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2015

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UNIVERSITY OF CALIFORNIA

Los Angeles

Epigenetic Mechanisms for Klotho Gene Silencing in Dystrophic Muscle

A thesis submitted in partial satisfaction of the requirements for the degree

Master of Science in Physiological Science

by

Zhenzhi Li

2015

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ABSTRACT OF THE THESIS

Epigenetic Mechanisms for Klotho Gene Silencing in Dystrophic Muscle

by

Zhenzhi Li

Master of Science in Physiological Science

University of California, Los Angeles 2015

Professor James G. Tidball, Chair

Duchenne muscular dystrophy (DMD) is an X-linked disease characterized by muscle membrane damage and muscle wasting that eventually causes death of the afflicted. Klotho is a powerful longevity protein that has been linked to lifespan and kidney function; in recent years, Klotho's role in muscle growth has been reported. Previous findings in our lab showed that Klotho expression is suppressed in DMD patients and mdx mice, an animal model of muscular dystrophy. In this study, we investigate the epigenetic mechanisms that regulate Klotho expression in dystrophic muscle. Our data suggest DNA hypermethylation and histone 3 lysine 9 dimethylation at the Klotho transcriptional start site are involved in the transcriptional suppression under oxidative stress. Furthermore, our data also show disrupted demethylation in oxidatively stressed muscle cells during differentiation that may also contribute to the hypermethylation seen in muscle.

The thesis of Zhenzhi Li is approved.

David William Walker

Kenneth A. Dorshkind

James G. Tidball, Committee Chair

University of California, Los Angeles

2015

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Introduction

Duchenne muscular dystrophy (DMD) is caused by mutations of the dystrophin gene in the X chromosome, leading to loss of the dystrophin protein from the cell membrane. DMD patients have progressive skeletal muscle degeneration and wasting. Mdx mice, with a point mutation of the dystrophin gene, are commonly used as a model to study DMD pathology (Sicinski et al., 1989). In DMD patients and mdx mice, the loss of dystrophin causes a secondary loss of neuronal nitric oxide synthase (nNOS) from muscle, which reduces nitric oxide (NO) production (Chang et al., 1996). The NO deficiency results in many features of the dystrophic pathology such as inflammation and deficiency in regeneration (Rando et al., 1998, Brenman et al., 1995, Grozdanovic et al., 1999, Terrill et al., 2013, Wehling et al., 2001). Because NO scavenges other free radicals, its loss can elevate oxidative stress in skeletal muscle (Tidball and Wehling-Henricks, 2007). The disrupted oxidation balance in dystrophic muscle has been documented to play important roles in dystrophic pathophysiology (Rando et al., 1998, Rando, 2002, Tidball and Wehling-Henricks, 2007). In particular, oxidative stress can activate the pro-inflammatory transcription factor NF- κ B, which facilitates proteolysis and wasting (Powers et al., 2007, Acharyya et al., 2007).

Dystrophin deficiency causes disruption in the expression of numerous genes, in addition to nNOS. Several high throughput studies have documented global transcriptional modifications in dystrophic muscles, including the suppression of genes that are important in muscle function and structure (Boer et al., 2002, Marotta et al., 2009, Porter et al., 2002). Although the mechanisms that underlie the transcriptional dysregulation in dystrophic muscle are largely

unknown, some relate to nNOS deficiency and oxidative stress. NO deficiency in dystrophic muscle leads to global chromatin modifications, which in turn alter the expression of genes that can influence muscle pathology and regeneration (Colussi et al., 2009). In particular, Colussi et al showed NO deficient mdx muscles have modifications in the activating histone code histone 3 lysine 9 acetylation (H3K9ac), the repressive histone code histone 3 lysine 9 dimethylation (H3K9me2) and the activating histone code histone 3 lysine 79 dimethylation (H3K79me2) that regulate the expression of genes such as c-Fos and tumor necrosis factor alpha (TNF-alpha). In fact, oxidative stress influences epigenetic landscape in many disease models (Szumiel et al., 2015, Andrezza et al., 2012). Although epigenetic modifications have been suspected to cause transcriptional modifications in dystrophic muscle, detailed, high-resolution studies to examine epigenetic modifications on single gene level are still lacking. Since gene expression changes are important contributors of dystrophic pathologies, identifying the mechanisms behind these changes is critical for understanding the disease progression and may offer potential avenues for therapeutic approach.

Oxidative stress can also lead to change in DNA methylation patterns by influencing the levels of expression of genes that encode specific methyltransferases or facilitate DNA demethylation. In particular, expression of DNA methyltransferase (DNMT), an enzyme that facilitates cytosine methylation, is increased by oxidative stress (Rubinek et al., 2011, Sun et al., 2012).

Furthermore, ten-eleven Translocation (Tet) family proteins, which facilitate DNA demethylation, can be suppressed by oxidative stress, suggesting another method for oxidative stress to affect DNA methylation (Niu et al., 2015).

Although expression of many genes is influenced by oxidative stress, perturbations of Klotho expression by oxidative stress may be particularly important. Klotho is a single-pass transmembrane protein that is predominantly expressed in the kidney, the choroid plexus of the brain, and at lower levels in skeletal muscle (Kuro-o et al., 1997). Transmembrane Klotho functions as a co-factor for fibroblast growth factor (FGF)-23 signaling pathway, which regulates vitamin D metabolism and phosphate balance (Razzaque, 2009). Klotho can also function as a hormone after post-transcriptional cleavage and release into the circulation where it can suppress insulin growth factor-1 and insulin (Saito et al., 2000, Imura et al., 2004, Matsumura et al., 1998). Klotho was originally identified as an anti-aging protein because the lifespan of Klotho-deficient mice is only 5% of the lifespan of wild-type mice and Klotho mutants demonstrate many pre-mature aging phenotypes. In addition, over-expressing Klotho in mice extends lifespan by 20-30% (Kurosue et al., 2005, Kuro-o et al., 1997).

Data from our laboratory revealed a significant loss of Klotho expression in mdx mice (Figure 1a). This defect may be important in the pathophysiology of muscular dystrophy because Klotho can inhibit the Wnt signaling pathway (Liu et al., 2007) and the Wnt pathway can influence muscle regeneration (Brack et al., 2007). In addition, Klotho deficient mice have a remarkable reduction of skeletal muscle strength, tongue muscle size and weight (Iida et al., 2011, Phelps et al., 2013). Thus, Klotho may play a functional role in muscle physiology, and the loss of Klotho may contribute to muscle wasting that is characteristic of muscular dystrophy.

Because *Klotho* expression is regulated through epigenetic mechanisms that can be influenced by oxidative stress, it may be an important target gene for which dysregulation contributes to the dystrophic pathology. For example, oxidative stress has been shown to cause DNA hypermethylation at the transcriptional start site (TSS) of *Klotho* that suppresses *Klotho* expression in a kidney disease model (Sun et al., 2012). Hypermethylation at *Klotho* TSS also correlates with decreased gene expression in aged brain and cancer models (Xie et al., 2013, King et al., 2012). Inhibition of DNMT is able to restore *Klotho* expression in vivo and in vitro (Rubinek et al., 2012, Sun et al., 2012). In addition to DNA methylation, histone modification is a well-established mechanism to regulate chromatin structure and accessibility that has also been reported to regulate *Klotho* gene expression (Moreno et al., 2011 and Rubinek et al., 2012).

In this investigation, we test the hypothesis that increased oxidative stress contributes to epigenetic changes in the *Klotho* gene that are associated with *Klotho* silencing and that those epigenetic changes also occur in dystrophic muscle. Validation of this hypothesis can provide an important mechanistic link between the increases in oxidative stress in dystrophic muscle and changes in gene expression that contribute to defects in muscle growth and regeneration.

