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Comprehensive Analyses of miRNA and DNA Methylation in 17β-Estradiol Exposed Juvenile Rainbow Trout Skeletal Muscle

Prasanthi P. Koganti

Dissertation submitted to the
Davis College of Agriculture, Natural Resources and Design
at West Virginia University
in partial fulfillment of requirements
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in
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ABSTRACT

Comprehensive Analyses of miRNA and DNA Methylation in 17β-Estradiol Exposed Juvenile Rainbow Trout Skeletal Muscle

Prasanthi P. Koganti

Reproductive development in female teleosts like rainbow trout starts with release of the sex steroid estrogen, necessary for synthesis of the egg yolk protein vitellogenin. Increase in estrogen secretion during these reproductive phases results in increased energy demands. Skeletal muscle serves as an immediate endogenous source to address energy demands during vitellogenesis and spawning due to which it undergoes rapid degradation resulting in water accumulation, poor fillet and low egg quality. An imbalance in protein turnover is observed not only because of rapid increase in degradative pathways but also due to decreased protein synthesis. These changes in protein metabolism in skeletal muscle were identified within 24 hours of E2 exposure and confirmed in both *in vivo* and *in vitro* models. Normally during adult myogenesis myogenic precursor cells proliferate and differentiate further to form a mature muscle fiber resulting in either hyperplasty or hypertrophy or both in fishes. Signaling pathways and MRFs need to function in coordination with epigenetic factors and non-coding RNAs to positively or negatively regulate muscle synthesis. Interestingly, estrogen also influences the expression of miRNA and in turn their target genes. However, the effects of estrogen regulating protein imbalance in rainbow trout skeletal muscle is not completely understood. Hence studies to understand the influence of estrogen were carried out using juvenile, sexually immature fish. Juvenile rainbow trout were treated with either estrogen or vehicle and their skeletal muscle collected after 24 hours and 72 hours of treatment to understand the role of miRNA and DNA methylation in regulation of myogenesis. Findings from the miRNA study revealed diluted effects of estrogen after 72 hours of treatment, hence further studies were carried out with samples from 24-hour post treatment.

miRNAs play a crucial role in regulation of gene expression along with epigenetics. These are small non-coding RNAs with length ranging from 21-23bp mostly functioning as post-transcriptional regulators of gene expression. Generally, they bind to the 3' region of transcribed mRNA with a complementary region called seed sequence. These genes are called target genes of miRNA. A single miRNA can target more than one mRNA. Binding of miRNA to its target gene results in either translational repression or deadenylation or both. Deadenylation results in loss of RNA stability, which leads to its degradation. miRNAs express in tissue specific manner and those that express specifically in muscle are called myomiRNA. Various myomiRNA have been identified and studied for their functions. Estrogen influence the expression of miRNA and in turn their target genes. Therefore, rainbow trout skeletal muscle samples were sequenced for small RNAs to understand the effects of estrogen. Differentially expressed miRNA were identified in estrogen treated samples when compared to control. A total of 36 miRNAs were either upregulated or down regulated in E2 exposed skeletal muscle of which two were novel. Target genes of these differentially expressed miRNAs were identified followed by gene ontology enrichment. These target genes are involved in various biological and molecular functions including their role in

signaling pathways, cell cycle, DNA methylation, signal transduction and transcription factor binding. Genes regulated by miRNAs were also involved specifically in proliferation and differentiation of myogenic precursor cells and degradative pathways. E2 induced expression of miR-17 and miR-20, which are important in regulation of cell cycle. Further gene expression analysis of myogeneic regulatory factor, Pax7 specific to MPCs confirms increase in their number with decreased expression of MyoD, specific to differentiation. Similarly, miR-23a revealed its role in maintenance of mitochondrial outer membrane permeability and post-transcriptional regulation of atrophic genes. Decreased expression of miR-23a with increase in permeability was supported by increased gene expressions including fbxo32, caspases 3a and 9 that are involved in atrophy and apoptosis respectively. Expression analysis of one of the novel genes, miR-nov-285, was performed to know its expression pattern in different tissues, highest expression was observed in testis. C-5 methylation of cytosine was one of the GO term that was enriched during the target gene analysis of this novel miRNA. Further regulation of *Dnmt1* gene expression by this novel miRNA was confirmed by luciferase assay. All together these observations indicate that E2 influences differential expression of various miRNAs which in turn regulate gene expression contributing to muscle metabolism.

Reduced expression of *MyoD* was observed in our previous study and direct regulation of this differentiation factor was not observed by any of the differentially expressed miRNA. Since gene expression is also regulated transcriptionally by epigenetic mechanisms including DNA methylation, efforts were made to understand its role in reduced MyoD expression. Generally, DNA methylation reduces gene expression by directly blocking transcription factor binding or by recruiting other epigenetic contributors like histone deacetylases or histone methyltransferases. Three different CpG rich regions were identified in MyoD gene which were further amplified after bisulfite treatment and analyzed to understand the influence of E2 on DNA methylation. Remarkable differential methylations were also observed in non-CpG sites in exon1 of MyoD in addition to CpG sites. Exposure to E2 resulted in increased methylation of all the CpH methylations, which were also observed to be present in conserved motifs: c-Myc and Estrogen Response Element (ERE). Relative gene expression study revealed an increase in DNA methyltransferases, Dnmt1, Dnmt3a and Dnmt3b in E2 exposed skeletal muscle. These observations directed our study to understand the influence of DNA methylation at whole genome level.

Whole genome bisulfite sequencing (WGBS) was performed with control and estrogen treated skeletal muscle samples using paired end sequencing. These analyses resulted in identification of differential methylations of various chromosomal regions in CG, CHG and CHH contexts. Most of the CHG and CHH methylations observed were less than 10% while CG methylations were well distributed across various rates per site ranging from 0%-10% to 90%-100% of the total genome. Most of the cytosine methylations were mapped to unknown chromosome of rainbow trout genome, which is currently 80% of the total genome. 43 million CG sites were identified in the whole genome of which 304 thousand were differentially methylated in E2 sample when compared to control. About 818 regions show significant differential methylation with a difference of at least 80 percentage. Fifty percent of these regions were mapped to 208 genes involved in primary and cellular metabolic processes, cell communication, signal transduction, ion binding, transferase activity and are parts of various cellular organelles. They also regulate metabolic pathways including steroid hormone and purine metabolism.

Overall these observations indicate the role of small RNA and epigenetic mechanism particularly DNA methylation involvement in regulation of rainbow trout skeletal myogenesis under the influence of E2. These gene regulations were observed within 24 hours of E2 treatment indicating rapid molecular changes to external stimuli.

DEDICATION

To my husband T. V. R. Chowdari, you encouraged me to pursue my dream. You believed in me more than I did and never let me give up. Your sacrifices and constant support helped in achieving my goals.

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Publication-2

Koganti, P. P., J. Wang, B. Cleveland and J. Yao, 2017b 17β-Estradiol Increases Non-CpG Methylation in Exon 1 of the Rainbow Trout (*Oncorhynchus mykiss*) MyoD Gene. *Marine Biotechnology 19: 321-327.*

Publication-3

J. Hand, K. Zhang, L.Wang, **P. P. Koganti**, K.Mastrantoni, S. Rajput, M. Ashry, G. W. Smith, J. Yao., 2017, Discovery of a novel oocyte-specific Krüppel-associated box domain-containing zinc finger protein required for early embryogenesis in cattle. *Mechanisms of Development: DOI: 10.1016/j.mod.2017.02.003.*

Publication-4

Fu, L., **P. P. Koganti**, J. Wang, L. Wang, C. L. Wang, J. Yao., 2017 Lhx8 interacts with a novel germ cell-specific nuclear factor containing an Nbl1 domain in rainbow trout (*Oncorhynchus mykiss*). *PLOS ONE 12(2): e0170760.*

Publication-5

Koganti, P. P., J. Wang, B. Cleveland, H. Ma, G. M. Weber *et al.*, 2017a Estradiol regulates expression of miRNAs associated with myogenesis in rainbow trout. *Molecular and Cellular Endocrinology 443: 1-14.*

Publication-6

Wang, J., L. Fu, **P. P. Koganti**, L. Wang, J. M. Hand *et al.*, 2016 Identification and Functional Prediction of Large Intergenic Noncoding RNAs (lincRNAs) in Rainbow Trout (Oncorhynchus mykiss). *Marine Biotechnology: 1-12.*

TABLE OF CONTENTS

ABSTRACT II

DEDICATION V

ACKNOWLEDGEMENTS VI

LIST OF PUBLICATIONS VIII

TABLE OF CONTENTS X

LIST OF FIGURES XIV

LIST OF TABLES XVI

ABBREVIATIONS XVII

LITERATURE REVIEW 1
- INTRODUCTION 2
- MYOGENIC REGULATORY FACTORS (MRFS) 2
- SIGNALING PATHWAYS INVOLVED IN MYOGENESIS 3
- INFLUENCE OF ESTROGEN 4
- MICRORNA 5
  - miRNA targeting genes involved in muscle synthesis 7
  - Muscle cell fate 8
  - Estradiol and dietary influence on miRNA that regulates myogenesis 9
- DNA METHYLATION 11
  - DNA methylation in fish species 12
  - DNA methylation during skeletal myogenesis 13
  - Influence of estrogen on DNA methylation 14
- FIGURES 15
- TABLES 16
- REFERENCES 17

CHAPTER-1: ESTRADIOL REGULATES EXPRESSION OF miRNAS ASSOCIATED WITH MYOGENESIS IN RAINBOW TROUT 33

ABSTRACT 34
INTRODUCTION 35
MATERIALS AND METHODS 37
Experimental design 37
RNA isolation 37
miRNA sequencing and data analysis 38
Quantitative real time PCR 39
Prediction of target genes 39
Gene ontology enrichment analysis 40
RESULTS 40
miRNA profile in rainbow trout skeletal muscle 40
Differentially expressed miRNAs 41
Validation of differentially expressed miRNAs 41
Gene ontology enrichment analysis 41
Validation of miRNA target genes 42
Tissue distribution of novel miRNAs identified 43
DISCUSSION 43
ACKNOWLEDGEMENTS 47
FIGURES 48
TABLES 55
SUPPLEMENTARY DATA 63
REFERENCES 75

CHAPTER-2: 17B-ESTRADIOL INCREASES NON-CPG METHYLATION IN EXON 1 OF THE RAINBOW TROUT (*Oncorhynchus mykiss*) *MYOD* GENE 87
ABSTRACT 88
INTRODUCTION 89
MATERIALS AND METHODS 90
Sample collection 90
Screening of CpG islands in silico 90
DNA isolation and bisulfite conversion 90
Bisulfite specific PCR and bisulfite amplicon sequencing 91

Identification of differentially methylated regions ... 91
RNA isolation and quantitative real-time PCR (qPCR) ... 91
RESULTS ... 92
DISCUSSION ... 93
ACKNOWLEDGEMENTS ... 95
FIGURES ... 96
SUPPLEMENTARY DATA ... 99
REFERENCES ... 103

CHAPTER-3: DNA METHYLATION IN RESPONSE TO ESTROGEN IN RAINBOW TROUT SKELETAL MUSCLE ... 108
ABSTRACT ... 109
INTRODUCTION ... 110
METHODS ... 112
Sample collection and DNA isolation ... 112
Methylation-specific restriction digestion assay and ELISA ... 112
Whole genome bisulfite sequencing ... 113
Mapping and identification of methylated cytosines ... 113
Identification of differentially methylated regions ... 114
RESULTS ... 114
Methylation-specific restriction digestion assay and ELISA ... 114
Whole genome bisulfite sequencing ... 114
CpG methylations ... 115
Genes with differentially methylated regions ... 116
DISCUSSION ... 117
ACKNOWLEDGEMENTS ... 123
FIGURES ... 124
TABLES ... 136
SUPPLEMENTARY DATA ... 140
REFERENCES ... 148

APPENDIX-1: miR-NOV-285-3P REGULATES DNA METHYLTRANSFERASE1 EXPRESSION 156
INTRODUCTION 156
CLONING AND PLASMID PURIFICATION 156
CELL CULTURE AND DUAL LUCIFERASE REPORTER ASSAY 157
LUCIFERASE ACTIVITY 157
FIGURES 158

CONCLUSIONS 161
FIGURES 164

PERSPECTIVES 169

RESUME 171

LIST OF FIGURES

Literature Review

Figure 1. Relative gene expression of estrogen receptors. 15

Chapter 1: Estradiol regulates expression of miRNAs associated with myogenesis in rainbow trout

Figure 1. miRNA profile of skeletal muscle from control and E2 treated sample. 48

Figure 2. Heat map showing differential expression of miRNAs. 49

Figure 3. Validation of miRNA sequencing results. 50

Figure 4. Omy-miR-23a-3p and its target genes. 51

Figure 5. Expression of myogenic regulatory factors (MRFs) defining cell cycle. 52

Figure 6. Novel miRNA, omy-miR-nov-285-5p. 53

Figure 7. Prospective response to E2. 54

Chapter 2: 17β-Estradiol increases non-CpG methylation in exon 1 of the rainbow trout (Oncorhynchus mykiss) MyoD gene

Figure 1. Heat maps showing methylation of CpG and CpH sites in MyoD gene and alignment of MyoD exon1. 97

Figure 2. Relative expression of DNMTs. 98

Chapter 3: DNA methylation in response to estrogen in rainbow trout skeletal muscle

Figure 1. Pipeline used to identify differentially methylated regions. 124

Figure 2. Methyl-specific restriction enzyme analysis of genomic DNA. 127

Figure 3. ELISA to identify global methyl cytosine levels. 128

Figure 4. Reverse cumulative plot of the methylated cytosine percentage. 129

Figure 5. Global methylated cytosine distribution. 131

Figure 6. Differentially methylated regions visualized using IGV. 133

Figure 7. Gene Ontology analysis of gene with differentially methylated regions (CG). 135

Appendix 1: miR-nov-285-3p regulates DNA methyltransferase1 expression

Figure 1. Binding of seed sequence to last exon of Dnmt1. 158

Figure 2. Cloning strategies. 159

Figure 3. Relative luciferase activity in comparison to control are presented. 160

Conclusions:

Figure 1. Regulation of gene expression at transcriptional and post-transcriptional levels. 164
Figure 2. Increase in self renewal of MPCs. 165
Figure 3. Increase in apoptosis and atrophy. 166
Figure 4. Identified regulatory mechanisms resulting in imbalance in protein turnover under the influence of E2. 167
Figure 5. Major mechanisms being regulated by DNA methylation. 168

LIST OF TABLES

Literature Review

Table 1. Role of DNA methylation on expression of myogenic regulatory factors 16

Chapter 1: Estradiol regulates expression of miRNAs associated with myogenesis in rainbow trout

Table 1. Summary of miRNA sequencing data. 55

Table 2. Differentially expressed miRNAs.. 56

Table 3. Enriched target genes of differentially expressed miRNAs.. 58

Table 4. Target genes of omy-miR-23a-3p.. 60

Table 5. Target genes involved in cell cycle and myogenesis.. 61

Chapter 3: DNA methylation in response to estrogen in rainbow trout skeletal muscle

Table 1. KEGG pathways analysis of genes with differentially CG methylated regions. 136

Table 2. DMRS with delta = ±100.. 138

ABBREVIATIONS

ER	Estrogen Receptor
MPC	Myogenic Precursor Cells
E2	17β-estradiol
MRFs	Myogenic Regulatory Factors
MOMP	Mitochondrial Outer Membrane Permeability
IGF-1	Insulin Growth Factor 1
GH	Growth Factor
HDAC	Histone Deacetylase
DNMT	DNA Methyltransferase
CpG	Cytosine followed by Guanine
Non-CpG/CpH	Cytosine followed by nucleotide other than Guanine
UTR	UnTranslated Region
ERE	Estrogen Response Element
^{m}C	Methylated cytosine
DMRs	Differentially Methylated Regions
IGV	Integrative Genome Viewer
SAM	S-Adenosyl Methionine
MIM or MTSS1	Missing-in-metastasis protein
PKN1	Serine threonine-kinase N1
ARAP1	Arf-GAP with Rho-GAP ANK repeat and PH domain-containing 1
KIF5B	Kinesin-1 heavy chain
NRIP2	Nuclear receptor-interacting 2
TNRC6B	Trinucleotide Repeat-Containing gene 6B
CHD7	Chromodomain-Helicase-DNA-binding 7

LITERATURE REVIEW

INTRODUCTION

Rainbow trout belongs to salmon family and is cultivated in America, with its importance in food, recreation and research. These fishes require enormous amount of energy during vitellogenesis and spawning to address the need for yolk proteins synthesis. The expression of sexual hormone genes and their secretion consequently promote catabolism in rainbow trout (ARMOUR *et al.* 1997; SALEM *et al.* 2006b; SALEM *et al.* 2010a). These demands in fish like rainbow trout are either addressed by exogenous sources (feed) or endogenous sources (skeletal muscle). Derivation of energy from endogenous sources results in rapid skeletal muscle degradation (SALEM *et al.* 2006b; CLEVELAND AND WEBER 2011a) creating an imbalance in protein turnover. Supportive evidence for the effect of the sex steroids on protein turnover was well studied (CLEVELAND AND WEBER 2011a). The imbalance in protein turnover is because of both increased catabolic pathways and decreased anabolic pathways resulting in poor muscle quality (LOVE 1960b).

Somites from mesodermal layer contribute to primary phase of myogenesis during embryonic development. These somites proliferate and differentiate to become myogenic precursor cells (MPCs), while acting as a scaffold for secondary phase of myogenesis during embryonic development. Adult myogenesis starts with activation, proliferation, differentiation and maturation of myogenic precursor cells present as a reservoir between the basal lamina and sarcolemma of mature muscle bundle (SEALE *et al.* 2001). Unlike mammals (ROWE AND GOLDSPINK 1969) MPC differentiation and maturation either contribute to hyperplasty (increase in muscle fiber number) or hypertrophy (increase in muscle fiber size) in rainbow trout hence most of the fish exhibit indeterminate growth pattern throughout their life (STICKLAND 1983; HIGGINS 1985; WEATHERLEY *et al.* 1988; KOUMANS *et al.* 1993; ALAMI-DURANTE *et al.* 1997; PATRUNO *et al.* 1998; MOMMSEN 2001). Myogenesis is a complex mechanism involving various signaling pathways, myogenic regulatory factors and epigenetic factors. During regeneration, general metabolism (turn over) or injury a coordinated interplay among different contributing mechanisms is required for maturation of MPC to muscle fiber.

MYOGENIC REGULATORY FACTORS (MRFS)

Myogenic regulatory factors are basic helix-loop-helix transcription factors which drive the process of myogenesis. Differential expression of these myogenic regulatory factors determine

progression of MPCs (WATABE 1999). Myf5 is an important MRF playing a role in committing the satellite cells to myogenic lineage. MPCs are mitotically inactive cells expressing satellite cell markers Pax7 (HOLLWAY *et al.* 2007; SEGER *et al.* 2011), HGF receptor c-met (HOLLWAY *et al.* 2007) and Syndecan-4 (FROEHLICH *et al.* 2013a). Several knockout studies were performed in mice to understand the importance of Pax7 expression in satellite cells. Collectively they conclude Pax7 is necessary for the maintenance of satellite cells state (SEALE *et al.* 2000; OUSTANINA *et al.* 2004; KUANG *et al.* 2006; RELAIX *et al.* 2006). Absence of Pax7 expression lead to defective muscle differentiation as an effect of cell cycle arrest and precautious differentiation (GÜNTHER *et al.* 2013; VON MALTZAHN *et al.* 2013). Increased expression of Pax7 is also observed in satellite cells (OLGUIN AND OLWIN 2004). Hence Pax7 is determined as a marker for MPCs. Mitotically inactive MPCs enter proliferative phase to either result in self-renewal or further differentiate to myoblasts. High levels of Pax7 expression is observed in self-renewing cells (OLGUIN AND OLWIN 2004) while differentiating cells exhibit increased expression of MyoD and decreased expression of Pax7 (ZAMMIT *et al.* 2004). Thus, MyoD is one of the major factor is determining the myogenic cell fate of MPCs (MEGENEY *et al.* 1996; WHITE *et al.* 2000). Expression of other myogenic regulatory factors including Mrf4 and Myogenin after proliferation and during differentiation marks the progression of differentiation process to form a myofiber (FÜCHTBAUER AND WESTPHAL 1992; YABLONKA-REUVENI AND RIVERA 1994). Signaling molecules play a significant role in regulating the expression of these MRFs thus determining the fate of myogenesis.

SIGNALING PATHWAYS INVOLVED IN MYOGENESIS

Muscle regeneration is triggered by transduction of the signals, eventually resulting in the activation of different signaling pathways which further activate expression of myogenic factors responsible for muscle growth. Maintenance of MPCs in satellite cell state is regulated by TGF-β family members while transition from proliferative to quantile mitosis by hedgehog signaling. These signaling molecules regulate differential expression of MRFs. Markers of MPCs, Pax7 and Pax3 are downregulated and differentiation factor MyoD is upregulated by hedgehog in teleosts thus functioning as one of the major determining factor for myogenic commitment. Growth hormone and insulin regulate myogenesis by binding to receptor tyrosine kinase and initiating signaling cascade. IGF-1 pathway is myogenic (FLORINI *et al.* 1996) activating the expression of the different genes responsible for the synthesis of muscle, which is synthesized either by liver or

specific tissues in response to the growth factors. IGF-1 pathway enhances myogenic proliferation and differentiation via RAS/RAf and PI3K pathways thus controlling MPC proliferation and differentiation respectively (BING-HUA JIANG *et al.* 1999).

INFLUENCE OF ESTROGEN

Response to estrogen in skeletal myogenesis of rainbow trout contrasts to that of rats, pigs and cow (REHFELDT *et al.* 2009b; KAMANGA-SOLLO *et al.* 2010b; CLEVELAND AND WEBER 2011a). Increased protein degradation with decreased protein synthesis was observed in rainbow trout. Activity of estrogen on a cell is either through genomic or non-genomic pathways. Estrogen transduces its signal in non-genomic means by binding to the estrogen receptors which in turn activate various signaling pathways or by binding to IGF-1 receptor (KAHLERT *et al.* 2000; TIAN *et al.* 2012). Estrogen activates various signaling pathways including MAPK (MIGLIACCIO *et al.* 1996; WATTERS *et al.* 1997; ENDOH *et al.* 1997) and phosphoinositol 3-kinase pathway (CASTORIA *et al.* 2001). These signaling pathways eventually regulate gene expression by initiating a cascade of phosphorylation and de-phosphorylation reactions. The GH/IGF axis plays a major role in myogenesis (GABILLARD AND RESCAN 2013) which is disrupted in salmonids (HOLLOWAY AND LEATHERLAND 1997a; NORBECK AND SHERIDAN 2011b; HANSON *et al.* 2012; HANSON *et al.* 2014) as an effect of estrogen release during sexual maturation and decrease in IGF-1 (TAYLOR *et al.* 2008). A disrupted GH/IGF axis partially explains the imbalance in protein turnover in sexually maturing fish. Genomic pathways of estrogen activity involve activation of estrogen receptors. Two subtypes of estrogen receptors were identified in rainbow trout; ERα and ERβ, with isoforms ERα1, ERα2 and ERβ1, ERβ2 respectively. Differential expression of these receptors in various tissues were reported during sexual development of fish (SABO-ATTWOOD *et al.* 2004; FILBY AND TYLER 2005; NELSON AND HABIBI 2010; CHAKRABORTY *et al.* 2011; SHI *et al.* 2011; NAGLER *et al.* 2012; TIAN *et al.* 2012). These estrogen receptors either form homo or hetero dimers and act as transcription factors by binding to estrogen response elements on DNA. Sexually maturing salmonids show increased in muscle catabolism with increased estrogen levels (ANDO *et al.* 1986; SALEM *et al.* 2006b; CLEVELAND AND WEBER 2011a; CLEVELAND *et al.* 2012) or after estrogen treatment (NAZAR *et al.* 1991a; OLIN *et al.* 1991). Increase in catabolism is an effect of increase in protein degradation with decrease in its synthesis. Three different degradative pathways contribute to the process of proteolysis in fish, ubiquitin-proteasome pathway, cathepsins and calcium-

dependent calpains along with protein degradation by caspases during apoptosis. Spawning rainbow trout shows an increase in ubiquitin ligases, cathepsins and apoptotic caspases (TOYOHARA *et al.* 1998; SALEM *et al.* 2006b) which might be the major degradative pathways during sexual maturation.

Molecular regulation of myogenesis is also governed through transcriptional, post-transcriptional and translational stages. A coordinated communication among these molecular mechanisms is necessary to regulate these mechanisms meticulously. miRNA and DNA methylation are key players in transcriptional and post-transcriptional regulation. Expression of certain miRNA is restricted to muscle and they are called myomiRs and these miRNAs regulate the gene expression of MRFs in different stages of myogenesis. miRNA not only targets the expression of genes (CHEN *et al.* 2006c) but also play a role in signaling pathways aiding in muscle cell proliferation and differentiation (ZANOU AND GAILLY 2013b). Similarly, gene expression is also regulated at transcriptional level by DNA methylation of carbon at 5th position of cytosine. Methylated cytosines either upregulate or down regulate gene expression depending on the region of gene structure. Majority of the studies reported DNA methylation to be a negative regulator of gene expression thus making it unavailable for transcription. Removal of these methyl groups through demethylating mechanisms would result in gene regulation (RAZIN AND RIGGS 1980). Role of DNA methylation in myogenesis was established in 1979, by using chemical inhibitors of DNA methylations which resulted in differentiation of fibroblasts to cells specific to muscle lineage (TAYLOR AND JONES 1979). The role of miRNA and DNA methylation in various aspects of myogenesis is discussed below.

microRNA

Small RNAs, particularly miRNAs are extensively studied as post-transcriptional microregulators of gene expression governing mRNA translation or its stability (NILSEN 2007). Recent studies provided evidence for their transport (HWANG *et al.* 2007) and nuclear existence (LIAO *et al.* 2010) suggesting functions other than translational inhibition. Similarly, the presence of miRNA in exosomes (VALADI *et al.* 2007) advocates their secretion and circulation through body fluids (GALLO *et al.* 2012; ZHOU *et al.* 2012; LV *et al.* 2013) to control gene expression in recipient cells (IGUCHI *et al.* 2010). Besides continuous efforts to understand various functional roles of miRNA, their biogenesis (reviewed in (KIM *et al.* 2009) is well established. Primary

transcripts of miRNA are either generated by transcription of individual miRNA genes or by processing of intronic regions. Most of the primary transcripts (pri-miRNA) are transcribed by RNA polymerase II which are further cleaved by Drosha to ~65bp hairpin precursor miRNA (pre-miRNA). The pre-miRNAs are then exported to the cytoplasm by exportin 5 for further cleavage by dicer to ~22bp RNA duplex that are loaded on to Ago proteins to form a RISC complex. One of the strand from the RNA duplex remains (mature miRNA) on the RISC complex while the other is released and degraded. The mature miRNA binds to target mRNA by either perfect or imperfect base-pairing leading to translational repression or mRNA degradation. The earliest reports of miRNA in rainbow trout was reported in an effort to understand the role of regulatory small RNA during early embryogenesis (RAMACHANDRA *et al.* 2008a) and further miRNA profiling from nine somatic tissues including muscle was performed (SALEM *et al.* 2010d). Recent availability of trout genome sequence (BERTHELOT *et al.* 2014) led to the establishment of microtrout database establishing genome wide prediction of miRNA-mRNA binding (MENNIGEN AND ZHANG 2016). Various factors, including embryonic temperature and (JOHNSTON *et al.* 2009), feeding (BIZUAYEHU *et al.* 2016) affect growth in fish through miRNAs. Johnston and his colleagues (JOHNSTON *et al.* 2009) showed the transition of hyperplastic to hypertrophic growth patterns in zebrafish involving differential expression of miRNA with varied embryonic temperatures. miRNA mediate the effects of nutrition on gene regulation, studies in atlantic cod and grass carp support such regulation. Differential expression of endogenous miRNA when fish were fed with different diets and supplementation with *Bacillus natto* are observed in atlantic cod and grass carp respectively, ultimately changing muscle metabolism in fish.

Functional regulation of miRNA in Muscle

Myogenesis involves orchestrated interaction of various mechanisms involving signaling pathways and coordinated gene expression of various myogenic regulatory factors (MRFs). The role of miRNA in myogenesis was first established in Dicer mutant mice exhibiting reduced muscle miRNA, perinatal death and decreased skeletal muscle with abnormal myofiber morphology and apoptosis of myoblasts (O'ROURKE *et al.* 2007). This study establishes the important role of miRNAs in vertebrate muscle development. miRNAs with muscle specific expression and function are designated as myomiRNAs. The involvement of these myomiRNA in muscle precursor cell proliferation and differentiation is established in various studies (reviewed

in (GE AND CHEN 2011). Nachtigall *et al* compared the evolution and organization of myomiRNAs in cartilaginous and bony-fish genomes through genome-wide comparative analysis and concluded synteny in myomiRs distribution (NACHTIGALL *et al.* 2014).

miRNA targeting genes involved in muscle synthesis

Transcription factors that play a central role in proliferation, differentiation and maturation of myogenic precursor cells are Myogenic Regulatory Factors (MRFs). Interactions of miRNAs with major MRFs and other metabolic genes fine-tune gene expressions consequently controlling myogenesis (CHEN *et al.* 2006b; RAO *et al.* 2006). Differential miRNA expression was observed during various growth stages in skeletal muscle of slow and fast growing Nile tilapia (HUANG *et al.* 2012a), within 17 and 29 days after hatching in Japanese flounder (FU *et al.* 2011), during various developmental stages from hatching to 2 years in common carp (YAN *et al.* 2012), and 30 days post hatching to 3 years in ray-finned fish (ZHANG *et al.* 2017), indicating a stage specific diverse miRNA profile in skeletal muscle of fish. Most abundant miRNA expressed in skeletal muscle are miR-1, miR-206 and miR-133a in various fish species including rainbow trout (SALEM *et al.* 2010d; KOGANTI *et al.* 2017a), zebrafish (MISHIMA *et al.* 2009), flounder (FU *et al.* 2011), sea bass (XIA *et al.* 2011), Nile tilapia (HUANG *et al.* 2012a), Chinese perch (CHU *et al.* 2013) and common carp (YAN *et al.* 2012) consistent with studies from other species. Interaction of miR-1, miR-206 and miR-133a with their target genes to regulate skeletal muscle cell proliferation and differentiation is well documented from studies conducted using mice and mouse cell lines C2C12 and MEF (CHEN *et al.* 2006b; ROSENBERG *et al.* 2006; CHEN *et al.* 2010). These studies shed light on the regulatory network among the miRNA and MRFs. Mature miR-1 and miR-133 are derived from one polycistronic pre-miRNA targeting two different genes, miR-1 and miR-133 target histone deacetylase 4 which negatively regulates the expression of muscle specific genes and serum response factor which regulates muscle cell proliferation respectively (CHEN *et al.* 2006b). Additionally, muscle specific expression of miR-206 is controlled by MyoD (ROSENBERG *et al.* 2006) which along with miR-1 targets and represses Pax7, muscle cells marker for proliferation (CHEN *et al.* 2010). Regulatory expression of miR-206 by MyoD is also associated with the negative regulation of *follistatin-like 1* and *utrophin* genes which are necessary for muscle cell differentiation (ROSENBERG *et al.* 2006). An increased expression of these three miRNAs (miR-1, miR206 and miR-133a) along with miR-21 was observed in common carp skeletal muscle with

increase in age from 30 day post-hatching to 2 years (CHEN *et al.* 2010; YAN *et al.* 2012). Continuous expression of these miRNAs indicates their functional importance in maintenance and growth of skeletal muscle. Same study (YAN *et al.* 2012)reported the existence of oocyte specific miRNAs which decreased expression after fertilization supporting the concept of maternal miRNA or dicer. miR-1 and miR-133 explain more than 54% of variation in regulating gene expression that influences myogenesis in zebrafish embryo. They mainly contribute to the disruption of actin organization in sarcomere assembly when down-regulated (MISHIMA *et al.* 2009). Few studies report miRNA involvement upstream to MyoD gene expression. Findings in tilapia and Chinese perch report regulation of MyoD expression by miR-203b and miR-143 respectively, both miR-203b and miR-143 bind to the 3'-untranslated region (UTR) of MyoD suppressing its expression (YAN *et al.* 2013a; CHEN *et al.* 2014). Additionally, expression of miR-27a, miR-214 and miR-222 after hatching and eventual loss in mature common carp indicates their role in myogenic precursor cell proliferation and differentiation (YAN *et al.* 2012). miR-222 is also reported to be highly expressed in muscle related tissues in Chinese perch (ZHU *et al.* 2015b). A member of transforming growth factor-β and a negative regulator of myogenesis, myostatin is also regulated by miRNA. miR-181a-5p targets myostatin and down regulates its expression, hence regulating the performance of red and white muscles (CHU *et al.* 2013). miRNA-206 is also involved in negative regulation of IGF-1 expression in tilapia skeletal muscle regulating its growth (YAN *et al.* 2013b). These findings suggest controlled regulation of myogenesis by miRNA in fish species.

