9 genes were co-regulated (n = 6). Modified from Wang (2016).

3.2 Rationale for the Selected miRNA-mRNA Pairs

During bioinformatics screening, a literature search was done to identify the functions of hypoxia-responsive genes that fulfill the first 4 requirements, and only 5 mRNAs were found to be related to reproductive functions, steroidogenesis, spermatogenesis, oogenesis, sexual development or apoptosis. The functions of the 5 transgenerationally co-regulated mRNA are explained in Table 1.1, along with the seed sequence of hypoxia-response miRNAs. The 5 candidate genes are neuron- and testis-specific protein 1 (PNMA1), coronin-3 (COR1C), type-II keratin Kb8 (K2C8), p53-induced protein phosphate 1 (PPM1D), and serine/threonine-protein kinase pim-1 (PIM1). PNMA1 is involved in the positive regulation of apoptosis (Chen & D'Mello, 2010) and spermatogenesis (Dalmau et al. 1999). COR1C is an established modulator of actin cytoskeleton dynamics (Behrens et al., 2016), and may have additional roles in spermatogenesis (Larance et al., 2011) and apoptosis. K2C8 is involved in ovarian tumorigenicity and apoptosis (Collodoro et al., 2012). PPM1D stimulates progesterone receptor activity and negatively regulate cell proliferation (Proia et al., 2006). PIM1 plays vital roles in cell cycle progression, apoptosis, tumorigenesis (Wang et al., 2001; Bachmann & Möröy, 2005) and oogenesis (Guzmán et al., 2014).

Table 3.1. Selected hypoxia-responsive miRNA-mRNA pairs in testis of marine medaka. Using the miRanda algorithm, 14 hypoxia-responsive miRNAs were predicted to control 3257 gene targets (mRNAs). Screening of miRNA-mRNA pairs identified 5 mRNAs that are targeted by hypoxia-responsive miRNAs. These 5 genes were recently reported in Wang et al. (2016) to be co-regulated transgenerationally and associated with reproductive functions such as steroidogenesis, spermatogenesis, oogenesis, sexual development or apoptosis. The seed sequence of hypoxia-response miRNAs are listed below: miR-125b-5p targets PNMA1 mRNA; miR-103b targets COR1C and K2C8 mRNAs; miR-204-5p targets PPM1D mRNA; and miR-451a targets PIM1 mRNA. The optimal complementary sequence between the seed sequence of the miRNAs and corresponding mRNA targets are marked with vertical bar (|). The less optimal complementary sequence (G:U wobble pair) are marked with colon (:) but it scores less than the optimal matches.

Pair	miRNA	Gene target	Gene symbol	Gene functions	Hypoxia-responsive miRNAs targeted sequences of selected genes associated with reproduction
1	miR-125-5b	Neuron- and testis-specific protein 1	PNMA1	Positive regulation of apoptosis (RefSeq, 2016; Chen & D'Mello, 2010); Spermatogenesis (Dalmau et al., 1999)	miR-125b-5p 3' AGUGUUCAAUCCCAGAGUCCCU 5' PNMA1 5' AACAAAATGGAAATCTCAGGGT 3'
2	miR-103b	Coronin-3	COR1C	Modulator of actin cytoskeleton dynamics (Behrens et al., 2016); Spermatogenesis (Larance et al., 2011); Apoptosis (RefSeq, 2016)	miR-103b 3' UCGUCGUAACAUGUCCCGAUACU 5' COR1C 5' ATCTCCCTTAAGAACGGCTATGT 3'
3	miR-103b	Type-II keratin Kb8	K2C8	Ovarian tumorigenicity; Apoptosis (Collodoro et al., 2012)	miR-103b 3' UCGUCGUAACAUGUCCCGAUACU 5' K2C8 5' ATCCACTTCCTTCTTGGCTATGA 3'
4	miR-204-5p	p53-induced protein phosphatase 1	PPM1D	Progesterone receptor activity (Proia et al., 2006), Negative regulation of cell proliferation	miR-204-5p 3' UCCGUAUCCUACUGUUUCCCUU 5' PPM1D 5' ATAAAGAACGTATTAAAGGGAT 3'
5	miR-451a	Serine/threonine -protein kinase pim-1	PIM1	Cell cycle progression, apoptosis and tumorigenesis (Wang et al., 2001; Bachmann & Möröy, 2005); Oogenesis (Guzmán et al., 2014)	miR-451a 3' UUGAGUCAUUACCAUUGCCAAA 5' PIM1 5' GTCAAAGGGAGTCTAACGGTTC 3'

3.3 Effect of Hypoxia on Sperm Motility in Marine Medaka

In an attempt to validate the observations by Wang et al. (2016) that prolonged exposure to hypoxia causes reproductive impairment, in particular, sperm motility in adult male medaka, I performed a normoxia and hypoxia exposure experiment for 6 weeks. Although a slight decrease in mean curvilinear velocity (VCL) was observed, the mean straight line velocity (VSL) and mean angular path velocity (VAP) of sperm in the hypoxic group were not significantly different from the normoxic group of fish (Figure 3.3). In other words, I was not able to repeat the observations on sperm motility in hypoxic marine medaka males. Some errors may have occurred during maintenance of medaka under hypoxia, or the water was not sufficiently hypoxic or there was an error of judgment on my part in the measurement of sperm motility. For this reason, testes samples from the normoxic and hypoxic male fish that were used in the study by Wang et al. (2016) were used here for qRT-PCR measurement of the four hypoxia-responsive miRNAs and 5 target mRNAs described above.