Materials and Methods

Cell culture and menadione treatment

C2C12 myoblasts were seeded on 100 mm x 20 mm cell culture dishes at 250,000 cells per dish. Myoblasts were kept in 10% fetal bovine serum (FBS) in Dulbecco's Modified Eagle Medium (DMEM) containing penicillin and streptomycin at 37°C in an atmosphere of 5% CO₂. Culture medium was changed daily until cells reached 90% confluence. The cells were differentiated overnight in FBS free DMEM containing, 0, 7.5 mM or 15 mM menadione as the source of oxidative stress as previously described (Franco et al., 1998). Menadione was selected to induce oxidative stress because it is a potent supplier of superoxide anion, the primary free radical in the body that induces oxidative stress (Vaziri et al., 2003). On the next day (Day 1), the culture medium was changed into 10% FBS DMEM with appropriate menadione concentration. For the following three days, 10% FBS was changed every 24 hours with appropriate menadione concentration. On Day 4, cells were collected for RNA, methylation and histone analysis.

mRNA analysis

Total RNA was isolated from either the C2C12 cells or quadriceps muscles using the Trizol RNA purification system (Invitrogen). One microgram of total RNA was then reverse transcribed with an oligo(dT) kit (SuperScript II; Invitrogen) and used as a template for quantitative real-time polymerase chain reaction (qRT-PCR) using the a real-time PCR detection system (MyiQ; Bio-Rad) according to manufacturer's specifications. For each mRNA examined, the cycle threshold

ct value ($2^{\text{cycle number}}$) was normalized by the Ct values of the geomean of housekeeping genes HPRT1 and SRP14, and then as a fold change from wild-type mice.

Methylation specific PCR

Primer design:

The methylation-specific and unmethylated-specific primers were designed with MethPrimer software using input sequence -1000 to +900 bp of Klotho gene start codon of mouse genome.

Previous investigators have demonstrated that hypermethylation of the Klotho gene in this region is strongly associated with its suppression (Azuma et al., 2012, Sun et al., 2012).

Genomic DNA isolation and bisulfite conversion:

100 mg of right hamstring muscles from C57 and mdx mice were lysed in 750 ul Tris-EDTA buffer with 100 µl Protease K in 55 °C for 48 hours. The genomic DNA samples were then purified by phenol-chloroform extraction. Genomic DNA samples were subjected to bisulfite conversion in which all the non-methylated cytosine residues were converted into uracil via deamination, whereas methylated cytosine remained as unreacted. The methylation specific primers were designed to be complementary to cytosine on CpG sites whereas unmethylation specific primers were complementary to uracil on CpG sites.

Real-time PCR reaction:

300 ng of bisulfite treated DNA were used for real-time PCR analysis using methylation and unmethylated specific primers. The methylation index was calculated as $2^{(Ct \text{ of input} - Ct \text{ of methylated or unmethylated primers})}$. MSP primer sequences can be found in Table 1.

Targeted bisulfite sequencing

Because prior research showed hypermethylation at Klotho transcriptional start site (TSS) is important in its silencing, we sequenced Ch5: 150952090-150953510 (-627 to +793 of Klotho start codon) (Azuma et al., 2012, Sun et al., 2012). All 116 CpG sites within this region were covered by targeted bisulfite sequencing. The hamstrings from five 3-month-old C57 and mdx mice were digested with 0.5 mg/ml proteinase K overnight at 55°C. Genomic DNA was purified using phenol-chloroform-iso amyl alcohol (25:24:1) before bisulfite treatment and sequencing.

Assay Design, Sample Preparation, and Multiplex Targeted Amplification:

Assays were designed targeting CpG sites in the specified regions of interest (ROI) using primers created with Rosefinch, Zymo Research's proprietary sodium bisulfite converted DNA-specific primer design tool. We chose parameters such that PCR amplicons would be ideally bigger than 100 bp but smaller than 300 bp. In addition, we designed primers such that they avoid annealing to CpG sites at the region of interest to the maximum extent possible. In the event that CpG sites were absolutely necessary for target amplification, we ordered that primers be synthesized with a pyrimidine (C or T) at the CpG cytosine in the forward primer, or a purine (A or G) in the reverse primer to minimize amplification bias to either a methylated or unmethylated allele.

All primers were resuspended or ordered in TE solution at 100 μ M. Primers were then mixed (if necessary) and diluted to 2 μ M each. All primers were then tested using Real-Time PCR with 1 ng of bisulfite-converted control DNA, in duplicate individual reactions. DNA melt analysis was performed to confirm the presence of a specific PCR product. The following guidelines were used to assess performance:

- Had average Cp values <40
- Duplicate Cps do not have a Cp difference >1 (within 5% CV)
- Reached the plateau phase before the run ended at cycle 45
- Produced melting curves in the expected range for PCR products
- Duplicate melts had calculated Tms within 10% CV

Following primer validation, samples were bisulfite converted using the EZ DNA Methylation-Lightning™ Kit (www.zymoresearch.com - catalog number D5030) according to the manufacturer's instructions. Multiplex amplification of all samples using ROI specific primer pairs and the Fluidigm Access Array™ System was performed according to the manufacturer's instructions. The resulting amplicons were pooled for harvesting and subsequent barcoding according to the Fluidigm instrument's guidelines. After barcoding, samples were purified (ZR-96 DNA Clean & Concentrator™ - ZR, Cat#D4023) and then prepared for massively parallel sequencing using a MiSeq V2 300 bp Reagent Kit and paired-end sequencing protocol according to the manufacturer's guidelines.

Targeted Sequence Alignments and Data Analysis:

Sequence reads were identified using standard Illumina base-calling software and then analyzed using a Zymo Research proprietary analysis pipeline, which is written in Python. Low quality nucleotides and adapter sequences were trimmed off during analysis QC. Sequence reads were aligned back to the reference genome using Bismark (<http://www.bioinformatics.babraham.ac.uk/projects/bismark/>), an aligner optimized for bisulfite sequence data and methylation calling (Krueger and Andrews, 2011). Paired-end alignment was used as default thus requiring both read 1 and read 2 be aligned within a certain distance, otherwise both read 1 and read 2 were discarded. Index files were constructed using the *bismark_genome_preparation* command and the entire reference genome. The *non_directional* parameter was applied while running Bismark. All other parameters were set to default. Nucleotides in primers were trimmed off from amplicons during methylation calling. The methylation level of each sampled cytosine was estimated as the number of reads reporting a C, divided by the total number of reads reporting a C or T.

Chromatin immunoprecipitation (ChIP)

Cultured cell collection and chromatin shearing:

C2C12 cells were first trypsinized to detach them from the culture dishes and then fixed in 1.1% formaldehyde for 15 min. The fixed chromatin then subjected to sonication. A Q800R1 sonicator was used to shear fixed chromatin with 20% amp, 15 s on, 15 s off for 24 cycles to reach fragment size of approximately 500 bp.

Skeletal muscle collection and chromatin shearing:

100 mg of muscle were homogenized with a mortar and pestle in the presence of liquid nitrogen. Samples were then transferred to a pre-chilled centrifuge tube and fixed with 1.1% formaldehyde for 15 min. Fixed chromatin was sheared using a Q800R1 sonicator with 40% amp, 15 s on, 30 s off for 26 cycles to reach fragment size of approximately 500 bp.

Immunoprecipitation:

10 μ g of fixed chromatin was incubated with 2 μ g anti-H3K9ac, anti-H3K9me2, anti-H3K79me2, or mouse IgG as negative control overnight. On the next day, Protein A beads (abcam69993) were washed and added to each sample per manufacturer's instructions. The beads-chromatin mix was then washed three times with wash buffer supplied by manufacturer and subjected to decrosslink and DNA purification.

Decrosslinking and DNA purification:

The sample was decrosslinked with 40 μ l of proteinase K for two hours in 55°C and then underwent phenol-chloroform-isoamyl alcohol (25:24:1) purification. DNA was precipitated in 2.6 x volume 100% ethanol and diluted with 23 μ l Tris-EDTA buffer. For an input control, 5 μ g of fixed chromatin were directly decrosslinked and purified with phenol-chloroform-isoamyl alcohol. DNA concentration of the final solution was measured; 50 ng of DNA were used in each PCR reaction.

qPCR and calculation:

11.5 µl of CHIP product or 50 ng of input DNA were added to each PCR well. The primers were designed to specifically target Klotho promoter region ch5: 150952159-150952358 (-558 to -359 of Klotho start codon). The results were calculated by percent input method as described previously (QIGEN, 2011). CHIP-qPCR primer sequences can be found in Table 1.

