THE EFFECT OF IRON STATUS ON FACTORS RESPONSIBLE FOR THE MAINTENANCE OF CIRCADIAN RHYTHM AND CELLULAR METABOLISM IN C2C12

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Abstract: In mammals iron deficiency (ID) may contribute to hyperglycemia and dyslipidemia. Similar changes in glucose and lipid metabolism are observed in response to abnormalities in circadian rhythm. The nuclear hormone receptors (NHR) REV-ERBα and REV-ERBβ are heme-regulated transcription factors that are critical to the maintenance of circadian rhythm [1], [2]. Cellular oscillations in circadian rhythm are controlled through a network of negative feedback and feed-forward loops that regulate the transcription of clock-related and metabolic genes. In the presence of heme, REV-ERBs repress transcription of *Bmal1*, *G6pase*, and *Pepck* in the liver and reduced heme levels leads to de-repression of these genes [3]. In skeletal muscle, targets of REV-ERBs such as *Bmal1* regulate the transcription of *MyoD, Pgc-1α* and *Pgc-1β,* proteins that are involved in cellular differentiation and metabolic function [4]. *Bmal1* expression is also controlled by another NHR, RORα which activates the transcription of *Bmal1*. Expression profiling in skeletal muscle of clock mutant mice showed that skeletal muscle specific gene expression *MyoD* and *Pdk4*, which are important for cell proliferation and glucose metabolism, were dyregulated. The aim of the study was to investigate the effect of iron status on the regulation of circadian rhythm and cellular metabolism in C2C12 myoblast and myotubes. C2C12 myoblasts and myotubes were treated with the iron chelator desferroxamine (DFO) or iron in the form of ferric ammonium citrate (FAC) or hemin. RNA was extracted for gene expression analyses. Rhythmicity in circadian genes was altered by iron chelation and iron loading in C2C12 myoblasts and myotubes. Iron chelation decreased iron availability for heme biosynthesis resulting in a de-repression of REV-ERBα activity and transactivation of *Bmal1* gene expression in both myotubes and myoblasts. These findings are consistent with studies in ID animals. Low cellular iron status may potentially dysregulate CLOCK:BMAL modulations of its target gene (*MyoD , Pgc-1α* and *Pgc-1β*). *Bmal1* and *Tfrc* response to a decrease in iron status in both C2C12 myoblasts and myotubes and are similar to observations in the skeletal muscle of ID animals. Additional studies may be required to understand the mechanisms that altered these circadian and metabolic gene expression.

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CHAPTER I

INTRODUCTION

Disruption of circadian rhythm by dietary changes, shift work, long distance air travel, and artificial lighting at night is increasingly prevalence in today's society [5]–[7]. Multiple studies have suggests that chronic disruption of circadian rhythmicity may lead to pathological consequences such as obesity, metabolic diseases, inflammatory disorders and cancer [5]–[7]. Skeletal muscle, like other tissues, expressed circadian rhythmicity. When circadian rhythms are disrupted in skeletal muscles, alteration in muscle composition and function are correlated with disease development such as diabetes cardiovascular disease and cancer [8]. These disorders are associated with decreased muscle mass, and impaired muscle metabolism [8]. Loss of skeletal mass is also associated with decreased insulin insensitivity in skeletal muscle which is involved in development of type 2 diabetes [8]–[10]. Thus, circadian disruption is a critical detrimental factor of skeletal muscle health [8], [11], [12].

Circadian rhythms (in Latin 'circa diem'; meaning 'about a day') were first observed in plants and over the past centuries, discoveries were extended to almost all life forms [13]–[15]. The circadian clocks are critical in coordinating the physiology and behavior of mammalian at a 24 hour light-dark cycle, allowing temporal regulation of body metabolism and homeostasis [16], [17]. Other than light, entrainment of circadian clock to the environment and food availability is essential [5], [13], [14], [16], [18]–[20]. In mammals, suprachiasmatic nucleus (SCN) is

programmed to control circadian time based on light-dark signal, as well as signals from feeding and sleeping [6], [4]. Even though the core circadian regulation is found in the SCN, peripheral tissues have cell autonomous circadian control [21], [22]. An iron-containing nutrient, heme, has demonstrated to affect regulation of circadian and metabolic pathways through activation of circadian repressor, REV-ERBα [2], [14], [23], [24]. Additionally, dietary iron has been recently shown to modulate circadian rhythm and heme biosynthesis in liver by repression of gluconeogenic gene expression *Pepck* and *G6pase* by REV-ERBα [3]. These observations suggest that nutritional signaling by heme is a critical factor in circadian regulation. Despite an understanding of interaction between circadian regulation and metabolism in response to dietary iron in the liver, the response of circadian clock to iron status in the skeletal muscle has yet to be clearly elucidated.

REV-ERBα is a nuclear hormone receptor that regulates circadian rhythm, lipid metabolism and cellular differentiation [16]. Heme, an iron containing porphyrin ring, is ligand of REV-ERBα that repressed the circadian gene expression *Bmal1*which is involved in core clock metabolism [2], [16], [24]–[26]. Other than *Bmal1* gene expression, REV-ERBα is important in regulating clock-controlled genes such as *Pgc-1α* for mitochondrial biogenesis and the muscle specific gene, *MyoD* [20], [4], [21], [22]. Disruption of circadian rhythm in *Bmal1* knockout and *Clock* mutant mice has led to diet-induced obesity, hyperlipidemia, hepatic steatosis and hypoinsulinemic hyperglycemia, diabetes and age-related myopathy [12], [14], [27]–[29]. These findings demonstrate the disruption of downstream gene expression regulates by heme-mediated REV-ERBα potentially lead to metabolic disorders. Iron is an essential micronutrient involves in metabolic and cellular processes such as oxygen transport, energy transformation, DNA synthesis, cellular proliferation and heme biosynthesis [30].

Iron deficiency is a pronounced problem in both developed and developing countries, affecting almost 30% of the world populations [31]. Despite advanced development of nutritional

sciences and improved standard of living, iron deficiency is estimated to impact nine million people and affecting mostly pregnant women and young adolescent in the United States [32], [33]. The disturbance of iron-dependent systemic and cellular homeostasis due to iron deficiency has been shown to enhance metabolic syndromes and diseases such as hyperglycemia, accumulation of lipid, weight gain, muscle fatigue and mitochondrial myopathy [12], [29], [33], [34]. Our preliminary studies on 21 days iron deficient rats showed that they exhibit a disruption in lipogenesis, glycolytic, iron metabolism as well as circadian gene expression. The effect of nutrition (iron) showed strong linked between alteration and dysfunction in metabolism and circadian gene expression[13]. Intriguingly, both iron deficiency and circadian disruption negatively affect skeletal muscle health. These studies reveal that there is a correlation between circadian and metabolic regulation that lead to these similar metabolic syndromes.

REV-ERBα regulates PGC-1α pathway which is important for heme homeostasis in the liver [3], [25]. The transcription of the coactivator PGC-1α is a potent transcriptional activator of the rate-limiting enzyme *Alas1* in heme biosynthesis [3], [25]. Moreover, PGC-1 α, regulator of energy metabolism and mitochondrial biogenesis, also coactivate the expression of *Bmal1* and *Rev-erbs* [35]. Iron is necessary for heme biosynthesis and heme plays a role in oxygen transport, ATP synthesis and circadian rhythm. In iron deficiency, heme and ISC biogenesis are compromised and dysregulate cellular and mitochondrial function [36], [37]. These studies showed that there is a possible regulation of iron status on circadian and metabolic pathway through heme biosynthesis. Despite an understanding of interaction between iron status and circadian-regulated heme biosynthesis in the liver, the iron-dependent mechanism that regulates circadian-regulated heme biosynthesis in skeletal muscle is not clearly elucidated.

Cellular iron is the key regulator of mitochondrial biogenesis [30]. Iron depletion in mouse muscle cells resulted in decrease in mitochondrial protein level and oxidative capacity [38]–[43]. Surprisingly, iron deficiency in the liver showed no significant change in mitochondria

activity [38]–[43]. The degree that iron status coordinates circadian metabolism and mitochondria function in the skeletal muscle has not been substantially elucidated.

MyoD is a known clock-controlled gene specific to skeletal muscle and function as regulator of myogenesis [12], [44], [45]. Iron deficiency stunt growth in adolescent and MyoD is one of the important regulator of muscle growth and differentiation that is controlled in a circadian manner by REV-ERBα [12], [45]–[47]. Co-activators PGC-1α and PPAR family, as well as HIF1α are important for up-regulation *Pdk4* which is involved in glucose metabolism [48], [49]. Circadian protein REV-ERBα binds upstream of *insig2* transcript which activates nSREBP-1c for fatty acid and cholesterol metabolism in the liver [50]. *Pdk4* and *Srebp1c* gene expression are altered in skeletal muscle of iron deficient rats and these genes have been shown to be regulated in a circadian manner and are involves in glucose metabolism and fatty acid and lipid production in skeletal muscles [50]–[55]. *Pdk4* alteration in gene expression may be due to the increased activity of HIF1α by hypoxia mimetic desferroxamine, which is commonly used as an iron chelator in cell culture experiments [56] . Despite various studies on muscle circadian regulation, the degree to which alteration in iron status could potentially regulate these critical circadian components remains unknown.

These findings lead to the proposed examination on impact of iron depletion and iron loading on circadian and metabolic gene expression in C2C12 synchronized myoblast and myotubes which model the physiological state of proliferating and differentiating muscle cells. The relationship of iron status and regulation of *Bmal1* and *Rev-erbα* gene expression may reveal the impact of iron status on circadian rhythm and its metabolic gene expression specific to skeletal muscle cells.

Our objectives were to further examine and characterize the effect of iron status on the alteration in circadian and metabolic gene expression in synchronized C2C12 myoblast *in vitro*.

Our central hypothesis was that iron chelation and iron loading would alter circadian and metabolic gene expression involved in mitochondrial function, myogenesis, macronutrient metabolism in C2C12 myoblast. Furthermore, we hypothesized that chelation and loading of iron would alter mitochondrial function and oxidative capacity. Thus, the results presented herein will provide insight to potential relationship between cellular iron status and circadian rhythm as well as metabolic gene expression.

CHAPTER II

LITERATURE REVIEW

Molecular Clock in Mammals, transcriptional control and regulation

Circadian rhythms (in Latin ‘circa diem’; meaning ‘about a day’) were first observed in plants and over the past centuries, discoveries were extended to almost all life forms such as drosophila, bacteria, yeast and mammalians [13]–[15]. The circadian clocks are critical in coordinating the physiology and behavior of mammalian at a 24 hour light-dark cycle, allowing temporal regulation of body metabolism and homeostasis [13], [14], [16]–[18]. The complexity of circadian rhythms within a cells involves the intracellular regulation of many other cellular processes such as transcription regulation, cell cycle and metabolism [57], [58]. Circadian rhythm is regulated by two clocks: the master clock in the brain and peripheral clocks in other tissues [59]. The master clock in the suprachiasmatic nucleus (SCN) of hypothalamus received the light input from the retina, resulting in the transmission of electrical signal through the nerves in hypothalamus which activate neuronal and hormonal pathway that synchronizes circadian timing in brain and other tissues such as liver, adipose tissues, pancreas, and skeletal muscles [14], [16], [59].

Over the past decades, mammalian clocks in the peripheral tissues were thought to occur through neuronal-hormonal signaling from SCN [60]–[64]. However, recent studies showed that

peripherals tissue are capable of adapting locally to the metabolic status of the tissues [14], [16], [60], [65]. The peripheral clocks can be entrained independent of signals from SCN through the regulation from external temperature and feeding cues [65]. The rhythmic changes observed in metabolic and cell signaling pathways occurred when the mammals adapt to environment changes by altering the body circadian rhythm through heat shock signaling and other hormonal and neuronal pathways [14]. Other than environmental temperature, feeding cues have been shown to entrain metabolic and hormonal pathways and can behave as the dominant factor, instead of SCN, for setting circadian clock in peripheral tissues [60]. The changes in circadian rhythm by environmental and feeding cues allow the body to adapt to the environmental changes and reset oscillating phase (i.e. length and amplitude) [14].

At the molecular level, the mammalian circadian clock is a highly conserved system involving the interaction of mRNA and protein in the circadian clock transcription-translation feedback loop (TTFL) **(Figure 1)** [4]. Within the TTFL, there are primary and secondary regulatory feedback loops. In the primary loop, the auto-regulatory feedback loop is involved in the core molecular CLOCK (Circadian Locomotor Output Cycles Kaput) and BMAL1 (Brain Muscle ARNT-like 1), which play important roles as a PAS-bHLH transcriptional activator in driving the time keeping mechanism in mammals [4], [60], [61], [64], [66]–[68]. The activation of other clock genes is mediated by the activation of histone acetyl transferase (HAT) of CLOCK protein [69], [70]. Dimerization and binding of CLOCK and BMAL heterodimers onto conserved E-box (CACGTG) sequences in the promoter region of its target genes drives the circadian gene expression *Period* (*Per1* and *Per2*) and *Cryptochrome* (*Cry1*, *Cry2*, *Cry3*) as well as the nuclear hormone receptor *Rorα* (RAR-related orphan receptor alpha), and *Rev-erbα* (*Nr1d1*; nuclear receptor subfamily 1 group D member 1) [4], [71].

The secondary regulatory loop involves the nuclear hormone receptors REV-ERBα and RORα which are negative and positive transcriptional regulator of their target genes *Bmal1*,

respectively. REV-ERBα recruits NcoR (nuclear receptor co-repressor) and histone deacetylase (HDAC3) upstream of *Bmal1* and prevents its transcription. Decreased in BMAL1 indicates binding of REV-ERBα onto RORE (AAAGTAGGTTA) sequences in the promoter region of *Bmal1* encoded gene, resulting in inactivation of *Bmal1* transcription [72], [73]. Decrease in BMAL1 for dimerization with CLOCK protein result in decrease in the transcription of downstream target genes, *Per, Cry, Rev-erba,* and *Rora*. Binding of activated RORα onto E-box upstream of *Bmal1* promotes Bmal1 transcriptional activation. As BMAL1 protein is made, hyperphosphorylation occurred in the PER:CRY and CLOCK:BMAL1 complexes which signal them for degradation [4]. CLOCK protein is not rhythmic, however, it is important circadian protein that has HAT activity and binding of BMAL1 to CLOCK enhance the activity of HAT for circadian regulation [74]. Despite the known mechanism of CLOCK protein in circadian regulation and the circadian disruption of *Clock-/-*, the regulation of *Clock* gene expression is not well understood at present.

This transcription-translation feedback loop is important in maintaining circadian regulation in the cell at different time of the day [4]. The well coordinate transcription and translation of this feedback loop maintain cell at different stages of its life, such as growth and differentiation, sustaining energy balance as well as during oxidative stress [4].

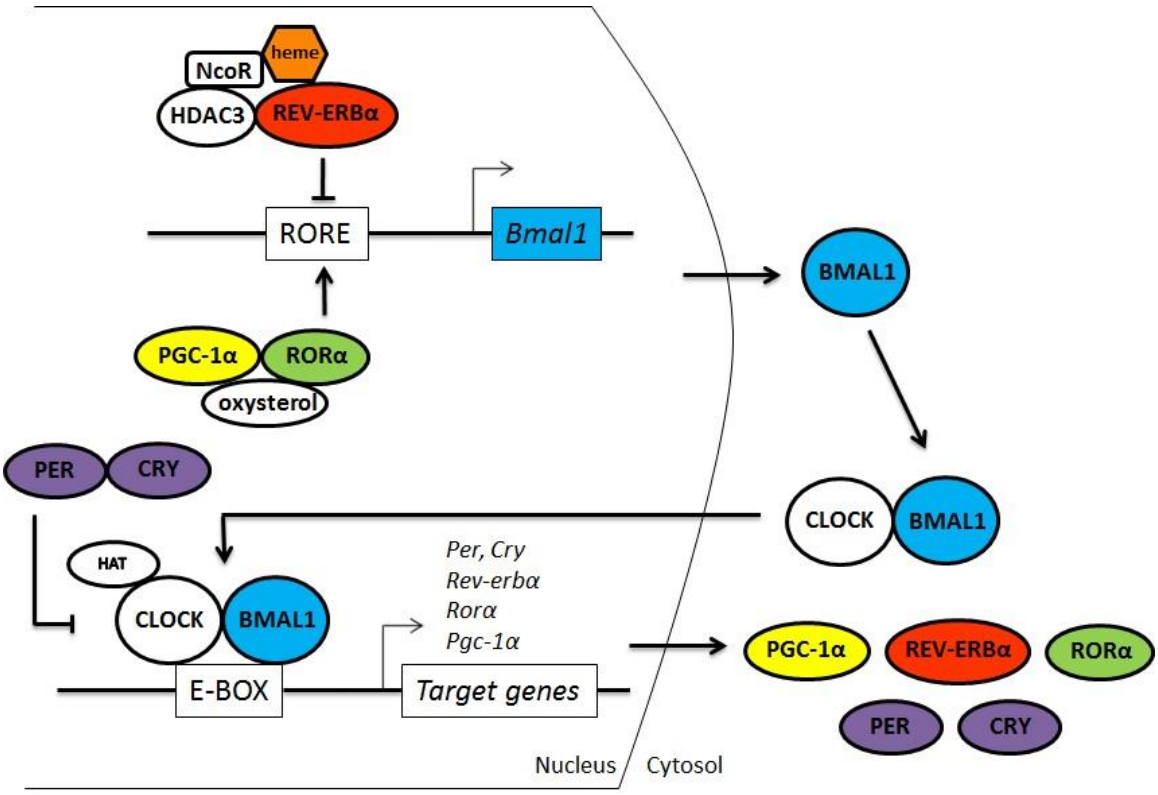


Figure 1: Molecular circadian clock in mammals (revised from *Golombek D.A. et al.*) [75]. This figure illustrates the primary and secondary feedback loop in the transcriptional and translational regulation in molecular circadian system. In the primary regulatory loop, the *Per* and *Cry* gene expression gets activated by CLOCK:BMAL1 heterodimer by binding to E-box sequence (CACGTG) upstream of the their target genes. As PER and CRY protein are made, they form a heterodimer and function by inhibiting their own transcription by repression of CLOCK:BMAL1 activity. In the secondary regulatory feedback loop, the nuclear hormone receptor, REV-ERBα and RORα function to repress and activate, respectively, by competitive binding to RORE on their target gene *Bmal1*.

REV-ERBα and RORα regulate circadian rhythm and metabolic function

Mammalian rhythmic regulations such as development and reproduction are dependent on changes in hormones and metabolites (i.e. vitamins, lipids, and heme) which bind to nuclear hormone receptor ligand binding domain (LBD) [73], [76]. Nuclear hormone receptor behaves as a transcription co-regulator that activates or represses target gene expression by chromatin remodeling via histone modification [77]. The multiple loop of regulation between nuclear hormone receptors (REV-ERBα and RORα) and the core clock mechanism suggest that circadian regulation is a complex process [76].

There are two types of nuclear hormone receptors: co-activators (RORα) and co-repressors (REV-ERBα). The regulation of gene expression occurs when specific ligand is bound to nuclear hormone receptor protein resulting in activation through a conformation change in protein structure [77]. Oxysterols and heme are specific ligands that bind to RORα and REV-ERBα, respectively. When oxysterol is bound to the coactivator RORα, the activity of HAT loosen the linkage of histones to DNA which in turn activates gene expression [77]. Moreover, PGC-1α acts as a co-activator of RORα [78], [79]. When heme is bound to corepressor REV-ERBα, a protein conformational change occurs resulting in recruitment of NcoR and HDAC3 which tightens the association of histones to DNA, thus preventing gene transcription [23], [77]. REV-ERBα and RORα are important nuclear hormone receptors that regulate core clock metabolism and gene expression.

REV-ERBα and heme homeostasis

Heme biosynthesis has been extensively examined in past decades, and the gene and structural form of the enzymes involved has been discovered [80]. Heme influences the metabolism and circadian rhythm by serving as a cofactor for REV-ERBα protein.