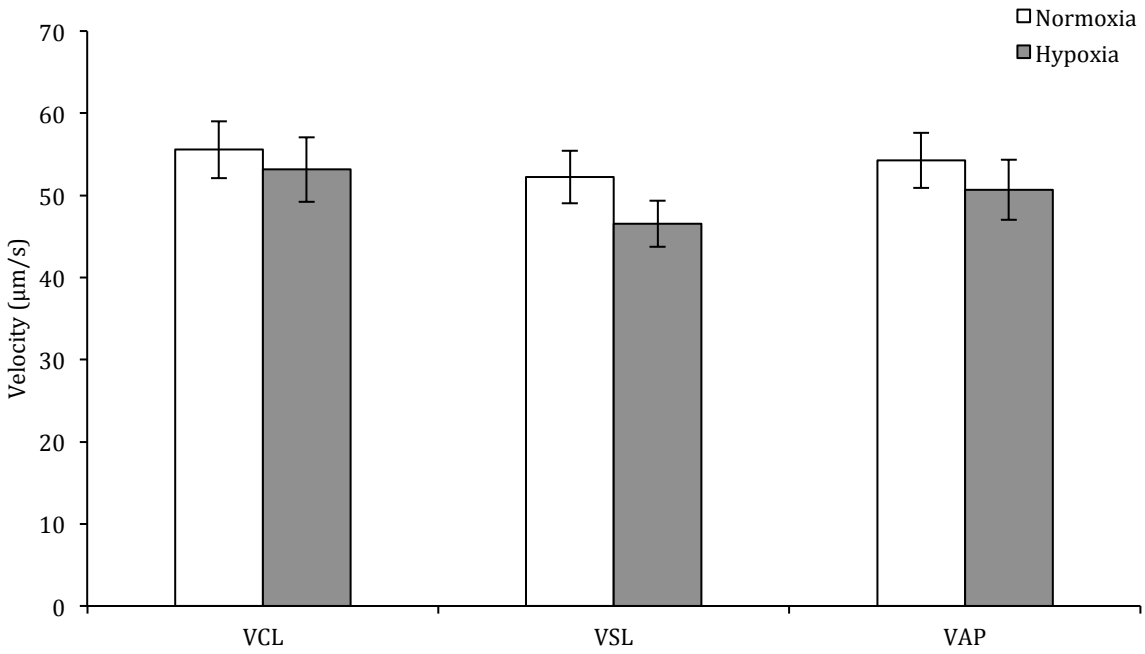

Figure 3.3. Sperm motility of marine medaka in normoxia and hypoxia groups. VCL, mean curvilinear velocity; VSL, mean straight line velocity; VAP, mean angular path velocity. Data are presented as the means ± S.E.M. (*$P < 0.05$) (n = 4).

3.4 Effect of Hypoxia on Transcriptome of Marine Medaka

The results of small RNA sequencing (Tse et al., 2016) and mRNA transcriptome sequencing (RNA seq) (Wang et al., 2016) have suggested differential expression of the selected miRNA-mRNAs in testis of normoxic and hypoxic marine medaka (Table 3.1). However, RNA-Seq is merely a high-throughput method used to provide an initial indication of the gene expression and variation The qRT-PCR technique provides higher sensitivity and is normally performed to validate RNA-seq transcriptome data to determine the trend and magnitude of fold-change. Figure 3.4 shows that the expression of 4 miRNAs in testes of normoxic and hypoxic fish. The qRT-PCR data from this project showed that only miR-204-5p expression was significantly reduced by 3.5 fold ($P < 0.05$) in hypoxic testes, and agreed with the small RNA transcriptome data reported by Tse et al. (2016).

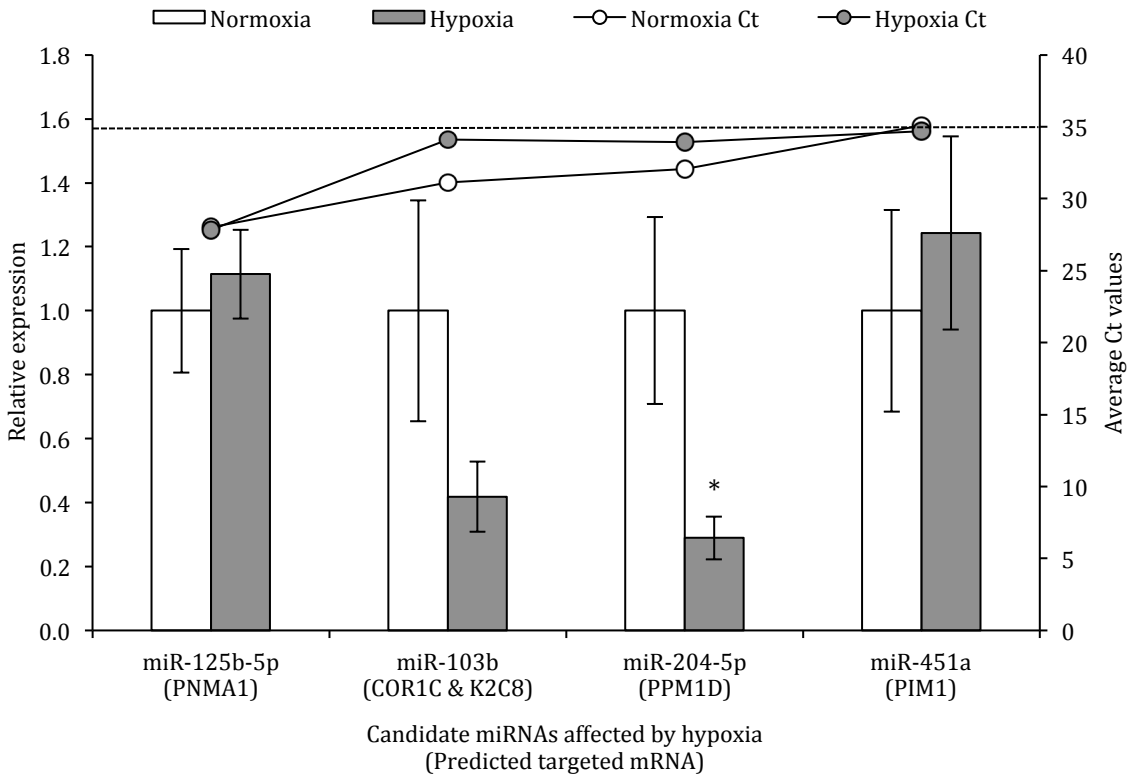

Figure 3.4. Validation of hypoxia-responsive miRNAs identified by small RNA sequencing using qRT-PCR. TaqMan® MicroRNA Assay was used to determine the deregulation of miR-125b-5p, miR-103b, miR-204-5p and miR-451a caused by hypoxia. Data are presented as the means ± S.E.M. (*$P < 0.05$) (n = 10).

Of the 5 mRNA targets measured by qRT-PCR, Figure 3.5 shows that only PIM1 mRNA expression was significantly reduced by 1.8 fold ($P < 0.05$) compared to the normoxic control., which was the only gene that yields significant difference. However, qRT-PCR analysis of its predicted regulatory miRNA (miR-451a) was not significantly upregulated in hypoxic testes (Figure 3.2). PIM1 is a highly conserved serine/threonine-protein kinase pim-1 that is involved in cell cycle progression, apoptosis, and tumorigenesis.