Muscle cell fate

miRNA determine muscle cell fate very early during somitogenesis (FLYNT *et al.* 2007). Differential expression of miRNA targeting various genes responsible for muscle cell fate are also influenced by embryonic temperature in zebra fish leading from hyperplastic to hypertrophic growth transition. Let-7 family of miRNAs were up-regulated and miR-19 family, miR-130 and miR-9 were downregulated in fish experiencing hypertrophic muscle growth (JOHNSTON *et al.* 2009). Fish exhibit distinct slow and fast muscles in comparison to mammals and studies using fish as model species report the role of miRNA in muscle cell type determination. Ubiquitously expressing miR-214 synchronizes Hedgehog signaling in zebrafish by translational repression of its negative regulator *Su(Fu),* thus coordinating a balance among slow and fast muscle cell types. Knockdown of miR-214 in zebrafish embryos led to the developed of less or no slow muscle cells

(FLYNT *et al.* 2007). Similarly, knockdown and overexpression of miR-3906 in zebrafish embryos changed fast muscle phenotype as miR-3906 maintains homeostasis of calcium ion concentration specifically in fast muscles. Knockdown of miR-3906 increases the expression of its target gene *homer-1b* which in turn up-regulates fast muscle specific gene *fmhc4* and calcium sensitive gene *atp2a1*. All together, these changes cause a surge calcium ions concentration causing disorganized sarcomeric actin in fast muscle and swimming abnormality. However, over expression of miR-3906 decreases calcium ion concentration, resulting in bent bodies and shortened tails (LIN *et al.* 2013). Another important miRNA extensively studied for its involvement in muscle cell fate determination is miR-499. It is highly expressed in red skeletal muscle in Nile tilapia compared to white skeletal muscle (NACHTIGALL *et al.* 2015). Target gene analysis revealed down regulation *sox6* and *rod1* genes associated with slow muscle phenotype. A regulatory network involving functional repression of *sox6* by miR-499 through Prdm1for the maintenance of slow-twitch muscle was also reported in zebrafish (WANG *et al.* 2011) allowing restricted expression of sox6 in fast-twitch muscle. miR-181a-5p and miR-143 target genes myostatin and MyoD to control performance of different muscle cell types in vertebrates (CHU *et al.* 2013; CHEN *et al.* 2014). miR-222 in Chinese perch is abundantly expressed in white muscle which is regulated by nutritional status (ZHU *et al.* 2015b). Varied expression patterns of miRNAs were observed in slow and fast muscles in Pacu fish (DURAN *et al.* 2015) and Chinese perch (CHU *et al.* 2013), suggesting their importance in muscle cell type specification. All together these studies emphasize the regulatory mechanisms of miRNA in determining muscle cell fate either through involvement in regulatory networks, regulating gene expression or by maintaining ion homeostasis.

Estradiol and dietary influence on miRNA that regulates myogenesis

The complex regulatory coordination of miRNA and MRF to dictate either muscle precursor cell fate and/or muscle cell fiber type and/or synthetic or degradative pathways of myogenesis is subjective to feed and hormone induced miRNAs. The influence of estradiol on differential miRNA expression was well studied in human breast cancers (reviewed in (COCHRANE *et al.* 2011)). Estradiol is released only during sexual maturation in teleosts (LUBZENS *et al.* 2010b) which is essential for the synthesis of vitellogenin for oogenesis. Recent studies focusing to understand differential expression of miRNA influenced by quality of eggs in relation to post-ovulatory age (MA *et al.* 2015), ploidy of female fish and its fertility (ZHOU *et al.* 2015), fish

follicular development and oocyte maturation (ABRAMOV *et al.* 2013), estrogen regulation of miRNA during vitellogenesis (COHEN AND SMITH 2014) and during gonadal development (PRESSLAUER *et al.* 2017) were reported. Physiological changes in skeletal muscle during these stages of sexual maturation is obvious and widely accepted (ANDO *et al.* 1986; CLEVELAND AND WEBER 2011a), though studies to understand the role of miRNA in fish are sparse. Our study focused to understand the direct influence of estradiol and reported differential expression of 36 miRNAs including miR-133, miR-499 and miR-145 in skeletal muscle of rainbow trout (KOGANTI *et al.* 2017a). Differentially expressed miRNA and their target gene analysis showed that estradiol affected various processes that govern muscle precursor cell proliferation, differentiation, muscle growth and atrophy. Efforts to understand the effects of thyroid hormone influence on expression of histone deacetylase, *HDAC4* lead to the discovery of miRNA regulation in muscle development of Japanese halibut (ZHU *et al.* 2015a). This study presents evidence of an interaction of signaling pathways, epigenetic regulators like *HDAC4* and miRNAs (miR-1 and miR-133). Further studies to functionally determine these identified miRNA-mRNA targets in skeletal muscle under the influence of estradiol are necessary. More focused species specific studies in other teleosts are crucial to determine and pinpoint the exact roles of miRNA during sexual maturation in skeletal muscle.

Skeletal muscle loss during sexual maturation in fish supports energy demands required for vitellogenesis. Proteins from skeletal muscle are mobilized to address these demands leading to poor quality fillet. Increase in feeding levels to 74% satiation in rainbow trout during its sexual maturation partially prevents total loss by replacing mobilized endogenous proteins (CLEVELAND *et al.* 2012). Therefore, it is crucial to comprehend the role of feed in expression profiles of miRNA involved in growth. Findings from different feed experiments conducted in Atlantic cod, rainbow trout and Chinese perch corroborate differential miRNA expression governing myogenesis and skeletal muscle maintenance (MENNIGEN *et al.* 2013; ZHU *et al.* 2015a; ZHU *et al.* 2015b; BIZUAYEHU *et al.* 2016). Growth and development of Atlantic cod first fed with zooplankton is better compared to aquaculture feed. Eight differentially expressed miRNAs were identified among Atlantic cod fed with either zooplankton or aquaculture feeds whose target genes were involved in various metabolic and signaling pathways (BIZUAYEHU *et al.* 2016). This study indicated nutritional influence on genomic regulation that influenced growth and development. Refeeding juvenile Chinese perch after fasting for one week induced immediate changes in

miRNA expression (within 1 hour) whose targets are involved in resuming myogenesis after feeding (ZHU *et al.* 2015a; ZHU *et al.* 2015b). miR-1/miR-133 expression decreased during ontogeneic transition from edogenous to exogenous feed transition in rainbow trout leading to muscle hypertrophy demonstrating the importance of miRNA in all crucial stages (MENNIGEN *et al.* 2013).

DNA METHYLATION

Chemical modification of DNA bases was first reported in *Tubercle bacillus* and later in calf thymus DNA (JOHNSON AND COGHILL 1925; HOTCHKISS 1948). Further the importance of DNA methylation in transcriptional regulation of eukaryotic gene expression was reported by two simultaneous studies (HOLLIDAY AND PUGH 1975; RIGGS 1975). Since then, studies report the presence of such modifications in various organisms including prokaryotes, fungi, plants and animals. These modifications are post-synthetic, as the methyl group is added after the nucleotides are incorporated into the DNA. Methylation of bases is carried out by DNA methyltransferases (DNMT) and the methyl group is donated by S-Adenosyl methionine. DNA methylation in mammals is carried out by three DNMTs: DNMT1, DNMT3a, DNMT3b. However, 8 dnmt genes were identified in zebrafish dnmt1, dnmt2 while the rest were similar to the mammalian DNMT3 (DONG *et al.* 2001; MHANNI *et al.* 2001; SHIMODA *et al.* 2005). DNMT1 is a maintenance methyltransferase mainly functioning in methylating hemi-methylated DNA strands during replication. DNMT3a and DNMT3b are *de novo* methyltransferases methylating nascent DNA or hemi-methylated DNA. *De novo* methyltransferases are essential for laying methylation marks during embryo implantation and early development (OKANO *et al.* 1998). The majority of base modifications in eukaryotes are on cytosines, while some of the unicellular organisms present methylation on adenosine (CUMMINGS *et al.* 1974; HATTMAN *et al.* 1978). Methyl group is added to the carbon at fifth position of cytosine resulting in 5^{m}C. Until recently it was believed that methylation of cytosine is restricted to CpG dinucleotide. Advanced sequencing techniques as well as the ability to map sequences at base pair resolution disclosed non-CpG methylations. Non-CpG methylations are less frequent than CpG methylations and were seen in oocytes (mammalian) (TOMIZAWA *et al.* 2011b), adult brain (Asian Seabass) (XIE *et al.* 2012) and embryonic stem cells (mammalian) (RAMSAHOYE *et al.* 2000). In general DNA methylation is associated with transcriptional silencing with exceptions (BELL AND FELSENFELD 2000). Introduction of the methyl

group on cytosine affects the binding of proteins such as repressors, histones and hormone receptors to the DNA indicating regulation of gene expression (LIN AND RIGGS 1972; LIN *et al.* 1976; KALLOS *et al.* 1978), by sterically preventing binding. The methylated regions act as binding sites for certain transcriptional repressors including MeCP2, MBD1 and MBD2 leading to closed chromatin (HENDRICH AND BIRD 1998).

DNA methylation in fish species

Vertebrate DNA is methylated at higher levels when compared to that of invertebrates (BIRD AND TAGGART 1980). Various studies report DNA methylation in fish under different physiological and environmental conditions. Vanyushin et al (VANYUSHIN *et al.* 1970) observed decreased DNA methylation in all tissues of salmon during its upstream migration. Same group later ascertained a positive correlation between DNA methylation and GC content in marine fishes (VANYUSHIN *et al.* 1973). Exposure to various pollutants, toxins as well as habitat conditions influence DNA methylation patters in fish; exposure to oestradiol and cadmium resulted in hypermethylation in male gonad tissue of *Gasterosteus aculeatus* and *Anguilla anguilla* liver respectively (ANIAGU *et al.* 2008; PIERRON *et al.* 2014). Toxins like tributyltin and triphenyltin lead to hypomethylation in *Sebastiscus marmoratus* liver (WANG *et al.* 2009). Fish species with differences in habitat temperatures exhibit differences in DNA methylation. Polar fishes are hypermethylated when compared to tropical and temperate species (VARRIALE AND BERNARDI 2006). Similar studies to understand the role of DNA methylation contributing to the physiological differences among hatchery reared and wild species of *Oncorhynchus mykiss* showed no significant differences in global DNA methylation with differences in specific sites (BLOUIN *et al.* 2010). Differences in methylation patterns were also observed in species with differences in migration ability (BAERWALD *et al.* 2016), maturation pattern (MORÁN AND PÉREZ-FIGUEROA 2011) and during metamorphosis (COVELO-SOTO *et al.* 2015). Sex reversal as an effect of environmental factors such as temperature is observed in various species including fish (DEVLIN AND NAGAHAMA 2002). DNA methylation is proved to negatively regulate gene expression of aromatase (cyp 19a), methylation in the cyp19a promoter was reported in female fish exposed to elevated temperatures which is generally a trait of male fish (NAVARRO-MARTÍN *et al.* 2011). Additionally, the role of DNA methylation in sex determination in fish was established in various studies using *Cynoglossus semilaevis* (SHAO *et al.* 2014) and *Paralichthys olivaceus* (WEN *et al.*

2014; SI *et al.* 2016). Also, evidence for transgene and repetitive sequence methylation was established in fish (WINN *et al.* 1995; KATO 1996). Collectively these studies indicate the important role of DNA methylation in fine tuning of gene expression in response to various physiological and environmental factors.

DNA methylation during skeletal myogenesis

Studies in humans confirmed the regulatory role of DNA methylation during myogenesis; including activation of muscle precursor cells, proliferation and differentiation. Comparative studies to understand the methylation landscape among myogenic differentiating cells and mature skeletal muscle suggested loss of methylation in mature fibers. Differentiating cells exhibit approximately 90% higher methylation rates when compared to mature myofibers (CARRIÓ *et al.* 2015). Differential expression of enzymes involved in DNA methylation was observed in stage specific manner, *Dnmt1* was upregulated during activation and downregulated during differentiation. Reduced expression of de novo methyltransferases *Dnmt3a* and demethylating enzymes *Tet1*, *Tet2* and *Tet3* during muscle precursor cell activation was reported while *Dnmt3b* remained unchanged (LIU *et al.* 1996; PALLAFACCHINA *et al.* 2010; LIU *et al.* 2013; RYALL *et al.* 2015). The role of DNA methylation in cell fate commitment and in progression of myogenesis was supported by various studies using 5-azacytidine and antisense RNA against DNMT1 (CONSTANTINIDES *et al.* 1977; SZYF *et al.* 1992). Proliferating myoblasts exposed to 5-azacytidine exhibited increased expression of myogenic genes including myogenin. This indicated a functional role of DNA methylation in allowing binding of myogenic transcription factors to their target genes promoting differentiation (SCARPA *et al.* 1996). Hyper and hypomethylation of various myogenic regulatory factors and genes involved in the process of myogenesis and their importance were reported in various studies listed in Table 1. Collectively these studies support the important role of DNA methylation in various stages on myogenesis. Although most of the studies represent CpG methylations, the importance of non-CpG methylations are yet to be identified. Additionally, technical methods used could not differentiate between methylated and hemi-methylated cytosines, hence studies understanding such differences are necessary to know the functional impact of DNA methylation in myogenesis.

Influence of estrogen on DNA methylation

Increased expression of estrogen receptors α and β was observed in skeletal muscle of rainbow trout exposed to 17β-estradiol within 24 hours of exposure (Fig 1). Supporting evidence from breast cancer and prostate cancer studies suggest a role for estradiol and its receptors α and β in regulation of gene repression through DNA methylation (ADJAKLY *et al.* 2014; JADHAV *et al.* 2015; ARIAZI *et al.* 2017). Expression of DNA methylating enzymes DNMT1, DNMT3a and DNMT3b were reported to be influenced by sex steroid hormone in females during menstrual cycle (YAMAGATA *et al.* 2009). Exposure to estrogenic compounds led to hypomethylation of various genes in mice (LI *et al.* 1997; TANG *et al.* 2008; BROMER *et al.* 2010). Similarly, studies to understand the effect of 17β-estradiol and soy phytoestrogens on DNA methylation in prostate cancer cells also showed decreased DNA methylation (ADJAKLY *et al.* 2014). Conversely, our studies indicate an increase in DNA methylation of myogenic differentiation factor MyoD1 (KOGANTI *et al.* 2017b) in exon 1. Mostly decreased methylations reported in mice and cancer cell lines under the influence of E2 are CpG while increased methylations observed in skeletal muscle of rainbow trout are CpH methylations. Availability and use of advanced sequencing techniques assisted in identification of these CpH methylations. In general, evidence suggest that gene expression is regulated by E2 and its receptors via DNA methylation.

Previous studies strongly suggest E2 leads to an imbalance of protein turnover, but direct evidence for the role of miRNA and DNA methylation in rainbow trout skeletal muscle and their contribution for imbalance in protein turnover under the influence of E2 is limited. Hence determination of the role of these molecular mechanisms is necessary. The current study aims in understanding the effect of E2 in rainbow trout skeletal muscle by firstly, determining differential expression of miRNAs and regulation of their target genes involved in muscle metabolism; secondly, understanding the role of DNA methylation in regulation of gene expression involved in myogenesis.

FIGURES

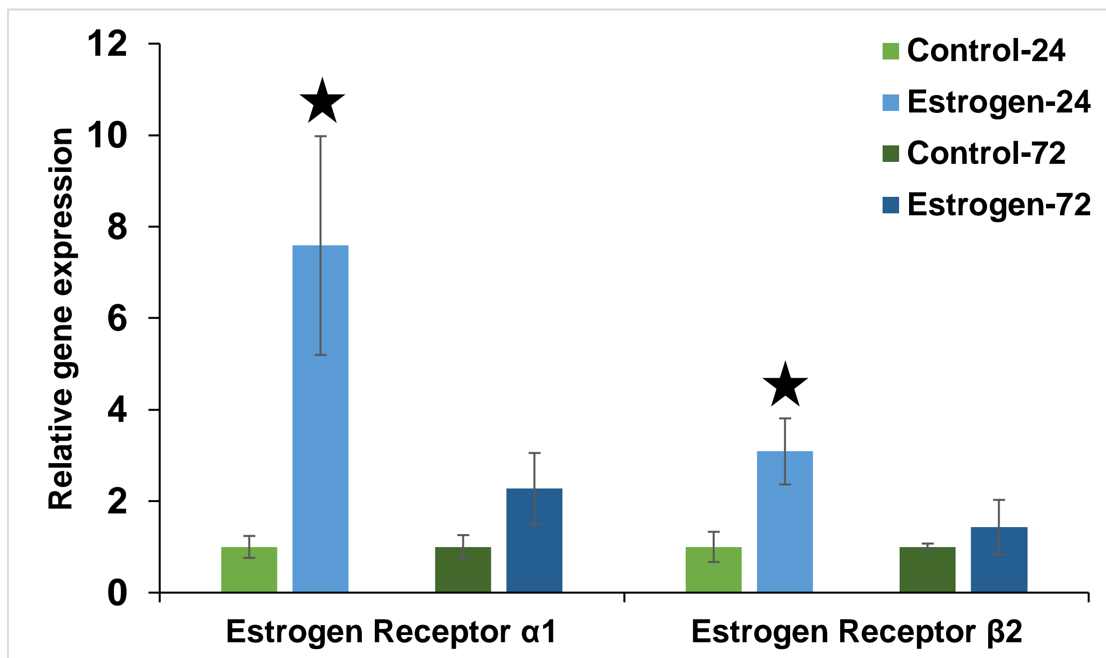

Figure 1. Relative gene expression of estrogen receptors.

Expression measured at 24 hours and 72 hours post treatment represented as mean values ± SEM (n = 6). * P < 0.05.

TABLES

Table 1. Role of DNA methylation on expression of myogenic regulatory factors

Methylation status of genes involved in myogenesis	Functional contribution of genes	Reference
Hypermethylation of Pax3 in myogenic cells and mature muscle fibers	Pax3 necessary for migration and early lineage commitment	(TSUMAGARI *et al.* 2013)
Hypomethylation of a giant muscle associated protein Obscn in muscle and hypermethylated in the same sites in myoblasts and myotubes	Important in the formation of skeletal muscle	(TSUMAGARI *et al.* 2013)
Hypomethylation of Myh7b in muscle	Important because of its intronic microRNA miR499 in skeletal and cardiac muscle	(TSUMAGARI *et al.* 2013)
hypermethylation in promoter regions of genes involved in muscle contraction and other muscle processes	Important during terminal differentiation	(MIYATA *et al.* 2015)
Hypermethylation of ID4 and ZNF238 binding sites	Important for myotube formation	(MIYATA *et al.* 2015)
Demethylation of myogenin promoter region in differentiated muscle cells which is hypermethylated in myoblasts and other non-myogenic cells	important for C2C12 muscle differentiation	(LUCARELLI *et al.* 2000)
Hypomethylation of Notch1 and its ligands Dll1 and Jag2 in skeletal lineage cells	Notch signalling is necessary for proliferation of muscle satellite cells	(TERRAGNI *et al.* 2014)
Lack of methylation in distal enhancer of myogenic differentiation factor MyoD1	Suffecient for gene expression during skeletal muscle development	(BRUNK *et al.* 1996)
Hypermethylation of Myf5 enhancer region in embryonic stem cells which is completely demethylated in muscle lineage cells including myoblasts, myotubes, and skeletal muscle	Myf5 necessary for muscle cell lineage	(CARRIÓ *et al.* 2015)

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CHAPTER-1

Estradiol regulates expression of miRNAs associated with myogenesis in rainbow trout

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ABSTRACT

17β-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 progression indicates that E2 leads to expansion of the quiescent myogenic precursor cell population to address atrophying mature muscle in rainbow trout during sexual development.

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 *et al.* 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 is estrogen receptors (ERs) which bind and are activated by 17β-estradiol (E2). Four isoforms of ERs belonging to two different subtypes, ERα and ERβ, are present in rainbow trout (NAGLER *et al.* 2007).

17β-Estradiol production and release is greatly increased during sexual maturation (LUBZENS *et al.* 2010a) in rainbow trout, driving high energy demanding metabolic processes during the follicular growth phase of vitellogenesis, including synthesis of vitellogenin by liver (ANDERSON *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 (LOVE 1960a; SALEM *et al.* 2010b; DAVIDSON *et al.* 2014; MANOR *et al.* 2015). Reduction in muscle growth during spawning in rainbow trout partially results from an increase in protein turnover (SALEM *et al.* 2006a; CLEVELAND AND WEBER 2011b; CLEVELAND *et al.* 2012) stimulated by maturation-related signals such as E2 (CLEVELAND AND WEBER 2011b; CLEVELAND AND WEBER 2016b). Also, the 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 *et al.* 2008) as well as disruption of the GH/IGF axis (HOLLOWAY AND LEATHERLAND 1997b; NORBECK AND SHERIDAN 2011a; HANSON *et al.* 2012; HANSON *et al.* 2014). 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 *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 *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; SOKOL 2012; HITACHI AND TSUCHIDA 2013; LUO *et al.* 2013; WANG 2013; SHENOY AND BLELLOCH 2014; ZHANG *et al.* 2014). Rainbow trout miRNA from various tissues were recently reported (JUANCHICH *et al.* 2016), and miRNA serve as important regulatory factors of energy metabolism in teleosts (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 by Drosha to a hairpin, which is transported to the cytoplasm and further processed into a mature miRNA by Dicer. The miRNAs 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 the *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 *et al.* 2007; MCCARTHY AND ESSER 2007; MCCARTHY 2008; YUASA *et al.* 2008; CHEN *et al.* 2010; TOWNLEY-TILSON *et al.* 2010). 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 *et al.* 2006a) but also play a role in signaling pathways controlling muscle cell proliferation and differentiation (ZANOU AND GAILLY 2013a).

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.

MATERIALS AND METHODS

Experimental design

Animal experiments were conducted at the USDA/ARS National Center for Cool and Cold Water Aquaculture (NCCCWA) in accordance with 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 µg/µL in ethanol and diluted to 2.5 µg/µl with vegetable oil. The control treatment contained an equal ratio of ethanol: vegetable oil. Fish were anesthetized with tricaine methylsulphonate (MS-222, 100 mg/l), weighed, and received intraperitoneal injections of E2 or the vehicle (2.0 µl/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.

RNA isolation

Total RNA was prepared 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 a NanoDrop 1000 Spectrophotometer (Thermo Scientific). Four biological replicates from each treatment were sequenced.

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 by an 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 (RAMACHANDRA *et al.* 2008b; SALEM *et al.* 2010c; MA *et al.* 2012; MA *et al.* 2015). 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 the 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.

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 iQ™ SYBR® Green Supermix (Bio-Rad) and 300 nM of forward and reverse primer in a final volume of 25 µl 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.

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.

Gene ontology enrichment analysis

R package GOstats (FALCON AND GENTLEMAN 2007) was used for gene ontology (GO) enrichment (ASHBURNER *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 . Gene ontologies with respect to molecular function and biological processes were identified.

RESULTS

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.1d 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.

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 log_2 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). A 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.

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 no significant difference between the E2 treated and control samples in qPCR analysis (Fig. 3).

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 and 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.

Validation of miRNA target genes

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 as well as 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).

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 led to a 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 *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 the two time points (Fig. 5).

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).

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 (SALEM *et al.* 2006a; CLEVELAND AND WEBER 2011b; CLEVELAND AND WEBER 2015) unlike rodent, bovine and porcine species in which E2 has anabolic effects (REHFELDT *et al.* 2009a; KAMANGA-SOLLO *et al.* 2010a). 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 *et al.* 2012; CRIST *et al.* 2012), proliferation (GAMMELL 2007; HUANG *et al.* 2012b; SHENOY AND BLELLOCH 2014) and differentiation (NAKAJIMA *et al.* 2006; LIU *et al.* 2010; GAGAN *et al.* 2011) are well studied in different physiological and disease states of muscle (CHEN *et al.* 2009; ROOIJ *et al.* 2009; GAMBARDELLA *et al.* 2010; NIELSEN *et al.* 2010; GOLJANEK-WHYSALL *et al.* 2012; KORNFELD *et al.* 2012; LIU *et al.* 2012; ROSALES *et al.* 2013; KIM *et al.* 2014). The roles of miRNAs during different metabolic states of muscle including apoptosis (GAMMELL 2007; HIRAI *et al.* 2010; WADA *et al.* 2011; HUDSON *et al.* 2014) and hypertrophy (MCCARTHY AND ESSER 2007; HITACHI AND TSUCHIDA 2013; JAVED *et al.* 2014) have also been established. Contribution of miRNAs in regulating different signaling pathways (GRANJON *et al.* 2009; WADA *et al.* 2011; HITACHI AND TSUCHIDA 2013; LUO *et al.* 2013) involved in myogenesis demonstrates 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 (NAGUIBNEVA *et al.* 2006; RAO *et al.* 2006; SWEETMAN *et al.* 2008; HIRAI *et al.* 2010; GAGAN *et al.* 2012).

Previous reports in humans, rodents, and zebrafish indicate E2 influenced the expression of several miRNAs, including miR-125b, miR-145, miR-17-5p, miR-196b, let-7h, miR-206, miR-20a and miR-21 (HOSSAIN *et al.* 2006; ADAMS *et al.* 2007; KOVALCHUK *et al.* 2007; SCOTT *et al.* 2007; COHEN *et al.* 2008; DAI *et al.* 2008; KONDO *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 ERα; miR-221, miR-222(ZHAO *et al.* 2008), miR-22 (SUN *et al.* 2008) and miR-206 (KONDO *et al.* 2008) negatively regulate the expression of ERα, 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 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 *et al.* 2016) . miR-499 is highly expressed in type I muscles, encoded by myosin genes and is thought to play a role in coordinating the expression of myosin heavy chain in slow muscle (VAN ROOIJ *et al.* 2008; MCCARTHY *et al.* 2009; VAN ROOIJ *et al.* 2009). Studies in humans with miR-125b-3p in different cell lines established its essential role in proliferation of differentiated cells (LEE *et al.* 2005). Besides the expression of MyoD and myogenin, myosatellite cell differentiation also corresponds to the activation of miR-214 (JUAN *et al.* 2009), and the significance of miR-214 is confirmed by embryonic lethality and severe defects in muscle development in mice functional knockout studies (WATANABE *et al.* 2008). Additionally miR-214 regulates IRS/AKT/PI3K pathway in C2C12 myocytes (HONARDOOST *et al.* 2015), and this also supports a role of miR-214 in the process of differentiation. In the present study, miR-214 is down regulated in fish 24 hours after injection with E2, suggesting that this mechanism is involved with negative effects of E2 on myogenesis in rainbow trout. An additional miRNA down regulated by E2 was miR-145, which can regulate the PI3K pathway in humans (ROSALES *et al.* 2013), and targets different factors involved in proliferation (MARTINEZ AND GREGORY 2010), including OCT4, SOX2 and KLF4 (XU *et al.* 2009), and its loss in smooth muscles resulted in increased proliferation with reduced differentiation (ZHANG 2009). Also down regulated by E2 in rainbow trout was miR-181a-3p, a miRNA known to regulate Nanog expression in humans (MINTZ *et al.* 2012), a protein that promotes myoblast pluripotency and reduces differentiation in C2C12 cells (LANG *et al.* 2009). Another important cluster of miRNA with a role in the cell cycle is the miR-17 cluster, of which miR-17 and miR-20a control c-Myc mediated cell proliferation in humans through reduction in E2F1 expression (O'DONNELL *et al.* 2005). In our study, these two miRNAs exhibited an E2-induced expression increase, suggesting this mechanism may also be important for E2-induced reductions in muscle growth in rainbow trout. Nearly all of the aforementioned findings occurred in mammals, most of which were human, and a functional role of these miRNAs in myogenesis cannot be assumed to be conserved between mammals and teleosts like rainbow trout. However, given 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 elucidate 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, 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 *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 *et al.* 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 aforemention 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 2011b; CLEVELAND AND WEBER 2015; CLEVELAND AND WEBER 2016b). Part of the E2 response is increased expression of E3 ubiquitin ligases, including muscle-specific atrophy gene Fbxo32/atrogin-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 (WADA *et al.* 2011; HUDSON *et al.* 2014), 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 *et al.* 2014). miRNAs belonging to same family imply that they have similar sequence or structural configuration, hence similar function (KACZKOWSKI *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 *et al.* 2016). Novel miRNA expressed in skeletal muscle after E2 exposure belongs to the same family with the probable function of chromatin remodeling.

These findings suggest that E2 can affect processes of 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 *et al.* 2013b). 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.

ACKNOWLEDGEMENTS

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FIGURES

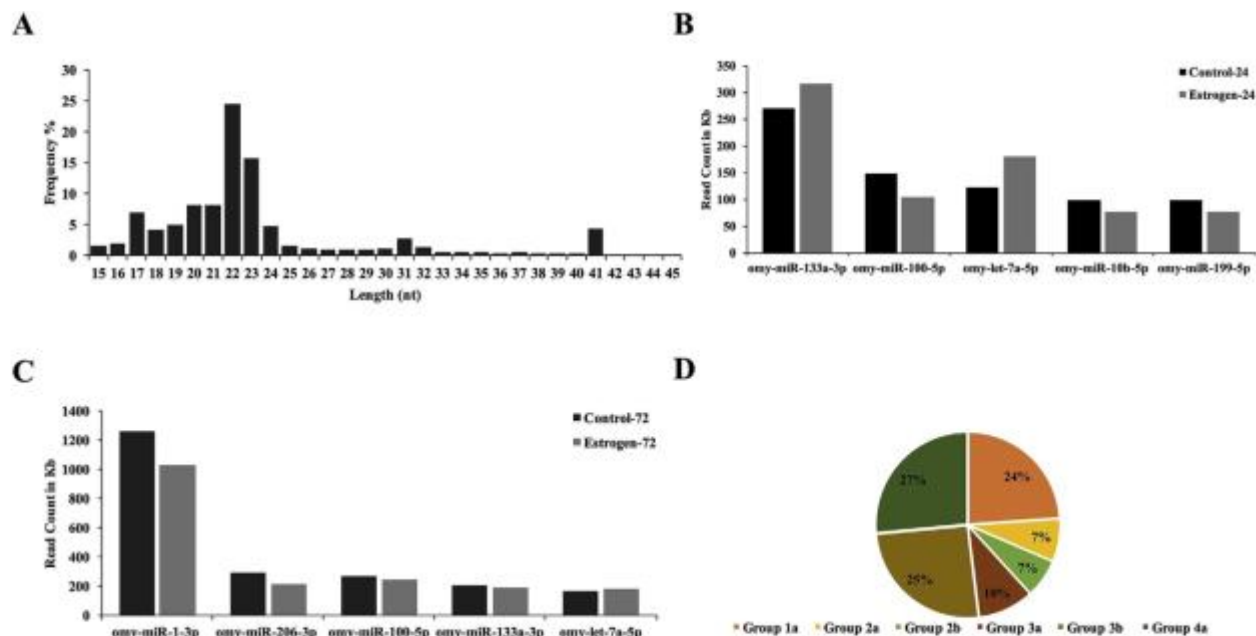


Figure 1. miRNA profile of skeletal muscle from control and E2 treated sample.

(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.

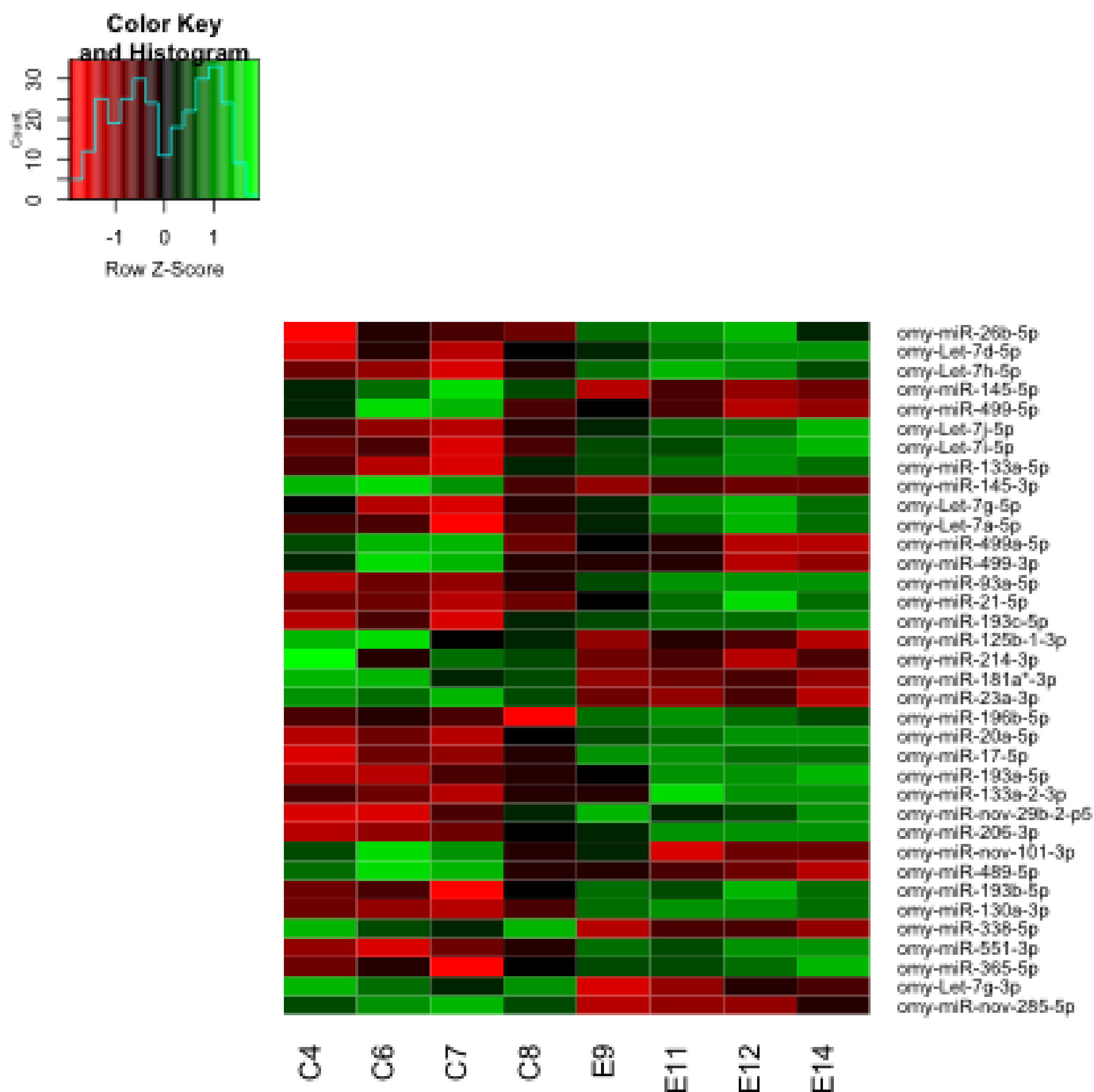


Figure 2. Heat map showing differential expression of miRNAs.

