Estradiol regulates expression of miRNAs associated with myogenesis in rainbow trout

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Abstract

17 β -Estradiol (E2) is a steroid hormone that negatively affects muscle growth in rainbow trout, but the mechanism associated with this response is not fully understood. To better characterize the effects of E2 on muscle, we identified differentially regulated microRNAs (miRNAs) and muscle atrophy-related transcripts in juvenile rainbow trout exposed to E2. Small RNA-Seq analysis of E2-treated vs. control muscle identified 36 differentially expressed miRNAs including those known to be involved in myogenesis, cell cycle, apoptosis, and cell death. Some important myogenic miRNAs, such as miR-133 and miR-206, are upregulated while others like miR-145 and miR-499, are downregulated. Gene Ontology analysis of the target genes regulated by the miRNAs involved in atrophy and cell cycle indicates that E2 influence leads to expansion of quiescent myogenic precursor cell population to address atrophying mature muscle in rainbow trout during sexual development.

Key Words: microRNA; myogenesis; rainbow trout; estradiol; apoptosis; cell cycle

Abbreviations: ER – Estrogen Receptor; MPC – Myogenic Precursor Cells; E2 - 17 β -estradiol; MRFs – Myogenic Regulatory Factors; MOMP – Mitochondrial Outer Membrane Permeability

1. Introduction

Skeletal muscle in rainbow trout accounts for more than 50% of the total weight and exhibits indeterminate growth unlike other vertebrates (Mommsen and Moon, 2001). Quiescent myogenic precursor cells (MPCs), destined to muscle cell lineage, lie underneath the basal lamina of muscle fibers and contribute to both hypertrophic and hyperplastic growth (Mommsen and Moon, 2001, Johnston, Bower and Macqueen, 2011). Myogenesis is initiated by activation of MPCs that are further proliferated and differentiated to form a mature muscle fiber or fuse to the existing fibers. This process involves coordinated communication among signaling pathways, epigenetic and different myogenic regulatory factors. Among several factors that control muscle synthesis and maintenance, hormonal regulation plays a vital role. Fish muscle harbors many hormone receptors capable of transducing signals into the cells and eventually contributing to changes in muscle metabolism. One example of such receptors are estrogen receptors (ERs) which bind and are activated by 17β -estradiol (E2). Four isoforms of ERs belonging to two different subtypes, ER α and ER β , are present in rainbow trout (Nagler, Cavileer, Sullivan et al., 2007).

17β -Estradiol production and release is greatly increased during sexual maturation (Lubzens, Young, Bobe et al., 2010) in rainbow trout, driving high energy demanding metabolic processes during the follicular growth phase of vitellogenesis, including synthesis of vitellogenin by liver (Anderson, Olsen, Matsumura et al., 1996). Earlier studies in cod and Pacific salmon indicate an increase in water content in muscle causing reduction in muscle quality during spawning (Davidson, Kenney, Manor et al., 2014, Love, 1960, Manor, Weber, Cleveland et al., 2015, Salem, Kenney, Rexroad Iii et al., 2010). Reduction in muscle growth during spawning in rainbow trout partially results from an increase in protein turnover (Cleveland and Weber, 2011, Cleveland, Kenney, Manor et al., 2012, Salem, Kenney, Rexroad et al., 2006) stimulated by maturation-related signals such as E2 (Cleveland and Weber, 2011, Cleveland and Weber, 2016). GH/IGF axis plays a major role in myogenesis (Gabillard and Rescan, 2013). Increase in

E2 levels during ovarian development in salmonids corresponds with decrease in IGF-1 (Taylor, Porter, Bromage et al., 2008) as well as disruption of the GH/IGF axis (Hanson, Kittilson, Martin et al., 2014, Hanson, Kittilson, McCormick et al., 2012, Norbeck and Sheridan, 2011, Holloway and Leatherland, 1997). Therefore, negative effects of E2 on muscle growth are partially a result of endocrine mechanisms regulating growth. Findings from a study with ovariectomized mice with estrogen implants showed reduced IGF-1 (a potent mediator of skeletal muscle growth) expression with an increase in myostatin, a negative regulator of muscle development (Tsai, McCormick, Brazeau et al., 2007).

Myogenic regulatory factors (MRFs) have a predominant role in the regulation of myogenic development. Regulatory noncoding RNAs (long noncoding and small RNAs) pose an additional level of gene regulation in the same process. A recent study of long noncoding RNAs in rainbow trout showed their role in skeletal muscle differentiation (Wang, Fu, Koganti et al., 2016). Additionally the function of miRNAs in skeletal muscle development is well addressed in a number of previous studies in vertebrates (Güller and Russell, 2010, Hitachi and Tsuchida, 2013, Luo, Nie and Zhang, 2013, Shenoy and Blelloch, 2014, Sokol, 2012, Wang, 2013, Zhang, Zuo, Yang et al., 2014). Rainbow trout miRNA from various tissues were recently reported (Juanchich, Bardou, Rué et al., 2016), and miRNA as important regulatory factors of energy metabolism in teleosts was well discussed (Mennigen, 2015). miRNAs are small 21-23 nucleotide molecules transcribed either independently or from the intronic regions of protein coding genes. The transcribed primary miRNA is processed successively by Droscha to a hairpin, which is transported to cytoplasm and further processed into a mature miRNA by Dicer. They are involved in post-transcriptional gene regulation by binding to the 3'UTR region of their target mRNAs and in blocking translational initiation resulting in decreased gene expression. Knockdown of *Dicer* gene in mice demonstrated the importance of miRNAs in skeletal muscle development. Both embryonic and postnatal skeletal muscle development were affected in the absence of miRNAs causing hypoplasia and death (O'Rourke et al., 2007). Small RNAs are essential components in the network of skeletal muscle development with very specific functions in proliferation, differentiation and maturation of myosatellite

cells. miR-1, miR-133, and miR-206 are well studied muscle-specific miRNAs (Callis, Chen and Wang, 2007, Chen, Tao, Li et al., 2010, McCarthy and Esser, 2007, McCarthy, 2008, Townley-Tilson, Callis and Wang, 2010, Yuasa, Hagiwara, Ando et al., 2008). They work in coordination with the MRFs and control cell fate. miR-1 promotes skeletal muscle differentiation, while miR-133 regulates proliferation. Along with these myogenic miRNAs, several other miRNAs have been identified for their prominent roles in myogenesis. They not only control the expression of different MRFs (Chen, Mandel, Thomson et al., 2006) but also play a role in signaling pathways controlling muscle cell proliferation and differentiation (Zanou and Gailly, 2013).

