Investigating the Relationship Between the Molecular Circadian Clock and Muscle Satellite Cell Fate

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Investigating the Relationship Between the Molecular Circadian Clock and Muscle Satellite Cell Fate

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Defense April 8, 2014

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Abstract

Skeletal muscle is a dynamic tissue capable of rapid regeneration. The functional unit of skeletal muscle, known as myofibers, contains terminally differentiated, post-myototic myonuclei. These myofibers are often damage as a result of injury, exercise, natural turnover, or disease. Upon trauma, a rare population of muscle stem cells, known as satellite cells, mount a coordinated regenerative response to repair the damage. The regenerative response is wellorchestrated and precisely coordinated in time such that the myofiber that exists after regeneration is identical to the myofiber that existed before trauma. The coordination of the individual events within the regenerative response is currently uncharacterized, but is potentially facilitated by the molecular circadian clock. The molecular clock is a tightly regulated feedback loop that oscillates over a 24 hour period and functions to coordinated various cellular functions. I am interested in understanding if the molecular clock coordinates the timing of the regenerative response by examining differentiation and quiescence. Using qRT-PCR, I examined the mRNA levels of three key genes expressed at different points throughout regeneration including the satellite cell specific gene Pax 7 and the differentiation markers myogenin and Myf6. In a myoblast cell line and in primary cells treated with chemical circadian inhibitors, Pax7 was unregulated whereas myogenin and My6 were down regulated. Using immunofluorescence, I found that myogenin was expressed less when the cells were treated with circadian inhibitors. I also used immunofluorescence to determine that circadian inhibition increased the number of undifferentiated cells that were not actively in the cell cycle. Overall, phenotypes of cells treated with circadian inhibitors are more undifferentiated than cells with a functioning clock, suggesting the clock plays a critical role in managing and coordinating regeneration in skeletal muscles.
Chapter 1: Introduction

Skeletal Muscle

Function

Skeletal muscle is a dynamic tissue involved in many essential activities including respiration, chewing, swallowing, and other types of mechanical motion (Sambasivan et al., 2011). Skeletal muscle tissue constitutes nearly 38% of body mass in men and 30% in women, making it the most abundant tissue in the body (Janssen et al., 2000). Skeletal muscle employs a remarkable regenerative capability allowing it to respond rapidly to physiological stimuli including injury, exercise, and natural turnover (Shi et al., 2006).

Structure

Structurally, skeletal muscles are composed of a highly organized arrangement of myofibers that are arranged in parallel from one tendon to another and generate a striated pattern (Sambasivan et al., 2011). Myofibers contain hundreds of terminally differentiated myonuclei that result from the fusion of myoblasts during embryonic development (Relaix and Zammit, 2012). Myofibers serve as the functional units of the skeletal muscle and are able to generate force by contraction of the internal units known as sarcomeres (Relaix and Zammit, 2012). As muscles grow, sarcomeres are added to the ends of existing myofibrils. Sarcomeres form a repeating transverse pattern and contain alternating segments of myosin light chain, known as thick filaments and actin filaments, known as thin filaments. Muscle cells contain thousands of multinucleated myofibrils that are comprised of alternating thick and thin filaments. At rest, mammalian sarcomeres are 2.4 um in length but can extend and contract to 3um and 2 um respectively. To contract, the head groups of myosin proteins reversibly bind to actin filaments
and hydrolyze adenosine triphosphate (ATP) resulting in the release of ADP and inorganic phosphate and the production of a mechanical impulse that transmits along the actin and myosin filaments within a sarcomere. Concurrent and successive contractions of sarcomeres shorten to generate force resulting in macroscopic movement.

**Satellite Cells**

*Discovery and Characterization*

Skeletal muscles are constantly stressed as a result of natural turnover, severe injury, and physiological stimuli including intensive exercise (Shi et al., 2006). In response to such stress, skeletal muscles mount a well orchestrated regenerative response to restore the mechanical function and cellular architecture of myofibers (Shi et al., 2006). The regenerative capacity of muscle is potent such that within 12 days post injury, skeletal muscle architecture is nearly resorted, and by 30 days post injury the muscle tissue appears completely repaired (Figure 1). The remarkable regenerative capacity of skeletal muscles is dependent upon a population of adult stem cells, deemed satellite cells, located peripherally on the myofiber between the plasma membrane and basil lamina (Mauro, 1961; Shi et al., 2006). The rare SC population was first visualized using electron microscopy in the tibialis anticus of an adult male frog and characterized as a unique cell population of mononucleated cells that adopt nuclear shape due to the lack of

![Image](image.jpg)

**Figure 1:** Skeletal muscle is capable of complete and rapid regeneration. 3 days post injury, the tissue is in disarray and the muscle cells are unorganized and dead. 12 days post injury the tissue is almost completely regenerated with highly organized architecture. By 30 days post injury, the muscle tissue is almost identical to the uninjured muscle tissue.
substantial cytoplasm (Maruo, 1961). The intimate association between satellite cells and the multinucleated myofibers suggest that these cells might play a critical role in muscle regeneration (Mauro, 1961). Examination of the satellite cell population in the web muscle of fruit bat wings and the hind leg muscles of mice and rats using microscopy led to further structural characterization including the identification of centriole, a critical protein involved in the organization of mitotic spindle during cellular division (Muir et al, 1965). Identification of undifferentiated cytoplasmic components, small mitochondria, and irrelevant Golgi apparatus suggested satellite cells have undifferentiated fate (Muir et al, 1965). In mammalian muscle, undifferentiated satellite cells located on the periphery of damaged myofibers resemble the morphology of “young myoblasts” (Shafiq and Gorycki, 1965). The satellite cells appeared to be more abundant in areas of myofibre trauma, specifically in areas where the myofibers had retracted such that only the basil lamina was present, than in uninjured muscle tissue (Shafiq and Gorycki, 1965). Satellite cell response to crush injuries in the web muscle of fruit bats results in a decreasing number of satellite cells that coincide with the emergence of myoblasts successively fusing into myofibers (Relaix and Zammit, 2012). Indication that the satellite cell pool had been repopulated was subsequently demonstrated by the reemergence of the seemingly depleted satellite cell population on the myotubes (Relaix and Zammit, 2012).

**Function**

Since their discovery, satellite cells have been shown to have potent regenerative capabilities while also employing the ability to self-renewal, thus fulfilling the requirements to be classified as a stem cell (Figure 2). Lineage tracing in growing muscles showed that a satellite cell division could give rise to one progeny that retained satellite cell characteristics while the other progeny differentiated into a myonucleus (Moss and Leblond, 1971). Grafting an isolated...
myofiber with associated satellite cells from an adult resulted in large numbers of donor derived satellite cells than originally transplanted suggesting extensive proliferation of the original satellite cells to produce satellite cell progeny (Collins et al., 2005). In an ablation study that eliminated any cell expressing Pax7, a protein characteristic of satellite cells, the regenerative capacity of skeletal muscles was completely diminished (Murphy et al., 2011). This data suggests skeletal muscle is unable to regenerate after injury in the absence of satellite cells.

