Aquatic Toxicology 177 (2016) 476–483

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Transcriptomic responses of marine medaka's ovary to hypoxia

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ARTICLE INFO

Article history:
Received 19 April 2016
Received in revised form 24 June 2016
Accepted 27 June 2016
Available online 28 June 2016

Keywords:
Transcriptome
Ovary
Hypoxia
Bioinformatics

ABSTRACT

Hypoxia, an endocrine disruptor, is pressing global problem affecting marine organisms in over 400 "Dead Zones" worldwide. There is growing evident demonstrated the disruptive effect of hypoxia on reproductive systems of marine fish through the impairments of steroidogenic gene expression, leading to the alteration of sex hormone production in gonads. But the detailed molecular mechanism underlying the responses of female reproductive systems to hypoxic stress remains largely unknown. In the present report, we used marine medaka *Oryzias melastigma* as a model, together with high-throughput transcriptome sequencing and bioinformatics analysis, aiming to determine the changes in transcriptional signature in the ovary of marine fish under hypoxic stress. Our result discovered over two hundred differential expressed genes in ovary in response to hypoxia. The bioinformatics analysis together with quantitative RT-PCR validation on the deregulated genes highlighted the dysregulations of a number of female reproductive functions including interruptions of ovarian follicle development, gonad development and steroid metabolic process. Additionally, we revealed that these deregulations are through the modulation of leukemia inhibitory factor (LIF), insulin-like growth factor 1 receptor (IGF1R) and follicle stimulating hormone (FSH). The result of this work complements previous studies and provides additional insights into the underlying molecular mechanism of hypoxia-induced impairment of female reproductive system.

1. Introduction

Hypoxia is pressing global problem affecting marine and freshwater organisms. Over 400 "Dead Zones" have been identified worldwide by the United Nation (Diaz and Rosenberg, 2008). Hypoxia was found to cause severe reproductive impairments in fish, leading to a heavy loss of fisheries production over large areas (Wu, 2002). Cumulative laboratory and field evidence demonstrated hypoxia can affect both male and female reproductive systems (Lu et al., 2014; Martinovic et al., 2009; Wu et al., 2003). For example, hypoxia was found to suppress spermatogenesis and decrease sperm motility in carp (Wu et al., 2003), impair testicular development and sperm production, thereby reducing reproductive success of Atlantic croaker (Thomas and Rahman, 2009, 2010). Retardation of gonadal development was also observed in ovary of both carp and zebrafish (Wu et al., 2003). Numerous reports attributed these reproductive impairments to the suppression of steroidogenic gene expression, leading to the alteration of sex hormone production in fish gonads (Shang and Wu, 2004; Wang et al., 2008; Wu et al., 2003). But, there is hitherto no genome-wide transcriptomic study to investigate the effect of hypoxia on reproductive impairments in fish. Notably, male and female may have differential responses to different environmental stresses. For example, our previous studies demonstrated a gender-specific transcriptional profiling of the liver in marine medaka upon BDE-47 exposure (Yu et al., 2013). More interestingly, adult males and females of the plainfin midshipman exhibited different hypoxia

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http://dx.doi.org/10.1016/j.aquatox.2016.06.023
0166-445X/

tolerance (LeMoine et al., 2014). In our previous report, we have demonstrated that hypoxia could dysregulate a cluster of genes that are involved in p53 signaling and cell cycle regulation (Wang et al., 2016), suggesting that hypoxia have disrupted normal sperm production through perturbations of cell cycle and apoptosis-related processes. However, the mechanism underlying the responses of female reproductive systems to hypoxic stress remains largely unexplored.

In this report, we conducted transcriptome sequencing on hypoxia-exposed ovary of the marine medaka (*Oryzias melastigma*), followed by comprehensive bioinformatics analysis, aiming to determine the molecular and functional responses of ovary to hypoxic stress. The results of functional gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and Ingenuity® Pathway Analysis (IPA) demonstrated dysregulation of different signaling pathways mediating female reproductive behavior in response to hypoxic stress. Our data revealed the dysregulation of ovarian follicle development, gonad development and steroid metabolic process through the modulation of activators including leukemia inhibitory factor (LIF), insulin-like growth factor 1 receptor (IGF1R) and follicle stimulating hormone (FSH) in the ovary under hypoxic stress. The result of this report not only provides valuable resources for the better understanding of the molecular networks of hypoxic stress responses, but also provides novel biomarkers for assessing the effects of hypoxia on female fish reproduction.