The main goal of the present study was to profile differentially expressed miRNAs in rainbow trout skeletal muscle under the influence of E2 relative to normal muscle using next generation sequencing (Illumina). Thirty-six differentially expressed miRNAs including three novel miRNAs were identified using DESeq2 analysis. Known important myogenic miRNAs including miR-206, miR-145 and miR-499, and ubiquitously expressed miRNAs having a role in myogenesis including miR-214, miR-181, miR-125b and let-7 family, were either up regulated or down regulated under the influence of E2. Target gene analysis followed by gene ontology (GO) term enrichment for their roles in molecular function and biological processes revealed their roles in cell cycle and apoptosis. Findings from this study suggest involvement the role of miRNAs and their regulation through E2 in myogenesis. Collectively, these observations assist in understanding the basic mechanisms regulating muscle growth and contribute to the development of markers associated with muscle quality and nutrient partitioning in rainbow trout.

2. Materials and Methods

2.1 Experimental design

Animal experiments were conducted at the USDA/ARS National Center for Cool and Cold Water Aquaculture (NCCCWA) in accordance to methods approved by the NCCCWA Institutional Animal Care and Use Committee, protocol #50. Prior to the study, fish were fed with a commercial feed (Finfish G, Zeigler Brothers, Inc, Gardners, PA) and reared according to standard operating procedures. Feed was withheld the day of hormone injection. Twenty all-female, diploid, juvenile fish weighing approximately 40 g were randomly selected for each treatment from an in-house population. Hormonal treatments included intraperitoneal injections of E2 and or the delivery vehicle to serve as the control. E2 was resuspended at 10 $\mu\text{g}/\mu\text{L}$ in ethanol and diluted to 2.5 $\mu\text{g}/\mu\text{L}$ with vegetable oil. The control treatment contained an equal ratio of ethanol: vegetable oil. Fish were anesthetized with tricaine methanesulphonate (MS-222, 100 mg/l), weighed, and received intraperitoneal injections of E2 or the vehicle (2.0 $\mu\text{L}/\text{g}$ body weight). Injected fish were divided between four tanks (10 fish per treatment per tank). One tank per treatment was harvested 24 and 72 hours post-injection. Skeletal muscle was collected, immediately frozen using liquid nitrogen, and stored at -80°C until analysis.

2.2 RNA isolation

Total RNA from skeletal muscle samples of fish treated with E2 and controls at 24 and 72 hours ($n = 6$). Approximately 100 mg of tissue were homogenized in Trizol reagent (Invitrogen, Carlsbad, CA) using a bench-top homogenizer followed by DNase treatment and column purification of total RNA using a Direct-zol™ RNA MiniPrep kit following the manufacturer's instructions (Zymo Research). The quantity and quality of the total RNA was evaluated by measuring absorbance at 260 nm and 280 nm using NanoDrop 1000 Spectrophotometer (Thermo Scientific). Four biological replicates from each treatment were sequenced.

2.3 miRNA sequencing and data analysis

Small RNA sequencing was performed using services provided by LC Sciences (Houston, TX). Four samples from each group with the highest $A_{260}:A_{280}$ ratios were used for sequencing. The quality of total RNA was confirmed with RNA Integrity Numbers greater than 9.0 determined from Agilent Technologies 2100 Bioanalyser. Nucleotide fractions (15-50) of small RNA was isolated from the total RNA using polyacrylamide gel and were ligated to the 3' adapter followed by 5' adapter (Illumina, San Diego, CA). The small RNA ligated to the adaptors was reverse transcribed to cDNA, PCR amplified and gel purified. The gel-extracted cDNA was used for library preparation, which was further used for cluster generation on Illumina's Cluster Station before sequencing using Illumina GAIIx. Raw sequence data was obtained from image data using Illumina's Sequencing Control Studio software version 2.8 (SCS v2.8) following real-time sequencing image analysis and base-calling by Illumina's Real-Time Analysis version 1.8.70 (RTA v1.8.70). ACGT101-miR v4.2 (LC Sciences) pipeline was used to analyze sequenced data. Sequences with low Q scores, reads mapped to mRNA, RFam, Repbase and piRNA database were deleted and unique families were generated from identical sequences. These filtered unique sequences were then mapped to pre-miRNA and miRNA in miRBase or to the genome of rainbow trout to identify conserved miRNA following ACGT-101 User's Manual. Unique or novel miRNAs were identified after the BLAST performed against Pisces miRNAs from miRbase database (release 19) and published miRNAs from rainbow trout (Salem, Rexroad, Wang et al., 2010, Ma, Hostuttler, Wei et al., 2012, Ma, Weber, Hostuttler et al., 2015, Ramachandra, Salem, Gahr et al., 2008). The miRNAs that do not match any sequences in the specified databases and have the propensity of forming hairpin structure with the extended sequences at the mapped positions were classified as unique miRNAs. These unique miRNAs were further classified into different groups depending on the mappable reads to selected miRNAs in miRbase. Details of the miRNAs and their respective groups are presented in Supplementary Table.1. Differentially expressed miRNAs were identified using statistical software R (Version 3.2.2) package DESeq2. Two different groups were considered for paired analysis: 1) E2 treated group compared to the

control group at 24 hours, and 2) E2 treated group compared to control group at 72 hours.