Statistically significant miRNAs after 24 hours of treatment. C4, C6, C7, C8 represent control samples and E9, E11, E12, E14 are E2 treated samples.

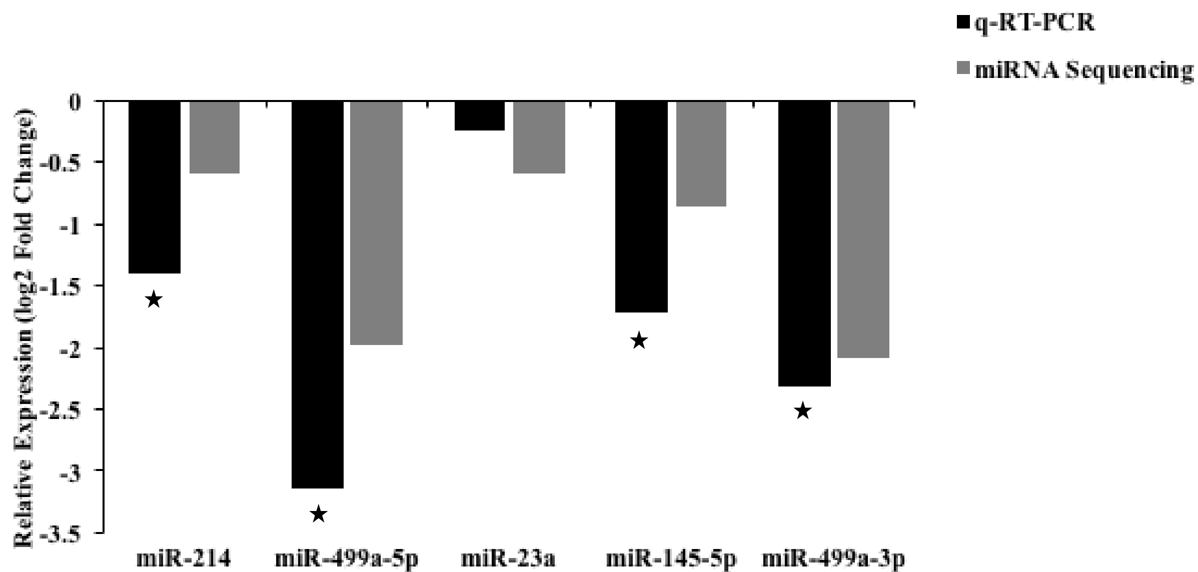


Figure 3. Validation of miRNA sequencing results.

Log_2 fold change of E2 samples compared to control from sequencing and qPCR. Results are expressed as mean ± SEM (n = 6 or 5). * P < 0.05.

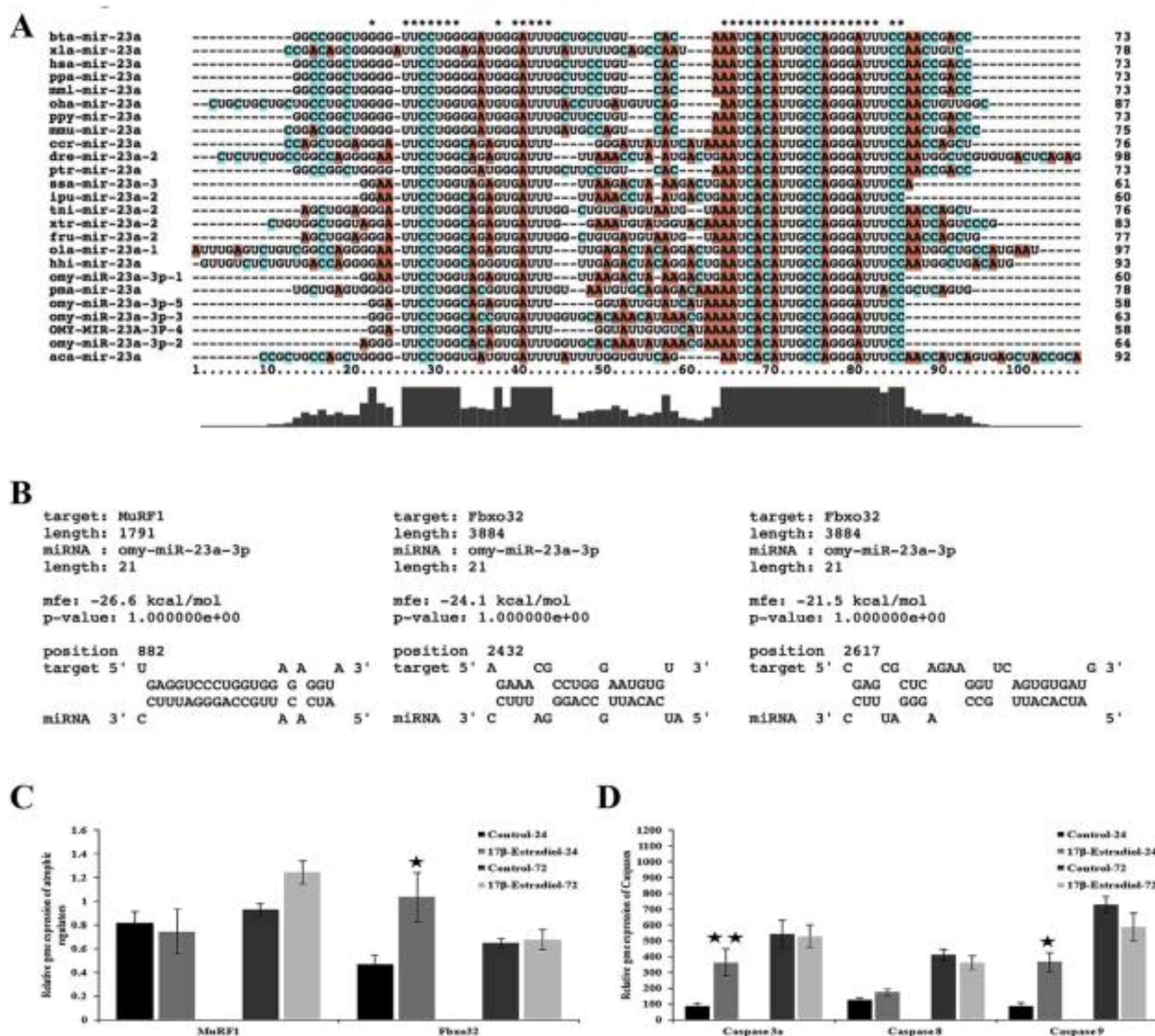


Figure 4. Omy-miR-23a-3p and its target genes.

(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 ± SEM (n = 6). * $P < 0.05$ and ** $P < 0.01$.

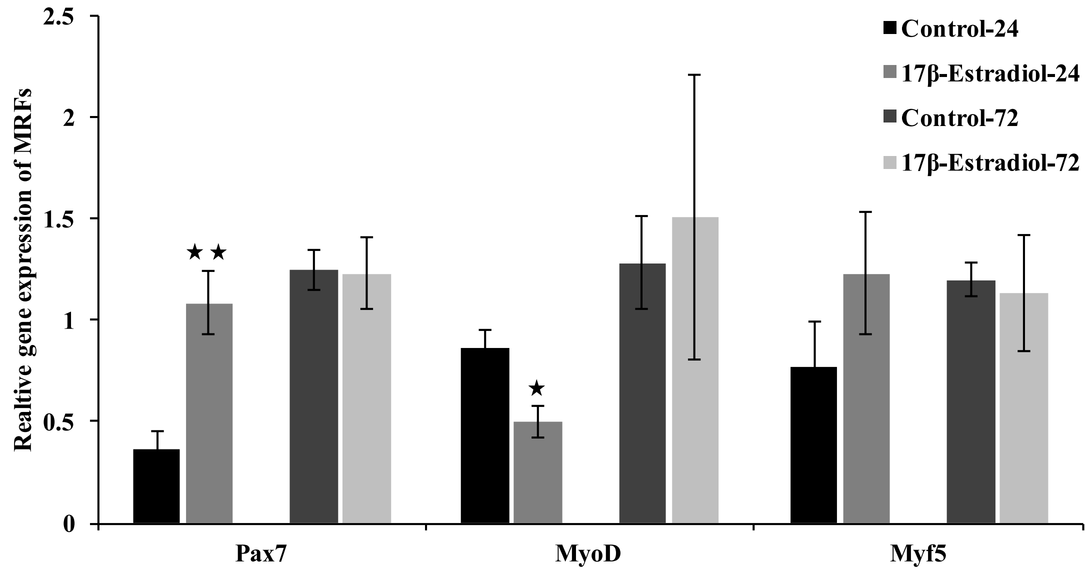


Figure 5. Expression of myogenic regulatory factors (MRFs) defining cell cycle.

Relative gene expression of MRFs in control and E2 treated samples after 24 and 72 hours, represented as mean values ± SEM (n = 6). ** $P < 0.01$.

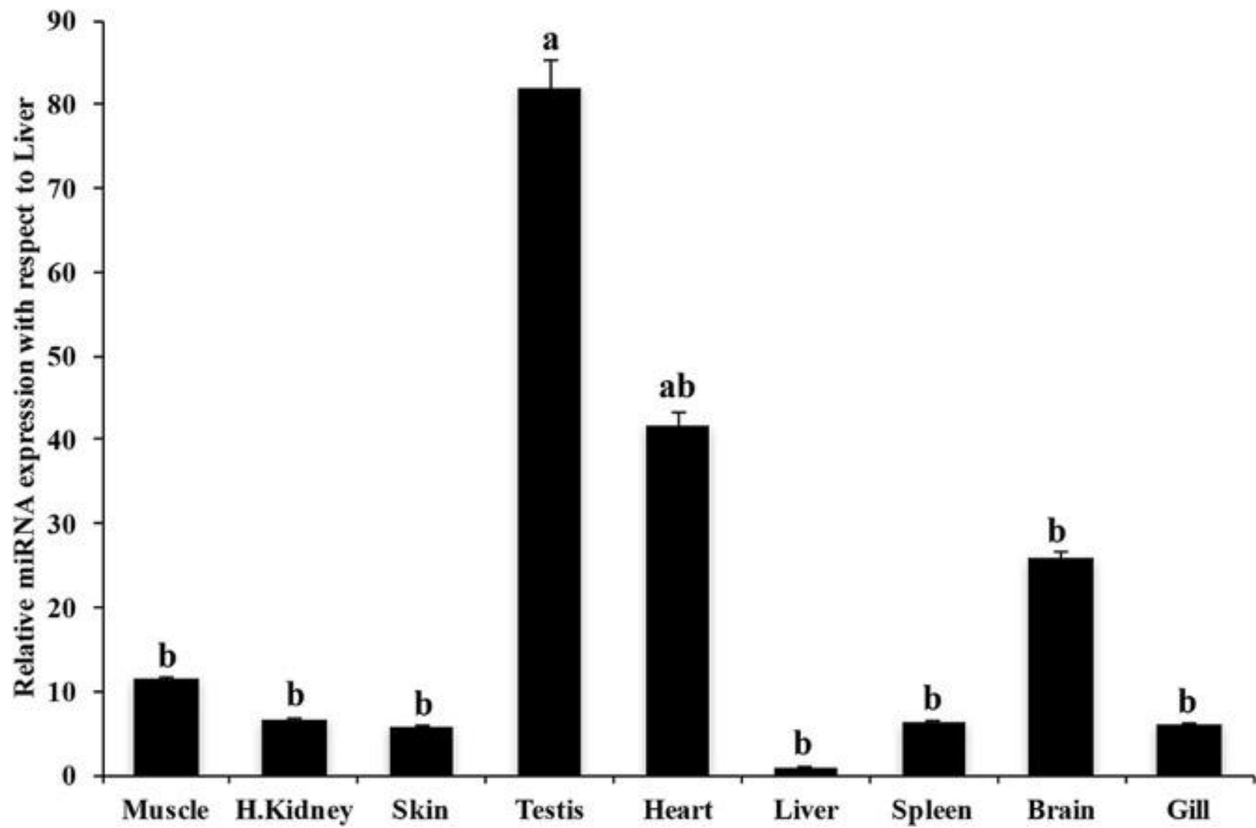


Figure 6. Novel miRNA, omy-miR-nov-285-5p.

Rainbow trout tissue distribution and expression of novel miRNA.

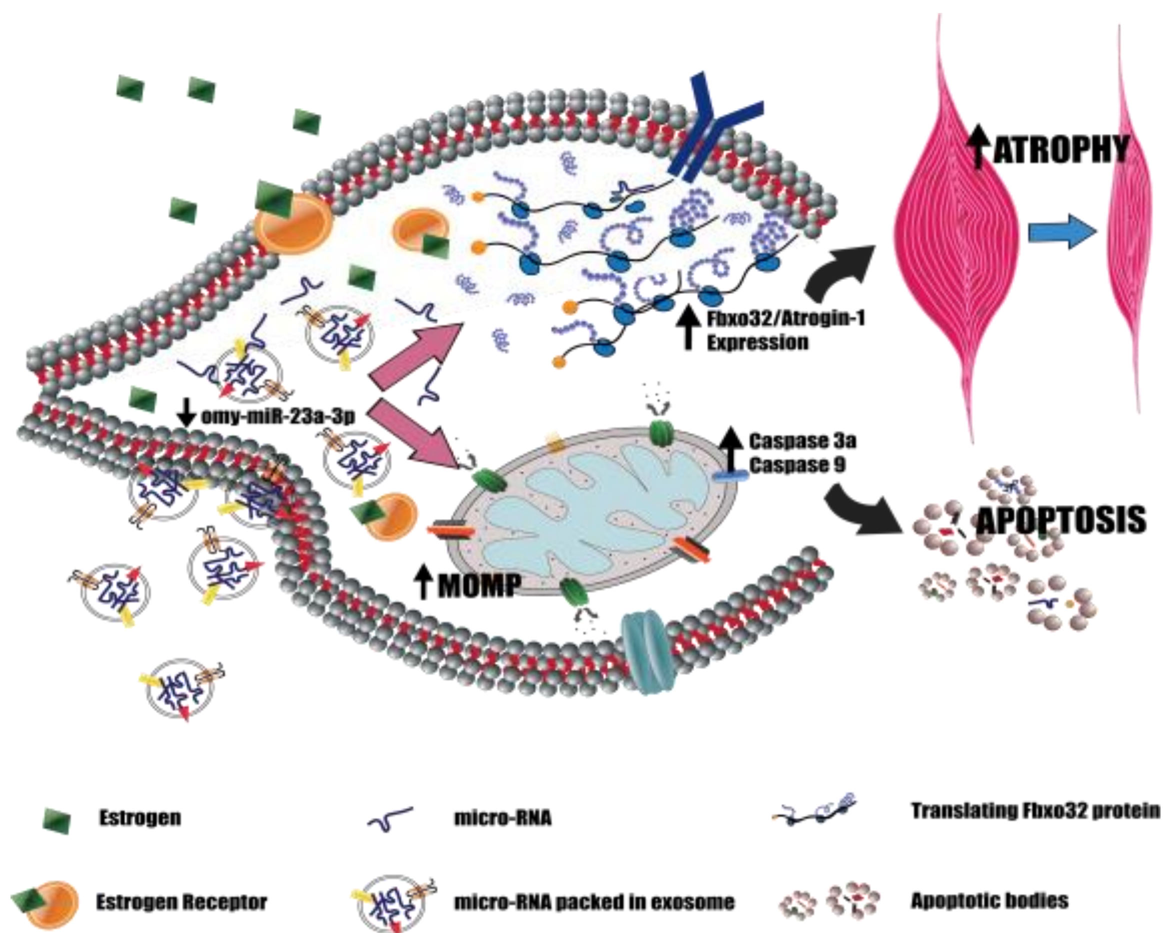


Figure 7. Prospective response to E2.

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.

TABLES

Table 1. Summary of miRNA sequencing data.

Reads obtained from E2 treated and control skeletal muscle samples at 24 and 72 hours.

	Number of reads	**% of mappable 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%

Table 2. Differentially expressed miRNAs.

Listed are the adjusted p-values < 0.05, their respective sequence and log_2 fold change

miRNA Name	Sequence	Log_2Fold Change	P-Value	P-Adjusted
omy-489-5p	UGGUCGUAUGUAUGACGUCAUU	-1.13	0.0032	0.0435
omy-let-7a-5p-1	UGAGGUAGUAGGUUGU/GAUAGUUU/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	GUCCAGUUUUCCCAGGAAUCCCU	-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	CGGGAUCUUGUGGGCGAGAUGA	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	UAAAGUGCUUAUAGUGCAGGUAG	1.35	<0.0001	0.0006
omy-miR-21-5p	UAGCUUAUCAGACUGGUGUUGGA	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	AACAACAUCCUGGUGCUGCCUGAGU	-0.71	0.0006	0.0134
omy-miR-365-5p	AGGGACUUUUAGGGGCAGCUGUG	1.09	0.0008	0.0154
omy-miR-499-3p	AACAUCACUUUAAGUCUG/CUGCU	-2.09	0.0001	0.0019
omy-miR-499-5p	UUAAGACUUA/GCAGUGAUGUUUCGGU	-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	AAAAGUGCUGUUUGUGCAGGUAG	0.95	<0.0001	0.0001
omy-miR-nov-101-3p	GCGACCCACUCAUGAUUUCAGA	-1.59	0.0022	0.0335
omy-miR-nov-285-5p	GCAGUGGUUGUCGUAAUAGGUUC	-2.25	<0.0001	<0.0001
omy-let-7g-3p	CUGUACAAGCCACUGCCUUGCU	-0.82	0.0032	0.0435
omy-nov-miR-29b-2-p5	GCUGGUUUCAGGUGGUGACUUAGA	1.33	0.0036	0.0444

Table 3. Enriched target genes of differentially expressed miRNAs.

The target genes involved in different biological processes and molecular functions are listed.

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
Down-Regulated miRNA				
GOBPID	**P-value**	**Count**	**Size**	**GO Term**
GO:0006259	7.32E-25	144	2158	DNA metabolic process
GO:0006310	2.95E-35	124	1311	DNA recombination
GO:0006313	3.07E-38	121	1169	Transposition, DNA-mediated
GO:0015074	2.87E-35	122	1272	DNA integration
GO:0032196	3.96E-38	121	1172	Transposition
GO:0055085	6.40E-12	263	2461	Transmembrane transport
GO:0044710	2.82E-11	285	7574	Single-organism metabolic process
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**
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-cystein 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

Table 4. Target genes of omy-miR-23a-3p.

Gene ontologies and p-values of target genes (predicted) of omy-miR-23a-3p involved in mitochondrial 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

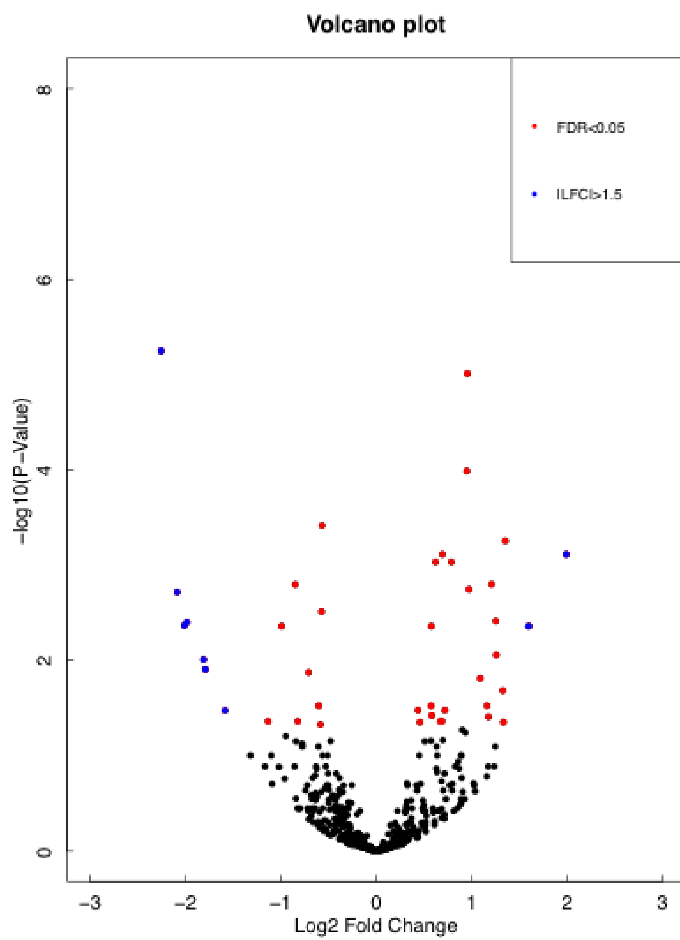
Table 5. Target genes involved in cell cycle and myogenesis.

Listed are the target genes of differentially expressed miRNAs that are involved in cell cycle and myogenesis

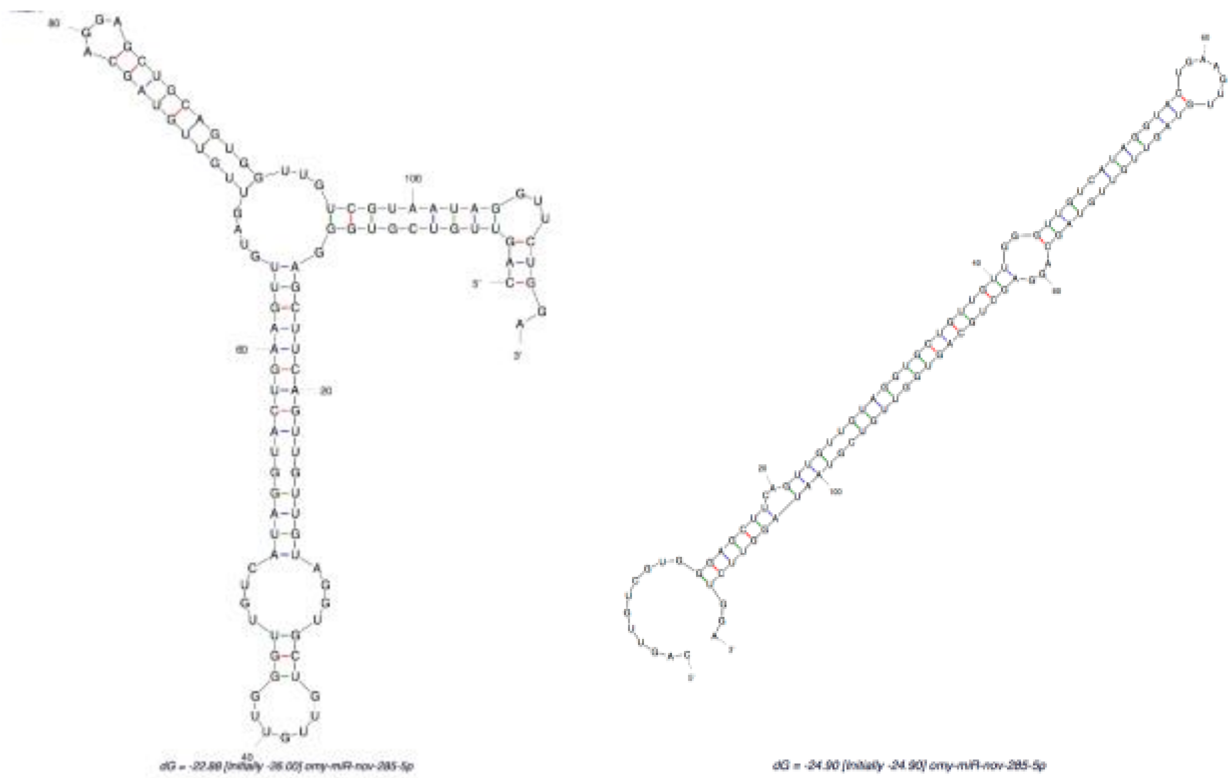
Target genes related to Cell Cycle				
GOBPID	**P-value**	**Count**	**Size**	**GO Term**
GO:0000083	0.0004	3	3	Regulation of transcription involved in G1/S transition of mitotic cell cycle
GO:0000086	0.0029	6	42	G2/M transition of mitotic cell cycle
GO:0000278	0.0096	28	553	Mitotic cell cycle
GO:0007093	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

SUPPLEMENTARY DATA



Supplementary Figure 1. Volcano plot of miRNAs from sequencing analysis: miRNAs expressed above and below average were used for generating volcano plot. Differentially expressed miRNAs with a P-value < 0.05 are represented in red and those with a log2 fold change of ± 1.5 in blue.



Supplementary Figure 2. Putative stem loop structures of the novel miRNA_omy-miR-nov-285-5p.

Supplementary Table 1. List of primers used for qPCR analysis

Gene Name	**Primer efficiency**	**Primer Name**	**Sequence**
Muscle Ring-finger Protein 1	97%	RT-Murf1F	5' CTGATTAGTGGCAAGGAGCTG 3'
		RT-Murf1R	5' GTAAGGTGCTCCATGTTCTCG 3'
Atrogin-1	95%	RT-Fbxo32F	5' TGCGATCAAATGGATTCAAA 3'
		RT-Fbxo32R	5' GATTGCATCATTTCCCCACT 3'
Paired Box protein-7	104%	RT-Pax7F	5' TACAGGTGTGGTTCAGCAACAG 3'
		RT-Pax7R	5' GCTGGTAGGTAGGTAGACTGG 3'
MyoD	100%	RT-MyoDF	5' CCTTACATGACCCAAACACCA 3'
		RT-MyoDR	5' GGCATGTTCTCTCCCCATAA 3'
Myf5	127%	RT-Myf5F	5' GACCTGTGTACCACGTATTGTG 3'
		RT-Myf5R	5' CCGAAATAGGAAGTCAAAGCGG 3'
Caspase 3A	87%	Casp3	5' TTTGGGAGTAGATTGCAGGG 3'
			5' TGCACATCCACGATTTGATT 3'
Caspase 8	87%	Casp8	5' CAGCATAGAGAAGCAAGGGG 3'
			5' TGACTGAGGGGAGCTGAGTT 3'
Caspase 9	105%	Casp9	5' TTCGAGACCTGGAGACTCGT 3'
			5' GCTATGCTGCCCTTTCTCAC 3'
Omy-miR-nov-285-5p	100%	omy-miR-nov-285-5p	5' GCAGTGGTTGTCGTAATAGGTTC 3'
Omy-miR-214-3p	98%	omy-miR-214-3p	5' ACAGCAGGCACAGACAGGCAGT 3'
Omy-miR-23a-3p	77%	omy-miR-23a-3p	5' ATCACATTGCCAGGGATTTCC 3'
Omy-miR-145-5p	112%	omy-miR-145-5p	5' GTCCAGTTTTCCCAGGAATCCCT 3'
Omy-miR-499a-3p	101%	omy-miR-499a-3p	5' AACATCACTTTAAGTCTCTGCT 3'
Omy-miR-499-5p	73%	omy-miR-499-5p	5' TTAAGACTTACAGTGATGTTTCGGT 3'
Omy-miR-133a-5p	NA	omy-miR-133a-5p	5' AGCTGGTAAAATGGAACCAAAT 3'

Supplementary Table 2. miRNA groups based on the region mapped

Group	**Mapped to**
Group 1a	Mapped to rainbow trout genome
Group 2a	Mapped to known mirs of Pisces and rainbow trout genome, with hairpins
Group 2b	Mapped to known mirs of Pisces and rainbow trout genome, without hairpins
Group 3a	Mapped to mirs and miRs of Pisces but unmapped to rainbow trout genome
Group 3b	Mapped to mirs of Pisces but unmapped to rainbow trout genome
Group 4a	Unmapped to known miRs but mapped to rainbow trout genome and with hairpins (novel)