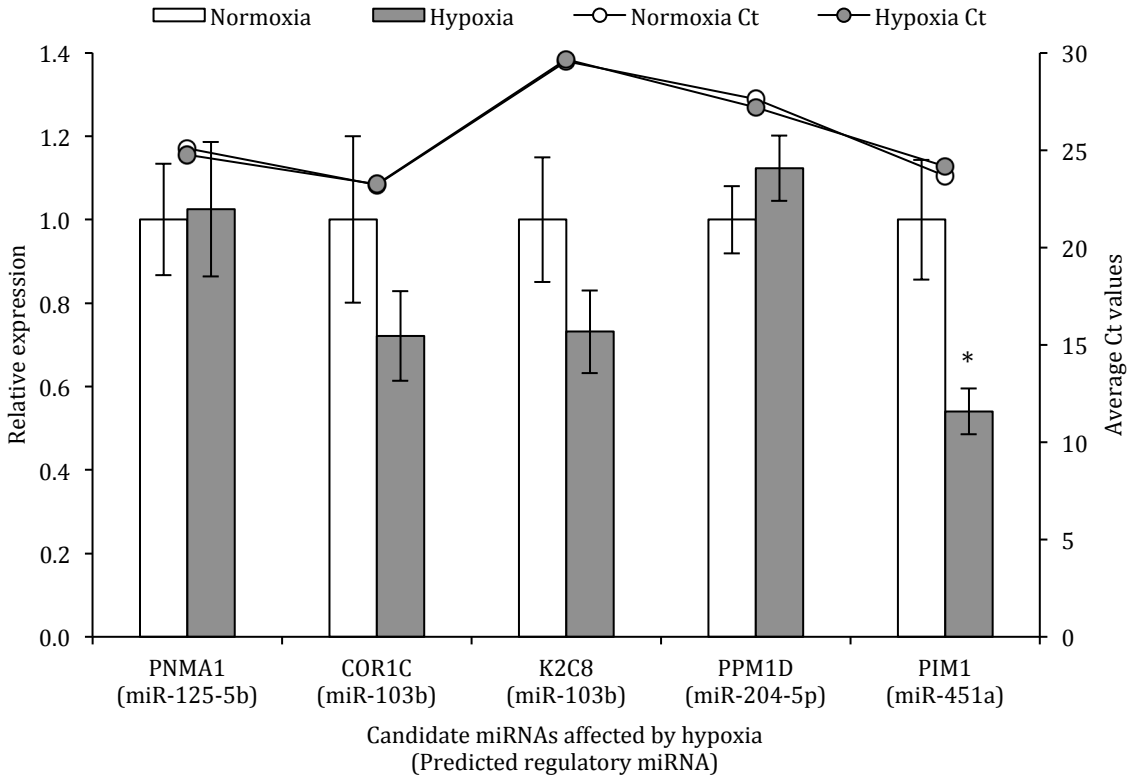

Figure 3.5. Validation of hypoxia-responsive genes identified by RNA-seq using qRT-PCR. GoTaq® qPCR Assay was used to examine the hypoxia-induced differential gene expression of PNMA1, COR1C, K2C8, PPM1D, and PIM1. Data are presented as the means ± S.E.M. (*$P < 0.05$) (n = 22).

Based on the qRT-PCR results, the expression pattern of miRNAs and mRNAs are only marginally consistent, respectively, with the previous results of small RNA sequencing by Tse et al. (2016) and RNA-seq (transcriptome) sequencing by Wang et al. (2016), and most of the results are not statistically significant. None of the predicted miRNA-mRNA pairs showed a significant inverse correlation expression pattern (Table 3.2). For example, although expression of miR-204-5p is significantly downregulated in hypoxic testes, up-regulation of its predicted mRNA target (PPM1D) is not significant. Similarly, even though the expression of PIM1 is significantly downregulated, its predicted regulatory miRNA (miR-451a) is not significantly upregulated in hypoxic testes as compare to the normoxic control.

Table 3.2. Validation of the small RNA sequencing and transcriptome sequencing result, and the inverse correlation between the expression of miRNAs and predicted target genes. Results of qRT-PCR that have a significant difference in expression are marked with asterisks (*). Results that are consistent and significant are highlighted in GREEN.

Pair	miRNAs	Results of small RNA sequencing	Results of qRT-PCR on miRNAs	mRNAs	Results of transcriptome sequencing	Results of qRT-PCR on mRNAs
1	miR-125-5b	Downregulated	Upregulated	PNMA1	Upregulated	Upregulated
2	miR-103b	Downregulated	Downregulated	COR1C	Upregulated	Downregulated
3	miR-103b	Downregulated	Downregulated	K2C8	Upregulated	Downregulated
4	miR-204-5p	Downregulated	Downregulated*	PPM1D	Upregulated	Upregulated
5	miR-451a	Upregulated	Upregulated	PIM1	Downregulated	Downregulated*

3.5 Characterization of Medaka PIM1 and Multiple Sequence Alignment Analysis

The qRT-PCR results showed that PIM1 is the only mRNA that has a significant difference in expression under hypoxia and studies on the functions of PIM1 proteins (Wang et al., 2001; Bachmann & Möröy, 2005) suggested it is associated with cell survival, cell cycle progression, apoptosis, differentiation, and proliferation, so characterization of medaka PIM1 was performed. The full-length *omPIM1* cDNA was obtained and assembled from the transcriptome sequencing data by Lai et al. (2015). The 2118-bp cDNA contains an open reading frame of 1506-bp, which encodes a polypeptide of 501 amino acids (Figure 3.4). Multiple sequence alignment showed that omPIM1 shares high sequence identity with the PIM1 of Japanese Medaka (63%) and moderate sequence identity with the PIM1s of other bonyfishes, rat, human, and frog (Table 3.2). The PIM1s of most fishes consist of 400-500 amino acids compared to around 300 amino acids in zebrafish, rat, human and frog (Table 3.2), but the significance of the additional amino acids before the catalytic domain (position 231-501) is unknown. Alignment result also indicated omPIM1 contains all of the functional domains that are highly conserved in PIM1s from other vertebrate species (Figure 3.5) (Hanks et al., 1988; Reeves et al., 1990; Icard-Liepkalns et al., 1999; Marchler-Bauer A, 2017). It is believed that these regions are involved in ATP binding, phosphorylation or substrate-polypeptide binding. Except for regions 6, 7 and 8 which are more conserved in bonyfishes than other vertebrates, there is no substantial difference between bonyfishes and other vertebrates (Table 3.3). Further investigation can be done to find out the functional importance of such difference. Based on these sequence identities, omPIM1 represents the marine medaka homolog of vertebrate PIM1 serine/threonine-protein kinase.

Table 3.3. The species of PIM1 proteins used for multiple sequence alignment. The cross-species identities were calculated using Clustal Omega (McWilliam et al., 2013) and the sources of the sequence are listed in the rightmost column. omPIM1 shared high sequence identity with the PIM1 of Japanese Medaka (63%) (highlighted in BLUE). The PIM1s of most fishes have 400-500 amino acids (highlighted in ORANGE). compared to around 300 amino acids in zebrafish, rat, human and frog (highlighted in TEAL). Some sequences of the selected PIM1 proteins are published (highlighted in GREEN) and some are not (highlighted in RED).

