THE ROLE OF HOTAIR IN THE MODULATION OF SKELETAL MUSCLE MYOGENESIS

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Abstract

THE ROLE OF HOTAIR IN THE MODULATION OF SKELETAL MUSCLE MYOGENESIS

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Skeletal muscle function is a vital requirement for an enriched healthy life. For years the molecular processes governing myogenesis and the development of skeletal muscle were primarily associated with proteins. In recent years, however, non-coding RNAs including microRNAs (miRNAs) and long non coding RNAs (lncRNAs) have emerged as integral components of gene regulation that take part in cellular differentiation, development and disease. *HOTAIR* (HOX antisense intergenic RNA) is a long non-coding RNA (lncRNA) that is transcribed from the antisense strand of the homeobox C (HOXC) gene locus present on chromosome 12. *HOTAIR* has been shown to have a regulatory role in gene silencing as it coordinates with chromatin-modifying enzymes. Although there is a substantial amount of data suggesting important functions of *HOTAIR* in several human and mouse cells and tissues, very little knowledge exists about *HOTAIR* functions in skeletal muscle. To explore the impact of *HOTAIR* on skeletal muscle cell growth and development, we knockdown *HOTAIR* in mouse skeletal muscle cells and analyzed the impact on cell proliferation and differentiation. *HOTAIR* expression was reduced with a synthetically designed *HOTAIR* antisense DNA and siRNA in separate experiments. To investigate the underlying effect of *HOTAIR* in C2C12 cells, a series of gene expression, gene array, immunostaining and calcium imaging analyses

were employed during cell differentiation. Partial knockdown of *HOTAIR* in C2C12 cells resulted in fewer and smaller myotubes as confirmed by immunostaining at days 3 and 4 of differentiation with Myosin Heavy Chain (MHC) which only stains myocytes and myotubes but not myoblasts. RT-qPCR revealed a dysregulation of myogenic genes expression that is reflective of abnormal myogenesis. Intracellular calcium (Ca^{2+}) measurements of the siRNA-treated C2C12 cells showed a decrease in maximum amplitude peak response to caffeine, reduced peak level and shorten relaxation phase, suggesting that less Ca^{2+} is available for release and re-uptake due to the partial silencing of *HOTAIR*, correlating with impaired myogenesis. To investigate the molecular signaling machinery induced by partial *HOTAIR* knockdown we used a real-time PCR gene array to monitor the activity of 10 signaling pathways. We discovered that *HOTAIR* knockdown modulated the NFκB and Oxidative Stress pathways (*Ccl5* and *Nqo1*). These results suggest that *HOTAIR* may exert its function on skeletal muscle via the regulation of the NFκB and Oxidative Stress pathways which are critical for skeletal muscle homeostasis. These studies provide the basis for in-depth studies of *HOTAIR* function in skeletal muscle by extending its exploration to primary murine and human models as well as animal models.

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Chapter 1

INTRODUCTION

1.1 AN OVERVIEW ON SKELETAL MUSCLE FUNCTION

Skeletal muscles are dynamic integrated tissues of the musculoskeletal system. As implied by the name, skeletal muscles play a vital role in positioning and moving the skeleton. They are involved in voluntary movement, execution of facial expressions, postural support and respiration.[1] Among their role in the musculoskeletal system, skeletal muscles also have interdependent links to other systems in the body including the nervous system and cardiovascular system.[2] Skeletal muscles attach to the bone through a mobile tendon-fascia connection. The flexible attachment facilitates the movement of the bone upon contraction of the muscle. Historically, the relationship between bone and skeletal muscle has been viewed as strictly mechanical in the sense that the bone provides the attachment site for muscle and muscle primarily loads bone.[3] However, recent investigations suggest that in addition to their mechanical involvement, skeletal muscle and bone engage in biochemical crosstalk in which the bone and skeletal muscle secrete factors that cross-regulate the growth and function of the other tissue.[4] In order to fully comprehend changing perspectives of the role of skeletal muscle, the development and function of skeletal muscle must be closely examined.

The theoretical framework of our thesis is skeletal muscle myogenesis, which is the process in which skeletal muscle fibers develop from myogenic progenitor cells (See Fig. 1-1 accompanying movie of C2C12 myoblasts differentiating in vitro). During myogenesis, undifferentiated satellite cells give rise to mono-nucleated proliferating myoblasts that can spontaneously fuse together to form multi-nucleated myotubes that undergo further development to form skeletal muscle fiber.[5] The fate of the muscle satellite cell is undetermined as it has the choice of remaining a satellite cell through a

continuous mitotic cycle or differentiating to form an enlarged, elongated and tubular myotube.[5] The myogenic differentiation process takes place during embryonic development as well as throughout adult life. For example, in response to skeletal muscle injury, because of activating satellite cells that align outside the myofiber under the basal lamina, skeletal muscle cells are able to regenerate.[5-6] The regenerative potential of satellite cells can also be replicated in-vitro, which provides a mechanism for recapitulating and translating the underlying science of skeletal muscle disorders from the bench to the bedside.

Figure 1-1 **Multimedia of C2C12 myoblasts differentiating in vitro**. The in vitro C2C12 differentiation process takes approximately 7 days for completion. The step-by-step differentiation process involving myoblast maturation, fusion of myoblasts and fusion of myotubes was recorded in the Brotto Lab by Dr. Chenglin Mo in collaboration with Dr. Sarah Dallas at the University of Missouri-Kanas City.

In addition to its regenerative capacity, muscular contractions are another distinct characteristic of skeletal muscle. Skeletal muscle contraction provides the primary force to generate movement in the body mediated by muscle fibers. A muscle fiber is a single muscle cell that resulted from the fusion of multiple myoblasts. Each muscle cell or muscle fiber consist of a sarcolemma (cell membrane), sarcoplasm (cytoplasm) and myofibrils which are intracellular structures that consist of the contractile thin filament, actin, thick filament, myosin, and the contractile facilitator proteins, tropomoyosin and troponins.[7] Each myofibril is closely enwrapped by a sarcoplasmic reticulum (SR), which

is a modified endoplasmic reticulum that stores Ca^{2+} ions and is juxtaposed to traversing tubules (t-tubules) that are involved in ion exchange.[7-8] When a skeletal muscle cell receives a signal to contract, it undergoes a dynamic transient change in its myofibril structure through shortening of the sarcomeres.

The signal for contraction begins when a motor neuron releases acetylcholine (Ach) at the neuromuscular junction.[9] As Ach is released and binds to the Ach receptor-channels of the muscle fiber, there is an influx of Na^{+} ions which generates an action potential that spreads along the sarcolemma and into the t-tubules.[8] The action potential causes the dihydropyridine receptor (DHPR) of the t-tubule to change conformation and open the SR Ca^{2+} release channels, also known as the ryanodine receptors (RyR).[7-8] The opening of RyR releases Ca^{2+} down its concentration gradient into the cytosol (Fig. 1-2).[9] In the resting state of a skeletal muscle, tropomyosin binds to the myosin binding sites of actin and this prevents the movement of myosin along actin and thereby inhibits contraction.[9] However, as cytosolic Ca^{2+} concentration increases, Ca^{2+} binds reversibly to troponin which allows it to pull tropomyosin off such that sarcomere shortening and skeletal muscle contraction are enabled.[9]

Calcium homeostasis is maintained after contraction through the re-entry of Ca^{2+} from the sarcoplasm to the SR through the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) which is embedded within the SR membrane.[10] Another mechanism for maintaining the normally low intracellular Ca^{2+} concentrations (100-200nM) is through active transport of Ca^{2+} from the cytosol to the extracellular space through the plasma membrane Ca^{2+} ATPase (PMCA).[10] Recent studies have also indicated that as the Ca^{2+} concentration in the SR depletes due to contraction, it is replenished by store operated channels (SOC) that are located on the transverse-tubule membrane and transport Ca^{2+}

back into the SR.[10] The mechanisms underlying calcium homeostasis are essential for proper cell signaling, gene regulation and myogenesis.[10-11]

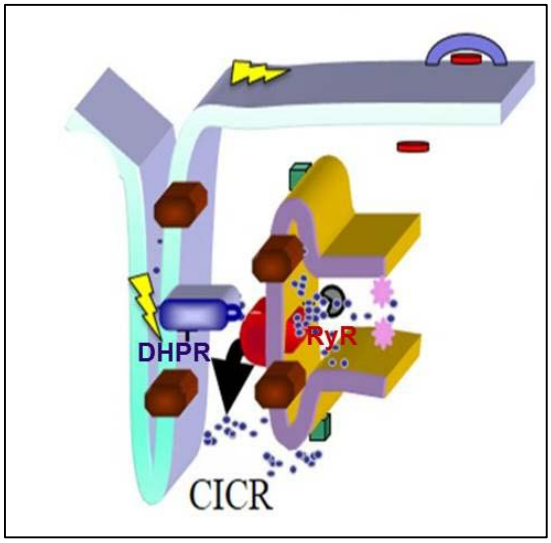


Figure 1-2 **Schematic drawing of the triad junction, chief site of the excitation-coupling (E-C) process in skeletal muscles.[12]** In skeletal muscle, depolarization of the sarcolemma and its t-tubules (represented by the lightning bolt in yellow) alters the conformation of DHPR and results in its modified interaction with RyR (shown in red) which enables the dominant type of calcium release observed in skeletal muscle, depolarization induced calcium release (DICR). The initial depolarization can further be amplified by a secondary mechanism, calcium-induced calcium release (CICR), which is the predominant mechanism in cardiac muscle.

The process of skeletal muscle contraction is what enables the range of fine delicate facial movements to that of forceful loading of the bone through aerobic exercise or lifting. Although the mechanism of skeletal muscle contraction appears reasonably thorough, there is still much to be learned about the function and development of skeletal muscle.

However, with innovative research, there is hope for sustainable contributions to therapies for skeletal muscle disorders and diseases.

1.2 MOLECULAR MECHANISMS OF SKELETAL MUSCLE DISEASE

The World Health Organization Global Burden of Disease Study (GBD) of 2010 estimated 1.7 billion people worldwide are affected by musculoskeletal disorders (MSDs).[13] Among the 289 diseases and injuries evaluated in the GBD study of 2010, MSDs rank as the second greatest cause of disability according to the calculated years lived with disability (YLDs) for affected individuals.[13] This equates to roughly 21.3% of all YLDs.[13] MSDs only rank below mental and behavioral disorders with respect to this measure.[13]

The state of muscle satellite cells determines to a large degree the regenerative capacity of skeletal muscle as a result of injury, aging or dystrophy. In adults, quiescent undifferentiated satellite cells are in direct contact with the myofiber under the basal lamina where they await activation in response to specific signals.[14] Upon activation, the satellite cells escape the basal lamina and can proliferate or differentiate into new myotubes and thereby regenerate skeletal muscle.[15] Skeletal muscle disorders due to muscular dystrophy and aging are often linked to a reduced regenerative capacity of satellite cells caused by excessive muscle-repair degeneration and regeneration cycles.[16] The importance of satellite cells for skeletal muscle regeneration was demonstrated in the Muscular Duchenne Dystrophy (DMD) study by Luz et al.[17] DMD is an x-linked recessive disorder that is characterized by a mutations in the *Dystrophin* gene resulting in progressive muscle degeneration, weakness, and early death.[18] Luz et al. showed that *mdx* mice harboring a point mutation in the Dystrophin gene that repeatedly underwent muscle degenerative-regenerative cycles by injection with a myotoxic agent experienced

a significant decrease in the population of regenerated muscle fiber that was proportional to the number of injections given.[17]

As with DMD, aging can also induce a repetitive cycling of muscle degeneration and regeneration.[1] From this perspective, aging induces sarcopenia, a decrease in muscle mass and strength. The residual loss of muscle mass that characterizes sarcopenia can result in denervation, mitochondrial dysfunction, inflammation and hormonal changes at the cellular level and lead to weakness, imbalance and susceptibility to falls, and the direst cases, morbidity at the systemic level.[1, 18] In the several documented cases of sarcopenia, the reduction in the size of muscle fibers suggest a triggering mechanism involving nutrition, physical activity, metabolic homeostasis, hormonal changes, oxidative stress, and age.[18-19] The therapies against sarcopenia are rather diverse and involve exercise, ingestion of amino acids and treatment with testosterone.[20] Inhibitors against myostatin, a negative regulator of myogenesis that has be shown to inhibit cell cycle progression and reduce levels of myogenic transcription factors, may also attenuate the symptoms of sarcopenia.[18, 20-21] Wehling et al. and Sakuma et al. showed that levels of myostatin increases with muscle atrophy due to unloading as shown in mice and humans and as a result of muscle wasting in patients with cancer cachexia, chronic health failure, chronic obstructive pulmonary disease (COPD), AIDS and diabetes.[22] In response to this finding, Siriett et al. showed that myostatin-null old mice had minimal atrophy and accelerated muscle regeneration compared to the wild-type.[21a] However, Gu et al. found that although deletion of myostatin in rats resulted in greater muscle mass, it did not result in greater fiber strength.[23] This finding is among other similar reports of myostatin in sarcopenia and contributes to the challenge of defining its role.[18]

Perhaps the uncertainty surrounding the role of myostatin in reducing/strengthening musce fiber in dystrophic skeletal muscle is due to a multifactorial combination of

mechanisms affecting fiber strength. As shown by recent studies, age-induced skeletal muscle decline could be the consequence of oxidative stress caused by an accumulation of reactive oxygen species (ROS).[11] ROS/RNS are natural byproducts of eukaryotic cellular metabolism.[24] In eukaryotic cells, ROS are typically produced by the membrane-bound NADPH oxidase complex and the mitochondrial electron transport chain (ETC) and monamine oxidases (MAO).[11, 25] Excessive production of ROS can be detrimental to cellular processes and therefore are tightly regulated by antioxidant enzymes that convert the byproducts to less reactive species such as hydrogen peroxide (H_2O_2) that can then be further reduced to water.[11] Defects that stimulate the overproduction of ROS and prevents their removal is an attribute of cancer, cellular aging, ischemic disorders and degenerative diseases.[11, 25-26] In skeletal muscle, ROS have been shown to serve a physiological purpose as they take part in signaling mechanisms that promote muscle adaptation to exercise.[11, 27] However, ROS can also have a pathological effect on skeletal muscle. Several studies on dystrophic human and mice muscles using DMD patients and *mdx* mice report elevated levels of antioxidant enzymes which is indicative of oxidative stress.[11, 28]

As a result of oxidative stress by ROS accumulation, several signaling mechanisms in skeletal muscle can be altered and disrupted. The loss of calcium homeostasis due to the emergence of leaky membrane calcium channels induced by ROS is a well-documented event.[29] The resulting influx of calcium ions into the cytosol is a speculated cause of myofiber necrosis in muscular dystrophy.[30] This hypothesis has been supported by mouse models with elevated calcium influx giving rise to muscular dystrophic symptoms and models with enhanced calcium clearance or inhibited calcium influx resulting in greater resistance to myofiber death and muscular dystrophy.[30] The emergence of calcium as an effector molecule in cases of muscular dystrophy brings it to

the forefront of considerable therapeutic solutions among alternatives such as NFκB pathway targets.