Supplementary Table 3. List of differentially expressed miRNA precursor coordinates, precursor sequence with their family name

miRNA Name	Family Name	Chromosome Number	Precursor Coordinate	Consensus precursor sequence
omy-miR-nov-285-5p	mir-7911	chrUn_10574	chrUn_10574:1102181477..1102181589:+	caguugucgugggagcuucaguuguuguaggugcuguuguugggguugucauagguacugaaguuguaguuguuguagcaggagcugcagugguugucguaauagguucugga
omy-miR-499-3p_chrUn_16	mir-7911	chrUn_16_25635	chrUn_16:306707..306767:-	uuaagacuuacagugauguuucgguaacucagcucaugaacaucacuuuaagucugugcu
omy-miR-499-3p_chrUn_9	mir-499	chrUn_9_37064	chrUn_9:20333537..20333597:+	uuaagacuugcagugauguuuagguaacucaacucaugaacaucacuuuaagucugugcu
omy-miR-499-3p_chrUn_7	mir-499	chrUn_7_35915	chrUn_7:18228420..18228480:-	uuaagacuugcagugauguuuagggaaaugaucacaugaacaucacuuuaagucugugcu
omy-miR-499a-3p_chrUn_17	mir-30	chrUn_17_26348	chrUn_17:42442523..42442583:+	uuaagacuuguagugauguuuagggaaauuaucacaugaacaucacuuuaagucucugcu
omy-miR-499-5p_chrUn_9	mir-499	chrUn_9_37064	chrUn_9:20333537..20333597:+	uuaagacuugcagugauguuuagguaacucaacucaugaacaucacuuuaagucugugcu
omy-miR-499-5p_chrUn_7	mir-499	chrUn_7_35915	chrUn_7:18228420..18228480:-	uuaagacuugcagugauguuuagggaaaugaucacaugaacaucacuuuaagucugugcu
omy-miR-499-5p_chrUn_16	mir-7911	chrUn_16_25635	chrUn_16:306707..306767:-	uuaagacuuacagugauguuucgguaacucagcucaugaacaucacuuuaagucugugcu
omy-miR-499a-5p_chrUn_17	mir-30	chrUn_17_26348	chrUn_17:42442523..42442583:+	uuaagacuuguagugauguuuagggaaauuaucacaugaacaucacuuuaagucucugcu
omy-miR-nov-101-3p	mir-399	chrUn_15152	chrUn:403699924..403699981:-	caaauggaauggcaucaaacaccuggaaacuauguuuuugauuucauuccacugauu
omy-489-5p_chrUn_18	mir-489	chrUn_18_26907	chrUn_18:19465087..19465148:+	uggucguauguaugacgucauuuacuucaauguuuggagugacaucauauguacggcugcu
omy-489-5p_chrUn_16150	mir-489	chrUn_16150	chrUn:519763126..519763187:-	uggucguauguaugacgucauuuacuucaacguuugaagugacaucauauguacggcugcu

omy-miR-125b-1-3p_chrUn_1999	mir-10	chrUn_1999	chrUn:202178785..202178847:+	ucccugagacccuaacuugugacguugugcucucaugucca cggguuaggcucucgggagcu
omy-miR-125b-1-3p_chrUn_8205	mir-10	chrUn_8205	chrUn:871557473..871557535:+	ucccugagacccuaacuugugacguugugcucucaugucca cggguuaggcucucgggagcu
omy-miR-145-5p_chrUn_14	mir-145	chrUn_14_24309	chrUn_14:7434334..7434395:+	guccaguuuucccaggaaucccuugggcaaucacaaggggg auuccuggaaauacuguucu
omy-miR-145-5p_chrUn_29	mir-7911	chrUn_29_32561	chrUn_29:7025223..7025284:+	guccaguuuucccaggaaucccuuggguaaucacaacgggg auuccuggaaauacuguucu
omy-let7g-3p	let-7	chrUn_15717	chrUn:475111092..475111171:-	ugagguaguuguuuguacaguuugagggucccgugauucu gcccgauaaaggagcuaacuguacaagccacugccuugcc
omy-mir-338-5p	mir-338	chrUn_18157	chrUn:740714712..740714771:-	aacaacauccuggugcugccugagugacucucaccaacucc agcaucagugauuuuguu
omy-miR-145-3p_chrUn_14	mir-145	chrUn_14_24309	chrUn_14:7434334..7434395:+	guccaguuuucccaggaaucccuugggcaaucacaaggggg auuccuggaaauacuguucu
omy-miR-145-3p_chrUn_29	mir-7911	chrUn_29_32561	chrUn_29:7025223..7025284:+	guccaguuuucccaggaaucccuuggguaaucacaacgggg auuccuggaaauacuguucu
omy-miR-214-3p_chrUn_28	mir-214	chrUn_28_32478	chrUn_28:15864311..15864374:-	ugccugucuacacuugcugugcagaacauccuggaaccugu acagcaggcacagacaggcaga
omy-miR-214-3p_chrUn_4815	mir-214	chrUn_4815	chrUn:522383727..522383790:+	ugccugucuacacuugcugugcagaacuuccuggaaccug uacagcaggcacagacaggcaga
omy-miR-214-3p_chrUn_10189	mir-214	chrUn_10189	chrUn:1059605857..1059605918:+	ugccugucuacacuugcugugcagaccuuccuauccuguac agcaggcacagacaggcaga
omy-miR-214-3p_chrUn_14546	mir-214	chrUn_14546	chrUn:335338672..335338733:-	ugccugucuacacuugcugugcagaccuuccuauccuguac agcaggcacagacaggcaga
omy-miR-181a*-3p	mir-181	chrUn_Sex_37389	chrUn_Sex:6760148..6760210:+	aacauucaacgcugucggugaguuugagccucugacagaaa ccaucgaccguugacuguacc
omy-miR-23a-3p_chrUn_13452	mir-23	chrUn_13452	chrUn:224625950..224626010:-	ggaauuccugguagagugauuuuuaagacuaaagacugaa ucacauugccagggauuucc
omy-miR-23a-3p_chr_5	mir-23	chr_5_39012	chr_5:2216705..2216769:-	aggguuccuggcacagugauuuggugcacaaauauaaacga aaaucacauugccagggauuucc

omy-miR-23a-3p_chrUn_1	mir-23	chrUn_1_21585	chrUn_1:38914562..38914625:+	ggguuccuggcaccgugauuuggugcacaaacauaaacgaaaaucacauugccagggauuucc
omy-miR-23a-3p_chrUn_28	mir-23	chrUn_28_32241	chrUn_28:8507544..8507602:+	ggauuccuggcagagugauuugguauugugucauaaaaucacauugccagggauuucc
omy-miR-23a-3p_chrUn_8	mir-23	chrUn_8_36536	chrUn_8:8993647..8993705:-	ggauuccuggcagagugauuugguauuguaucauaaaaucacauugccagggauuucc
omy-miR-26b-5p_chrUn_3	mir-26	chrUn_3_33348	chrUn_3:7814465..7814526:-	uucaaguaauccaggauaggcuuguuuaagugggcacagccuauucgggaugacuugguuc
omy-miR-26b-5p_chrUn_9579	mir-7911	chrUn_9579	chrUn:1009543714..1009543775:+	uucaaguaauccaggauaggcuguguguauccugguuggccuguucaugauuacuugaacu
omy-miR-26b-5p_chrUn_17	mir-26	chrUn_17_25985	chrUn_17:4064179..4064240:+	uucaaguaauccaggauaggcugguucccgccagcacggccuauucucgauuacuuguuuc
omy-miR-26b-5p_chrUn_7	mir-26	chrUn_7_35749	chrUn_7:39514929..39514990:+	uucaaguaauccaggauaggcugguucucaccagcacggccuauucucgauuacuuguuuc
omy-miR-26b-5p_chrUn_16136	mir-26	chrUn_16136	chrUn:518325708..518325769:-	uucaaguaauccaggauaggcugguuaacaucugcacggccuauucuugauuacuuguuuc
omy-miR-26b-5p_chrUn_16	mir-26	chrUn_16_25367	chrUn_16:5301608..5301669:+	uucaaguaauccaggauaggcugguuaacacaugcacggccuguucuugauuacuuguuuc
omy-miR-26b-5p_chrUn_11369	mir-7911	chrUn_11369	chrUn:85451983..85452037:-	uucaaguaauccaggauagguuuguucagaaaccuauucugcaugacuugguuc
omy-miR-26b-5p_chrUn_592	mir-7911	chrUn_592	chrUn:71977043..71977104:+	uucaaguaauccaggauagguuuguucagaaagguaaagccuauucugcaugacuugguuc
omy-let-7g-5p_chrUn_9	let-7	chrUn_9_37319	chrUn_9:28477036..28477108:-	ugagguaguaguuuguauaguuuuaggaucacaccagaucugggagaugacuauacagucuacugucuuucc
omy-let-7g-5p_chrUn_16	let-7	chrUn_16_25873	chrUn_16:30748005..30748077:-	ugagguaguaguuuguauaguuuuaggaucacaccagaucugggagauaacuauacagucuacugucuuucc
omy-let-7a-5p_chrUn_2	let-7	chrUn_2_27956	chrUn_2:22287570..22287642:-	ugagguaguagguuguauaguuuuagggucauacccuuccugucagauaacuauacaacuuacugucuuucc
omy-let-7a-5p_chr_4	let-7	chr_4_38888	chr_4:111962..112034:-	ugagguaguagguuguauaguuuuagggucauacccuuccugucagauaacuauacaacuuacugucuuucc

omy-let-7a-5p_chrUn_15	let-7	chrUn_15_24975	chrUn_15:18670635..18670707:+	ugagguaguagguuguauaguuuuagggucacacccaagcugucagaugacuauacaacauacugucuuucc
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omy-let-7a-5p_chrUn_15644	let-7	chrUn_15644	chrUn:462238477..462238553:-	ugagguaguagguuguauaguuugugaagggauagaauccuauucaggugauaacuauacagucuauugccuuccu
omy-let-7a-5p_chrUn_7	let-7	chrUn_7_35858	chrUn_7:10444498..10444575:-	ugagguaguagguuguauaguuugugggagggauuuaauccuauucaggugauaacuauacagucuauugccuuccu
omy-let-7a-5p_chrUn_24	let-7	chrUn_24_31544	chrUn_24:8493389..8493456:+	ugagguaguagguuguauaguugagaauuacaccccgggagauaacuauacaaccuccuagcuuucc
omy-let-7a-5p_chrUn_27	let-7	chrUn_27_32175	chrUn_27:17620732..17620799:-	ugagguaguagguuguauaguugagaauuacaccccgggagauaacuguacagccuccuagcuuucc
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omy-let-7j-5p_chrUn_15717	let-7	chrUn_15717	chrUn:475111092..475111171:-	ugagguaguuguuuguacaguuugaggguccgugauucugcccgauaaaggagcuaacuguacaagccacugccuugcc
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omy-let-7a-5p_chrUn_15_24975	let-7	chrUn_15_24975	chrUn_15:18670635..18670707:+	ugagguaguagguuguauaguuuuagggucacacccaagcugucagaugacuauacaacauacugucuuucc
omy-let-7a-5p_chrUn_12122	let-7	chrUn_12122	chrUn:161015221..161015293:-	ugagguaguagguuguauaguuuuagggucacucccaagcugucagaugacuauacaacauacugucuuucc
omy-miR-17-5p_chrUn_22	mir-17	chrUn_22_31011	chrUn_22:5010277..5010336:-	caaagugcuuacagugcagguagucuucuaauaucuacugcagugaaggcacuuuaagc

omy-miR-17-5p_chrUn_3	mir-17	chr_3_38843	chr_3:57328..57387:-	caaagugcuuacagugcagguagucaucuaauaucuacugcagugaaggcacuuuaagc
omy-miR-551-3p_chrUn_9041	mir-7911	chrUn_9041	chrUn:962874869..962874928:+	gaaaccaagauugggguguggccugggcugacaauauggcgacccauccuugguuucuga
omy-miR-551-3p_chrUn_24	mir-7911	chrUn_24_31596	chrUn_24:1359393..1359452:-	gaaaccaagugugggguguggccuggaaugacaauauggcgacccauccuugguuucuga
omy-let-7d-5p_chrUn_7	let-7	chrUn_7_35542	chrUn_7:12937205..12937275:+	ugagguaguugguuguaugguuucgcauaauaaacaguccggagauaacuguacaaccuucuagcuuucc
omy-let-7d-5p_chrUn_3	let-7	chrUn_3_33484	chrUn_3:29575540..29575620:-	ccuggccggcugguggaagacguggcagagggagugcuacuacagagugugcugcggugugagguaguugguuguaugguu
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omy-let-7i-5p_chrUn_14529	let-7	chrUn_14529	chrUn:333187428..333187506:-	ugagguaguaguuugugcuguugguugguuaugacauuucccgcuauggagaugacugcgcaagcuacugccuugcc
omy-let-7i-5p_chrUn_14530	mir-7911	chrUn_14530	chrUn:333187484..333187560:-	uugugauuucugcuauuucaguguguuucuaucggcauuugaccauguacuggcugagguaguaguuugugcuguu

omy-miR-93a-5p_chrUn_14	mir-17	chrUn_14_24643	chrUn_14:20239223..20239285:-	agcugguaaaaaggaaccaaaucaccucuugaauggauuugguccccuucaaccagcugu
omy-miR-93a-5p_chrUn_2900	mir-17	chrUn_2900	chrUn:305563007..305563068:+	agcugguaaaaaggaaccaaaucaccucuugaauggauuugguccccuucaaccagcugu
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omy-miR-21-5p_chrUn_12	mir-21	chrUn_12_23569	chrUn_12:16027409..16027468:-	acucuuucccuguugcacuacugugggagcuguagcuagcagugcaacaaugaaagggcau
omy-miR-365-5p_chrUn_12052	mir-365	chrUn_12052	chrUn:153600496..153600558:-	uagcuuaucagacugguguuggcuguuacauugcaaggcgacaacagucuguaggcugu
omy-miR-365-5p_chrUn_13	mir-365	chrUn_13_23831	chrUn_13:391873..391935:+	uagcuuaucagacugguguuggcuguuacauugcaaggcgacaacagucuguaggcugu
omy-mir-133a-2-3p_chrUn_203	mir-133	chrUn_203	chrUn_203:22107348..22107459:+	agggacuuuuaggggcagcugugguuuuauuaacccagucauaaugccccuaaaaaauccuuau
omy-mir-133a-2-3p_chrUn_204	mir-133	chrUn_204	chrUn_204:22107398..22107509:+	agggacuuuuaggggcagcugugguuuaauuaacccagucauaaugccccuaaaaaauccuuau
omy-let-7a-5p_chrUn_24_31544	let-7	chrUn_24_31544	chrUn_24:8493389..8493456:+	ugagguaguagguuguauaguugagaauuacaccccgggagauaacuauacaaccuccuagcuuucc
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omy-miR-196b-5p_chrUn_9004	mir-7911	chrUn_9004	chrUn:957643003..957643061:+	uagguaguuucaaguuguugggcuggacgcuuaucacaggaaccugaaacuaccugaa
omy-miR-196b-5p_chrUn_12	mir-7911	chrUn_12_23128	chrUn_12:6650412..6650470:+	uagguaguuucaaguuguugggcuggacguauaucacaggaaccugaaacuaccugaa
omy-miR-193-5p_chrUn_12	mir-7911	chrUn_12_23309	chrUn_12:28388114..28388173:+	cgggaucuugugggcgagaugaguuugaucagaauccaacuggcccgcaaagucccgcu
omy-miR-193-5p_chrUn_13	mir-7911	chrUn_13_23971	chrUn_13:10837366..10837425:+	cgggaucuugugggcgagaugaguuugauuagaauccaacuggcccgcaaagucccgcu

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omy-let-7a-5p_chrUn_16889	let-7	chrUn_16889	chrUn:597852171..597852242:-	ugagguaguagguuggauaguuuuaggguuacaccuacacugggagauaacuguacaaccuacugucuuuc
omy-nov-miR-29b-2-p5_chrUn_16889	mir-29	chrUn_16899	chrUn:598903297..598903360:-	gcugguuucagguggugacuuagaguguuguagucauuaucuagcaccauuugaaaucagugu
omy-miR-20a-5p_chrUn_22	mir-17	chrUn_22_31007	chrUn_22:5009840..5009899:-	uaaagugcuuauagugcagguaguuuuauggauaucuacugcagugugagcacuugaag
omy-miR-20a-5p_chrUn_16	mir-17	chrUn_16	chrUn:2032150..2032212:+	uaaagugcuuauagugcagguaguguauuuucuccaucuacugcaaugugagcacuuccagu
omy-miR-206-3p_chrUn_5343	mir-7911	chrUn_5343	chrUn_5343:567865408..567865516:+	ggagcagauccugucguugccucccugugaugacgugcuuccuuauaucccccauaugaauacugccguuauggaauguaaggaagugugugguuacaaggggacugga
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omy-mir-193-5p_chrUn_10117	mir-7911	chrUn_10117	chrUn:1055620475..1055620537:+	ugggucuuuguuugcaaggugaguaguguucucuccucucaacuggccuacaaagucccagu

Supplementary Table 4. Predicted target gene ontologies of novel miRNA Omy-miR-nov-285-5p

Top 10 target genes of novel miRNA (biological processes)				
GOBPID	**P-value**	**Count**	**Size**	**GO Term**
GO:0016042	1.17E-07	23	196	Lipid catabolic process
GO:0060395	1.47E-07	10	36	SMAD protein signal transduction
GO:0010862	1.13E-06	10	44	Positive regulation of pathway-restricted SMAD protein phosphorylation
GO:0060393	1.42E-06	10	45	Regulation of pathway-restricted SMAD protein phosphorylation
GO:0060389	1.76E-06	10	46	Pathway-restricted SMAD protein phosphorylation
GO:0090100	1.50E-05	11	70	Positive regulation of transmembrane receptor protein serine/threonine kinase signaling pathway
GO:0090116	3.42E-05	3	3	C-5 methylation of cytosine
GO:1990009	3.42E-05	3	3	Retinal cell apoptotic process
GO:0090092	8.81E-05	14	130	Regulation of transmembrane receptor protein serine/threonine kinase signaling pathway
GO:0002934	0.000122	4	9	Desmosome organization
Top 10 target genes of novel miRNA (molecular function)				
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:0004435	3.72E-11	19	84	Phosphatidylinositol phospholipase C activity
GO:0016409	3.88E-11	16	57	Palmitoyltransferase activity
GO:0004629	7.15E-11	19	87	Phospholipase C activity
GO:0016417	2.62E-10	16	64	S-acyltransferase activity
GO:0004620	1.90E-07	20	151	Phospholipase activity
GO:0005160	4.48E-06	10	49	Transforming growth factor beta receptor binding
GO:0016298	4.83E-06	20	185	Lipase activity
GO:0008083	1.59E-05	21	217	Growth factor activity

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CHAPTER-2

17β-Estradiol increases non-CpG methylation in exon 1 of the rainbow trout (*Oncorhynchus mykiss*) *MyoD* gene

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ABSTRACT

MyoD is an important myogenic transcription factor necessary for the differentiation of myogenic precursor cells (MPC) to form mature myotubes, a process essential for muscle growth. Epigenetic markers such as CpH methylation are known gene regulators that are important for the differentiation process. In this study, we investigated whether DNA methylation is a potential mechanism associated with the ability of 17β-estradiol (E2) to reduce *MyoD* gene expression and muscle growth in rainbow trout. Rainbow trout received a single intraperitoneal injection of E2 or the injection vehicle (control). Skeletal muscle was collected 24 hours post-injection and analyzed for DNA methylation within the *MyoD* gene and the expression of DNA methyltransferases was quantified. CpG islands of the *MyoD* gene were predicted using MethPrimer software and these regions were PCR amplified after bisulfite treatment and sequenced. The percent methylation of the targeted CpG did not differ between control and E2-treated fish. However, percent CpH methylation in *MyoD* exon 1 region was elevated with E2 treatment. Two of the methylated CpH sites were located in conserved transcription factor binding motifs, an estrogen response element (ERE) and a *Myc* binding site. Quantitative real time PCR analysis revealed a significant increase in expression of DNA methyltransferases, Dnmt3a and Dnmt3b, in E2-treated muscle, suggesting an increased genome methylation. Differential CpH methylation in the *MyoD* gene of control and E2-treated fish suggests an epigenetic mechanism through which E2 decreases *MyoD* gene expression and contributes to reduced muscle growth.

INTRODUCTION

DNA methylation is one of the epigenetic mechanisms regulating gene expression. Cytosines in DNA are methylated at C5 by DNA methyltransferases (Dnmt) to form 5-methylcytosines. These modified bases interact with and recruit different proteins collectively involved in chromatin remodeling and regulation of gene expression (CHENG AND BLUMENTHAL 2010). Even though the existence of this modification was identified immediately after the discovery of DNA as hereditary material (AVERY *et al.* 1944; MCCARTY AND AVERY 1946), its role in gene regulation and cell differentiation was not reported until later (HOLLIDAY AND PUGH 1975; COMPERE AND PALMITER 1981). Dnmt1 is a maintenance methyltransferase, which functions during replication by adding a methyl group to cytosines of the new strand, whereas Dnmt3a and Dnmt3b are *de novo* methyltransferases, transferring methyl groups to un-methylated cytosines. Methylation of cytosines in the contexts of CpA, CpT, and CpC is known as CpH (non-CpG) methylation. It is mediated by Dnmt3a and Dnmt3b, and is also strongly correlated with CpG methylation in flanking regions (ARAND *et al.* 2012). The availability of whole genome sequencing techniques assisted in identification of CpH methylation in mammalian cells (LISTER *et al.* 2013). Epigenome and gene expression studies using various tissues from humans revealed tissue specificity of CpH methylation and its role in gene regulation (SCHULTZ *et al.* 2015).

Skeletal muscle development and regeneration is the result of both hyperplastic and hypertrophic growth in fish, including rainbow trout (*Oncorhynchus mykiss*). Myogenic precursor cells (MPCs) proliferate and differentiate to form mature myotubes. These processes are regulated by different transcription factors including Pax7 and myogenic regulatory factors (MRFs). MRFs are basic helix loop helix transcription factors binding to the E-box motifs of DNA to regulate gene expression. MyoD and Myf5 are considered as primary MRFs that direct the differentiation of MPCs, of which MyoD is considered as a master regulator of muscle precursor cell differentiation. In rainbow trout, 17β-estradiol (E2) is a maturation-related signal that mediates reductions in muscle accretion during sexual maturation (NAZAR *et al.* 1991b; NORBECK AND SHERIDAN 2011b; CLEVELAND AND WEBER 2016a). We recently demonstrated that E2 treatment reduces the expression of *MyoD* gene in rainbow trout muscle, and this likely occurs independent of mechanisms directly involving miRNAs (KOGANTI *et al.* 2017a). Given the significance of DNA methylation as a significant regulator of gene expression, we investigated whether this mechanism is associated with transcriptional regulation of *MyoD* by E2 in rainbow trout muscle.

DNA bisulfite conversion and PCR of CpG rich regions of *MyoD* gene were performed using skeletal muscle samples from the previous study (KOGANTI *et al.* 2017a). Amplicons were sequenced using Illumina technology and differential methylations were analyzed. Our data showed that percent methylation of the CpG sites did not differ between control and E2-treated fish. However, percent CpH methylation in *MyoD* exon 1 region was elevated in E2-treated muscle. In addition, the expression of two DNA methyltransferases, Dnmt3a and Dnmt3b, was significantly increased in E2-treated muscle. These observations explain reduced expression of *MyoD* under the influence of estrogen, providing additional evidence for the role of gene body CpH methylation in maintenance of MPCs.

MATERIALS AND METHODS

Sample collection

Skeletal muscle samples used in this study were from a previous study conducted at the USDA/ARS National Center for Cool and Cold Water Aquaculture (KOGANTI *et al.* 2017a). Briefly, ten juvenile rainbow trout (~40 g) were used for each group (treatment or control). They received intraperitoneal injections of E2 (5.0 μg per g body weight) or the delivery vehicle (control). Skeletal muscle samples were collected 24 hours post-injection. Four samples from each group were randomly selected and used in this study.

Screening of CpG islands in silico

The coding sequence of *MyoD* was obtained from NCBI (accession number – Z46924.1), which was further used to obtain *MyoD* gene sequence from the recently published rainbow trout genome sequence. Methprimer software (http://www.urogene.org/methprimer/) was used to predict CpG islands and design bisulfite specific primers. Three separate regions with 9 CpG sites (upstream region), 14 CpG sites (exon1-region1) and 6 CpG sites (exon1-region2) were further chosen for amplification and sequencing.

DNA isolation and bisulfite conversion

DNA was isolated from E2-treated and control skeletal muscle samples (n = 4 per group) using Quick-DNA™ Universal kit (Zymo Research) following the manufacturer's protocol. The quantity and quality of the DNA was estimated using NanoDrop 1000 Spectrophotometer (Thermo

Scientific) by measuring absorbance at 260 nm and 280 nm. Bisulfite conversion of DNA (500 ng) was performed using EZ DNA Methylation–Lightning™ kit from Zymo Research and converted DNA stored at -80 °C until further use.

Bisulfite specific PCR and bisulfite amplicon sequencing

Bisulfite-specific PCR was performed with 5 µL of 1:50 diluted bisulfite-converted DNA in a 25-µL reaction. Three regions with amplicon sizes 281 bp, 251 bp, and 185 bp were amplified using bisulfite specific primers (Table S1) following standard conditions: 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s with a final 72 °C extension for 7 min. PCR products were separated using agarose gel electrophoresis and eluted using QIA-Quick Gel Extraction kit (Qiagen) following manufacturer's protocol. The concentration of DNA (PCR amplicon) in the final elution was determined using NanoDrop. Equal concentrations of PCR amplified DNA were pooled from each of the four replicates within a treatment to produce two samples (control and E2) for next generation sequencing (Mr. DNA, Shallowater, TX).

Identification of differentially methylated regions

The reads obtained from control and E2 treated samples were mapped to *MyoD* genomic sequence, sorted, and indexed before using them in Integrative Genome Viewer (ROBINSON *et al.* 2011a) to identify differentially methylated regions. Percentage of cytosines methylated in control and E2 treated samples were calculated based on the number of unconverted cytosines relative to the number of reads. Chi-square tests were used to statistically determine the differentially methylated sites and those with a P-value < 0.01 and < 0.0001 are considered significant and highly significant, respectively.

RNA isolation and quantitative real-time PCR (qPCR)

Total RNA was isolated from approximately 100 mg skeletal muscle (n = 6 per treatment) using Trizol reagent (Invitrogen, Carlsbad, CA) and Direct-zol™ RNA MiniPrep kit following the manufacturer's instructions (Zymo Research). cDNA was synthesized from 2 µg of DNase-treated total RNA using miScript II (Qiagen, Valencia, CA). qPCR was performed using gene specific primers, 5 µL of 1:1,024 diluted cDNA and iQ™ SYBR® Green Supermix (Bio-Rad) in a final volume of 25 µL. Cycling conditions used were 95 °C for 3 min followed by a denaturation at 95

^{o}C for 40 s, annealing at 58 ^{o}C for 30 s, extension at 72 ^{o}C for 30 s for 40 cycles and a final extension for 10 min at 72 ^{o}C followed by a melting curve analysis. β-actin was used as an endogenous control for normalization. Efficiency of qPCR was calculated using a standard curve generated using a 10-time serial dilution of a pooled cDNA sample. Primers used for qPCR are listed in Table S1. The expression of DNA methyltransferases was expressed as relative fold changes, which were calculated by dividing the means of normalized gene expression values for the E2 treated samples by the means of normalized gene expression values for the control samples. Statistical analysis was performed using the Student's t-test with significant differences indicated as P-value < 0.05.

RESULTS

Three CpG rich regions were identified in the *MyoD* genomic sequence (Fig. S1). One of the regions is located -3308 to -3161 upstream of the translation start site, represented as upstream region. The second and third CpG rich regions are within the first exon, +14 to +186 represented as exon1-region1 and +256 to +340 represented as exon1-region2. More than 1,000 reads for each region were obtained through Illumina sequencing.

Methylation percentage of the 9 CpG sites in the upstream region ranged from 27% to 47% (no significant difference). Eleven of 14 CpG sites in exon1-region1 exhibited methylations; percent methylation of 3 sites was less than 50% while the remaining 8 sites ranged from 50% to 97%. Four CpG sites are differentially methylated in exon1-region1, one showing higher percentage in control (50%) than E2 treated samples (4%) with a (P-value < 0.0001) while the others exhibiting 6%, 78% and 81% in control and 26%, 93% and 94% in E2 samples (P-value < 0.01). All 6 CpG sites in exon1-region2 presented lower methylation levels (< 11%) with no significant difference in methylation levels. Heat map comparing methylation percentages of control and E2 treated samples of different regions are presented in Fig 1. None of the other CpG sites in three regions studied are differentially methylated. Details of percent methylation of CpG and CpH sites in three regions are presented in Table S2.

In addition to the CpG methylations, exon1-region1 exhibited unexpected differential methylation of CpH sites, with a higher methylation percentage in E2 versus control muscle (Fig. 1b). Seven CpH sites in exon1-region1 presented methylation on both control and E2 treatment samples. Methylation percentage ranged from 0% to 38% in control and 0% to 97% in E2-treated

samples. Among the methylated CpH (^{m}CpH) sites, CpA dinucleotides are predominant, followed by CpC and CpT. Five of the ^{m}CpH sites showed significant differential methylation with higher percentage of methylation in E2 samples than in controls. Comparison of highly methylated CpG and CpH sites in *MyoD* exon1-region1 across species using Clustal Omega revealed that most of the ^{m}CpH sites are well conserved. Sequence alignment of exon1-region1 with methylated cytosines are presented in Fig 1e. Comet software was used to find motif clusters and those motifs that overlap with ^{m}C are highlighted. Exon 1 of rainbow trout *MyoD* gene harbors one estrogen response element (ERE) and two *Myc* binding motifs (Fig. 1e). Both CpG and non-CpG methylations were observed within and neighboring ERE and *Myc* transcriptional elements. In the two *Myc* motifs, the first element showed methylation in a central CpG site and the second in a CpA upstream of the central CpG. Fifty percent of the central CpGs were methylated in control with 4% in E2 treated samples and 87% and 0% CpA methylations in E2 and control samples, respectively. Similarly, ERE also presented methylations in both CpG and CpH context, with 42% and 0% in control and 50% and 88% in E2 samples.

qPCR was performed to analyze the expression profiles of enzymes mediating DNA methylations. A 3.7-fold increase in the expression levels of *Dnmt1*, 2.6-fold increase in *Dnmt3a*, and 8-fold increase in *Dnmt3b* were detected in skeletal muscles 24 hours post E2 treatment. Relative gene expression of methyltransferases is presented in Fig 2.

DISCUSSION

Novel approaches involving next generation sequencing have been used to identify CpG rich regions in various studies (MASSER *et al.* 2015; AKAGI *et al.* 2016). Although E2 did not affect CpG methylation substantially, various studies have confirmed site-specific DNA methylation as a mechanism in regulating gene expression. CpG methylation in regulatory and promotor regions largely downregulate gene expression while most CpG methylations in the gene body enhance gene expression. For example, over expression of Dmnt1 in C2C12 myoblasts increased methylation of CpG islands across *MyoD* exon 1 and exon 2, elevated *MyoD* gene expression, and accelerated terminal differentiation (TAKAGI *et al.* 1995). Evidence of *MyoD* gene methylation in 10T1/2 fibroblasts, adipocyte derivatives, and demethylation in myogenic derivatives was reported during treatment with the demethylating agent, 5-azacytidine, further supporting the role of DNA CpG methylation as a mechanism that regulates *MyoD* gene expression (JONES *et al.* 1990).

However, further investigation revealed that methylation of CpG islands around the *MyoD* promoter region occurs in 10T1/2 fibroblasts, but not in myogenic derivatives. Later Brunk et al. identified a distal enhancer region conserved in humans and mice that is methylated at all CpG sites in non-muscle cells, contrasting to myogenic cells (BRUNK *et al.* 1996). Collectively, these studies indicate the prominent role of DNA methylation in *MyoD* gene expression, although there are mechanistic differences between tissue types.

Elevated CpH methylation in E2-treated samples suggests an effect of E2 on *de novo* methylation of *MyoD in vivo*. Existence of CpH methylation in gene exons and flanking regions has been previously reported (HAINES *et al.* 2001; KOUIDOU *et al.* 2005). In fact, myogenin, a second MRF, exhibits DNA methylation of both CpG and non-CpG elements in 5' flanking regions, with demethylation of non-CpG elements occurring more rapidly than CpG elements (FUSO *et al.* 2010). In the present study, significant reduction of CpH methylation in control samples could represent demethylation activity and aid in differential *MyoD* expression. Studies from humans (TOMIZAWA *et al.* 2011a; ZILLER *et al.* 2011; GÓMEZ *et al.* 2015) and Drosophila (RAMSAHOYE *et al.* 2000) reported the presence of CpH methylations in pluripotent cells and oocytes, which were lost upon differentiation of cells or embryo formation. Previously, we reported reduced *MyoD* gene expression in rainbow trout skeletal muscle 24 hours after E2 treatment (KOGANTI *et al.* 2017a). Collectively, these findings support E2 induced negative regulation of *MyoD* gene expression, partially via increased gene body CpH methylation.

Transcriptional gene regulation is controlled by various regions including promoters, enhancers and repressors. These elements are often harbored in both exons and introns. ERE and *Myc* elements, which can regulate gene expression, were identified in exon1-region1 of *MyoD*. Supporting our findings is a positive transcriptional regulatory element that was reported within exon 1 of the mouse elastin gene (PIERCE *et al.* 2006) and a negative transcriptional regulatory element in an intron of the bovine elastin gene (MANOHAR AND ANWAR 1994). To regulate gene expression, ligand (E2) bound estrogen receptors bind to EREs (WALLACE 1985) and Myc family proteins bind to *Myc* elements. The presence of CpG and non-CpG methylations within and neighboring *Myc* and ERE transcriptional elements suggests probable interaction between these two mechanisms to regulate *MyoD* gene expression. Furthermore, differential CpH methylation by E2 introduces epigenetic modification as a mechanism through which it regulates *MyoD* expression. Supporting this concept are previous findings indicating that Myc protein binding is

negatively affected by methylation of the central CpG present in its motif GACCACGTGGTC (PRENDERGAST *et al.* 1991). Likewise, methylations observed in the current study might regulate Myc binding to regulate gene expression. Therefore, treatment-dependent methylation patterns in *Myc* motifs indicate their probable role in regulation of gene expression. Further studies to understand the effects of these methylations on *Myc* and ER binding are warranted.

DNA methyltransferases are enzymes responsible for the transfer of methyl group to cytosines. Given the role for Dnmt3 in *de novo* methylations, up-regulation of *Dnmt3a* and *Dnmt3b* in E2-treated samples is consistent with the observed increase in CpH methylation in exon1-region1. Whole genome bisulfite sequencing would give better understanding of genome wide methylations and the epigenetic consequences of increased expression of *Dnmt1* and *Dnmt3a* under the influence of E2.

Collectively, these observations expand our understanding of the effects of E2 on DNA methylation of the *MyoD* gene. The presence of both CpG and CpH methylations in the *MyoD* gene indicates DNA methylation is a regulator of *MyoD* expression in rainbow trout muscle. Furthermore, the E2-induced increase in CpH methylation in transcriptional regulatory regions of *MyoD* exon 1 suggests a mechanism through which *MyoD* expression is reduced in E2-treated fish.

ACKNOWLEDGEMENTS

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COMPETING INTERESTS

The authors declare that they have no competing interests.