Heme binds to REV-ERB ligand binding domain and stabilized the REV-ERB proteins, resulting in the repression of target genes by the recruitment of NcoR co-repressor and HDAC3 [23], [77]. Heme binding is specific to REV-ERB LBDs as no effect of heme on other nuclear receptor such as LXR was observed [81]. The affinity of heme for REV-ERB LBD (K_d = 3 μM) suggests that these receptors are 'metabolite' sensor such as FXR (bile acid), LXR (oxysterol), RORα (oxysterol) and PPAR (fatty acids) [77], [81]. Heme binding to REV-ERB ligand binding proteins is reversible and REV-ERBs function as heme sensor rather than modulator in redox condition or diatomic gases (i.e. NO) [23], [81]. A study had shown that histidine602 (H602) is crucial for the binding of heme on REV-ERBs and it is conserved in REV-ERBα [23]. Structural mutant REV-ERBα (H602F) had been shown to impair heme binding to LBDs [2], [23]. Hence, we know that heme is specific to the binding domain in REV-ERBα.

Heme biosynthesis is control in a circadian manner [3], [25], [82]. Intracellular heme have been shown to oscillate in a circadian manner in NIH3T3 cells, however, their significance was unknown [82]. The rate-limiting enzyme, aminolevulenic acid (ALAS1), is induced during heme biosynthesis by nuclear receptor coactivator PGC-1α by binding to NRF1 and FOXO1 which activates *Alas1* gene expression [25]. Heme controls its own synthesis by feedback inhibition of *Alas1* gene expression [3], [83]. Moreover, increase in intracellular heme induces its own degradation through heme-oxygenase (HMOX-1) whose gene expression is induced by free heme as well as oxidative stress [84]. *Wu et al.* has identified two RORE binding site within the first intron region *Pgc-1α* gene and they are conserved in mouse and human [25]. Moreover, the study showed that in HepG2 cells, REV-ERBα directly represses *Pgc-1α* transcription via binding of RORE and heme induces *Alas1* and *Pgc-1α* gene expression in HepG2 cells [25].

These studies demonstrate that heme is a specific metabolite that binds to the LBD on nuclear hormone receptor REV-ERBα and rhythmic variation in intracellular heme modulates *Alas1* and *Pgc-1α* gene expression, via binding of heme to REV-ERB, which is important for

homeostasis of heme biosynthesis. Moreover, heme-mediated REV-ERBs modulate repression of their target genes such as *Bmal1* and *Pgc-1α* therefore, shaping the appropriate amplitude for its circadian rhythm and heme biosynthesis. These demonstrate the important of heme in circadian and heme biosynthesis regulation.

Disruption of Circadian Rhythm

Disruption of circadian rhythm by dietary changes, shift work, long distance air travel, and artificial lighting at night is increasingly prevalence in today's society [5]–[7]. Multiple studies have suggested that chronic disruption of circadian rhythmicity may lead to pathological consequences such as obesity, metabolic diseases, inflammatory disorders and cancer [5]–[7]. Mice lacking REV-ERBα experienced disorganized circadian rhythm [14], [72], [85]. In C57BL/6 mice, loss of both *Rev-erbs* in the liver result in decrease in circadian gene expression and hepatic steatosis [85], [86]. Recently, *Simcox et al.* have demonstrated that dietary iron affects circadian glucose metabolism in the liver through regulation of heme biosynthesis [3]. Free heme was suggested to induce production of ROS and activates PGC-1α which in turn up-regulates *Alas1* gene expression and heme biosynthesis [3].

Skeletal muscle, like other tissues, expressed circadian rhythmicity. Circadian study in skeletal muscle is still in the early stages. When circadian rhythms are disrupted in skeletal muscles, alteration in muscle composition and function are correlated with disease development such as diabetes cardiovascular disease and cancer [8]. These disorders are associated with decrease muscle mass, reduced mitochondria volume and impaired muscle metabolism [8]. Loss of skeletal mass is also associated with decreased insulin insensitivity in skeletal muscle which is involved in development of type 2 diabetes [8]–[10]. Thus, circadian disruption is a critically detrimental factor to skeletal muscle health [8], [11], [12].The metabolic disorders observed in disrupted circadian rhythm are similar to those observed in iron deficient rat's skeletal muscle (i.e. low muscle mass, reduced mitochondria volume and function) . Therefore, further

investigation is required to understand the relationship between iron status and circadian rhythm in skeletal muscle.

Circadian Rhythm in Skeletal Muscles

In the past decades, skeletal muscle circadian transcriptome, diurnal gene expression and bioinformatics analysis have been reported, therefore, assisted the understanding and future studies of circadian rhythm in skeletal muscles [87]–[90]. Approximately 7% of skeletal muscle transcriptome have been identified to express in circadian manner [4], [89], [91]. Within the 7% transcriptome, 18% are involved in the biosynthesis and metabolism and 17% are involved in the regulation of gene transcription [87], [91].

The bioinformatics analysis by *McCarthy et al.* showed that known core clock genes *Bmal1, Per2* and *Cry2* as well as metabolic genes, *Pdk4,* expressed circadian rhythmicity in skeletal muscle [89]. In *Cry1-/-* and *Cry2-/-* mice, complete loss of circadian gene oscillation is observed, therefore *Cry1* and *Cry2* are both essential in maintaining circadian rhythmicity in mammals [92]. The tissue-specific metabolic genes expressed in skeletal muscle such as *MyoD* (myogenic differentiation) has also been identified and regulate by REV-ERB and CLOCK:BMAL1 heterodimer [89]. SREBP-1c, a regulator of cholesterol and fatty acid metabolism, has been identify to be under circadian control of REV-ERBs through regulation of INSIG2 in the skeletal muscle [93]–[95].

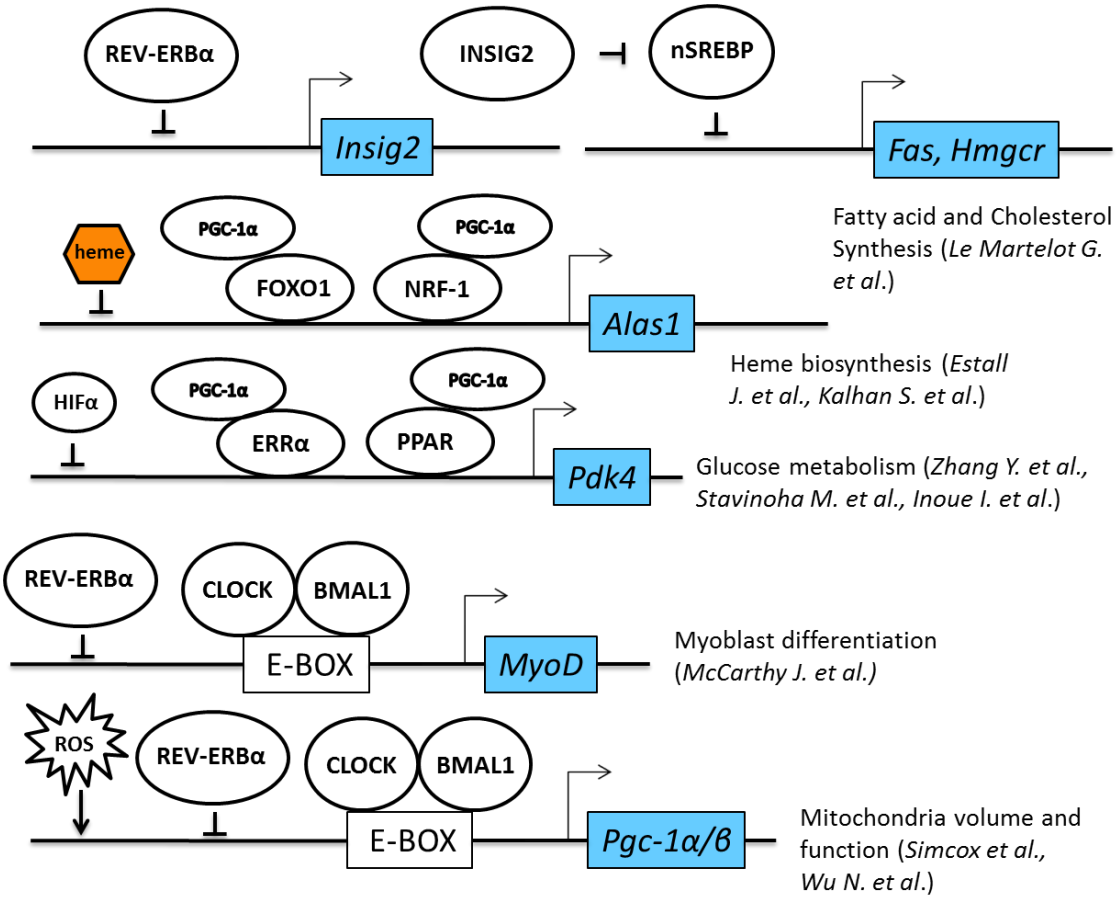


Figure 2: Regulation of metabolic genes (*Insig2*, *Srebp1c*, *Fas*, *Hmgcr*, *Alas1*, *Pdk4*, *MyoD*, *Pgc-1α* and *Pgc-1β*) by circadian regulators and circadian mediated regulators such as REV-ERBα, CLOCK:BMAL1 and PGC-1α [48], [78], [87], [89], [96], [97]. *Insig2*: Insulin induced gene 2; *Srebp1c*: Sterol regulatory element binding transcription factor 1; *Fas*: Fatty acid synthase; *Hmgcr*: HMG-CoA reductase; *Alas1*: Aminolevulinic acid; *Pdk4*: Pyruvate dehydrogenase kinase, isozyme 4; *MyoD*: Myogenic differentiation; *Pgc-1α* and *Pgc-1β*: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha and beta.

REV-ERB and myogenesis

REV-ERBα has specific function in different tissues such as liver, adipose tissue, pancreas, brain and macrophages [98]. *Rev-erbα* is highly expressed in oxidative skeletal muscle and REV-ERBα protein modulates myogenesis by repression *MyoD* gene expression required for muscle cell differentiation [98].

The myogenic basic helix-loop-helix (myo-bHLH) *MyoD* is a known clock-controlled gene in skeletal muscle and function as muscle regulator of myogenesis [4], [12], [46], [47]. During myogenesis, cell cycle arrest occurs in the mononucleated proliferating myoblasts together with increased production of MYOD protein, resulting in fusion into myotubes [47]. Isolation of undifferentiated synchronized myoblast have shown that the ability for cell to differentiate is linked to *MyoD* expression level [47], [99]. A certain level of MyoD protein must be expressed before myoblast undergoes differentiation [47]. Other than growth factors, upstream regulation of *MyoD* is also controlled by core clock genes CLOCK and BMAL and the nuclear hormone receptor REV-ERBα [89].

REV-ERB and energy metabolism

PGC-1α is a transcription coactivator that involved in the activation of gene coding for mitochondrial protein and biosynthesis in skeletal muscle [100]. *Woldt et al.* has shown that deficiency in *Rev-erbα* reduced mitochondrial content, impaired mitochondrial biogenesis and increased autophagy [91], [96], [101]. Overexpression of *Rev-erbα in vivo* increases exercise capacity; and *in vitro, Rev-erbα* overexpression increases the number of mitochondria and improves respiratory capacity [91], [96]. Previous studies have established that PGC-1α is the main regulator of mitochondrial biogenesis and increased in PGC-1α in skeletal muscle up-regulates the mitochondrial number and function [91], [96], [101]. These transcript changes were

observed in the muscle but not in liver or WAT suggesting that it is muscle specific regulation of REV-ERBα [36].

PGC-1α has been shown to up-regulate *Bmal1* and *Rev-erbα* gene expression through the activation of RORα transcriptional activity [102], [103]. Deficiency in *Pgc-1α* results in similar metabolic syndrome as deficiency in *Rev-erbα*, such as a decrease in mitochondrial number and muscle oxidative capacity [91], [96], [104]–[106]. Moreover, PGC-1β showed similar modulation in circadian and metabolic regulation [103]. These findings suggest that PGC-1α, and possibly PGC-1β are important factors that linked circadian clock with energy metabolism through modulation of *Rev-erbα* and *Rorα* [103].

The nuclear receptor coactivator PGC-1α has been shown to bind to PPAR family of nuclear hormone receptors as well as RORα and RORγ [76]. Moreover, *Pparα* transcription is directly regulated by CLOCK and BMAL1 [76], [97], [107], [108]. PGC-1α enhances *Bmal1* transcription by activating RORa and RORγ [12], [4], [35]. This interaction occurred as p300 histone acyltransferase is translocated to *Bmal1* promoter by PGC1α and it is dependent on the levels of REV-ERBα [4]. It was shown that PGC-1α protein is at a high concentration when the *Bmal1* transcription expression is at its highest and this correlates with the repression of CLOCK:BMAL1 dependent transcription [4], [35]. *Pgc-1α* knockout mice had shown to lost their daily cycle pattern of their rate-limiting enzymes in oxidative phosphorylation and TCA cycle. Other metabolic signals such as SIRT and AMPK have shown to activate PGC-1α and alters PER:CRY activity [78]. These studies demonstrated the association between a variety of metabolic signals to REV-ERBs and PGC-1 α in regulation of energy metabolism [78].

Glucose metabolism by Pdk4 in skeletal muscle

Pyruvate dehydrogenase kinase 4 (*Pdk4*) gene expressions is known to regulate by PPARα-binding upstream of its promoter [109]. PDK4 is involved in glucose metabolism by

inhibiting the activity of pyruvate dehydrogenase complex from converting pyruvate to acetyl-CoA for TCA cycle. Moreover, the reduction of acetyl-CoA results in up-regulation of beta-oxidation to make up for the decrease in acetyl-CoA. The PPARγ-regulated *Pdk4* expressed circadian pattern in muscle and oscillate in CLOCK mutant mouse but the phase was shifted by 12 h suggesting indirect circadian regulation due to CLOCK mutation [110]. Other than PPARγ, estrogen-related receptor α (ERRα) is one of the important regulators of energy metabolism in skeletal muscle [48], [111]. In C2C12 myotubes, overexpression of PGC-1α has been shown to co-activate *Pdk4* transcript by binding to ERRα on the promoter and activates its gene expression, resulting in decrease in glucose oxidation [48]. *Pdk4* is also regulated by HIF1α activity, which can be induced by hypoxia mimetic desferroxamine and functions as iron chelator in cell culture model [56].

Circadian rhythm is disrupted in mouse strain with muscle-specific *Bmal1* knockout [112]. Skeletal muscle in *Bmal1* knockout mice has impaired insulin sensitivity, reduced GLUT4 protein and translocation of GLUT4 [112]. Moreover, circadian gene *Pdk4* gene expression is up-regulated, inhibiting pyruvate dehydrogenase activity leading to reduced glucose oxidation in *Bmal1* knockout mice [112]. Glycolytic substrates are mediated through other pathways [112]. Decreased in *Pdk4* gene expression has been observed in ID rat's skeletal muscle suggesting an alteration in gluconeogenesis similar to *Bmal1* knockout mice [51]. These studies demonstrate that there are correlation between iron deficiency and circadian rhythm disruption leading to alteration in glucose metabolism, however, the mechanism that regulate these pathway has not been clearly elucidated.

Lipid homeostasis by Srebp-1c in skeletal muscle

The role of SREBP-1C in cholesterol and fatty acid metabolism has been extensively studied in liver and adipose tissue, however, studies in the skeletal muscle had just began in the

last decade [93], [94]. In the liver, SREBP-1c responses to circadian and nutritional cues and regulates genes involved in cholesterol regulation and fatty acid metabolism such as glucokinase, fatty acid synthase, acetyl-CoA carboxylase, and glycerol-3-phosphaste acyltransferase [94], [95]. In muscle cell culture, *Srebp-1c* gene expression decreased in diabetic-induced cells and increased with insulin-induced cell culture, indicating that insulin may regulate skeletal muscle [94]. SREBP-1C is identify to function as a transcription factor in the regulation of myogenesis [93]. The overexpression of SREBP-1C protein in skeletal muscle inhibits differentiation of myoblast to myotubes and also results in muscle degeneration both *in vivo* and *in vitro* by downregulating the myogenic regulatory factors (*MyoD* and *MyoG*) [93]. Overexpression of *Srebp-1c* genes has been observed in ID rat's skeletal muscle, suggesting an alteration in lipogenesis [51].

Rhythmicity in cholesterol and lipid metabolism is known to alter by feeding and fasting cycles, however, studies have demonstrate that the nuclear hormone receptor REV-ERBα and REV-ERBβ participates in circadian regulation of SREBP-1c and its target genes [50], [52], [113].

REV-ERBα involves in circadian SREBP signaling activity by targeting *Insig2* gene expression which in turn exhibits circadian control on nuclear SREBP that are involved in cholesterol and lipid metabolism [50]. REV-ERBα has been shown to control the nuclear accumulation of SREBP and the transcription of SREBP target genes [50]. REV-ERBβ was demonstrated to control lipid and energy homeostasis in skeletal muscle [113]. Overexpression of *Rev-erbβ* in C2C12 myotubes have shown to decrease gene expression involved in fatty acid and lipid absorption (i.e. *Cd36*, *Fabp-3*, *Fabp-4*) and lipid metabolism (*Pdk4*↓1.4 fold)while increase lipogenic gene expression (*Srebp-1c* ↑2.8 fold) [52], [113]. ChIP analysis in C2C12 cells have shown that REV-ERBβ is recruited to *Srebp-1c* promoter for activation, however, REV-ERBβ repressed Rev-erbα promoter [52]. Moreover, treatment of C2C12 cells with hemin have shown

to increase gene expression of *Rev-erbβ* and *Srebp-1c* in skeletal muscles [52]. In iron deficient rat, *Srebp1c* gene expression has been shown to up-regulate in skeletal muscle [51]. These studies showed that heme-mediated REV-ERBs altered circadian regulation in *Pdk4* and *Srebp-1c* which correlates with symptoms observed in iron deficient animals. However, detailed connection between iron status and these metabolic genes have not been shown.

Iron as an essential micronutrient

Iron is an essential micronutrient involves in metabolic and cellular processes such as oxygen transport, energy transformation, DNA synthesis, and heme biosynthesis [30], [114], [115]. The human body contains approximately 3-5 g of iron and mostly found integrated in hemoprotein for oxygen binding and transport such as hemoglobin and myoglobin in erythroid cells and skeletal muscles, respectively [116].

Iron deficiency has affected people worldwide resulting in anemia, restlessness, muscle and cognitive dysfunction in adult and children. Iron deficiency has been a known problem in both developed and developing countries, affecting almost 30% of the world populations and many are women and children [31]. The demand for iron is high in adolescence due to the increase of blood volume and muscle mass [117]. Moreover, in general population, the disturbance in iron systemic and cellular homeostasis due to iron deficiency has been shown to enhance metabolic syndrome and diseases such as hyperglycemia, accumulation of lipid, cognitive dysfunction, weight gain, muscle fatigue and mitochondrial myopathy [12], [29], [33], [34]. Animal studies have shown that there is a correlation between iron deficiency and alteration in lipid and glucose metabolism due to the lowered muscle oxidative capacity, thus utilizing more glucose over fat [51], [118]. Iron deficiency alters metabolic homeostasis of insulin signaling resulting in hyperglycemia, hyperinsulinemia, and hyperlipidemia [51], [118]. Decreased in PGC-

1α protein has been observed in iron-deficient diet, however, the mechanism for this changes is unknown [3], [119].

Iron overload leads to the generation of reactive oxygen species (ROS) which damages structure of DNA, protein and lipid membrane [120]. The mammalian system is efficient at regulating iron homeostasis, however, excess iron in individuals with inheritable disease hemochromatosis can result iron overload in the peripheral tissues and contribute to the development of metabolic disorders such as cirrhosis, cardiomyopathy as well as diabetes mellitus [115]. Moreover, iron toxicity also lead to neurodegenerative diseases such as Friedreich's ataxia, Alzheimer's disease and Parkinson's disease [51], [121].

Iron homeostasis is therefore an important cellular regulation in maintaining normal body function. Iron homeostasis is maintained at a systemic and cellular level by the tight regulation of iron uptake, storage, and export via the hormone hepcidin and iron regulatory proteins 1 and 2 (IRP1 and IRP2).