NFκB is a transcription factor that is involved in immune and inflammatory response as well as cellular growth, differentiation and apoptosis.[31] Recent studies indicate that skeletal muscle degeneration induced by oxidative stress is mediated by NFκB. In *mdx* mice, NFkB is activated prior to the onset of muscular dystrophy.[11] Kumar et al. and Acharrya et al have shown that when *mdx* mice are treated with the N-acetyl cysteine (NAC) antioxidant, NFkB activation is inhibited, suggesting that oxidative stress lies upstream of NFkB and takes part in the upregulation of NFκB in DMD myopathy.[32] Other factors within the NFκB pathway such as TNFα, which activates NFκB, may also play a causal role in muscular dystrophy induced by oxidative stress but the oxidative stress-muscular dystrophy mechanism in its entirety remains to be unequivocally affirmed.

1.3 IMPLICATION OF NON-CODING RNAS ON SKELETAL MUSCLE

Adequate skeletal muscle function is a vital requirement for an enriched healthy life. Thus the regulatory processes that govern skeletal muscle development are essential for promoting health. For years the molecular processes governing myogenesis and the development of skeletal muscle were primarily associated with the “Central Dogma of Molecular Biology,” that is DNA makes RNA make protein.[33] Resultantly, the regulation or dysregulation of myogenesis was exclusively attributed to protein transcriptional factors. Despite the completion of the Human Genome Project and the revelation that protein-coding genes account for only 1.5% of the genome, the view that proteins are sole regulatory molecules continued and non-coding genomic DNA was disregarded as “junk DNA.”[34] In recent years, however, non-coding RNAs including microRNAs (miRNAs) and

long non coding RNAs (lncRNAs) have emerged as integral components of gene regulation that take part in cellular differentiation, development and disease.[34b]

miRNAs are a class of evolutionarily conserved, relatively short non protein-coding RNAs that are approximately 22 nucleotides long.[35] miRNas typically play a role in post-transcriptional regulation by targeting messenger RNAs (mRNAs) for cleavage or translational repression.[35] Conversely, lncRNAs typically are not conserved, have transcripts that are longer than 200 nucleotides and have low or no protein-coding potential.[36] lncRNAs have been shown to also play a role in transcriptional and epigenetic regulation via co-interactions with transcriptional complexes or by recruitment of chromatin modifying complexes post-transcription.[34b] In the case of skeletal muscle disease, recent studies indicate that dysregulation of miRNAs and lncRNAs in skeletal muscle may result in muscle hypoplasia, increased apoptosis and muscular dystrophy.[37]

The recent discoveries of lncRNAs in skeletal muscle prompt interest in their function. In 2010 De Santa et al. and Kim et al. discovered several lncRNAs from the enhancer region of skeletal muscle genes that appeared to regulate chromosome structure or transcription machinery through cis and trans mediated-mechanisms.[38] In 2011, the first muscle-specific lncRNA, linc-MD1, was identified by Gabellini et al. and since then has been found to be required for proper muscle differentiation.[39] Interestingly, reduced levels of linc-MD1 have been observed in DMD patients and overexpression of linc-MD1 has restored normal expression of myogenic genes which includes Myogenin (*MyoG*)and Myosin Heavy Chain (*MHC*).[39a] Moreover, in 2013 Mousavi et al. identified two enhancer RNAs from the Myogenic Determination gene (*MyoD*), Distal Regulatory Regions (*DDR*) and Core Enhancer (*CE*), which reportedly function in stimulating the expressions of the myogenic transcription factors, Myogenic Determination Protein-1 (MyoD1) and MyoG.[40] In summary, the role of ncRNAs in skeletal muscle is a continuous

development. However the exciting discoveries and research on ncRNAs open doors and pave avenues for promising and innovative therapies against skeletal muscle disease.

1.4 IMPLICATION OF HOTAIR ON SKELETAL MUSCLE

HOX Transcript Antisense Intergenic RNA (*HOTAIR*) is a spliced and polyadenylated 2.2 kb long non-coding RNA (lncRNA) that is transcribed from the antisense strand of the homeobox C (HOX C) gene locus between chromosomes 11 and 12.[41] *HOTAIR* was discovered in 2007 as a result of a series of transcriptomic and proteomic analyses of the HOX loci on primary adult human fibroblasts.[41] A high resolution tiled microarray was designed for the four human HOX loci (HOXA, HOXB, HOXC and HOXD) to investigate their transcriptional and epigenetic activities.[41] To reconcile the characteristic of human adult fibroblasts to differentiate exclusively based on their anatomical sites of origin and maintain their embryonic features, samples were obtained from 11 distinct anatomical positions and analyzed.[41] The length of *HOTAIR* as well as its splicing and polyadenylation was confirmed by molecular cloning and northern blot analysis.[41] Additionally, strand-specific RT-PCR confirmed the antisense transcription of *HOTAIR* from the HOXC locus.[41] To investigate correlations between methylation marks and the locations that the non-coding RNAs were discovered, chromatin immunoprecipiation (ChIP) microarray experiments were performed.[41] The results of the ChIP assay followed by HOTAIR knockdown revealed that *HOTAIR* targets and represses the HOXD locus through a trans mechanism.[41]

Since its discovery *HOTAIR* has been described as a transcriptional and epigenetic gene regulator due to its interaction with the Polycomb Repressive Complex 2 (PRC2) and Lysine Specific Demethylase 1 (LSD1) chromatin modification complexes.[42] *HOTAIR* bridges PRC2 and LSD1 and guides the silencing complexes to different sites along the genome to repress transcription in trans mode.[41, 42d, 43] *HOTAIR* is hence a

molecular scaffold as well as a chaperone in gene silencing. The 5' end of *HOTAIR* binds to PRC2 which consists of three major subunits, Histone-lysine N-methyltransferase EZH2 (EZH2), Polycomb Protein Suz12 (Suz12) and Polycomb Protein EED (EED).[44] The EZH2 subunit of PRC2 lays down deactivated trimethylation marks on histone H3 lysine-27 (H3K27).[44] The 3' end of *HOTAIR* binds to LSD1 which removes active methylation marks from H3K4.[44] Collectively, the actions of PRC2 and LSD1 in association with *HOTAIR* contribute to repressive chromatin structure.[45]

The dysregulation of *HOTAIR* has been linked to several cancers including colorectal, pancreatic, gastric, breast, cervical and prostate.[42a, 44, 46] Surprisingly, there is limited exploration of HOTAIR in skeletal muscle considering that its counterparts, PRC2 and LSD1 have significant effects on skeletal muscle. In 2004, Caretti et al. postulated that EZH2 methytransferase regulates gene expression and skeletal muscle differentiation based on the finding that its downregulation coincided with myogenic activation in mouse somites.[47] Subsequent studies showing that ablation of EZH2 in satellite cells and myoblasts impairs muscle regeneration and inhibits myoblast differentiation have provided further evidence to the importance of EZH2 in skeletal muscle development.[48] Similar to studies of EZH2, recent investigations of LSD1 in skeletal muscle also suggest a regulatory importance. In 2010, Choi et al. suggested that LSD1 regulates myogenic factors and is required for skeletal muscle differentiation. This hypothesis was supported by the observation of enhanced LSD1 protein expression during myogenic differentiation and impairment of myoblast differentiation upon LSD1 depletion.[49] The association of EZH2 and LSD1 with skeletal muscle regulation is intriguing. Although there is still much to be resolved about the roles of EZH2 and LSD1 on skeletal muscle, the evidence of a relationship coincident with the role of *HOTAIR* in

the recruitment of PRC2 and LSD1 to chromatin stimulates thought on the function and effect of *HOTAIR* on skeletal muscle.

1.5 STUDY PURPOSE AND WORKING HYPOTHESIS

Chapter 2 The ***overall objective*** of this study was to decipher the role of *HOTAIR* in skeletal muscle and elucidate mechanisms by which it may function. This research ***applied*** established knowledge of skeletal muscle regulation to expand the investigation to the influence of an lncRNA (*HOTAIR*) on skeletal muscle. The ***research question*** for this study was what is the overall molecular, chemical and physical effect of *HOTAIR* silencing on C2C12 cells? The ***working hypothesis*** for this study was that treatment of the mouse skeletal muscle myogenic stem cells (C2C12) with *HOTAIR* antisense DNA and siRNA would disrupt skeletal muscle myogenesis compared to untreated C2C12 cells as evident by fewer nuclei within myotubes, reduced fusion index, down regulation of myogenesis regulatory genes and decreased sarcoplasmic reticulum calcium release response to calcium stimulation with caffeine. The hypothesis was ***tested*** by investigating the effects of *HOTAIR* antisense DNA and siRNA on C2C12 cells using fluorescent microscopy, performing quantitative Real-Time Polymerase Chain Reactions (qRT-PCR) and gene arrays as well as measuring intracellular calcium concentration. The ***significance*** of this study is embedded within its potential to provide better understanding of the function of lncRNAs on skeletal muscle development which under pathophysiology conditions could expedite the delivery of creative and effective therapeutic solutions to skeletal muscle disorders and diseases. This work also provides a foundation for continued collaboration between the College of Nursing and Health Innovation and Department of Chemistry at the University of Texas-Arlington.

Chapter 2

THEORETICAL FRAMEWORK AND METHODOLOGY

2.1 THEORETICAL FRAMEWORK

The conceptual framework for this study is the skeletal muscle myogenesis model (Fig 2-1). Skeletal muscle development commences with myogenic quiescent satellite cells that await activation.[14, 50] Upon activation, the satellite cells undergo asymmetric division by which they can self-renew or enter the myogenic pathway and differentiate into muscle.[14, 50] As myogenesis ensues, committed satellite cells become myoblasts.[50] Myoblasts are mononucleated cells that can continually proliferate via mitosis in response to specific signals, some of which have yet to be identified.[50-51] Myoblasts in response to differentiation signals may also exit the cell cycle and fuse with other myoblasts to become myotubes.[50, 51b, 52]As myoblasts fuse they become enlarged and take on the tubular elongated shape of myotubes.[50, 52] Myogenesis takes place not only during embryonic development but also throughout the life span in response to growth and muscle injury.[14, 50]

Aging and disease can introduce many physiological as well as molecular changes in skeletal muscle which can disrupt the regulation of myogenesis.[20] Recent studies have shown that factors other than proteins such as non-coding RNAs (ncRNAs) can alter myogenesis as a result of aging and disease.[53] With the recent discovery of Hox Transcript Antisense Intergenic RNA (*HOTAIR*) which has been observed as an oncogenic molecule in several cancers, discovering its role in skeletal muscle is entailing.[43, 54] One way to further explore the role of *HOTAIR* in skeletal muscle is to compare muscle formation in skeletal muscle cells lacking *HOTAIR* with those that have normal expression. This study is therefore an in vitro design in which the myogenesis of

HOTAIR-silenced mouse skeletal muscle cells was compared with the myogenesis of mouse skeletal muscle cells with unperturbed expression of *HOTAIR*.

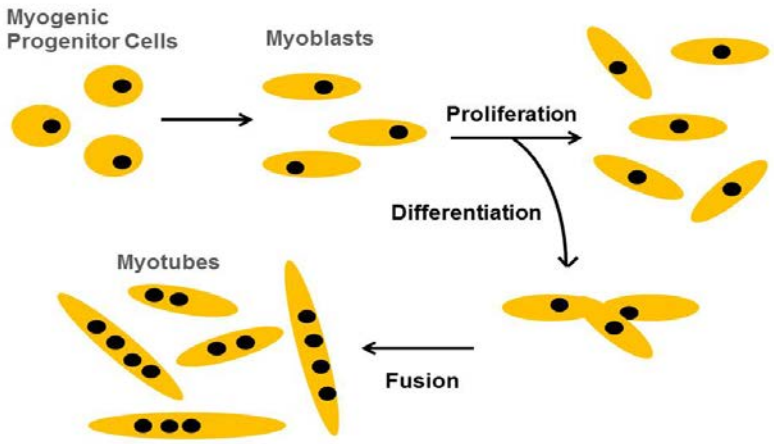


Figure 2-1 **Skeletal Muscle Myogenesis Model**. Schematic drawing of myogenic progenitor cells activating to become myoblasts that proliferate or differentiate into myotubes via fusion with other myoblasts.