Statistical Analysis

Bisulfite sequencing data analysis:

R package Biseq was used to detect differential methylated region (DMR) and methylKit package was used to detect differential methylated cytosine (DMC) as described (Akalin et al., 2012, Hebestreit et al., 2013).

mRNA data analysis:

The Students t-test and one-way ANOVA were used.

Results

Klotho is suppressed in mdx muscle and oxidatively stressed cells

Because our previous data (Figure 1a) showed a marked down-regulation of Klotho expression in 4 weeks old mdx muscle, at the stage of the pathology when mdx muscles experience extensive oxidative stress (Disatnik et al., 1998), we tested whether oxidative stress was sufficient to induce Klotho gene silencing. We treated myotubes with menadione, a source of oxidative stress, to investigate whether Klotho expression is suppressed. As shown in Figure 1b, menadione treatment suppresses Klotho expression in myotubes in a dosage depend manner, suggesting oxidative stress indeed causes Klotho gene silencing.

Klotho gene is hypermethylated in mdx muscle during oxidative stress in vivo

Because previous investigators have shown that hypermethylation near the Klotho TSS is associated with Klotho transcriptional silencing (Azuma et al., 2012, Sun et al., 2012), we conducted targeted bisulfite sequencing (TBS) to assay cytosine methylation from -627 to +793 of Klotho start codon. As shown in Figure 2a, among the 116 CpG sites sequenced, the average methylation level of mdx mice is significantly higher than the wild type controls. We also used the Biseq R package to assay for the occurrence of differentially methylated regions (DMR), defined as segments of DNA in which all the CpG sites are significantly hyper or hypomethylated in relative to the control group. Figure 2b shows the DMR, located in Klotho exon 1. Among all 16 CpG sites in this region, mdx mice have significantly higher methylation levels than controls. We extended our investigation to find differentially methylated cytosines (DMC). As shown in Figure 2c, three CpG sites are significantly hypermethylated (meth.diff >

25%; $q < 0.0001$) in mdx mice. Single clustering analysis based on methylation correlation also suggests control and mdx group possess distinct methylation patterns (Figure 2d). Analysis of total methylation levels, methylation correlation, DMR and DMC all support the hypothesis that hypermethylation occurs at the Klotho TSS in mdx mice, which provides an association between Klotho expression level and methylation status in skeletal muscle.

Oxidative stress does not cause increases in Klotho gene methylation in muscle cells in vitro

TBS data linked the Klotho suppression with DNA hypermethylation in dystrophic skeletal muscle. Thus, we further investigated whether oxidative stress increased DNA methylation in vitro using C2C12 cell model. As shown above, menadione-induced oxidative stress is sufficient to suppress Klotho gene expression in myotubes. We used methylation specific PCR (MSP) to exam whether methylation differences can be observed in oxidatively stressed myotubes relative to the no treatment controls. Surprisingly, MSP results show high methylation levels in both control and treatment groups, with no significant difference between the groups observed (Figure 3a). We thus speculated that myotubes in vitro might possess high baseline methylation levels so that any further increase in methylation would be undetectable. Indeed, analysis of TBS data showed that the CpG sites TSS region of Klotho were more than 75% methylated in myoblasts and myotubes but only 6% methylated in muscle tissue from 3-month-old mice (Figures 3b, c). This suggests that epigenetic mechanisms other than DNA methylation can regulate Klotho expression.

Oxidative stress is associated with chromatin remodeling at Klotho transcriptional start site in muscle in vivo and in vitro

Because our in vitro findings showed that oxidative stress reduced Klotho expression without causing a significant increase in DNA methylation, we tested whether other epigenetic modifications of the Klotho gene were associated with its silencing in muscle. We extended our investigation to histone modifications, another well-established mechanism to regulate gene expression via controlling the structure and accessibility of chromosomes. We tested whether histone tail modifications could be involved in Klotho silencing by performing CHIP-qPCR to assay for enrichment of H3K9ac, H3K9me2 and H3K79me2, three histone modifications that have been demonstrated in dystrophic muscle (Colussi et al., 2009). Both oxidatively stressed C2C12 cells and mdx skeletal muscles have higher H3K9me2 enrichment levels relative to the control groups, suggesting repressed transcriptional activity (Figure 4a and 4b). Unlike H3K9me2, no significant changes were observed in H3K9ac and H3K79me2. Since Klotho transcriptional activity is relatively low in skeletal muscle and its progenitors that are not experiencing oxidative stress, changes in activating markers such as H3K9ac and H3K79me2 during oxidative stress may not be detectible.

Oxidative stress is associated with increased expression of DNMT1 and HDAC1 in muscle in vivo and in vitro

Since many previous investigations have shown that oxidative stress induces global epigenetic modifications, we investigated the relationship between oxidative stress and the epigenetic enzymes DNA methyltransferase-1 (DNMT1) and histone deacetylase-1 (HDAC1) which regulate

DNA methylation and histone acetylation, respectively. We observed that expression of DNMT1 and HDAC1 is upregulated in oxidatively stressed cells and dystrophic muscle, compared to controls (Figures 5a-d). Because DNA hypermethylation and histone deacetylation have been associated with transcriptional silencing, elevated DNMT1 and HDAC1 expression indicates that transcriptional activities are suppressed by oxidative stress in muscle.

Oxidative stress is associated with reduced expression of Tet enzymes in muscle in vitro

Because DNA demethylation via Tet family proteins occurs during muscle differentiation (Tsumagari et al., 2013), understanding the expression of Tet family proteins may offer additional insights into methylation regulation under oxidative stress. Tet family proteins are key enzymes that facilitate the transformation of methyl-cytosine to hydroxymethyl-cytosine, which eventually undergoes base excision repair to remove the methyl group (Ito et al., 2011). Thus, we investigated the expression of Tet1, Tet2 and Tet3 in oxidatively stressed myotubes. Figures 6a-c show that Tet2 and Tet3 expression is suppressed by oxidative stress in muscle, suggesting that elevations of DNA methylation in oxidatively stressed muscle progenitor cells may also result from reductions in Tet-mediated demethylation.

Discussion

Our findings establish a novel linkage between DNA hypermethylation and Klotho transcriptional silencing in mdx mice. Among the 116 CpG sites sequenced, we demonstrated an increase in methylation level in mdx muscle. We also found one DNA region and three CpG sites located at the Klotho transcriptional start site with significantly elevated methylation levels in mdx muscle. The methylation status at Klotho TSS has been associated previously with its transcriptional silencing, particularly in kidney disease models (Sun et al., 2012). Although the specific sites and region of differential methylation that we identify differ from those identified in previous research on kidney, the differences may be attributable to cell-lineage specific methylation patterns. Furthermore, despite differences between human and murine Klotho TSS sequence, the link between hypermethylation and transcriptional silencing is preserved. In particular, previous investigators demonstrated that highly elevated methylation levels at the Klotho TSS in human carcinoma cell lines lead to Klotho silencing (Dallol et al., 2015). Collectively, the cross-model and cross-species data support the conclusion that DNA methylation is an important negative regulator for Klotho gene expression.

Increased Klotho DNA methylation is associated with oxidative stress in vivo, but not in vitro

Our data showing that Klotho silencing in mdx mouse muscle occurs at the stage of mdx pathology when the tissue experiences high levels of oxidative stress suggests that oxidative stress may drive Klotho gene silencing. This inference is supported by the work of previous investigators who have demonstrated in other tissues that Klotho gene silencing follows increases in oxidative stress (Sun et al., 2012; Xin et al., 2015). However, we did not detect

changes in Klotho methylation in oxidatively stressed myoblasts or myotubes in vitro, despite the suppression of Klotho expression caused by the stress. We believe that the lack of significant increases in DNA methylation in oxidatively stressed myoblasts and myotubes in vitro is attributable to the extremely high levels of methylation in control muscle cells in vitro; baseline methylation for the 116 CpG sites we sequenced exceeded 75% in vitro. Nevertheless, the findings indicate that general increases in Klotho gene methylation are not sufficient for gene silencing, at least in undifferentiated muscle cells in vitro. Interestingly, previous investigators have shown that there are significant global elevations of CpG site hypermethylation in myoblasts and myotubes in vitro when compared to muscle tissue ex vivo (Tsumagari et al., 2013) and others have shown that forced DNA demethylation of myoblasts in vitro promotes their differentiation in vitro (Hupkes et al., 2011). Those observations suggest that epigenetic regulation of Klotho expression in muscle may vary with the developmental stage of the muscle cells.