Systemic regulation of iron metabolism

There is no designated pathway to excrete iron; hence, intestinal absorption of iron must be regulated to provide sufficient iron to meet the demand of the body [114], [122]. In mammals, small amount of iron (1-2mg) is absorbed by the human body per day to replace the loss of iron from mucosal sloughing, loss of blood, desquamation or sweat [114], [116]. The absorption of iron depends on the body need which increases during growth and pregnancy while decreases during iron overload condition [114]. Transferrin-bound iron in the blood contributes to the majority of iron pool in the body and gets replenished mainly by the recycling of iron by reticuloendothelial macrophages via erythrophagocytosis and small portion from dietary iron through duodenal enterocyte.

In the intestine, inorganic iron in the lumen undergo reduction from ferric to ferrous iron by ascorbate or ferrireductase on the intestinal membrane such as duodenal cytochrome b (DcytB). Duodenal enterocyte then absorb the reduced iron via divalent metal transporter (DMT1/SLC11A2) located on the apical membrane. The absorption of heme is not well defined and was hypothesized to be absorbed via heme transporter or endocytosis [123]. The enterocyte and macrophages contain heme oxygenase which breaks down heme and release ferrous iron which is then exported via ferroportin (SLC40A1). The ferrous iron then gets oxidized by hepaestin or ceruplasmin to form ferric iron which binds to transferring to form diferric transferrin for distribution to other peripheral tissues for utilization.

Iron trafficking in the body is regulated by the hormone hepcidin that is produced by the liver and is influenced by iron concentration in the body as well as conditions such as ER stress, inflammation, erythropoiesis and hypoxia. Hepcidin regulates the efflux of iron by regulating the stability of ferroportin and the inactivation of feroportin result in the retention of iron in the cells. Ferroportin also degrades DMT1 [124].

Cellular regulation of iron and Iron homeostasis

Iron homeostasis involves complex regulatory mechanisms to meet the demand of the human body and to prevent toxicity from iron overloading [125]. Maintaining optimal iron level is crucial as disruption in iron status potentially result in cellular dysfunction and metabolic disorder [37], [114], [122]. In mammals, increase in serum ferritin and transferrin saturation are indicators of iron status. In cell culture, gene expression of *Tfrc* is commonly used to measure the increase or decrease of iron within the cell as *Tfrc* directly regulated by iron sensor iron regulatory proteins (IRPs). However, the most sensitive marker for iron status in the cell is the mRNA binding form of IRPs.

Transferrin bound to two ferric iron atoms binds to Tfrc on the cell surface where the Tf-[Fe(III)2–TFRC] complex is endocytosed. As the complex gets endocytosed, hydrogen is pumped into the endosomes and this result in an increased in pH which then release of ferric iron from transferrin. The ferric iron gets reduced to ferrous iron by steap3 oxidoreductase before getting export by DMT1 into the cytosol. Apo Tf/Tfrc complex is then returned to the cell surface where it dissociates and initiates another round of iron uptake. DMT1 is also localized on the apical membrane of duodenal enterocytes where it transports ferrous iron after reduction to ferric iron by membrane reductases, DCYTB. Iron taken up by either Tfrc or DMT1 enters labile iron pool consist of ferrous iron. The iron in this pool is then sensed by iron regulatory proteins (IRPs). IRPs regulate the translation of mRNA coding for ferritin, ALAS, m-aconitase, ferroportin, Tfrc and DMT1 which are important for maintaining iron homeostasis in the cells. Iron that is not utilized or stored in ferritin is exported by ferroportin. Export of iron from cells is done through the oxidation of iron by hephaestin (membrane) or ceruloplasmin (serum).

Iron regulatory proteins and transferrin receptor

Transferrin receptor (Tfrc) is responsible for the uptake of transferring bound iron found in the blood [114]. The uptake of iron contributes to the labile iron pool (LIP) and is used by the cells for DNA synthesis, mitochondrial function and heme biosynthesis [114]. The LIP also contributes to the iron storage in ferritin and export for use by the body via ferroportin [114]. These receptors and transporters are regulated tightly in the cell by iron regulatory proteins (IRP1 and IRP2) and iron regulatory element (IRE) [37], [114].

Cellular irons are maintained by IRP1 and IRP2 which bind to IRE sequence upstream or downstream of the target genes [115]. The interaction between IRP and IRE are important in maintaining the iron homeostasis, cellular iron utilization, mitochondrial biogenesis as well as heme biosynthesis [115], [126]. IRP biding to IRE either regulate or repress the expression of

proteins involved in iron import, export, usage and storage [114]. IRP binding to the 5` UTR inhibits transcription of mRNA while binding to 3` UTR stabilizes mRNA [114]. Ferritin and ferroportin contain IRE in the 5` UTR while *Tfrc* and *Dmt1* have IRE located on the 3` UTR [37]. Iron status in the cell can be measured by the binding activity of IRP to IRE which is the optimal indicator of iron pool in the cell.

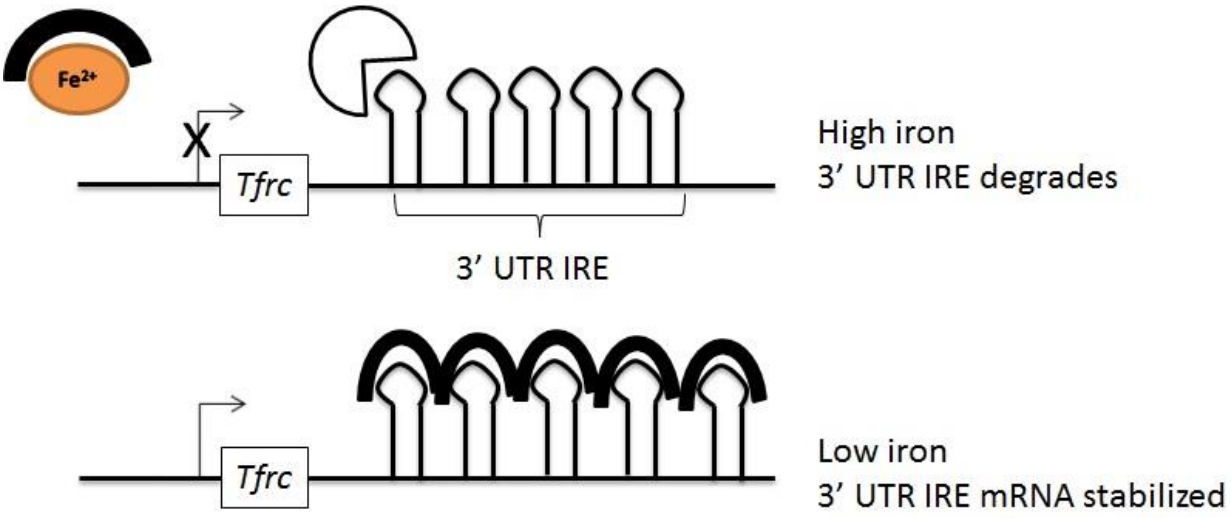


Figure 3: Regulation of *Tfrc* by IRPs during high iron and low iron condition (revised from *Muckenthaler M.U. et al.*) [37]. Iron-regulatory protein 1 and 2 (IRP1 and IRP2) are essential regulatory proteins for maintaining cellular iron homeostasis. IRPs bind to iron-response elements (IREs) in the 5′ or the 3′ untranslated region (UTR) of their target mRNAs. Ferritin (Ft), ferroportin (Ftn) and hypoxia-inducible factor 2α (Hif2α) contains IRE in the 5′ UTR of mRNAs. Transferrin receptor (Tfrc) and divalent metal transporter 1 (DMT1) has IREs in the 3′ UTR of mRNAs. During low iron, IRPs bind to IRE and inhibit5′ IREs translation and stabilizes 3’ IREs. Under high iron condition, IRP1 binds to iron and form cytosolic aconistase while IRP2 is degraded. IRE–IRP regulatory function to increase iron uptake by increase transferrin receptor and DMT1 during low iron and decrease iron storage and export by inhibiting translation of ferritin and ferroportin.

Heme oxygenase

Heme is an essential co-factor for cellular processes such as oxygen transport, electron transport, and signal transduction [127]. However, excess heme is toxic due to production of H_2O_2, and released of free iron which leads to cellular membrane damage and apoptosis [127]. Myoglobin may release free heme in skeletal muscle during severe ischemia-reperfusion injury [127].

Heme oxygenase (encoded by *Hmox-1* genes), a microsomal protein, play a critical role in recycling iron and maintaining cellular homeostasis under stress condition [128]. Under oxidative stress condition, free heme can catalyze the production of free radicals [128], [129]. Free iron can react in a Fenton reaction to produce highly toxic radicals derived from hydrogen peroxides [128], [129]. Activation of *Hmox-1* is stimulated by various factors such as heme, ultraviolet radiation, heavy metals, hydrogen peroxides, endotoxins, and inflammation cytokines [128]. Heme oxygenase has been reported to be induced in rat skeletal muscle following exhaustive exercise, stimulation by electricity and hemin treatment [127].

To remove the pro-oxidant caused by free heme, heme oxygenase breaks down free heme into free iron (Fe^{2+}), carbon monoxide (CO) and biliverdin [127]–[129]. Biliverdin and its reduced form bilirubin are efficient free radical scavengers that behave as anti-oxidants [127]. Moreover, CO functions as vasodilator. The importance of heme oxygenase in cyto-proctection and heme metabolism may be one of the indicators of stress within the cells due to effect of iron chelation or iron loading treatment.

C2C12 in circadian *in vitro* model

In mammals, skeletal muscle is composed of several muscle fibers (i.e. myocytes) and the formation involves the differentiation of myoblast (embryonic progenitor cells) into myotubes during fetal and postnatal development. During muscle damage or degradation, skeletal muscle

resident stem cells, known as the satellite cells, gets activated by entering the cell cycle, proliferate and form myoblasts [130]–[132]. The activation of satellite cells is due to extracellular signal associated with damage in skeletal muscle, contribute to the pool of myoblasts available and provide differentiation-competent myoblast for growth and repair [130]–[132]. These myoblasts will replace the damaged muscle fibers by differentiating and fusing with other myofibers through the process of myogenesis [130]–[132].

The C3H mice dystrophic thigh muscle-derived C2C12 mouse myoblast has the capability to differentiate into myotubes which is closer replication of the physiological state of muscle cells by displaying the increase in metabolic flux and mitochondrial respiration [133], [134]. C2C12 is an immortalized cell line derived from muscle progenitor cells [135]. They can differentiate rapidly, forming contractile myotubes and producing characteristic of muscle proteins. The advantage of examining circadian metabolism via *in vitro* method is that it allows the elimination of upstream factors that impact the circadian regulation such as diet and hormonal regulation [133]. Nevertheless, the purpose of study is to further comprehend the cellular regulation at the tissue and organism level and therefore, provides a holistic portrait of physiology and metabolism [133].

Objectives and hypothesis

These findings lead to the proposed examination on impact of iron depletion and overloading on circadian and metabolic gene expression in C2C12 synchronized myoblast which model the physiological state of proliferating muscle cells. The relationship of iron status and regulation of *Bmal1* and *Rev-erbα* gene expression may reveal the impact of iron status on circadian rhythm and its related metabolic gene expression specific to skeletal muscle cells.

Our objective was to further examine and characterize the effect of iron status on the alteration in circadian and metabolic gene expression in synchronized C2C12 myoblast *in vitro*.

Our central hypothesis was that iron chelation and iron loading would alter circadian and metabolic gene expression (in mitochondrial, myogenesis, macronutrient metabolism) in C2C12 myoblast. Furthermore, we hypothesized that chelation and loading of iron would alter mitochondrial function and oxidative capacity. Thus, the results presented herein provide insight to potential relationship between cellular iron status and circadian rhythm as well as metabolic gene expression.

CHAPTER III

METHODS

Maintenance of C2C12 myoblast cells

Mouse myogenic cell line C2C12 was obtained from a subclone of dystrophic thigh muscle of C3H mice C2C12 (ATCC®; CRL-1772™, Manassas, VA). C2C12 is an immortalized cell line derived from muscle progenitor cells (also known as muscle satellite cells) [135]. These muscle satellite cells enter cell cycle and proliferate into myoblast which replace damaged cells by fusing with each other via differentiation [133]–[135].

C2C12 myoblast cells were maintained in complete media containing Dulbecco's Modified Eagle's Medium (DMEM) with 4.5g/L glucose, 2.5 mM L-glutamine and sodium pyruvate (Cellgro; Catalog # 10-013-CV; Henderson, VA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Atlanta Bio; Catalog # S11150H, Lawrenceville, GA), 2 mM L-glutamine (Cellgro; Catalog # 25-005-c1; Henderson, VA) and penicillin-streptomycin in antibiotic-antimycotic solution containing 100 IU penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B with 8.5 g/L NaCl (Cellgro; Catalog # 30-004-c1;Henderson, VA). The cells were grown for no more than 80% confluence in 100 mm plate in 10 mL of complete media and maintained at 37°C in a humidified incubator with 5% CO_2 supply. Cells were passage up to 25 times, and frequency of every 48 hours at 1:20 dilution or every 36 hours at 1:40 dilution. To

determine the concentration of viable cells, cells were stained with 0.40% trypan blue and counted under the microscope for unstained live cells; dead cells are stained blue.

In vitro circadian assay

Synchronized C2C12 myoblasts and myotubes were used as models for studying circadian rhythm by serum shock using a method described by *Peek, CB et al* [13] [136].

For *in vitro* circadian study in myoblast, $1x10^5$ cells/mL C2C12 myoblast cells were seeded in 6-well plate in complete media and incubated for 24 hours (grown to 80-90% confluence). After 24 hours, the cells were serum shock with DMEM supplemented with 50% heat-inactivated donor horse serum (HS) for 2 hours (Atlanta Bio, Catalog #S12150H; Lawrenceville, GA). Media was switched back to complete media after serum shock and treatments (described below) were introduced for 18 or 24 hours followed by RNA and protein extraction.

For myotubes *in vitro* experiments, myoblast cells were seeded in a 6-well plate at 1 x 10^5 cells/mL in 10% FBS supplemented DMEM media and incubated for 24 hour (grown to 80-90% confluence). After 24 hours, media was replaced with DMEM supplemented with 2% heat-inactivated horse serum to facilitate cell differentiation and media was replaced every other day. Differentiation medium (DM) consisted of DMEM supplemented with 4.5 g/l glucose, 2% heat-activated horse serum (Atlanta Bio; catalog # S12195H,Lawrenceville, GA), 2.5 mM glutamine, 1 mM sodium pyruvate, and 100 IU penicillin and 100ug/mL streptomycin. After 5 days in DM, C2C12 myotubes were serum shock with DMEM supplemented with 50% horse serum for 2 hours. Treatments (described below) were introduced at time 24 hour after changing to 0.5% horse serum + DMEM. Total RNA and protein were harvested at 18 hour after treatment following serum stimulation.

DFO, FAC and hemin treatments

C2C12 myoblast and myotube cells were treated after serum shock for 18 hours with iron chelator using 100 μM deferoxamine (DFO) mesylate salt (Sigma-Aldrich; Catalog # D9533, St. Louis, MO). Iron loading treatment used were 100 μM iron using ferric ammonium citrate (FAC) (Sigma-Aldrich; Catalog # F5879, St. Louis, MO) solubilized in dimethylsulfoxide (DMSO) (Sigma-Aldrich; Catalog # D8418, St. Louis, MO) and 100 μM of iron from hemin (Frontier Scientific; Catalog # H651-9, Logan, UT). To ensure diurnal rhythmicity in C2C12 myoblasts, these cells were treated and harvested every 6 hours for a period of 24 hours. C2C12 myotubes were treated for 18 hours with 200 μM DFO, 200 μM FAC, and 50 μM hemin. The gene expression of *Tfrc* is used as a measurement for iron depletion or iron loading.

RNA extraction

To study the alterations in circadian and metabolic gene expression following treatments, total RNA was extracted from C2C12 myoblasts. Media was aspirated and C2C12 adherent cells were lysed by resuspension with 1 mL RNA STAT-60 (Tel-test, Inc.; Catalog # CS-502, Friendswood, TX) and transfer to 1.7 mL sterile microcentrifuge tubes. The lysed cells were incubated at room temperature for 5 minutes to allow dissociation of ribonucleoprotein complexes. After 5 minutes incubation, 200 μL $CHCl_3$ was added (Sigma; Catalog # C2432, San Francisco, CA) and shaken vigorously for 30 seconds to homogenize the cells. The cells were incubated at room temperature for 3 minutes and following the incubation, the cells were centrifuge at 12,000xg for 15 minutes at 4°C. The protein disassociated into the organic phase and the RNA is in the aqueous phase. The aqueous phase was transferred into new 1.7 mL micro-centrifuge tubes and 500 μL of isopropanol was added and vortex briefly. RNA was precipitated overnight at -80°C and followed by centrifugation at 12,000xg for 30 minutes at 4°C. Supernatant was decant and the RNA pellet was wash with 75% ethanol and centrifuge at 12,000xg for 10 minutes at 4°C. Ethanol was discard and pellet was air dried for 7 minutes. The RNA pellet was incubated in DEPC water (diethyl pyrocarbonate) for 30 minutes before resuspending. The

concentration and purity were determined using nanospectrometer Nanodrop-1000 (Thermo Scientific; Wilmington, DE). The RNA integrity was determined by 1% agarose gel electrophoresis (Sigma; Catalog #A9539, San Francisco, CA) in a solution of 1xTAE [40 mM Tris-acetate acid and 1 mM ethylenediaminetetraacetic acid (EDTA)] using a 1:10000 dilution of Gel Star Nucleic Acid Gel Stain (Lonza; Catalog # 50535; Rockland, ME).

cDNA synthesis for gene expression analysis

cDNA was synthesized from C2C12 total RNA for analysis of gene expression via quantitative polymerase chain reaction (qPCR). Total RNA (2 μg) was treated with DNase I (Roche Applied Science; Catalog # 04716728001, Indianapolis, IN) at 37°C for 30 minutes followed by heat inactivation at 75°C for 10 minutes and soak at 4°C in a BioMetra T-Gradient thermocycler (Biometra,; Goettingen, Germany). DNase I treated RNA was then reverse-transcribed with SuperScript™ II Reverse Transcriptase (Invitrogen; Catalog # 18064071, Carlsbad, CA), random hexamers (Roche Applied Science; Catalog # 11034731001, Indianapolis, IN), 1 mM dNTP mix (Promega Catalog # U1240, Madison, WI), and 10 mM DTT (Invitrogen, Carlsbad, CA). Samples were incubated at 25°C for 10 minutes, 42°C for 50 minutes, and 72°C for 15 minutes and soaked at 4°C in a thermocycler. cDNA was used for qPCR analysis or stored at -20°C until future use up to six months.

Quantitative PCR analysis

Quantitative polymerase chain reaction (qPCR) was used to examine alterations in gene expression in response to treatment conditions. Each reaction was performed in triplicate with a final volume of 10 μL per reaction. Each reaction contained SYBR Green (SA Biosciences; Catalog # 4368706, Frederick, MD), 50 ng cDNA, and 2.5 μM of the primer mix obtained from Integrated DNA Technology (IDT, Coralville, IA). PrimerExpress2.0 software (Applied Biosystems; Foster City, CA) was use to design qPCR primers and the sequences were designed

with amplicon spanning an intron whenever possible. Reactions were performed on a MULTIMAX 384 well PCR Plate in triplicate (Bioexpress, Catalog # T-3138-1, Kaysville, UT) using a 7900HT Fast Real-time PCR System (Applied Biosystems, Foster City, CA). mRNA expression levels were analyzed using relative quantification $2^{-\Delta\Delta C_T}$ method analyzed via SDS 2.4 (Applied Biosystems; ABI software, Foster City, CA) and Cq values where obtained for the genes measured [137], [138].

Accurate analysis of circadian and metabolic gene expression requires appropriate housekeeping genes for normalization. Housekeeping genes *36b4, β-actin, Cyclo, Hprt1, Rpl19* mRNA expression were assessed using real-time qPCR. The determination of housekeeping gene was calculated via NormFinder using log of CT values to plot the most stable genes for C2C12 [139]. Genes of interest that were analyzed are listed in **Table 2**.