2.2 RESEARCH DESIGN

In vitro Studies

The studies were performed using an in vitro experimental design. In vitro designs involve the investigation of a living system in a controlled environment outside of the living organism.[55] The in vitro experimental design provides a reliable and often feasible method for exploring and manipulating a living system.[55b, 56] The in vitro design has been used historically in a variety of techniques for amplifying DNA, purifying proteins, fertilizing eggs and differentiating myogenic cells.[55a, 56-57] The advantages of using an in vitro design are plentiful in regards to reduced cost, direct manipulation of the

intricate parts of the living system and bypass of several ethical considerations stipulated by human or animal subjects. While some may argue that in vitro studies provide a skewed view of the mechanisms occurring within an intact living organism, a particular advantage of using in vitro differentiation of myogenic cells in this study is that it is a replica of the in vivo process that takes place during muscle repair and damage.[55, 58] In vitro studies are also considered an initial important step before animals are used due to ethical considerations.

C2C12 Cell Line

The commercially available C2C12 cell line was used in the in vitro experimental design. C2C12 cell line was originally obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). C2C12 myoblast cells proliferate rapidly in cell culture and they can differentiate rapidly (5-7 days) from myoblasts to form myotubes, also known as muscle cells. The C2C12 cell line is useful for studying myoblast proliferation and differentiation, as well as basic functional and biochemical studies. The culture and effective manipulation of these cells has been firmly established in the Brotto laboratory where these studies were conducted.[59]

Scope of Investigation

HOTAIR is a long non coding RNA (lncRNA) that is transcribed from the antisense strand of the HOXC gene locus of chromosome 12. Recent studies identify *HOTAIR* as an epigenetic gene repressor. Although there is a substantial amount of data suggesting important functions of *HOTAIR* in several human and mouse cells and tissues, very little knowledge exists about *HOTAIR* functions in skeletal muscle.[43-44] To explore the impact of *HOTAIR* on skeletal muscle cell growth and development, we

knockdown *HOTAIR* in mouse skeletal muscle cells and analyzed the impact on cell proliferation and differentiation. To knockdown *HOTAIR*, a synthetically designed *HOTAIR* antisense DNA and siRNA were used in separate experiments. To investigate the underlying effect of *HOTAIR* in C2C12 cells, a series of gene expression, gene array, immunostaining and calcium imaging analyses were employed during cell differentiation.[59]

2.3 C2C12 CELL CULTURE

Myoblast Proliferation

Cryopreserved C2C12 cells were thawed in a 37^{o}C water bath. The cells were then transferred to a 15mL canonical tube containing 5mL of pre-warmed complete growth media [CGM, high-glucose Dulbecco's Modified Eagle Medium (DMEM, Mediatech Inc., Manassas, VA) with 10% fetal bovine serum (FBS from Thermo Fischer Scientific Inc. Waltham, MA), and 100 U/mL penicillin/100 µg/mL streptomycin (penicillin-streptomycin (P/S) 10,000 U/mL each from Mediatech Inc. (Manassas, VA, USA)] and gently mixed. Cells were then centrifuged at 500 rpm for 5 minutes to remove the dimethyl sulfoxide (DMSO) used in cryopreservation. Upon removal of the supernatant, the cells were re-suspended in 4 mL of warm CGM. From this suspension, a 10µL aliquot was used for cell counting using a hemacytometer. For cell proliferation, 100 x 10^4 cells were plated in 6 six-well plates and incubated at 37^{o}C and 5% CO_2. As C2C12 cells can spontaneously differentiate when fully confluent, the cells were passaged after 48 hours at 60% confluency to ensure that they remained undifferentiated.[59a, 59b]

Myogenic Differentiation

To induce the differentiation of C2C12 myoblasts, the same protocol for maintaining myoblasts was employed but with the substitution of CGM with differentiation

medium [DM, high-glucose DMEM with 2% horse serum (horse serum from Thermo Fischer Scientific Inc. Waltham, MA), and 100 U/mL penicillin/ 100 µg/mL streptomycin].[59b]

2.4 HOTAIR ANTISENSE DNA TRANSFECTION ASSAY

To knock down *HOTAIR* expression in C2C12 cells, $10x10^4$ cells/well were plated in 6-well plates and grown to 70% confluency in complete growth media (CGM) before transfection with *HOTAIR* antisense DNA. C2C12 cells were transfected with the *HOTAIR* antisense DNA designed by the laboratory of Dr. Subhrangsu Mandal, Department of Chemistry, University of Texas-Arlington (sequence is shown in Figure 1) with concentrations of either 200nM or 500nM using the Lipofectamine RNAiMax reagent (Invitrogen, Grand Island, NY). The transfection reagent cocktail of a total volume of 1mL was applied to the cells followed by incubation for 24 hours at 37°C. After 24 hours, the cells were supplemented with 1mL of CGM and allowed to grow another 24 hours prior to harvesting the cells for RNA extraction and immunofluorescence staining. To detect the effect of *HOTAIR* on C2C12 cell differentiation, 48 hours after transfection, the media of the C2C12 cells was changed from CGM to DM and C2C12 cells were allowed to differentiate for 72 hours prior to harvesting the cells for RNA extraction and Myosin Heavy Chain antibody (MHC)/DAPI staining. Cell in the presence of just the Lipofectamine RNAiMAX reagent or without any additives was used as internal controls, while the negative control also contained a scrambled sequence of the probe.

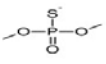
5'-T*C*T*C*T*C*T*G*T*A*C*T*C*C*C*G*T*T*C*C*C-3' * = 

Figure 2-2 **HOTAIR antisense DNA sequence**

2.5 HOTAIR siRNA TRANSFECTION ASSAY

Although transfections with antisense DNA can provide satisfactory results, siRNAs often have more intracellular stability and are more specific to the target RNA. To achieve potentially greater knockdown of *HOTAIR* in C2C2 cells, $6x10^4$ cells were plated in 6-well plates and grown to 50% confluency prior to transfection with the *HOTAIR* Silencer Select n519437 siRNA (ThermoFisher Scientific, Carlsbad, CA). The siRNA transfection assay mirrored that of the antisense DNA transfection with the usage of 2nM, 5nM, 10nM, 25nM, 50nM or 100nM siRNA. After 24 and 48 hours of proliferation following the transfection, C2C12 cells were harvested for RNA extraction. After 72 hours of differentiation following the transfection, the cells were harvested for RNA extraction and MHC/DAPI staining. For calcium imaging experiments, the cells were allowed to differentiate for 5 days post-transfection prior to analysis to allow these experiments to be conducted in myotubes.[59b-d]

2.6 *HOTAIR* siRNA TRANSFECTION EFFICIENCY

To evaluate the efficiency of the HOTAIR siRNA transfection in C2C12 cells, BLOCK-iT™ Fluorescent Oligo (ThermoFisher Scientific) was used as a negative control of the *HOTAIR* siRNA transfection. BlOCK-iT™ is a fluorescein-labeled double stranded RNA that is non-specific to any known genes and is of the same dimensions and configurations of standard siRNAs. The transfection procedure was followed according to the manufacturer's instructions using the Lipofectamine RNAiMAX reagent at the recommended concentration of 100nM (Invitrogen). The transfection efficiency was subsequently assessed via fluorescence imaging after 24 and 48 hours following the transfection using the Olympus IX73 Inverted Research microscope and camera. The cellSens Dimension Imaging software was used for the imaging acquisition and

calculation of the percentage of the total number of cells with the fluorescent tag within the total number of cells to obtain the transfection efficiency.

2.7 QUANTITATIVE REAL-TIME PCR (RT-qPCR)

An aliquot of the purified RNA sample (500ng/µL) with the A260/280nm absorbance ratio at 1.8 or above was reverse transcribed into Complementary DNA (cDNA) in a 20µL reaction volume using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Foster City, CA) according to the manufacturer's instructions. The cDNA was amplified using the RT^2 SYBR Green qPCR Mastermix (SABioscience, Frederick, MD) on the ABI 7300 Real-Time PCR instrument using primers specific for the myogenic marker genes, Myoblast Determination Protein (*MyoD*), Myogenin (*MyoG*), Myosin Heavy Chain (*MHC*) and *Myostatin*. The expressions of *HOTAIR* and its target, Homeobox D10 (*HOXD10*), and coordinate enzyme partners, Enhancer of Zeste 2 Polycomb Repressive Complex 2 subunit (*EZH2*) and Lysine-specific Histone Demethylase 1 (*LSD1*) were also analyzed for additional insight of *HOTAIR* knockdown. The RT-qPCR reactions were triplicated for each treated and control samples and a no-template and minus reverse transcriptase (RT) controls were included to detect contamination or genomic DNA presence. The specificity of the *HOTAIR* RT-qPCR reaction was further validated using a 12µL aliquot of the PCR product for gel electrophoresis (30 minutes, 100 volts, 400mA). Ct values were normalized to *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase) and gene expression was determined as up/down regulation of the gene of interest compared to the control.

Table 2-1 Primer Sequences

MyoG	F: TGAGCATTGTCCAGGCCAG R: GCTTCTCCCTCAGTGTGGCT
Myostatin	F: ATCCACCACGGTGCTAATG R: GGGAGAGAGAGACTCGTACTT
MHC	F:CAAGTCATCGGTGTTTGTGG R: TGTCGTACTTGGGCGGGTTC
MyoD	F:CCCCGGCGGCAGAATGGCTACG R:GGTCTGGGTTCCCTGTTCTGTGT
HOTAIR 1	F: TCCAGATGGAAGGAACTCCAGACA R: ATAGATGTGCGTGGTCAGATCGCT
HOTAIR 2	F: CCTTATAAGCTCATCGGAGCA R: CATTTCTGGGTGGTTCCTTT
EZH2	F: CTAATTGGTACTTACTACGATAACTTT R: ACTCTAAACTCATACACCTGTCTACAT
LSD1	F: AGCGGGCCAAGGTAGAATACA R: ATGGGGAAGTCGGCTTTGAAA
HOXD10	F: AACAGATCTTGTCGAATAGAGCAAC R: GGGCTGTTATTGTACTCTTGGGTTT
GAPDH	F: TCTGATGGGTGTGAACCACGAGAA R: GAGCCCTTCCACAATGCCAAAGTT

2.8 RT-PCR GENE ARRAY

To investigate potential molecular pathways affected by *HOTAIR* knockdown, the gene expression of C2C12 cells transfected with 25nM of *HOTAIR* siRNA after 24 hours of proliferation was analyzed using the Mouse Signal Transduction PathwayFinder PCR array (PAMM-014Z) (SABioscience). The total RNA extracted from the treated and untreated C2C12 cells was purified and quantitated as previously described. A 500ng aliquot of the RNA was used to synthesize the cDNA using the RT^2 First Strand Kit (From Qiagen) and amplified by real time PCR using the RT^2 SYBR Green qPCR Mastermix on the ABI Step One Plus instrument (SABioscience). The array profiled the expression of 84 genes within 10 pathways that are important for developmental, metabolic, oxidative

stress, immunological and stress-activated processes and that are responsive to signal transduction pathway activation or deactivation (Table 2-1). The PCR reaction was triplicated and validated against 6 housekeeping genes and assay controls on the array plate. Data were analyzed using the web-based RT² Profiler™ PCR Array Data Analysis Software (SABscience); Ct values were normalized to six built-in reference house-keeping genes, genomic DNA control, reverse transcription control, and positive PCR control. We used this analytical software to set the statistical significance of up/down regulation of all tested genes at 2-fold difference. Some major advantages of this methodology are the reliability, accuracy and the validation of gene expression by the manufacturer at the protein level.

Table 2-2 Mouse Signal Transduction Pathway Finder PCR Array Genes

NFκB Pathway	*Ccl5 (Rantes), Bcl2a1a (Bfl-1/A1), Bcl2l1 (Bcl-x), Birc3 (c-IAP1), Csf1 (Mcsf), Icam1, Ifng, Stat1, Tnf*
Oxidative Stress Pathway	*Nqo1, Fth1, Gclc, Gclm, Gsr, Hmox1, Sqstm1,Txn1, Txnrd1*
p53 Pathway	*Bax, Bbc3, Btg2, Cdkn1a (p21Cip1/Waf1), Egfr, Fas (Tnfrsf6), Gadd45a, Pcna, Rb1*
Notch Pathway	*Hes1, Hes5, Hey1, Hey2, Heyl, Id1, Jag1, Lfng, Notch1*
Hedgehog Pathway	*Bcl2, Bmp2, Bmp4, Ptch1, Wnt1, Wnt2b, Wnt3a, Wnt5a, Wnt6*
PPAR Pathway	*Acsl3, Acsl4, Acsl5, Cpt2, Fabp1, Olr1, Slc27a4, Sorbs1*
Hypoxia Pathway	*Adm, Arnt, Car9, Epo, Hmox1, Ldha, Serpine1 (PAI-1), Slc2a1, Vegfa*
TGFß Pathway	*Atf4*, *Cdkn1b* (p27Kip1), *Emp1*, *Gadd45b, Herpud1, Ifrd1, Myc, Tnfsf10*
WNT Pathway	*Axin2, Ccnd1, Ccnd2, Dab2, Fosl1 (Fra-1), Mmp7 (Matrilysin), Myc, Ppard, Wisp1*
JAK/STAT Pathway	
JAK1, 2 / STAT1:	*Irf1*
STAT3:	*Bcl2l1 (Bcl-x), Ccnd1, Cebpd, Lrg1, Mcl1, Socs3*
STAT5:	*Bcl2l1 (Bcl-x), Ccnd1, Socs3*
JAK1, 3 / STAT6:	*Fcer2a, Gata3*

2.9 IMMUNOSTAINING AND FUSION INDEX

MHC Staining

To further investigate the phenotypic changes induced by *HOTAIR* knockdown on C2C12 differentiation, myosin heavy chain (MHC) was stained following 72 hours of differentiation after transfection. For staining MHC, the culture medium (DM) was removed, followed by washing each well in the 6-well plate with 2mL 1X phosphate buffered saline/well (PBS, Fisher Scientific International Inc., Fair Lawn, NJ). Following the removal of the PBS, the cells were fixed with 1mL/well of 10% neutral buffered formalin solution (NBF, Sigma-Aldrich, St. Louis, MO) for 10 minutes. After removal of the NBF, the cells were washed 3 times with 2mL/well PBS and placed on a shaker for 5 minutes. The cells were subsequently permeabilized on the shaker with 1mL/well of 0.1% Triton X-100 for 10 minutes followed by 3 more washes in PBS. Cells were stained with 20µL/ml of carboxy fluorescein (CFS) conjugated MHC antibody (R&D system, Minneapolis, MN, USA) in TBST (PBS with 0.1% Tween20 and 0.1% Triton X-100) in the dark, wrapped with foil and gently shaken for 1 hour at room temperature.