2. Materials and methods

2.1. Medaka maintenance and hypoxic exposure

All animal research procedures were approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR, #2714-12), the University of Hong Kong. The stock of marine medaka used in our experiment was obtained from Interocean Industries (Taiwan) and has been reared in our laboratory for over 10 generations. The marine medaka were maintained under optimal growth and breeding conditions (5.8 mg O_2/L, 28 ± 2 °C, pH 7.2 in a 14-h light: 10-h dark cycle) as previously described (Lau et al., 2014). The fish were fed with hormone-free Aquatox Feed (Zeigler Bros. Inc. Gardners, PA) and live artemia. Fifty sexually mature female marine medaka (3 months-old) were reared under normoxia (5.8 ± 0.2 mg O_2/L) or hypoxia (1.5 ± 0.2 mg O_2/L) for one month. Hypoxic condition was generated by pumping nitrogen into water through a mass flow meter (Voegtlin, Aesch, Switzerland). The dissolved oxygen level was measured twice daily, and fine-tuned by the mass flow meter.

2.2. RNA isolation and qualification

After the exposure, the fish were anesthetized on an ice bath and the ovary tissues were dissected from randomly selected female fish (n = 6 from normoxia and n = 6 from hypoxia). Three ovaries per treatment group were used to prepare one pooled RNA sample. Two replicate pooled RNA samples were prepared from the normoxic control and hypoxia treatment. Total RNA from the pooled tissue samples was extracted using the mirVana™ RNA isolation kit (Applied Biosystems). RNA quality was assessed using the Agilent 2100 Bioanalyzer system and samples with a RNA Integrity Number (RIN) greater than 9 were used for RNA library construction.

2.3. RNA library construction and sequencing

Four RNA (cDNA) libraries of ovaries were constructed (2 for the normoxic group, 2 for the hypoxic group) using the TruSeq Stranded RNA LT Sample Prep Kit (Illumina, San Diego, USA), each prepared from 300 ng of total RNA. The cDNA libraries were prepared according to manufacturer's instruction as previously described (Gu et al., 2015). Briefly, Index codes were ligated to identify individual samples. mRNA was purified from the total RNA using poly-T oligo-attached magnetic beads (Illumina), and was then fragmented using divalent cations in the Illumina fragmentation buffer under 94 °C for 1 min. First and second strand cDNAs were synthesized using random oligonucleotides and SuperScript II, followed by DNA polymerase I and RNase H. Overhangs were blunted by using exonuclease/polymerase and, after 3′ end adenylation, Illumina PE adapter oligonucleotides were ligated. DNA fragments that ligated with adaptor molecules on both ends were enriched using the Illumina PCR Primer Cocktail in a 15-cycle PCR reaction. Products were purified and quantified using the AMPure XP and the Agilent Bioanalyzer 2100 systems, respectively. Then the concentration of libraries was quantified using KAPA Library Quantification Kits. Paired-end reads, each of 150 bp read-length, were sequenced on the Illumina MiSeq sequencer.

2.4. Illumina sequencing data analysis

Adapters and reads containing poly-N were first trimmed and the sequence-reads were dynamically trimmed according to BWA's −q algorithm. Briefly, a running sum algorithm was executed in which a cumulative area-plot is plotted from 3′-end to the 5′-end of the sequence reads and where positions with a base-calling Phred quality lower than 30 cause an increase of the area and vice versa. Such plot was built for each read individually and each read was trimmed from the 3′-end to the position where the area was greatest. Read-pairs were then synchronized such that all read-pairs with sequence on both sides longer than 35 bp after quality trimming were retained, and any singleton read resulting from read trimming was removed (Lai et al., 2015; Li et al., 2014). All the downstream analyses were based on quality-trimmed reads. Sequencing reads were mapped to the assembled transcripts using Novoalign (v3.00.05) with parameter −r ALL to report all multi-mapped reads (http://www.novocraft.com/). Alignment files were sorted using Samtools (http://samtools.sourceforge.net/) to generate a read-name sorted BAM file. Then, "Samtools view −F 0 × 4" was used to parse the mapped reads from the BAM file and the number of read-pairs mapping to each transcript in each sample were summarized to generate a count table (http://seqanswers.com/forums/showthread.php?t=29745) (Li et al., 2012). Ambiguously mapped read-pairs with each end mapped to different transcripts were discarded. Read-count data were then subjected to differential expression analysis using the edgeR package (Robinson et al., 2010).

2.5. Differential gene expression and comparative pathway analysis

Samples with identical treatments (normoxic or hypoxic) were considered to be biological replicates. Genes with B&H corrected p-value < 0.05 and |log2 (fold change)|>1 were considered to show statistically significant differential expression. The differentially expressed genes were subjected to GO functional enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis using GeneCodis tool to determine molecular functions, biological processes and biochemical pathways of these deregulated genes (Carmona-Saez et al., 2007; Nogales-Cadenas et al., 2009; Tabas-Madrid et al., 2012). Furthermore, Ingenuity Pathway Analysis (IPA®,QIAGEN Redwood City, www.qiagen.com/ingenuity) was used to decipher the molecular interaction networks that are deregulated by hypoxic stress.