Table 1: Primer sequences for qPCR

Gene	Sense	Antisense	Accession no.
36b4	5' CACTGGTCTAGGACCCGAGAAG 3'	5' GGTGCCTCTGAAGATTTTCG 3'	NM_007475.5
Alas1	5' TGCAGAAGGCAGGAAAGTCT 3'	5' AGGGGTTTCTTTGACCTGCT 3'	NM_001291835.1
Bmal1 (Arntl)	5' TGTCACAGGCAAGTTTTACAGAC 3'	5' ACAGTGGGATGAGTCCTCTTTG 3'	NM_007489.4
Clock	5' GAAGAAACTTTTACAGGCGTTGTTG 3'	5' ACGCAAGGCCGTCTTCTG 3'	NM_007715
Cry1	5' TCG CCG GCT CTT CCA A 3'	5' TCA AGA CAC TGA AGC AAA AAT CG 3'	NM_007771.3
Hmox-1	5' TCAGGTGTCCAGAGAAGGCTTT 3'	5' TCTTCCAGGGCCGTGTAGAT 3'	NM_010442
Rev-erbα (Nr1d1)	5' TCCAACAGAATATCCAGTACAAACG 3'	5' GCGATTGATGCGAACGAT 3'	NM_145434.4
Rev-erbβ (Nr1d2)	5' TGGGACTTTTGAGGTTTTAATGG 3'	5' GTGACAGTCCGTTCCTTTGC 3'	NM_011584.4
Rorα (Nr1f1)	5' ACCGTGTCCATGGCAGAAC 3'	5' TTTCCAGGTGGGATTTGGAT 3'	NM_001289916.1
MyoD	5' TACAGTGGCGACTCAGATGC	5'GAGATGCGCTCCACTATGCT 3'	NM_010866.2
Per2	5' AAGAACGCGGATATGTT	5' GGCGTCCTTCTTACAGTGA 3'	NM_011066
Pdk4	5' CAAAGACGGGAAACCCAAGC3'	5' CGCAGAGCATCTTTGCACAC3'	NM_013743
Srebp1c	5' GGAGCCATGGATTGCACATT 3'	5' GGCCCGGGAAGTCACTGT	NM_011480
Pgc-1α	5` AACCACACCCACAGGATCAGA 3`	5` TCTTCGCTTTATTGCTCCATGA 3`	NM_008904
Pgc-1β	5' GAGGGCTCCGGCACTTC 3'	5' CGTACTTGCTTTTCCCAGATGA 3'	NM_133249
Tfrc	5' TCATGAGGGAAATCAATGATCGTA 3'	5' GCCCCAGAAGATATGTCGGAA 3'	NM_011638

XF96 Seahorse Assay

Seahorse XF96 analyzer measured mitochondrial respiration and glycolysis in living cells. The Seahorse XF technology has sensor that measures both oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). For our study purpose, mitochondria respiration was analyze and OCR was measure via mitochondria stress test.

Seeding C2C12 in Seahorse XF96 mircoplate

C2C12 myoblast cells were seeded in a Seahorse 96 well XF cell culture microplate (Seahorse Bioscience, Catalog # 101085-004; North Billerica, MA) at 80 μL in complete media at final concentration of $1x10^5$cells/mL. Background correction wells (A1, A12, H1, H12) are fill with 100 μL complete media only. Plate is rest in the cell culture hood at room temperature for one hour to allow even distribution of cells before incubating at 37°C for 24 hours. After 24 hours, media is replaced with synchronization media (50% horse serum/50%DMEM) for 2 hours. After serum shock, media was replaced with complete media and cells are treated with 100 μM DFO, FAC and hemin treatments (described above) for 18 hours before running Mito stress test.

XF96 Assay Cartridge and Assay Media Preparation

XF96 sensor cartridge (Seahorse Bioscience, Catalog # 101085-004; North Billerica, MA) has solid sensors for oxygen consumption and extracellular acidification which also contains four ports per well for compounds injection during the analysis. The day before running assay, the XF96 sensor cartridge is hydrated. Each well of the Utility Plate is filled with 200 μL of Seahorse XF Calibrant. Sensor Cartridge (Seahorse Bioscience, Catalog # 100840-000; North Billerica, MA) is gently lowered onto the Utility Plate submerging the sensors in XF Calibrant. The cartridge was contained in a sealed box to prevent evaporation in non-CO_2 37 °C incubator overnight.

XF base medium (Seahorse Bioscience, Catalog # 102353; North Billerica, MA) was pre-warmed in 37°C water bath until ready to use. To make mito stress test assay medium, in 150 mL of XF base media, 25 mM of d-glucose (Sigma-Aldrich; # G7528, St. Louis, MO), 1 mM sodium pyruvate (Sigma-Aldrich; # S8636, St. Louis, MO) and 2 mM L-glutamine (Life Technologies; Catalog #25030-081, Carlsbad, CA) were added and adjusted pH to 7.4 using 1 M NaOH.

After 18 hours of treatments of C2C12, media was removed and microplate was gently washed three times with 200 μL of pre-warmed mito stress test assay medium. Finally, the cells were incubate with 175 μL cell mito stress test assay medium in 37°C incubator without CO_2 for 1 hours to allow cells to equilibrate with the assay medium and ready for XF96 Seahorse Assay.

Mitochondrial Respiration Measurement Using XF96 Cell Mito Stress Test Kit

XF96 Mito stress test kit (Seahorse Bioscience; Catalog # 101706-100, MA) was utilized. Compounds for mito stress test were added to pre-warmed mito stress test assay medium prior to loading sensor cartridge. The mito stress test compounds are 25 μL of 1 μM oligomycin, 1 μM FCCP (carbonilcyanide p-triflouromethoxyphenylhydrazone) and 1μM rotenone/antimycin A. The compounds were loaded in proper column with a multi-channel pipette according to assay lay out into ports A, B, and C respectively. The sensor cartridge with compounds was loaded on XF96 Extracellular Flux Analyzer for calibration prior to Mito stress assay. Once calibration process is done, microplate containing C2C12 cells was loaded onto the XF96 Extracellular Flux Analyzer and start the mito stress test assay. The compounds were loaded sequentially as described in **Table 3**.

Oligomycin is an inhibitor of ATP synthase (complex V), FCCP is a mitochondrial uncoupler, and antimycin A/rotenone are electron transport inhibitor (complex III and complex I, respectively). Mito stress test reveals the key parameters of mitochondrial function by measuring the oxygen consumption rate (OCR) in the media to assess basal respiration, ATP turnover,

proton leak and maximal respiration. The rate of OCR is proportional to mitochondrial respiration. The test was carried out by sequentially loading of Oligomycin, FCCP and rotenone/antimycin into port A, B and C respectively to obtain OCR measurement using XF96 WAVE program (Seahorse Biosciences, Software Version 2.2 Wave controller, MA). Small changes in O_2 level and pH can be readily detected by Seahorse analyzer. Proteins were extracted from the microplate and concentrations were obtained via BCA assay. OCR measurements were normalized with protein concentration.

Table 2: Mix and measurement cycle time for XF96 assay

Command	Time (min)	Port	# Repeat
Calibrate	20		
Equilibrate	12		
Mix	3		3
Wait	0		
Measure	3		
Inject		A	
Mix	3		3
Wait	0		
Measure	3		
Inject		B	
Mix	3		3
Wait	1		
Measure	3		
Inject		C	
Mix	3		
Wait	0		
Measure	3		
End of Assay			

Protein Extraction

Cytosolic proteins were extracted for normalization of protein concentration with OCR from Seahorse XF96 mitochondrial stress test. XF buffer media was removed from XF96 microplate and the cells were gently washed three times with ice-cold phosphate buffered saline (PBS) (Cellgro, Herndon, VA; Catalog # 21-031-CV). Cells were resuspended in cell lysis buffer (20 mM HEPES pH 7.4, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 50 mM β-glycerol phosphate, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM guanosine triphosphate (GTP), 1 mM sodium orthovanadate, and 0.5% v/v NP-40) containing protease inhibitors (0.1 mg/mL leupeptin, 0.1 mg/mL pepstatin, 0.25 mg/mL soybean trypsin inhibitor, 0.2 mM phenylmethylsulfonylfloride, and 0.1 mM MG-132), 1 mM citrate, and 1 mM dithiothreitol (DTT). Samples were incubated on ice on a shaker for 1 hour. After 1 hour, protein concentrations of cell extracts were measured using Pierce™ BCA Protein Assay Kit (Thermo Sci, Catalog # 23227; Grand Island, NY) using Synergy HT microplate reader (Biotek; Winooski, VT).

Statistical Analysis

Data from C2C12 cells were analyzed with either one-way ANOVA or student's T-test using SPSS software version 17.0 (IBM-SPSS, Chicago, IL) to determine statistically significant changes. Gene expression between control and treatment groups at same time point or control at 0h and treatment group at different time point was determined using student's *t*-test. Dose response experiment and mitochondrial stress test were analyzed with one-way ANOVA. All tests were measured at a 95% confidence interval with means ± SEM.

CHAPTER IV

RESULTS

Effects of treatment in C2C12 Myoblast

Effects of DFO, FAC, and hemin concentrations on *Tfrc* and *Hmox-1*gene expression

To determine the appropriate concentration of DFO, FAC and hemin, C2C12 cells were treated in varying concentrations for 18 h. Based on various literatures that studied *Tfrc* gene expression with effect of iron source and iron chelator treatments, treatment time ranges from 6, 12, 16, 18 and 24 h [3], [140]–[143]. The optimal time ranges from 12-24 h for observation of significant changes of *Tfrc* gene expression [140]. Studies on cell culture on DFO, FAC and hemin treatment commonly ranges from 50-200 μM for DFO, while FAC is 20-500 μM and hemin is 10-200 μM and ranging period from 16 h-24 h treatment [3], [141]–[146].

The dose response experiment measured *Tfrc* and *Hmox-1* gene expression after 18 h of treatment. C2C12 myoblasts were treated with different concentration of DFO (0, 50, 100 and 200 μM) and significant differences were observed with *Tfrc* gene expression in 100 μM ($p = 0.025$) and 200 μM ($p = 0.012$) treatment **(Figure 4A)**. No significant changes in *Hmox-1* was observed, however, the gene expression was trending upward **(Figure 4A)**. Incubating C2C12 cells in 100 μM is sufficient to significantly induced *Tfrc* gene expression.

Tfrc gene expression in FAC (0, 100, and 200 μM) treated cells was trending downward but no statistical significant different was observed **(Figure 4B)**. *Hmox-1* gene expression was

upregulated by 3- and 2.9- fold in 100 μM and 200 μM. Higher concentration of FAC (400 μM) resulted in cell death in C2C12 myoblast (data not shown due to cell death). 100 μM of FAC was used in the study based on 40% decreased in *Tfrc* gene expression despite insignificant statistical difference.

In hemin (0, 100, 200, and 400 uM) treated cells, there was a significant repression of *Tfrc* gene expression at 100 μM (p =0.014), 200 μM (p = 0.005) and 400 μM (p = 0.002) **(Figure 4C)**. As expected, high induction of *Hmox-1* gene expression was observed in hemin treated cells (100 μM: p = 0.004; 200 μM: p = 0.000; 400 μM: p = 0.000). Interestingly, the induction of *Hmox-1* went slightly down in 400 μM hemin when compared to 200 μM treated cells.

These findings demonstrated that DFO and hemin are effective to stimulate a change in *Tfrc* gene expression. Therefore, treatment concentration for the study was decided at 100 μM for DFO, 100 μM FAC and 100 μM hemin. FAC treatment needs further validation by measurement of iron regulatory protein (IRP) RNA binding activity to ensure iron loading. Moreover, cell viability is needed to ensure the changes in gene expression are not resulted from cell death.

Effect of DFO, FAC, and hemin on *Alas1*, *Bmal1*, *Rev-erbα* and *Tfrc* gene expression

To determine the treatment effect of iron chelator (DFO) and iron sources (FAC and hemin) on circadian rhythmicity in C2C12. Cells were harvest at every 6 h for a period of 24 h starting from time of treatment for qPCR analysis.

In DFO treated cells, no change in oscillation of *Alas1* gene expression however, there was a 2.6-fold increase of peaked ZT12 (p= 0.003) **(Figure 5A)**. *Bmal1* and *Rev-erbα* gene expression exhibit circadian oscillation and treatment with DFO significantly increased the amplitude of the oscillation. Regulation of *Bmal1* gene expression is still in phase and was significantly regulated at ZT24 (p = 0.011) **(Figure 5B)**. *Rev-erbα* gene expression is trending toward a constant increased and significantly up-regulated at ZT18 (p = 0.028) and ZT24 (p =

0.025) **(Figure 5C)**. *Tfrc* gene expression is upregulated at ZT12 ($p = 0.017$) and peaked at ZT18 ($p = 0.019$). Despite a slight decrease, *Tfrc* was still significantly up-regulated at ZT24 ($p = 0.030$), indicating higher demand of iron by the cell **(Figure 5D)**.

In FAC treated cells, *Alas1* gene expression loses rhythmicity and significantly regulate over 24 h period ($p \leq 0.05$) **(Figure 6A)**. *Bmal1* gene expression was similar to control cells at ZT6 but trend toward repression in gene expression from ZT12 – ZT24 **(Figure 6B)**. Despite the downward trend in *Bmal1* from ZT12 to ZT24, the gene expression was higher than the control at ZT12 ($p = 0.005$) and ZT18 ($p = 0.013$). The oscillation of *Rev-erbα* is out-of-phase by 6 h despite similar amplitude and trough (ZT12: $p = 0.017$) **(Figure 6C)**. *Tfrc* gene expression was trending downward at ZT6 and ZT 12 by 40%, however, at ZT18 and ZT 24 ($p = 0.025$), *Tfrc* gene expression in FAC treated cells was similar to the control **(Figure 6D)**.

In hemin treated cells, *Alas1*gene expression was down regulated over the 24 h period, and was significantly different from the control at ZT6 ($p = 0.010$) **(Figure 7A)**. *Bmal1*gene expression was down regulated over 24 h period, however, no significant difference was observed **(Figure 7B)**. In the case of *Rev-erbα* gene expression, the oscillations in mRNA between control and hemin treated cells had similar period, however, hemin treated cells have higher amplitude at ZT12 ($p = 0.022$) and lower trough at ZT6 than the control **(Figure 7C)**. *Tfrc* gene expression was significantly repressed at ZT6 ($p = 0.024$), ZT12 ($p = 0.011$), ZT18 ($p = 0.013$), and ZT24 ($p = 0.014$) **(Figure 7D)**.

These results confirm our hypothesis that iron status based on changes in *Tfrc* gene expression regulates circadian (*Bmal1 and Rev-erbα*) and metabolic (*Alas1*) gene expression. In FAC treated cells, *Tfrc* gene expression was not significantly repressed, however, changes circadian rhythm in *Alas1, Bmal1* and *Rev-erbα* were observed.

Effect of DFO, FAC, and hemin on circadian gene expression

Core circadian clock gene expression in C2C12 treated cells was assessed by qPCR. After 18 h of iron chelator and iron loading treatment, cells were harvested for RNA extraction and qPCR analysis to measure circadian gene *Bmal1, Clock, Cry1, Per2, Rev-erbα, Rev-erbβ* and *Rorα* expression.

At ZT18, *Bmal1* and *Rev-erbα* gene expression was significantly up-regulated in DFO treated cells by 1.7- ($p = 0.010$) and 3.4- ($p = 0.010$) folds respectively **(Figure 8A)**. *Per2* gene expression was down-regulated ($p = 0.039$). No significant change was observed in *Clock, Cry1,* and *Rev-erbβ.* Interestingly, in DFO treated cells, *Rorα* was increased by 22.4-fold ($p = 0.001$).

In FAC treated cells, *Rev-erbβ* gene expression was down-regulated at ZT18, however, only changes in *Rev-erbβ* ($p = 0.020$) were statistically significant **(Figure 8B)**. No significant change was observed in *Bmal1, Clock, Cry1,* and *Per2* gene expression **(Figure 8B)**. In hemin treatment at ZT18, no significant change was observed in *Bmal1, Clock, Cry1, Per2, Rev-erbα, Rev-erbβ* and *Rorα* gene expression **(Figure 8C)**. Interestingly, despite no statistically significant result, *Rev-erbα* and *Rev-erbβ* gene expression were trending towards repression, which is similar to treatment with FAC.

Effect of DFO, FAC, and hemin on iron metabolism, heme biosynthesis and mitochondrial gene expression

To determine the effect of iron treatment and iron chelation on gene expression involves in energy metabolism, mitochondrial biogenesis, heme biosynthesis, and iron metabolism, *Pgc1α, Pgc1β, Alas1, Hmox-1,* and *Tfrc* gene expression were analyzed via qPCR.

In DFO treated cells, *Pgc1α* ($p = 0.006$) and *Tfrc* ($p = 0.002$) gene expression were significantly up-regulated at ZT18 **(Figure 9A)**. Interestingly, *Pgc1β* gene expression was significantly repressed ($p = 0.012$). No change in *Hmox-1* and *Alas1* gene expression was observed.

In FAC treated cells, *Pgc1α* (p = 0.009) and *Alas1* (p = 0.011) gene expression were significantly up-regulated while gene expression was significantly repressed **(Figure 9B)**. However, no significant change in *Pgc1β, Hmox-1* and *Tfrc* was observed.

In hemin treated cells, *Hmox-1* (p = 0.012) was significantly up-regulated while *Pgc1β* (p = 0.032) gene expression was significantly repressed **(Figure 9C)**. As expected, *Tfrc* (p = 0.015) is down regulated with hemin treatment. However, no change in *Pgc1α* and *Alas1* gene expression was observed.

Effect of DFO, FAC, and hemin on metabolic gene *MyoD, Pdk4* and *Srebp1c* expression

MyoD, Pdk4 and *Srebp-1c* are genes that are involves in skeletal muscles differentiation, glucose metabolism and lipid metabolism, respectively. The changes in genes expression were assessed by qPCR.

In DFO treated cells, a dramatic increase in *Pdk4* (p = 0.004) gene expression with 9.9 fold was observed at ZT18 **(Figure 10A)**. No change in *MyoD* and *Srebp-1c* gene expression was observed.

In FAC treated cells, *MyoD* (p = 0.05), *Pdk4* (p = 0.024) and *Srebp-1c* (p = 0.019) gene expression were significantly repressed at ZT18 **(Figure 10B)**. In hemin treated cells, *MyoD* (p = 0.017) and *Pdk4* (p = 0.052) gene expression were significantly repressed at ZT18 **(Figure 10C)**. Despite insignificant difference, *Srebp-1c* gene expression was trending towards repression**.**

Effect of DFO, FAC, and hemin on maximal mitochondria respiration capacity

To study the effect of iron status on mitochondrial function in C2C12 myoblast, XF96 seahorse mitochondrial stressed test was utilized. Cell density (5k, 10k and 20k) and oligomycin concentration (0, 0.5, 1, and 2 μM) were optimized **(Figure 11A & 11B).** Cell density at 10k and 1 μM of oligomycin were used for the study. Next, FCCP concentration (0, 0.75, 1.5, 3, 4.5 and 6

μM) were optimized **(Figure 12).** The optimal concentration for FCCP was 0.75 μM. Mitochondrial stress tests were performed on C2C12 myoblast treated with DFO, FAC and hemin **(Figure 13)**. Despite insignificant difference, oxygen consumption rate (OCR) for DFO treated cells were trending downward compare to control cells. FAC ($p = 0.000$) and hemin ($p = 0.011$) treated cells experienced significant increase in OCR **(Figure 13)**.

Effects of treatment in C2C12 Myotubes

Effect of DFO, FAC, and hemin on circadian gene expression

Core circadian clock gene expression in C2C12 myotubes treated cells was assessed by qPCR. After serum shock, cells were treated at ZT6. After 18 h of iron chelator and iron loading treatment, cells were harvested at ZT24 for RNA extraction and qPCR analysis to measure *Bmal1, Rev-erbα, Rev-erbβ and Rorα* gene expression.

At ZT24, *Bmal1* and *Rorα* gene expression was significantly up-regulated in DFO treated cells by 1.7- ($p = 0.043$) and 3.4- ($p = 0.001$) folds respectively **(Figure 14A)**. No significant change was observed in FAC or hemin treatment in these genes except for an increased in *Rev-erbα* ($p = 0.020$) with hemin treatment.

Effect of DFO, FAC, and hemin on iron metabolism, heme biosynthesis and mitochondrial gene expression

To determine the effect of iron treatment and iron chelation on gene expression involves with energy metabolism, mitochondrial biogenesis, heme biosynthesis, and iron metabolism, *Pgc1α, Pgc1β, Alas1, Mfrn2, Hmox-1,* and *Tfrc* gene expression was analyzed via qPCR.