Histone modifications in oxidatively stressed cells and mdx muscle

In addition to DNA methylation, histone modifications are important regulators of gene expression. Our data show significant elevation of the histone marker H3K9me2 in our in vivo and in vitro models during increased oxidative stress, while the histone modifications H3K9ac and H3K79me2 remain unchanged. H3K9ac and H3K79me2 are usually enriched in active promoters (Barski et al., 2007, Steger et al., 2008). However, Klotho has a weak promoter lacking a TATA box (Wang et al., 2009) and its expression in muscle lineage cells occurs at a relatively low level (Kuro-o et al., 1997). Thus, H3K9ac and H3K79me2 levels on Klotho would be

expected to be low, even in unstressed cells. In contrast, H3K9me2, a prominent marker for transcriptional repression (Rosenfeld et al., 2009), is significantly enriched in both oxidatively stressed cells and mdx muscles relative to control groups. Taken together, the findings indicate that the weak transcriptional activity of Klotho in muscle cells is further suppressed by repressive histone modification H3K9me2, and not significantly affected by changes in H3K9 acetylation or H3K79 methylation. Our conclusion that dimethylation of H3K9 is an important negative regulator of Klotho expression in dystrophic and oxidatively stressed muscle is consistent with previous reports which showed that oxidative stress can cause H3K9me2 enrichment in other cell type (Tausendschön et al., 2011).

Loss of Klotho amplifies the effects of oxidative stress in cells

Although oxidative stress can cause Klotho gene silencing (Wang et al., 2012; Xin et al., 2015; present investigation), Klotho itself can influence the effects of oxidative stress on cells. For example, neuronal cells experiencing elevated oxidative stress showed increases in DNA methylation and increased apoptosis that could be prevented by DNA methyltransferase inhibitors (Xin et al., 2015). However, when Klotho was knocked down in the stressed cells, the rescue effects of the demethylase inhibitors was diminished, suggesting a role for Klotho in protecting against cell damage caused by oxidative stress (Xin et al., 2015). Whether similar, positive feedback occurs in dystrophic muscle in which there is an increase in oxidative stress and Klotho gene silencing is unknown. If such feedback did occur in muscular dystrophy, then therapeutic restoration of Klotho to the dystrophic muscle could feasibly reduce multiple defects that are attributable to oxidative stress in muscular dystrophy. Perhaps reduction of

muscle cell apoptosis, which is a component of the dystrophic pathology (Tidball et al., 1995), could be an additional benefit of Klotho based therapies in muscular dystrophy.

Oxidative stress may alter the epigenetic landscape of muscle cells

Genome-wide studies of transcriptome activity in mdx mouse muscle clearly show expression of numerous genes that are responsible for muscle structure, regeneration and function are disrupted (Boer et al., 2002, Marotte et al., 2009, Porter et al., 2002). However, the contribution of epigenetic mechanisms to those changes in transcriptome in mdx mice remains largely unknown. Nevertheless, high throughput data obtained in other models link changes in the transcriptome with epigenetic modifications (Brookes and Shi, 2013), which suggest that epigenetic modifications may be similarly responsible for the transcriptional changes in dystrophic muscle. Our data support that expectation and indicate that oxidative stress may drive at least some of those epigenetic modification in gene expression. For example, the increased expression of HDAC and DNMT that we observed in oxidatively stressed cells and mdx muscle may be important because deacetylation and DNA methylation both result in transcriptional repression; up-regulation of these two genes will lead to a shift in epigenetic landscape, which ultimately results in changes in many genes' expression. Furthermore, our TBS data and the work of previous investigators show that myoblasts and myotubes have very high methylation levels, and indicate that demethylation via Tet family proteins is an active process during muscle differentiation (Tsumagari et al., 2013, Carrió et al., 2015). Our data show that upon oxidative stress, muscle cells have suppressed Tet2 and Tet3 expression in vitro. This

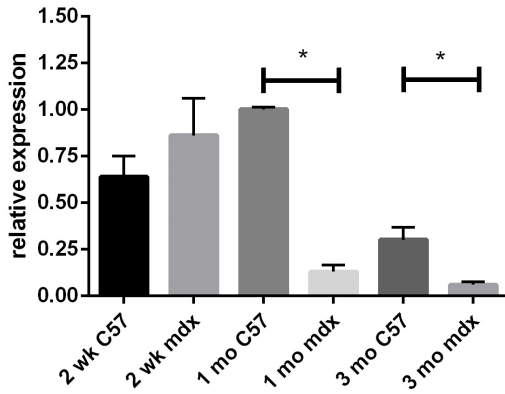
finding suggests that under oxidative stress, the process of demethylation may be disrupted as the expression of Tet family proteins is downregulated.

Our data show that oxidative stress will suppresses Klotho expression via epigenetic mechanisms including DNA hypermethylation and enrichment of H3K9me2 at Klotho TSS. Because Klotho may reduce the effects of oxidative stress on cells, loss of Klotho will worsen the oxidative damage and further influence the epigenetic modifications on large scale by elevating the epigenetic enzyme expression such as DNMT1, HDAC1, and by suppressing expression of Tet family proteins. The expressional changes of those epigenetic enzymes will modify epigenetic landscape and transcriptional activities on the whole genome scale, ultimately contributing to the dystrophic pathology.

Figures and Table

Figure 1.

1a



1b

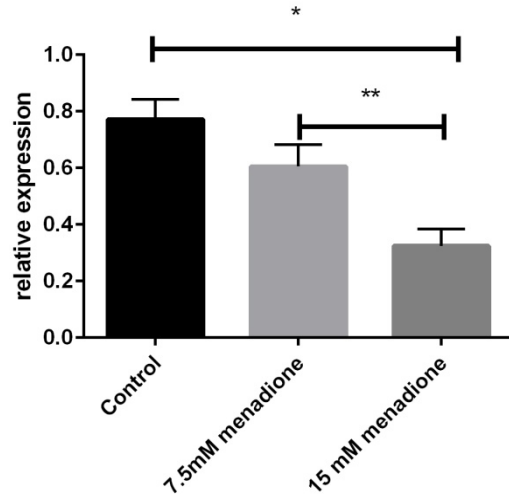
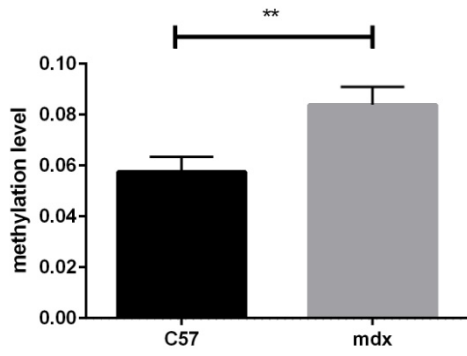
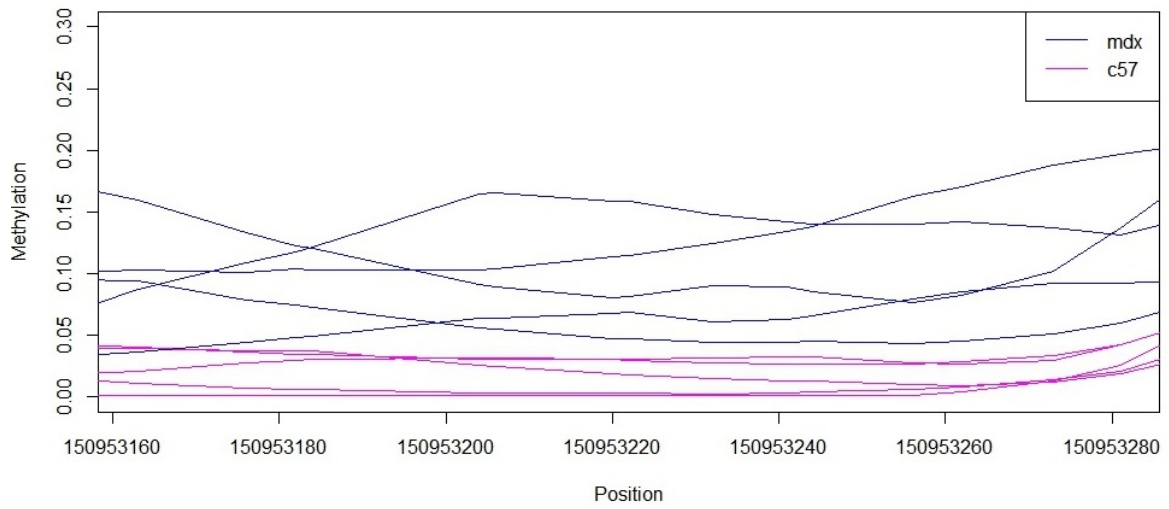