DAPI Staining

For nuclei staining, 4',6-diamidino-2-phenylindole hydrochloride hydrate was used (DAPI, Sigma-Aldrich, St. Louis, MO), after removing the Triton X-100 solution from the wells, DAPI at 1:1000 dilution from stock (1mg/ml) in 1XTBST was used in the dark at 1ml/well. The cells were wrapped with foil and gently shaken for 5 minutes prior to washing in the dark 3 times with PBS. For cells transfected with the *HOTAIR* antisense DNA, imaging of the cells was done with 1ml of PBS in each well using the 20X objective of the Nikon Eclipse TS100 microscope and acquired using the NIS Elements Advanced Research imaging software. For cells transfected with the *HOTAIR* siRNA, imaging was

done using the 20X objective of the Olympus IX73 Inverted Research Microscope and acquired using the cellSens Dimension imaging software.

Fusion Index

The fusion index is a quantitative measurement that determines the in vitro efficiency of myoblast fusion in myotube formation and is defined by the number of nuclei in myotubes divided by the total number of nuclei.[59d] Following MHC and DAPI staining, six randomly selected fields in each sample were imaged and acquired. Myotubes with two or more nuclei were identified by the expression of MHC (green). The number of nuclei (blue) in myotubes and total nuclei in the six fields were counted in each field by manual and automatic calculation using ImageJ and the cellSens Dimension software. The fusion index was calculated as the percentage of total nuclei incorporated into myotubes compared to the total number of nuclei.

2.10 INTRACELLULAR CALCIUM IMAGING

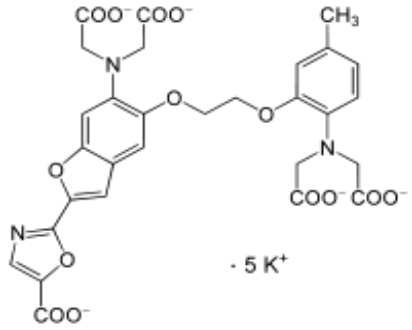
Calcium is a critical signaling molecule in skeletal muscle cells due to its stimulatory effect on muscle contraction and gene expression. To investigate the effects of knockdown of *HOTAIR* on muscle function, since changes in intracellular calcium release can provide insight on the force and speed of muscle contractions, and calcium homeostasis is a hallmark of muscle health and disease.[10, 60] Calcium imaging studies were conducted on C2C12 cells transfected with 25nM of *HOTAIR* siRNA. C2C12 cells were seeded with $6x10^4$ cells/dish in Nunc™ glass bottom dishes (Thermo Scientific) in CGM and transfected with 25nM of *HOTAIR* siRNA at 50% confluency as previously described.[59d] After 48 hours in culture, the CGM was exchanged with DM to differentiate myoblasts into myotubes. When mature myotubes were formed at day 5 of differentiation, DM was aspirated and the cells were gently washed 5 times with 1mL of Ringer solution

containing 2.5mM Ca^{2+} (145mM NaCl, 5mM KCl, 1.6 mM $MgCl_2$, 10mM HEPES and 2.5mM $CaCl_2$ adjusted to a pH of 7.4). The fluorescent dye Fura-2 AM (acetoxymethyl) is a highly specific calcium indicator that is ratiometric and UV excitable. To load the Fura-2AM dye into the cells, a 4µL (1mM in DMSO) aliquot of the dye was added to a 500µL solution of Ringer and gently mixed several times by pippetting. The solution was sonicated for 90 seconds to allow for thorough separation of Fura-2AM from DMSO. A subsequent addition of 500 µL of Ringer was added to the Fura-2AM solution and gently mixed several times by pipetting. The final Ringer wash was removed from the dishes prior to slow drop-wise addition of Fura-2AM around the dish. The cells were then incubated for 30 minutes at 37^{o}C to promote Fura-2AM entry, after which they were placed at room temperature for 25 minutes to allow for de-esterification of Fura-2AM to the active Fura-2 form.

After 25 minutes of de-esterification, the dish was mounted on the stage of the Nikon Eclipse Ti Microscope connected to a spectrofluorometer system (Photon Technology International Inc., PTI, London, Ontario). The 10X objective was then used to locate a field with several robust and mature myotubes. The “in” and “out” perfusion tubes of the perfusion system (Bioscience Tools, San Diego, CA) were then placed directly in the vicinity of the myotubes of interests to allow for specific and quick delivery of caffeine during the stimulatory step. The calcium imaging was then performed under the 40x objective. With the use of an automatic monochromator (PTI) with excitation wavelengths set at 340 and 380 to detect bound Fura and unbound Fura-2, ratiometric measurements at 340/380nm were taken in response to fluctuations of intracellular calcium concentration using the EasyRatio Pro software (PTI). For each experimental dish, 5-6 myotubes were selected in one field of view for simultaneous analysis. To ensure that background changes did not perturb measurements, a region of interest with

low fluorescence intensity was selected. A baseline reading was performed for 2 minutes prior to activating the perfusion system for caffeine delivery. The caffeine perfusion was stopped after observation of intracellular calcium release indicated by an increase of fluorescence excitation at 340nm along with decreased excitation intensity at 380nm. Perfusion with Ringer solution was initiated as the caffeine perfusion ended to re-acquire a baseline Ca^{2+} measurement.[59b-d]

A



B

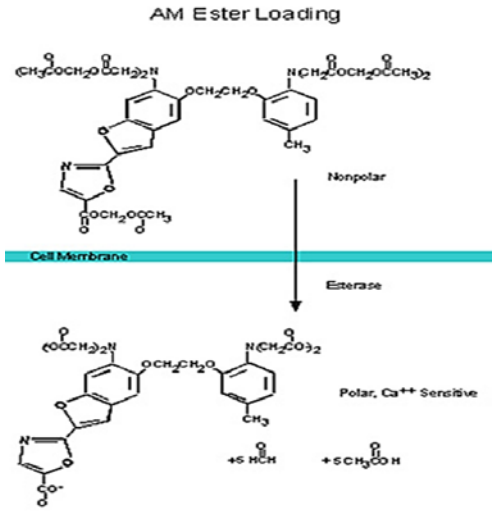


Figure 2-3 **Molecular structure and schematic drawing of Fura-2AM entry into the cell**. (*A*) Molecular structure of Fura-2. (*B*) The non-polar property of the Fura-2AM allows

for easy diffusion through the cellular membrane de-esterification of Fura-2AM by intracellular esterases yields the polar Fura-2 which remains in the cell.[61]

Chapter 3

RESULTS

3.1 INTRODUCTION

Skeletal muscle development from myogenic progenitor cells to myoblasts and myotubes is an important process to investigate for the purposes of developing targeted therapies against muscle illness and disorder. Furthermore, while our understanding of the basic biology of myogenesis has greatly advanced, many challenges remain unsolved.[62] Given that myogenesis is a dynamic cellular interdependent process, it is reasonable to hypothesize that there are additional factors at interplay with the myogenic transcription factors, MyoG, MyoD, MHC and myostatin. A relevant example that supports this hypothesis is the recent study that identified LSD1 as a critical myogenic regulatory factor as it removes repressive histone epigenetic marks during myoblast differentiation into myotubes.[49] Ezh2 is another histone modifying factor that is associated with myogenesis. Down regulation of *Ezh2* in mouse somites has been shown to coincide with activation of muscle gene expression and myoblast differentiation.[47]

As discussed in chapter 1, *HOTAIR* is a long non-coding RNA that can act as an epigenetic transcription repressor when it tethers the PRC2 and LSD1 repressive enzymatic complexes. Thus, genomic regions where *HOTAIR* is found due its association with PRC2 and LSD1 will result in repressive chromatin structure and low transcription. Due to its silencing effect on cell cycle regulatory genes, *HOTAIR* has been implicated in several cancers. Although it has been shown that *HOTAIR* is preferentially expressed in human myogenic progenitor cells compared to human lung fibroblasts (NHLF), lymphoblasts (LCL), umbilical vein endothelial cells (HUVEC), embryonic stem cells (ESC), its association with *LSD1* and *EZH2* and overall effect in skeletal muscle has yet to become entirely deciphered.[63] This section provides the results of testing the

hypothesis that *HOTAIR* knockdown will result in dysregulation of myogenesis due to de-recruitment of Ezh2 and LSD1 from chromatin, which should lead to changes in the expression of these genes. To knockdown *HOTAIR* we employed the use of antisense DNA and siRNA in separate yet procedurally parallel experiments. We also examine dose response of *HOTAIR* antisense DNA and siRNA on C2C12 cells quantitatively and qualitatively. We monitored the efficiency of the knockdown with RT-qPCR and PCR gel electrohopresis. We assessed the effects of *HOTAIR* knockdown on the gene expression of myogenic markers, *HOTAIR* coordinate partners, *Ezh2* and *Lsd1* and its known target, *HOXD10*. In addition to gene expression, we performed gene arrays on canonical mouse signal transduction pathways and examined myotube morphological and biochemical changes by immunofluorescence, Fusion Index, and calcium imaging as a surrogate of calcium homeostasis status.

3.2 HOTAIR ANTISENSE DNA TRANSFECTION RESULTS

HOTAIR antisense DNA partially inhibits HOTAIR in C2C12 cells

As a preliminary experiment for our studies on *HOTAIR* in C2C12 cells, *HOTAIR* antisense DNA was used to knockdown *HOTAIR*.[46c] Subsequent RT-qPCR was done to examine the effectiveness of *HOTAIR* knockdown by *HOTAIR* antisense DNA on gene expression. C2C12 cells were treated with varying concentrations (200nM and 500nM) of *HOTAIR* antisense DNA prior to RNA isolation at day 2 of proliferation and day 3 of differentiation. The relative expression of *HOTAIR* in cells treated with 200nM and 500nM of antisense DNA during day 2 of proliferation is respectively 33% and 38% less than the blank controls when normalized to *GAPDH* expression, which was unaltered in the blank and reagent controls (not shown) (Fig 3-1*A*). *HOTAIR* expression was measured again on day 3 of differentiation to investigate the extent of the knockdown. Interestingly,

whereas *HOTAIR* gene expression remains relatively unchanged with only a 9% decrease in expression in cells treated with 200nM of *HOTAIR* antisense DNA, there is a 53% decrease in *HOTAIR* expression with cells treated with 500nM of *HOTAIR* antisense DNA (Fig 3-1*B*).