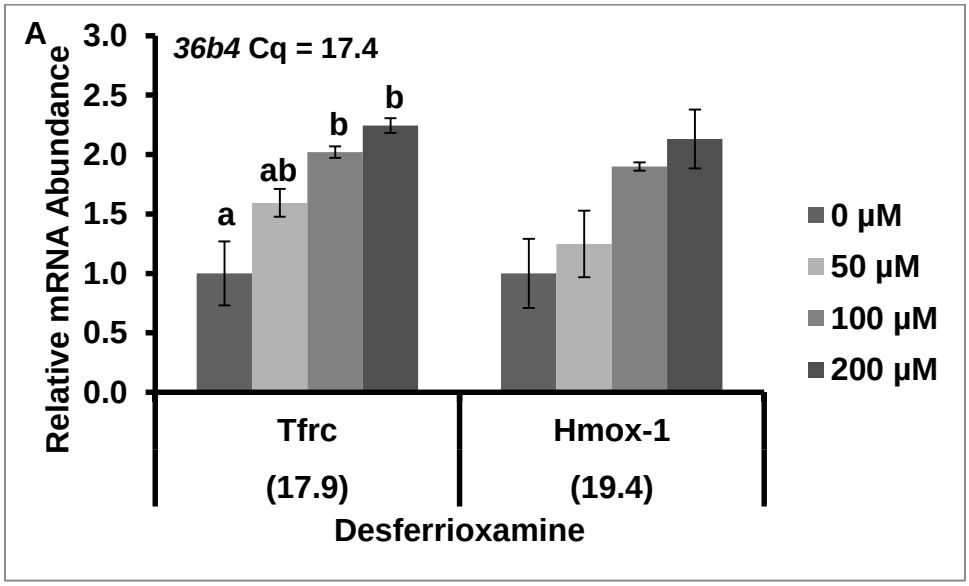
Pgc-1α gene expression was significantly inhibited in DFO ($p = 0.000$) and hemin ($p = 0.000$) treatment ZT24 **(Figure 14B)**. No changes in *Alas1* and *Mfrn2* gene expression was observed except for *Alas1* gene expression in hemin treated cells. *Tfrc* was significantly

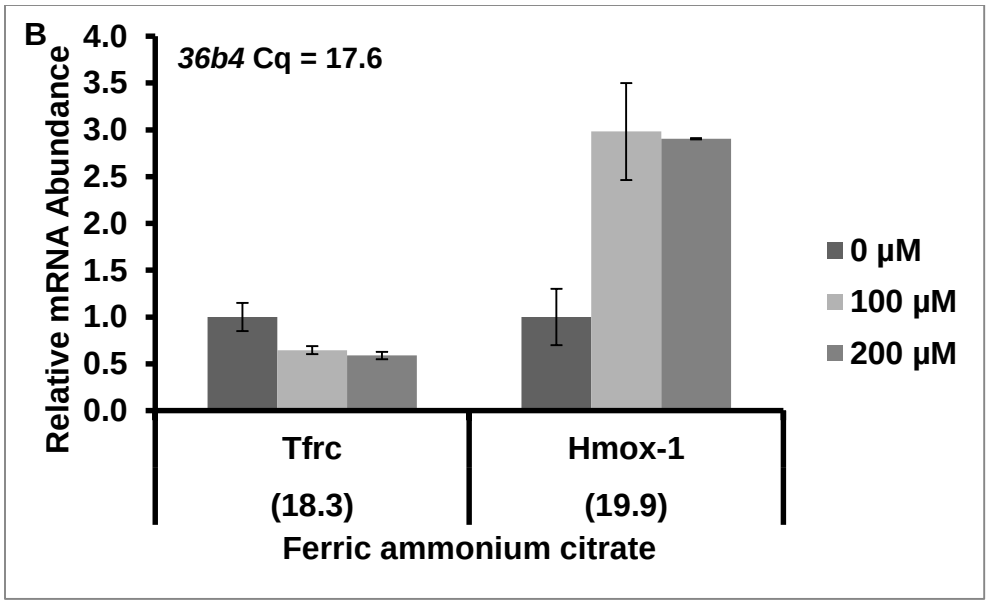
expressed in DFO (p = 0.006) and inhibited in FAC (p = 0.000) and hemin (p = 0.000) treatment. *Hmox-1* was upregulated with hemin treatment (p = 0.003) **(Figure 14C)**.

Effect of DFO, FAC, and hemin on metabolic gene *MyoD, Fasn* and *Srebp1c* expression

MyoD, Fasn and *Srebp-1c* are genes that are involved in skeletal muscles differentiation, glucose metabolism and lipid metabolism, respectively. The changes in genes expression were assessed by qPCR.

MyoD gene expression was significantly down regulated in all treatment (DFO, p = 0.024; FAC, p = 0.038, Hemin, p = 0.003) **(Figure 14D)**. Intriguingly, *Fasn* gene expression was upregulated with FAC treatment but went down with hemin. No change in *Srebp1c* gene expression was observed.





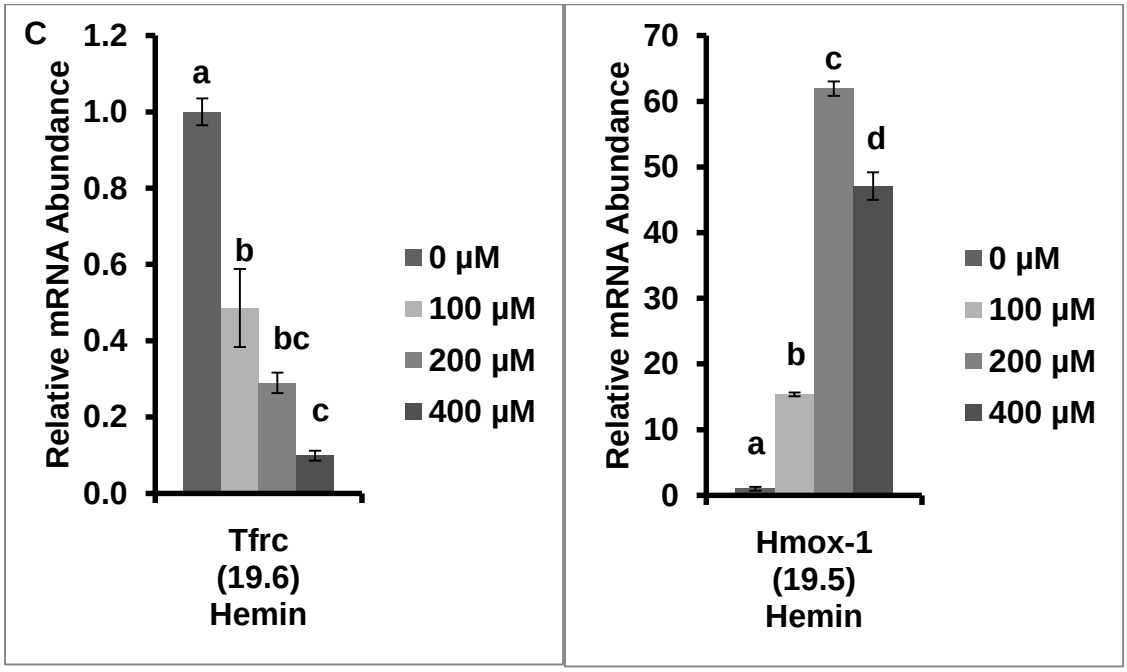
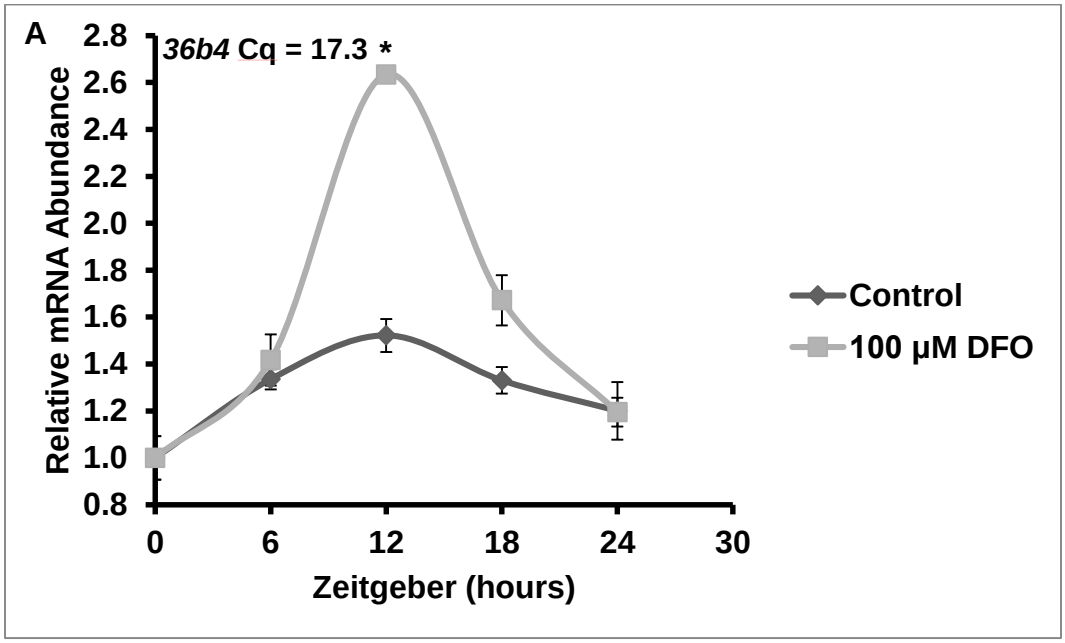
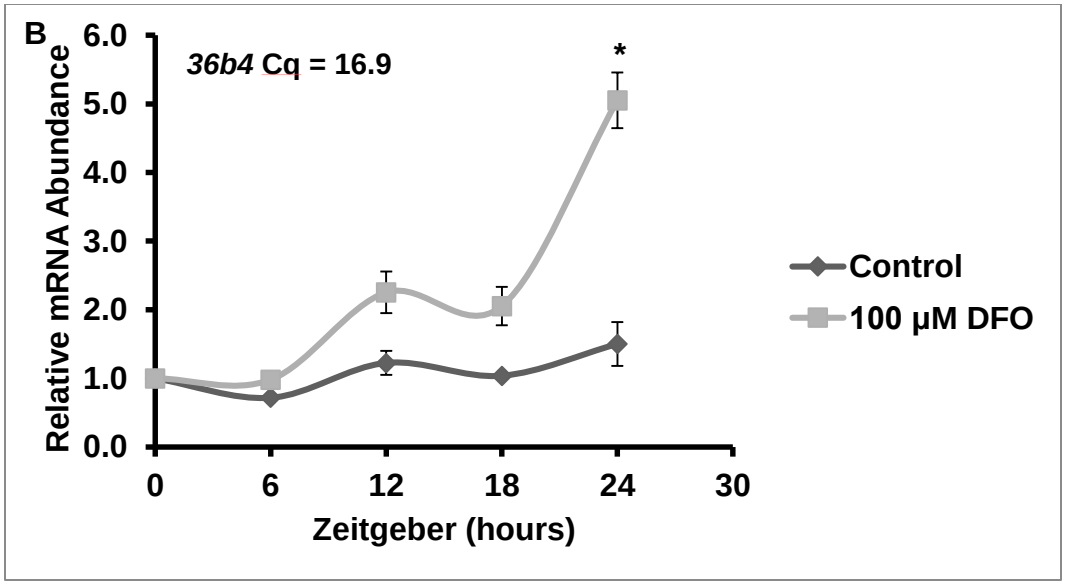


Figure 4: Dose dependent response of (A) DFO, (B) FAC and (C) hemin over 18 h treatment with C2C12 myoblast in complete media. *Tfrc* and *Hmox-1* mRNA abundance was determined by qPCR. All treatments concentration were compared with control. Data is representative of at least three independent experiments conducted in duplicate and are expressed as fold changes ± SEM. Significant difference was determined using one-way ANOVA; Significance is set at $P < 0.05$ and the letters represent significance different between group.


A
36b4 Cq = 17.3 *
Relative mRNA Abundance
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0
6
12
18
24
30
Zeitgeber (hours)
Control
100 µM DFO


B
36b4 Cq = 16.9
*
Relative mRNA Abundance
6.0
5.0
4.0
3.0
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1.0
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0
6
12
18
24
30
Zeitgeber (hours)
Control
100 µM DFO

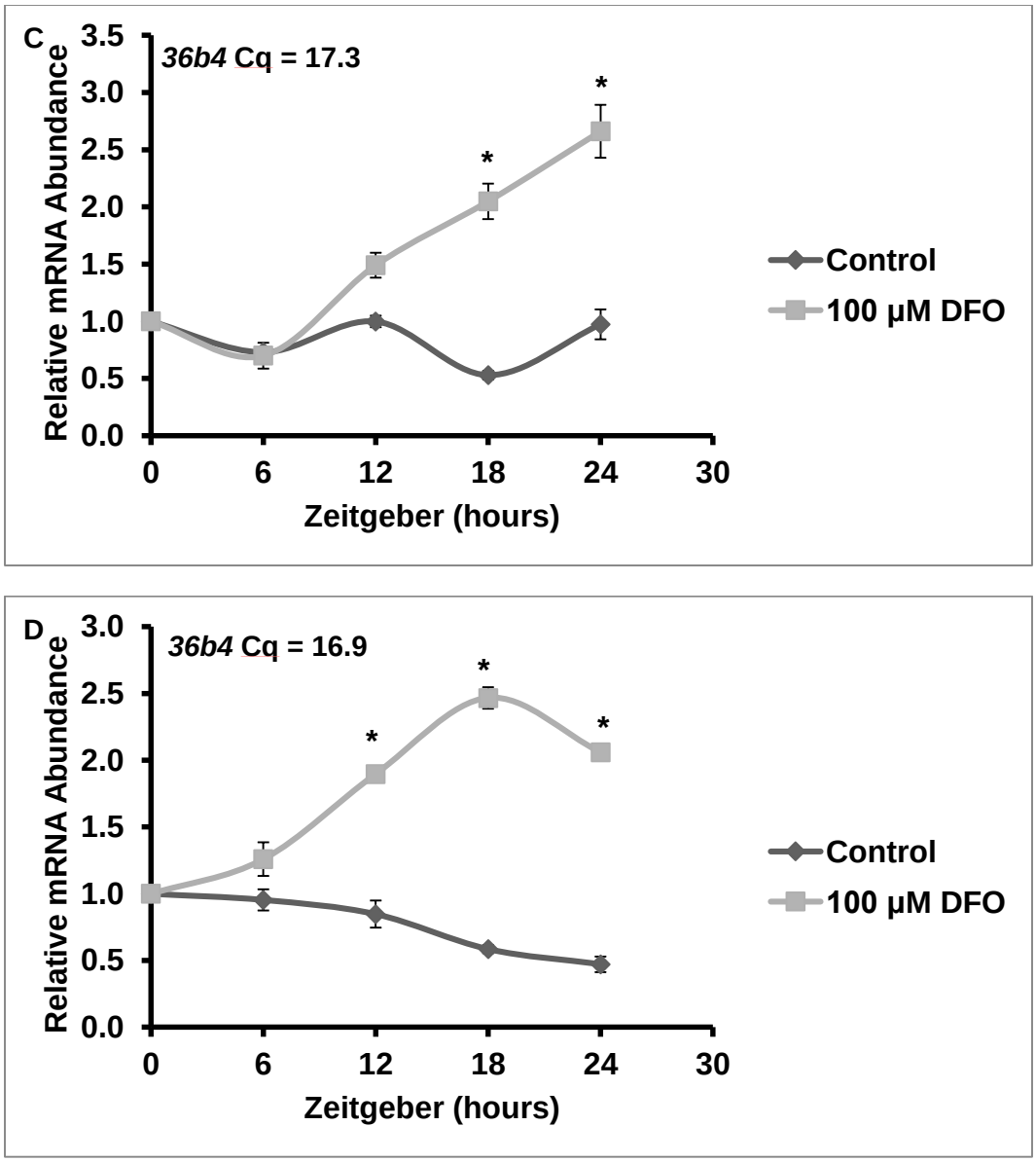
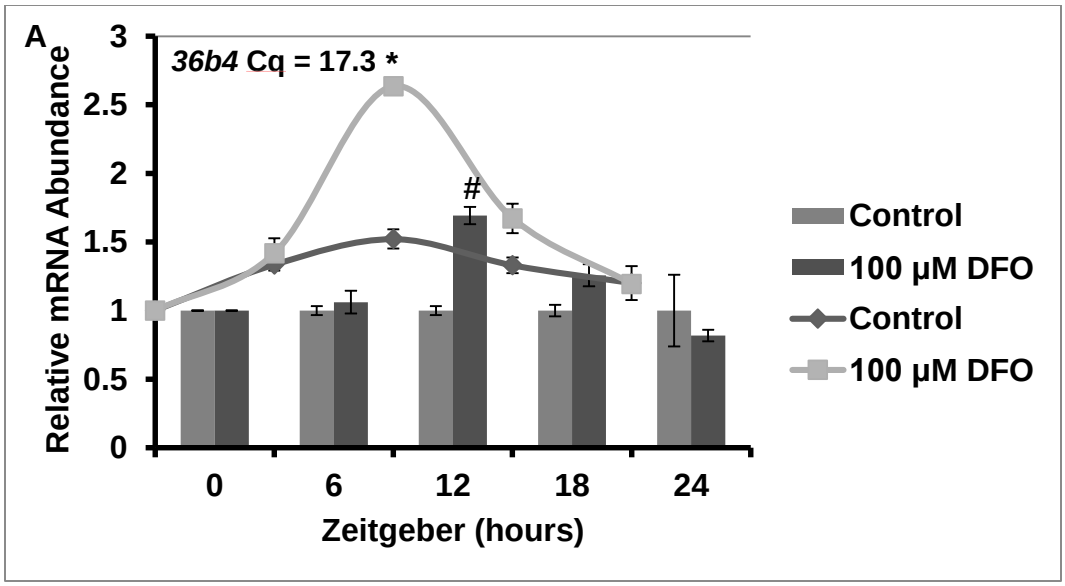
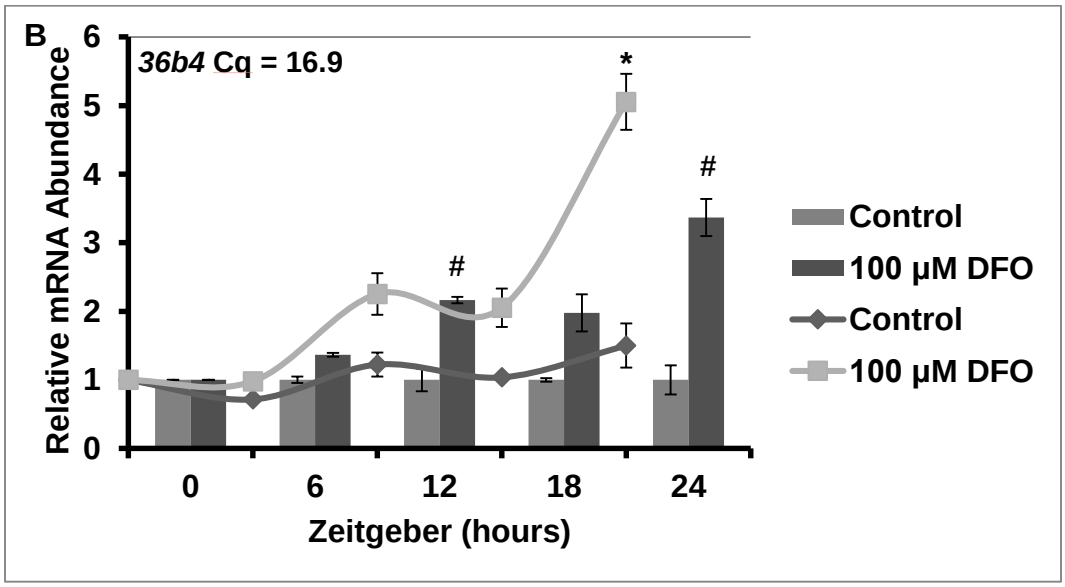


Figure 5.1: Diurnal rhythmicity of (A) *Alas1* (B) *Bmal1* (C) *Rev-erbα* and (D) *Tfrc* gene expression in C2C12 myoblasts post serum shock. C2C12 myoblasts were treated with DFO after serum shock for a period of 24 h and RNA was harvested every 6 h. mRNA abundance was measured by qPCR and normalized to 36b4. Data is representative of three independent experiments conducted in duplicate and are expressed as fold changes ± SEM. Significant different was determined using student's *t*-test.; *P < 0.05 when compared to untreated control at 0h.