Figure 1. Klotho expression is suppressed in mdx mice and oxidatively stressed muscle cells.
1a. Klotho gene expression in 2-week (2wk), 1-month (1 mo) and 3-month (3 mo) old wild type (C57) and mdx mouse muscle. 1b. Klotho gene expression in C2C12 myotubes under menadione-induced oxidative stress. Myotubes were treated with culture medium only (control), or media supplemented with 7.5 mM or 15 mM menadione for four days. Error bars represent standard error of the mean (S.E.M.).

Figure 2.
2a



2b



2c

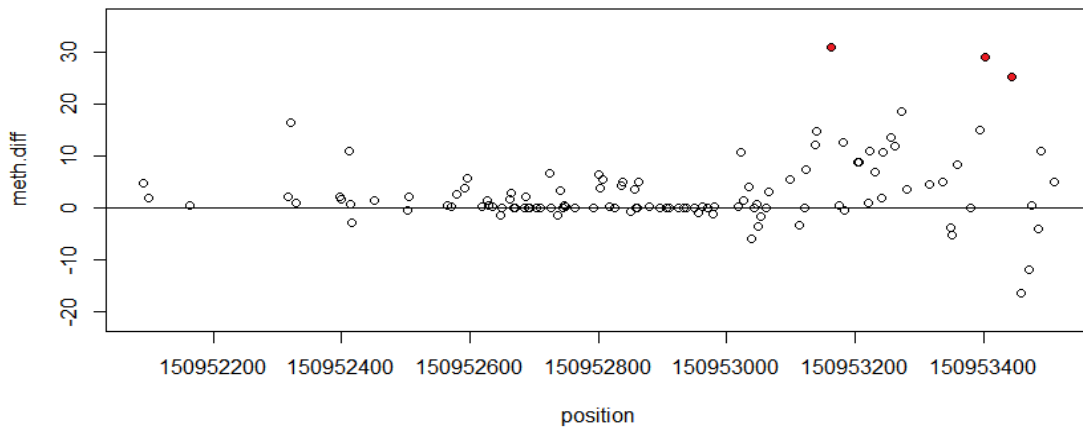


Figure 2. (cont.)
2d

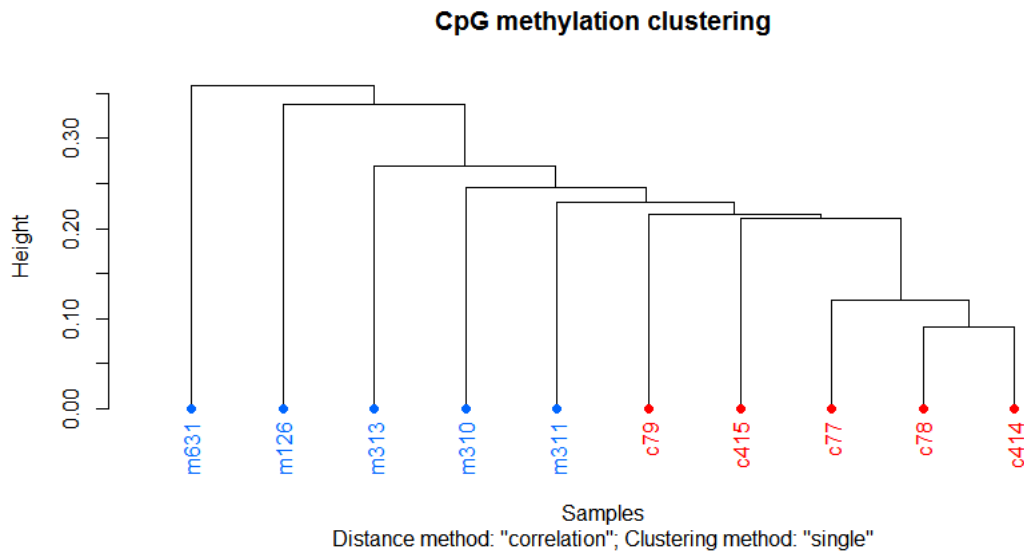


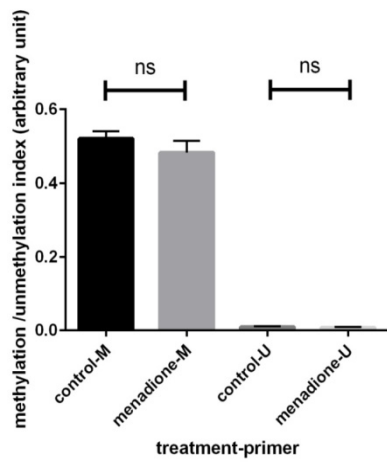
Figure 2. DNA hypermethylation at Klotho transcriptional start site in mdx mice.

2a. The % methylation near Klotho transcription start site (Ch5:150952090-150953510) was determined by comparing the average % methylation across all 116 CpGs for five samples within each group. An overall 0.02633 increase was observed in mdx mice relative to wild type controls (mean \pm S.E.M.; Student's *t*-test, $**p < 0.01$). 2b. Differentially methylated region (DMR) detected by Biseq package. The lines represent methylation level at each CpG site within the DMR in mdx (black) or wild-type (pink) samples. The DMR is located on chr5 [150953163, 150953281]; 16 CpG sites are located within this region. The median methylation level of C57 control group in this region is 0.01942, whereas the mdx group has median methylation of 0.09686 in this region. 2c. Differentially methylated cytosine (DMC). X-axis shows the position of CpG sites, Y-axis shows the average methylation difference between mdx and C57. Positive methylation difference means higher methylation in mdx whereas negative means higher methylation in C57 control samples. Three DMCs are detected using methylKit package. The locations of DMCs are ch5: 150953163, 150953402, 150953444. Three DMCs are colored in red. 2d. CpG methylation clustering analysis by single clustering method to group the controls and mdx mice based on the methylation pattern correlation. Red color represents the control group (C57), blue color represents mdx mice.

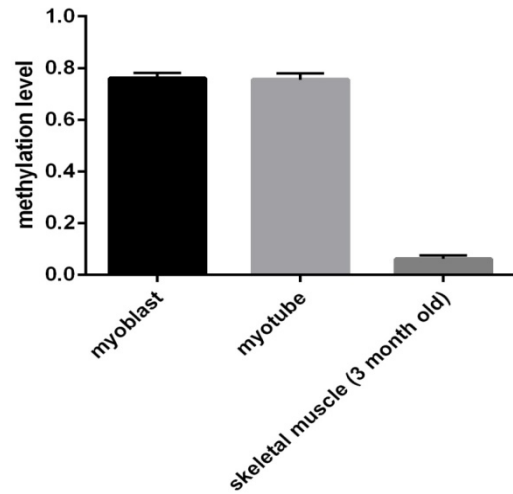
Figure 3.