FIGURES

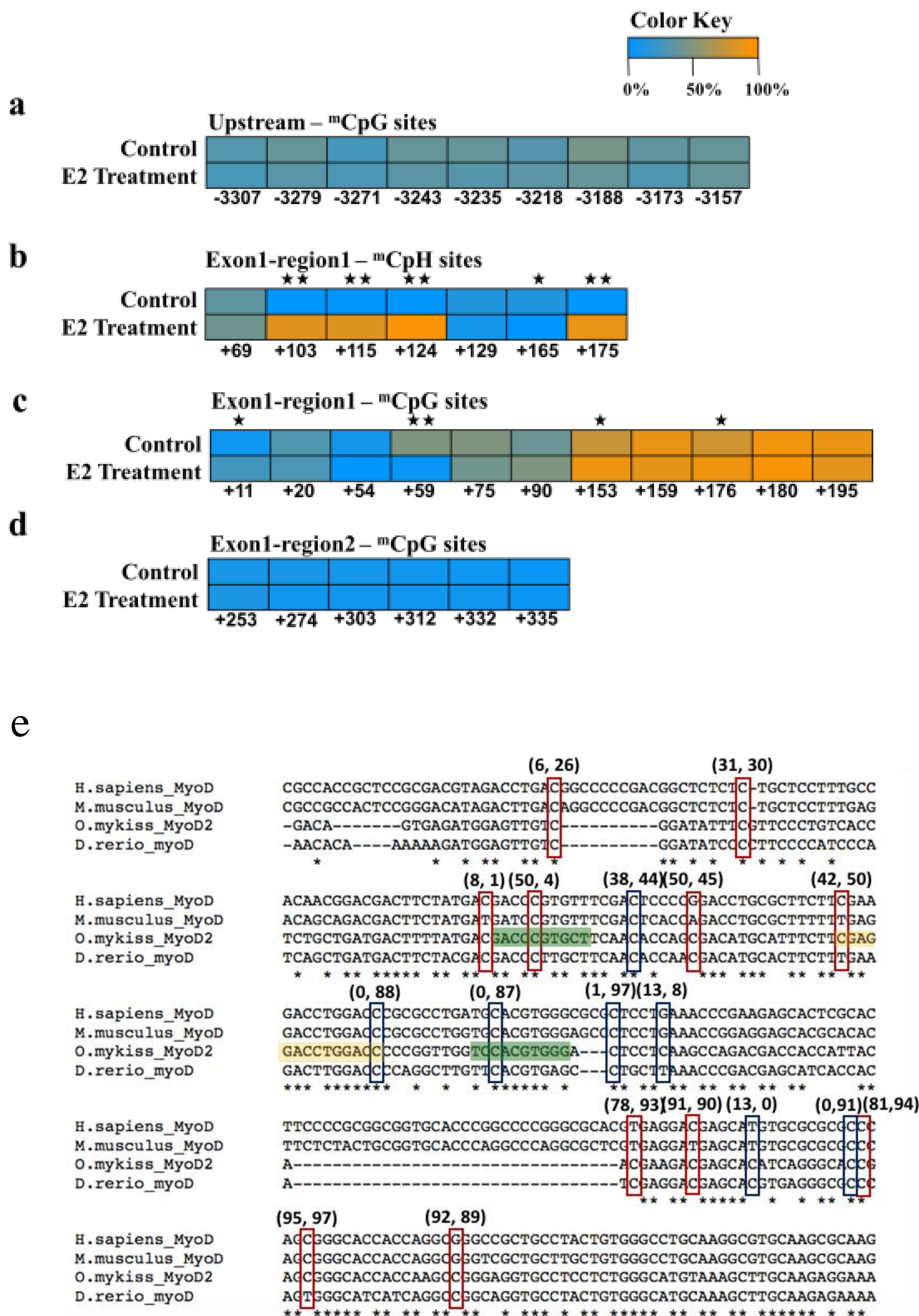


Figure 1. Heat maps showing methylation of CpG and CpH sites in MyoD gene and alignment of MyoD exon1.

(**a**) CpG sites in upstream region, (b) CpH sites in exon1-region1, (**c**) CpG sites in exon1-region1 and (**d**) CpG sites in exon1-region2. Top row represents methylated sites from control samples and bottom row represents methylated sites from E2-treated samples. Differentially methylated sites with statistical significance were indicated *$P < 0.01$ and **$P < 0.0001$. Below each block is the position of methylated cytosines relative to translation start site ATG, A considered as position +1. (**e**) Alignment of the *MyoD* exon 1 region of selected species. Methylated CpG and CpH sites were shown in red and blue boxes, respectively, with percent methylation indicated (control, E2) in parenthesis above each box. ERE site is highlighted in yellow and *Myc* motifs are highlighted in green.

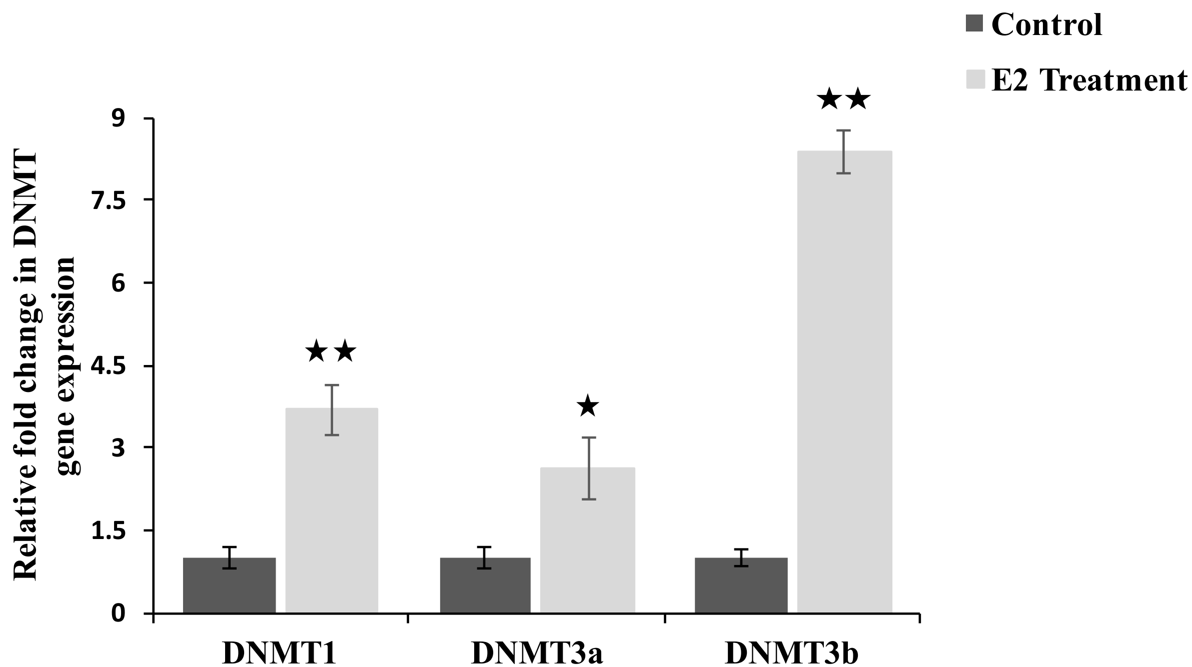
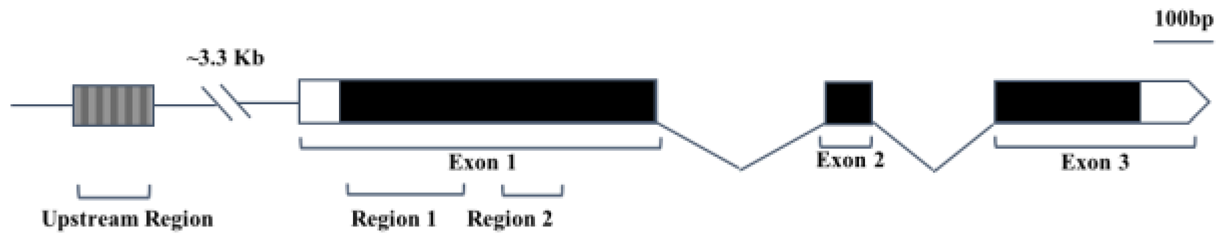


Figure 2. Relative expression of DNMTs.

qPCR analysis of mRNA expression of *Dnmt 1, Dnmt 3a* and *Dnmt 3b* in E2 treated samples relative to control samples. Results are expressed as fold change ± SE (n = 6). *P < 0.05 and **P < 0.01

SUPPLEMENTARY DATA



Supplementary Figure 1. Schematic map showing three CpG rich regions in *MyoD* gene. Upstream region: -3308 to -3161 upstream of the translation start site. Exon1-region1 and Exon1-region2: +14 to +186 and +256 to +340 in the first exon, respectively.

Supplementary Table 1. List of primers used for bisulfite specific PCR and qPCR.

Gene /Gene Region	**Primer Name**	**Sequence**
Primers used for bisulfite specific PCR		
MyoD-Upstream Region	MyoD-Up-Methyl F	5' GGGGTTTAGGTTTATTGTTTGTTAG 3'
	MyoD-Up-Methyl R	5' AAACTTATTCAAAACACATCATTTACC 3'
MyoD-Exon1-Region1	MyoD-Exon1-R1 F	5' TAAGGTTGGTTTTGGAATTTGATAG 3'
	MyoD-Exon1-R1 R	5' TTTACATACCCAAAAAAAACACCT 3'
MyoD-Exon1-Region2	MyoD-Exon2-R1 F	5' GAGGTGTTTTTTTTGGGTATGTAAA 3'
	MyoD-Exon2-R1 R	5' CATTTCTCAAAATCTCCACCTTAAA 3'
Primers used for qPCR (PCR efficiency in parenthesis)		
DNA Methyltransferase 1 (96.7%)	DNMT1 F	5' CACCCTGACAAGAAGAATGGTC 3'
	DNMT1 R	5' CTCTGGATGCAGGACACGAC 3'
DNA Methyltransferase 3a (95.3%)	DNMT3a F	5' GAGGACCATCACCACTCGCTC 3'
	DNMT3a R	5' ACACCCTCTCCATCTCAGTGC 3'
DNA Methyltransferase 3b (105.2%)	DNMT3b F	5' CTGGATGTTCGAGAATGTGGTC 3'
	DNMT3b R	5' CAGAAGTACCTAGCCCTGTGAGC 3'

Supplementary Table 2. Percent methylation of CpG and CpH sites in three regions of *MyoD* gene

Methylation site	**Percent methylation (Control)**	**Percent methylation (E2 treatment)**	**P-value (Chi-square test)**
Up-stream Region			
^{m}CpG	33	29	5.41E-01
^{m}CpG	40	35	4.65E-01
^{m}CpG	27	30	6.38E-01
^{m}CpG	40	36	5.60E-01
^{m}CpG	40	34	3.80E-01
^{m}CpG	34	36	7.67E-01
^{m}CpG	47	40	3.18E-01
^{m}CpG	38	31	2.98E-01
^{m}CpG	40	40	1.00E+00
Exon 1-Region 1			
^{m}CpG	6	26	1.15E-04
^{m}CpG	31	30	8.78E-01
^{m}CpG	8	1	1.70E-02
^{m}CpG	50	4	2.36E-13
^{m}CpH	38	44	3.88E-01
^{m}CpG	50	45	4.79E-01
^{m}CpG	42	50	2.56E-01
^{m}CpH	0	88	4.76E-36
^{m}CpH	0	87	2.34E-35
^{m}CpH	1	97	5.32E-42
^{m}CpH	13	8	2.49E-01
^{m}CpG	78	93	2.59E-03
^{m}CpG	91	90	8.09E-01
^{m}CpH	13	0	1.92E-04
^{m}CpH	0	91	3.39E-38
^{m}CpG	81	94	5.44E-03
^{m}CpG	95	97	4.70E-01
^{m}CpG	92	89	4.69E-01
Exon 1-Region 2			
^{m}CpG	10	11	8.18E-01
^{m}CpG	10	7	4.47E-01
^{m}CpG	8	7	7.88E-01

^{m}CpG	7	7	1.00E+00
^{m}CpG	6	7	7.74E-01
^{m}CpG	4	5	7.33E-01

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CHAPTER-3

DNA methylation responds to 17β-estradiol in rainbow trout skeletal muscle

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ABSTRACT

Cytosine methylation is one of the epigenetic mechanisms important in determining gene expression. Current understanding of the role of DNA methylation in regulating an imbalance of protein turn over in rainbow trout skeletal muscle exposed to 17β-estradiol (E2) remains limited. Our previous study suggests a role for DNA methylation in muscle precursor cell differentiation. Therefore, we further investigated and characterized the whole genome methylation landscape in control and E2 treated samples, identifying differentially methylated regions (DMRs). Whole genome bisulfite sequencing was performed at single nucleotide resolution, and cytosine methylations in CG, CHG and CHH contexts were identified. A total of 43 million CG sites were identified in the trout genome, of which 304,000 sites were differentially methylated in E2 treated samples compared to the control. The difference in methylation between control and E2 treated samples was estimated, and those regions that are differentially methylated at ≥ 80% rate were documented for further analysis. About 818 differentially methylated regions were differentially methylated at ≥ 80% and 408 were further mapped to 208 protein coding genes. Gene ontology and KEGG pathways analysis of these 208 genes were performed. Enriched genes were parts of various cellular organelles, besides those involved in various primary and cellular metabolic processes, cell communication, signal transduction, ion binding and transferase activity. Pathways regulating different amino acids metabolism were enriched in KEGG pathway analysis including steroid hormone and purine metabolism. Collectively, these observations describe the influence of E2 on CG DNA methylation in rainbow trout skeletal muscle. Further analysis of CHG and CHH methylations were in progress. Likewise, availability of completely annotated rainbow trout genome would enhance our capability in evaluation of gene expression guided by DNA methylation.

INTRODUCTION

Rainbow trout skeletal muscle is an immediate energy source during gonadogenesis and increase in estrogen triggers these processes driving nutrient partitioning leading to increased protein degradation and decreased protein synthesis, depending on plane of nutrition. Various degradative pathways are highly expressed in rainbow trout as an influence of estrogen. During rainbow trout growth, hyperplasty and hypertrophy contribute to myogenesis that starts with proliferation of myogenic precursor cells (MPCs) present under the basal lamina. These MPCs further differentiate and mature to form a myofiber. Various signaling molecules and molecular factors regulate the differentiation of MPCs in development of mature muscle fibers. Epigenetics and non-coding RNA add an extra layer of regulation and play an important role in gene expression at transcriptional and post-transcriptional levels. Various studies emphasized specific DNA methylation in determining muscle cell lineage (COLLAS *et al.* 2008; SØRENSEN *et al.* 2009; SØRENSEN *et al.* 2010). Cells determined to commit to myogenic lineage demonstrate hypermethylation of genes responsible for adipogenic, osteogenic, pancreatic, neurogenic and epidermal lineage commitment (SØRENSEN *et al.* 2010). Concurrent hypomethylation of contractile muscle protein Obscurin, Myh7 and Myotilin was reported (CALVANESE *et al.* 2012). Demethylation was detected in many studies during the progression of myogenesis, thus activating muscle cell genes with specific changes that determine cell fate and terminal differentiation (CHIU AND BLAU ; TSUMAGARI *et al.* 2013; MIYATA *et al.* 2015). Most studies relative to DNA methylation and myogenesis focus primarily on global changes in DNA methylation and those that are specific to MyoD and myogenin genes. Expression of MyoD, an important myogenic regulatory factor, is necessary for myogenic cell fate decision is partially determined by DNA methylation at its distal regulatory region. This distal regulatory region, present 20kb upstream of TSS of MyoD is highly methylated in presomatic mesoderm cells but unmethylated in C2C12 cells, as well as hindlimb and forelimb muscles (BRUNK *et al.* 1996). These experiments suggest that demethylation of the distal enhancer is necessary for MyoD gene expression. Similarly, demethylation of CpG, 340bp upstream of TSS of myogenin, is essential for the onset of myoblast differentiation. Methylation of this region allows binding of a repressor protein CIBZ that further blocks binding of the activator complex. Additional studies to understand gene regulation by DNA methylation of myogenin revealed non-CpG methylations in the myogenin promoter and these sites exhibit rapid demethylation when compared to CpG methylations. Furthermore, regulation

of MyoD expression is also influenced by non-CpG methylations in its exon1 region. E2 exposure led to increased non-CpG methylation thus inversely regulating MyoD gene expression (RAMSAHOYE *et al.* 2000). Also, Pax7 and myostatin gene expression is negatively correlated with methylation in their promoter and exon1 regions respectively. A gradual increase in Pax7 methylation was reported in differentiating C2C12 cells. Collectively these studies implicate the role of DNA methylation in timely regulation and precocious activation of gene expression in different stages of myogenesis. DNA methylation is not only confined to the myogenic genes but also regulates those involved in signaling pathways regulating myogenesis. Hypermethylation of IGF1 promoter is observed in slow-growing chickens with reduced gene expression. Most of the studies investigating regulation of gene expression revolved around CpG methylations. Availability of advanced sequencing techniques and bioinformatics tools allow exploration at base pair resolution, thereby revealing the occurrence of methylation at cytosines in CG, GHH and CHG contexts. CHH and CHG methylations are called non-CpG methylations, and differential methylation of such cytosines was observed as an effect of E2 in skeletal muscle of rainbow trout. Additionally, increased gene expression of maintenance and de novo methyltransferases DNMT1, DNMT3a and DNMT3b was observed in skeletal muscle exposed to E2 within 24hrs.

Controlled regulation of DNA methylation is important to various contributing pathways and MRFs involved in myogenesis. We predict changes in the global methylation signature that further regulates gene expression in E2 exposed skeletal muscle. These skeletal muscle tissues were sequenced after bisulfite treatment, to describe global changes. DNA methylation is a dynamic process occurring at various stages of cell development, however no visible drastic changes in global methylation levels were observed in control and E2 treated samples. To further identify methylation changes differentially methylated regions were identified in CG/CHH/CHG contexts. Gene ontology and KEGG pathway analysis were performed for identified differentially methylated regions in CG context. A total of 201 genes were either hypo or hypermethylated with 80% difference between control and E2 samples, 25 showed 100% difference in methylation rates. Though our previous studies report an extensive role of miRNAs and long non-coding RNAs in myogenesis of rainbow trout exposed to estrogen, not all differential gene expressions were explained by non-coding RNAs. Therefore, current study aimed at understanding the roles of DNA methylations that regulate changes in gene expressions influencing muscle synthesis in E2 exposed skeletal muscle.

METHODS

Sample collection and DNA isolation

Animal experiment was conducted at the USDA/ARS National Center for Cool and Cold Water Aquaculture (KOGANTI *et al.* 2017a) including treatments with 17β-estradiol, testosterone and DHT. Our current study was performed with samples treated with either vehicle or 17β-estradiol. The juvenile fish (each ~40 g) were treated with either vehicle or 17β-estradiol, and their skeletal muscle collected after 24 hours. Treatments were carried out by intraperitoneal injections. Four skeletal muscle samples from each treatment were randomly selected and DNA isolated using Quick-DNA™ Universal kit (Zymo Research) following manufacturer's instructions. Briefly, skeletal muscle tissues were incubated overnight in solid tissue buffer provided in the kit and Proteinase K at 55°C for digestion followed by column purification. Purity was evaluated using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). Equal concentrations of four control samples were pooled and its purity checked using Qubit (Life Technologies and Thermo Fisher Scientific); likewise, four estrogen samples were pooled and DNA purity checked before sending it for sequencing. These two samples, labelled control and E2 treatment, were sent for pair end (PE150) sequencing to Novogene (Beijing, China). Raw sequences obtained were filtered and differentially methylated regions were identified following pipeline presented in Fig. 1

Methylation-specific restriction digestion assay and ELISA

Restriction digestion of 2 μg of genomic DNA was performed at 37°C for 4 hours in a 50 μl reaction. Individual reactions with restriction enzymes Hha I, Hpa II, HpyCH4 IV and Dnp I were performed. Hha I, Hpa II and HpyCH4 IV identify cytosines that are not methylated but are followed by guanine, cytosine and adenine respectively. They cannot restrict DNA when the cytosines are methylated. Dnp I identified methylated adenine in DNA. The recognition sites were presented in Fig. 2. One percent agarose gel, with TAE buffer, was used to separate digested DNA and visualized using GelRed.

ELISA was performed to understand global methylation of cytosines using a 5-^{m}C DNA methylation kit (Zymo Research) following manufacturer's instructions. Briefly, 100 ng of DNA from 5 control and 5 E2 treated samples (24 hr samples) were denatured in a PCR machine at 98°C for 5 min using 5-^{m}C coating buffer and immediately cooled on ice. Then, denatured DNA was

plated on an ELISA plate and incubated at 37°C for 1 hour. The plate was washed 3 times with 5-^{m}C ELISA buffer after discarding the DNA followed by incubation in the same buffer at 37°C for 30 min. Subsequently, 5-^{m}C ELISA buffer was discarded and primary antibody was added to the plate before incubating at 37°C for 1 hour. After incubation, the primary antibody was discarded, and the plate was washed three times using 5-^{m}C ELISA buffer. Lastly, secondary antibody was added after washing primary antibody and the plate was incubated for 30 min before the absorption was measured at 405-450nm using an ELISA plate reader. A standard curve was made using the standards provided with the kit and is used to calculate percentage methylation in control and E2 treated samples. Finally, cytosine methylation was calculated relative to control samples. A T-test was performed to determine significant differences in global methylation among the two treatments.

Whole genome bisulfite sequencing

Library Construction and bisulfite treatment was performed at Novogene before sequencing. Briefly, genomic DNA was fragmented and purified before adaptor ligation and bisulfite conversion. The bisulfite converted DNA was further amplified by PCR and subjected to illumina sequencing. Paired-end sequencing was performed with a fragment size of 150bp each.

Mapping and identification of methylated cytosines

BSseeker2 (GUO *et al.* 2013) is a Python-based programming tool to filter raw sequences obtained, map to the reference genome, and identify methylated cytosines. The sequences were first filtered for unique reads, this step helps to identify extremely amplified reads during PCR and eventually remove them. A reduced three letter approach (A, G, C/T) is used to map reads to the reference genome because unmethylated cytosines are converted to thymines during bisulfite treatment. Reference genome is indexed and converted using the three-letter approach. Simultaneously filtered unique reads are converted using the same approach and then mapped to the indexed reference genome. Once mapped, methylated cytosines were identified. ^{m}C in contexts of CpG, CpHpH and CpHpG along with their genomic coordinates were recognized. Lastly, identified methylated cytosines were processed using MethGo to characterize the coverage distribution and methylation profiling at the genome level.

Identification of differentially methylated regions

Differentially methylated regions (DMRs) for control and E2 treated samples were identified using another python based package histogram of methylation, HOME (https://github.com/Akanksha2511/HOME#citation). Pairwise comparison of these regions was performed by HOME. The DMRs were identified depending on type of ^{m}C (CG, CHH or CHG). Downstream analysis after this step is performed with differential methylations of CG. DMRs with differences between control and E2 that are more than two times were filtered. Genomic coordinates of these DMRs were obtained to associate with available 46585 coding regions using RGmatch (FURIÓ-TARÍ *et al.* 2016). Gene ontology and KEGG pathways analysis was performed using Blast2Go for genes harboring DMRs.

RESULTS

Methylation-specific restriction digestion assay and ELISA

Hha I and Hpa II recognize cytosines in 5' GCGC 3' and internal cytosine in 5' CCGG 3' respectively, and they restrict DNA in regions that are not methylated. Similarly, Msp I recognize and restricts DNA with internal methylated cytosine in the sequence 5' CCGG 3'. High molecular weight fragments were present after digestion with Hha I and Hpa II, these fragments were absent in Msp I digestion. This finding indicates the presence of CpG methylation in both control and estrogen treated samples. Distinct changes among the digestion patterns between control and E2 treated samples were not observed (Fig. 2A, B and C). Likewise, digestion with HpyCH4 IV resulted in fewer high density DNA fragments when compared to Hha I and Hpa II. DNA was not significantly digested with Dnp I.

Relative, global methyl cytosine level was calculated between control and E2 samples using ELISA. The difference in DNA methylation between control and E2 treated samples is not significantly different at the global level (Fig. 3).

Whole genome bisulfite sequencing

Erwin Chargaff's experiments to characterize DNA composition revealed that the number of guanines are equal to number of cytosines and the number of adenines to thymines (CHARGAFF 1950; CHARGAFF 1951; CHARGAFF *et al.* 1952). Bisulfite treatment converts unmethylated cytosines to uracil that results in loss of complementarity between opposite strands of DNA. These

single strands can be further amplified using PCR, this amplification process replace uracils by thymines, consequently resulting in reduced GC content after bisulfite treatment (FROMMER *et al.* 1992). Hence, distribution of these bases was checked for both reads that were sequenced. Cytosine content of read 1 and guanine content of the complementary read were very low because of the cytosine methylation as shown in Supplementary Fig. 1.

A total of 669,056,192 reads were obtained from paired end sequencing of control and estrogen treated samples with an average of 30X coverage. Approximately 81% of reads in control and 87% in estrogen treated samples were retained after filtering the raw reads. Further processing of these reads by mapping to the reference genome and methylated cytosines in contexts of CG, CHH and CHG were identified. MethGo was used to understand changes in methylation at the global level; total coverage of methylation sites was represented by reverse cumulative plots (Fig. 4A and B). Control and estrogen treated samples exhibited 40% genome coverage with approximately around 7X depth. These changes in methylation were also plotted in CG/CHH/CHG contexts. Graphs revealed an overlap of CG and CHG methylation, while CHH is comparatively lower across the genome. Additionally, fractions of total cytosine methylations are plotted relative to total percentage of CG/CHG/CHH methylations (Fig. 5A and B). The fractions of CG methylations in control and estrogen treated samples range from 0%-10% to 90%-100%; about 40% of total identified CG methylations showed 90-100% methylations. Whereas, more than 99% of total CHH and CHG methylations in control and estrogen treated muscle samples presented lower levels of methylation levels ranging from 0-10%. Further analysis was performed individually for different methylation patterns. Evaluation of CG methylations are presented as differential methylation in CHH and CHG contexts are still being processed.

CpG methylations

A total of about 43 million CG sites were identified in pooled DNA samples from 4 individual rainbow trout skeletal muscle samples from control and estrogen treatments. Differentially methylated regions were identified using HOME program; the difference in percent methylation between control and estrogen samples was recorded as a change is methylation or delta. A total of 304 thousand regions were differentially methylated; of these regions, 140 thousand were hypermethylated and 164 thousand were hypomethylated in the control. Methylation in these regions varied from 5% methylation to 100%, because the percentage of

methylation affects gene expression, regions with a delta value more than or equal to 80% were identified. There are 818 such regions that are either hyper or hypomethylated in E2 treated samples. Additionally, these regions were compared to the available rainbow trout transcriptomic data to identify differential methylation in regions common to those in coding genes. Of 818 regions, 408 regions were matched to the genomic coordinates of transcriptomics data after removal of redundant regions; these regions were eventually mapped to different region in 208 genes. Differential CG methylation of individual genes can also be visualized using Integrative Genome Viewer (IGV) (ROBINSON *et al.* 2011b) (Fig. 6).

Genes with differentially methylated regions

Gene ontology and KEGG pathway analysis were performed using Blast2Go (CONESA *et al.* 2005) to identify the roles of 208 differentially methylated genes. GO terms that were obtained during gene ontology analysis were further scanned using InterProScan to obtain any of those missing in GO. The resultant identities from GO and InterProScan were merged for gene annotation. Genes involved in various biological processes, molecular functions, and cellular components were identified as differentially methylated between control and E2 treated samples (Fig. 7A, B and C). Genes involved in cellular and primary metabolic processes, cellular response to stimulus, cell communication, signal transduction, cellular component organization and anatomical structure development in biological processes differential methylation in E2 treated samples. Several differentially methylated regions associated with molecular functions include hydrolase, transferase, and binding to various proteins, carbohydrates, ions, cyclic and heterocyclic compounds. A large number of genes are also contributing to cellular components including membrane bound and non-membrane bound intracellular organelles, membrane components, parts of plasma membrane, and the endomembrane system. These genes were later mapped to reference KEGG pathways. Thirty-eight KEGG pathways were identified (Table 1). These KEGG pathways include steroid hormone biosynthesis and degradation, phosphatidylinositol signaling, inositol phosphate metabolism, and different amino acid metabolic pathways including lysine, phenylalanine, tyrosine, beta-alanine, glycine, serine, threonine, cysteine and methionine. Genes involved in cysteine and methionine metabolism were specifically regulating transfer of the adenosyl group to methionine, thus synthesizing s-adenosyl methionine (SAM), the primary methyl donor for DNA methylation (supplementary figure 2). Purine metabolism, nicotinate and

nicotinamide metabolism, ascorbate metabolism, and glycosphingolipid and sphingolipid metabolism are among the enriched KEGG pathways. Enriched KEGG pathways also include the phosphatidylinositol signaling system, Th1 and Th2 cell differentiation, the T cell receptor signaling pathway, and cell calcium signaling.

Twenty-five of 208 differentially methylated genes were either 100% methylated in control or estrogen treated samples; 15 genes were hypermethylated and 10 were hypomethylated in control compared to E2 treated muscle. The list of the gene IDs, function and their chromosomal location were listed in Table 2. Hypermethylated genes in control include MTSS1 isoform X4, kinesin-1 heavy chain and hypomethylated genes are type 1 phosphatidylinositol 4,5-bisphosphate 4-phosphatase and integrin beta-5.

DISCUSSION

Global cytosine methylation was first identified using ELISA and methyl sensitive restriction digestion. Results from ELISA indicated no difference in DNA methylation percentage between E2 and control samples; it is necessary to consider the disadvantage of the technique with regard to DNA loading and its concentrations. Because DNA methylation is dynamic, it is advantageous to look at changes in DNA methylation at the gene level even when significant global methylation changes were not observed. Methyl sensitive digestion indicated cytosine methylations. Similarly, ELISA data do not show significant changes between control and E2 treatment.

Methylation of cytosine is studied in contexts of CG, CHH and CHG while the epigenetic marks on CG is best characterized. These chemical modifications on cytosine contribute to regulation of the highly orchestrated process, gene expression. In this study, WGBS was performed to reveal changes in methylation as a direct or indirect effect of E2 on muscle development. WGBS gives an opportunity to detect cytosine methylation at a single-base-pair resolution. Bsseeker2 was used to align and map reads obtained from sequencing, which is believed to be more accurate and efficient. It uses a three-letter approach where all cytosines are converted to thymines in the reference genome and in filtered paired-end reads before mapping, compared to wild card approach followed in other available mapping software. A total of 43 million CG sites were identified, and methylation levels at each site were noted. Most of the CG sites were mapped to an unknown chromosome because the rainbow trout genome is not well annotated. Since the percent

methylation regulates gene expression, CG sites that show at least 80% hypo or hypermethylated were categorized.

Gene ontology of DMRs revealed changes in CG methylation patterns in genes including kinesin-1 heavy chain, MTSS isoform and integrin β-5. Regions of 100% hyper or hypo methylation was observed in 25 genes and the remainder were more than 80%. Missing-in-metastasis protein (MIM or MTSS1) is believed to be important in cytoskeletal remodeling, mostly acting downstream to tyrosine kinases and Rho-family GTPases (MATTILA *et al.* 2003; WOODINGS *et al.* 2003; BOMPARD *et al.* 2005; GONZALEZ-QUEVEDO *et al.* 2005; LIN *et al.* 2005). Mouse studies show that MTSS1 is capable of binding to the actin monomer, and inhibits actin filament nucleation by efficiently inhibiting nucleotide exchange (MATTILA *et al.* 2003). Overexpression of MTSS1 in bladder uroepithelium cell carcinoma reduced cell proliferation and cell cycle progression with no influence on cell apoptosis (DU *et al.* 2017). Also, MTSS1 mediates ubiquitination of chemokine receptor CXCR4 and facilitates degradation by E3 ubiquitin ligase (LI *et al.* 2017). This CXCR4 protein expression is repressed post-transcriptionally by the estrogen receptor, thus controlling cell migration in zebrafish (GAMBA *et al.* 2010). Together these studies indicate differential methylation of MTSS1due to E2 treatment, and control samples might regulate actin nucleation and cell migration. Current findings were also supported by our mRNA expression studies; downregulation was observed for genes involved in actin cytoskeleton, actin binding, and those involved in mesenchymal (precursors for MPCs) cell migration (WANG *et al.* 2017). Additional proteins with DMRs that are involved in actin polymerization and stabilization include dematin, serine threonine-kinase N1, LIM domain and actin-binding 1, and dystrophin. Knockout experiments in mouse embryonic fibroblasts found dematin is important in actin stress fiber formation, cell mobility and adhesion. LIM domain and actin-binding 1 binds to actin monomers and filaments, and it inhibits depolymerization of actin filaments. Dematin and actin binding proteins with a LIM domains bind to actin monomers and filaments, playing an important role in actin cytoskeleton regulation and maintenance of actin stability (MOHSENI AND CHISHTI 2008). On the other hand, dystrophin is enriched in costamers that connects sarcomeres to cell membranes in striated muscle (ERVASTI AND CAMPBELL 1991; DMYTRENKO *et al.* 1993; RYBAKOVA *et al.* 2000). Muscular dystrophies were observed both in mouse and humans due to lack of dystrophin or its reduced expression (MINETTI *et al.* 1998; WILLIAMS AND BLOCH 1999; CLAFLIN AND BROOKS 2008).

Serine threonine-kinase N1 (PKN1) is activated by small G proteins belonging to the Rho family (PALMER AND PARKER 1995), phosphoinositides (PALMER *et al.* 1995), and fatty acids (MUKAI *et al.* 1994). Over expression and expression of kinase defective PKN1 in fibroblasts and Rat1-IR cells indicate its role in cytoskeleton reorganization (DONG *et al.* 2000) through the insulin signaling pathway. PKN1 also interacts and phosphorylates a microfilament protein, α-actinin, in a phosphatidylinositol 4,5 bisphosphate dependent manner (MUKAI *et al.* 1997); it affects contraction of myosin by phosphorylation of CPI-17, an inhibitor of myosin phosphatase (HAMAGUCHI *et al.* 2000). PKN1's role in cell migration is suggested by studies confirming its function upstream to MAP kinase,s JNK and p38 (TAKAHASHI *et al.* 2003; KATO JR *et al.* 2008) and its association with changes in focal adhesions (LACHMANN *et al.* 2011). Supportive evidence for a functional role of PKN1 in regulation of muscle contraction/relaxation and force transmission is presented by studies in roundworm (QADOTA *et al.* 2011). Moreover, PKN1 regulates activity of various transcription factors necessary for myogenic terminal differentiation; differentiation of C2C12 cells was inhibited in the presence of mutated PKN1 (CHEN *et al.* 2015). Furthermore, PKN1 regulate a nuclear import of class IIa histone deacetylases (HDAC) by phosphorylation of nuclear localization signal in HDAC5, 7 and 9, thereby partially regulating gene expression by impairing the import of transcriptional repressors (HARRISON *et al.* 2010). Similar observations in prostate cancer cells reveal that phosphorylation of histone 3 at threonine 11 regulates transcription of genes that are also regulated by the androgen receptor. Androgen receptor induced cell proliferation is inhibited by PKN1 repression (METZGER *et al.* 2008).