A
36b4 Cq = 17.3 *
Relative mRNA Abundance
3
2.5
2
1.5
1
0.5
0
#
0
6
12
18
24
Zeitgeber (hours)
Control
100 µM DFO
Control
100 µM DFO


B
36b4 Cq = 16.9
Relative mRNA Abundance
6
5
4
3
2
1
0
*
#
#
0
6
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18
24
Zeitgeber (hours)
Control
100 µM DFO
Control
100 µM DFO

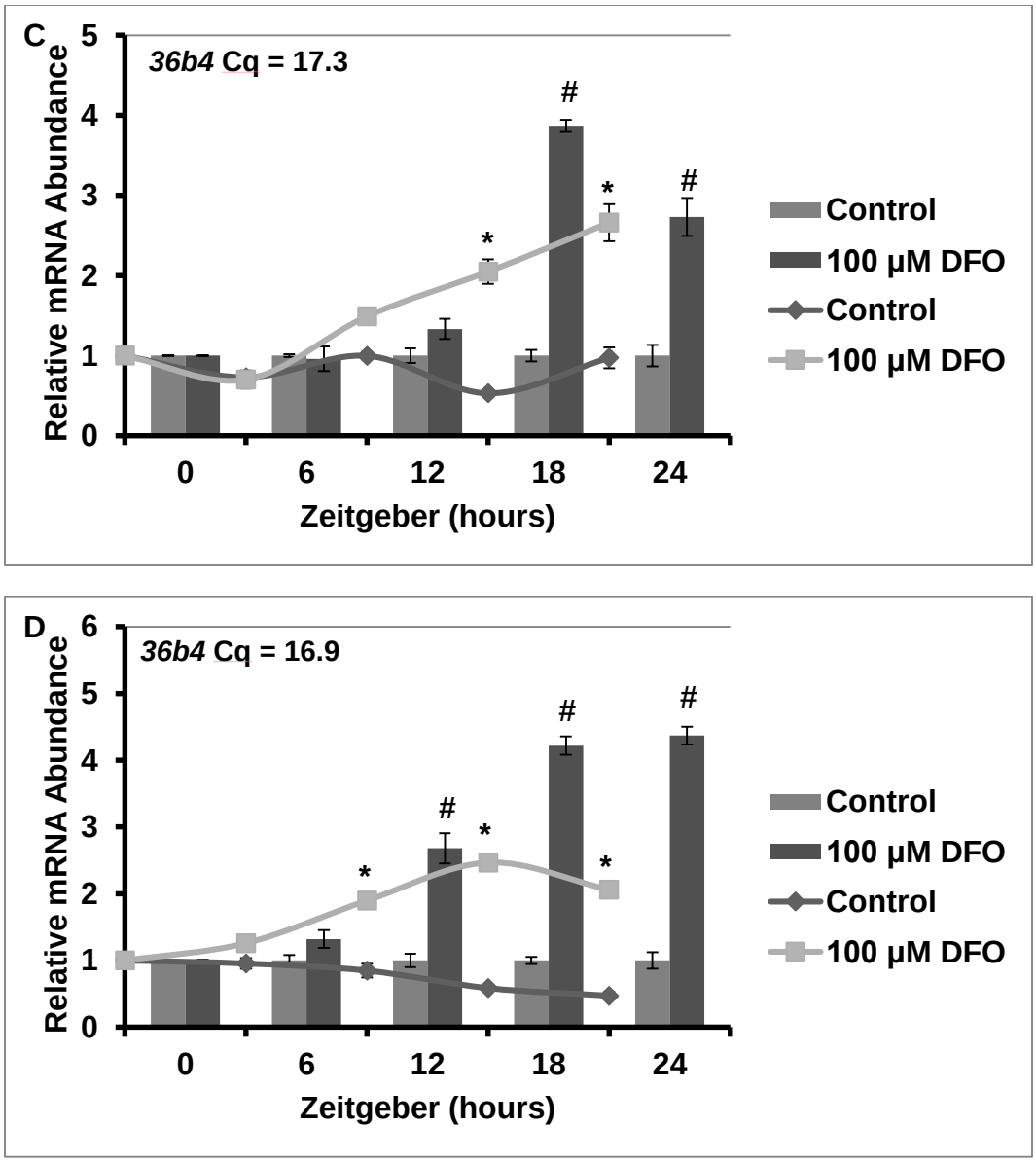
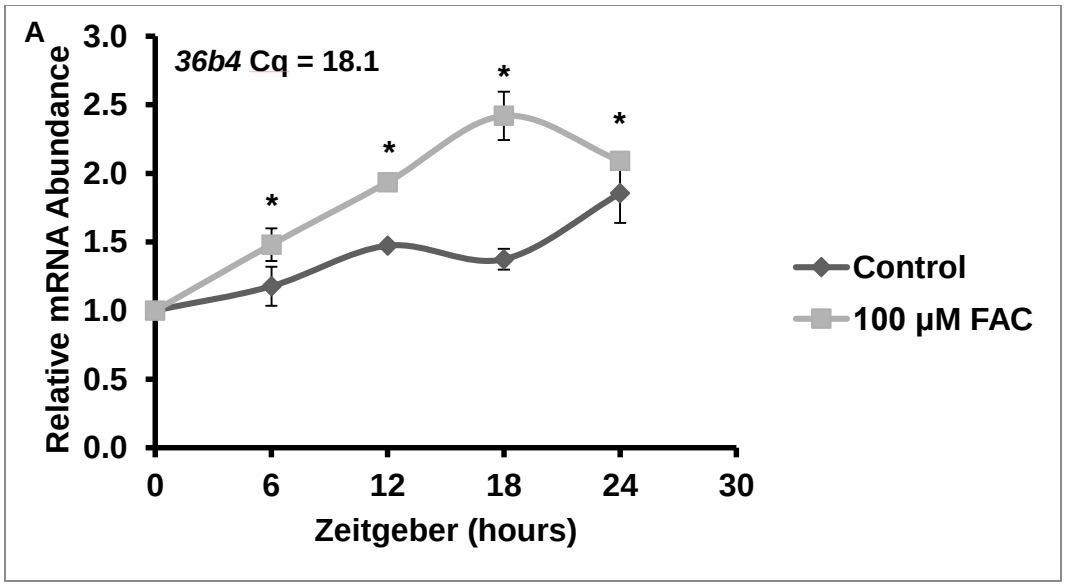
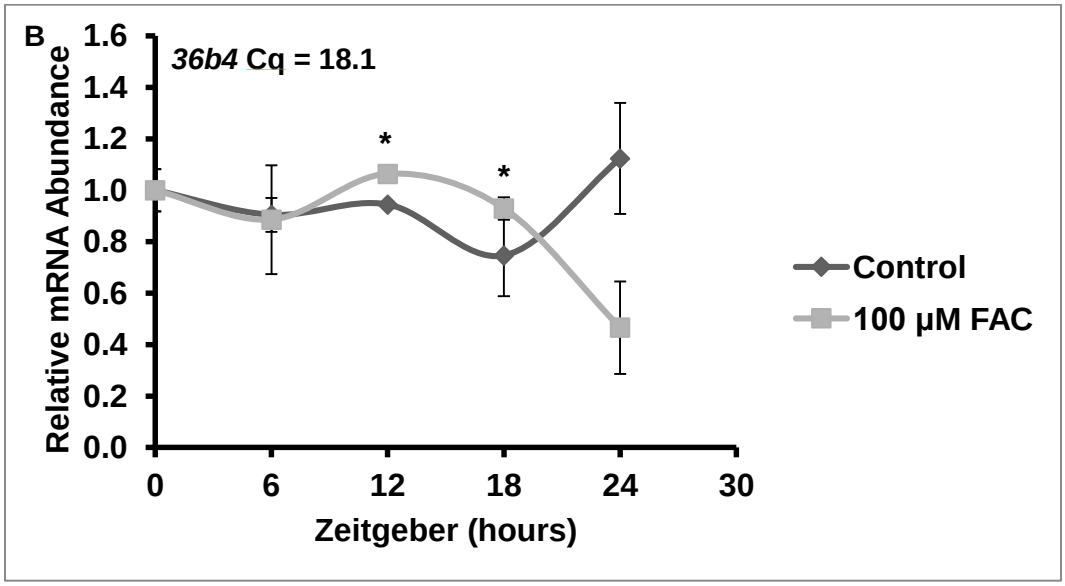


Figure 5.2: Diurnal rhythmicity of (A) *Alas1* (B) *Bmal1* (C) *Rev-erbα* and (D) *Tfrc* gene expression in C2C12 myoblasts post serum shock. The line graph (from Figure. 5.1) represents gene expression of treatment compared to 0h control. Bar graph is a representative of untreated control compare with treated cells at the same time point. C2C12 myoblasts were treated with DFO after serum shock for a period of 24 h and RNA was harvested every 6 h. mRNA abundance was measured by qPCR and normalized to 36b4. Data is representative of three independent experiments conducted in duplicate and are expressed as fold changes ± SEM. Significant different was determined using student's *t*-test. *P < 0.05 when compared to untreated control at 0h. # P < 0.05 when compared to untreated control at same time point.


A
36b4 Cq = 18.1
Relative mRNA Abundance
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
6
12
18
24
30
Zeitgeber (hours)
*
*
*
*
Control
100 µM FAC


B
36b4 Cq = 18.1
Relative mRNA Abundance
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
6
12
18
24
30
Zeitgeber (hours)
*
*
Control
100 µM FAC

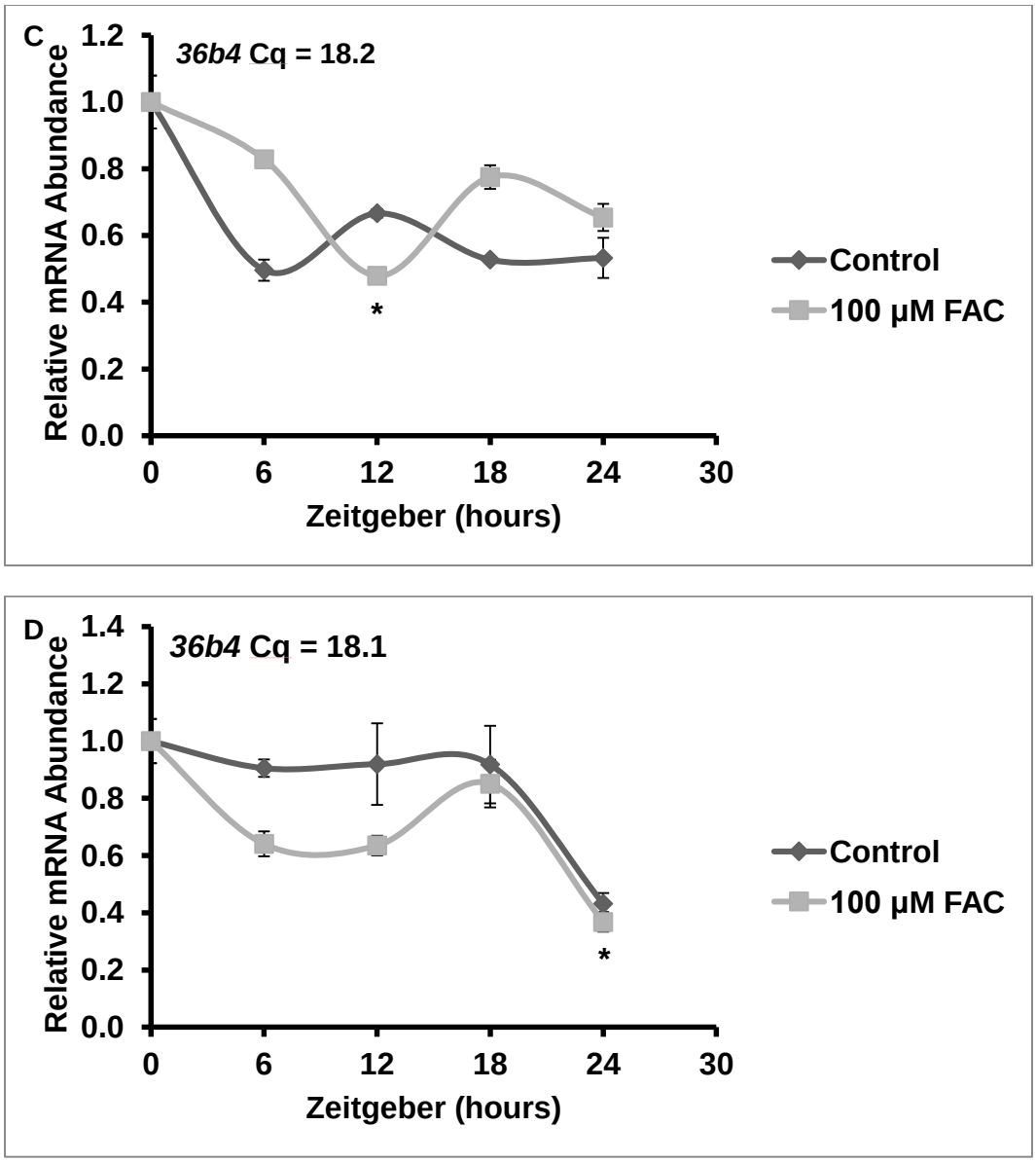
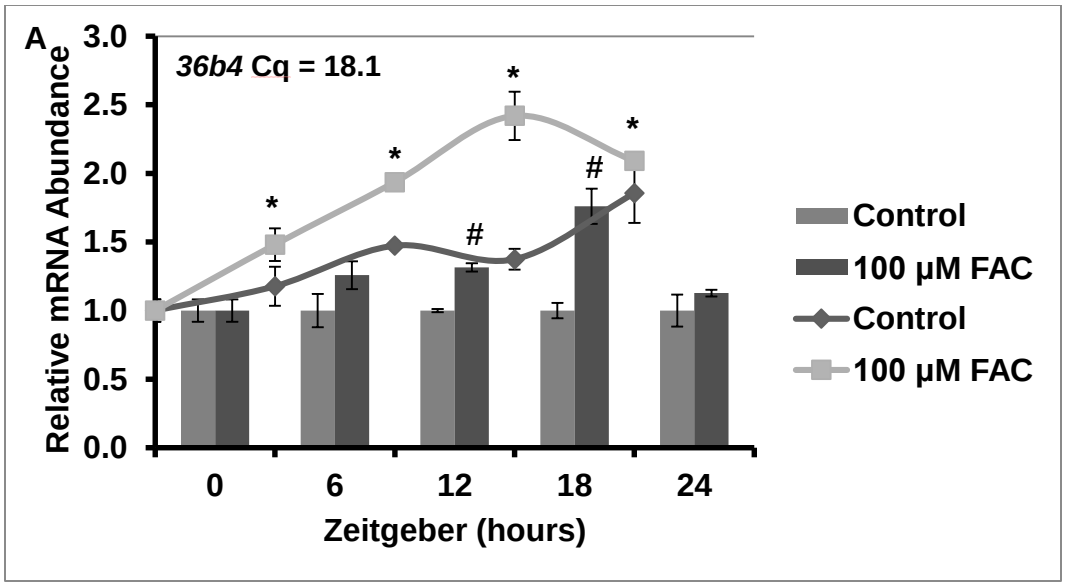
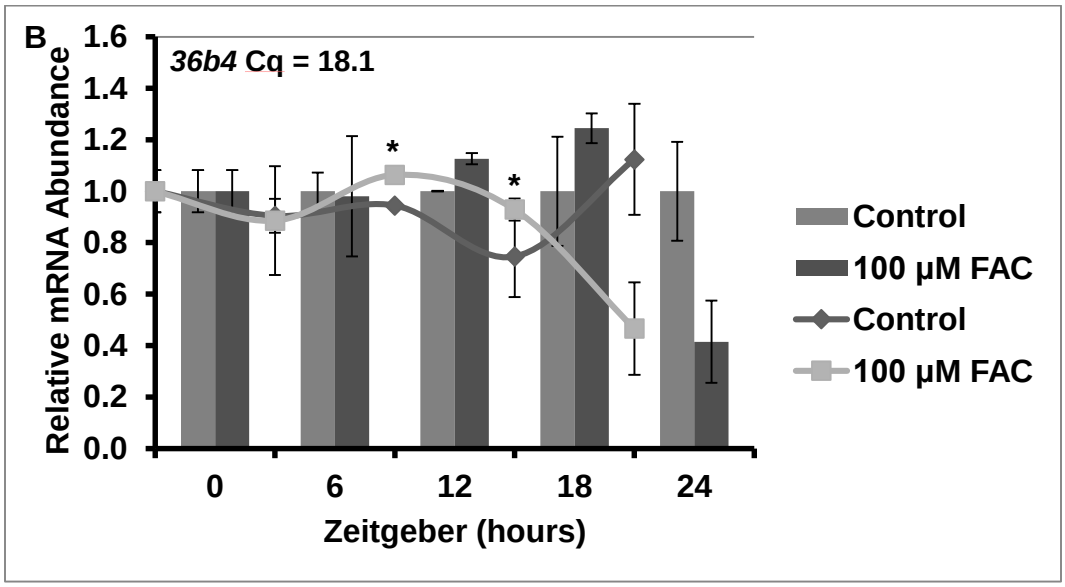


Figure 6.1: Diurnal rhythmicity of (A) *Alas1* (B) *Bmal1* (C) *Rev-erbα* and (D) *Tfrc* in C2C12 myoblasts post serum shock. C2C12 myoblasts were treated with 100 µM FAC after serum shock for a period of 24 h and RNA was harvested every 6 h. mRNA abundance was measured by qPCR and normalized to 36b4. Data is representative of three independent experiments conducted in duplicate and are expressed as fold changes ± SEM. Significant different was determined using student's *t*-test.; *P < 0.05 when compared to untreated control at 0h.