Therefore, satellite cells are necessary for regeneration of skeletal muscles and severe muscular pathologies can result from perturbation of satellite cell function or a diminished satellite cell pool.

**Pathologies**

In muscle pathologies including Duchene muscular dystrophy (DMD) and polymyositis, satellite cells are over active, as suggested by their expansive cytoplasm and large amounts of ribosomes and rough endoplasmic reticulum (Laguens, 1963; Shafiq et al., 1967). Extensive activation of the satellite cells results in exhaustion of the regenerative capacity and ultimate depletion of the pool, resulting in progression of these pathologies and overall muscular

---

**Figure 2: Schematic of muscle regeneration.** A.) The muscle fiber is myonucleated with rare quiescent satellite cells located peripherally. B.) Upon injury, satellite cells activate. C.) Upon activation, the satellite cells proliferate giving rise to committed myoblasts. D.) The committed myoblasts fuse to the myofiber at the point of injury. Satellite cells undergo self-renewal to replenish the satellite cell pool.
deterioration (Shi et al., 2006). In age associated muscle wasting known as sarcopenia, the satellite cell pool is diminished also resulting in a crippled ability to regenerate (Wagers and Conboy, 2005). In healthy, uninjured skeletal muscle tissue, satellite cells are maintained in a low metabolic, non-dividing state known as quiescence. Upon injury, satellite cells activate rapidly and a subset undergo asymmetric division resulting in two daughter cells. One daughter cells returns to quiescence to replenish the satellite cell pool while the second daughter cell forms a transit-amplifying population of myoblasts that eventually differentiate and fuse to repair the damaged myotube. Activation of satellite cells occurs when endogenous signals, primarily hepatocyte growth factor (HGF) and tumor narcosis factor alpha (TGFα) bind to receptors specific to quiescent satellite cells (Janich et al., 2011).

Circadian Rhythm

Organismal and Molecular Function

Circadian rhythms are near daily cycles that regulate change in metabolism, physiology, and behavior that function to prepare organisms for daily changes in the environment. The master clock is the suprachismatic nucleus located in the hypothalamus and controls organism wide circadian behaviors most notably sleep and feeding. Specialized ganglion cells located in the retina express photoreceptors that project directly to the SCN and help to coordinate organismal behaviors with the light/dark cycle of the environment. Light represents the most well studied circadian time cue, also known as a zeitgeber, while other stimuli including feed and exercise are being studied as altering the molecular clock. Though destruction of the SCN results in a disrupted sleep/wake cycle, humans kept in complete darkness are able to maintain a sleep/dark cycle based on their internal clock and are not reliant on environmental cues.
In addition to the SCN, peripheral tissues and nearly all cell types, including skeletal muscle, employ an autonomous, intrinsic molecular clock that is thought to prepare cells for daily environmental changes. The molecular clock is maintained by a tightly regulated feedback loop (Figure 3). In the SCN and many peripheral tissues, a core component of the clock, Bmal1 (Brain muscle arnt-like 1), is expressed in a circadian pattern, oscillating over 24 hours. The other core component of the clock, CLOCK (Circadian Locomoter Output control kaput), does not exhibit circadian expression patterns in the SCN or in peripheral tissues. However, the translocation of CLOCK occurs most frequently during the day as compared to at night suggesting the nuclear to cytosolic distribution of CLOCK is circadian in nature. The core components of the molecular clock, CLOCK and Bmal1, are transcribed then heterodimerize to initiate transcription of their negative regulators Period (Per1/2/3) and Cryptochrome (Cry1/2) by binding to the E box of these genes’ regulatory regions. In the second regulatory loops, CLOCK and Bmal1 initiate transcription of REV-ERBa/b and ROR genes that feedback to negatively and positively regulate transcription of Bmal1, respectively. Recently, the core components of the molecular clock have been found to have a broad variety of down stream transcriptional targets (Figure 4). Additionally, many genes not involved in time keeping, known as clock controlled

**Figure 3: Schematic of core circadian clock network.** The core components of the clock. Bmal1 and CLOCK heterodimerize to activate transcription of Period and Cryptochrome. The CRY and PER proteins form a complex that negative regulates the clock by inhibiting Clock:Bmal1 activity. The Rev-Erb and ROR genes repress and activate Bmal1 transcription, respectively.
genes (CCG) are being characterized as having circadian rhythmicity, meaning the expression of these genes changes across a 24 hour time period, but these expression levels repeat every 24 hours.

**Role in Skeletal Muscles**

In skeletal muscles, whole tissue assays revealed hundreds of genes that exhibit circadian-like oscillations while nearly 30% of those transcripts lost their rhythmicity in Clock-mutant mice indicating the clock plays a central role in maintaining proper expression of these genes (Miller et al., 2007; Zhang et al., 2009). Myogenic differentiation 1 (MyoD), a gene activated during the early stages of differentiation and considered a key regulator of muscle differentiation, emerged as a CCG initially because of its distinctly circadian expression pattern in adult muscle (figure from mmb and paper with MyoD RNA levels) (Andrews et al., 2010). Knocking out core components of the molecular circadian clock causes defects in MyoD levels, expression of MyoD target genes, and disruption in myofiber organization and overall muscle function (Andrews et al., 2010; Mendez-Ferrer et al., 2008, Miller et al., 2007). CLOCK and Per1 mutant mice displayed perturbed expression patterns of MyoD and consequently disorganized myofibers and diminished muscle function (Figure 5). Additionally, Bmal1 mutant mice displayed reduced life span and age-associated sarcopenia, though interestingly, de novo muscle development was...
not hindered suggesting Bmal1 is not pivotal in muscle development (Kondratov et al., 2006). CLOCK, Per2, and ROR mutants exhibited pathologies including mitochondrial abnormalities, contractile and locomotor defects, and muscle weakness (Kondratov et al., 2006).

Role in Stem Cell Fate in Other Tissue Systems

In other systems, stem cells exhibit sensitivity to perturbations in the molecular clock. In adipose derived stem cells, clock genes exhibit circadian oscillations and are responsive to clock perturbations (Wu et al., 2007). RNAi mediated inhibition of Bmal1 prevents cells from differentiating into mature adipose cells while overexpression of Bmal1 causes the cells to increase expression levels of transcripts signifying early stages of adipose differentiation (Shimba et al., 2005). In the epidermis, the circadian clock had been shown to control epidermal stem cell responsiveness to the activation associated signaling pathway Tgf-beta (Janich et al., 2011). Genome-wide screening data suggests Fgfr1 and Igf2 knockdown, two key regulators of satellite cell function, results in molecular clock defects (Zhang et al., 2009).