2.4 Quantitative real time PCR

Validation of differentially expressed miRNAs was performed using quantitative real time PCR (qPCR) using RNA isolated from 6 biological replicates. Two μg of DNase treated total RNA were used in cDNA synthesis using miScript II (Qiagen, Valencia, CA). U6 was used as an endogenous control for normalization. miRNA-specific forward primers (Supplementary Table 1) and a universal reverse primer were used for qPCR. DNA melting curve analysis was performed to determine the specificity of the amplicons. Standard curves with 10-time serial dilutions of a pooled cDNA sample were generated to calculate the efficiency of qPCR. Five μl of 1,024 times diluted cDNA was used with iQTM SYBR[®] Green Supermix (Bio-Rad) and 300 nM of forward and reverse primer in a final volume of 25 μl for the reaction. Cycle conditions were the same for all the primers except the annealing temperatures for different primers. Initial denaturation at 95 °C for 3 minutes followed by a denaturation at 95 °C for 40 sec, annealing for 30 sec, extension at 72 °C for 30 seconds for 40 cycles and a final extension for 10 min at 72 °C. Melt curve analysis was performed with an increase of 0.5 °C increase every cycle. Quantification cycle (Cq) values were used for quantification of expression using the log-linear equation of standard curve. Relative fold changes were calculated by setting the values of the controls to 1.0 and comparing the respective treatment groups. Statistical analysis was performed using Student's t-test and those with a p-value < 0.05 were considered statistically significant.

Relative quantification of target gene transcripts of respective miRNA was determined using qPCR and statistically analyzed following the protocol mentioned above. Gene specific primers used in the analysis are listed in Supplementary Table 2.

2.5 Prediction of target genes

The potential target genes for the differentially expressed miRNAs were predicted using miRanda and PITA algorithms. 3'UTR, 5'UTR and the coding regions were used for prediction of the targets from the protein coding gene database of rainbow trout. Target genes predicted by both algorithms were used for further analysis.

2.6 Gene ontology enrichment analysis

R package GOstats (Falcon and Gentleman, 2007) was used for gene ontology (GO) enrichment (Ashburner, Ball, Blake et al., 2000) of target genes for respective miRNA. The target genes of each miRNA were tested against a gene reference created using the rainbow trout transcriptome sequence (Salem, Rexroad, Wang et al., 2010). Gene ontologies with respect to molecular function and biological processes were identified.

3. Results

3.1 miRNA profile in rainbow trout skeletal muscle

The skeletal muscle samples used for sequencing are from E2 treated and control fish at two time points, 24 hours and 72 hours after injections. A total of 246,336,230 raw reads were generated from 16 samples sequenced (4 biological replicates of each treatment at respective time points), of which 73% are mapped to rainbow trout genome. The mapped reads were further grouped depending on their similarity to the selected miRNAs from miRbase. The reads mapped to the rainbow trout genome and miRbase were further grouped as known miRNAs accounting for about 35.8% of the mapped reads. About 34.4% of the

mapped reads also map to mRNA, RFam and Repbase (Table 1). The size of the reads ranged from 17 to 24 nucleotides, majority being around the size of 22 to 23 (Fig. 1a) with Phred scores higher than 35 indicating 99.9% accuracy in sequencing read.

Myogenic miRNAs including miR-1, miR-133a and miR-206 are among the top five abundantly expressed miRNAs. Other highly expressed miRNAs include miR-100-5p, let-7a-5p, mir-10b-5p and miR-199-5p (Fig. 1b and 1c). The number of unique reads identified account for 1,401, which were further grouped into known and predicted miRNAs. Group 1a in (Fig.1c and Supplementary Table 2) represents known miRNAs in rainbow trout, accounting for approximately 24 % of the total unique miRNAs. The novel miRNAs accounts for about 27 % and are represented as Group 4a. The other major groups are pre-miRs mapped to Pisces but not to the rainbow trout genome (Group 3b). An incomplete rainbow trout genome sequence likely prevents mapping ability.

3.2 Differentially expressed miRNAs

DESeq2 analysis indicated 36 differentially expressed miRNAs between E2 treated and control fish at 24 hours and one differentially expressed miRNA at 72 hours, all with an adjusted p-value less than 0.05. The log₂ fold change ranged from -2.2 to 1.99, with 14 miRNAs downregulated and 22 upregulated. Supplementary Figure 1 is a Volcano plot representing all identified miRNAs. Red dots represent differentially expressed miRNAs (adjusted P-Value < 0.05) identified by DESeq2 and blue dots represent those with a log₂ fold change of ± 1.5 .

Three of the differentially expressed miRNAs at 24 hours are novel miRNAs, including two that were identified in rainbow trout eggs previously (Ma et al., 2012). Further analysis was performed with the differentially expressed miRNA at 24 hours. Pre-miRNA sequences were obtained and the 36

differentially expressed miRNAs were classified into 19 families using miRClassify. Details of the differentially expressed miRNAs (Table 2) including their families and precursor coordinates are presented (Supplementary Table 3). Clustered heatmap (Fig. 2) was generated for the differentially expressed miRNAs using R showing a significant difference in the expression between the E2 treated and control samples.

3.3 Validation of differentially expressed miRNAs

The sequencing data was verified using qPCR for the selected 5 miRNAs (omy-miR-214-3p, omy-miR-499a-5p, omy-miR-23a-3p, omy-miR-145-5p, omy-miR-499-3p) for their role in myogenesis and related processes. The expression of these miRNAs were normalized using U6 snRNA as an internal control. The expression of all 5 miRNAs was down regulated in rainbow trout skeletal muscles under the influence of E2 by RNA-Seq analysis (Fig.3). These results were consistent with qPCR results except for omy-miR-23a-3p, which showed similar expression trend with no significant difference between the E2 treated and control samples in qPCR analysis (Fig. 3).

3.4 Gene ontology enrichment analysis

A significant number of enriched target genes with various molecular functions and biological processes were observed for both up and down regulated miRNAs with a P-value < 0.01. R package GOstats was used for gene ontology enrichment analysis. Highly enriched genes involved in different biological processes include those that have significant roles in different signaling pathways, cell cycle, mitotic phase transition, quiescent stem cell regulation, proliferation and differentiation, muscle cell chemotaxis, contraction and migration, programmed cell death, epigenetic regulation involving DNA methylation and histone modifications. Enriched GO terms in molecular function include phosphoprotein

phosphatase activity, signal transduction, different histone and DNA modifying enzymes, DNA and nucleoside binding, cytoskeleton binding, transcription factor binding, helicase and proteases including ubiquitination, sumoylation activities. The top 10 enriched gene ontologies for biological processes and molecular function for both up-regulated and down-regulated miRNAs are presented in Table 3.