Abbreviation	Common Name	Scientific Name	Identity	Length (bp)	Sources
omPIM1	Marine Medaka	*Oryzias melastigma*	-	501	Lai et al., 2015
olPIM1	Japanese Medaka	*Oryzias latipes*	63%	443	Unpublished, Genbank: XP_011492215
lcPIM1	Barramundi Perch	*Lates calcarifer*	42%	455	Unpublished, Genbank: XP_018548135
mzPIM1	Zebra Mbuna	*Maylandia zebra*	42%	495	Unpublished, Genbank: XP_014269581
onPIM1	Nile Tilapia	*Oreochromis niloticus*	44%	414	Unpublished, Genbank: XP_019217914
croakerPIM1	Large Yellow Croaker	*Larimichthys crocea*	38%	524	Genbank: KKF24757
zebraPIM1	Zebrafish	*Danio rerio*	34%	336	Genbank: NP_001116186
ratPIM1	Rat	*Rattus norvegicus*	35%	313	Genbank: NP_058730
humanPIM1	Human	*Homo sapiens*	34%	313	Genbank: AAH20224
frogPIM1	Frog	*Xenopus laevis*	32%	337	Genbank: NP_001088619

Table 3.4. Sequence identity of conserved domains of omPIM1 to different PIM1 proteins. Region 6, 7 and 8 are more conserved in bonyfishes (the sequence identity is higher than 50%; highlighted in GREEN) than other vertebrates (the sequence identity is lower than 40%; highlighted in RED), there is no substantial difference between bonyfishes and other vertebrates in the conserved domains.

Region	Percent sequence identity of omPIM1 and:								
	olPIM1	**lcPIM1**	**mzPIM1**	**onPIM1**	**croakerPIM1**	**zebraPIM1**	**ratPIM1**	**humanPIM1**	**frogPIM1**
1	58.33	58.33	58.33	50.00	58.33	66.67	66.67	66.67	50.00
2	100.00	100.00	100.00	75.00	100.00	100.00	100.00	100.00	100.00
3	100.00	85.71	57.14	57.14	57.14	71.43	57.14	57.14	57.14
4	80.00	70.00	80.00	70.00	70.00	50.00	70.00	70.00	70.00
5	87.50	100.00	87.50	87.50	100.00	50.00	62.50	62.50	62.50
6	75.00	75.00	62.50	75.00	50.00	62.50	37.50	37.50	37.50
7	70.59	58.82	68.75	68.75	64.71	47.06	35.29	35.29	35.29
8	72.73	63.64	36.36	45.45	54.55	45.45	36.36	36.36	36.36

```
GCAACACGCTTCCAC AACACAAAGAGTTTT AGTCGGTAGCTGACC ATTCGCAAGTAGCTG AGAAAAACTTTGATC        1
GTTTCACTCGTGTTT GGGAAAAAACAGCAA AAGATCATGGTTAAA AATTCTGCTAAGGAT AATCCAAAATGTACT       76
                                        M  V  K    N  S  A  K  D    N  P  K  C  T
GATTCCAGCAGTAAA ACACGTCAAAAACCT TTTGACAATAAACAA ACCAAAAAGGTTGAG AGGAAACCCAAGTCT      151
 D  S  S  S  K    T  R  Q  K  P    F  D  N  K  Q    T  K  K  V  E    R  K  P  K  S
GAAACCAAGAAGCAG AAGAAAAAAGTGAAA TTCTTGAAACCACGT GAGTCTCCAGAACCT GAACCAACACGGCCA      226
 E  T  K  K  Q    K  K  K  V  K    F  L  K  P  R    E  S  P  E  P    E  P  T  R  P
TCTACCAGCACCCAA GACAACCCCCCAGTG AGAGGACTAAAGAGA AAACGACAGGAAGAC CAACAGGAAGAAGAA      301
 S  T  S  T  Q    D  N  P  P  V    R  G  L  K  R    K  R  Q  E  D    Q  Q  E  E  E
ATCATTGAGCCAAAG AAGAGGAGACCTTGT CTTAAAGCTTCCAGC AGCAGTGCAGCCCAA CAAAGCAGCAAAACG      376
 I  I  E  P  K    K  R  R  P  C    L  K  A  S  S    S  S  A  A  Q    Q  S  S  K  T
TTTTCTGATGACCAA ACCAAAGAAGTCAAG AGAAAGCCCAAACCT GAACCAAAGAAAAAG AAGAAAAAAGTGAAA      451
 F  S  D  D  Q    T  K  E  V  K    R  K  P  K  P    E  P  K  K  K    K  K  K  V  K
CACTGTGAACAGACC GAGTCTGATATCGGT CAGGACAACACCCGA GTGAGAGGACTAAAG AGGAAAAGACAGGAG      526
 H  C  E  Q  T    E  S  D  I  G    Q  D  N  T  R    V  R  G  L  K    R  K  R  Q  E
GACGAAGACGAAGAC ACAAGAGACCAACCA AAGATAATGAAGCCA TCTTTGAAGAGCTAC CAAGAACTGAGGGAT      601
 D  E  D  E  D    T  R  D  Q  P    K  I  M  K  P    S  L  K  S  Y    Q  E  L  R  D
TTATACAATAAGATG AAAGGAAACAAAACG AGTGGCCAAGAAAAG ACACTTTTAAAGAGT TCAGCAAATGAAAAA      676
 L  Y  N  K  M    K  G  N  K  T    S  G  Q  E  K    T  L  L  K  S    S  A  N  E  K
GAATCAAACCAAGAA ATCCCAATCAAAAAG ACGGAGAGAGACGAC GTGATCGACCAAAAA GCCGAGTTTGAAGCC      751
 E  S  N  Q  E    I  P  I  K  K    T  E  R  D  D    V  I  D  Q  K    A  E  F  E  A
AAATATGTGGAAGAA GATCAGTTTGGATCA GGAGGTTTTGGATCC ATTCATGCCGGCTTT CGCAGATCTGACAAT      826
 K  Y  V  E  E    D  Q  F  G  S    G  G  F  G  S    I  H  A  G  F    R  R  S  D  N
CTACCAGTAGCCATC AAACACATTCTTAAA TGCTACATTAGAAAC AAAGAGCTGGATGAG AATGGGAACTTCATC      901
 L  P  V  A  I    K  H  I  L  K    C  Y  I  R  N    K  E  L  D  E    N  G  N  F  I
CCTTCTGAAGTGGCC ATTCTGCTAAAGCTA CGAGATGAATCCCTT CAGTCCAAAGACAAA GCAGCACCAGTACCG      976
 P  S  E  V  A    I  L  L  K  L    R  D  E  S  L    Q  S  K  D  K    A  A  P  V  P
CTGTTGGACTGGTAT GAGATAGGCAGAGAA GTCATTCTAGTCATG GAGAGACCAATCCCT TGTGAGGACATGTAC     1051
 L  L  D  W  Y    E  I  G  R  E    V  I  L  V  M    E  R  P  I  P    C  E  D  M  Y
GATTACATCGAAAAC AAAGGAGGGAAACTG CAAGAAGAAGAAGCC AAGATCATAATGAAA CAACTAATTCACACA     1126
 D  Y  I  E  N    K  G  G  K  L    Q  E  E  E  A    K  I  I  M  K    Q  L  I  H  T
GCCATAGATCTTGAG GAGAGAAACATCTTT CATCAGGACATCAAA ATTGATAACATCCTC ATTCAGACCAGCTCA     1201
 A  I  D  L  E    E  R  N  I  F    H  Q  D  I  K    I  D  N  I  L    I  Q  T  S  S
GACGTCCCTCGCGCT CGTCTGATCGACTTT GGACTGAGCTGCATG GCGGAAAAAGATACC ATCTTCAGAGTCTTT     1276
 D  V  P  R  A    R  L  I  D  F    G  L  S  C  M    A  E  K  D  T    I  F  R  V  F
TCAGGAACTCCAGCA CATGGCCCTCCAGAA TATTTTAAAGGTTTT TCTAGTCCGGGATCA ACCACAGTCTGGCAA     1351
 S  G  T  P  A    H  G  P  P  E    Y  F  K  G  F    S  S  P  G  S    T  T  V  W  Q
CTCGGAGTAGTTTTG TATGAAAGCCTCCAT GACCCTATGAGCTTC TCAACGCGGGATTTT GTCAAAGGGAGTCTA     1426
 L  G  V  V  L    Y  E  S  L  H    D  P  M  S  F    S  T  R  D  F    V  K  G  S  L
ACGGTTCACGATAAA CTGTCAGAAGAGTGC CAGGACTTCTTTCGA GCATGCTTGAACTTA AACCAGGAGAGACGC     1501
 T  V  H  D  K    L  S  E  E  C    Q  D  F  F  R    A  C  L  N  L    N  Q  E  R  R
GCCCAGCTGAAGGAC CTCCTGCATCATCCA TGGCTAAGATGAATT CACAACACTGCACAT GAAACAGTCAAGACA     1576
 A  Q  L  K  D    L  L  H  H  P    W  L  R  *
CTAAAACACACATGA GGACAGGGAACTGGA AACCTTGAAACATTA GAGCTGGAATAGGTC AAAAAGGGGCGTTTC     1651
CCCTCATTTTTGGTC GTGTTTACCAACCCT TTTCTTAAAGTACCC CAAACTTAAATGATG CGCTCCGTCTTTTAC     1726
TGTTTATTTCCTTTG GTTTTCTGGTTTATG CTTCACCGATCATGA AAATTTAAACTTTTT TATATATATGTATAT     1801
TATTCAATTGACAGT GTATATCTCTGTTTT GTTATTTTAAACTTA AAATGTATATGTAGA GGGTAAAGGAAATAG     1876
ATTGAGATACTATGC TTTAGGTTTCGACTC TGATCAATATGCGTG ATTTAGATTTAGAAC TGGAATTGGTCAAAA     1951
GTGAAGGTTCTCCTA ATTTTAGGTCCTTTA CCCCAAACTTAAATG ATACGCTGTATTCTG TATTCTCATTTCTGA     2026
GATAACCTCCACTTT                                                                    2101
```