Preliminary Data

Clock Manipulation Affects Myogenic Differentiation

Examination of the clock perturbation in MM14s, an FGF-dependent immortal myoblast cell line, uses chemical and genetic inhibitors. The chemical inhibitors used were the small molecule drugs KL001 and KL002 (Cayman Chemical), both of which prevent ubiquitin

Figure 5: MyoD mRNA expression exhibits circadian oscillations. Expression of MyoD transcript in wildtype (blue) and clock-deficient mice (red) over circadian time (CT). Data was collected every four hours starting at CT 18. Data was curated from GEO dataset GSE3751 (Miller et al. 2007).
dependent degradation of Cryptochrome 1 and 2, and GSK 4112, an agonist of REV-ERB alpha and inhibitor of Bmal1.

Importantly, all of these drugs disrupt circadian oscillations by potentiating negative regulators of the core machinery. In the first assay, MM14 cells were either a) transduced with short hairpin RNAs (shRNAs) targeting clock components, or b) treated with the above-mentioned small molecule drugs and briefly stimulated to differentiate. Differentiation was examined using flow cytometry to quantify expression of myogenin, a late differentiation marker in myogenesis. shRNA knockdown of Bmal1 and Clock yielded similar results to pathway inhibition by small molecules. As shown in figure 6, both chemical and genetic inhibition approaches resulted in a decrease in the percentage of cells expressing myogenin.

Immunostaining and subsequent quantification of differentiation capacity yielded similar findings (Figure 7). The MM14 cells line was transfected with a vector encoding a shRNA targeting Bmal1 as well as the florescent marker mCherry. Importantly, mCherry expression permits rapid identification and tracking of individual cells expressing the Bmal1 shRNA. In the cells infected with the shRNA Bmal1 construct, 23% of cells fused into myotubes while 42% of cells with the scramble control shRNA construct differentiated and fused into myotubes. The
decreased percentage of infected cells fusing into myotubes suggests cells with perturbed circadian clocks are unable to differentiate as efficiently as cells in which the clock is functioning properly.

_Differentiation commitment assay_

To more precisely interrogate the differentiation defect observed using immunofluorescence and flow cytometry, a clonal differentiation commitment assay was preformed. This assay takes advantage of the fact that MM14 cells irreversibly commit to differentiation if FGF is not present during the G1 phase of the cell cycle. Additionally, as MM14 cells detach from culture dishes during mitosis, a ‘mitotic shake off’ can be used to collect and synchronize cells just prior to G1 entry (Figure 8). Thus, following a mitotic shake off, all MM14 cells supplemented with FGF2 will form colonies of cells when plated at clonal density 24 hours following shake off, whereas cells...

**Figure 7: Clock manipulation affects myogenic differentiation.** A.) Immunofluorescence staining of MM14 cells infected with Bmal1 knockdown shRNA construct after 72 hours. mCherry+ cells (red) have been infected with shBmal1. Visualization of mCherry+ single cells and mCherry+ cells fused into myotubes. B.) Quantification of mCherry+ single cells and mCherry+ cells fused into myotubes in scramble shRNA, shBmal1-1, and shBmal1-2.

**Figure 8: Schematic of differentiation commitment assay.** Mitotic shake off synchronizes cells in G1. Synchronized cells floating in media are replated into 6 plates. Cells grow in media after the addition of circadian inhibitor for 24 hours. Cells are washed and re-plated in new media containing FGF2 for 6 days. Differentiated cells do not form colonies.
cultured for 24h in the absence of FGF2 will not form colonies as they have committed to a post-mitotic differentiation state. To test if a functional clock was required for differentiation commitment, ‘mitotic shake off’ MM14 cells were then treated with either FGF2, a p38 MAPK inhibitor (SB203580) or the circadian drugs GSK 4112, KL001, and KL002. The cells treated with circadian inhibitors were able to partially prevent differentiation similar to the p38 inhibitor (a mediator of FGF2 signal transduction and thus a positive control). This suggests that circadian perturbation may play a critical role in either preventing differentiation or otherwise maintaining the primitive state (Figure 9).

Central Aims and Hypothesis

The regenerative process of skeletal muscle is well orchestrated in time such that the myofiber after healing is identical to the myofiber that existed before trauma. This requires the regenerative response to be precisely coordinated in time. In order to determine if the clock is controlling cell fate throughout the regenerative process, I will examine differentiation and quiescence. More specifically, I will examine the effect of circadian perturbation on the percentage of cells that are differentiated and quiescent. The central aim of this thesis is to
determine if the circadian clock regulates satellite cell differentiation and/or quiescence. Given the preliminary data from the MM14 myoblast cell line, I expect that circadian perturbation will affect satellite cell function in primary muscle satellite cell cultures. Using two primary satellite cell culture techniques, I will examine SC differentiation capacity as well as regulation of quiescence using immunofluorescence and confocal microscopy based approaches.

Chapter 2: Methods and Materials

**RNA Extractions**

To extract RNA for qRT-PCR, we use the RNeasy Mini Kit from Qiagen to extracted RNA from MM14 myoblasts grown in growth media, differentiation media, and treated with DMSO (vehicle), KL001 (1 uM, 10 uM), GSK 4112 (2 uM, 5 uM, 20 uM), and KL002 (2 uM, 20 uM) for 72 hours. RNA from primary cell cultures is extracted from samples treated with DMSO (vehicle), GSK (20 uM), and KL001 (20 uM) for 72 hours. A maximum of $10^7$ cells are harvested from the respective samples, pelleted, then resuspended in 350 uL Buffer RLT. One volume of 70% ethanol is added to lysate and thoroughly mixed via pipetting. Up to 700 uL of the sample is transferred to RNeasy mini spin columns in 2 mL collection tubes and centrifuged at 10,000 rpm for 15 seconds. To digest any remaining DNA in the sample, add 350 uL of Buffer RW1 to RNeasy column and centrifuge at 10,000 rpm for 15 seconds. 10 uL of DNase 1 stock is mixed to 70 uL of Buffer RDD is mixed and added to the RNeasy column followed by the addition of 350 uL Buffer RW1 and centrifugation at 10,000 rpm for 15 seconds. 700 uL of Buffer RW1 is added followed by the addition of 500 uL Buffer RPE. The sample is again centrifuged at 10,000 rpm for 15 seconds. 500 uL of Buffer RPE is again added and centrifuged at 10,000 rpm for 15 seconds. The RNeasy column is placed into a new column and 40 uL of
RNase-free water is added followed by centrifugation at 10,000 rpm for 15 seconds. The elute contains the RNA extract from the cell sample.

**Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)**

The RNA extract is used to make cDNA in qRT-PCR to determine the relative amounts of RNA in the sample. RNA is used because it gives an accurate representation of gene expression of a given gene. Because we want to determine the extent to which circadian inhibitors are altering transcription of particular circadian drugs, qRT-PCR is the appropriate method. One reaction examines the levels of GAPDH (control) and Bmal1 RNA transcripts present in DMSO (veh) and GSK (5 uM and 20 uM) treated MM14s for 72 hours. The second reaction examines transcript levels of Pax 7, MyoG, and Myf6 present in MM14 myoblasts in growth media and differentiation media (baseline control), DMSO (vehicle), GSK (2 uM and 20 uM), KL001 (1 uM, 10 uM), and KL002 (2 uM and 20 uM) for 72 hours. The final reaction examines transcript levels of Pax7, MyoG, and Myf6 present in primary cells in the presence of DMSO (vehicle), GSK (20 uM), and KL001 (20 uM) after 72 hours. Each sample undergoes reverse transcription reaction to make cDNA which can be used for qRT-PCR. The reaction contains 1 uL of random hexomers that binds RNA all over, 1 uL of 10 uM dNTPs, 2 uL of RNA (determined based on RNA concentration), and 9 uL of water. This reaction is placed into the PCR machine that has a particular cycle specifically constructed to make cDNA. A master mix containing 10x buffer, DTT, MgCl2, RNase out, and SS III Reverse transcriptase is divided evenly among the number of samples. After the cDNA is made, we add the reverse transcriptase mixture to the respective wells containing the master mix. The plate containing the samples is placed into the qRT-PCR machine and ran. After the reaction is complete, the numerical values of each sample is normalized to the concentration of GAPDH and graphed accordingly.
Primary Cell Culture: Mass Preps

The primary cell cultures are composed of myofibers and satellite cells isolated from wildtype C57BL6/J mice under 6 months old. The mouse is sacrificed by an overdose of isofluorine followed by the separation of the vertebrate. The hind limb muscles are removed from the mouse and mechanically digested with scissors for four minutes until the slurry tissue is homogeneous. The muscle is added to 10mL of Ham’s F12 (+pen/strep) growth media followed by the addition of 1mL of collagenase I from Worthington to enzymatically break down the remaining muscle tissue. The tissue and collagenase mixture is incubated at 37°C for 60 minutes. During the incubation period, the mixture is vortexed for 20 seconds every 10 minutes. After the incubation period is complete, 10mL of Ham’s F12C+15% horse serum is added to the mixture in order to inactivate collagenase activity. The digested tissue and media mixture is then filtered sequentially through 100um, 70um, and 40um cell strainers in order to remove debris and remaining non-digested tissue. After the final passage through the 40um cell strainer, the muscle cells are pelleted by centrifuging the remaining mixture at 1500rpm for 4 minutes. The supernatant is discarded and the cells are resuspended in 10mL of Ham’s F12C+15% horse serum. As a wash step to remove remaining debris, the cells are again pelleted by centrifugation at 1500 rpm for 4 minutes. The supernatant is again removed and discarded and the pelleted cells are resuspended in 10mL of Ham’s F12C+15% horse serum. The cell containing media is plated onto an uncoated dish for 2 hours. Fibroblast Growth Factor (FGF) is added to the cells during the pre-plate step because of FGF’s ability to promote quiescence in satellite cells. This pre-plate step removed fibroblasts because they characteristically attach to the plate over the 2 hour period, leaving muscle cells to remain suspended in media. The muscle cell containing media is then removed and distributed equally among a 6 well dish. Each dish contains a collagen coated
coverslip that the muscle cells will attach to. Each well receives a different chemical inhibitor of the molecular clock. KL001, KL002, and GSK 4112 (Cayman Chemical) are dissolved in the vehicle DMSO and are added to their respective wells at 1:1000. Preliminary data using the drugs at 1:1000 suggests efficacy and minimal toxicity. Higher concentrations of DMSO can be toxic to the cells. The control includes cells treated with DMSO at 1:1000 in order to eliminate any adverse effects of the cells as a direct result of DMSO addition. After the cells grow for 72 hours, they are washed with PBS. The cells are then fixed with the addition of 2mL of 4% paraformaldehyde (PFA) into each well. The cells incubate in 4% PFA for 10 minutes at room temperature. The 4%PFA is removed and disposed of in the toxic aqueous waste container because of its carcinogenic properties. The cells are washed with 1x PBS 3 times. The cells can be stored in PBS at 4°C for several months.

**Primary Cell Culture: Fiber Preps**

The fiber cultures are established by removing the extensor digitorum longus (EDL) muscle from wildtype C57BL6/J mice under 6 months old. Both EDLs are placed into 1710uL of Ham’s F15 (+pen/strep) growth media followed by the addition of 190uL of 10x collagenase from Worthington. The mixture is incubated for 90 minutes at 37°C, including moderate shaking every 15 minutes. Before incubation is over, 3mL of Ham’s F12C+15% horse serum spiked with 5nm FGF is placed in a single well of a 6 well plate. 4mL of Ham’s F12C+15% horse serum spiked with 5nm FGF is placed into a second well on the same 6 well plate. After the incubation period is complete, the digested muscle is removed and placed into the first well containing 3mL of growth media to inactivate collagenase. Under the dissection microscope, detached individual fibers are transferred to the second well containing 4mL growth media using a transfer glass pipet. Fibers that are still attached to debris or muscle tissue can be shaken lose using forceps.
During this transfer, fibers being transferred with forceps must be held at the ends because grabbing fibers in the middle will result in death of the fiber. After transferring all detached fibers to the second well, the fibers will be divided evenly into fourths and each fraction of the fibers will be transferred into its own well. Chemical inhibitors including KL001 (5uM), KL001 (20uM), and GSK (20uM) dissolved in DMSO are added to their respective wells at 1:1000. The remaining well is treated with DMSO at a concentration of 1:1000. After growing for 72 hours, the fibers are removed from their well and transferred into an empty well. The four samples of fibers are kept separate throughout the remainder of the experiment. Remove as much media as possible then flush the fibers with 2mL PBS. The fibers are transferred to another empty well and flooded with 5mL 4% paraformaldehyde and incubate at room temperature for 10 minutes. Following the incubation, the fibers are again transferred to an empty well. As much 4% PFA as possible is removed and is discarded in the toxic aqueous waste bin because of its carcinogenic properties. The fibers are again flooded with 5mL PBS. The fibers are transferred again, removing as much PBS as possible, then flooded with 5mL of fresh PBS. This transfer is repeated once more. The fibers can be stored in PBS at 4°C for several months.