3a

Klotho gene methylation in menadione treated cells



3b



3c

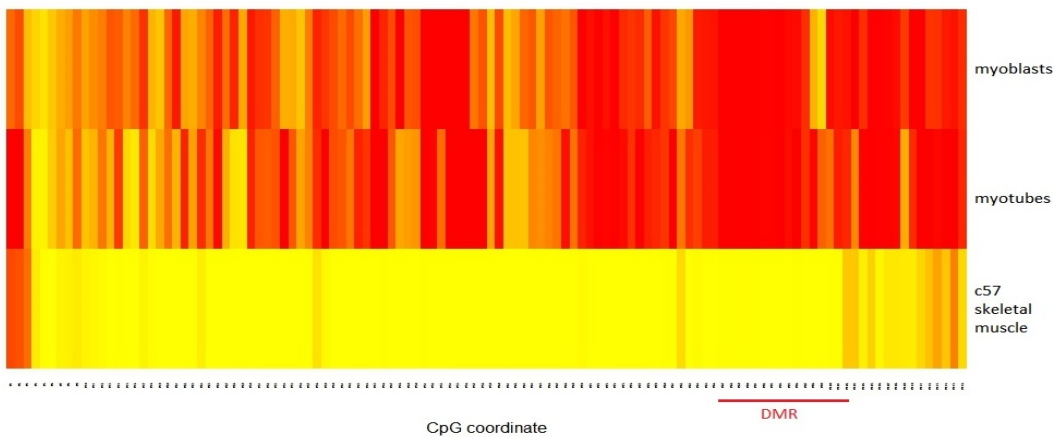
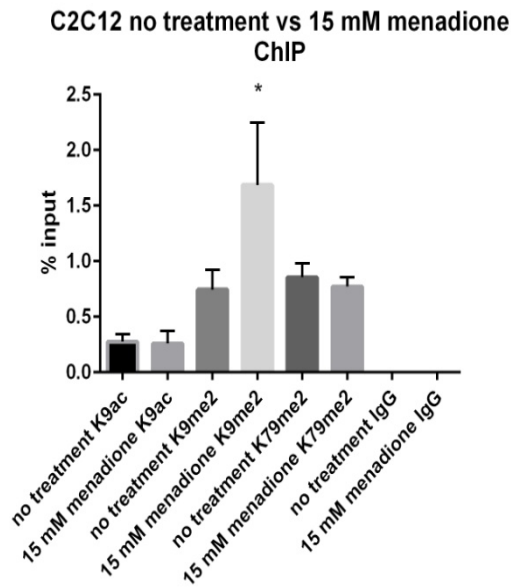


Figure 3. Hypermethylation in muscle progenitor cells.

3a. The methylation index of menadione-treated myotubes. Each bar represents methylation/unmethylation index of control/menadione treated cells. No significant methylation change occurred. M: methylation specific primer; U: unmethylation specific primer. The unit of methylation/unmethylation is arbitrary and should not be compared with methylation level from other figures. 3b. Bar graph representation of average methylation level of 116 CpG sites sequenced. Myoblast, myotube and 3 month old C57 skeletal muscle data are shown with the average methylation level of 0.7522, 0.7611 and 0.0614, respectively. Error bar represents S.E.M. 3c. Heatmap representation of the methylation levels of myoblasts, myotubes and C57 skeletal muscle at Ch5:150952090-150953510. 116 CpG sites are shown for each sample. Yellow color represents 0 methylation whereas red means 100% methylation. On the CpG coordinate, the red line indicates the DMR detected in C57 vs mdx comparison.

Figure 4.
4a



4b

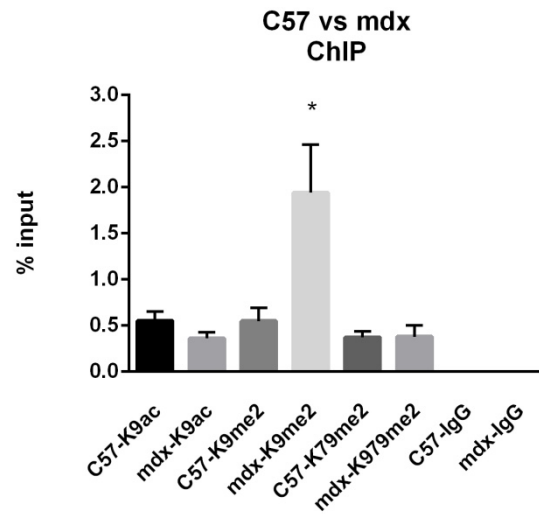
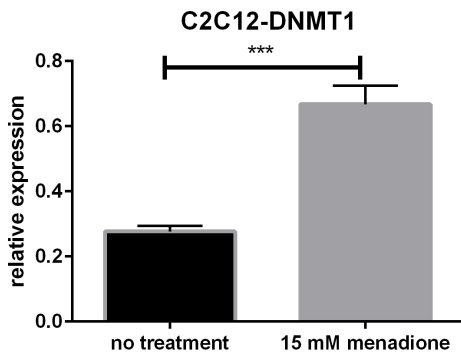


Figure 4. Histone modifications in mdx muscle and oxidatively stressed muscle cells.

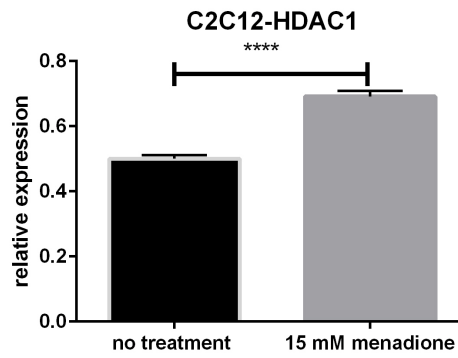
4a. The repressive histone mark H3K9me2 is enriched upon menadione treatment whereas activating H3K9ac and H3K79me2 remain unchanged in the Klotho promoter region. 4b. The repressive histone mark H3K9me2 is enriched in mdx muscle whereas activating H3K9ac and H3K79me2 remain unchanged in the Klotho promoter region. *p<0.05.

Figure 5.

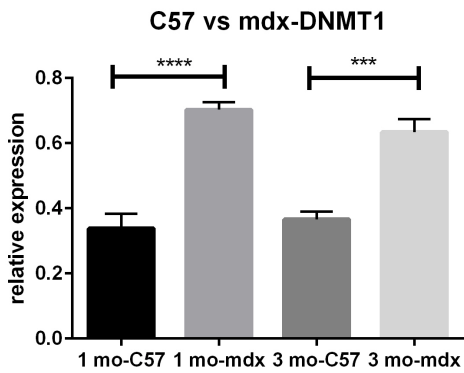
5a



5b



5c



5d

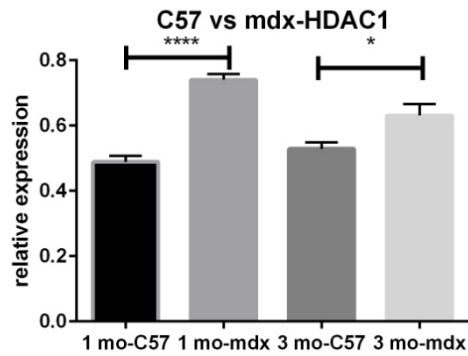
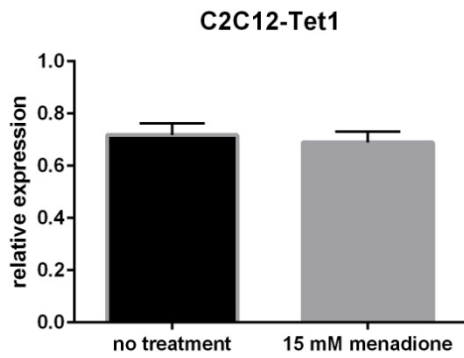


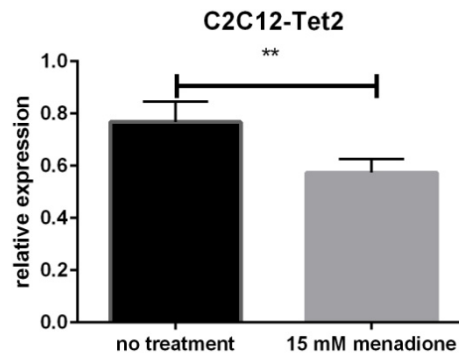
Figure 5. DNMT1 and HDAC1 expression are suppressed in mdx muscle and oxidatively stressed cells. 5a-b. mRNA expression of HDAC1 and DNMT1 in no treatment and menadione-treated myotubes. *** $p < 0.001$, **** $p < 0.0001$. 5c-d. mRNA expression of HDAC1 and DNMT1 in one and three month old C57 and mdx mice.