A
36b4 Cq = 18.1
Relative mRNA Abundance
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
6
12
18
24
Zeitgeber (hours)
Control
100 µM FAC
Control
100 µM FAC


B
36b4 Cq = 18.1
Relative mRNA Abundance
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
6
12
18
24
Zeitgeber (hours)
Control
100 µM FAC
Control
100 µM FAC

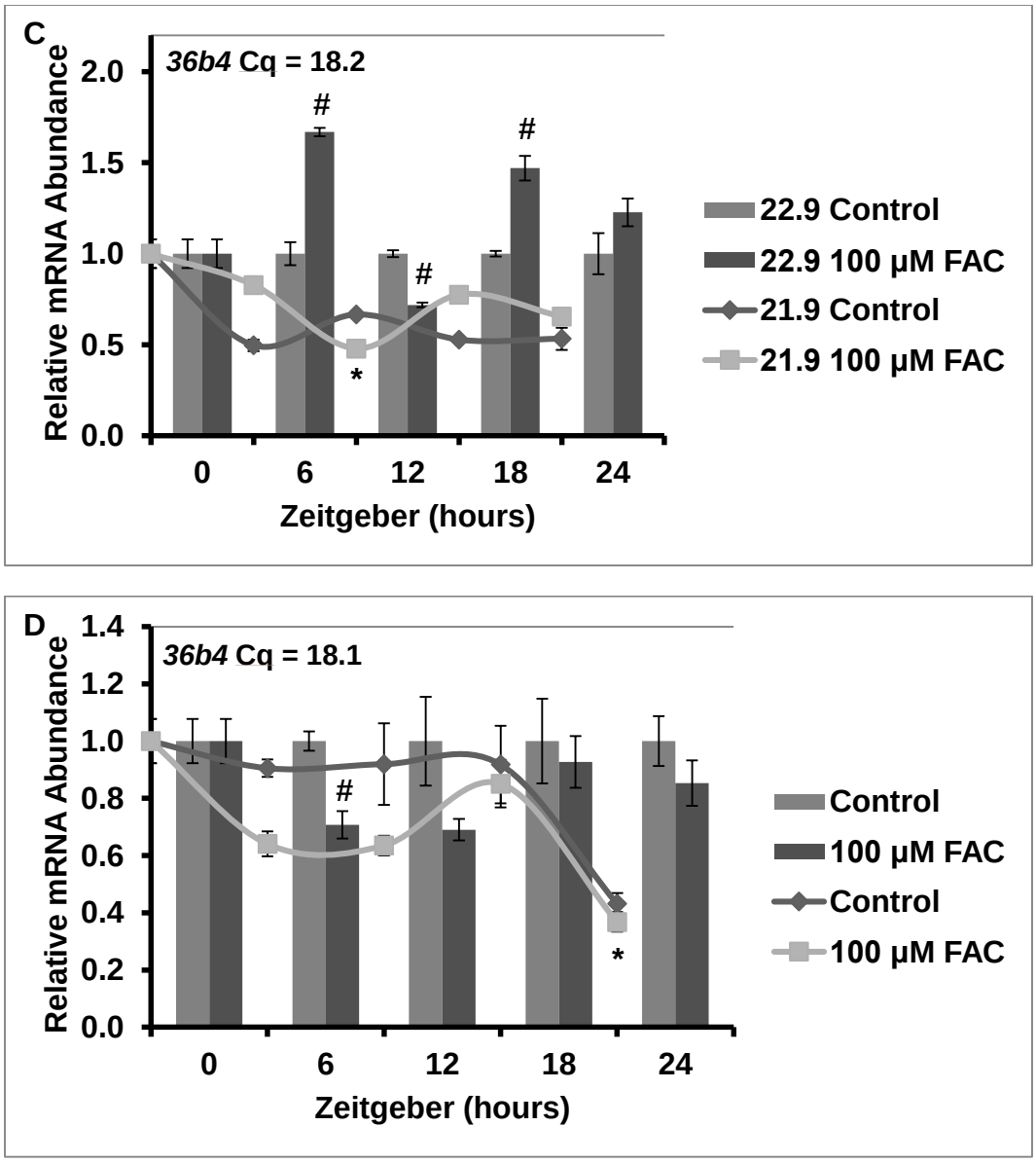
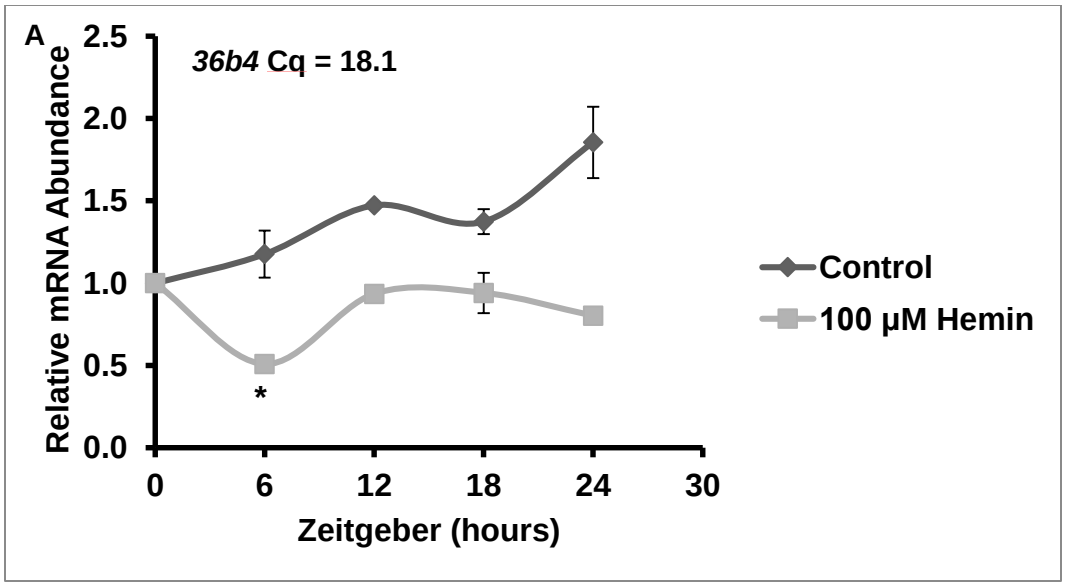
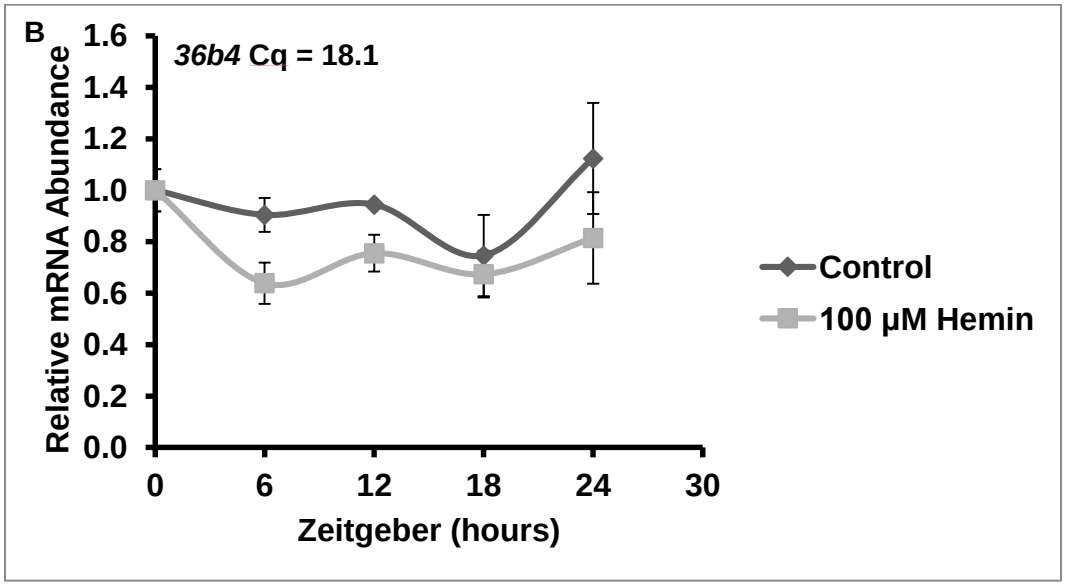


Figure 6.2: Diurnal rhythmicity of (A) *Alas1* (B) *Bmal1* (C) *Rev-erbα* and (D) *Tfrc* gene expression in C2C12 myoblasts post serum shock. The line graph (from Figure. 6A) represents gene expression of treatment compared to 0h control. Bar graph is a representative of untreated control compare with treated cells at the same time point. C2C12 myoblasts were treated with FAC after serum shock for a period of 24 h and RNA was harvested every 6 h. mRNA abundance was measured by qPCR and normalized to 36b4. Data is representative of three independent experiments conducted in duplicate and are expressed as fold changes ± SEM. Significant different was determined using student's *t*-test. *$P < 0.05$ when compared to untreated control at 0h. # $P < 0.05$ when compared to untreated control at same time point.


A
36b4 Cq = 18.1
Relative mRNA Abundance
2.5
2.0
1.5
1.0
0.5
0.0
*
0
6
12
18
24
30
Zeitgeber (hours)
Control
100 µM Hemin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36b4 Cq = 18.1
Relative mRNA Abundance
1.6
1.4
1.2
1.0
0.8
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0.2
0.0
0
6
12
18
24
30
Zeitgeber (hours)
Control
100 µM Hemin

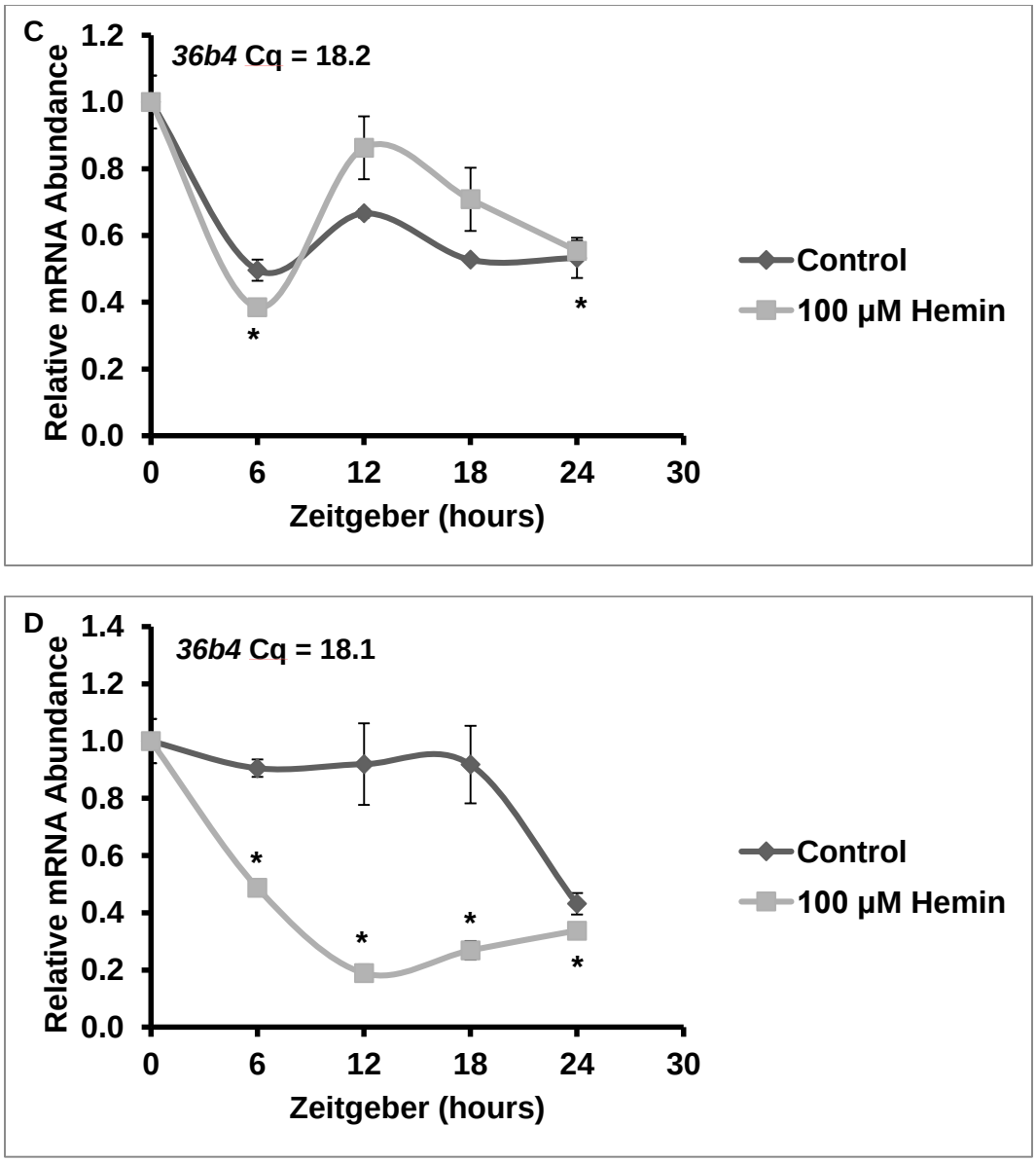
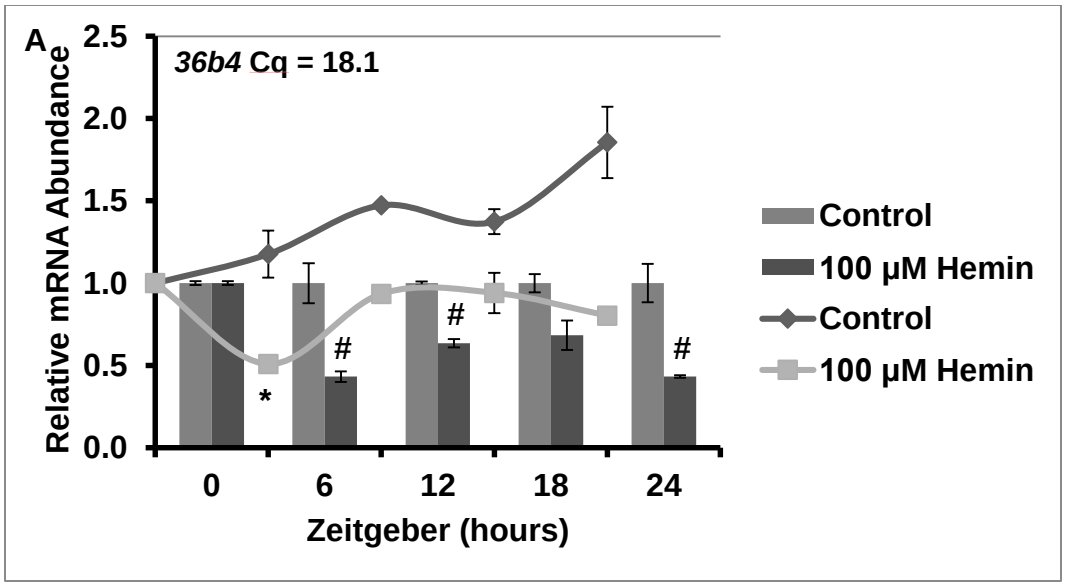
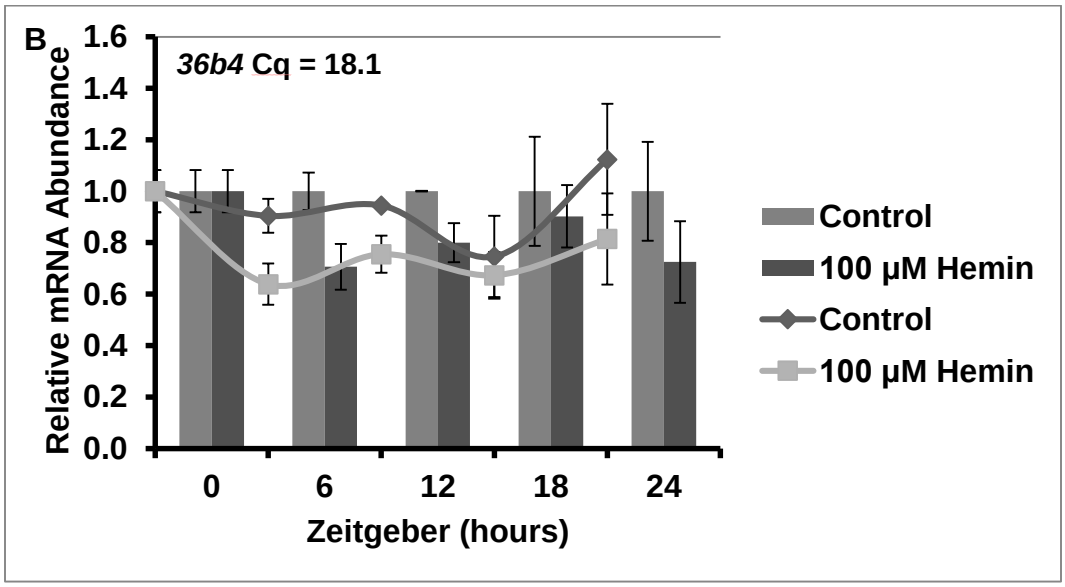


Figure 7.1: Diurnal rhythmicity of (A) *Alas1* (B) *Bmal1* (C) *Rev-erbα* and (D) *Tfrc* in C2C12 myoblasts post serum shock. C2C12 myoblasts were treated with 100 µM hemin after serum shock for a period of 24 h and RNA was harvested every 6 h. mRNA abundance was measured by qPCR and normalized to 36b4. Data is representative of three independent experiments conducted in duplicate and are expressed as fold changes ± SEM. Significant different was determined using student's *t*-test.; *P < 0.05 when compared to untreated control at 0h.


A
36b4 Cq = 18.1
Relative mRNA Abundance
2.5
2.0
1.5
1.0
0.5
0.0
*
#
#
#
0
6
12
18
24
Zeitgeber (hours)
Control
100 µM Hemin
Control
100 µM Hemin


B
36b4 Cq = 18.1
Relative mRNA Abundance
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
6
12
18
24
Zeitgeber (hours)
Control
100 µM Hemin
Control
100 µM Hemin

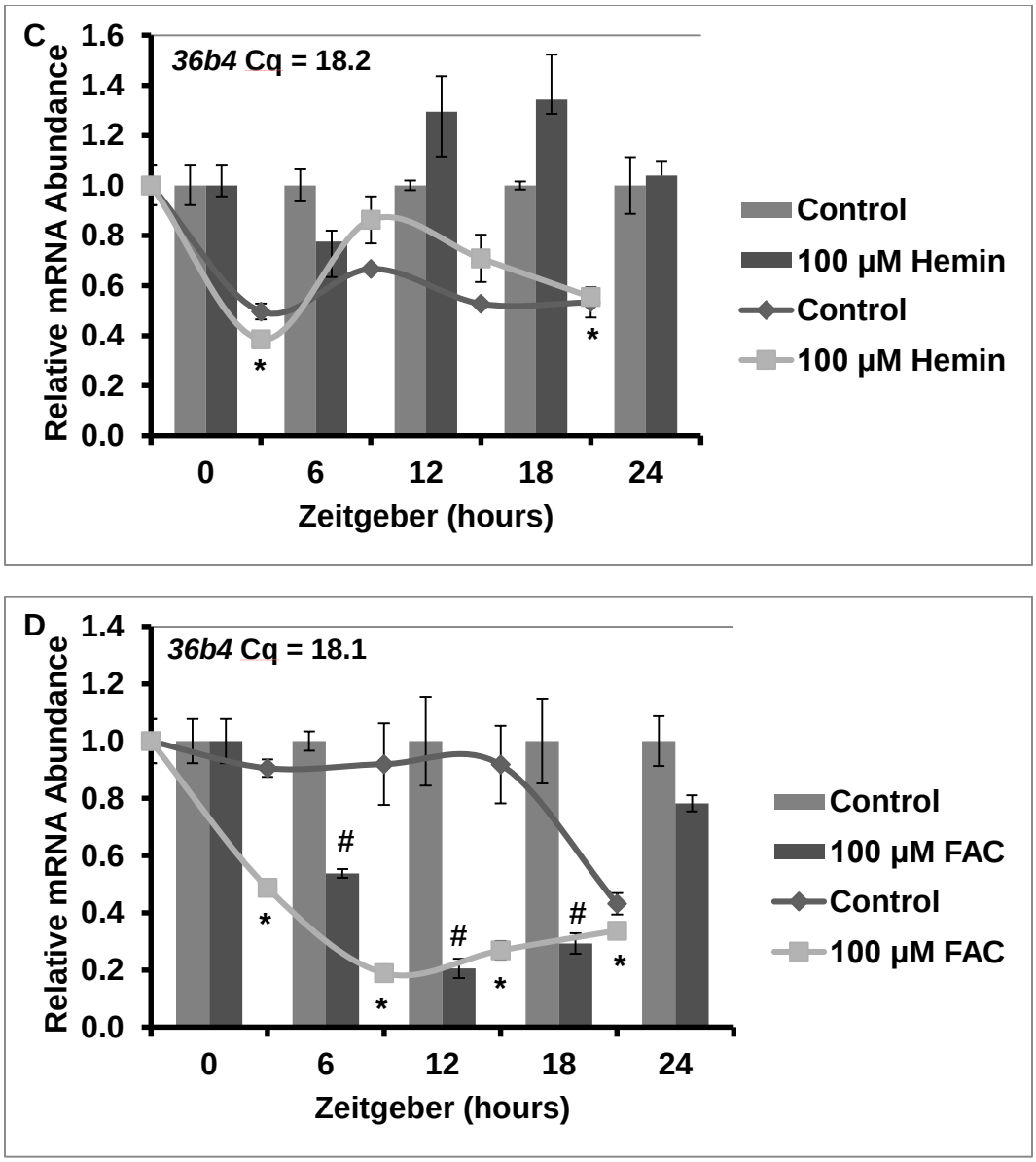
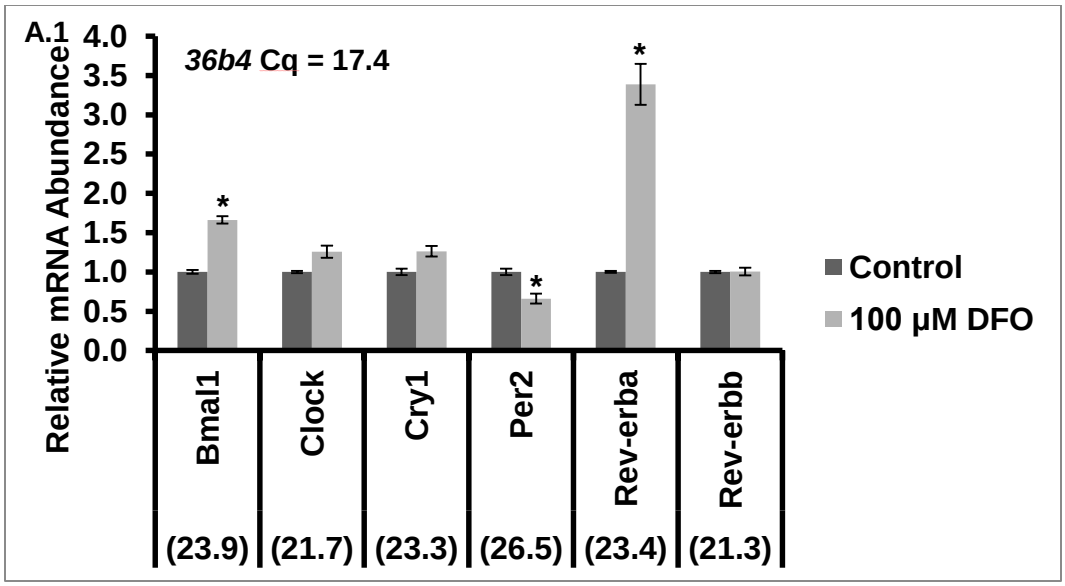
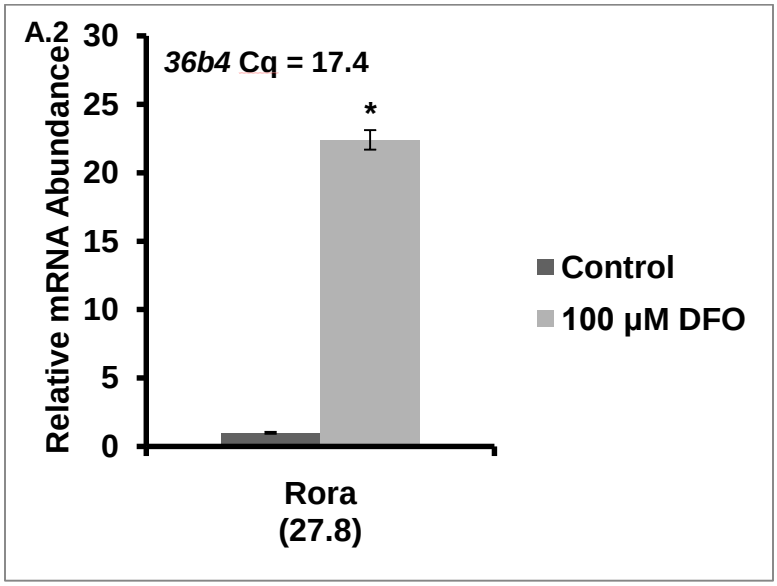