**Immunofluorescence: Differentiation in Primary Cell Culture**

The differentiation assay stains cells based on their expression of syndecan-4, a cell surface protein characteristic of satellite cells, and myogenin, a late differentiation marker in muscle cells. Chicken anti-syndecan-4 antibody (abcam) is used at 1:1000, and rabbit anti-myogenin antibody (Santa Cruz, M-20) is used at 1:250. The cells attached to the collagen coated coverslips and are stores at 4 °C in PBS. The coverslips are removed and placed onto a piece of hydrophobic parafilm in a 15cm uncoated plate. Before adding these antibodies, 3% PBS in 1x PBS 0.2% Triton is used to block nonspecific epitopes. The block is added to the cells and
incubates for 60 minutes at room temperature. The blocker is then removed using the bench top vacuum and the cover slip is washed using 1x PBS 0.2% Triton. The primary antibodies, diluted in 1% BSA in 1x PBS 0.2% Triton, are added to the cover slips and incubate in the 4 °C cold room overnight. The primary antibody is removed via bench top vacuum and the cover slip was washed 3 times with 1x PBS 0.2% Triton. The secondary antibodies conjugated to AlexaFluor dyes diluted in 1% BSA 1x PBS 0.2% Triton are added to the coverslips and incubate for 60 minutes at room temperature. The secondary antibodies include Invitrogen anti-chicken 488 used at 1:500 and Invitrogen anti-rabbit 647 at 1:500. The coverslips are again washed using 1x PBS 0.2% Triton. DAPI is a nuclear stain that is excited by UV light. DAPI is diluted in 1% BSA 1X PBS 0.2% Triton at 1:1000. The coverslips incubate with DAPI for 10 minutes at room temperature. The coverslips are washed with 1x PBS 3 times. The coverslips are then dipped in water and allowed to air dry for 20 seconds, and are then mounted onto glass slides using 40uL of MOWIOL. The slides are stored away from light for up to 1 month.

**Immunofluorescence: Differentiation in Muscle Fibers**

The differentiation assay stains cells on the muscle fiber based on their expression of sydecan 4, a cell surface protein characteristic of satellite cells, and myogenin, a late differentiation marker in muscle cells. Chicken anti-syndecan4 antibody (abcam) is used at 1:1000, and rabbit anti-myogenin antibody (Santa Cruz, M-20) is used at 1:250. Fibers are removed from the 6 well dish using a glass transfer pipet and are transferred into a strainer made from (yellow things) in a 12 well plate to prevent the fibers from passing through while allowing them to be exposed to the respective reagents. Fibers are first blocked using 3% BSA in 1x PBS 0.2% Triton and incubate for 60 minutes at room temperature. The basket containing the fibers is transferred to a new well and washed with 500 uL 1x PBS 0.2% Triton. The basket is transferred to an empty well.
followed by the addition of the primary antibody diluted in 1% BSA 1x PBS 0.2% Triton. The fibers incubate in the primary antibody overnight in the 4 ºC cold room. Following overnight incubation, the fibers are transferred to an empty well and washed with 500 uL 1x PBS 0.2% Triton 3 times. The secondary antibodies are conjugated AlexaFluor dyes diluted in 1% BSA 1x PBS 0.2% Triton. The secondary antibodies include Invitrogen anti-chicken 488 used at 1:500 and Invitrogen anti-rabbit 647 at 1:500. The fibers are incubated in the secondary antibody at room temperature for 60 minutes. The fibers are then washed with 1x PBS 0.2% Triton then transferred to a new well. DAPI is diluted in 1% BSA 1X PBS 0.2% Triton at 1:1000. The fibers incubate with DAPI for 10 minutes at room temperature. The fibers are transferred to new wells and rinsed with 1x PBS 3 times. The fibers are then removed from the basket and transferred into a 6 well dish. The fibers are then transferred to a glass slide using a glass transfer pipet. After removing remaining PBS from the slide, the fibers are mounted using 80 uL of mowoil. The slides are stored away from light for up to 1 month.

**Immunofluorescence: Proliferation/Quiescence in Primary Cells**

The proliferation assay stains cells based on their expression of Pax7, a nuclear protein present in satellite cells, and Ki67, a protein present when a cell is actively in the cell cycle. Mouse anti-pax7 antibody (abcam) is used at 1:250, and rabbit anti-Ki67 antibody (Abcam) is used at 1:1000. The cells attached to the collagen coated coverslips and are stores at 4 ºC in PBS. The coverslips are removed and placed onto a piece of hydrophobic parafilm in a 15cm uncoated plate. Before adding these antibodies, 3% PBS in 1x PBS 0.2% Triton is used to block nonspecific epitopes. The block is added to the cells and incubates for 60 minutes at room temperature. The blocker is then removed using the bench top vacuum and the cover slip is washed using 1x PBS 0.2% Triton. The primary antibodies, diluted in 1% BSA in 1x PBS 0.2%
Triton, are added to the cover slips and incubate in the 4 °C cold room overnight. The primary antibody is removed via bench top vacuum and the cover slip was washed 3 times with 1x PBS 0.2% Triton. The secondary antibodies conjugated to AlexaFluor dyes diluted in 1% BSA 1x PBS 0.2% Triton are added to the coverslips and incubate for 60 minutes at room temperature. The secondary antibodies include Invitrogen anti-mouse 488 used at 1:500 and Invitrogen anti-rabbit 647 at 1:500. The coverslips are again washed using 1x PBS 0.2% Triton. DAPI is diluted in 1% BSA 1X PBS 0.2% Triton at 1:1000. The coverslips incubate with DAPI for 10 minutes at room temperature. The coverslips are washed with 1x PBS 3 times. The coverslips are then dipped in water and allowed to air dry for 20 seconds, and are then mounted onto glass slides using 40uL of MOWIOL.

**Imaging**

The slides are imaged using a laser-scanning Zeiss confocal microscope located in the MCDB Light Microscopy Core Facility. 8 images were gathered from each slide. The images were processed using ImageJ and then coded to ensure non-biased scoring. During scoring, the number of cells were quantified based on the number of nuclei stained with DAPI. Additionally, the number of cells in each of the following category for the differentiation assay are quantified: Syndecan4+/myogenin-, syndecan4+/myogenin+, and syndecan4-/myogenin+. For the proliferation assay, the number of cells in each of the following category are quantified under: Pax7+/Ki67-, Pax7+/Ki67+, and Pax7-/Ki67+. All scoring was completed via manual auditing and then decoded.

**DNA Extractions**

Tails from mice are clipped and placed in sterile 1.5 mL conical tubes. 90 uL of Tail Lysis buffer (0.1 M NaCl, 10mM Tris pH 8.0, 1mM EDTA pH 8.0, 1% SDS) and 12.5 uL of OB
Protease (20 mg/mL) is added to the tail clip and vortexted thoroughly. The digestion reaction is placed in a 56 ºC waterbath over night. Following incubation, alloquates of elution buffer (10mM Tris pH 8.0) is heated to 70 ºC. The tubes containing protease digestion reaction are removed from the waterbath and centrifuged for 2 minutes at 15000rpm to pellet the insoluble tissue debris. The supernatant is removed and placed into a new sterile 1.5 mL conical tube containing 200 uL Buffer BL/EtOH 1:1 mixture (4M Guanidinium hydrochloride, 50% 100% EtOH). The mixture is vortexed thoroughly and then pipetted into a DNA column coated with Equllibration Buffer (750 mM NaCl, 50 mM MOPS pH 7.0, 15% isopropanol, 0.15% Triton X-100) in a vacuum manifold. The column is washed with 500 uL of HB Buffer (5M Guanidinium hydrochloride, 30% 100% EtOH) followed by 2 washes with 700 uL DNA Wash Buffer (10 mM Tris-HCl p.h 7.5, 80% EtOH). The column is then spun down in the centrifuge to remove any residual EtOH. The column is placed into a new 1.5 mL conical tube, followed by the addition of 100 uL elution buffer. The column is spun for 1 minute in the centrifuge. The column can be removed and disposed of; the remaining liquid contains the mouse DNA sample. It can be stored in the 4 ºC cold room until genotyping.