Figure 3.6. Nucleotide and deduced amino acid (below) sequences of marine medaka PIM1. The number on the right denotes the nucleotide position.

```
omPIM1          MVKNSAKDNPKCTDSSSKTRQKPFDNK-QTKKVERKPKSETKKQKKKVKFLKPRESPEPE     59
olPIM1          MDKDSAMDNP-------------------------KHTVERKKRKKKGTFPVPIETPESE     35
lcPIM1          ------------------------------------------------------MDK-      3
mzPIM1          MKKETRKNT------NSGDKKDPGDQNSKSRMAKRKASPEKKTPVKRR------RVTEQ-     47
onPIM1          MNKETRKTT------SPADNKD-------------------------------------     16
croakerPIM1     MDDQT-------------------------RRGKRKASPAPKKAKRKR------TRVSK-     28
zebraPIM1       ------------------------------------------------------------      0
ratPIM1         ------------------------------------------------------------      0
humanPIM1       ------------------------------------------------------------      0
frogPIM1        ------------------------------------------------------------      0

omPIM1          PTRPSTSTQDNPPVRGLKRKRQEDQQEEEIIEPKK--------RRPCLKASSSSAAQQSS     111
olPIM1          PSQQSTSTQDNTPVRKLKRKKQEDKEEEEIIEPKK--------VKTPLKDDGQTK-----     82
lcPIM1          --EPAKRIKTH---RCLAR--RRDQERSGSTTQQS----TASAMDGRMRST----K-REA     47
mzPIM1          --AAPSTSSDTHRVKAVKRKAQQDGAGTTSPPK-KIKLAEPTSGDREQASSSVDAGKD--     102
onPIM1          ------------------------------------------------PGDQN--         21
croakerPIM1     --SPPRNTPEKNVNRRVKRKVREDGEESPKTKKRAFDITHLTQDDDEMASSSADTGRDSS     86
zebraPIM1       ------------------------------------------------------------      0
ratPIM1         ------------------------------------------------------------      0
humanPIM1       ------------------------------------------------------------      0
frogPIM1        ------------------------------------------------------------      0

omPIM1          KTFSDDQTKEVKRKPKPEPKKKKKKV-KHC--------EQTESDIGQDNTRVRGLKRKRQ     162
olPIM1          ---------NAERKSFPRKKKQKKKHLNHKPIESSAPEPSQPSASTQDKTQVTTLKRKRQ     133
lcPIM1          SPVKNTQSKK-----------------------TKVSRMTEPCSNSEEDSSVRGKKRKDR     84
mzPIM1          -----SKSRMAKRKASPEKKTPVKRRRVTE------QAAPSTSSDTHRVKAV---KRKAQ     148
onPIM1          -----CESRMAKRKASPERKTPVKRRRVTE------EAAPSTSSDTHEVKGV---KRKLQ     67
croakerPIM1     RPFKEVSERKRKRKASNDGGKPKRKRSVDI-VETTQDVTINACRDKFEDKYVQQNQLGE-     144
zebraPIM1       ------------------------------------------------------------      0
ratPIM1         ------------------------------------------------------------      0
humanPIM1       ------------------------------------------------------------      0
frogPIM1        ------------------------------------------------------------      0

omPIM1          EDEDEDTRDQ-------------------------------------------PKIMK     177
olPIM1          EDKEEEEVIG-------------------------------------------PKKRK     148
lcPIM1          DNGDAPSKKTKKQKGGLLNQYNREST----------------------SSS--------     113
mzPIM1          Q-----------------------------------------DGAG---TTSPPKKIK     162
onPIM1          Q-----------------------------------------DAAG---TTSPAKKSK     81
croakerPIM1     -----------GGCGSVFAGYRKADNLPVAIKHVPNEKVFCKHVDNNGKKISVEVATMLK     193
zebraPIM1       ------------------------------------------------------MAQ      3
ratPIM1         -------------------------------------------------------ML      2
humanPIM1       -------------------------------------------------------ML      2
frogPIM1        -------------------------------------------------------ML      2

omPIM1          -------------------PSLKSYQELRDLYNKMKGNKTSGQE------KT--LLKSSA     210
olPIM1          --------------------TTLKDS---------------------------SS      156
lcPIM1          ----VETDRESSSN-----PLEGCSTALEHVNKRSSKRKAAADADGQ-TRKTKRSLDLKE     163
mzPIM1          LAEPTSGDREQASSSV--DAGKDCNQNTKGCTKNGR-SKSAGDSKE--SPKKK-------     210
onPIM1          LAEPTSGDEEQASSSL--DAGKDCSQSTNGCMKNGK-RKAAGDSKE--PPERR-------     129
croakerPIM1     LAEGTTGVGTSASV-----ALLDCSRPFKEVSERKRKRKAS--NDGG-KPKRKRSVDIVE     245
zebraPIM1       MAERGSRSRSRSAAFTQTAVQDTETR------------DASGGLHGYLAPLSSLLADSVS     51
ratPIM1         LSKIN--------SL-------------------------AHL---RAAPCN--------     18
humanPIM1       LSKIN--------SL-------------------------AHL---RAAPCN--------     18
frogPIM1        LAAQG--------RFTEPHMALQCYY------------RQGHSPTGAATPSSV-------     35
```