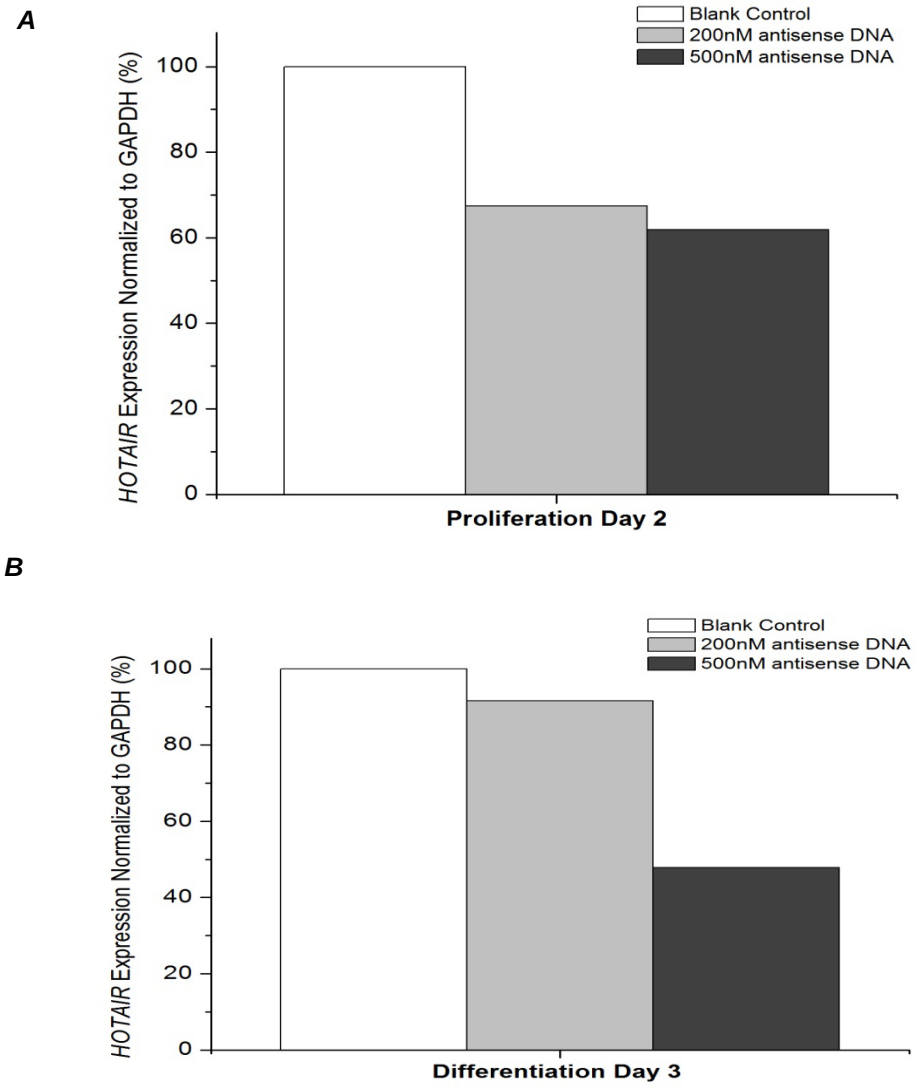
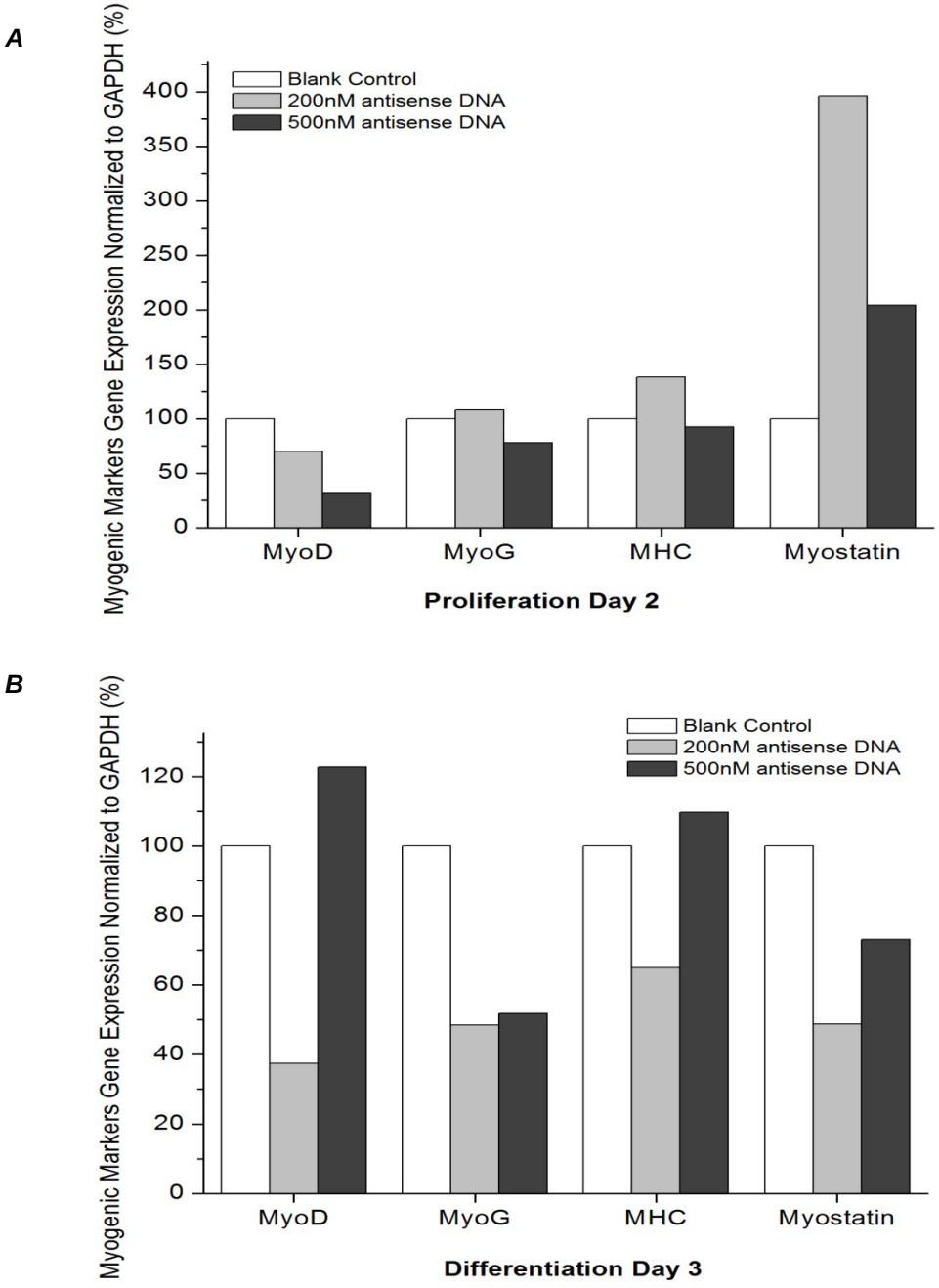


Figure 3-1 ***HOTAIR* gene expression relative to *GAPDH after transfection with HOTAIR antisense DNA*.** (*A*) *HOTAIR* gene expression on day 2 of proliferation after transfection with *HOTAIR* antisense DNA decreased in cells treated with *HOTAIR*

antisense DNA relative to *GAPDH*, which was unaltered in the blank control. N=1 (*B*) *HOTAIR* gene expression on day 3 of differentiation returned to baseline in cells treated with 200nM of antisense DNA but remained low in cells treated with 500nM of antisense DNA relative to *GAPDH*, which was unaltered in the blank control. N=1

Partial knockdown of HOTAIR alters myogenic gene expression

To assess the effect of partial *HOTAIR* knockdown on the gene expression of the myogenic markers, RT-qPCR was performed on day 2 of proliferation and on day 3 of differentiation. The expression of the myogenesis activating factors, *MyoG*, *MyoD* and *MHC,* decreased in expression in cells treated with 500nM of antisense DNA (Fig 3-2*A*). For cells treated with 200nM of antisense DNA, only *MyoD* decreased in expression. *Myostatin*, a negative regulator of muscle mass, increased in expression in cells treated with both 200nM and 500nM of antisense DNA. On day 3 of differentiation, the gene expression of all of the myogenic markers decreases in cells treated with 200nM of antisense DNA (Fig. 3-2*B*). The results for cells treated with 500nM of antisense DNA is much more complex; *MHC* and *MyoD* increased in expression while *MyoG* and *Myostatin* decreased in expression, perhaps reflecting the potential unspecific effects of a much higher dose of the antisense.



3-2 **Gene expression of Myogenic Determination Protein (*MyoD*), Myogenin (*MyoG*), Myosin Heavy Chain (*MHC*) and *Myostatin* relative to *GAPDH* in C2C12 cells after transfection with *HOTAIR* antisense DNA** (*A*) The gene expression of the myogenic

markers on day 2 of proliferation relative to *GAPDH*, which was unaltered in the blank control. (*B*) The gene expression of the myogenic markers on day 3 of differentiation relative to *GAPDH*, which was unaltered in the blank control. (N=2)

Partial Knockdown of HOTAIR inhibits myoblast differentiation

To further explore the function of *HOTAIR* in C2C12 myoblast differentiation, after two days of proliferation following the transfection with *HOTAIR* antisense DNA, the growth media of the cells was exchanged with differentiation media containing horse serum to induce myoblast fusion into myotubes. The cells were visualized daily for phenotypic changes compared to the blank control and analyzed by immunofluorescence after three days of differentiation. Six random image areas were used for all quantifications with 60-120 myotubes being analyzed per specific condition/treatment. DAPI and MHC staining (Fig. 3-3*A*) revealed a significant decrease in myotube formation in the cells treated with *HOTAIR* antisense DNA. The number of mature myotubes with three or more nuclei as indicated by the fusion index (Fig. 3-3*B*) in the treated cells is nearly half of the mature myotube count in the untreated cells. Whether the fusion index disparity between the treated and untreated cells is a direct consequence of *HOTAIR* knockdown or cellular death due to the toxicity of the antisense DNA cocktails remains unknown given that a scrambled control was not assessed in parallel.

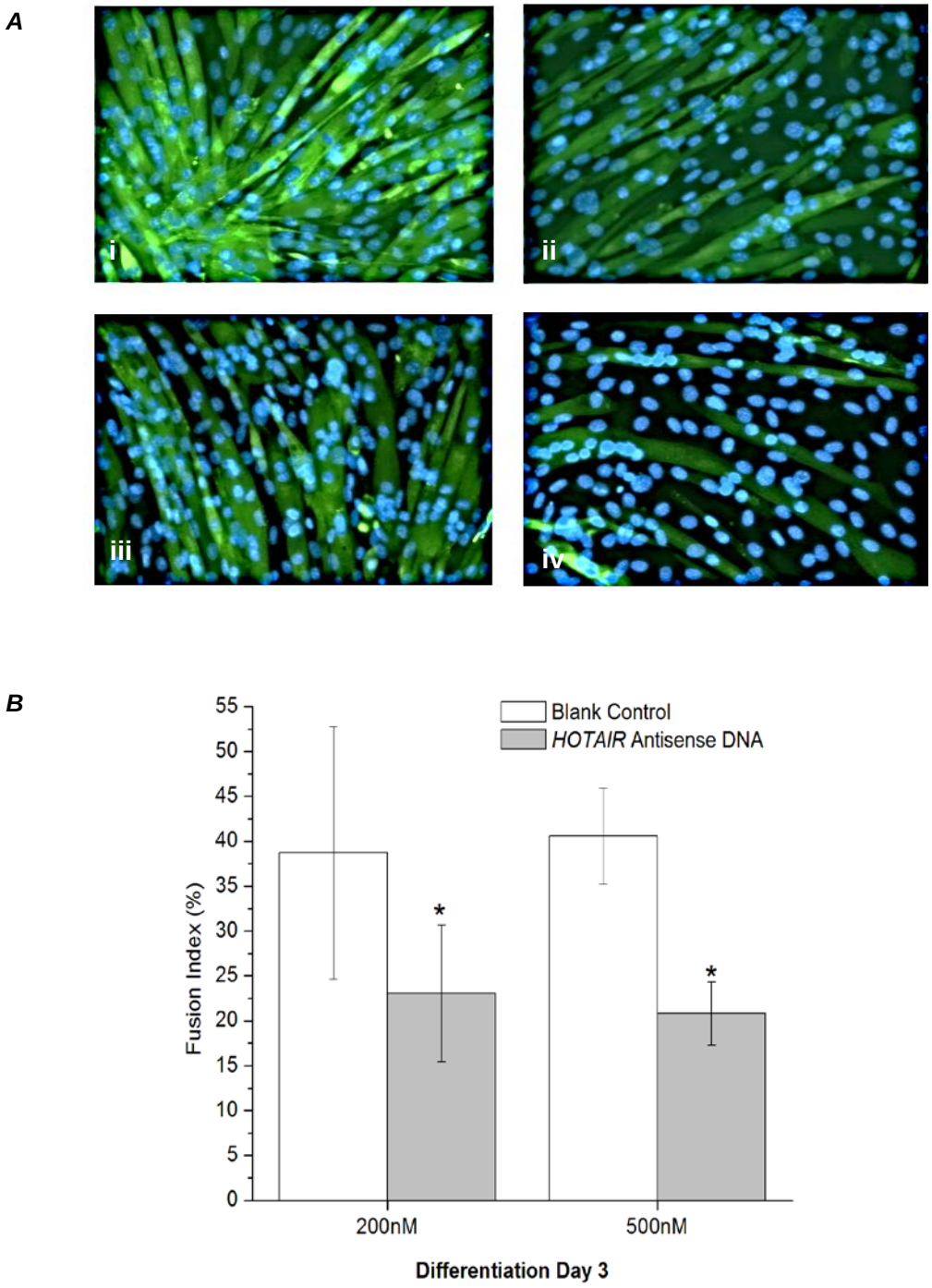


Figure 3-3 **Partial knockdown of *HOTAIR* in C2C12 cells effectively reduced fusion index after transfection with *HOTAIR* antisense DNA** (*A*) Representative

immunofluorescence images of DAPI-stained nuclei (blue) and Myosin Heavy Chain (MHC) antibody (green) of C2C12 cells on day 3 of differentiation after transfection: (i, 200nM; iii, 500nM) blank controls and (ii, iv) 200nM and 500nM *HOTAIR*-antisense DNA-treated cells. (B) Fusion index data shows a significant decrease in mature myotubes expressing MHC after treatment with *HOTAIR* antisense DNA compared to the blank control. *N=6*, *p<0.05*

3.3 HOTAIR siRNA TRANSFECTION RESULTS

Transfection efficiency confirms HOTAIR siRNA intracellular delivery in C2C12 myoblasts

The studies with the anti-sense probe were preliminary in nature but paved the way for the next phase of our studies, where we aimed to achieve a better understanding of the purpose and function of *HOTAIR* in C2C12 cells. The preliminary studies using antisense DNA to knockdown *HOTAIR* revealed interesting changes in the genetic and morphological phenotype of the treated C2C12 cells. However, the use of the high concentrations of antisense DNA in the absence of a scrambled control to assess the full effect of the experimental conditions on the cells might challenge the validity of our results. To achieve *HOTAIR* knockdown at a potentially higher degree with less cellular toxicity, we substituted the *HOTAIR* antisense DNA with *HOTAIR* siRNA. The efficiency of the siRNA uptake in the C2C12 myoblasts was assessed using 100nM of the BLOCK-iT Flourescent Oligo negative control siRNA. The imaging and detection of fluorescent-positive cells was done twenty-four hours after transfection (Fig. 3-4*A*). The transfection efficiency was determined as 88.6% ± 3.52% (Fig. 3-4*B*). When compared to a blank control, the morphology of the cells treated with the negative control did not differ in any regard (Fig. 3-4A i, iii). To further examine the effectiveness of the knockdown by

HOTAIR siRNA (25nM) on gene expression we performed RT-qPCR and report a 38.1% ± 7.47% reduction in *HOTAIR* expression compared to the negative control when normalized to *GAPDH,* which was unaltered in the negative control.