3.5 Validation of miRNA target genes

3.5.1 Regulation of proteolytic enzymes by Omy-miRNA-23a-3p

ClustalW alignment of pre-miR-23a-3p sequences revealed a well-conserved mature miR-23a-3p across different species (Fig. 4a). The miRNA was tested for binding with rainbow trout MuRF1 (HM357611.1) and fbxo32 (HM189693.1) sequences using RNA hybrid (Krüger and Rehmsmeier, 2006). The results showed that Omy-miR-23a-3p targets MuRF1 in the coding region, and fbxo32 at the 3' untranslated region (Fig. 4b). qPCR analysis of the target genes MuRF1 and fbxo32 was performed and the results presented in Fig. 4c. The expression of fbxo32 at 24 hours was increased in E2 treated samples with a fold change of 2.2 while no significant difference in the expression of MuRF1 in 24-hour samples. Additionally, the expression levels of both genes showed no significant difference in 72-hour samples.

Gene ontology enrichment of Omy-miR-23a-3p also revealed its role in regulating mitochondrial outer membrane permeability implicating its role in apoptosis, innate immunity involving natural killer cells, mast cell activation, postsynaptic regulation, leucocyte and lymphocyte mediated immunity, muscle tissue morphogenesis, DNA mediated transposition, pyrimidine metabolism, DNA recombination and integration. Gene ontologies involved in mitochondrial membrane permeability and apoptosis are presented in Table 4. Thirteen target genes of this miRNA regulate mitochondrial organization, regulation and outer membrane permeabilization. These results suggest the role of mitochondrial permeability in

muscle cells under the influence of E2. As the downstream effects of mitochondrial outer membrane permeability, the expression of caspases genes (caspase 3, caspase 8 and caspase 9) were determined by qPCR. The expression of the initiator caspase 9 and executor caspase 3 were higher in E2 treated samples compared to the control samples at 24 hours (P-value < 0.05), while the expression of initiator caspase 8 did not show any difference (Fig. 4d).

3.5.2 Cell cycle control by differentially expressed miRNAs

GO enrichment analysis of the target genes of the differentially expressed miRNAs disclosed their roles in stem cell proliferation, maintenance and phase transition control in mitotic division (Table 5). These results lead to focus on the regulation of proliferation of myosatellite cells in treated samples in comparison to controls. Paired box transcription factor (Pax7) is a marker for quiescent stem cells and is necessary for the commitment of pluripotent cells to myogenic origin (Seale, Sabourin, Girgis-Gabardo et al., 2000), whereas MyoD and/or Myf5 are necessary for myosatellite cells to progress further to differentiate and eventually form a mature muscle cell. Expression profiles of myogenic regulatory factors were determined using qPCR. At 24 hours, the expression of Pax7 in treated samples is significantly higher in E2 treated samples compared to the controls opposing to the expression of the MRF's MyoD. The expression levels of MyoD were decreased at 24 hours under the influence of E2. No significant differences were observed in the expression of Myf5 at two time points (Fig. 5).

3.6 Tissue distribution of novel miRNAs identified

The pre-miRNA sequences of the novel miRNAs were obtained and stem loop structures were predicted using Mfold (Zuker, 2003) (Supplementary Fig. 2). This novel miRNA was named as omy-miR-nov-285-5p. qPCR results showed that this miRNA is present in all tissues analyzed with a

difference in expression levels. The expression of this miRNA is the lowest in liver and highest in testis of the tissues analyzed. Tissue specific relative expression of this miRNA with respect to liver is presented in Fig. 6. The top 10 predicted target genes of this miRNA are presented (Supplementary Table 4).

4. Discussion

Myogenesis is a complex process involving a network of signaling pathways of epigenetic and myogenic regulatory factors. In salmonids, the impact of E2 during ovarian growth contributes to loss in muscle mass or reductions in muscle growth (Cleveland and Weber, 2011, Salem et al., 2006, Cleveland and Weber, 2015) unlike rodent, bovine and porcine species in which E2 has anabolic effects (Rehfeldt, Kalbe, Nürnberg et al., 2009, Kamanga-Sollo, White, Hathaway et al., 2010). Understanding these differences contributing to muscle loss has been an area of emphasis in rainbow trout aquaculture. Noncoding RNAs and post-transcriptional control of miRNA sets an additional layer of control over gene expression. The importance of miRNAs in myosatellite cell maintenance (Cheung, Quach, Charville et al., 2012, Crist, Montarras and Buckingham, 2012), proliferation (Shenoy and Blelloch, 2014, Gammell, 2007, Huang, Chen, Yu et al., 2012) and differentiation (Gagan, Dey, Layer et al., 2011, Liu, Luo, Xiong et al., 2010, Nakajima, Takahashi, Kitamura et al., 2006) are well studied in different physiological and disease states of muscle (Chen, Callis and Wang, 2009, Gambardella, Rinaldi, Lepore et al., 2010, Goljanek-Whysall, Sweetman and Münsterberg, 2012, Kim, Park, Lee et al., 2014, Kornfeld, Biggar and Storey, 2012, Liu, Williams, Maxeiner et al., 2012, Nielsen, Scheele, Yfanti et al., 2010, Rooij, Quiat, Johnson et al., 2009, Rosales, Malik, Sneh et al., 2013). The roles of miRNAs during different metabolic states of muscle including apoptosis (Gammell, 2007, Hirai, Verma, Watanabe et al., 2010, Hudson, Woodworth-Hobbs, Zheng et al., 2014, Wada, Kato, Okutsu et al., 2011) and hypertrophy (Hitachi and Tsuchida, 2013, McCarthy and Esser, 2007, Javed, Jing, Yang et al., 2014) have also been established.

Contribution of miRNAs in regulating different signaling pathways (Hitachi and Tsuchida, 2013, Luo et al., 2013, Granjon, Gustin, Rieusset et al., 2009, Ge, Sun and Chen, 2011) involved in myogenesis states their diverse roles in any living cell. Besides these prominent roles, miRNA also regulate different myogenic regulatory factors (MRFs) and are in turn regulated by different MRFs (Hirai et al., 2010, Gagan, Dey and Dutta, 2012, Naguibneva, Ameyar-Zazoua, Polesskaya et al., 2006, Sweetman, Goljanek, Rathjen et al., 2008, Rao, Kumar, Farkhondeh et al., 2006).