**Genotyping**

The DNA extracted from mouse tail clippings is used to determine the genotype of the mice. This is essential in setting up breedings that will give rise to pups with the desired genotype. Our knockout mice are generated using the Cre lox system such that gene expression is altered only skeletal muscles. Cre is a protein endogenous to E. Coli that recognized lox p sites in the genome and cleaves at these sites. The cellular machinery then repairs the double stranded break in the DNA, effectively removing the DNA strand that existed between the two lox p sites. Cre is driven by the estrogen receptor such that high levels of estrogen cause Cre to dissociate.
and translocate to the nucleus where it can scan the DNA for lox p sites. The knockout mice are generated by flanking the gene of interest with two lox p sites. This mouse is then crossed with a mouse expressing Cre in order to generate a mouse that express Cre and has the gene of interested flanked by the lox p sites. In order to determine which mouse expresses which allele, genotyping protocol from Jax Institute consists of a polymerase chain reaction that uses genomic DNA extracted from mouse tails and specific primers developed by Jax. The reaction is then run on an agarose gel to visualize the absence or presence of the desired gene. Based on these results, we are able to set up specific breeding that will yield pups with our desired genotype.

Chapter 3: Results

**Immunofluorescence-based differentiation assay using circadian inhibitors and MM14 cells**

Initially, I set out to confirm the preliminary flow cytometry and ‘mitotic shake off’ data in MM14 cells using immunofluorescence. In this assay, I treated MM14 cells with either circadian inhibitors or a control vehicle for 72 hours, allowing them to briefly differentiate. I then fixed and stained the samples of MM14 cells for myogenin, a differentiation marker of myogenesis that is localized to the

![Figure 10: Myogenin is decreased in MM14 cells treated with circadian inhibitors.](image)
nucleus. I costained with DAPI, a DNA nuclear stain that allows us to visualize the total number of cells present in a culture and to determine which myogenin signals were ‘real’ by comparing the localization of myogenin to that of DAPI. In a cell expressing myogenin and DAPI, both DAPI and myogenin stains are localized to the nucleus. Overall, the drug treated cells exhibited a lower percentage of myogenic cells in comparison to the vehicle, suggesting inhibition of the circadian clock influences satellite cell differentiation in MM14s cell (Figure 10).

**mRNA expression analysis in drug treated MM14 and primary SC cultures**

To confirm the circadian inhibitors were hitting their appropriate target, a portion of the vehicle and drug treated cells from the previous experiment (GSK 4112) were examined for mRNA expression analysis. In particular, qRT-PCR analysis of the bulk population of cells treated with GSK 4112 demonstrates a decreased expression of Bmal1 mRNA transcripts (Figure 11). The decreased expression of Bmal1 confirms that GSK 4112 is working properly because GSK4112 is an agonist of REV-ERBa which works to directly block transcription of Bmal1. Therefore, cells treated with GSK 4112 have increased inhibition of Bmal1 transcriptional activity and thus a decreased amount of Bmal1 mRNA. Additionally, the amount of Bmal1 transcripts present in the MM14s appears to be dosage dependent such that cells treated with a higher dose (20 uM) of GSK 4112 express a smaller amount of Bmal1 transcription more than

![Figure 11: Bmal1 mRNA transcript levels decrease in MM14 cell lines in the presence of circadian inhibitor. qRT-PCR data from MM14 myoblasts cultures measuring Bmal1 mRNA transcripts in presence of DMSO (veh, 20 uM) and GSK 4112 (5 and 20 uM) for 72 hours. Bmal1 transcript levels normalized to GAPDH transcript levels.](image-url)
the lower dose (5 uM). KL001 and KL002 prevent ubiquitin dependent degradation of Bmal1’s negative regulator Cryptochrome. However, cryptochrome prevents the Clock:Bmal1 heterodimer from functioning properly, therefore Bmal1 mRNA expression is not expected to change significantly in the KL001 treated cells.

In MM14s treated with circadian inhibitors (GSK 4112, KL001, and KL002), qRT-PCR was used to measure the amount of mRNA transcripts of key differentiation markers: Pax7, a nuclear protein present in quiescent satellite cells, Myogenin, a late differentiation marker of myogenesis, and Myf6, a differentiation inducing protein. The control for this experiment includes RNA extracted from MM14 cells treated with DMSO in growth media and in differentiation media (Figure 12A). The amount of mRNA transcripts of the four factors in the growth media and differentiation media provides a baseline for the expression pattern of each gene under growth or differentiation conditions. In the drug treated cells, expression of the differentiation markers Myogenin and Myf6 decreased (Figure 12 B,C). In primary cell cultures,
drug treatment with GSK and KL001 similarly results in reduced expression pattern of differentiation-associated transcripts indicating the drugs are hitting the same targets as in MM14 cells (Figure 13).

Taken together, these data show circadian drug exposure promotes mRNA expression patterns indicative of a more undifferentiated state.

**Primary cell culture assays: Mass prep and fiber culture**

Primary cultures include primary cells isolated from the hind limb muscle of mice. The satellite cells are isolated from the muscle and are treated with FGF to prevent differentiation in culture (Figure 14). Importantly, satellite cells are synchronized in the cell cycle in primary cells. While in the mouse, the satellite cells are resting in the G0 phase of the cell cycle. Once the muscle is removed from the mouse, the satellite cells sense stress and active. Upon activation, the satellite cells enter the cell cycle at G1. Upon isolation, the cells are treated with circadian inhibitors and grow for 72 hours. This ensures that the clock is being inhibited during G1 and therefore during the cell fate deacon in the cell cycle. The control sample is treated with DMSO,
the vehicle in which the circadian inhibitors are dissolved. The three remaining samples are
treated with KL001 (5 μM and 20 μM) and GSK 4112 (20 μM). The samples are then fixed and
stained for syndecan-4, a cell surface protein characteristic of satellite cells, and myogenin, a late
differentiation marker of myogenesis. Immunofluorescence staining is manually quantified to
determine the number of cells that are single positive for syndecan-4 and all cells positive for
myogenin. The quantification of the three cell samples in the vehicle offers a baseline from
which we can determine if the circadian inhibitors affect satellite cell differentiation
independently of the affect of DMSO. In the drug treated cells, circadian inhibition does not
have a significant effect on the percentage of cells expressing myogenin (Figure 15).