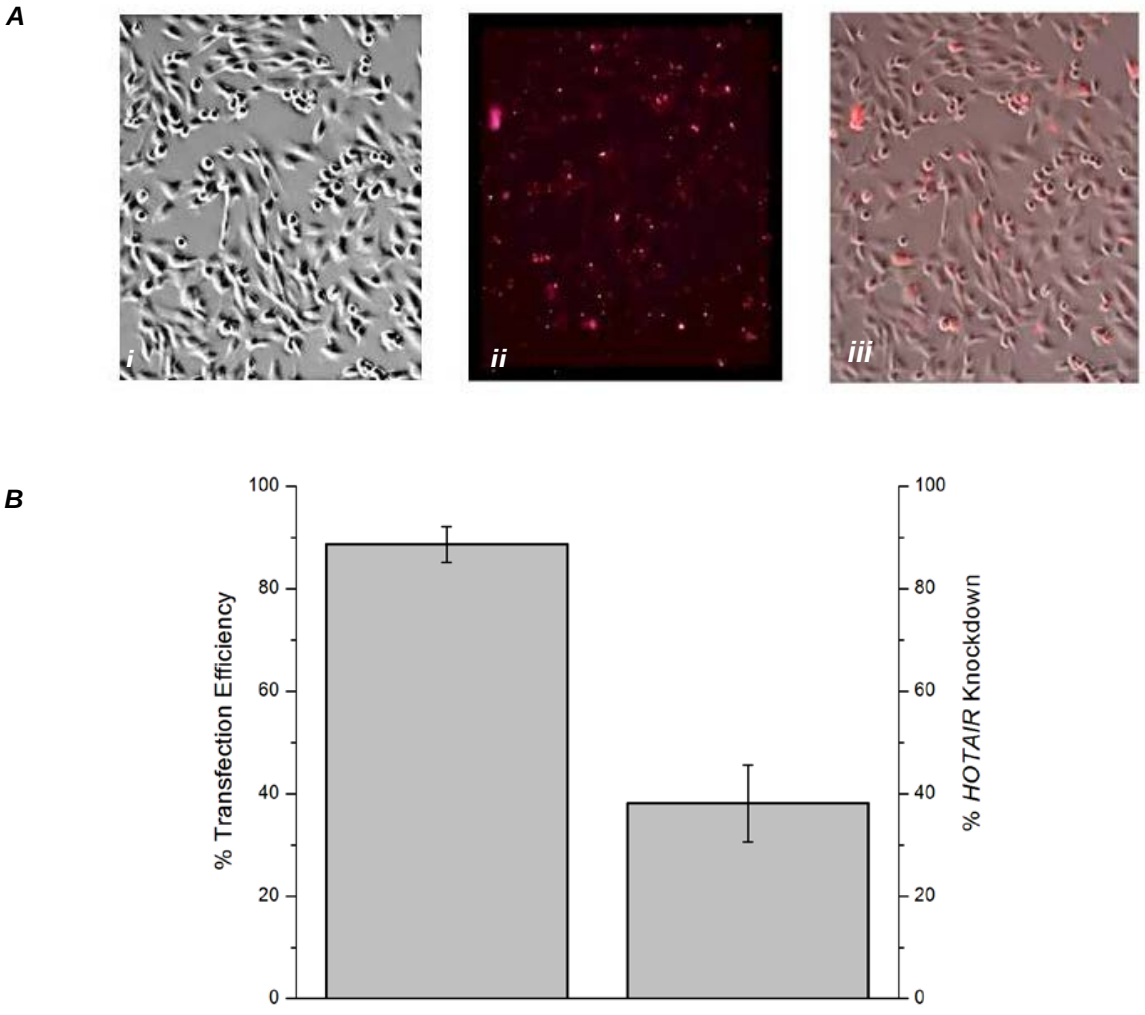


Figure 3-4 **High transfection efficiency of negative control siRNA in C2C12 cells yields partial knockdown of *HOTAIR***. (*A*) Representative images of C2C12 myoblasts 24 hours after transfection with BLOCK-iT Flourescent Oligo negative control siRNA (100nM): (i) phase contrast image, (ii) fluorescent image, (iii) merged images

taken from the same representative area. To quantify the positive uptake, five different areas (average of 290 cells) were analyzed. Under these conditions, the transfection efficiency was determined as 88.6% ± 3.52% which resulted in a 38.1% ± 7.47% lower expression of *HOTAIR* as detected by RT-qPCR (25nM siRNA). (N=3)

RT-qPCR reveals prolonged reduction of HOTAIR expression in proliferating C2C12 cells

To assess the specificity, extent, and duration of *HOTAIR* knockdown in C2C12 cells, we first confirmed the specificity of the *HOTAIR* primers used for the RT-qPCR reactions with gel electrophoresis (Fig. 5-3A)*. Next, HOTAIR* expression was quantitated using RT-qPCR on days 1 and 2 of proliferation and on day 3 of differentiation after transfection with varying concentrations of siRNA (2nM, 5nM, 10nM, 25nM, 50nM, 100nM). On day 1 of proliferation, *HOTAIR* expression was significantly reduced in all treatment groups (25nM, 50nM, and 100nM of siRNA when normalized to *GAPDH)*, which was unaltered in the negative control (Fig. 3-5*B*). On day 2 of proliferation, similar levels of reduction in *HOTAIR* expression to those seen at day 1 are still present. On day 3 of differentiation, *HOTAIR* expression returned to baseline for all treatments when normalized by *GAPDH*, which was unaltered in the negative control,(Fig. 3-5*C*) likely reflecting the exhaustion of the siRNA. The knockdown efficiency determined by the RT-qPCR results from days 1 and 2 of proliferation provided the basis for continuing subsequent gene array, immunofluorescence and calcium imaging experiments selectively with 25nM of *HOTAIR* siRNA. Furthermore, the levels of reduction with 25nM of approximately 40% were very similar to the levels of reduction with 200nM in the antisense series of experiments.

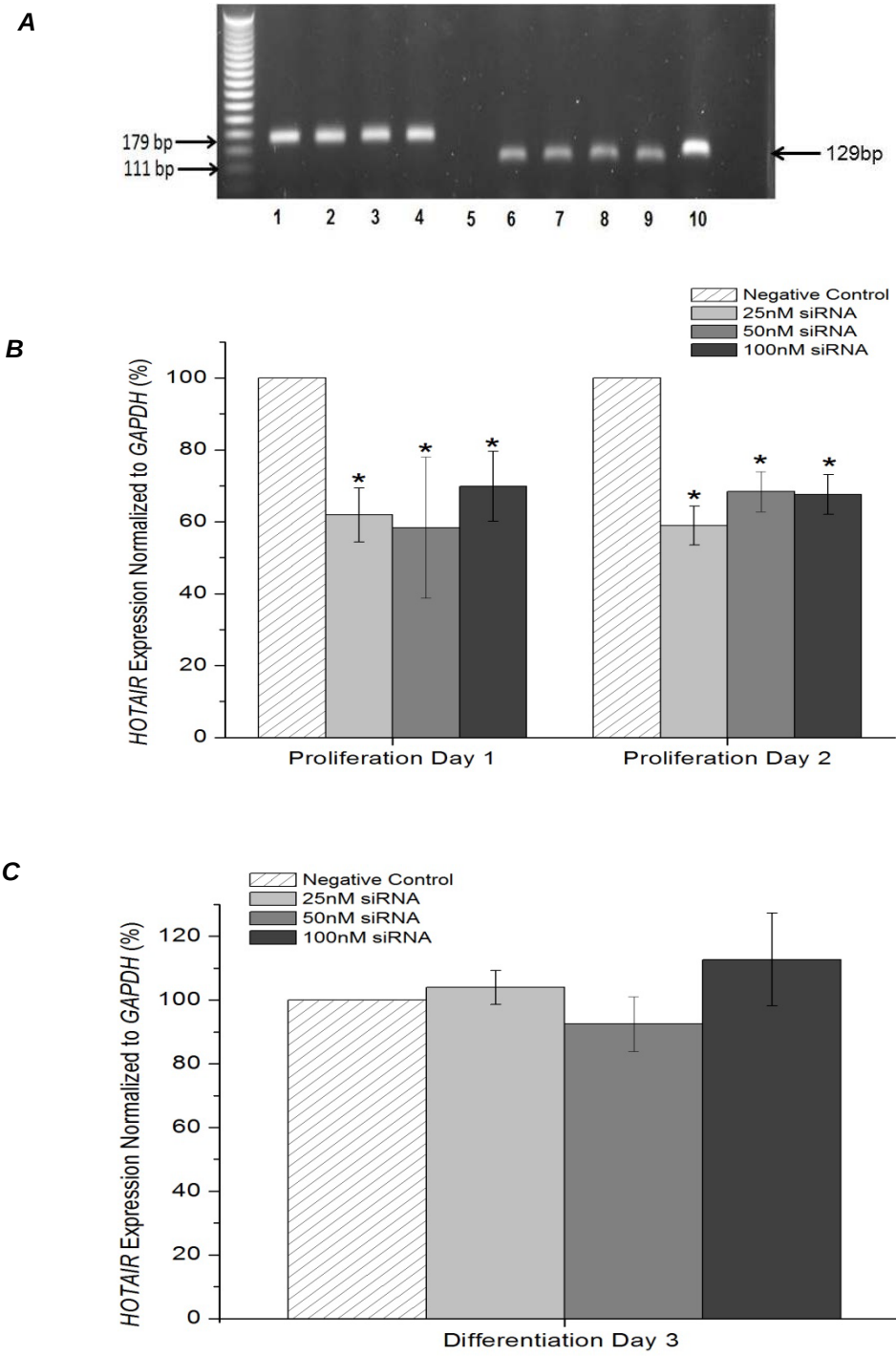


Figure 3-5 ***HOTAIR* specificity and expression in C2C12 cells during proliferation and differentiation after transfection with *HOTAIR* siRNA**. (*A*) Gel electrophoresis

confirmed the specificity of the *HOTAIR* primers used for the RT-qPCR reactions. The HOTAIR-1 primer, used in the RT-qPCR experiments with the *HOTAIR* antisense DNA amplifies a 179 base pair fragment from the mouse *HOTAIR* gene. The HOTAIR-2 primer used primarily in the siRNA experiments amplifies a 111 base pair fragment: (1, 6) 25nM *HOTAIR* siRNA treatment; (2, 7) blank controls; (3, 8) internal reagent controls; (4, 9) internal negative controls; (5) No cDNA blank control; (10) *GAPDH* positive control. (*N=3*, *p<0.05*). (*B*) *HOTAIR* expression on day 1 was reduced to 61.8% ± 7.47%, 58.3% ±19.6%, and 69.8% ± 9.78% for cells treated with 25, 50 and 100nM of siRNA when normalized to *GAPDH*, which was unaltered in the negative control. Similar results were achieved when the treatments were compared against blank and vehicle controls. (*C*) *HOTAIR* expression returned to baseline on day 3 of differentiation for all treatments.

Partial HOTAIR knockdown dysregulates myogenic gene expression

HOTAIR, through its interactions with PRC2 and LSD1 is an epigenetic regulator and its deletion can result in the activation of hundreds of genes.[64] To investigate the potential genetic modulations in C2C12 cells induced by *HOTAIR* knockdown (25nM siRNA), RT-qPCR was performed using primers specific for the myogenic markers, *MyoD*, *MyoG*, *MHC* and *Myostatin*. When *HOTAIR* is activated, it transcriptionally represses *HOXD10* in trans mode.[41] As a secondary confirmation of *HOTAIR* knockdown, the expression level of *HOXD10* was measured in addition to that of *EZH2* and *LSD1* (Fig. 3-6). Gene expression was measured on day 1 of proliferation (Fig. 3-6*A*) and day 3 of differentiation (Fig. 3-6*B*). On day 1 of proliferation, in the case of the expression levels of the myogenic markers, *MyoD* and *MHC* significantly decreased in expression, *MyoG* remained at baseline and Myostatin significantly increased. As predicted by the reduction in *HOTAIR* expression after knockdown, *HOXD10* significantly

increased in expression. Interestingly, whereas *EZH2* was significantly reduced in expression, *LSD1* expression was significantly increased. On day 3 of differentiation, with the exception of *MyoD* and *MyoG* which remained at baseline, *MHC*, *Myostatin*, *HOXD10* and *LSD1* significantly increased in expression. *EZh2* expression, however, was significantly reduced. RT-qPCR experiments with C2C12 cells treated with 50nM and 100nM of *HOTAIR* siRNA were also conducted in tandem and yielded similar results as 25nM *HOTAIR* siRNA-treated cells. (*N=3*, *p<0.05*)