Arf-GAP, with Rho-GAP ANK repeat and PH domain-containing 1-like (ARAP1), is specific to vertebrates, it exerts control in intracellular signaling and cytoskeleton structure. Overexpression of ARAP resulted in increased expression of a cell division protein 42 homolog which helps in extension and maintenance of filopodia, actin rich surface projections, and the homolog also mediates cell migration (GAUTHIER-CAMPBELL *et al.* 2004; MODZELEWSKA *et al.* 2006). ARAP1 serves as a node in a signaling network that facilitates actin remodeling and thus cell movement (MIURA *et al.* 2002). In total, these studies indicate the role of differentially methylated genes in actin, cytoskeleton maintenance and also in cell migration, terminal differentiation of myoblasts, and regulation of histone modifications. Cellular components, enriched using Blast2GO, revealed DMR in genes that regulate membrane bound and non-

membrane bound intracellular organelles, plasma membrane and those that are involved in maintaining cell periphery.

Kinesin-1 heavy chain (KIF5B) is expressed ubiquitously with abundant expression in heart, skeletal muscle, and intestine (HOLLENBECK 1989). KIF5B significantly regulates myofibril assembly and muscle tendon junction stability. Not surprisingly, KIF5B knock out mice exhibited aggregates of myosin and actin, detachment of myofibrils from the sarcolemma, and mis-localized desmin and nestin (WANG *et al.* 2013). Moreover, KIF5B is necessary for positioning nuclei during myoblast differentiation and for distribution of mitochondria and lysosomes (WANG AND SCHWARZ 2009; METZGER *et al.* 2012). In addition to genes regulating different structural aspects of myogenesis, genes involved in signaling pathways were also differentially methylated. Differentially methylated genes, with delta value ≥ ±80, are listed in Supplementary Table 1.

Nuclear receptor-interacting 2-like (NRIP2) is well studied in cancer cells because of increased expression in colorectal cancers. Overexpression of NRIP2 resulted in self-renewal of colorectal cancer initiating cells and increased Wnt activity (PAN *et al.* 2017; WEN *et al.* 2017). Differential methylation of NRIP2 is also observed in breast cancer (ZHANG *et al.* 2015). Moreover, estrogen regulates expression of NRIP2, and a significant decrease in NRIP2 was observed in ovariectomized mice (PIAO *et al.* 2017). The same study reported a critical role for NRIP2 in estrogen mediated bone resorption, osteoclasts differentiation, and apoptosis. This interacting protein is capable of functioning as a transcriptional coactivator or corepressor besides being a transcriptional regulator of estrogen signaling (KISKINIS *et al.* 2007; PIAO *et al.* 2017). Its role is well documented in adipogenesis and bone metabolism, further investigation is needed to understand its regulation in myogenesis.

Genes that are core components of ubiquitin ligases and different ubiquitin ligases were differentially methylated in the current study. These genes include E3 ubiquitin-ligase CHIP, cullin-9-like isoform X4, E3 ubiquitin-ligase MYCBP2-like, pre-mRNA-processing factor 19, and E3 ubiquitin-ligase RAD18-like isoform X1. E3 ubiquitin-ligase CHIP acts as cochaperone interacting with various proteins to deliver misfolded and partially folded proteins to proteasomes (DEMAND *et al.* 2001; MEACHAM *et al.* 2001). This ubiquitin ligase is highly expressed in tissues with high metabolic activity and protein turnover, such as skeletal muscle (BALLINGER *et al.* 1999). Cullin-9 is an essential element of the Cul9-RING ubiquitin protein ligase complex that mediates ubiquitination and degradation. Cul9 is involved in maintenance of microtubule integrity and

dynamics; it also binds to survivin for further ubiquitination and degradation (LI *et al.* 2014). Survivin is a multifunctioning protein necessary during cell division. E3 ubiquitin-ligase MYCBP2-like interacts with E2 enzymes, mediating ubiquitination and eventually proteasomal degradation. Besides, it may play a role in synaptogenesis and facilitating transcriptional activation by MYC (HAN *et al.* 2008). Two other aforementioned factors, pre-mRNA-processing factor 19, and E3 ubiquitin-ligase RAD18-like isoform X1, are also involved in repairing DNA damage, mRNA splicing, and other repair mechanisms including ubiquitination and degradation (HATAKEYAMA *et al.* 2001; UNK *et al.* 2006; SONG *et al.* 2010; COTTA-RAMUSINO *et al.* 2011). Increased expression of genes involved in ubiquitin conjugating enzyme complex was observed in our previous study (WANG *et al.* 2017). Our current findings and these observations together implicate DNA methylation as a major regulator of ubiquitin conjugating enzyme complex gene expression. Additionally, anti-apoptotic NR13-like and deleted in malignant brain tumors 1–like genes with roles in apoptosis are differentially methylated.

Furthermore, differentially methylated regions were identified in genes involved in regular cell mechanisms; these genes include TATA box-binding-associated factor RNA polymerase I subunit B, elongation factor 1-delta isoform X1, and eukaryotic translation initiation factor 3 subunit B. TATA box-binding-associated factor RNA polymerase I subunit B is involved in various steps of transcription, including assembly of pre-initiation complex and postpolymerase recruitment events during rRNA transcription (NAIDU *et al.* 2011). Eukaryotic translation initiation factor 3 subunit B and elongation factor 1-delta isoform X1 play essential roles in translation (MASUTANI *et al.* 2007). Small RNAs regulate gene expression by translational repression in animals and this process is further regulated by DNA methylation. Differential methylation of trinucleotide repeat-containing gene 6B-like (TNRC6B) was observed (Supplementary Table 1.) with a delta value ≥ (±80) between control and E2 treated samples. TNRC6B is necessary for miRNA dependent translational repression and eventual deadenylation (MEISTER *et al.* 2005; ZIPPRICH *et al.* 2009; BRAUN *et al.* 2011). Genes that regulate gene expression through DNA and histone methylation were also differentially methylated. Activating transcription factors, 7-interacting 1-like and histone-lysine N-H3 lysine-36 and H4 lysine-20 specific-like, regulates transcription through their epigenetic mechanisms. These two genes can act as transcriptional repressors and activators either aiding in gene expression or chromatin formation (QIAO *et al.* 2011; TIMMS *et al.* 2016). S-adenosylmethionine synthase-like isoform X2 is necessary for the

formation of S-adenosylmethionine (SAM) from methionine and ATP, with release and subsequent hydrolysis of triphosphate. Differential methylation of this gene would either increase or decrease SAM availability, which is a methyl donor for DNA methylation. Even though, significant changes were not observed in DNA methylation at the genome level, differential methylation of S-adenosylmethionine could explain changes in DNA methylation at the gene level. Chromodomain-helicase-DNA-binding 7-like (CHD7) is another gene that is differentially methylated and involved in cell fate decisions. CHD7 modulates epigenetic and signaling pathways and regulates gene expression by binding to active enhancer elements and recruiting transcription factors in stem cells increasing their pluripotency (ZENTNER *et al.* 2011; MICUCCI *et al.* 2015).

Previous studies focused on describing differential expression of miRNA, mRNA and lncRNAs (KOGANTI *et al.* 2017a; WANG *et al.* 2017a), and they indicate changes in calcium ion and associated signaling pathways. Increased mitochondrial outer membrane permeability was believed to be a consequence of increased reactive oxygen species and calcium signaling. Similarly, lncRNAs-pathway network suggested 15 differentially expressed lncRNAs among control and E2 treated samples; these lncRNAs were linked to calcium signaling pathways. Supporting our previous studies, more than 10 genes that are differentially methylated were directly or indirectly involved in cellular calcium homeostasis and calcium signaling (Supplementary Table 1). These genes collectively are regulators of Ca^{2+} influx (LIOU *et al.* 2005; ROOS *et al.* 2005) and release of and Ca^{2+} dependent exocytosis; they are calcium-binding transmembrane components. Many of these genes are involved in release of calcium into the cytoplasm of muscle cells thus regulating their contraction (SCHWARZMANN *et al.* 2002).

Collectively, these observations indicate a significant role for DNA methylation in maintenance of muscle cell structure, protein turnover, calcium signaling and ubiquitination. All these, directly or indirectly, contribute to the physiological changes observed in E2 treated rainbow trout skeletal muscle. Further functional analysis of DNA methylation at the individual gene level should be conducted to understand the regulation of gene expression by these modifications.

ACKNOWLEDGEMENTS

This project was supported by USDA Project No 8082-31000-012-00D. We would like to acknowledge Roshan Abeyratne for his timely help with perl script to obtain a subset of differentially methylated genes from the rainbow trout transcriptomic database. Mention of trade names is solely to provide accurate information and does not imply endorsement by the USDA. The USDA is an equal opportunity employer and provider.

FIGURES

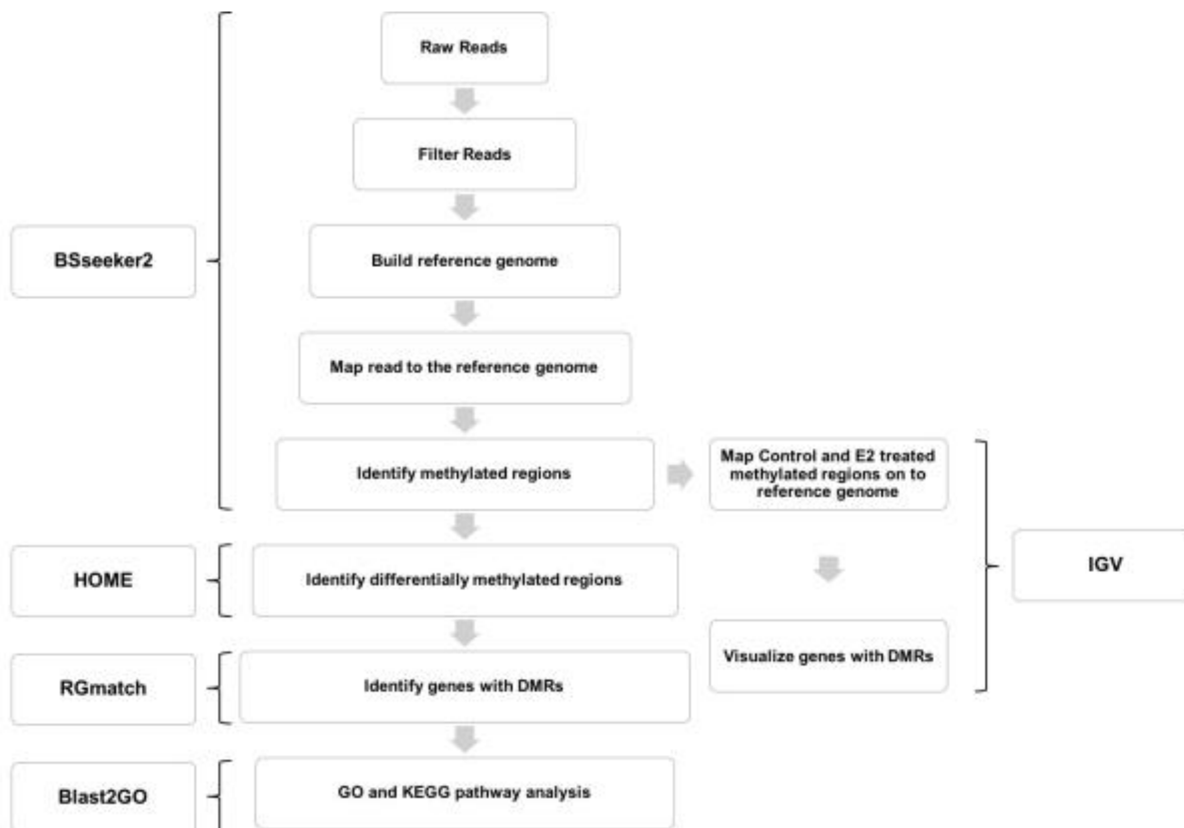
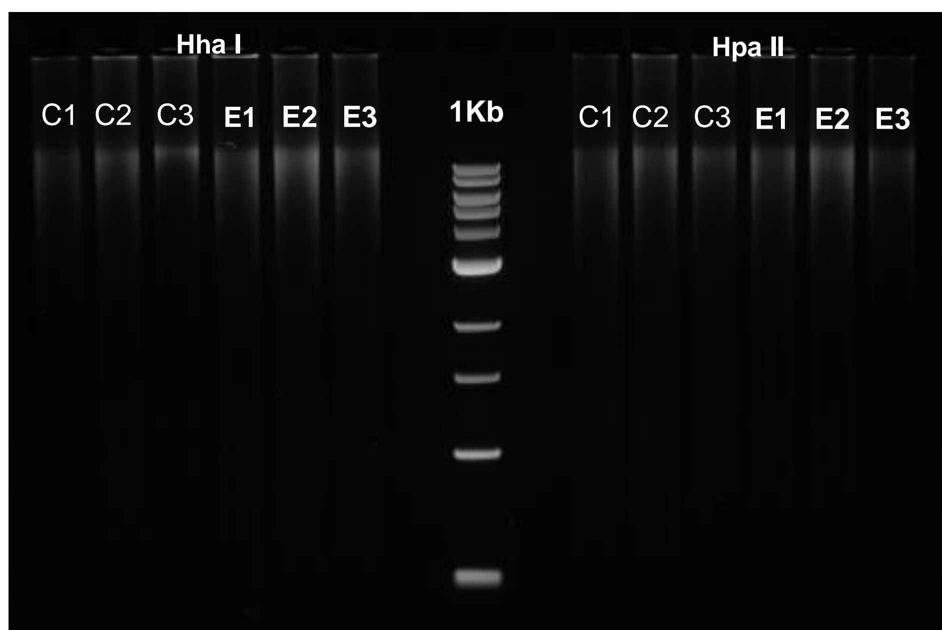


Figure 1. Pipeline used to identify differentially methylated regions.

The flow chart indicates the sequence of steps followed to identify DMRs, the software tools and packages used for those steps are shown in the boxes.

A



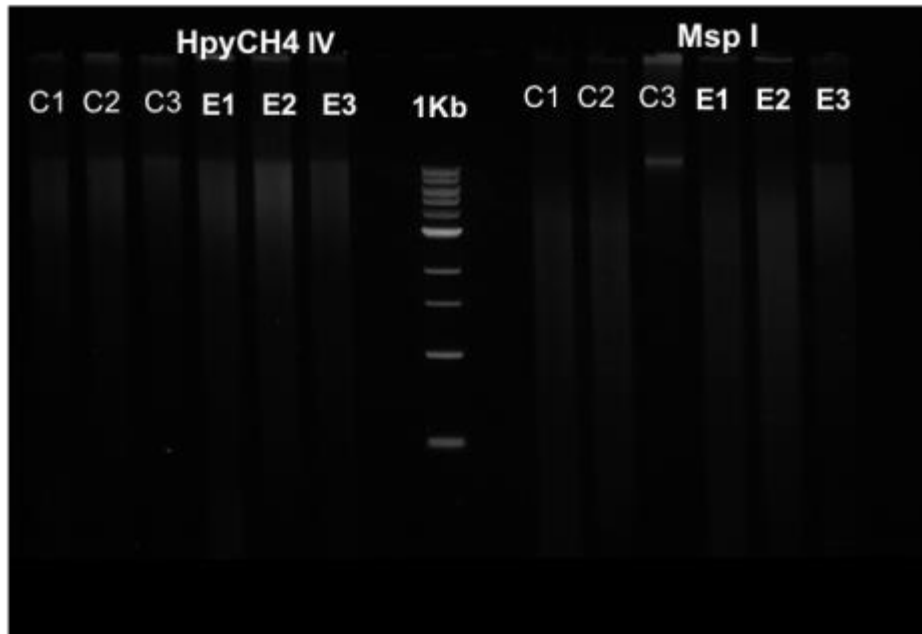
5′. . . GCGC . . . 3′
3′. . . CGCG . . . 5′

Methylation blocks

5′. . . CCGG . . . 3′
3′. . . GGCC . . . 5′

Methylation blocks

B

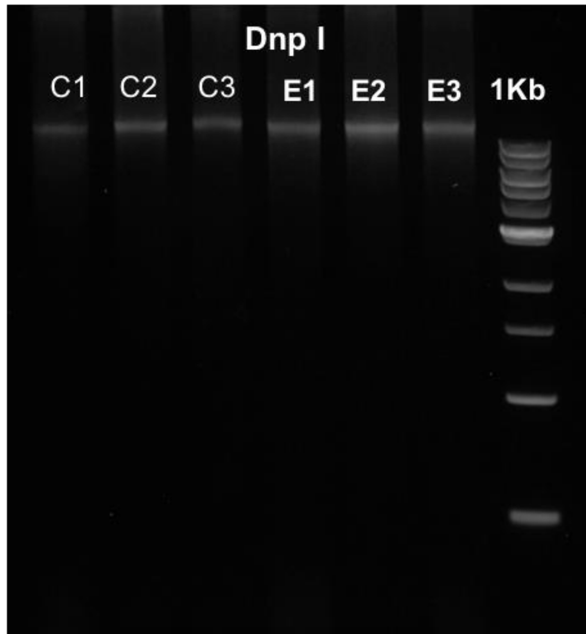


5′. . . A▼CG T . . . 3′
3′. . . T G C▲A . . . 5′

5′. . . C▼C G G . . . 3′
3′. . . G G C▲C . . . 5′

Methylation blocks

C



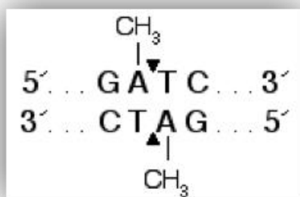


Figure 2. Methyl-specific restriction enzyme analysis of genomic DNA.

Restriction digestion of control and E2 treated samples (n = 3) with (A) Hha I and Hpa II (B) HpyCH4 IV and Msp I (C) Dnp I. C1, C2 and C3 represent control samples; E1, E2 and E3 represent estrogen treated samples. Below the restriction digestion gel picture the recognition site of the respective enzyme is presented.

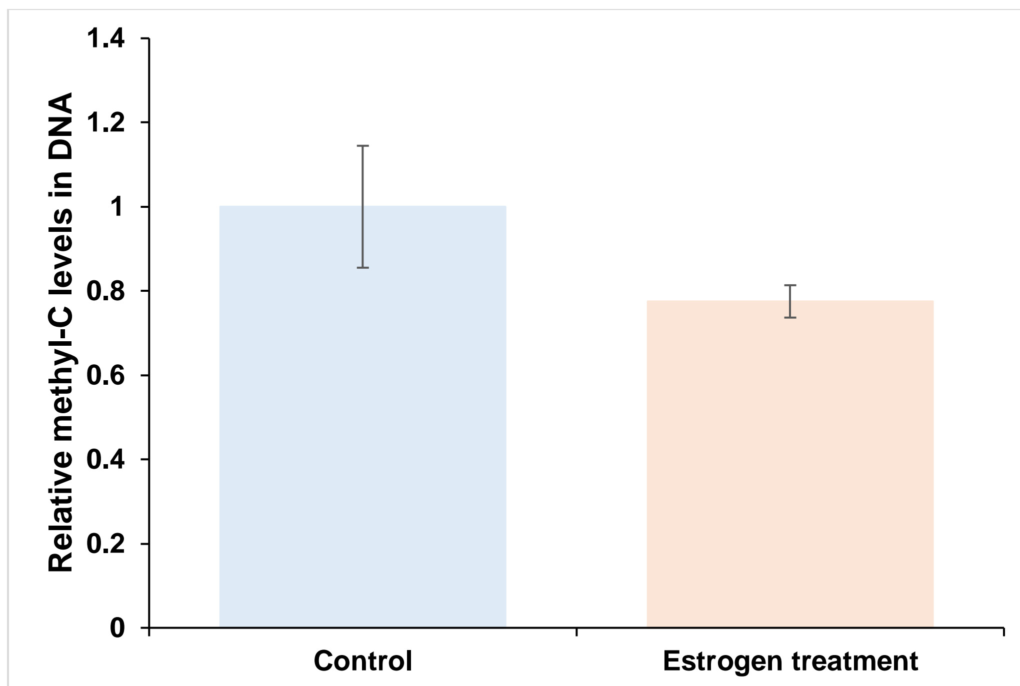


Figure 3. ELISA to identify global methyl cytosine levels.

Results are expressed as fold change ± SE (n = 5). Student's T-test was performed and no significant changes in global DNA methylation was observed.

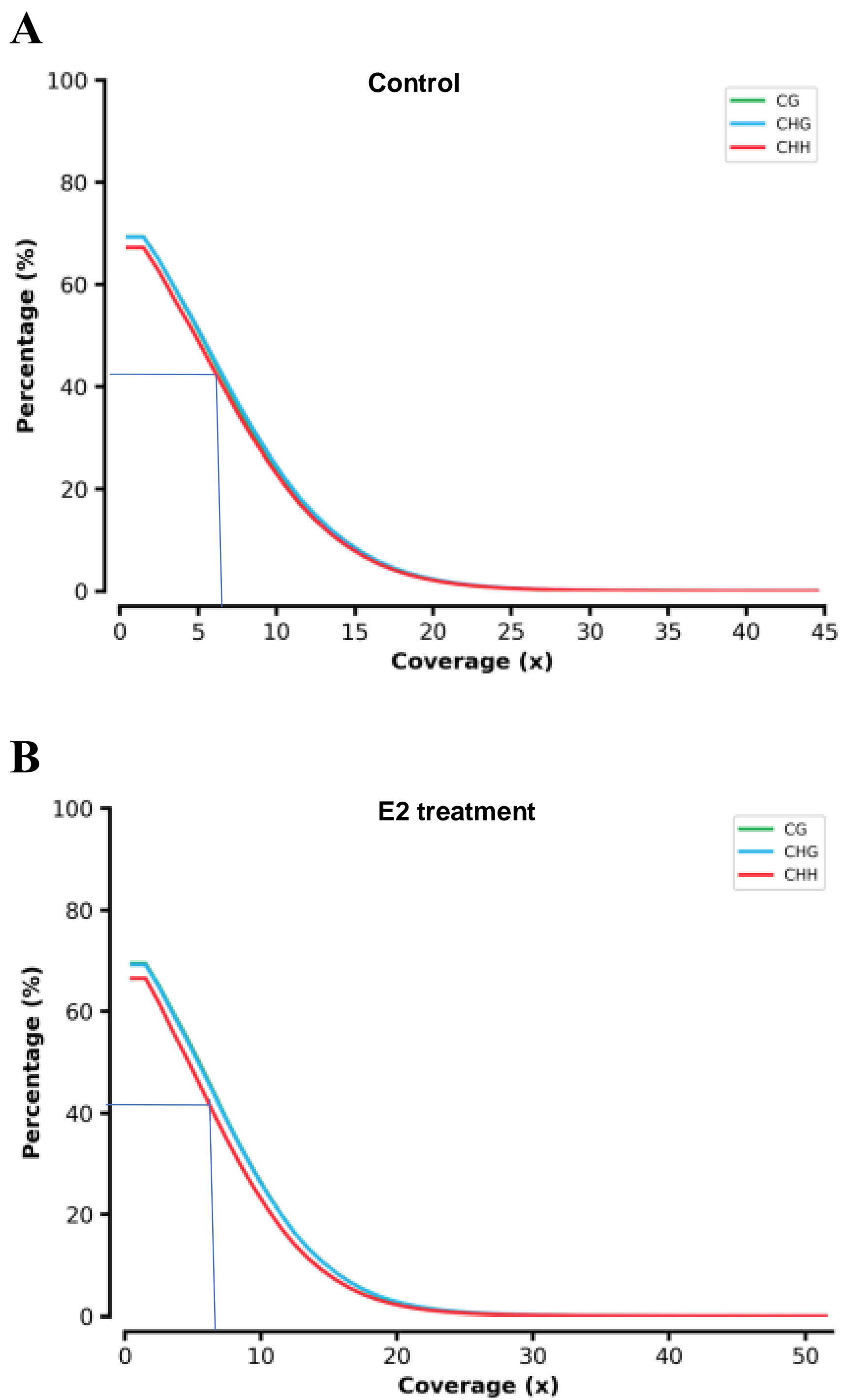


Figure 4. Reverse cumulative plot of the methylated cytosine percentage.

Percentage of respective sites (y-axis) and their coverage (x-axis) during sequencing are plotted in a reverse cumulative plot. The green represents CG methylation which is overlapped by CHG (blue line). CHH methylation is represented in red. (A) Control (B) E2 treated sample.

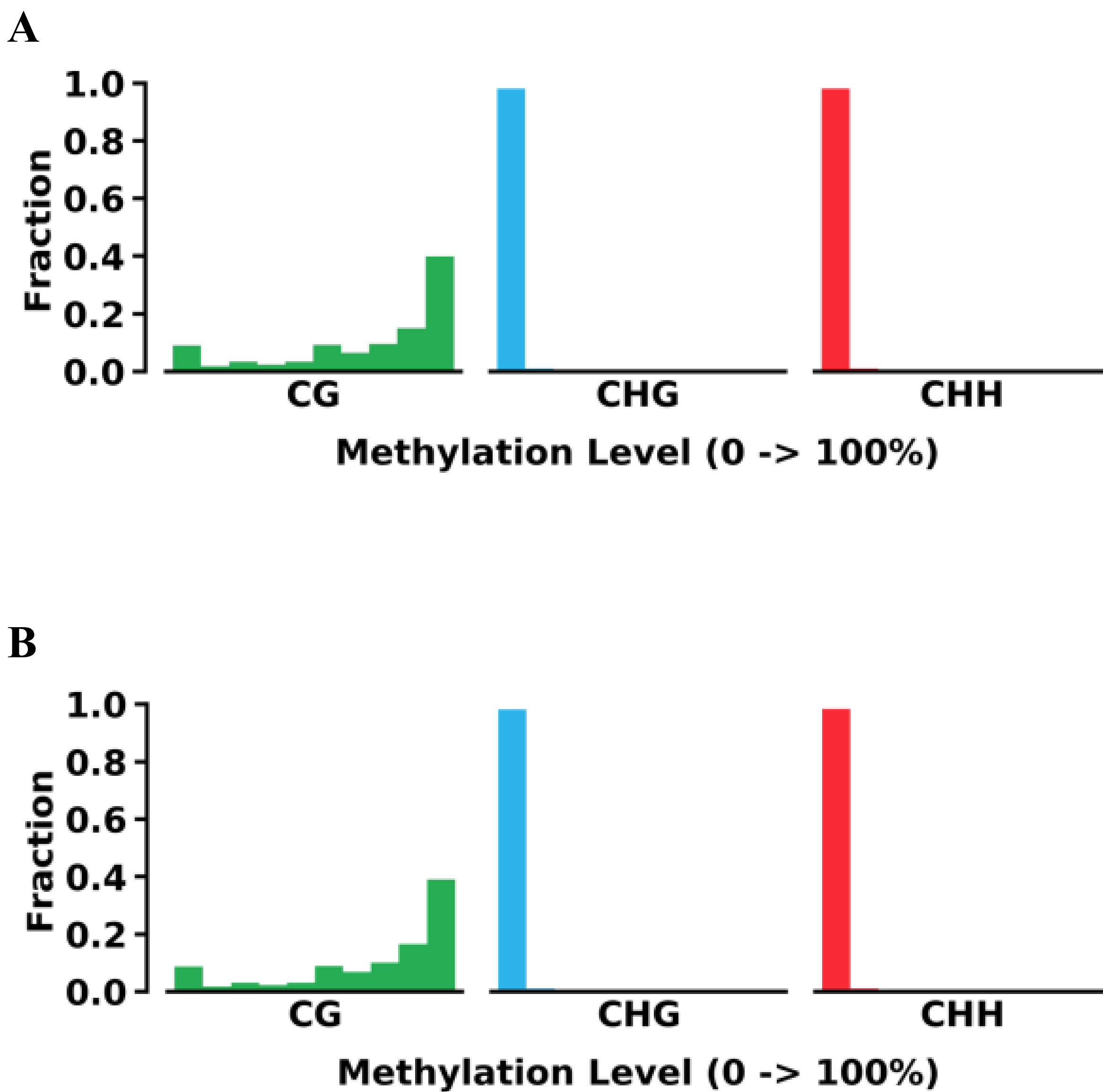
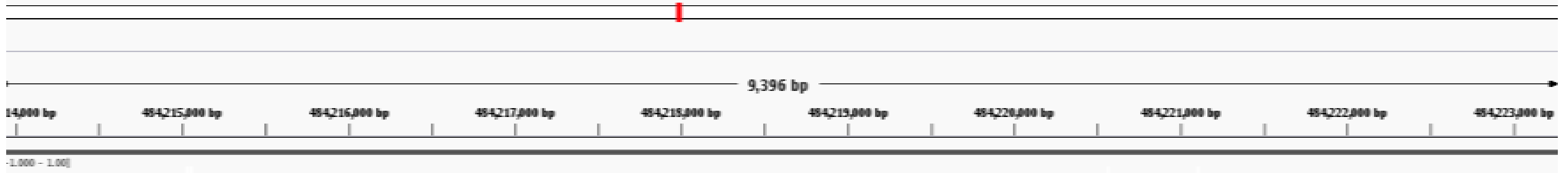


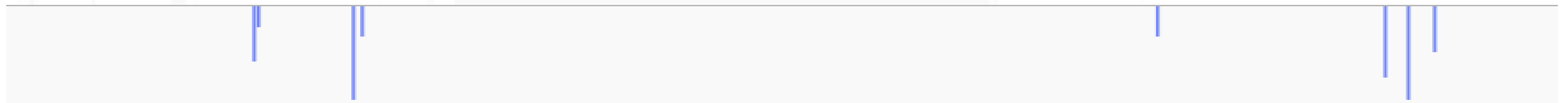
Figure 5. Global methylated cytosine distribution.

Distribution of global cytosine methylation in control (A) and E2 treated sample (B). The fraction of total CG, CHG and CHH methylation (y-axis) and the percentage of each fraction (x-axis) divided in 10% increments from 0%-10% to 90%-100%.