Catalytic Domain

ATP Binding

```
omPIM1          NEKESNQEIPIKKTERDDVIDQKAEFEAKYVEEDQFGSGGFGSIHAGFRRSDNLPVAIKH     270
olPIM1          SDA----E-QTATSSEKTEITDKDKFEAKYVEKYQFAEGGYGSIYGGFRKSDNLPVAIKH     211
lcPIM1          SKNNTQEETKKETKEESLFRARRAQFEAKYTEQNQLGEGGCGSVFAGYRNADNLPVAIKH     223
mzPIM1          -----KKDKTKNVVQ-NSVVDQQREYQARYVQEHHLGNGGFGAVFAGYRITDHFPVAIKH     264
onPIM1          -----KRDKTKHSAQ-NSAADQRREFQARYVEEHHLGGGGCGAVFAGYRITDHFPIAIKH     183
croakerPIM1     ------------TTQDVTINACRDKFEDKYVQQNQLGEGGCGSVFAGYRKADNLPVAIKH     293
zebraPIM1       -TDQRKRKRKQQTASTSSRRPAKRSRRDQYAKGPLLGRGGFGSVFAGIRRSDGLPVAIKY     110
ratPIM1         ----------DLHANKLAPGKEKEPLESQYQVGPLLGSGGFGSVYSGIRVADNLPVAIKH     68
humanPIM1       ----------DLHATKLAPGKEKEPLESQYQVGPLLGSGGFGSVYSGIRVSDNLPVAIKH     68
frogPIM1        ---------FSAAADQGSVCEVKEPFENCYQVGPVIGTGGFGTVYSGVRISDKLPVAIKH     86
                                :   .  *     :. ** *::..* * :* :*:***:
```