Previous reports in humans, rodents, and zebrafish indicate E2 influence alters the expression of several miRNAs, including miR-125b, miR-145, miR-17-5p, miR-196b, let-7h, miR-206, miR-20a and miR-21 (Dai, Phillips, Zhang et al., 2008, Scott, Goga, Bhaumik et al., 2007, Hossain, Kuo and Saunders, 2006, Kovalchuk, Tryndyak, Montgomery et al., 2007, Cohen, Shmoish, Levi et al., 2008, Adams, Furneaux and White, 2007, Kondo, Toyama, Sugiura et al., 2008), which were also identified to be responsive to E2 treatment in rainbow trout in this study. The expression of miRNA is reported to be also regulated by estrogen receptor alpha and estrogen receptors in turn regulating the expression of miRNAs (Klinge, 2009). Four miRNA were reported to reduce the stability and translation of ER α ; miR-221, miR-222 (Zhao, Lin, Yang et al., 2008), miR-22 (Sun, Estrov, Ji et al., 2008) and miR-206 (Kondo et al., 2008) negatively regulate the expression of ER α , which in turn regulates the expression of miR-206 (Kondo et al., 2008). Conclusively, the data presented here indicate that E2 dependent regulation of miRNA in rainbow trout muscle is supported by numerous studies that identify a significant role of miRNA-related mechanisms in E2-induced responses across different tissue types.

miR-206 is up regulated along with a down regulation of miR-145, miR-181, miR-214, miR-125b-3p and miR-499 after 24 hours in E2 treated fish. In this study, the rainbow trout miR-206 sequence lacks the seed sequence which is conserved among other species. Therefore, additional studies are warranted to further analyze the functional role of this miRNA, however, in rats fast muscle atrophy induced by denervation is also associated with increased miR-206 expression (Li, Li, Li et al., 2016). miR-499 is

344 highly expressed in type I muscles, encoded by myosin genes and is thought to play a role in coordinating
 345 the expression of myosin heavy chain in slow muscle (McCarthy, Esser, Peterson et al., 2009, van Rooij,
 346 Liu and Olson, 2008, van Rooij, Quiat, Johnson et al., 2009). Studies in humans with miR-125b-3p in
 347 different cell lines established its essential role in proliferation of differentiated cells (Lee, Kim, Chung et
 348 al., 2005). Besides the expression of MyoD and myogenin, myosatellite cell differentiation also
 349 corresponds to the activation of miR-214 (Juan, Kumar, Marx et al., 2009), and the significance of miR-
 350 214 is confirmed by embryonic lethality and severe defects in muscle development in mice functional
 351 knockout studies (Watanabe, Sato, Amano et al., 2008). Additionally miR-214 regulates IRS/AKT/PI3K
 352 pathway in C2C12 myocytes (Honardoost, Soleimani, Arefian et al., 2015), and this also supports a role
 353 of miR-214 in the process of differentiation. In the present study, miR-214 is down regulated in fish 24
 354 hours after injection with E2, suggesting that this mechanism is involved with negative effects of E2 on
 355 myogenesis in rainbow trout. An additional miRNA down regulated by E2 was miR-145, which can
 356 regulate the PI3K pathway in humans (Rosales et al., 2013), and targets different factors involved in
 357 proliferation (Martinez and Gregory, 2010), including OCT4, SOX2 and KLF4 (Xu, Papagiannakopoulos,
 358 Pan et al., 2009), and its loss in smooth muscles resulted in increased proliferation with reduced
 359 differentiation (Zhang, 2009). Also down regulated by E2 in rainbow trout was miR-181a-3p, a miRNA
 360 known to regulate Nanog expression in humans (Mintz, Sætrum, Reebye et al., 2012), a protein that
 361 promotes myoblast pluripotency and reduces differentiation in C2C12 cells (Lang, Lin, Teng et al., 2009).
 362 Another important cluster of miRNA with a role in the cell cycle is the miR-17 cluster, of which miR-17
 363 and miR-20a control c-Myc mediated cell proliferation in humans through reduction in E2F1 expression
 364 (O'Donnell, Wentzel, Zeller et al., 2005). In our study, these two miRNAs exhibited an E2-induced
 365 expression increase, suggesting this mechanism may also be important for E2-induced reductions in
 366 muscle growth in rainbow trout. Nearly all of the aforementioned findings occurred in mammals, most of
 367 which were human, and functional role of these miRNAs in myogenesis cannot be assumed to be
 368 conserved between mammals and teleosts like rainbow trout. However, using these previous studies we

can speculate that these miRNAs-related mechanisms that are regulated by E2 may be involved with the myogenic response, and additional studies can more clearly implicate these candidate mechanisms.

An E2-induced increase in Pax7 expression occurred after 24 hours of E2 treatment. In mammals, Pax7 is a marker of quiescent myogenic stem cells in mammals, however in indeterminate growing species like rainbow trout and Atlantic salmon, expression of Pax7 continues in differentiating myotubes in culture, potentially reflecting a population of self-renewing MPCs (Bower and Johnston, 2010, Seiliez, Froehlich, Marandel et al., 2015). Therefore, increased Pax7 expression during E2 treatment may represent reduced differentiation rates and/or an increased rate of MPC self-renewal. However, coordinated down-regulation of MyoD by E2 is suggestive of reductions in cell differentiation. Interestingly, in mammals, miR-206 directly down-regulates Pax7 expression during cell differentiation by binding to its 3' untranslated region (Dey, Gagan and Dutta, 2011). However, in the current study the expression of both miR-206 and Pax7 was increased by E2 treatment, suggesting this interaction may not be conserved across species. Indeed, the aforementioned absence of the miR-206 seed sequence in rainbow trout supports this concept.