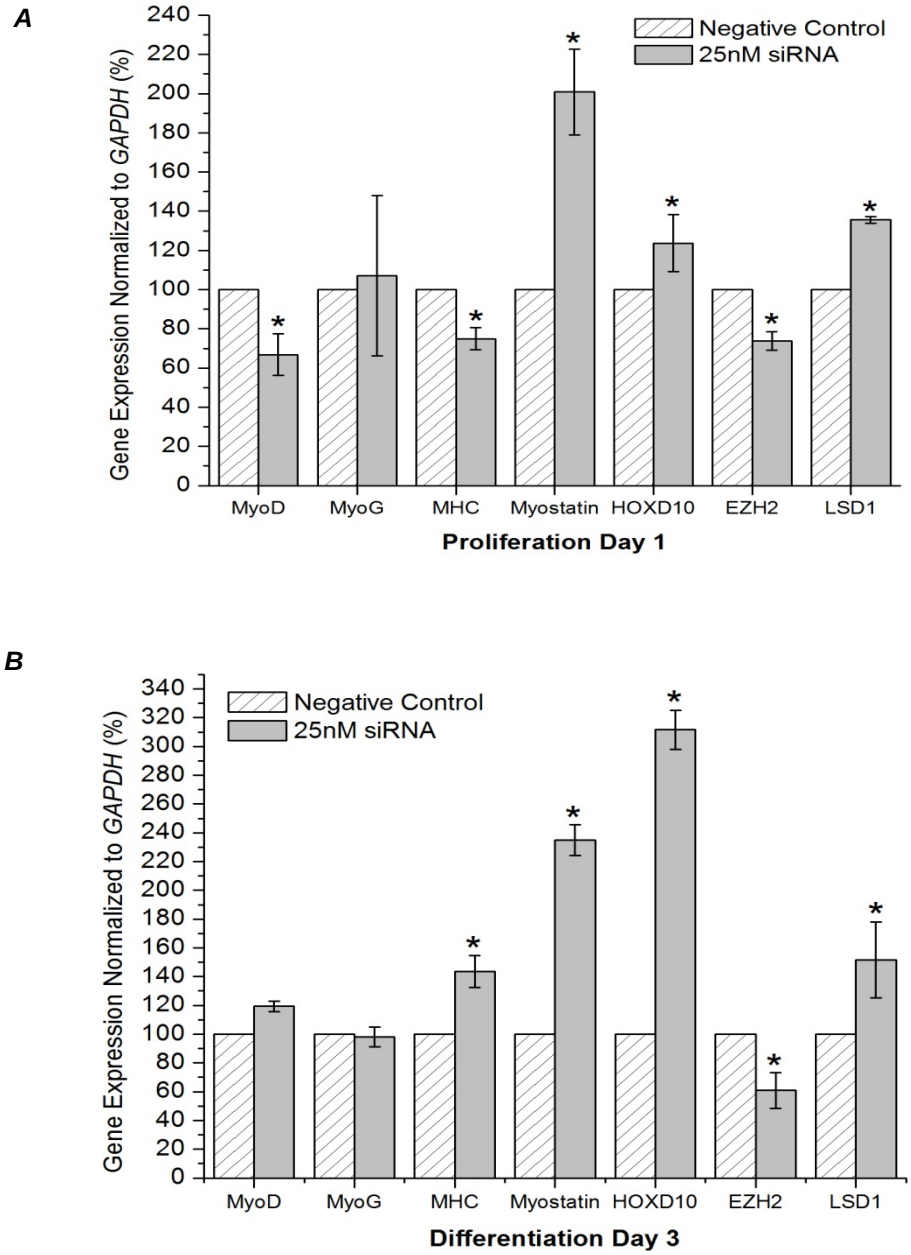


Figure 3-6 **RT-qPCR results of myogenic markers (*MyoD*, *MyoG*, *MHC*, *Myostatin*), *HOXD10*, *EZH2* and *LSD1* on day 1 of proliferation and day 3 of differentiation after transfection with 25nM of HOTAIR siRNA.** (*A*) Gene expression of myogenic markers

(with the exception of *MyoG*) and *HOTAIR* associated genes significantly changed in expression on day 1 of proliferation when normalized to GAPDH, which was unaltered in the negative control: *MyoD*, *MHC*, and *EZH2* decreased; *Myostatin*, *HOXD10* and *LSD1* increased. (*B*) The RT-qPCR experiment was repeated on day 3 of differentiation. With the exception of *MyoD* and *MyoG*, all of the measured genes were significantly changed: *MHC*, *Myostatin*, *HOXD10* and *LSD1* increased; *EZH2* decreased. (*N=3*, *p<0.05*)

Partial knockdown of HOTAIR with siRNA confirms the inhibition of myoblast differentiation observed in the antisense DNA studies

To further validate the preliminary finding that *HOTAIR* knockdown by antisense DNA inhibits myoblast differentiation, we replicated the MHC and DAPI staining after transfecting C2C12 cells with *HOTAIR* siRNA (25nM). Additionally, given that the RT-qPCR results indicated enhanced up-regulation of *Myostatin* we were interested in discovering the effect of gene expression on the MHC protein phenotype. As conducted in our preliminary experiment with antisense DNA, the growth media was exchanged with differentiation media containing horse serum to stimulate myoblast fusion into myotubes. The cells were visualized daily for phenotypic changes compared to the negative control and systemically analyzed by immunofluorescence on days 3 and 4 of differentiation (Fig 3-7). Six random image areas were used to quantify 40-100 myotubes per specific condition/treatment. As observed in the antisense DNA preliminary experiment, cells treated with *HOTAIR* siRNA experienced a significant reduction in the expression of MHC compared to the negative control on days 3 and 4 of differentiation as measured by the fusion index. Additionally, as observed in the preliminary experiment, the myotubes expressed by the transfected cells appeared thinner and less robust compared to the negative control.

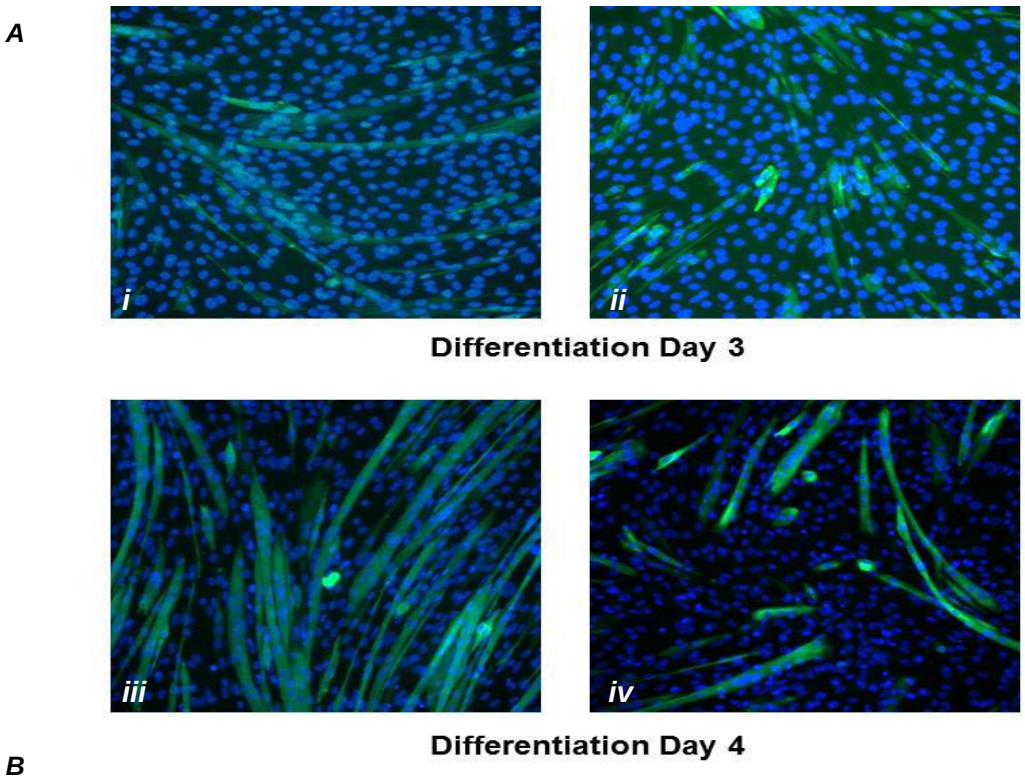


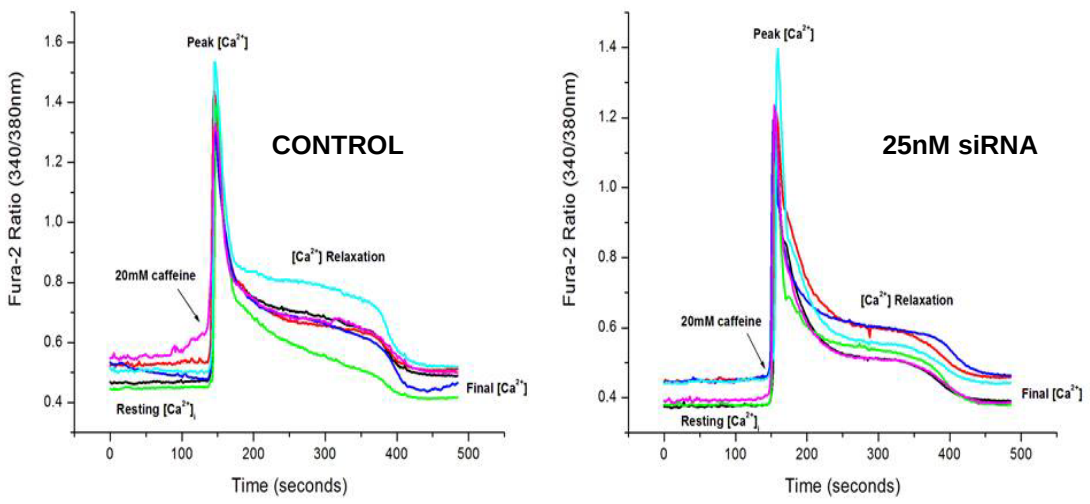
Figure 3-7 **Partial knockdown of *HOTAIR* in C2C12 cells effectively reduced fusion index after transfection with *HOTAIR* siRNA** (*A*) Representative immunofluorescence

images of DAPI-stained nuclei (blue) and Myosin Heavy Chain (MHC) antibody (green) of C2C12 cells on days 3 and 4 of differentiation after transfection: (i, day 3; iii, day 4) negative controls (ii, day 3; iv, day 4) 25nM *HOTAIR* siRNA treatment. (B) Fusion index data shows a significant decrease in mature myotubes expressing MHC after treatment with *HOTAIR* siRNA compared to the negative control. (*N=6*, *p<0.05)*

Partial HOTAIR knockdown reduces resting levels of intracellular calcium and calcium release from the sarcoplasmic reticulum (SR)

Calcium is an important intracellular signaling molecule and essential for skeletal muscle contractions and movement. Disturbance of calcium homeostasis can result in a number of pathological disorders and dysregulation of skeletal muscle functioning.[60e, 65] To investigate the influence of *HOTAIR* on resting intracellular calcium and SR calcium release, we monitored Fura-2 intracellular calcium in responses to caffeine-induced SR calcium release. In *HOTAIR*-siRNA treated C2C12 myotubes (25nM siRNA) at day 5 of differentiation, compared to the blank control, resting Ca^{2+} levels of the HOTAIR-siRNA treated cells decreased 14% (0.442 ± 0.08 versus 0.514 ± 0.08), the amplitude peak Ca^{2+} response to caffeine (i.e. time measured between the initial caffeine response to amplitude peak) was reduced by 33% (5.59 ± 2.19 versus 4.20 ±1.64), the peak level was reduced by 8.1% (0.723 vs 0.788) and the relaxation phase (ie. time to return from peak to baseline) as measured by exponential decay was 5.35% shorter (23.5 ± 0.876 versus 24.7 ± 0.769). Integration of the calcium transient peak correlates with total intracellular calcium levels. For the HOTAIR-siRNA treated cells, the intracellular calcium level as indicated by the area under the curve was reduced by 10% (262.2 vs. 291.4) (Fig 3-8).

A



B

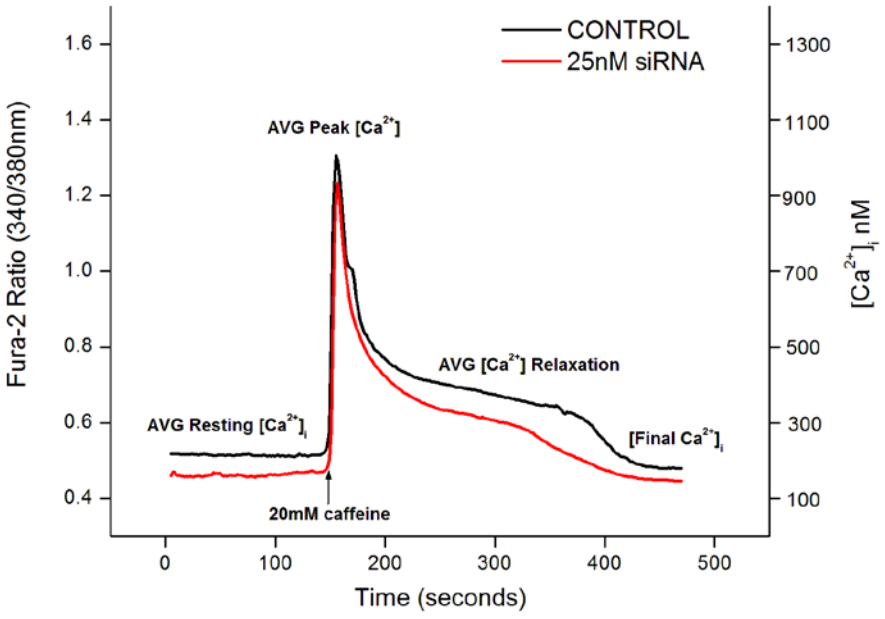


Figure 3-8 ***HOTAIR* knockdown alters the calcium transients induced by 20mM caffeine of C2C12 cells loaded with Fura-2/AM**. (A) In *HOTAIR*-siRNA treated cells on day 5 of differentiation, the resting calcium baseline was reduced, the time to peak was

longer and the relaxation phase of the transient was significantly shorter from that of the blank control. (*B*) Average calcium transients of the control and 25nM *HOTAIR*-siRNA treated cells loaded with Fura-2/AM. For *HOTAIR*-siRNA treated cells, the intracellular calcium concentration as measured by integration of the area under the curve is 10% less than the control. (N=16 blank control, N=12 siRNA, $p<0.05$)

Partial HOTAIR knockdown in C2C12 cells up-regulates genes within the NFκB and Oxidative Stress pathways

To explore the molecular mechanisms behind the significant alteration of myogenic gene expression and calcium homeostasis in *HOTAIR*-siRNA treated cells, a real-time PCR gene array was employed using the Mouse Signal Transduction Pathway PCR array. Differences in the gene expression of the control and the siRNA treated cells were measured across 10 different pathways as shown in Chapter 2, Table 2-2. Among the 84 genes profiled, we found two genes significantly altered (Fig. 3-9). In the *HOTAIR*-siRNA-treated cells the expression of Chemokine (C-C Motif) Ligand 5 (*Ccl5)* and NAD(P)H Dehydrogenase Quinone 1 (*Nqo1)* were significantly increased by 3.36 and 2.25 fold, respectively. These genes are directly related to the NFκB and Oxidative Stress pathways. These results suggest that *HOTAIR* may function through the NFκB and Oxidative Stress pathways to regulate myoblast differentiation and calcium homeostasis.