Region 1 **Region 2**

Identification and characterization of hypoxia-responsive microRNAs and target genes potentially associated with regulation of reproductive functions in male marine medaka

```
omPIM1          ILKCYIRNKELDE-NGNFIPSEVAILLKLRDESLQSKDKAAPVPLLDWYEIGREVILVME     329
olPIM1          ILKCFIRNKQLDDGSGKMVPSEVAIMLKLHDESIPSDGKAAPVALLDWYDIGREILLVME     271
lcPIM1          IPNDKVLCKEVDH-NGRQLSVEVAVMLKLAAKNNGSVGTSAPVSLLDWYDLGKELILVQE     282
mzPIM1          IPKDKIPIQVTDE-NGKEVSVEVAILLKLAAEADGSVGTSAPVSLLDWFDFGTELILVQE     323
onPIM1          IPKDRIPIKVRDE-NGKLVSVEVAIMLKLAAEADGSVGTSAPVSLLDWFDLGTELILVLE     242
croakerPIM1     VPNEKVFCKHVDN-NGKKISVEVATMLKLAEGT-TGVGTSASVALLDWYDLDKELILVME     351
zebraPIM1       VSKEPTDTRLKVD-GQGRLPLEVALMTRVSSA----PVCPSVLQLLDWFDHRRRYVLILE     165
ratPIM1         VEKDRISDWGELP-NGTRVPMEVVLLKKVSS------GFSGVIRLLDWFERPDSFVLILE     121
humanPIM1       VEKDRISDWGELP-NGTRVPMEVVLLKKVSS------GFSGVIRLLDWFERPDSFVLILE     121
frogPIM1        VSRDRIGEMKH-M-NGTLVPLEIYLLKKVSN------GCRGVIRLLDWYERPDGFIIIME     138
                : .           .   :  *:  : ::          . : ****::     ::: *

                Active Site                                  ATP Binding

omPIM1          RPIPCEDMYDYIENKGGKLQEEEAKIIMKQLIHTAIDLEERNIFHQDIKIDNILIQTSSD     389
olPIM1          RPIPCEDLFHYIDTKGGTLEEEEAKIIMTQLIRTAIDLEDRNIFHQDIKSENILIQSHSN     331
lcPIM1          RPVPCEDLLQYIEDNGGSLQEDEAKIILKQLVYAATELQEMKIFHRDIKVENILIETDSE     342
mzPIM1          RPVPAVDLFEYIRENGGCLPEGKAKVILKQLVDAAKDLEEKQIFHRDIKSDNILIETGSD     383
onPIM1          RPVPAVDLHDYITENGGCLPEEKAKVILKQLVDAAKDLEDKHIFHRDIKSENILIETGSD     302
croakerPIM1     RPVPAVDLLKYIQGKGGSIDENEAKIIMKQLVDAAKELKDKCIFHRDIKVENILIETGSG     411
zebraPIM1       RPAPCQDLQSFCEEN-GCLDEPLAKKVLVQLIAALKHCESRRVLHRDVKPENLLISTDSH     224
ratPIM1         RPEPVQDLFDFITER-GALQEELARSFFWQVLEAVRHCHNCGVLHRDIKDENILIDLNRG     180
humanPIM1       RPEPVQDLFDFITER-GALQEELARSFFWQVLEAVRHCHNCGVLHRDIKDENILIDLNRG     180
frogPIM1        RPEPVQDLFDFITER-GALGEELASNFFRQVVEAVRHCHSCDVVHRDIKDENILVDLRTA     197
                ** *  *:  :   . * : *  *  .: *:: :  . ..  :.*:*:* :*:*:.

                Region 3                                     Region 4

                          Autophosphorylation                          Catalytic
                                                                       Domain
omPIM1          VPRARLIDFGLSCMAEKDTIFRVFSGTPAHGPPEYFKGF-SSPGSTTVWQLGVVLYESLH     448
olPIM1          TPQARLIDFGVSCMAEKDSILRGFSGTPINAPPEAFKGF-CSPGSTTVWQLGVVLYETLH     390
lcPIM1          VPRVRLIDFGLSCFFKKRSLYRVFYGTPAHIPPEFYSRKTYWAGPTTVWQVGVVLFEMLH     402
mzPIM1          VPRVRIIDFGLSCFATEQSQFCFFYGTPIHSPPECYWGKKYRPGPTTVWQMGVVLYEALH     443
onPIM1          VPRVRIIDFGLSCFAKARSLYRVFYGTPIHTPPECYGCKKYKAGPTTVWQMGVVLYEALH     362
croakerPIM1     TPRLRLIDFGLSCFVKEKSRYHIFYGTPDHIPPEWFRHNTYHAGPTTVWQVGVVLYESVH     471
zebraPIM1       --DIKLLDFGCGDLM-KDSAYRYFAGTPAFAPPEWFRHHRYHASPLTVWSIGVTLYNILC     281
ratPIM1         --ELKLIDFGSGALL-KDTVYTDFDGTRVYSPPEWIRYHRYHGRSAAVWSLGILLYDMVC     237
humanPIM1       --ELKLIDFGSGALL-KDTVYTDFDGTRVYSPPEWIRYHRYHGRSAAVWSLGILLYDMVC     237
frogPIM1        --ELKLIDFGSGALL-RDAVYTDFDGTRVYSPPEWIRFHKYHGKSATVWSLGILLYDMVC     254
                    :::*** . :    :    * **    ***            :**.:*: *:: :

                Region 5          Region 6                     Region 7

                                Autophosphorylation
omPIM1          DPMSFSTRDF--VKGSLTVHDKLSEECQDFFRACLNLNQERRAQLKDLLHHPWLR-----     501
olPIM1          SHGDFSTMDF--LEGDLMIDSELSEECQDFFQACLNTSEEQRPYLQDLLHHPWLR-----     443
lcPIM1          RNTQFETPRF--LRDELKISNTLSEDCQDFLQMCLTKVPEERATLEQLLLHPWLR-----     455
mzPIM1          VG-DFNIMTF--IENELKFNEHLSPHCRNFLDACLTEVPEKRPTLGDLQRHPWLR-----     495
onPIM1          VG-DFNTVTF--LQKELIFNKDLSPHCQNFLDACLTNVPEKRPTLGDLQLHPWLR-----     414
croakerPIM1     GETRFETESF--TRNKQKISNELSKKCQDFLKGCLAKVPEDRPTLEQLKLHPWFR-----     524
zebraPIM1       DCFPFRGAQRVTSKSRLHFPRKLSTECRQLIRWCLSAAPADRPSLDDIERHPWLQ-----     336
ratPIM1         GDIPFEHDEEI-VKGQVYFRQRVSSECQHLIRWCLSLRPSDRPSFEEIQNHPWMQDVLLP     296
humanPIM1       GDIPFEHDEEI-IRGQVFFRQRVSSECQHLIRWCLALRPSDRPTFEEIQNHPWMQDVLLP     296
frogPIM1        GDIPFEHDDEI-LKGKIQYRCRVSRECQHLIEWCLSKRPSDRPSLEQILAHPWMSQDNFL     313
                    *         .         :* .*:.::  **      *  : ::  ***:

                                Region 8

omPIM1          -------------------------  501
olPIM1          -------------------------  443
lcPIM1          -------------------------  455
mzPIM1          -------------------------  495
onPIM1          -------------------------  414
croakerPIM1     -------------------------  524
zebraPIM1       -------------------------  336
ratPIM1         QATAEIHLH-SLS---PSPSK---  313
humanPIM1       QETAEIHLH-SLS---PGPSK---  313
frogPIM1        DKKDNGKVSRKLDQEATTTSKDSL  337
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Figure 3.7. Multiple sequence alignment of the deduced amino acid sequence of omPIM1 with selected PIM1 homologues. The number on the right denotes the amino acid position. Amino acids fully conserved by all protein sequences are marked with asterisks (*). Conservation between groups of strongly and weakly similar properties are marked with colon (:) and period (.) respectively. Dashes (--) indicate gaps inserted for improved alignment. Conserved regions (1-8) of vertebrate PIM1s and their presumptive functions are labeled underneath and above respectively.

Chapter 4
Discussion

4.1 Effect of Hypoxia on Reproductive Functions of Marine Medaka

The negative effect of hypoxia on fish reproductive physiology has strong experimental support, such as retarded gonad development and gametogenesis, decreased number and quality of gametes (Thomas et al., 2006, 2007). In this study, I exposed male medaka (3 months old) to normoxic and hypoxic conditions for 6 weeks and then compared the sperm motility of the fish using the method as described by Wang et al. (2016). Although there was a slight decrease in sperm motility in the hypoxic group of fish, the data was not statistically significant. The dissolved oxygen level of normoxia group in this study (4.5 ± 0.5 mg L^{-1}) is lower than the level in the previous study (5.8 ± 0.5 mg L^{-1}) and the normal in the marine environment (7-8 mg L^{-1}). Fishes in normoxia group may live in a suboptimal condition such that they also suffered from hypoxia and expressed a certain degree of reproductive response. In fact, the sperm motility (VCL, VSL, and VAP) of normoxia group in this study (53-55 μm/s) is lower than that of F0H group in the previous study (70-80 μm/s). Since behavioral adaptation to hypoxia, like aquatic surface respiration (ASR), were observed in the hypoxic group and its sperm motility is similar to the previous study, the insignificant result may not be because hypoxia has no effect on sperm motility but a biased normoxia (control) group.

4.2 Effect of Hypoxia on Transcriptome of Marine Medaka