Figure 6.

6a



6b



6c

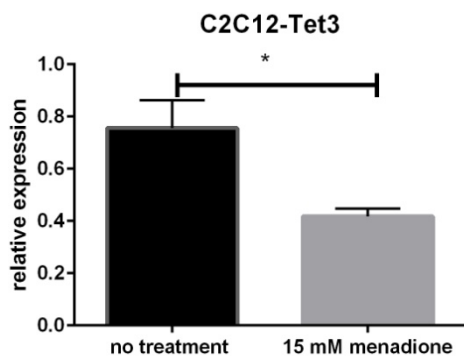


Figure 6. Tet family genes are suppressed in oxidatively stressed muscle cells in vitro. 6a-c. mRNA expression of Tet family genes Tet1, Tet2, and Tet3 in control and menadione-treated myotubes, (mean \pm S.E.M.; Student's *t*-test, **p* < 0.05, ***p* < 0.01).

Table 1. Primer sequences for methylation specific PCR and CHIP-qPCR

Methylation specific primer (MSP)	F: CGT TGT TTG AGC GTT GAG TC
	R: AAA CCG TCG AAA AAA ATA TCG TA
Unmethylation specific primer (MSP)	F: TTT GTT GTT TGA GTG TTG AGT TG
	R: AAA AAC CAT CAA AAA AAA TAT CATA
Input control primer (MSP)	F: TAG TTT TAG GAA GGT AAA GGG AGT G
	R: AAA TAC CCA AAA AAA ACA CAA CAA A
CHIP qPCR primer	F: AAA CCT CGC AAA GTT CCA CC
	R: CAG AAA CAG CTG CCC AAC TT

References

- Acharyya S, Villalta SA, Bakkar N, Bupha-Intr T, Janssen PM, Carathers M, Li ZW, Beg AA, Ghosh S, Sahenk Z, Weinstein M, Gardner KL, Rafael-Fortney JA, Karin M, Tidball JG, Baldwin AS, Guttridge DC. Interplay of IKK/NF-kappaB signaling in macrophages and myofibers promotes muscle degeneration in Duchenne muscular dystrophy. *J Clin Invest.* 2007; 117(4):889-901.
- Akalin A, Kormaksson M, Li S, Garrett-Bakelman FE, Figueroa ME, Melnick A, Mason CE. methylKit: a comprehensive R package for the analysis of genome-wide DNA methylation profiles. *Genome Biol.* 2012; 13(10):R87.
- Andreazza AC. Combining redox-proteomics and epigenomics to explain the involvement of oxidative stress in psychiatric disorders. *Mol Biosyst.* 2012;8(10):2503-12.
- Azuma M, Koyama D, Kikuchi J, Yoshizawa H, Thasinas D, Shiizaki K, Kuro-o M, Furukawa Y, Kusano E. Promoter methylation confers kidney-specific expression of the Klotho gene. *FASEB J.* 2012; 26(10):4264-74.
- Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K. High-resolution profiling of histone methylations in the human genome. *Cell.* 2007; 129(4):823-37.
- Boer JM, De meijer EJ, Mank EM, Van Ommen GB, Den Dunnen JT. Expression profiling in stably regenerating skeletal muscle of dystrophin-deficient mdx mice. *Neuromuscul Disord.* 2002; 12 Suppl 1:S118-24.

- Brack AS, Conboy MJ, Roy S, Lee M, Kuo CJ, Keller C, Rando TA. Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis. *Science*. 2007; 317(5839):807-10.
- Brenman JE, Chao DS, Xia H, Aldape K, Brettschneider DS. Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy. *Cell*. 1995; 82(5):743-52.
- Brookes E, Shi Y. Diverse epigenetic mechanisms of human disease. *Annu Rev Genet*. 2014; 48:237-68.
- Carrió E, Díez-Villanueva A, Lois S, Mallona I, Cases I, Forn M, Peinado MA, Suelves M. Deconstruction of DNA methylation patterns during myogenesis reveals specific epigenetic events in the establishment of the skeletal muscle lineage. *Stem Cells*. 2015; 33(6):2025-36.
- Chang WJ, Iannaccone ST, Lau KS, Masters BS, McCabe TJ, McMillan K, Padre RC, Spencer MJ, Tidball JG, Stull JT. Neuronal nitric oxide synthase and dystrophin-deficient muscular dystrophy. *Proc Natl Acad Sci USA*. 1996; 93(17):9142-7.
- Colussi C, Gurtner A, Rosati J, Illi B, Ragone G, Piaggio G, Moggio M, Lamperti C, D'Angelo G, Clementi E, Minetti G, Mozzetta C, Antonini A, Capogrossi MC, Puri PL, Gaetano C. Nitric oxide deficiency determines global chromatin changes in Duchenne muscular dystrophy. *FASEB J*. 2009; 23(7):2131-41.
- Dallol A, Buhmeida A, Merdad A, Al-Maghrabi J, Gari MA, Abu-Elmagd MM, Elaimi A, Assidi M, Chaudhary AG, Abuzenadah AM, Nedjadi T, Ermiah E Alkhayyat SS, Al-Qahtani MH.

- Frequent methylation of the KLOTHO gene and overexpression of the FGFR4 receptor in invasive ductal carcinoma of the breast. *Tumour Biol.* 2015.
- Disatnik MH, Dhawan J, Yu Y, Beal MF, Whirl MM, Franco AA, Rando TA. Evidence of oxidative stress in mdx mouse muscle: studies of the pre-necrotic state. *J Neurol Sci.* 1998; 161(1):77–84.
- Franco AA, Odom RS, Rando TA. Regulation of antioxidant enzyme gene expression in response to oxidative stress and during differentiation of mouse skeletal muscle. *Free Radic Biol Med.* 1999; 27(9-10):1122-32.
- Grozdanovic Z, Baumgarten HG. Nitric oxide synthase in skeletal muscle fibers: a signaling component of the dystrophin-glycoprotein complex. *Histol Histopathol.* 1999; 14(1):243-56.
- Hebestreit K, Dugas M, Klein HU. Detection of significantly differentially methylated regions in targeted bisulfite sequencing data. *Bioinformatics.* 2013; 29(13):1647-53.
- Hupkes M, Jonsson MK, Scheenen WJ, van Rotterdam W, Sotoca AM, van Someren EP, van der Heyden MA, van Veen TA, van Ravestein-van Os RI, Bauerschmidt S, Piek E, Ypey DL, van Zoelen EJ, Dechering KJ. Epigenetics: DNA demethylation promotes skeletal myotube maturation. *FASEB J.* 2011;25(11):3861-72
- Iida RH, Kanko S, Suga T, Morito M, Yamane A. Autophagic-lysosomal pathway functions in the masseter and tongue muscles in the klotho mouse, a mouse model for aging. *Mol Cell Biochem.* 2011; 348(1-2):89-98.