Previous studies conducted by Cleveland *et al.*, in rainbow trout demonstrate that E2 increases muscle catabolism, partially through elevations in protein degradation via up regulation of components of the ubiquitin-proteasome and autophagic-lysosome systems (Cleveland and Weber, 2011, Cleveland and Weber, 2016, Cleveland and Weber, 2015). Part of the E2 response is increased expression of E3 ubiquitin ligases, including muscle-specific atrophy gene Fbxo32/atrogen-1 and decreased expression of Omy-miR-23a-3p as shown in this study. It has been demonstrated in mice and C2C12 myocytes that miR-23a exhibits translational suppression by binding to the 3' untranslated regions of Fbxo32 (Hudson et al., 2014, Wada et al., 2011), subsequently reducing protein expression. Therefore, the E2-induced reduction in Omy-miR-23a-3p likely contributes to elevated Fbxo32 expression and increased proteolytic capacity in rainbow trout, suggesting a conserved mechanism. Further contributing to muscle catabolism may be

increases in mitochondrial outer membrane permeability (MOMP), the integrity of which is maintained by Omy-miR-23a-3p (Tait and Green, 2010) . Therefore, E2-induced reductions in miR-23a-3p may increase MOMP, a mechanism associated with caspase-induced apoptosis (Tait and Green, 2010), which themselves were affected by E2 treatment in rainbow trout.

The identified novel miRNA, omy-miR-nov-285-5p, belongs to the miRNA family miR-7911 with an accuracy of 72.74% (Zou, Mao, Hu et al., 2014). miRNAs belonging to same family imply that they have similar sequence or structural configuration, hence similar function (Kaczkowski, Torarinsson, Reiche et al., 2009). Studies in insects showed that miR-7911c-5p is expressed in immature testis and targets the chromobox protein which is involved in chromosomal DNA packaging (Tariq, Peng, Saccone et al., 2016). Novel miRNA expressed in skeletal muscle after E2 exposure belongs to the same family with probable function in chromatin remodeling.

These findings suggest that E2 can affect processes affecting myogenesis and muscle growth, partially through regulation of miRNA-related mechanisms even within 24 hours of exposure in rainbow trout skeletal muscle. Figure 7 shows the proposed model highlighting the effects of E2 in muscle cells. Omy-miR-23a-3p, a major regulator of muscle atrophy (Hudson et al., 2014) is down regulated by E2. Hudson et al. also demonstrated the exosomal transport of miR-23a from the cell. This reduction in cellular levels of miR-23a resulted in increased expression of atrophic genes leading to cell death and atrophy. It is possible that increased cell death triggers the replacement of myogenic cells, which explains significant expression of MPC specific myogenic regulatory factor Pax7 in support to studies in zebrafish (Froehlich, Galt, Charging et al., 2013). It is possible that E2 contributes to quiescent cell proliferation rather than differentiation, while the mature myotubes are prone to atrophy or cell death after 24 hours of treatment. Further emphasis on the increase of caspases and their effects should be considered to understand their potential role as protein degrading enzymes or regulators of cell division in the presence of E2.

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Figure legends

Figure 1. miRNA profile of skeletal muscle from control and E2 treated sample. (A) Length distribution of all miRNA sequenced, major percentage of miRNA are of sizes 22 and 23 nucleotides. (B and C) Abundantly expressed five miRNAs in control and treatment samples at 24 and 72 hours. (D) Percentage of miRNAs identified in each group.

Figure 2. Heat map showing differential expression of miRNAs. Statistically significant miRNAs after 24 hours of treatment. C4, C6, C7, C8 represent control samples and E9, E11, E12, E14 are E2 treated samples.

Figure 3. Validation of miRNA sequencing results: Log₂ fold change of E2 samples compared to control from sequencing and qPCR. Results are expressed as mean $\pm$ SEM (n = 6 or 5). * P < 0.05.

Figure 4. Omy-miR-23a-3p and its target genes. (A) ClustalW alignment of miR-23a primary transcript from selected species. (B) Putative duplex between the miRNA and its target genes MuRF1 (coding region) and Fbxo32 (3'UTR). (C and D) Relative expression of atrophic genes and caspases in control and E2 treated samples after 24 and 72 hours. Results are expressed as mean $\pm$ SEM (n = 6). * P < 0.05 and ** P < 0.01.

Figure 5. Expression of myogenic regulatory factors (MRFs) defining cell cycle: Relative gene expression of MRFs in control and E2 treated samples after 24 and 72 hours, represented as mean values $\pm$ SEM (n = 6). ** P < 0.01.

Figure 6. Novel miRNA, omy-miR-nov-285-5p: Rainbow trout tissue distribution and expression of novel miRNA.

Figure 7. Prospective response to E2. E2 and estrogen receptors could result in exosomal packaging of omy-miR-23a-3p, resulting in decrease in miRNA in the cell thus resulting in high expression of its target gene Atrogin-1/Fbxo32 and increased mitochondrial outer membrane permeability further leading to increased caspases 3a and 9. Fbxo32 drives the cell to atrophy while caspases to apoptotic damage.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

800 **Table 1**

801 Summary of miRNA sequencing data from E2 treated and control skeletal muscle samples at 24 and 72
 802 hours

	Number reads	% reads
Total mappable reads	181,005,496	100%
Known miRNA	64,697,879	35.8%
Predicted miRNA	37,264,799	20.6%
Mapped to mRNA	28,508,740	15.8%
Mapped to other RNAs (RFam: rRNA, tRNA, snRNA, snoRNA and others)	32,206,146	17.8%
Mapped to Repbase	1,402,438	0.8%
Nohit	24,583,755	13.6%

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804

805 **Table 2**

806 Differentially expressed miRNAs with adjusted p-values < 0.05, their respective sequence and log₂ fold
 807 change

miRNA Name	Sequence	Log ₂ Fold Change	P-Value	P-Adjusted
omy-489-5p	UGGUCGUAUGUAUGACGUCAUU	-1.13	0.0032	0.0435
omy-let-7a-5p-1	UGAGGUAGUAGGUUGU/GAUAG UUU/GU	1.33	0.0011	0.0206
omy-let-7d-5p	UGAGGUAGUUGGUUGUAUGGUU	0.69	0.0033	0.0435
omy-let-7g-5p	UGAGGUAGUAGUUUGUAUAGUU	0.46	0.0035	0.0444
omy-let-7h-5p	UGAGGUAGUAAGUUGUGUUGUU	0.69	0.0000	0.0008
omy-let-7i-5p	UGAGGUAGUAGUUUGUGCUGUU	0.79	0.0000	0.0009
omy-let-7j-5p	UGAGGUAGUUGUUUGUACAGUU	0.58	0.0002	0.0044
omy-miR-125b-1-3p	ACGGGUUAGGCUCUCGGGAGCU	-0.99	0.0002	0.0044
omy-miR-130a-3p	CAGUGCAACAAUGAAAGGGCA	0.96	<0.0001	0.0000
omy-mir-133a-2-3p	UCAACUGUUGAAUGGAUU	1.16	0.0016	0.0299
omy-miR-133a-5p	AGCUGGUAAAAUGGAACCAAAU	0.72	0.0021	0.0333
omy-miR-145-3p	GGAUUCCUGGAAAUACUGUUCU	-0.60	0.0017	0.0300