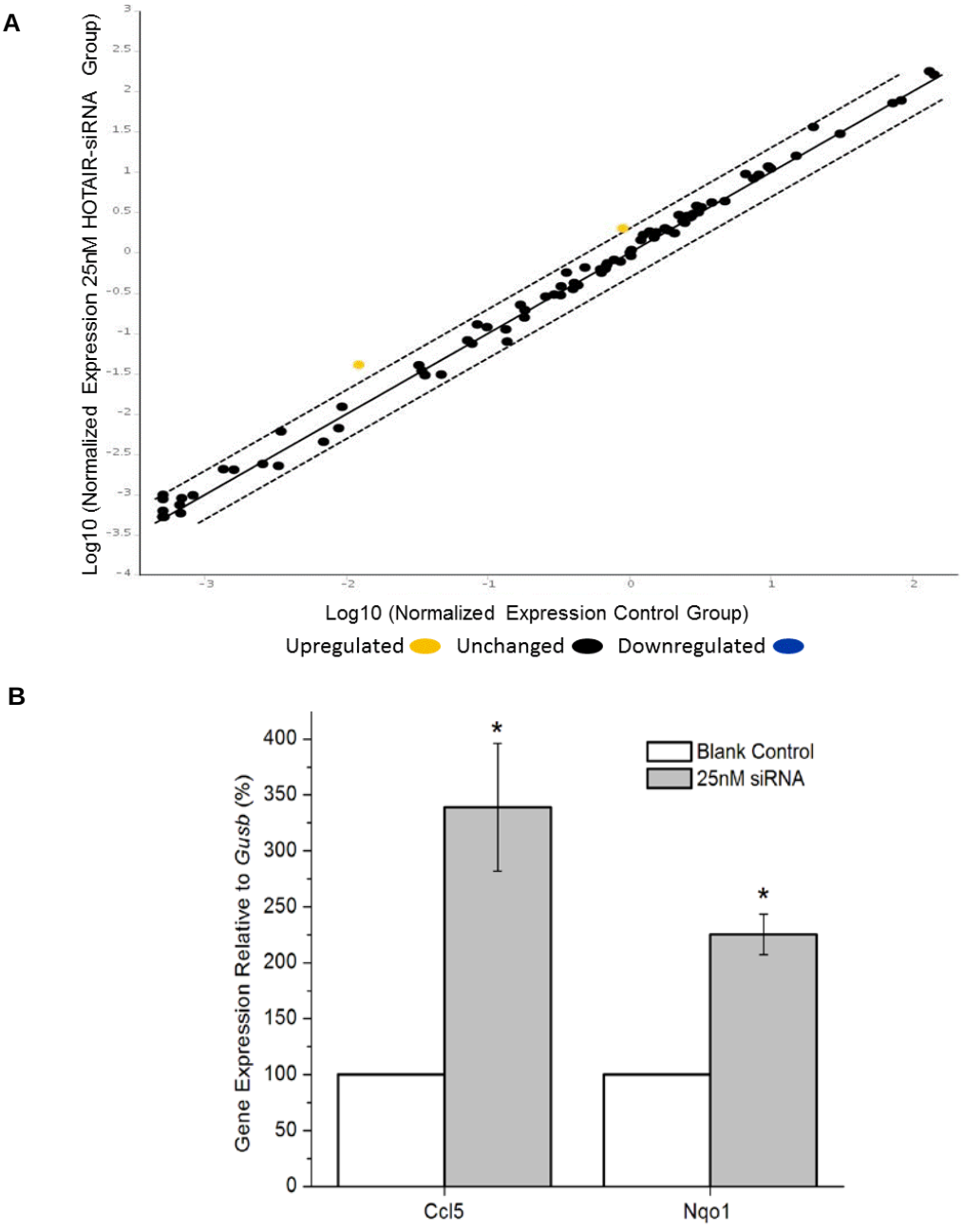


Figure 3-9 **Gene array profiling results of 84 genes belonging to 10 canonical pathways monitored in *HOTAIR*-siRNA treated cells.** (*A*) Normalized gene expression of all 84 gene array genes compared between the *HOTAIR*-siRNA treated cells and the

blank control. The central line indicates unchanged gene expression. Significantly up-regulated genes (fold change greater than 2.00) are shown in yellow, unchanged genes are shown in black and down regulated genes are shown in blue. (*B*) Gene expression of *Ccl5* and *Nqo1* show significant up-regulation in the *HOTAIR*-siRNA treated cells compared to the blank control when normalized to *Gusb* (Beta-D-Glucuronidase), which was unaltered in the blank control. ($N=3$, $p<0.05$)

Chapter 4

DISCUSSION AND FUTURE DIRECTIONS

Long non-coding RNAs (lncRNAs) are an extensive class of molecules that were dismissed for several years as non-functional cellular "junk" entities on account of their ambiguous sequence function and poor conservation across species.[66] In recent years, however, lncRNAs have stimulated increased interest due to developing insights on their functional intracellular roles. Several studies have cited lncRNAs as transcriptional, translational and epigenetic regulators.[41, 66-67] Hox Transcript Antisense Intergenic RNA (*HOTAIR*) is one example of a lncRNA that has been reported as an epigenetic transcriptional regulator. *HOTAIR* functions as a molecular scaffold through recruitment of Polycomb Repressor Complex 2 (PRC2) and Lysine-Specific Demethylase 1 (LSD1) to chromatin for repression of the homeobox gene D (HOXD) cluster.[41-42, 43, 64, 68] While HOX genes are crucial in embryonic development, coincident with its pivotal role in gene regulation, *HOTAIR* has been extensively studied as an oncogenic molecule.[42a, 44, 46a, 54, 69] Although there are several studies on the dysregulation of *HOTAIR* in cancer progression, there is still much to be understood about its overall systemic and metabolic effect.

Previous studies on lncRNAs in skeletal muscle reveal that transcription regulation through chromatin reorganization is critical during myogenesis, the concerted process of skeletal muscle generation and formation.[70] For example, current evidence suggest that the muscle-specific long noncoding RNAs, *linc-MD1* and *LncMyod* work together during the terminal differentiation stage of myogenesis to enhance myoblast fusion into multinucleated myotubes.[71] The terminal differentiation stage in myogenesis is a tightly regulated process that is necessary for proper development and one that may involve collaboration with additional unexplored factors like *HOTAIR*. Recent studies on

LSD1 and EZH2, a subunit of PRC2, suggest a potentially critical role for *HOTAIR* in skeletal muscle. Reportedly, silencing *EZH2* in C2C12 cells significantly impairs muscle cell growth and reduces differentiating myotubes.[5, 46c, 48b] Similarly, inhibiting *LSD1* in C2C12 cells hinders the activation of myogenic factors such as MEF2D that are involved in myogenic gene up-regulation during differentiation.[72] Given the reported effects of EZH2 and LSD1 on skeletal muscle and their close association with *HOTAIR*, in the present study we aimed to decipher the mechanistic role of *HOTAIR* in skeletal muscle and we demonstrate that it is a functional entity required for myoblast differentiation.

In our pilot study we used antisense DNA to silence *HOTAIR* in C2C12 cells., Decreased fusion index and reduced expression of Myosin Heavy Chain (*MHC*) was found upon *HOTAIR* knockdown. Coincident with the decrease in MHC expression in differentiated myotubes was the increase in gene expression of the myogenic inhibitor, *Myostatin*. This observation provided the impetus for developing our final, systematic studies with *HOTAIR* siRNA. Remarkably, as we observed in the pilot studies, C2C12 cells treated with *HOTAIR* siRNA experienced a significant reduction in fusion index and in MHC expression compared to the negative control. In parallel, there was a significant reduction of the myogenic regulatory gene, Myogenic Determination Protein (*MyoD*) in proliferating myoblasts and an up-regulation of *Myostatin* in differentiating myotubes. Interestingly, *EZH2* expression was also significantly reduced in proliferating myoblasts and differentiating myocytes treated with *HOTAIR* siRNA. In accord with substantiating work that signifies the role of HOTAIR in gene regulation through its recruitment of PRC2 to chromatin, a reduction in *HOTAIR* expression may explain the reduction of *EZH2* expression given that the EZH2 protein is a subunit of PRC2. Moreover, the reduction of *EZH2* expression in relation to the observed inhibitory effects of *HOTAIR* knockdown on myoblast differentiation aligns with current evidence that suggests the regulatory

importance of EZH2 in myogenic differentiation.[47, 48b] Conversely, whereas *EZH2* expression was reduced upon *HOTAIR* knockdown, LSD1 expression significantly increased in myoblast proliferation and differentiation. This result may potentially be a compensatory effect induced by *EZH2* reduction or myogenic inhibition. Additionally this finding provides more clinical insight to recent reports that suggest that mutations in LSD1 contribute to deficiencies involving mental retardation and skeletal anomalies.[73]

We end our systematic studies with an examination of the effect of *HOTAIR* knockdown on intracellular calcium homeostasis and the potential molecular mechanisms that might help begin to explain the roles of *HOTAIR* in skeletal muscle myogensis. Our calcium imaging results show that intracellular calcium homeostasis was altered in *HOTAIR*-siRNA treated myotubes. Resting levels of calcium, calcium-release and re-uptake from the sarcoplasmic reticulum (SR) are largely dependent on myotube functionality.[74] As noted from the fusion index of HOTAIR-siRNA treated C2C12 cells, the inhibition of differentiating myocytes is consistent with the reduced intracellular calcium release and re-uptake observed in our calcium imaging studies, in that, it would be expected that less mature myotubes would release less calcium from the SR. To begin to study the molecular mechanism of the effects of *HOTAIR* on C2C12 cells, we used the Mouse Signal Transduction PathwayFinder PCR array and found that the expression of two genes (*Ccl5* and *Nqo1*) were significantly altered. Chemokine (C-C Motif) Ligand 5 (*Ccl5*) is located on murine chromosome 11 downstream from NFκB.[75] Chemokines are typically involved in immunoregulatory and inflammatory processes. The earliest evidence of chemokine influence on skeletal muscle showed that *Ccl5* increased myoblast migratory activity.[59b, 76] Later work suggested that upregulaton of *Ccl5* expression in Duchenne muscular dystrophy could potentially attenuate myofiber necrosis.[77] In very recent work, the Brotto and Karasik Laboratories (Huang et al) tested

the in vitro data originating from 40,000 humans from Genome Wide Association Studies (GWAS) and found that *Mettl21C* appeared to be a pleiotropic gene for bone and muscle.[59b] They also found that silencing of *Mettl21C* resulted in the upregulation of Ccl5 and very similar phenotypic changes in C2C12 myotubes as we find in our study.[59b] Therefore, the upregulation of *Ccl5,* a key gene of the NFκB Pathway*,* in C2C12 cells after *HOTAIR*-siRNA mediated knockdown may suggest a potential mechanism through which *HOTAIR* modulates skeletal muscle myogenesis.

As for NAD(P)H Quinone Oxidoreductase 1 (*Nqo1*), which expresses one of the phase-II antioxidant enyzmes that catalyzes the two-electron reduction of quinones, the observed upregulation caused by *HOTAIR*-siRNA knockdown may be the result of an increased production of oxidative species.[78] Perhaps less of a coincidence, the disturbance in the observed higher levels of resting calcium and calcium release in *HOTAIR*-siRNA treated cells is symptomatic of perhaps defective excitation-contraction coupling at the ryanodine receptor (RyR) due to oxidative stress, known to induce a "leaky" channel.[79] Moreover, the observed upregulation of *Ccl5* and *Nqo1* correlates with trends observed in skeletal muscle dystrophy. In dystrophic muscles, as time progresses, the repetitive cycling of muscle damage and repair can exhaust the regenerative capacity of the tissue and result in persistent inflammation.[80] Previous studies that examined oxidative stress in DMD patients and muscular dystrophic mice (mdx) have suggested that dystrophic muscles may have increased susceptibility to oxidative stress which may trigger an inflammatory response and result in NFκB activation.[81] The implication for *HOTAIR* in this process is an unexplored area that can direct future studies. In summary, we explored for the first time the role of *HOTAIR* in skeletal muscle differentiation, calcium homeostasis, and signaling pathways. We demonstrate that *HOTAIR* inhibition reduces myoblast differentiation and alters calcium

homeostasis in mouse skeletal muscle cells, potentially via two genes related to the NFκB and Oxidative Stress pathways. These studies should provide the basis for in-depth studies of *HOTAIR* function in skeletal muscle by extending its exploration to primary murine and human models as well as animal models.

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Biographical Information

Fritzlaine Roche was born in Port-au-Prince, Haiti during a turbulent political unrest that threatened the safety of her family. As a result, Fritzlaine immigrated to the United States in 1994 as a refugee. Her family settled in Kansas City, Missouri where she lived for the majority of her life. Fritzlaine attended the prestigious public high school, Lincoln Preparatory Academy in Kansas City, Missouri and graduated in the top 5% of her class with a full merit scholarship to Wesleyan University in Middletown, Connecticut. At Wesleyan, Fritzlaine deeply immersed herself in the liberal arts curriculum and took interesting courses in French, Japanese, Spanish, music, tennis, literature and science while also participating in multiple collegiate activities.

While in college, Fritzlaine also engaged in diverse research projects during her summer breaks. Most notably, she was one of few students selected among thousands of applicants to participate in the Summer Medical Dental & Education Program at Case Western University in Cleveland, Ohio and the Summer Research Opportunity Program at Northwestern University in Evanston, Illinois. Her summer experiences stimulated her motivation to continue in the field of research. Thus, during her two post baccalaureate years of teaching high school science through the Teach For America program in Kansas City, Missouri, Fritzlaine, also volunteered in research labs, most notably that of her mentor, Dr. Marco Brotto.

Fritzlaine, along with the majority of the Brotto Lab moved to the University of Texas-Arlington in the Fall of 2015. Shortly after her move Fritzlaine commenced her Master studies in Chemistry. Upon her completion of her Master studies, Fritzlaine plans to matriculate to the University of Rochester Medical School in Rochester, New York in hopes of contributing to the holistic practice of medicine involving health, research and social justice.