Among the 4 miRNAs that were found to be differentially expressed in hypoxic testis in an earlier study (Tse et al. 2016), the expression pattern of only 1 miRNA (the downregulated miR-204-5p) was reproducible by qRT-PCR. However, the average Ct values of two miRNAs (miR-103b and miR-451a) are close to the 35-cycle detection limit and most are beyond 30. The low expression level some of the miRNAs may render the qRT-PCR results inaccurate. A large variation in the expression level was also observed in several miRNAs (see the S.E.M in Figure 3.2). This is very likely due to the dynamic profile of miRNAs in testis, epididymis, sperm precursors and spermatozoa during different stages of development, such phenomenon has been found in mouse and primates (Yan et al., 2007, 2009; Rodger et al., 2013; Nixon et al., 2015a, 2015b). In the case of marine medaka maintained under laboratory conditions, reproduction (spawning) occurs very frequently (daily to weekly), which may result in a very dynamic profile of miRNAs between individuals' testis. If possible, the spatial and temporal miRNA profile in testis should be characterized.

For mRNAs, qRT-PCR confirmed that the expression pattern of only 1 mRNA target (the downregulated PIM1) is consistent with the mRNA transcriptome data of Wang et al. (2016). One of the objectives of this project is to establish the regulatory role of miRNA-mediated gene silencing in hypoxic-induced reproductive impairment by identifying the miRNA-mRNA pairs that show an inverse correlation in the expression level in hypoxic testes. MiRNAs are known to inhibit gene expression at the post-transcriptional level by one of two mechanisms: deadenylation promotes mRNA decay, and translational repression inhibits translation, i.e. the latter would reduce the protein level without changing the expression of mRNA. Early study found that miRNAs can repress translation of mRNAs with little or no influence on their abundance (Nottrott, Simard, & Richter, 2006). In this study, quantification of gene expression level by qRT-PCR only characterize the effect on mRNA level, while that of protein level is not considered. Although the transcripts of PPM1D, the predicted target gene of the significantly downregulated miR-204-5p, are not significantly upregulated under hypoxia, its protein expression could be upregulated if there are less miR-204-5p inhibiting the translation. Further investigation on the miRNA-mRNA interaction could be done *in vivo*, for example, luciferase reporter assay to confirm whether those miRNAs specifically interact with the predicted mRNA targets (Yu et al., 2015) and western blot analysis to measure the expression level of proteins (PPM1D, PIM1, etc.) using specific antibodies.

4.3 PIM1 Multiple Sequence Alignment and its Possible Roles

The result of PIM1 multiple sequence alignment showed the deduced omPIM1 proteins contains all of the functional motifs that are conserved in other vertebrate PIM1s (Figure 3.5) and shares especially high sequence identity with the PIM1 from Japanese Medaka (63%) and moderate sequence identity (32-42%) with PIM1s from other vertebrate species. Except for regions 6, 7 and 8, the sequence identity of the other functional domains is highly similar between fish and other vertebrates (Table 3.3). Overall, the functional domains of PIM1 are highly conserved from fish to human (Icard-Liepkalns et al., 1999) but the structure and regulation of PIM1 in fish were not characterized. Existing knowledge concludes that PIM1 is associated with apoptosis and cell proliferation. PIM1 has been proved to play an important role in the critical processes of hematopoiesis and lymphopoiesis by repressing apoptosis, promoting cell cycle progression and mediating transcription repressing proteins (Bachmann & Möröy, 2005). It is very likely that PIM1 has the same role in the testis of marine medaka, therefore its down-regulation during hypoxia may impair spermatogenesis, ultimately leading to a decrease in sperm number and motility. However, overexpression and knockdown of PIM1 experiments will have to be carried out to test this hypothesis.

4.4 Future Studies

In this study, the results of the qRT-PCR experiments are quite inconclusive (due to large error bars) and have raised more questions. It is recommended to first confirm the sequence of amplified products using Sanger DNA sequencing and perform the experiment again to see if it is reproducible. In addition, there are several questions that have to be considered and they are important in filling the knowledge gap on how hypoxia leads to reproductive impairments. First, how hypoxia leads to the described transcriptome changes (the differential expression of miRNAs and mRNAs). Although the regulatory role of the miRNAs on the predicted mRNAs has not been established in this study, other miRNAs have been proved to be a critical downstream mediator of hypoxia stress (Lai et al., 2015; Tse et al., 2015, 2016), so it is important to understand how miRNA expression is regulated. Hypoxia has been identified as an endocrine disruptor that can affect steroidogenesis through the HPG axis so it is possible that the change in steroid levels could eventually lead to transcriptomic changes through signal transduction. On the other hand, since histone methylase was found be downregulated under hypoxia (Wang et al., 2016), the involvement of other epigenetic changes cannot be omitted. Second, experiments in this study was carried out on testes of the first generation of fish exposed to hypoxia (F0H) with the aim to identifying hypoxia-responsive miRNA-mRNA pairs that may have a role in reproductive functions, qRT-PCR analysis of the miRNAs and target mRNAs should also be performed on F1H, F2H, F1T, and F2T males to determine whether the change is transgenerational effect. If such effect was found to be transgenerational, the next question is how are those epigenetic modifications (differential expression of miRNAs) inherited through sperms, i.e. the exact mechanism of RNA-mediated paternal inheritance.

Chapter 5
Conclusion

An important recent discovery about the effect of hypoxia on fish reproduction was made by Wang (2016), who reported that hypoxia can cause transgenerational reproductive impairments in male marine medaka through inheritable epigenetic changes in testis, indicating that the adverse impact of aquatic hypoxia is potentially more serious than we previously thought. A follow-up study further suggested the possible role of hypoxia-responsive miRNAs in targeting epigenetic changes that regulate testicular functions (Tse et al., 2016), and apoptosis and steroidogenesis in the ovary (Tse et al., 2015; Lai et al., 2016). This project was initiated as a follow-up of these earlier work to identify hypoxia-responsive miRNA-mRNA pairs in medaka testes that show an inverse correlation expression pattern using the qRT-PCR technique. In conclusion, qRT-PCR confirmed that expression of the PIM1 gene is downregulated in hypoxic testes and may serve as an intermediate gene that mediates the adverse effect of hypoxia to impair spermatogenesis.

Acknowledgement

First, I would like to thank Dr. Richard Kong and Dr. Ball Lai for their continuous guidance, support and contribution throughout the research, particularly on experimental design, data analysis, data interpretation and result presentation. Also, I would like to thank Dr. Cindy Tan, Mr. Michael Chiang, Dr. Frauke Seemann, Mr. Peterson Drew Ryan, and Miss Amy Chong for their patience, assistance and valuable advice on various aspects of this project.

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