omy-miR-145-5p	GUCCAGUUUUCCAGGAAUCCC U	-0.85	<0.0001	0.0016
omy-miR-17-5p	CAAAGUGCUUACAGUGCAGGUA	0.62	<0.0001	0.0009
omy-miR-181a*-3p	ACCAUCGACCGUUGACUGUGCC	-0.57	0.0001	0.0031
omy-mir-193-5p-1	UGGGUCUUUGUUGGCAAGGUGA	1.99	<0.0001	0.0008
omy-miR-193-5p-2	CGGGAUCUUGUGGGGCGAGAUGA	1.25	0.0001	0.0039
omy-mir-193-5p-3	UGGGUCUUUGCGGGCAAGGUGA	1.26	0.0004	0.0088
omy-miR-196b-5p	UAGGUAGUUUCAAGUUGUUGGG	1.21	<0.0001	0.0016
omy-miR-206-3p	GUAAGGAAGUGUGUGGA	1.60	0.0002	0.0044
omy-miR-20a-5p	UAAAGUGCUUAUAGUGCAGGUA G	1.35	<0.0001	0.0006
omy-miR-21-5p	UAGCUUAUCAGACUGGUGUUGG A	0.97	<0.0001	0.0018
omy-miR-214-3p	ACAGCAGGCACAGACAGGCAGU	-0.58	0.0039	0.0471
omy-miR-23a-3p	AUCACAUUGCCAGGGAUUUCC	-0.57	<0.0001	0.0004
omy-miR-26b-5p	UUCAAGUAAUCCAGGAUAGG	0.44	0.0021	0.0333
omy-mir-338-5p	AACAACAUCCUGGUGCUGCCUG AGU	-0.71	0.0006	0.0134
omy-miR-365-5p	AGGGACUUUUAGGGGCAGCUGU	1.09	0.0008	0.0154

	G			
omy-miR-499-3p	AACAUCACUUUAAGUCUG/CUGC U	-2.09	0.0001	0.0019
omy-miR-499-5p	UUAAGACUUA/GCAGUGAUGUUU CGGU	-1.98	0.0001	0.0040
omy-miR-499a-5p	UUAAGACUUGUAGUGAUGUUU	-1.79	0.0006	0.0125
omy-miR-551-3p	GCGACCCAUCCUUGGUUUCUGU	0.67	0.0033	0.0435
omy-miR-93a-5p	AAAAGUGCUGUUUGUGCAGGUA G	0.95	<0.0001	0.0001
omy-miR-nov-101-3p	GCGACCCACUCAUGAUUUCAGA	-1.59	0.0022	0.0335
omy-miR-nov-285-5p	GCAGUGGUUGUCGUAAUAGGUU C	-2.25	<0.0001	<0.0001
omy-let-7g-3p	CUGUACAAGCCACUGCCUUGCU	-0.82	0.0032	0.0435
omy-nov-miR-29b-2-p5	GCUGGUUUCAGGUGGUGACUUA GA	1.33	0.0036	0.0444

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809

810 **Table 3**

811 Enriched target genes of differentially expressed miRNAs involved in different biological processes and
 812 molecular functions

Top 10 enriched gene ontologies (biological processes)

Up-Regulated miRNA

GOBPID	P-value	Count	Size	GO Term
GO:0006310	1.41E-12	49	1311	DNA recombination
GO:0006313	1.27E-12	46	1169	Transposition, DNA-mediated
GO:0016311	5.29E-16	55	741	Dephosphorylation
GO:0030041	1.04E-11	20	237	Actin filament polymerization
GO:0030838	4.02E-13	20	199	Positive regulation of actin filament polymerization
GO:0031334	7.04E-13	20	205	Positive regulation of protein complex assembly
GO:0032196	1.39E-12	46	1172	Transposition
GO:0032273	4.42E-13	20	200	Positive regulation of protein polymerization
GO:0045010	1.98E-14	20	170	Actin nucleation

GO:0051495	2.86E-12	20	221	Positive regulation of cytoskeleton organization	813
					814
Down-Regulated miRNA					815
GOBPID	P-value	Count	Size	GO Term	816
GO:0006259	7.32E-25	144	2158	DNA metabolic process	817
GO:0006310	2.95E-35	124	1311	DNA recombination	818
GO:0006313	3.07E-38	121	1169	Transposition, DNA-mediated	819
GO:0015074	2.87E-35	122	1272	DNA integration	820
GO:0032196	3.96E-38	121	1172	Transposition	821
GO:0055085	6.40E-12	263	2461	Transmembrane transport	822
GO:0044710	2.82E-11	285	7574	Single-organism metabolic process	823
GO:0006811	1.01E-10	247	2339	Ion transport	
GO:0044765	7.25E-10	397	4216	Single-organism transport	
GO:0007186	9.75E-10	170	1506	G-protein coupled receptor signaling pathway	

Top 10 enriched gene ontologies (molecular function)

Up-Regulated miRNA

GOMFID	P-value	Count	Size	GO Term
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GO:0004721	4.69E-20	50	482	Phosphoprotein phosphatase activity
GO:0004721	3.59E-16	55	482	Phosphoprotein phosphatase activity
GO:0004803	3.38E-12	46	1169	Transposase activity
GO:0005217	2.90E-12	13	56	Intracellular ligand-gated ion channel activity
GO:0015278	2.26E-12	13	55	Calcium-release channel activity
GO:0016788	1.59E-16	83	1419	Hydrolase activity, acting on ester bonds
GO:0016791	1.28E-18	55	626	Phosphatase activity
GO:0042578	1.03E-18	67	897	Phosphoric ester hydrolase activity
GO:0005220	3.59E-10	8	20	Inositol 1,4,5-trisphosphate-sensitive calcium-release channel activity