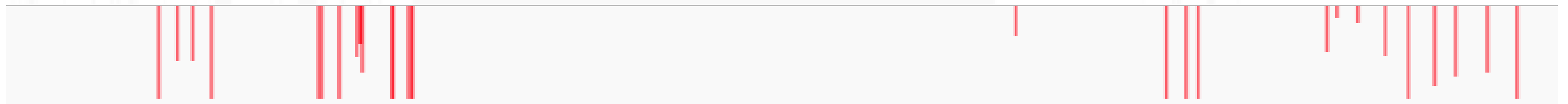
E3 ubiquitin-ligase CHIP



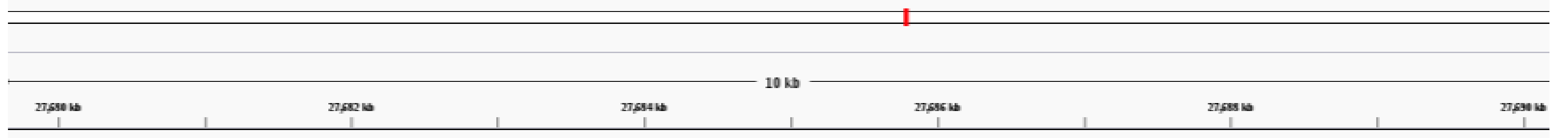
Control



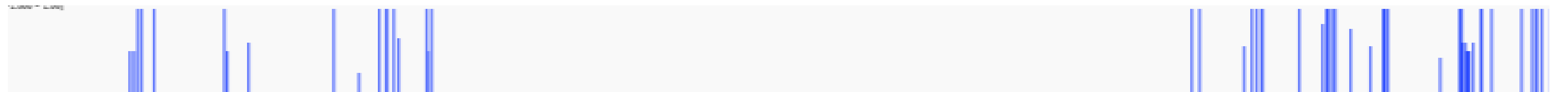
E2 treatment



Signal peptide peptidaselike 2B



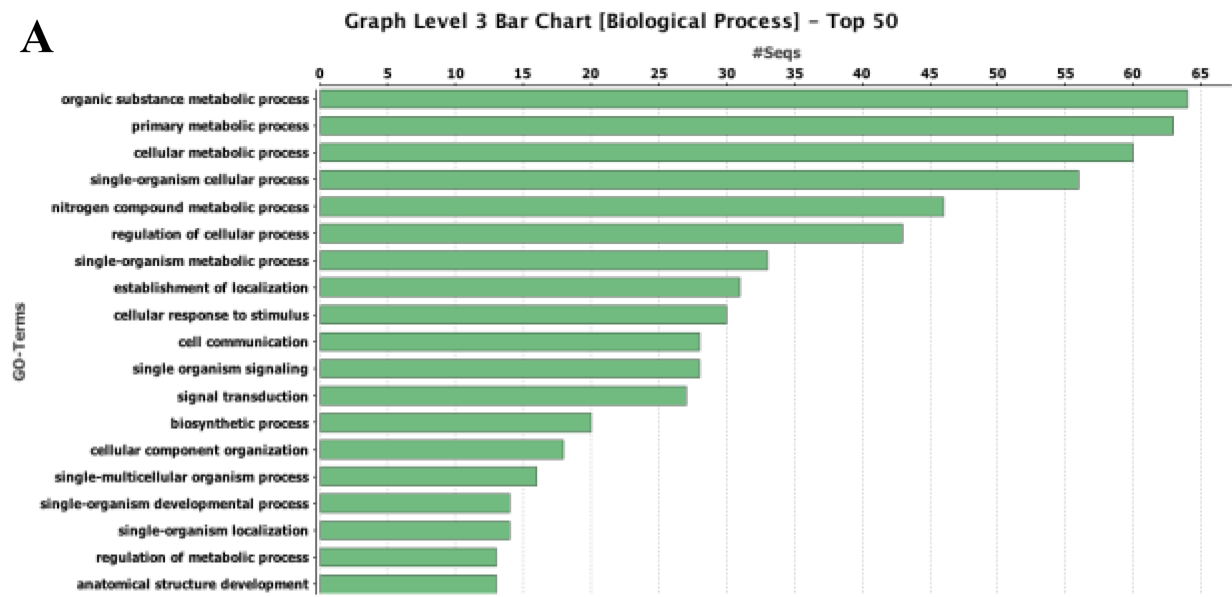
Control

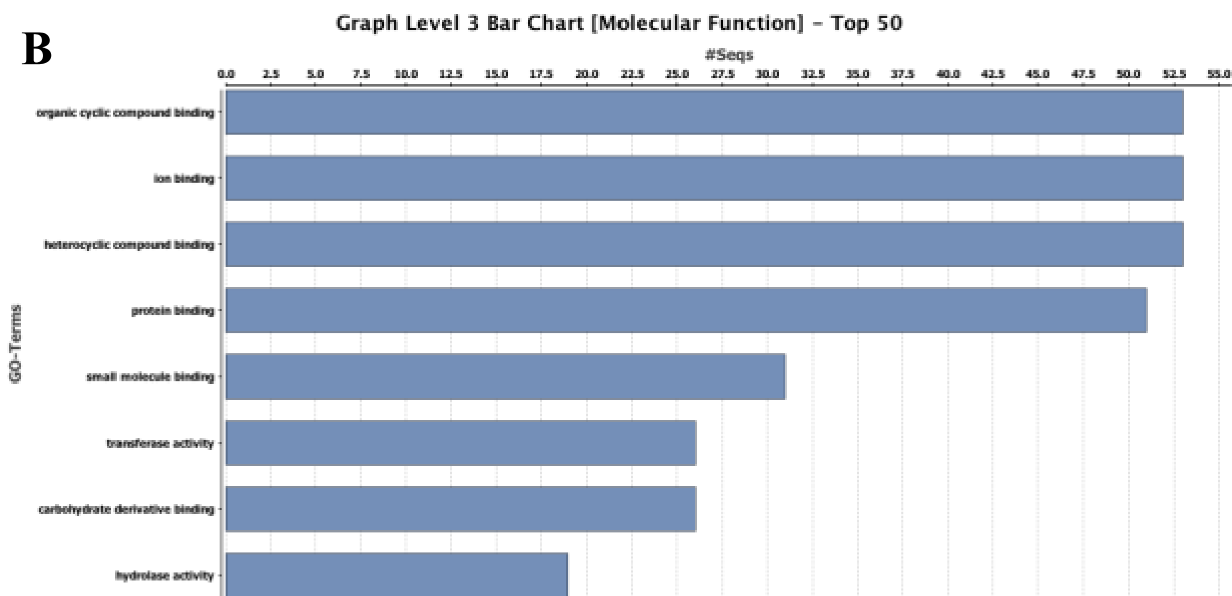


E2 treatment

Figure 6. Differentially methylated regions visualized using IGV.

Gene E3 ubiquitin-ligase CHIP is on the negative strand while Signal peptide peptidase like 2B is on the positive strand. The axis was set to 50% methylation which allows us to visualize methylations > 50%. The length of the bar indicates percentage of methylation.


A
Graph Level 3 Bar Chart [Biological Process] - Top 50
#Seqs
0
5
10
15
20
25
30
35
40
45
50
55
60
65
GO-Terms
organic substance metabolic process
primary metabolic process
cellular metabolic process
single-organism cellular process
nitrogen compound metabolic process
regulation of cellular process
single-organism metabolic process
establishment of localization
cellular response to stimulus
cell communication
single organism signaling
signal transduction
biosynthetic process
cellular component organization
single-multicellular organism process
single-organism developmental process
single-organism localization
regulation of metabolic process
anatomical structure development


B
Graph Level 3 Bar Chart [Molecular Function] - Top 50
#Seqs
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
25.0
27.5
30.0
32.5
35.0
37.5
40.0
42.5
45.0
47.5
50.0
52.5
55.0
GO-Terms
organic cyclic compound binding
ion binding
heterocyclic compound binding
protein binding
small molecule binding
transferase activity
carbohydrate derivative binding
hydrolase activity

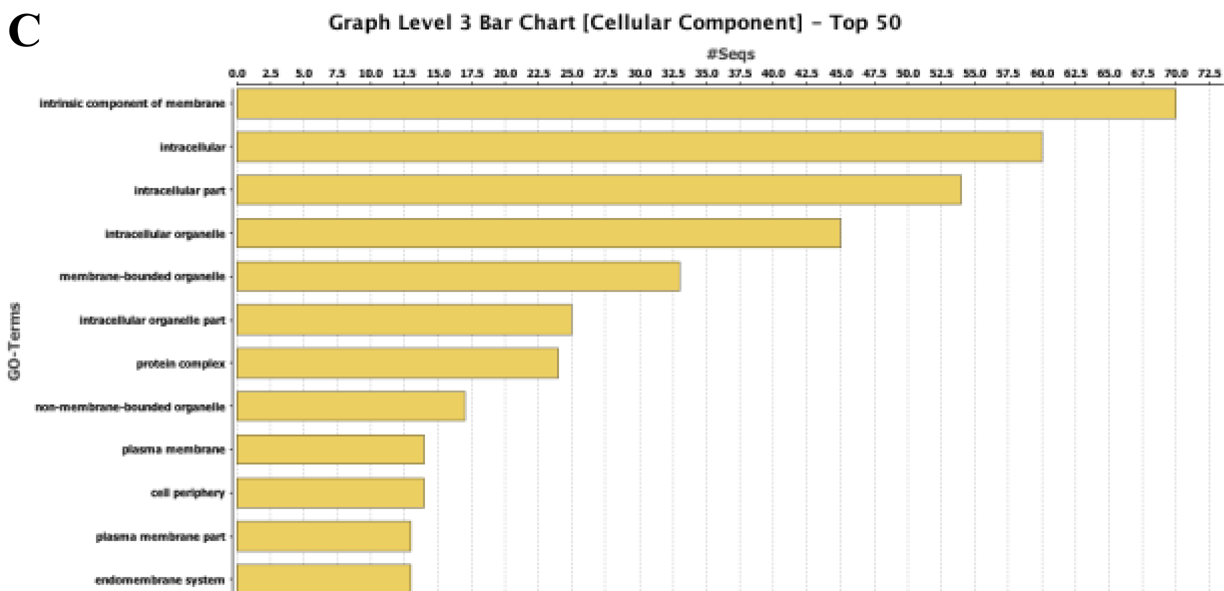


Figure 7. Gene Ontology analysis of gene with differentially methylated regions (CG). Representative GO terms in (A) Biological Processes (B) Molecular Function and (C) Cellular Components.

TABLES

Table 1. KEGG pathways analysis of genes with differentially CG methylated regions.

Pathway	**Pathway ID**
Mannose type O-glycan biosynthesis	map00515
Lysine degradation	map00310
Glycosaminoglycan biosynthesis - heparan sulfate / heparin	map00534
Nicotinate and nicotinamide metabolism	map00760
Steroid hormone biosynthesis	map00140
Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate	map00532
Purine metabolism	map00230
Steroid degradation	map00984
Glycerolipid metabolism	map00561
Glycosphingolipid biosynthesis - ganglio series	map00604
Drug metabolism - cytochrome P450	map00982
Retinol metabolism	map00830
Ether lipid metabolism	map00565
Metabolism of xenobiotics by cytochrome P450	map00980
Phenylalanine metabolism	map00360
Sphingolipid metabolism	map00600
Nitrogen metabolism	map00910
Tyrosine metabolism	map00350
Pentose and glucuronate interconversions	map00040
Aminobenzoate degradation	map00627
Tropane, piperidine and pyridine alkaloid biosynthesis	map00960
Mucin type O-glycan biosynthesis	map00512
Glycerophospholipid metabolism	map00564
Phosphatidylinositol signaling system	map04070
Glycosaminoglycan biosynthesis - keratan sulfate	map00533
Thiamine metabolism	map00730
Cysteine and methionine metabolism	map00270
Inositol phosphate metabolism	map00562
beta-Alanine metabolism	map00410
Glycine, serine and threonine metabolism	map00260
Drug metabolism - other enzymes	map00983
Ascorbate and aldarate metabolism	map00053
Th1 and Th2 cell differentiation	map04658
T cell receptor signaling pathway	map04660
Glycosphingolipid biosynthesis - globo and isoglobo series	map00603

Porphyrin and chlorophyll metabolism	map00860
Glycosphingolipid biosynthesis - lacto and neolacto series	map00601
Isoquinoline alkaloid biosynthesis	map00950

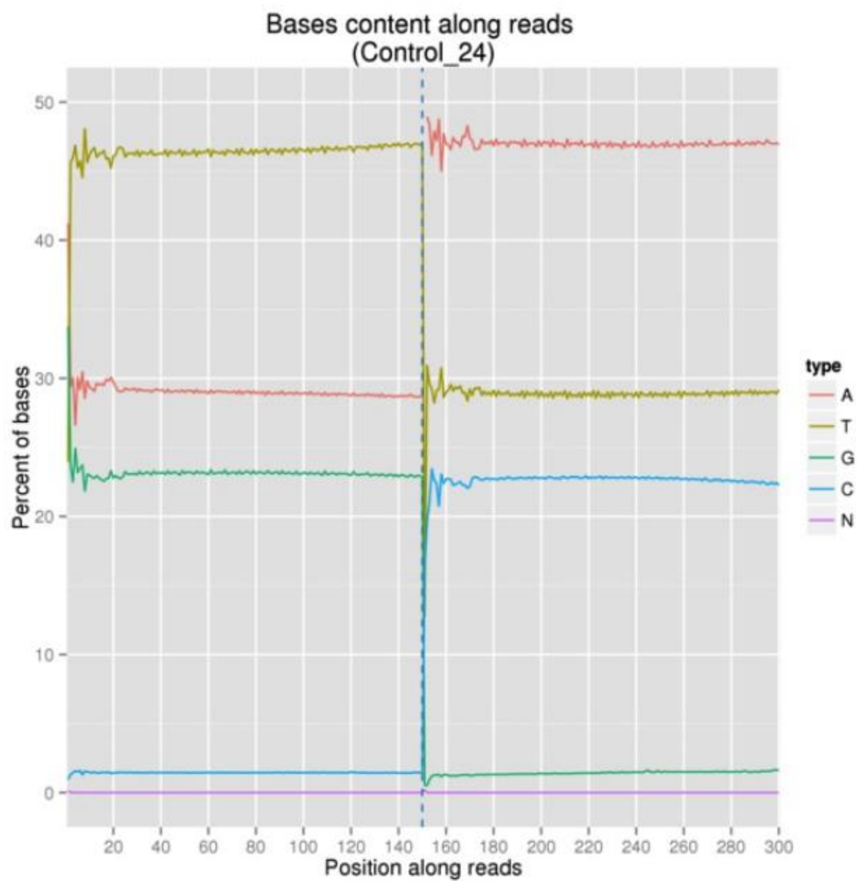
Table 2. DMRS with delta = ±100.

List of Gene IDs, their description and chromosomal location of genes with differentially methylated regions (delta value = ±100).

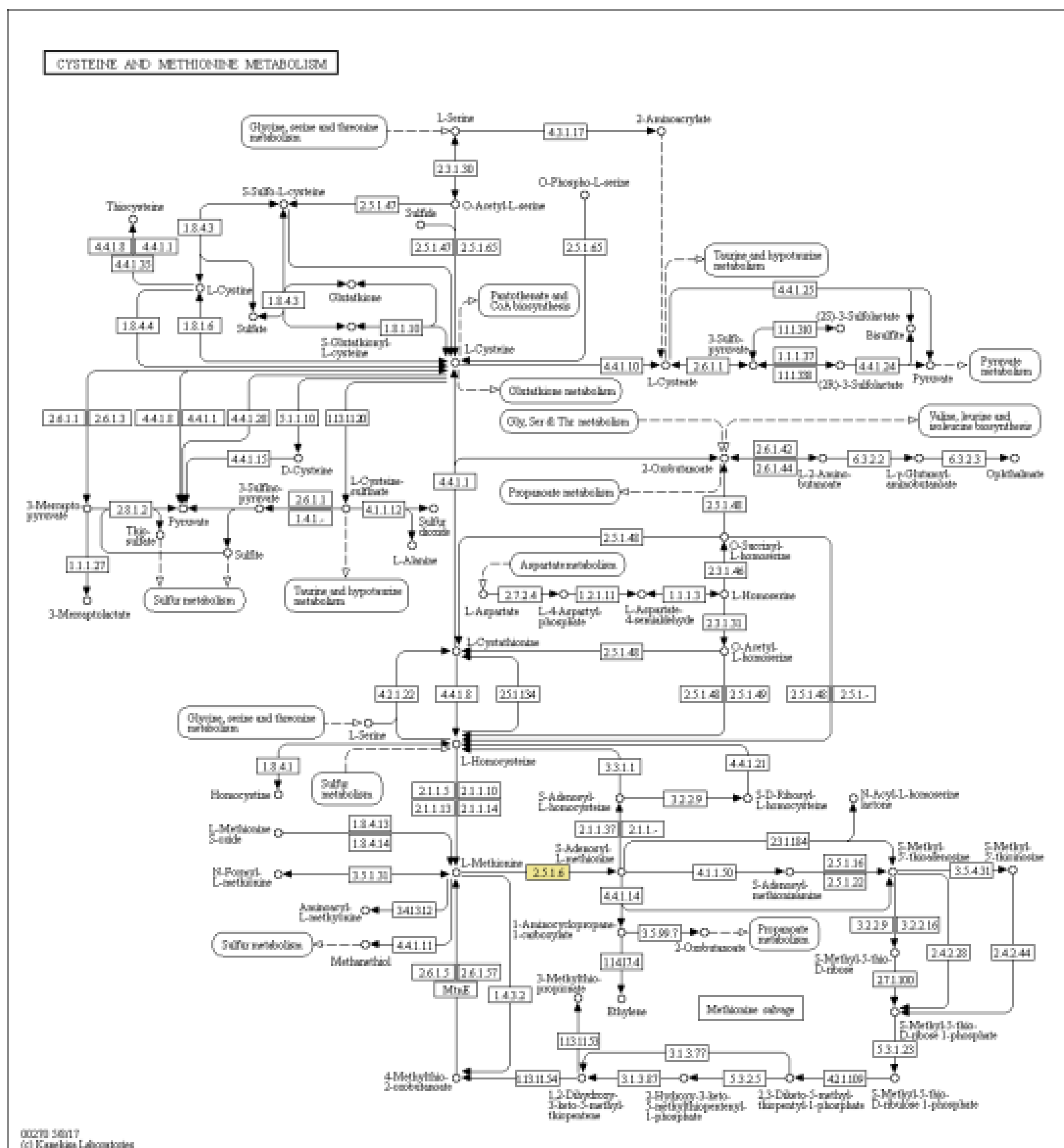
Gene ID	**Gene Description**	**Chromosomal location**
GSONMT00043412001	MTSS1 isoform X4	chrUn_177795616_177795843
GSONMT00047294001	NLR family CARD domain-containing 3-like	chrUn_52742408_52742790
GSONMT00052262001	T-lymphoma invasion and metastasis-inducing 2	chrUn_100101932_100102003
GSONMT00049850001	SH3 and multiple ankyrin repeat domains 3-like	chrUn_466526627_466526752
GSONMT00037508001	type 1 phosphatidylinositol 4,5-bisphosphate 4-phosphatase	chrUn_8_2184766_2184820 2184793
GSONMT00063837001	cell wall DAN4-	chrUn_516413858_516413932
GSONMT00009689001	oocyte zinc finger 6-like	chrUn_891648_891791
GSONMT00049306001	dymeclin isoform X2	chrUn_334685344_334685472
GSONMT00046774001	integrin beta-5	chrUn_1065978247_1065978298
GSONMT00063536001	RNA binding fox-1 homolog 2-like isoform X2	chrUn_1065978247_1065978298
GSONMT00045523001	zinc-binding alcohol dehydrogenase domain-containing 2	chrUn_183919601_183919676
GSONMT00015086001	desmoglein-2-like	chrUn_5_25492875_25493239
GSONMT00017342001	beta-galactosidase-1 2	chrUn_24_4446695_4446767
GSONMT00017343001	galactosylgalactosylxylosyl 3-beta-glucuronosyltransferase 1	chrUn_24_4446695_4446767
GSONMT00031753001	serine threonine- kinase D3-like	chrUn_29_2363855_2364050
GSONMT00031754001	unnamed protein product	chrUn_29_2363855_2364050
GSONMT00008846001	forkhead box P3	chrUn_16_35686473_35686576
GSONMT00013218001	unnamed protein product	chrUn_20_17993397_17993466
GSONMT00036928001	nuclear receptor coactivator 6-like isoform X1	chrUn_142652331_142652448
GSONMT00030380001	kinesin-1 heavy chain	chrUn_11_45310313_45310530
GSONMT00036502001	Golgi SNAP receptor complex member 2	chrUn_12_1756822_1756929
GSONMT00013489001	arf-GAP with Rho-GAP ANK repeat and PH domain-containing 1-like	chrUn_12_22210482_22210695

GSONMT00013490001	arf-GAP with Rho-GAP ANK repeat and PH domain-containing 1-like	chrUn_12_22210482_22210695
GSONMT00072683001	hepatic leukemia factor-like	chrUn_1055141136_1055141201
GSONMT00069684001	polypeptide N-acetylgalactosaminyltransferase 5	chrUn_3_4451088_4451147

SUPPLEMENTARY DATA



Supplementary figure 1. Distribution of GC content. Position of a base on the read is on x-axis and the percentage of each base on y-axis. A-T content of each read is more than the G-C content due to bisulfite conversion.



Supplementary figure 2. Gene with differentially methylated regions in enzyme (yellow box) necessary for conversion of methionine to s-adenosyl methionine (SAM), a methyl donor for DNA methylation.

Supplementary table 1. List of Gene IDs and their description with differentially methylated regions (delta value ≥ ±80)

Gene ID	Gene Description
GSONMT00038395001	neurobeachin-like isoform X2
GSONMT00040894001	serine threonine- kinase N1-like isoform X2
GSONMT00018560001	otopetrin-3-like isoform X2
GSONMT00033489001	voltage-dependent L-type calcium channel subunit alpha-1S
GSONMT00047032001	ATP synthase subunit mitochondrial-like
GSONMT00000329001	GMP synthase [glutamine-hydrolyzing]
GSONMT00032945001	matrix metallo ase-9-like
GSONMT00024979001	eukaryotic translation initiation factor 3 subunit B
GSONMT00008583001	RNA-directed DNA polymerase from mobile element jockey
GSONMT00062010001	unnamed protein product, partial
GSONMT00009581001	splicing arginine serine-rich 15-like
GSONMT00024985001	circularly permutated Ras 1-like
GSONMT00016042001	amine oxidase [flavin-containing]-like
GSONMT00003645001	zinc finger MIZ domain-containing 2-like isoform X3
GSONMT00043422001	unnamed protein product
GSONMT00060724001	poly [ADP-ribose] polymerase 11-like
GSONMT00046724001	stromal interaction molecule 1-like isoform X1
GSONMT00028745001	calcium-activated potassium channel subunit alpha-1-
GSONMT00045996001	DNA replication licensing factor MCM4
GSONMT00015659001	sodium-dependent neutral amino acid transporter B(0)AT1-like
GSONMT00003976001	nuclear receptor-interacting 2-like
GSONMT00062759001	T-cell receptor beta
GSONMT00062760001	T-cell receptor beta
GSONMT00023748001	sciellin isoform X1
GSONMT00052213001	anti-apoptotic NR13-like
GSONMT00042290001	ataxin-1-like
GSONMT00005606001	potassium voltage-gated channel subfamily H member 4
GSONMT00028833001	CYR61-like
GSONMT00031243001	cysteine serine-rich nuclear 3-like isoform X1
GSONMT00005364001	TC1-like transposase
GSONMT00043412001	MTSS1 isoform X4
GSONMT00020618001	unnamed protein product
GSONMT00016279001	spidroin-1-like isoform X1
GSONMT00002804001	cell migration-inducing and hyaluronan-binding
GSONMT00002504001	dmX 2

GSONMT00001099001	and PH domain-containing 5-like isoform X2
GSONMT00061970001	cytochrome P450 26A1
GSONMT00047294001	NLR family CARD domain-containing 3-like
GSONMT00056448001	unconventional myosin-IXa-like
GSONMT00002151001	LIM domain and actin-binding 1-like isoform X1
GSONMT00042327001	RNA-directed DNA polymerase from mobile element jockey-
GSONMT00030193001	low-density lipo receptor-related 4 isoform X1
GSONMT00025849001	phospholipid phosphatase-related type 5-like isoform X1
GSONMT00003392001	zinc finger
GSONMT00052262001	T-lymphoma invasion and metastasis-inducing 2
GSONMT00010108001	RNA-directed DNA polymerase from mobile element jockey-
GSONMT00010098001	membrane-associated phosphatidylinositol transfer 3
GSONMT00061299001	dystrophin-related 2-like
GSONMT00049335001	inosine-uridine preferring nucleoside hydrolase-like
GSONMT00044284001	Bardet-Biedl syndrome 4 -like isoform X2
GSONMT00049850001	SH3 and multiple ankyrin repeat domains 3-like
GSONMT00037508001	type 1 phosphatidylinositol 4,5-bisphosphate 4-phosphatase
GSONMT00009207001	U6 snRNA-associated Sm LSm3
GSONMT00063837001	cell wall DAN4-
GSONMT00009689001	oocyte zinc finger 6-like
GSONMT00062700001	LIM domain and actin-binding 1-like isoform X1
GSONMT00004147001	E3 ubiquitin- ligase CHIP
GSONMT00027340001	inter-alpha-trypsin inhibitor heavy chain H5
GSONMT00000030001	interleukin-21 receptor isoform X1
GSONMT00049306001	dymeclin isoform X2
GSONMT00005828001	cysteine-rich motor neuron 1
GSONMT00046774001	integrin beta-5
GSONMT00063536001	RNA binding fox-1 homolog 2-like isoform X2
GSONMT00062936001	piggyBac transposable element-derived 3-like
GSONMT00062937001	hepatocyte cell adhesion molecule-
GSONMT00025140001	zinc finger MIZ domain-containing 1-like isoform X5
GSONMT00018442001	peroxisomal carnitine O-octanoyltransferase-like
GSONMT00029013001	CMP-N-acetylneuraminate-beta-galactosamide-alpha-2,3-sialyltransferase 1-like
GSONMT00055168001	rho GTPase-activating 21-like isoform X1
GSONMT00035139001	transmembrane 94-like isoform X1
GSONMT00055431001	tetratricopeptide repeat 22
GSONMT00013110001	cAMP-dependent kinase type II-alpha regulatory subunit-like isoform X2

GSONMT00041814001	collagen alpha-1(V) chain-like isoform X2
GSONMT00014867001	repressor of yield of DENV
GSONMT00044531001	desmoplakin isoform X2
GSONMT00010477001	ATP-binding cassette sub-family E member 1
GSONMT00045523001	zinc-binding alcohol dehydrogenase domain-containing 2
GSONMT00001103001	Golgi SNAP receptor complex member 1
GSONMT00003333001	bicaudal D homolog 1-like
GSONMT00044390001	kelch 29
GSONMT00006838001	U6 snRNA-associated Sm LSm7
GSONMT00006841001	signal peptide peptidase-like 2B
GSONMT00041952001	interferon-induced very large GTPase 1-like
GSONMT00022513001	thyroid hormone receptor-associated 3 isoform X4
GSONMT00010472001	immmunoglobulin light chain
GSONMT00015086001	desmoglein-2-like
GSONMT00022139001	cullin-9-like isoform X4
GSONMT00022140001	cullin-9-like isoform X3
GSONMT00032029001	PDZ and LIM domain 5-like
GSONMT00006955001	zinc finger 813-like
GSONMT00062416001	deoxyribodipyrimidine photo-lyase-like
GSONMT00034452001	phospholipid phosphatase 2-like
GSONMT00029414001	dnaJ homolog subfamily C member 3-like
GSONMT00017342001	beta-galactosidase-1 2
GSONMT00017343001	galactosylgalactosylxylosyl 3-beta-glucuronosyltransferase 1
GSONMT00019877001	gastrula zinc finger -like
GSONMT00010391001	striatin-4 isoform X2
GSONMT00010392001	serine threonine- kinase D2
GSONMT00010318001	lutropin-choriogonadotropic hormone receptor-like
GSONMT00050171001	116 kDa U5 small nuclear ribonucleo component
GSONMT00050175001	60S ribosomal L19
GSONMT00040445001	vacuolar sorting-associated 13A isoform X1
GSONMT00015115001	insulin receptor substrate 2-like isoform X1
GSONMT00007270001	carbonic anhydrase 4-like
GSONMT00037482001	TCR-gamma constant region
GSONMT00031753001	serine threonine- kinase D3-like
GSONMT00031754001	unnamed protein product
GSONMT00009908001	spondin-1-like isoform X1
GSONMT00061869001	activating transcription factor 7-interacting 1-like
GSONMT00061870001	activating transcription factor 7-interacting 1-like
GSONMT00008846001	forkhead box P3

GSONMT00013218001	unnamed protein product
GSONMT00056810001	trinucleotide repeat-containing gene 6B -like
GSONMT00056812001	casein kinase I
GSONMT00038125001	coiled-coil domain-containing 174
GSONMT00014754001	E3 ubiquitin- ligase MYCBP2-like
GSONMT00052207001	NHS 1 isoform X3
GSONMT00036928001	nuclear receptor coactivator 6-like isoform X1
GSONMT00036934001	solute carrier family 26 member 6-like
GSONMT00040610001	MTSS1 isoform X3
GSONMT00010157001	SRSF kinase 3-like isoform X3
GSONMT00051319001	calcium-dependent secretion activator 1-
GSONMT00044026001	grainyhead 1 homolog isoform X2
GSONMT00044027001	TATA box-binding -associated factor RNA polymerase I subunit B
GSONMT00050547001	long-chain-fatty-acid-- ligase 1-like isoform X1
GSONMT00042791001	beta-galactoside alpha-2,6-sialyltransferase 2
GSONMT00042793001	RNA-directed DNA polymerase from mobile element jockey
GSONMT00043680001	B-cell receptor CD22-like
GSONMT00059137001	zinc finger 19-like
GSONMT00049831001	LIM domain transcription factor -like isoform X1
GSONMT00025587001	manganese-transporting ATPase 13A1
GSONMT00034033001	Krueppel-like factor 6
GSONMT00029702001	F-actin-methionine sulfoxide oxidase mical1-like isoform X1
GSONMT00030380001	kinesin-1 heavy chain
GSONMT00001799001	HRAS-like suppressor 2
GSONMT00001800001	deleted in malignant brain tumors 1 -like
GSONMT00036502001	Golgi SNAP receptor complex member 2
GSONMT00016924001	hepatocyte cell adhesion molecule-like isoform X2
GSONMT00016934001	transmembrane and coiled-coil domain-containing 6
GSONMT00044813001	secretin receptor-like isoform X1
GSONMT00044814001	cilia- and flagella-associated 221 isoform X1
GSONMT00055123001	ryanodine receptor 3-like isoform X2
GSONMT00044303001	CLIP-associating 1-B-like
GSONMT00044314001	disrupted in renal carcinoma 2
GSONMT00009799001	paired box Pax-6-like
GSONMT00021806001	dematin-like isoform X1
GSONMT00040393001	pre-mRNA-processing factor 19
GSONMT00019027001	contactin-4-like isoform X1
GSONMT00013489001	arf-GAP with Rho-GAP ANK repeat and PH domain-containing 1-like

GSONMT00013490001	arf-GAP with Rho-GAP ANK repeat and PH domain-containing 1-like
GSONMT00024682001	calcium-binding tyrosine phosphorylation-regulated isoform X1
GSONMT00027156001	rho GTPase-activating 32-like isoform X1
GSONMT00012762001	paired box and transposase domain containing
GSONMT00037200001	polypyrimidine tract-binding 2-like
GSONMT00034929001	unnamed protein product
GSONMT00058462001	T-cell receptor alpha chain V region HPB-MLT precursor
GSONMT00039234001	latent-transforming growth factor beta-binding 2 isoform X1
GSONMT00010006001	dynein heavy chain axonemal
GSONMT00076862001	ATP-binding cassette sub-family A member 12
GSONMT00076439001	teneurin-2 isoform X1
GSONMT00075587001	rhotekin-2 isoform X2
GSONMT00075537001	chromodomain-helicase-DNA-binding 7-like
GSONMT00074889001	tetratricopeptide repeat 28-like
GSONMT00074843001	phospholipase D3
GSONMT00074457001	voltage-dependent L-type calcium channel subunit alpha-1S
GSONMT00074458001	voltage-dependent L-type calcium channel subunit alpha-1S
GSONMT00074178001	casein kinase I isoform X1
GSONMT00073410001	Fanconi anemia group M
GSONMT00073089001	serine threonine- kinase TNNI3K
GSONMT00072683001	hepatic leukemia factor-like
GSONMT00072116001	nucleo TPR-like isoform X1
GSONMT00072085001	exostosin-1c isoform X1
GSONMT00072044001	myosin-binding cardiac-type-like
GSONMT00071418001	histone-lysine N- H3 lysine-36 and H4 lysine-20 specific-like
GSONMT00071319001	transmembrane 168-A-like
GSONMT00070672001	E3 ubiquitin- ligase RAD18-like isoform X1
GSONMT00070160001	S-adenosylmethionine synthase-like isoform X2
GSONMT00069974001	rootletin isoform X1
GSONMT00068112001	potassium voltage-gated channel subfamily S member 3
GSONMT00067850001	V-type proton ATPase 116 kDa subunit a-like
GSONMT00067851001	V-type proton ATPase 116 kDa subunit a-like
GSONMT00067164001	serine threonine- phosphatase 2B catalytic subunit alpha isoform isoform X1
GSONMT00067165001	serine threonine- phosphatase 2B catalytic subunit alpha isoform isoform X2
GSONMT00067170001	NXPE family member 3-like
GSONMT00066366001	F-box LRR-repeat 17
GSONMT00065409001	dysbindin-like isoform X1

GSONMT00065314001	elongation factor 1-delta isoform X1
GSONMT00065336001	desmoplakin-like isoform X1
GSONMT00065337001	desmoplakin-like isoform X1
GSONMT00082648001	fibroblast growth factor receptor 2 isoform X1
GSONMT00082323001	B-cell receptor-associated 29-like
GSONMT00082036001	3-oxo-5-alpha-steroid 4-dehydrogenase 2-like
GSONMT00081780001	pleckstrin homology domain-containing family A member 6-like isoform X6
GSONMT00080026001	SNF-related serine threonine- kinase-like
GSONMT00079425001	serine threonine- kinase D3-like isoform X1
GSONMT00079375001	serine threonine- phosphatase 2A 55 kDa regulatory subunit B alpha isoform
GSONMT00079136001	transcriptional activator GLI3 isoform X2
GSONMT00078403001	rab11 family-interacting 3 isoform X2
GSONMT00076795001	collectin-11 isoform X2
GSONMT00074009001	zinc finger 629-like
GSONMT00072782001	phosphatase 1 regulatory subunit 12A
GSONMT00072163001	FAM184A-like isoform X1
GSONMT00072208001	band 2 isoform X1
GSONMT00069684001	polypeptide N-acetylgalactosaminyltransferase 5
GSONMT00067744001	peptidyl-prolyl cis-trans isomerase FKBP7
GSONMT00077787001	MAGUK p55 subfamily member 7-like
GSONMT00070911001	plexin domain-containing 2
GSONMT00064098001	diacylglycerol kinase zeta-like isoform X1
GSONMT00066506001	tyrosine- kinase Yes
GSONMT00066508001	tyrosine- kinase yes

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miR-nov-285-3p regulates DNA methyltransferase1 expression

INTRODUCTION

miRNAs regulate gene expression post transcriptionally either by deadenylation or translational repression. They functionally reduce gene expression by binding to the 3' region of mRNA generally besides regulating gene expression by binding to 5' UTR and coding regions. Target gene prediction of this novel miRNA and subsequent gene ontology enrichment analysis revealed roles in biological processes and molecular functions involving DNA, RNA and histone methylation and mRNA capping as shown is table 1. In general, these processes directly or indirectly regulate gene expression. This novel miRNA showed reduced expression in E2 exposed skeletal muscle in comparison to control (Chapter 1, Figure 2). Supplementary to this study, expression analysis of DNA methyltransferases also confirmed an increase in all three DNMTs in skeletal muscle under the influence of estrogen after 24 hours (Chapter 2, Figure 2). Collectively, these findings directed our interest to investigate and functionally validate the role of this novel miRNA in Dnmt1 gene expression.