- Imura A, Iwano A, Tohyama O, Tsuji Y, Nozaki K, Hashimoto N, Fujimori T, Nabeshima Y.
Secreted Klotho protein in sera and CSF: implication for post-translational cleavage in release of Klotho protein from cell membrane. *FEBS Lett.* 2004; 565(1-3):143-7.
- Ito S, Shen L, Dai Q, Wu SC, Collins LB, Swenberg JA, He C, Zhang Y. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science.* 2011; 333(6047):1300-3.
- King GD, Rosene DL, Abraham CR. Promoter methylation and age-related downregulation of Klotho in rhesus monkey. *Age (Dordr).* 2012; 34(6):1405-19.
- Krueger F, Andrews SR. Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. *Bioinformatics.* 2011; 27(11):1571-2.
- Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T, Ohyama Y, Kurabayashi M, Kaname T, Kume E, Iwasaki H, Iida A, Shiraki-Iida T, Nishikawa S, Nagai R, Nabeshima YI. Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature.* 1997; 390(6655):45-51.
- Kurosu H, Yamamoto M, Clark JD, Pastor JV, Nandi A, Gurnani P, McGuinness OP, Chikuda H, Yamaguchi M, Kawaguchi H, Shimomura I, Takayama Y, Herz J, Kahn CR, Rosenblatt KP, Kuro-o M. Suppression of aging in mice by the hormone Klotho. *Science.* 2005; 309(5742):1829-33.
- Liu H, Fergusson MM, Castilho RM, Liu J, Cao L, Chen J, Malide D, Rovira II, Schimel D, Kuo CJ, Gutkind JS, Hwang PM, Finkel T. Augmented Wnt signaling in a mammalian model of accelerated aging. *Science.* 2007; 317(5839):803-6.

- Marotta M, Ruiz-Roig C, Sarria Y, Peiro JL, Nuñez F, Ceron J, Munell F, Roig-Quilis M. Muscle genome-wide expression profiling during disease evolution in mdx mice. *Physiol Genomics*. 2009; 37(2):119-32.
- Matsumura Y, Aizawa H, Shiraki-iida T, Nagai R, Kuro-o M, Nabeshima Y. Identification of the human klotho gene and its two transcripts encoding membrane and secreted klotho protein. *Biochem Biophys Res Commun*. 1998; 242(3):626-30.
- Moreno JA, Izquierdo MC, Sanchez-Niño MD, Suárez-Alvarez B, Lopez-Larrea C, Jakubowski A, Blanco J, Ramirez R, Selgas R, Ruiz-Ortega M, Egido J, Ortiz A, SanzAB. The inflammatory cytokines TWEAK and TNF α reduce renal Klotho expression through NF κ B. *J Am SocNephrol*. 2011; 22(7):1315-25.
- Niu Y, Desmarais TL, Tong Z, Yao Y, Costa M. Oxidative stress alters global histone modification and DNA methylation. *Free Radic Biol Med*. 2015; 82:22-8.
- Phelps M, Pettan-brewer C, Ladiges W, Yablonka-Reuveni Z. Decline in muscle strength and running endurance in klotho deficient C57BL/6 mice. *Biogerontology*. 2013; 14(6):729-39.
- Porter JD, Khanna S, Kaminski HJ, Rao JS, Merriam AP, Richmonds CR, Leahy P, Li J, Guo W, Andrade FH. A chronic inflammatory response dominates the skeletal muscle molecular signature in dystrophin-deficient mdx mice. *Hum Mol Genet*. 2002; 11(3):263-72.
- Powers SK, Kavazis AN, McClung JM. Oxidative stress and disuse muscle atrophy. *J Appl Physiol*. 2007; 102(6):2389–2397.
- QIGEN. EpiTect[®] ChIP qPCR Array Handbook. 2011; P15-16

- Rando TA. Oxidative stress and the pathogenesis of muscular dystrophies. *Am J Phys Med Rehabil.* 2002; 81(11 Suppl):S175–S186.
- Rando TA, Disatnik MH, Yu Y, Franco A. Muscle cells from mdx mice have an increased susceptibility to oxidative stress. *Neuromuscul Disord.* 1998; 8(1):14-21.
- Razzaque MS. FGF23-mediated regulation of systemic phosphate homeostasis: is Klotho an essential player? *Am J Physiol Renal Physiol.* 2009; 296(3):F470-6.
- Rosenfeld JA, Wang Z, Schones DE, Zhao K, Desalle R, Zhang MQ. Determination of enriched histone modifications in non-genic portions of the human genome. *BMC Genomics.* 2009; 10:143.
- Rubinek T, Shulman M, Israeli S, Bose S, Avraham A, Zundeleovich A, Evron E, Gal-Yam EN, Kaufman B, Wolf I. Epigenetic silencing of the tumor suppressor klotho in human breast cancer. *Breast Cancer Res Treat.* 2012;133(2): 649-57.
- Saito Y, Nakamura T, Ohyama Y, Suzuki T, Iida A, Shiraki-Iida T, Kuro-o M, Nabeshima Y, Kurabayashi M, Nagai R. In vivo klotho gene delivery protects against endothelial dysfunction in multiple risk factor syndrome. *Biochem Biophys Res Commun.* 2000;276(2): 767-72.
- Sicinski P, Geng Y, Ryder-cook AS, Barnard EA, Darlison MG, Barnard PJ. The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. *Science.* 1989; 244(4912):1578-80.
- Steger DJ, Lefterova MI, Ying L, Stonestrom AJ, Schupp M, Zhuo D, Vakoc AL, Kim JE, Chen J, Lazar MA, Blobel GA, Vakoc CR. DOT1L/KMT4 recruitment and H3K79 methylation are

- ubiquitously coupled with gene transcription in mammalian cells. *Mol Cell Biol.* 2008; 28(8):2825-39.
- Sun CY, Chang SC, Wu MS. Suppression of Klotho expression by protein-bound uremic toxins is associated with increased DNA methyltransferase expression and DNA hypermethylation. *Kidney Int.* 2012; 81(7):640-50.
- Szumiel I. Ionizing radiation-induced oxidative stress, epigenetic changes and genomic instability: the pivotal role of mitochondria. *Int J Radiat Biol.* 2015; 91(1):1-12.
- Tausendschön M, Dehne N, Brüne B. Hypoxia causes epigenetic gene regulation in macrophages by attenuating Jumonji histone demethylase activity. *Cytokine.* 2011; 53(2):256-62.
- Terrill JR, Radley-crabb HG, Iwasaki T, Lemckert FA, Arthur PG, Grounds MD. Oxidative stress and pathology in muscular dystrophies: focus on protein thiol oxidation and dysferlinopathies. *FEBS J.* 2013; 280(17):4149-64.
- Tidball JG, Albrecht DE, Lokensgard BE, Spencer MJ. Apoptosis precedes necrosis of dystrophin-deficient muscle. *J Cell Sci.* 1995;108 (Pt 6):2197-204.
- Tidball JG, Wehling-Henricks M. The role of free radicals in the pathophysiology of muscular dystrophy. *J Appl Physiol.* 2007; 102(4):1677–1686.
- Tsumagari K, Baribault C, Terragni J, Varley KE, Gertz J, Pradhan S, Badoo M, Crain CM, Song L, Crawford GE, Myers RM, Lacey M, Ehrlich M. Early de novo DNA methylation and prolonged demethylation in the muscle lineage. *Epigenetics.* 2013; 8(3):317-32.

- Vaziri ND, Dicus M, Ho ND, Boroujerdi-Rad L, Sindhu RK. Oxidative stress and dysregulation of superoxide dismutase and NADPH oxidase in renal insufficiency. *Kidney Int.* 2003; 63(1):179-85.
- Wei D, Loeken MR. Increased DNA methyltransferase 3b (Dnmt3b)-mediated CpG island methylation stimulated by oxidative stress inhibits expression of a gene required for neural tube and neural crest development in diabetic pregnancy. *Diabetes.* 2014; 63(10):3512-22.
- Xie B, Zhou J, Yuan L, Ren F, Liu DC, Li Q, Shu G. Epigenetic silencing of Klotho expression correlates with poor prognosis of human hepatocellular carcinoma. *Hum Pathol.* 2013; 44(5):795-801.
- Wang Y, Kuro-o M, Sun Z. Klotho gene delivery suppresses Nox2 expression and attenuates oxidative stress in rat aortic smooth muscle cells via the cAMP-PKA pathway. *Aging Cell.* 2012; 11(3):410-7.
- Wang Y, Sun Z. Current understanding of Klotho. *Ageing Res Rev.* 2009; 8:(1)43-51.
- Wehling M, Spencer MJ, Tidball JG. A nitric oxide synthase transgene ameliorates muscular dystrophy in mdx mice. *J Cell Biol.* 2001; 155(1):123-31.