Down-Regulated miRNA

GOMFID	P-value	Count	Size	GO Term
GO:0003677	3.25E-15	194	4260	DNA binding
GO:0004803	6.88E-39	121	1169	Transposase activity
GO:0004872	5.55E-13	257	2391	Receptor activity
GO:0004888	3.49E-13	211	1851	Transmembrane signaling receptor activity

GO:0004930	9.98E-15	148	1109	G-protein coupled receptor activity
GO:0019706	2.89E-12	16	49	Protein-cysteine S-palmitoyltransferase activity
GO:0019707	2.89E-12	16	49	Protein-cysteine S-acyltransferase activity
GO:0022857	1.16E-12	238	2185	Transmembrane transporter activity
GO:0038023	1.72E-12	231	2112	Signaling receptor activity
GO:0019706	2.89E-12	16	49	Protein-cysteine S-palmitoyltransferase activity

824

825

826 **Table 4**

827 Gene ontologies and p-values of target genes (predicted) of omy-miR-23a-3p involved in mitochondrial
 828 membrane permeability and apoptosis.

Target gene ontologies of omy-miR-23a-3p regulating mitochondrial permeability and apoptosis

GOBPID	P-value	Count	Size	GO Term
GO:1901029	0.0001	2	2	Negative regulation of mitochondrial outer membrane permeabilization involved in apoptotic signaling pathway
GO:0010823	0.0010	2	6	Negative regulation of mitochondrion organization
GO:1901028	0.0024	2	9	Regulation of mitochondrial outer membrane permeabilization involved in apoptotic signaling pathway
GO:0008637	0.0041	3	39	Apoptotic mitochondrial changes
GO:0097345	0.0058	2	14	Mitochondrial outer membrane permeabilization
GO:0035794	0.0067	2	15	Positive regulation of mitochondrial membrane permeability
GO:1902110	0.0067	2	15	Positive regulation of mitochondrial membrane permeability involved in apoptotic process

GO:1902686	0.0067	2	15	Mitochondrial outer membrane permeabilization involved in programmed cell death
GO:1902108	0.0076	2	16	Regulation of mitochondrial membrane permeability involved in apoptotic process
GO:0046902	0.0085	2	17	Regulation of mitochondrial membrane permeability
GO:0090559	0.0085	2	17	Regulation of membrane permeability
GO:0010821	0.0208	2	27	Regulation of mitochondrion organization
GO:0007006	0.0321	2	34	Mitochondrial membrane organization

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831 **Table 5**

832 Target genes of differentially expressed miRNAs involved in cell cycle and myogenesis

Target genes related to Cell Cycle				
GOBPID	P-value	Count	Size	GO Term
GO:0000083	0.0004	3	3	Regulation of transcription involved in G1/S transition of mitotic cell cycle
GO:0000086	0.0029	6	42	G2/M transition of mitotic cell cycle
GO:0000278	0.0096	28	553	Mitotic cell cycle
GO:0007093	0.0050	7	54	Mitotic cell cycle checkpoint
GO:0007346	0.0083	17	226	Regulation of mitotic cell cycle
GO:0010971	0.0067	2	4	Positive regulation of G2/M transition of mitotic cell cycle
GO:0022402	0.0015	35	650	Cell cycle process
GO:0033260	0.0032	3	5	Nuclear cell cycle DNA replication
GO:0033262	0.0011	2	2	Regulation of nuclear cell cycle DNA replication
GO:0044770	0.0060	12	132	Cell cycle phase transition
GO:0044772	0.0056	12	131	Mitotic cell cycle phase transition

GO:0044786	0.0061	3	6	Cell cycle DNA replication
GO:0044839	0.0029	6	42	Cell cycle G2/M phase transition
GO:0051726	0.0054	51	497	Regulation of cell cycle
GO:0071156	0.0004	3	5	Regulation of cell cycle arrest
GO:0071157	0.0004	3	5	Negative regulation of cell cycle arrest
GO:1901988	0.0082	7	59	Negative regulation of cell cycle phase transition
GO:1901991	0.0082	7	59	Negative regulation of mitotic cell cycle phase transition
GO:1902751	0.0067	2	4	Positive regulation of cell cycle G2/M phase transition
GO:1903047	0.0027	24	409	Mitotic cell cycle process

Target genes involved in proliferation of stem cells

GOBPID	P-value	Count	Size	GO Term
GO:0008283	0.0011	33	571	Cell proliferation
GO:0042127	0.0002	28	415	Regulation of cell proliferation
GO:0048144	0.0067	1	4	Fibroblast proliferation
GO:0048145	0.0067	1	4	Regulation of fibroblast proliferation

GO:0048146	0.0067	1	4	Positive regulation of fibroblast proliferation
GO:0048145	0.0070	2	4	Regulation of fibroblast proliferation
GO:0048146	0.0070	2	4	Positive regulation of fibroblast proliferation
GO:0072089	0.0024	5	29	Stem cell proliferation
GO:0072091	0.0004	4	17	Regulation of stem cell proliferation

Target genes involved in the fate of MPC cells

GOBPID	P-value	Count	Size	GO Term
GO:0014717	0.0004	2	2	Regulation of satellite cell activation involved in skeletal muscle regeneration
GO:0014719	0.0013	2	3	Skeletal muscle satellite cell activation
GO:0014901	0.0004	2	2	Satellite cell activation involved in skeletal muscle regeneration
GO:0033002	0.0062	3	33	Muscle cell proliferation
GO:0042693	<0.0001	4	6	Muscle cell fate commitment
GO:0043403	0.0013	2	3	Skeletal muscle tissue regeneration
GO:0043416	0.0013	2	3	Regulation of skeletal muscle tissue regeneration
GO:0051146	0.0099	18	250	Striated muscle cell differentiation

GO:0014812	0.0011	2	9	Muscle cell migration
GO:0042692	0.0051	18	281	Muscle cell differentiation
GO:0055001	0.0076	15	227	Muscle cell development