Cloning and plasmid purification

Dnmt1 gene and pre-miR-nov-285 sequences were obtained and their intramolecular hybridization was confirmed using RNA hybrid software (Figure 1). The seed sequence of this novel miRNA binds to the last exon of Dnmt1. Primers were designed with Pme1 and Xho1 restriction enzymes at the 5' and 3' ends respectively, to amplify a 252bp fragment from E2 exposed skeletal muscle cDNA samples. This 252bp fragment is from the last exon of Dnmt1 that harbors sequence complementary to the seed sequence. The amplified fragment was successively cloned to pmirGLO dual-luciferase miRNA target expression vector (Promega, Madison, WI, USA) downstream of firefly luciferase (Figure 2a). Concurrently, a 253 bp fragment of pre-miR-285-3p was amplified from testis cDNA and cloned to pcDNA3.1a using BamH1 and Xho1 (Figure 2b). Primers used for cloning were listed in table 2. Further, these clones were confirmed using Sanger's sequencing. Both the clones, pmiRGLO and pcDNA3.1a plasmids were later purified using Qiagen plasmid purification midi kit following manufacturer's instructions.

Cell culture and dual luciferase reporter assay

Human embryonic kidney cells line (HEK-293) was used for the luciferase reporter assay. The cells were maintained in 10mm plate at 37°C in 5% CO_2 using 10% fetal bovine serum (Gibco-BRL, Grand Island, NY, USA) supplemented DMEM/High glucose medium. These cells were plated in a 96-well plate with a density of 2 X 10^4 cells/well. Transfection was performed using Lipofectamine LTX transfection reagent following manufacturer's instructions (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA). PmiRGLO-3'DNMT is cotransfected with increasing concentrations of pcDNA3.1a-pre-miR285. The total amount of plasmid used for transfection was 100ng/well. Transfection was repeated 4 times in 3 independent experiments. Fortyeight hours post-transfection cells were collected and luciferase activity measured using Dual-Glo System (Promega, Madison, WI, USA). Firefly and renilla luciferase activity was measured following Dual-Glo Luciferase Assay protocol and statistical significance of differential luciferase activity was determined by Students t-test.

Luciferase activity

Firefly luciferase activity was normalized by dividing with renilla luciferase activity. Luciferase activity was represented as relative expression to pmiRGLO-3'Dnmt which is set as 1. pmiRGlo vector cotransfected with pcDNA3.1 was used as a control, to compare the luciferase activity of pmiRGLO-3'Dnmt with pcDNA3.1-pre-miR-285 at different concentrations. (Figure 3). Significant reductions in gene expression were observed with increasing pre-miR285 concentrations. These observations support the idea that miR-nov-285-3p regulates Dnmt1 gene expression consistent with the observations of miR-nov-285-3p and gene expressions in E2 treated samples.

FIGURES

```
Position: 4337
target 5' G           C          G 3'
           GAACC    GC ACAACCAUUG
           CUUGG    UG UGUUGGUGAC
miRNA  3'       AUAA  C          G 5'
```

Figure 1. Binding of seed sequence to last exon of Dnmt1.

10 bases of seed sequence at 5’ end of miRNA are complementary to the target gene with one G:U pair.

Figure 2. Cloning strategies.

Cloning of the 3' exon region of Dnmt1 with complementary sequence for mature miRNA in pmiRGLO vector (A), cloning of pre-miR285-3p with seed sequence in pcDNA3.1a (B). The primers used to amplify are in black and underlined, the target region and mature miRNA are in red and underlined.

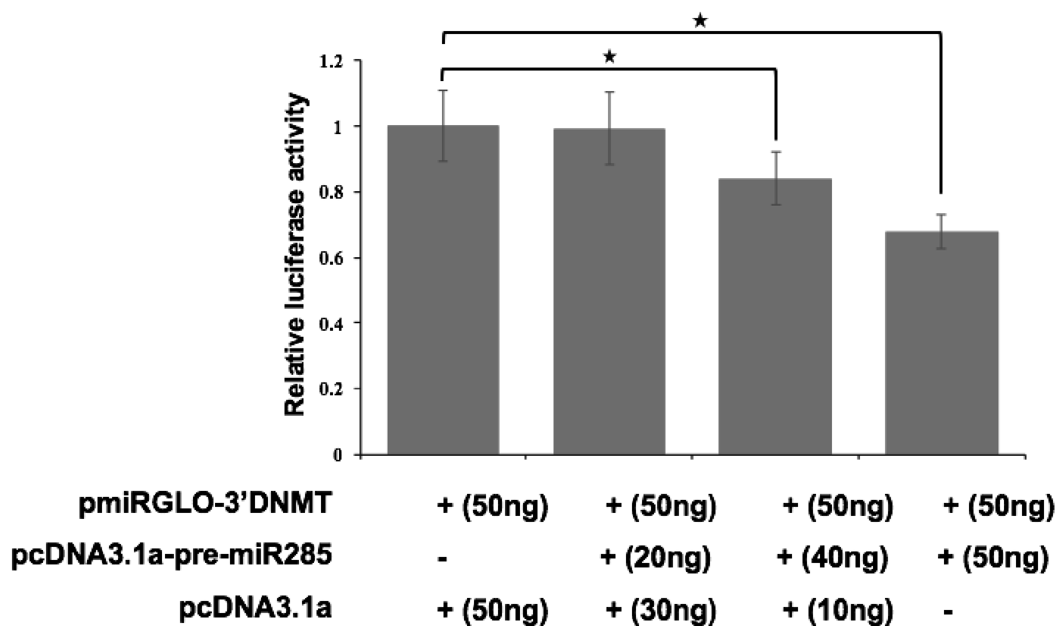

Figure 3. Relative luciferase activity in comparison to control are presented.

With increase in pre-miR285-3p reduction in luciferase activity was observed. Results are expressed in relative to control ± SE.

CONCLUSIONS

The role of non-coding RNA and epigenetic mechanisms can help explain details of regulation of gene expression. Fig. 1 briefly explains the various mechanism actively controlling gene expression at both transcriptional and post-transcriptional levels. Besides established regulatory mechanisms including cis and trans elements, splicing, alternative splicing, poly (A) length, mRNA export and decay; epigenetic mechanisms and non-coding RNA govern gene expression at additional levels. The current study to understand how non-coding RNAs and DNA methylation contribute to imbalance in protein turnover in E2 treated rainbow trout. The observations support that these molecular elements are highly influenced by external stimuli like E2 at 24 hours. Thirty-six miRNAs were differentially expressed between control and E2 treated samples within 24-hours of treatment. These effects were diluted after 72 hours of treatment as determined by miRNA expression and gene expression experiments. These pieces of evidence signify the rapid influence of external stimuli and their regulation of molecular mechanisms. Further, the previous observations of increased protein degradation and decreased protein synthesis were because of changes at the molecular levels. Differential expression of miRNA contributing to protein degradation and protein synthesis were observed (Fig. 4). Target genes of these differentially expressed miRNA are involved in stem cell proliferation, maintenance and control of phase transition in mitosis, maintenance of mitochondrial outer membrane permeability, apoptosis and atrophy. Increased expression of Pax7 involved in muscle precursor (stem) cell proliferation (Fig. 2), caspase 9, caspase 3 (downstream enzymes expressed as an effect of MOMP leading to apoptosis) and fbxo32 (Fig. 3) was observed. All together miRNA study indicates the role of small RNA in regulation of gene expression contributing to available evidences of protein metabolism in E2 exposed skeletal muscle (Fig. 4).

Further no differentially expressed miRNA directly explain decreased expression of MyoD, that helps in differentiation of MPCs. Hence efforts to understand the contributing factors affecting regulation MyoD gene expression were performed by focusing on DNA methylation (Fig. 4). Differential non-CpG methylations were observed between control and E2 samples in exon 1 of MyoD. These methylations were also part of transcription factor binding elements Myc and ERE indicating their role in gene regulation. Moreover, one of the novel differentially expressing miRNA identified regulate expression DNA methyltransferases (DNA methylation enzymes). These observations directed our focus to understand changes in global DNA methylation after 24 hours of E2 treatment. Cytosine methylations were identified in CG, CHG

and CHH contexts at global level but further analysis was carried out by CG methylations. More than 200 genes were identified to be differentially methylated between control and E2 samples. These genes are involved in signal transduction, ion binding, cellular metabolism, cell communication, development of cellular anatomical structures, response to stimuli and enzyme activity (Fig. 5). Some of the interesting genes that are differentially methylated include TATA box-binding -associated factor RNA polymerase I subunit B, pre-mRNA-processing factor 19, 60S ribosomal L19, E3-ubiquitin ligases etc. To better understand the direct effects of DNA methylation on independent genes additional studies are necessary. Such studies would help in gaining knowledge to identify specific role of these genes that are collectively contributing to physiological changes observed in E2 exposed rainbow trout.

FIGURES

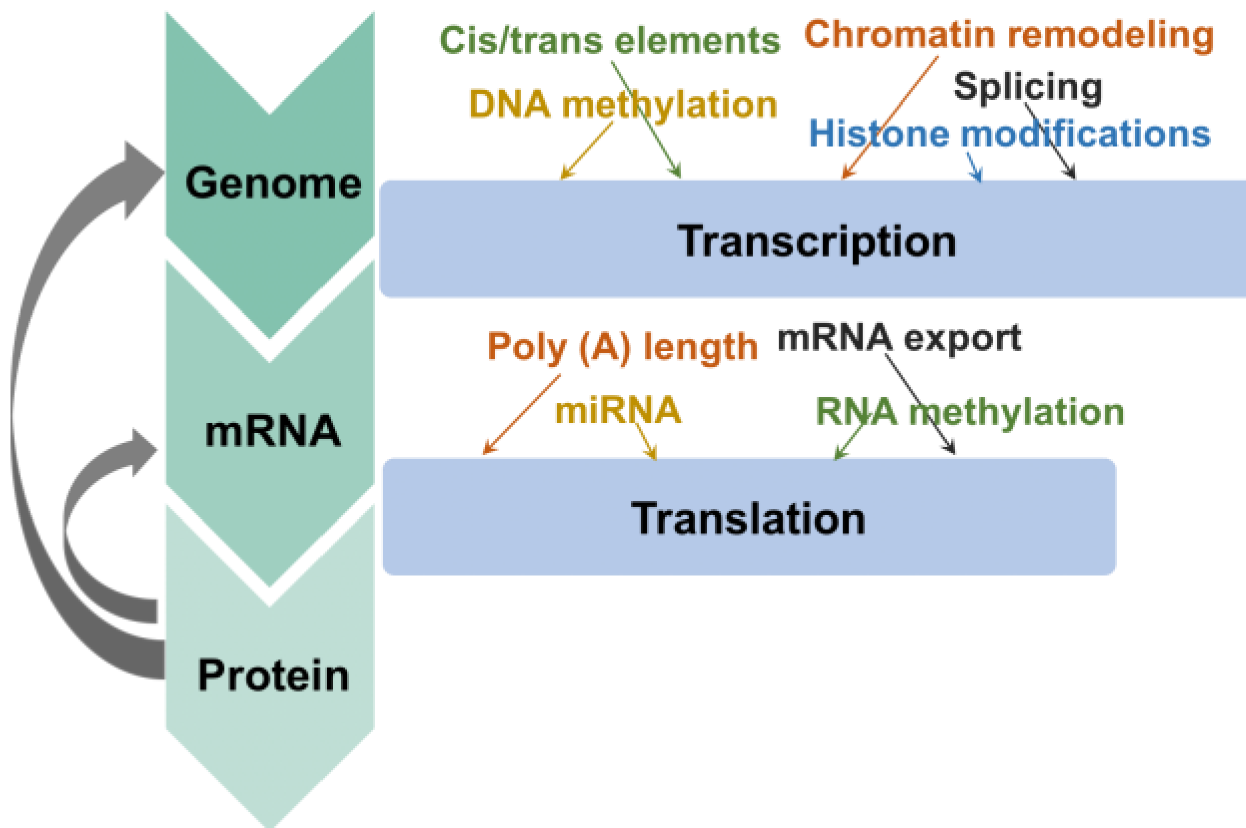

Figure 1. Regulation of gene expression at transcriptional and post-transcriptional levels. Genetic and epigenetic mechanisms regulate gene expression which are independently studies but they function in an interdependent manner.

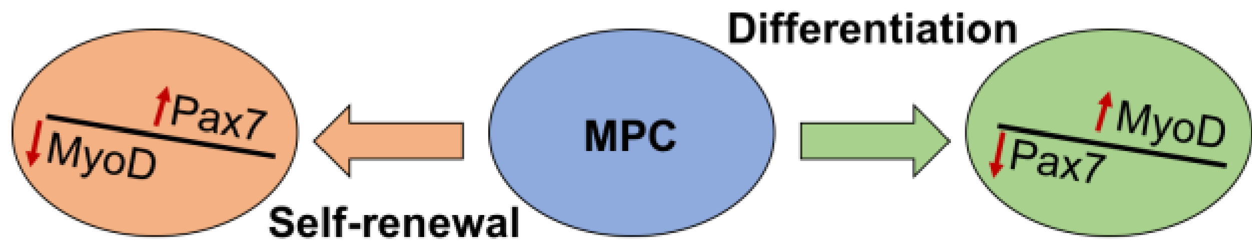

Figure 2. Increase in self renewal of MPCs.

An increase of MyoD and decrease in Pax7 is necessary for MPCs to differentiate further to form a mature muscle fiber. In our study, an increase in Pax7 is observed with decrease in MyoD indicating self-renewal of MPCs without differentiating

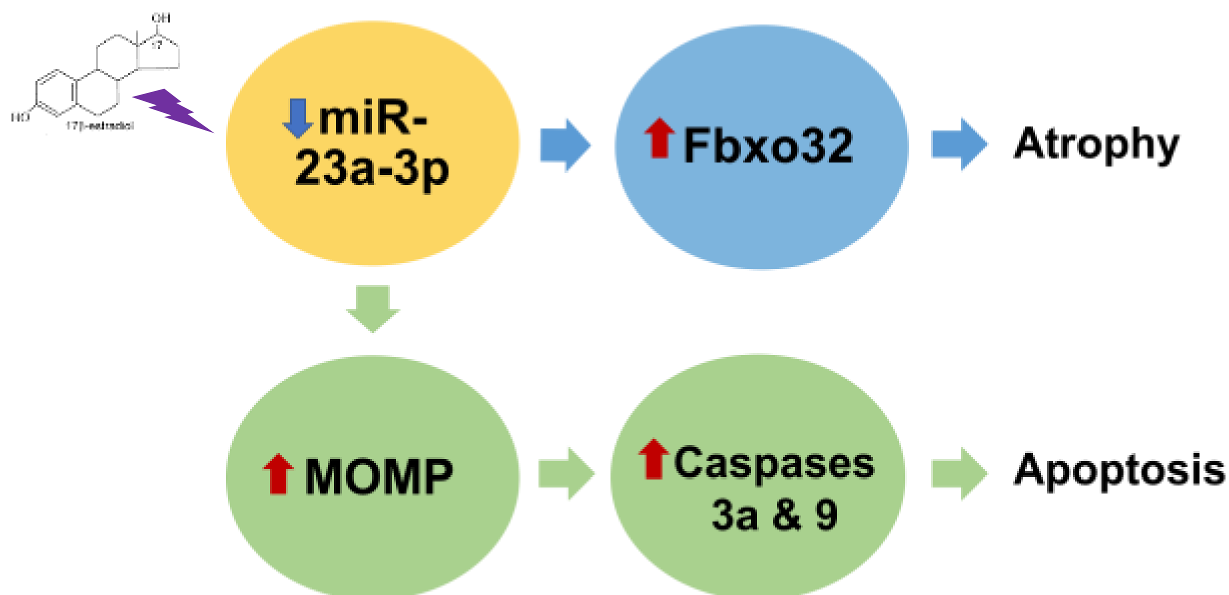

Figure 3. Increase in apoptosis and atrophy.

miR-23a-3p is predicted to regulate the expression of fbxo32 and outer membrane integrity. In the presence of E2 decrease in this miRNA resulted in increased fbxo32 expression and MOMP leading to increased caspases resulting in atrophy and apoptosis

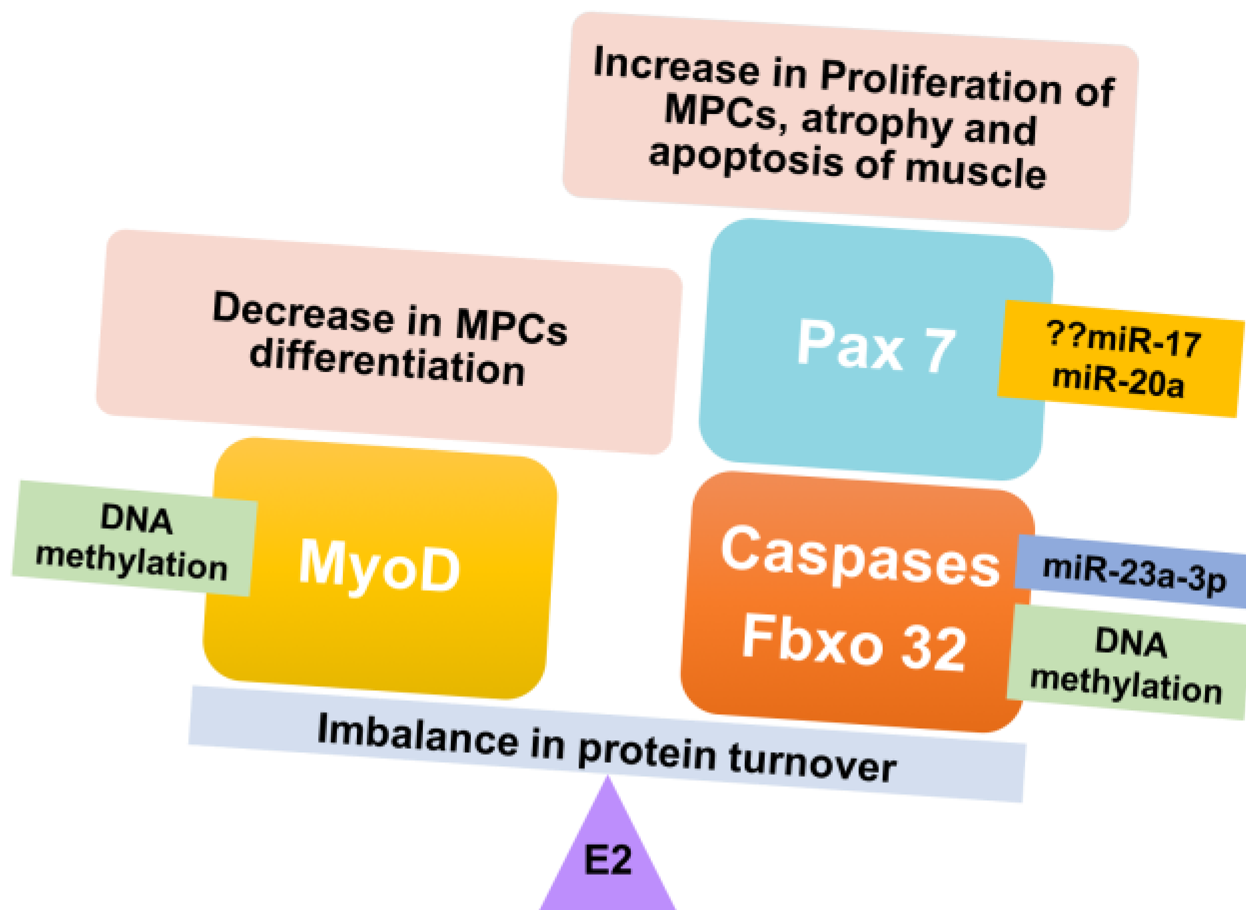

Figure 4. Identified regulatory mechanisms resulting in imbalance in protein turnover under the influence of E2.

Shown are different genes contributing to imbalance and their direct or indirect regulatory mechanisms. No direct evidence for regulation of Pax 7 by miR-17 and 20a cluster was found, these miRNAs are proven to be important in cell cycle contributing to proliferation.

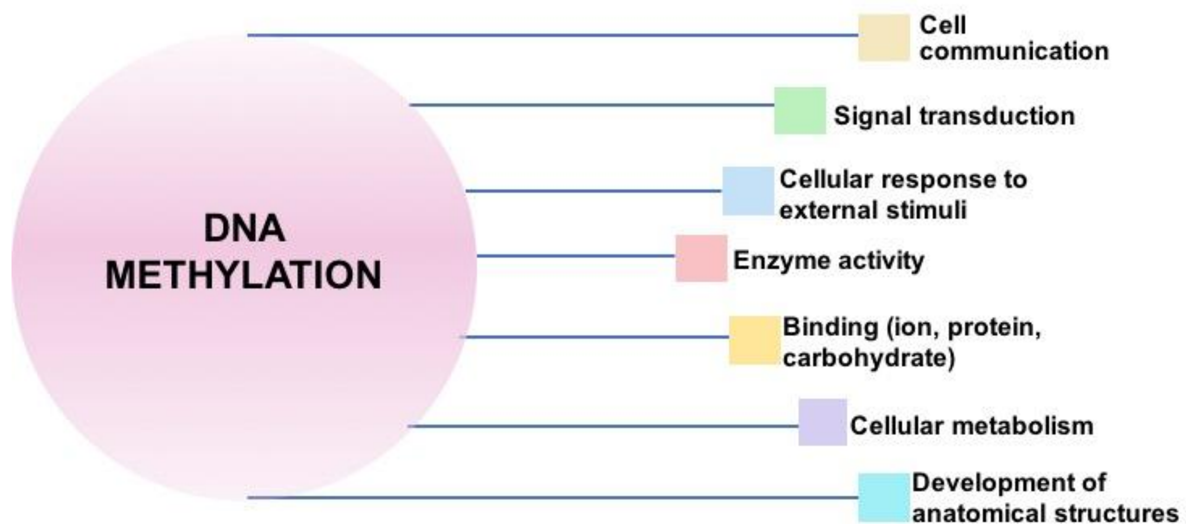

Figure 5. Major mechanisms being regulated by DNA methylation.

PERSPECTIVES

In the past gene expression was believed to be regulated solely by the order of nucleotide bases in a genome and their cis and trans regulatory factors. Recent advancement in the field established the role of additional modifications occurring on the genome, referred to as epigenome. These chemical modifications are inheritable but reversible and include DNA methylation, histone modifications and chromatin remodeling which are influenced by environmental, nutritional, physical and developmental changes. Convincing pieces of evidence are available indicating that these epigenetic modifications alter gene expression. Added to the list of epigenetic modifications are microRNAs and long noncoding RNAs with similar function.

Observations from current study indicates the influence of environmental factors (feed, estrogen) on gene expression though epigenetics and microRNAs. An initial understanding of epigenetic landscape in skeletal muscle was achieved during our study. Increase in MPC proliferation and decrease in differentiation was observed as an influence of E2 on skeletal muscle. MyoD expression in E2 treated samples at 72 hours reached to similar levels of control probably due to loss of methylation and activation of gene. It will be interesting to know if these changes in fish are exceptional due to energy demand during spawning or a signal to address recovery after spawning or both. Increase in degradative pathways support the energy needs to be addressed during these phases. Our experiments detail about muscle exclusively without considering other organs and their interaction during these processes. As muscle is believed to be one of the major available energy sources besides fat during these phases, assessing the expression of these molecular markers in various reproductive phases of fish will help gain further understanding.

Metabolism of various amino acids were differentially regulated in E2 skeletal muscle compared to control. Genes involved in metabolism of amino acids including lysine, phenylalanine methionine, threonine, cysteine, glycine, serine, alanine and tyrosine were differentially methylated. Understanding the role of DNA methylation in regulating these pathways would primarily lead to our understanding in the role of external stimuli on amino acid metabolism. Further its important to also know if supplementing any of these amino acids will be sufficient to address the energy demands by rainbow trout. Some of the above listed amino acids are essential which are to be supplemented by diet. Insufficient supply of those amino acids during vitellogenesis and spawning through feed might lead to protein breakdown to address the amino acid demands.

Overall these reproductive phases cause stressful situations for fish that modify epigenetic landscape. Another area to explore is to see the effects of transgenerational epigenetics in fish. Does any of these epigenetic effects pass over to next generation and if so how are these changes changing the muscle quality? The identified epigenetic markers supporting rapid muscle degradation are not only confined to effects of E2 on skeletal muscle in fish but can also be tested during stress or disease conditions leading to muscle loss. Furthermore, this study also provided understanding of basic epigenetic mechanisms in rainbow trout skeletal muscle development. Teleost fish like rainbow trout is capable of regenerating muscle by both hyperplasty and hypertrophy, unlike mammals. Furthering an investigation of the molecular mechanisms underlying and regulating differences in hypertrophy and hyperplasty will be fascinating. These findings will help explain the inability of increase in muscle fibers number during adult myogenesis in mammals, with possible applications in addressing mammalian muscular diseases and injury.

Availability of advanced and cost-effective techniques are necessary to take complete advantage of epigenetics and close assessment of specific stages to understand these modifications is important. Likewise, the availability of a well annotated genome would help better understand the changes and their importance. This knowledge would also help in designing new feed and feeding strategies. Similarly, assessing the available commercial feed in terms of available protein composition. Determination of these changes from early stages and their effects in development to maturation where heritable changes might affect subsequent generations. It is important to understand added epigenetic effects to genetic effects for more accuracy in selective breeding and genetic improvement. Finally, advances in epigenetic studies may lead to the development of biomarkers to determine diseases, nutritional and environmental changes that control fillet quality.

RESUME

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RESEARCH EXPERIENCE

2012-September 2017:

Graduate Research Assistant at WVU

Efforts to understand the regulation of miRNA and DNA methylation by estradiol in rainbow trout skeletal muscle. Gained research and teaching experience. Published and communicated scientific findings.

2008-2012:

Senior Research Fellow at ICRISAT

Efforts to developed and molecular characterize putative transgenic pulse crops, pigeonpea and chickpeas resistant to pod borer, peanuts for enhanced ß-carotene.

Planned and set up an entomology lab to carry out insect experiments on putative transgenic pigeonpea developed against pod borer

2006-2007:

Research Fellow at ICRISAT

Standardized pigeonpea tissue culture.

Efforts to develop and molecular characterize putative transgenic plants, pigeonpea for enhanced ß-carotene and sorghum resistant to spotted stem borer *Chilo partellus*.

TECHNICAL STRENGTHS

Isolation of myogenic precursor cells from muscle of rainbow trout; Mammalian cell cultures; Luciferase assay; miRNA target gene functional validation; Bacterial cloning and transformation; Microbial culture maintenance; DNA and RNA extraction; cDNA synthesis; Bisulfite conversion and target gene PCR amplification; Real time PCR (mRNA, miRNA); Expression of recombinant proteins using inducible bacterial systems (pET); Western blotting; Spectrophotometric estimation of β-carotene in the seed samples of pigeonpea; Reporter gene expression studies (GUS assay); Statistical software JMP; Packages Deseq2 and EdgeR using R tool; Basic SAS; Agrobacterium mediated transformation and tissue culture of pulse crops; Molecular characterization of putative transgenics for transgene integration and Transcription; Rearing of the insect pest *Helicoverpa armigera.*

TEACHING EXPERIENCE

Teaching Assistant

August 2013-December 2016:

Assisted in organizing Animal Biotechnology lab in Fall semesters since 2013.

August 2016-December 2016:

Worked as a teaching assistant in Department of Biology, WVU. Conducted two lab sessions every week for introductory biology for biology majors (Biology 115).

2004 - 2005:

Lecturer in Biotechnology at Raja Bhadur Venkata Rama Reddy Women's college. Taught genetics to undergraduate students and trained them in basic lab techniques.

EDUCATION

2012 – 2017:

PhD in Genetics and Developmental Biology, West Virginia University, WV (Graduation Date: December 2017)

Dissertation on "Comprehensive Analyses of miRNA and DNA Methylation in 17β-Estradiol Exposed Juvenile Rainbow Trout Skeletal Muscle"

2001-2003:

Master of Science, Biotechnology, Barkatullah University, MP, India

Thesis on "Various tests for standardization and control of Tetanus vaccine (adsorbed) as per Indian pharmacopoeia, British/ European pharmacopoeia and as per the requirements of World Health Organization"

1998-2001:

Bachelor of Science, Microbiology, Biochemistry and Nutrition, Andhra University, AP, India

Thesis on "The effect on different types of oils and fats on lipid profile of atherosclerotic patients"

BIOINFORMATICS TRAINING

2008: 3-day training program on In-silico Analysis and Annotation of ESTs organized by the Bioinformatics Centre, Indian Institute of Spices Research (IISR), Calicut, Kerala.

2016: Bioinformatics workshop conducted by Genomics Core Facility at Health Sciences Library, West Virginia University.

PUBLICATIONS

Wang, J., L. Fu, **P. P. Koganti**, L. Wang, J. M. Hand, H. Ma, J. Yao., 2016, Identification and Functional Prediction of Large Intergenic Noncoding RNAs (lincRNAs) in Rainbow Trout (*Oncorhynchus mykiss*). Marine Biotechnology 18:271-282.

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Fu, L., **P. P. Koganti**, J. Wang, L. Wang, C. L. Wang, J. Yao., 2017, Lhx8 interacts with a novel germ cell-specific nuclear factor containing an Nbl1 domain in rainbow trout (*Oncorhynchus mykiss*). PLOS ONE 12(2): e0170760.

J. Hand, K. Zhang, L. Wang, **P. P. Koganti**, K. Mastrantoni, S. Rajput, M. Ashry, G. W. Smith, J. Yao., 2017, Discovery of a novel oocyte-specific Krüppel-associated box domain-containing zinc finger protein required for early embryogenesis in cattle. Mechanisms of Development: DOI: 10.1016/j.mod.2017.02.003.

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Koganti, P. P., Wang, J, J. Yao, S. Wei and B. Cleveland, 2017 Comprehensive analysis of lncRNAs and mRNAs in skeletal muscle of rainbow trout (Oncorhynchus mykiss) exposed to estradiol. Scientific Reports 7**:** 11780.

CONFERENCE PROCEEDINGS

Presenter (poster), 2009, "Engineering pigeonpea, *Cajanus cajan* for resistance to legume pod borer, *Helicoverpa armigera*", Symposium on Biosafety and Environmental Impact of Genetically Modified Organisms and Conventional Technologies for Pest Management.

Presenter (poster), 2009, "Development and evaluation of the transgenic pigeonpea resistant to legume pod borer *Helicoverpa armigera*", 7th Pacific rim Conference on the Biotechnology of *Bacillus thuringensis* and its environmental impact.

Presenter (poster), 2015, "Differential expression of miRNA in skeletal muscle of rainbow trout under the influence of estrogen", Plant and Animal Genome Conference.

Presenter (Oral), 2015, "Estrogen affects miRNA expression in skeletal muscle of rainbow trout", Nineteenth Annual Davis College Student Research and Creative Scholarship Day.

Presenter (Oral), 2016, "Estrogen treatment results in non-CpG methylation of myogenic transcription factor MyoD in rainbow trout skeletal muscle", Aquaculture.

Presenter (Poster), 2017, "17β-Estradiol increases CpH methylations in first exon of *MyoD* gene in rainbow trout (*Oncorhynchus mykiss*)", Twenty-First Annual Davis College Student Research and Creative Scholarship Day.

Presenter (Poster), 2017, "Identification and Functional Prediction of Bovine Oocyte-Specific Long Non-Coding RNAs", Society for the Study of Reproduction.

AWARDS and MEMBERSHIPS

Awarded **Institutional scholarship** for one year by ICRISAT during 2006-2007.

Awarded **First position** and **Gold medal** for the poster presented at Symposium on Biosafety and Environmental Impact of Genetically Modified Organisms and Conventional Technologies for Pest Management, 2009.

Awarded **Student Travel Grant** from Aquaculture Conference, 2016.

Awarded **WVU Davis College Travel Grant** to attend Aquaculture Meeting, 2016.

Awarded a competitive graduate student enrichment fund award, **CoBank Graduate Student Travel Award Fund** for research and travel, 2017.

Student member of the Epigenetics Society since 2015.

REFERENCES

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