In order to determine if this phenotype was accurate, we examined the phenotypes in
muscle fibers derived from the EDL from the hind legs of mice (Figure 16). The muscle fibers
offer a more physiologically relevant setting, as the stem cell niche and extracellular matrix is
preserved more fully in the fiber preps. This *in vitro* model simulates an *in vivo* model more
effectively and allows us to examine the role of the molecular clock in satellite cells in a

**Figure 14: Schematic of primary cultures assays.** For mass preps, muscle from the hind limbs of wildtype mice are harvested, mechanically and enzymatically digested using scissors and collagenase, respectively. For fiber preps, the EDL is excised and enzymatically isolated using collagenase. The muscle cells are plated onto collagen coated cover slips; the fibers are placed in a 6 well dish. Each sample is treated with the vehicle (DMSO, 20 uM) or a circadian inhibitor (KL001, 5 uM or 20 uM; GSK, 20 uM) for 72 hours. The cells/fibers are fixed and stained for myogenin and syndecan-4 or Ki67 and pax7 then imaged using a confocal microscope.
physiologically relevant setting. In the fiber preps, the drug treated fibers contained a higher percent of quiescent satellite cells as marked by syndecan-4 staining. There was also decrease in the number of myogenin positive cells in the drug treated cells as compared to the vehicle. The fiber prep quantifications suggest that circadian inhibition influences satellite cell homeostasis by promoting quiescence or

![Figure 15: Primary cells treated with circadian inhibitors](image)

A.) Vehicle (row 1), KL001 (row 2), and GSK 4112 (row 3) samples cultured in DMSO (20 uM), KL001 (20 uM), and GSK 4112 (20 uM) respectively for 72 hours and stained with Myogenin (left), Syndecan-4 (middle left), and DAPI (middle right). Myogenin and Syndecan-4 composites (right). Examples of myogenin single positive cells (^), syndecan-4 single positive cells (*), and myogenin and syndecan-4 double positive cells (+). B.) Percentages of cells from primary culture (n=2) that are differentiating (Myogenin+) and undifferentiated (Syndecan-4+/Myogenin-).
Figure 16: Primary muscle fibers treated with circadian inhibitors increases percentage of quiescent satellite cells. A.) Vehicle (row 1), KL001 (row 2), and GSK 4112 (row 3) samples cultured in DMSO (20 uM), KL001 (20 uM), and GSK 4112 (20 uM) respectively for 72 hours and stained with Myogenin (left), Syndecan-4 (middle left), and DAPI (middle right). Myogenin, Syndecan-4, and DAPI composites (right). Examples of myogenin single positive cells (^), syndecan-4 single positive cells (*), and myogenin and syndecan-4 double positive cells (+). B.) Percentages of cells from primary culture (n=2) that are differentiating (Myogenin+) and undifferentiated (Syndecan-4+/Myogenin-).
inhibiting differentiation in a physiologically relevant culture.

In order to further examine the subset of cells that is single positive for syndecan-4, we examined the percent of Pax7+ cells that are actively in the cell cycle to cells that are quiescent and therefore not actively in the cell cycle (Figure 17). Ki67 is a protein present in all cells that are active in the cell cycle. Staining for Ki67 and Pax7 allows us to visualize undifferentiated cells (Pax7 positive) that are actively in the cell cycle and therefore undifferentiated myoblasts to cells in G0 of the cell cycle and therefore truly quiescent satellite cells. Ki67 and pax7 double positive cells represent undifferentiated myoblasts that have exited quiescence and are actively proliferating in the cell cycle. Immunofluorescence staining of primary cell cultures shows that the drug treated cells have fewer double positive cells.

**Figure 17: Primary cells treated with circadian inhibitors increases percentage of quiescent satellite cells.** A.) Vehicle (row 1) and KL001 (row 2) samples cultured in DMSO (20 uM) and KL001 (20 uM) respectively for 72 hours and stained with Ki67 (left), Pax7 (middle left), and DAPI (middle right). Ki67 and Pax7 composites (right). Examples of Pax7 single positive cells (▲) and Pax7 and Ki67 double positive cells (+). B.) Percentages of cells from primary culture (n=2) that are active in the cell cycle (Ki67+/Pax7-) and quiescent (Ki67-/Pax7+), and proliferating myoblasts (Ki67+/Pax7+).
indicates the circadian inhibitors increase the percent of satellite cells not in the cell cycle and therefore quiescent. This assay compliments the differentiation assay because it supports the same hypothesis from a different angle. Active satellite cells and undifferentiated myoblasts undergo rapid proliferation in the cell cycle whereas quiescent satellite cells remain in G0 of the cell cycle. Therefore, staining for cells actively in the cell cycle allows quantification of quiescent satellite cells. Overall, cells treated with circadian inhibitors have a decreased percentage of cells expressing Ki67 suggesting circadian inhibition plays a role in preventing satellite cell activation and entrance into the cell cycle.

Chapter 4: Discussion

Inhibition of the circadian clock appears to have an effect on satellite cell activation because primary cultures treated with circadian inhibitors have a smaller number of myogenin+ cells as compared to the vehicle. These data suggest that the clock inhibits differentiation of myoblasts. Primary cultures treated with circadian inhibitors also have a smaller population of satellite cells actively in the cell cycle in comparison to the vehicle. This suggests that inhibition of the clock is maintaining satellite cells in a quiescent state such that they are not actively in the cell cycle. These observations suggest the molecular clock plays a vital role in satellite cell quiescence and differentiation. More importantly, clock inhibition appears to play a vital role in coordinating the timing of the regenerative process. Because perturbations in the clock with chemical inhibitors appears to affect satellite cell fate, further investigation of the clock is required in the context of injury, disease, aging, and natural turnover.

Future Directions

The molecular clock has a wide range of gene targets and is particularly well characterized in metabolism. The increased amount of satellite cells in the presence of circadian
inhibitors potentially results from the metabolic control of the clock. Specifically, perturbing the molecular clock could result in metabolic dysfunction. In satellite cells, the mechanism by which perturbations in the clock increase satellite cell quiescence could be a direct result of metabolic dysfunction. For example, satellite cells that are more metabolically active might be more likely to activate in relation to satellite cells in a lower metabolic state. This could cause more metabolically active cells to activate and enter the cell cycle. Perturbations in the clock could alter the metabolic activity of satellite cells such that decreased clock activity leads to a lower metabolic rate and thus a decreased ability to activate. Since the molecular clock is cell autonomous, satellite cells can be at a different point in the circadian clock than one another. That is, the molecular clock may contribute to heterogeneity among the satellite cell population. It is necessary to further characterize the link between metabolism and the molecular clock in satellite cells in order to determine the mechanism by which the clock regulates satellite cell state. Additionally, further characterization of the molecular clock and its implications regarding heterogeneity among the satellite cell population is necessary.

Preliminary investigations of the relationship between the clock and metabolic state suggest that cells with higher RNA content also have a higher rate of Bmal1 transcription in Bmal1-GFP MM14 clones (unpublished). In these clones, GFP is placed under control of the Bmal1 promoter such that Bmal1 and GFP transcription are simultaneous. This clone is useful because GFP is transcribed any time Bmal1 is transcribed thus we can visualize Bmal1 transcription based on the amount of GFP present in a given cell. The clones were stained using Hoescht 33342, a live cell DNA stain, followed by staining with Pyronin Y, a RNA label. This particular form of GFP has a short half-life (~2 hours), allowing the expression levels of GFP to accurately portray the activity of the Bmal1 promoter and RNA transcription at large. Flow
cytometry sorted the Bmal1-GFP MM14 clones, separating cells based on amount of GFP. The separated cells were further characterized as being GFP<sup>HI</sup> (top 25%) or GFP<sup>LO</sup> (bottom 25%) and then examined for RNA content. Using flow cytometry, the GFP<sup>HI</sup> cells were characterized as having greater RNA content, suggesting these cells are more metabolically active. The relationship between transcription of Bmal1 and metabolic activity is unclear and requires further investigation, as it is unknown whether high levels of Bmal1 correlate to a high metabolic state or a low metabolic state. However, there appears to be a distinct relationship between GFP/Bmal1 transcription with the amount of RNA in the cell suggesting metabolic activity is correlated to the molecular clock.

Another possible mechanism by which the molecular clock works to affect satellite cell state is the downstream targets of the clock. The clock has a wide range of downstream gene targets including genes directly required for myogenesis, such as MyoD. Therefore, the effect of the clocks in regards to satellite cells may not be limited to metabolic targets but rather targets of the clock may themselves be critical components of satellite cell maintenance and activation. In skeletal muscles, Igf1 and Fgfr1 signaling are two other important factors that play a role in maintenance and activation of satellite cells and might be subject to clock control. Conditional deletion of Fgfr1 impairs muscle regeneration and satellite cell self renewal while a constitutively active Fgfr1 partially rescues aged satellite cell phenotypes in culture (unpublished). SiRNA-mediated knockdown of Fgfr1 and Igf1 resulted in defects in circadian rhythmicity though a related Fgf receptor (Fgfr3) did not suggesting a direct relationship between the clock and Fgfr1 and Igf1 signaling (Zhang et al., 2009). Investigating clock targets in muscle satellite cells such as Fgfr1 and Igf1 could yield valuable insights into the mechanisms by which the clock affects satellite cell homeostasis and activation.
Further examination of the clock in an *in vivo* model is necessary to further characterize the degree to which clock manipulation can control satellite cell state. We are currently developing a line of Bmal1 conditional knock out mice that use a cre lox system to knockout Bmal1 in skeletal muscles upon nuclear translocation of Cre. When tomaxifin is injected into the hind limbs of the Bmal1 mutant mice, it binds to the estrogen receptor thus releasing Cre which translocates to the nucleus and cleaves DNA around the lox p sites which flank the Bmal1 locus. This system will allow us to examine the effects of Bmal1 knock out in specific skeletal muscles at particular time points. At this point, we are working on crossing Cre positive mice to mice with the Bmal locus floxed by two lox p sites, both of which were originally acquired from Jax Institute. The conditional knock out will ultimately allow us to examine the extent to which clock manipulation alters satellite cell state. Additional overexpression experiments can be done to see if turning on Bmal1 expression in these conditional knock outs restores a normal regenerative capacity. Knocking out Bmal1 entirely results in decreased life expectancy and age related muscle waste (Kondratov et al., 2006). However, conditionally knocking out Bmal1 in skeletal muscles may result in a less severe phenotype and perhaps increase the satellite cell regenerative capacity, mimicking the phenotype observed in wild type primary cells treated with circadian inhibitors. Characterizing the phenotype of the Bmal1 conditional knockouts is critical because it will provide valuable insight into the types of clock manipulation that can prove valuable versus those that are destructive. For example, knocking out Bmal1 may yield crippling phenotypes whereas perturbations of the clock via circadian inhibitors may have a positive effect on muscle regeneration and satellite cell state wildtype mice. Overall, the Bmal1 conditional knockout will provide valuable insight into the extent to which clock perturbation affects satellite cell homeostasis.
Following the development of the Bmal1 conditional knock down mice, we will be able to examine the clock-perturbed muscle following injury and in the context of transplants. Injuring the muscles in the hind limbs of the mice using Barium Chloride will allow us to further examine the regenerative capacity of skeletal muscle in a clock perturbed mouse as opposed to wildtype. Transplanting the satellite cells from a clock perturbed muscle to a healthy wild type muscle will allow us to examine the extent to which these cells can function in the context of a wildtype microenvironment.

The Bmal1 conditional knockout can also be used in a microarray study to examine the difference between gene expression in wildtype and clock perturbed muscle. This study could examine known targets of the clock as well as factors known to be associated with satellite cell homeostasis, function, and activation such as Fgfr1 and Igf1. Genes that have altered expression in the clock mutant mice could potentially be a target of the clock that loses rhythmicity with clock perturbation. Therefore, it is necessary to further characterize the clock and particularly how the clock mechanistically works to control satellite cells.

In regards to muscular pathologies including Duchene’s muscular dystrophy (DMD) and sarcopenia characteristically have a diminished satellite cell population. Because circadian inhibition appears to promote quiescence and inhibit differentiation of satellite cells, clock manipulation can potentially control satellite cell state and thus decrease disease severity. By promoting quiescence, circadian inhibition can prevent muscle cells from rapidly proliferating. This would prove significant, particularly in muscular dystrophy, because it employs a rapidly proliferating and highly active satellite cell population. Therefore, preventing a subset of these cells from activating via circadian inhibition will, in theory, preserve the satellite population more effectively. Another important step in regards to pathologies would be to examine whether
or not the clock is perturbed in diseased samples. This would be particularly interesting in muscular dystrophy patients and could give potential insight as to the role the clock plays in such pathologies. Additionally, we have an mdx mouse line that models DMD, creating possibilities for in vivo studies investigating the relationship between clock perturbation and DMD. Crossing the mdx DMD mouse with Bmal1 knock out mouse could create an opportunity to examine to effects of clock perturbation and DMD particularly in regards to disease severity and muscle regeneration in this system. Further examination of the relationship between muscle pathologies and the molecular clock will provide valuable insight into disease characterization and potential therapeutics.

Overall, inhibition of the circadian clock appears to play an important role in stem cell homeostasis and activation. In order to better characterize the role of the molecular clock, it is important to continue examining the clock in satellite cells particularly in experiments that have been discussed throughout this chapter. A more profound characterization of the clock in satellite cells can potentially aid in understanding the mechanisms behind muscular pathologies and perhaps lead to therapeutics in the future.

Chapter 5: Acknowledgments

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Chapter 6: References