Figure 7.2: Diurnal rhythmicity of (A) *Alas1* (B) *Bmal1* (C) *Rev-erbα* and (D) *Tfrc* gene expression in C2C12 myoblasts post serum shock. The line graph (from Figure. 7A) represents gene expression of treatment compared to 0h control. Bar graph is a representative of untreated control compare with treated cells at the same time point. C2C12 myoblasts were treated with FAC after serum shock for a period of 24 h and RNA was harvested every 6 h. mRNA abundance was measured by qPCR and normalized to 36b4. Data is representative of three independent experiments conducted in duplicate and are expressed as fold changes ± SEM. Significant different was determined using student's *t*-test. *P < 0.05 when compared to untreated control at 0h. # P < 0.05 when compared to untreated control at same time point.


A.1
Relative mRNA Abundance
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
36b4 Cq = 17.4
*
*
*
Control
100 µM DFO
Bmal1
Clock
Cry1
Per2
Rev-erba
Rev-erbb
(23.9)
(21.7)
(23.3)
(26.5)
(23.4)
(21.3)


A.2
Relative mRNA Abundance
30
25
20
15
10
5
0
36b4 Cq = 17.4
*
Control
100 µM DFO
Rora
(27.8)

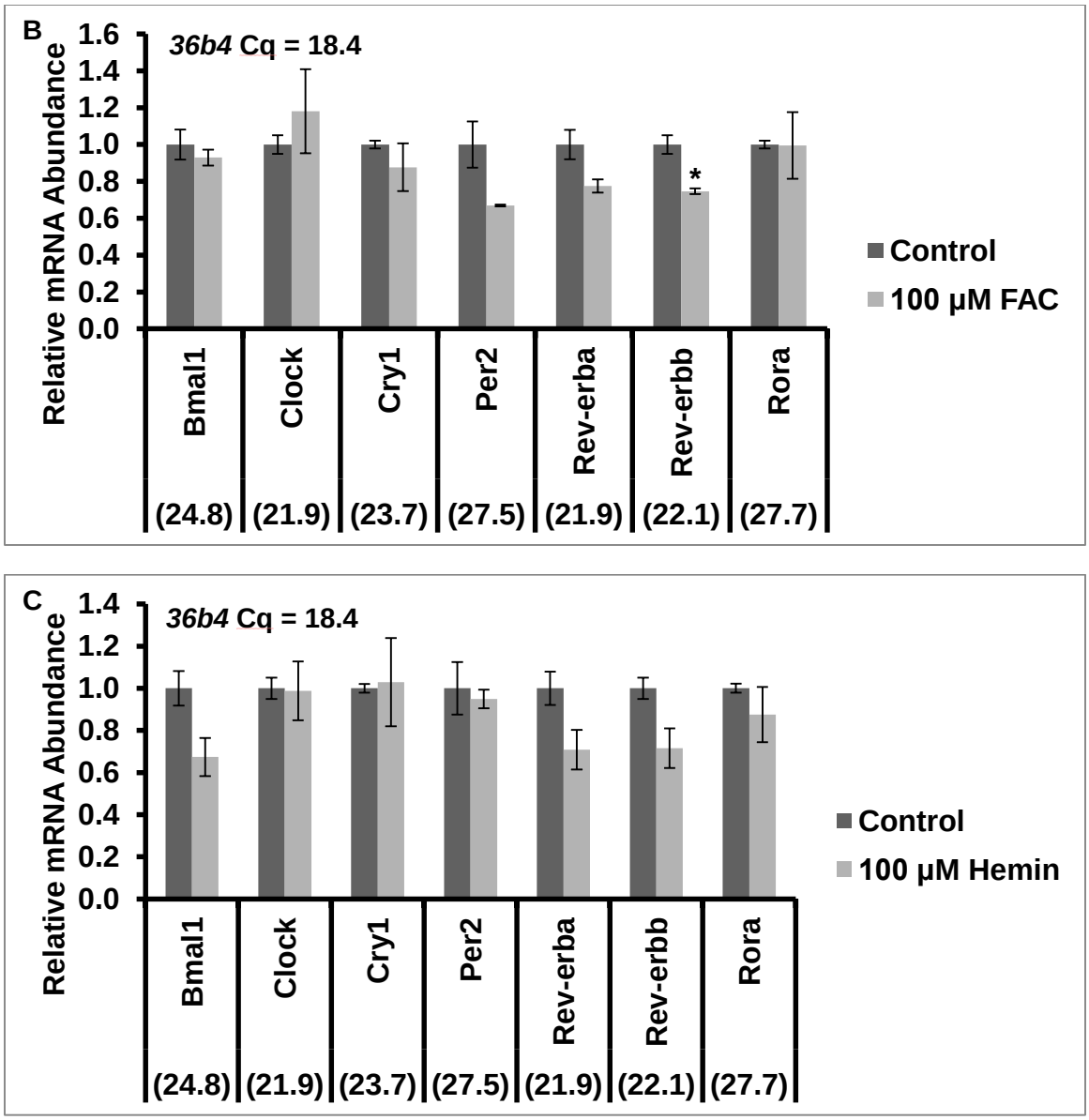
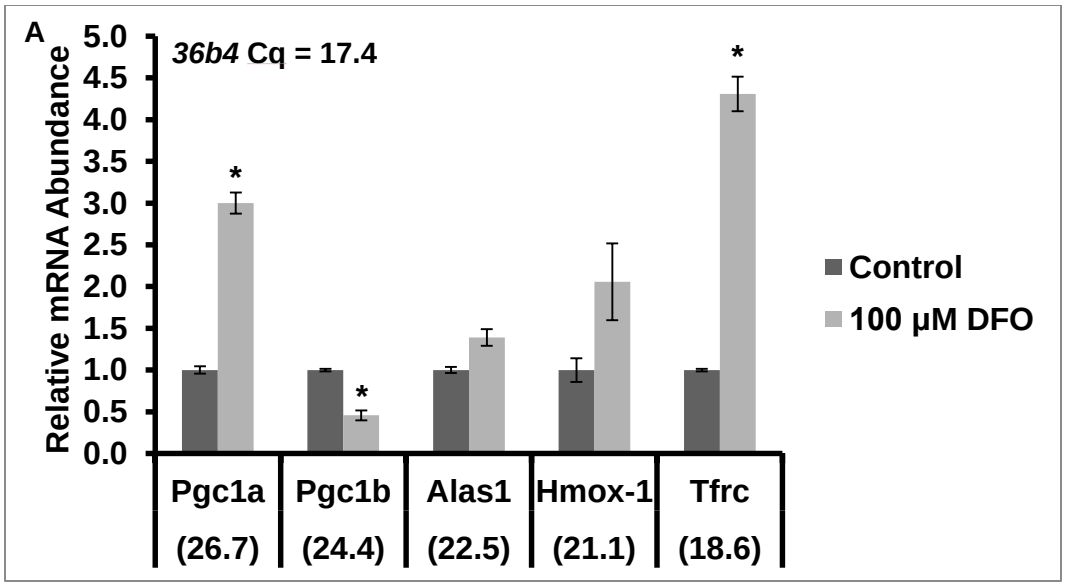
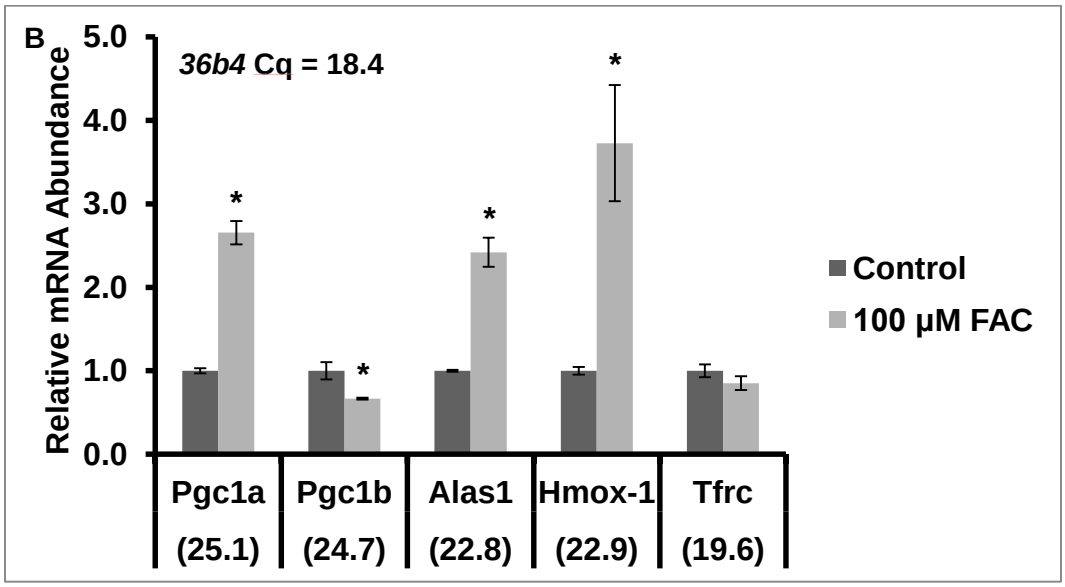


Figure 8: Circadian *Bmal1, Clock, Cry1, Per2, Rev-erbα, Rev-erbβ and Rorα* gene expression in C2C12 synchronized myoblast at 18h post serum shock treated with (A) 100 µM DFO (B) 100 µM FAC (C) 100 µM hemin for 18h post serum shock. Gene expression was measured by qPCR and normalized to 36b4. Data is representative of three independent experiments conducted in duplicate and are expressed as fold changes ± SEM. Significant different was determined using student's *t*-test; *P < 0.05 when compared to untreated control at same time point.


A
Relative mRNA Abundance
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
36b4 Cq = 17.4
*
*
*
Control
100 μM DFO
Pgc1a
(26.7)
Pgc1b
(24.4)
Alas1
(22.5)
Hmox-1
(21.1)
Tfrc
(18.6)


B
Relative mRNA Abundance
5.0
4.0
3.0
2.0
1.0
0.0
36b4 Cq = 18.4
*
*
*
*
Control
100 μM FAC
Pgc1a
(25.1)
Pgc1b
(24.7)
Alas1
(22.8)
Hmox-1
(22.9)
Tfrc
(19.6)

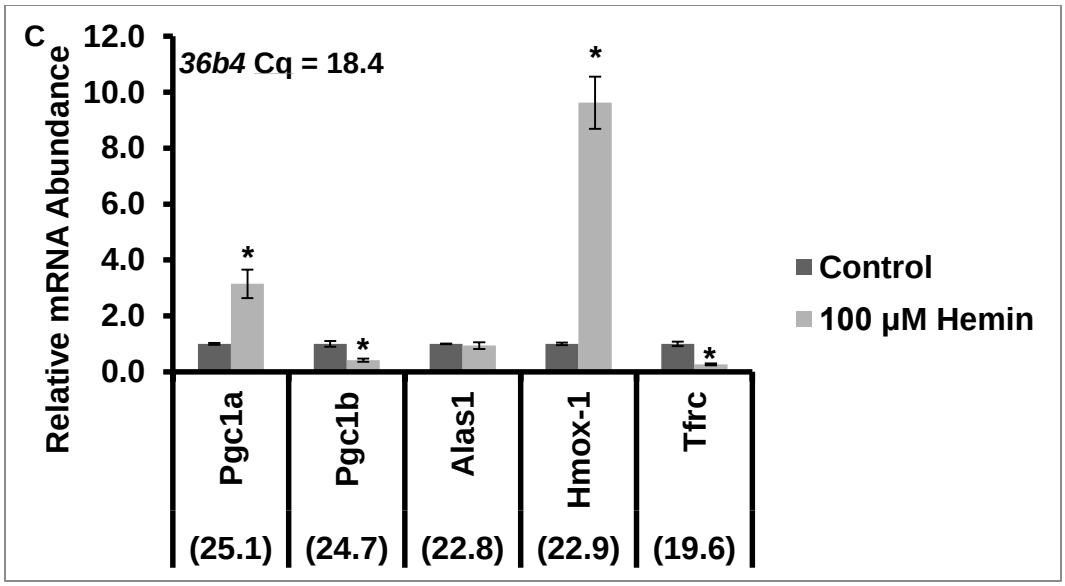
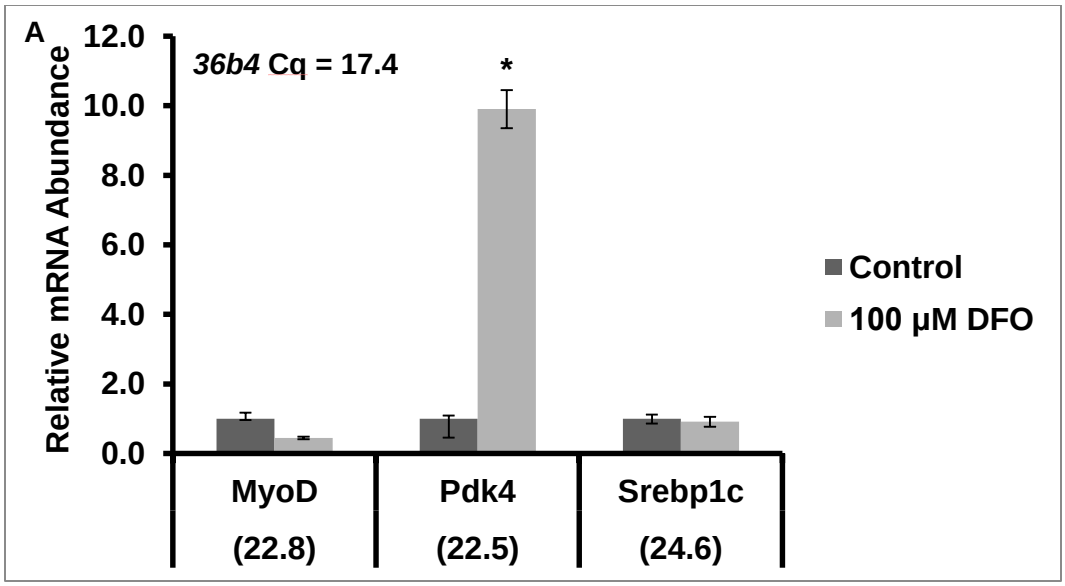
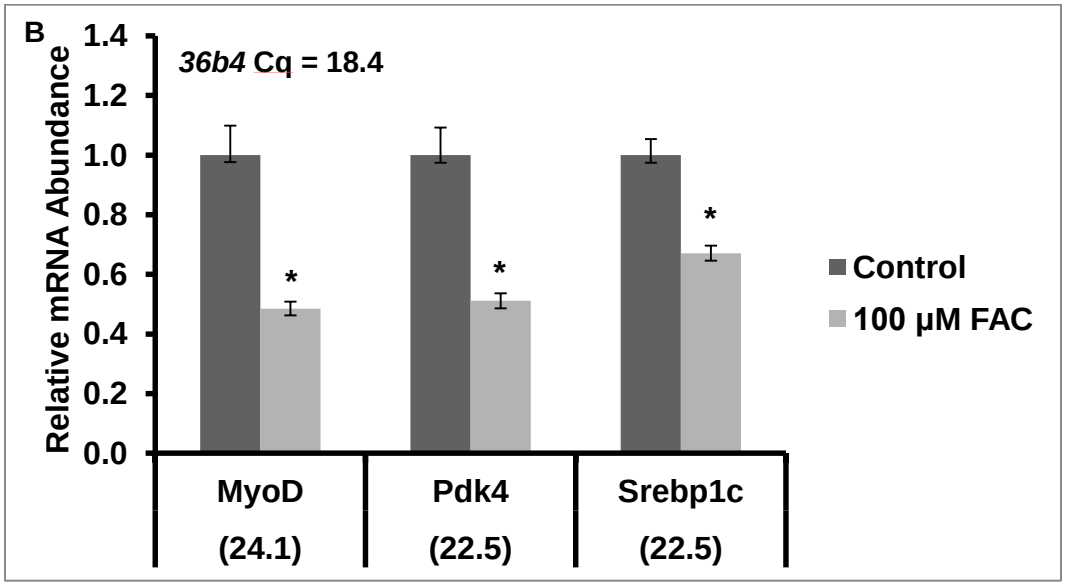


Figure 9: Metabolic gene expression in C2C12 synchronized myoblast treated (A) 100 µM DFO (B) 100 µM FAC (C) 100 µM hemin. Genes involves in mitochondrial biogenesis (*Pgc1α, Pgc1β),* heme biosynthesis *(Alas1),* and iron metabolism (*Hmox-1* and *Tfrc*) was analyzed via qPCR. Gene expression was measured by qPCR and normalized to 36b4. Data is representative of three independent experiments conducted in duplicate and are expressed as fold changes ± SEM. Significant different was determined using student's *t*-test; *P < 0.05 when compared to untreated control at same time point.


A
Relative mRNA Abundance
12.0
10.0
8.0
6.0
4.0
2.0
0.0
36b4 Cq = 17.4
*
Control
100 μM DFO
MyoD
(22.8)
Pdk4
(22.5)
Srebp1c
(24.6)


B
Relative mRNA Abundance
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
36b4 Cq = 18.4
*
*
*
Control
100 μM FAC
MyoD
(24.1)
Pdk4
(22.5)
Srebp1c
(22.5)

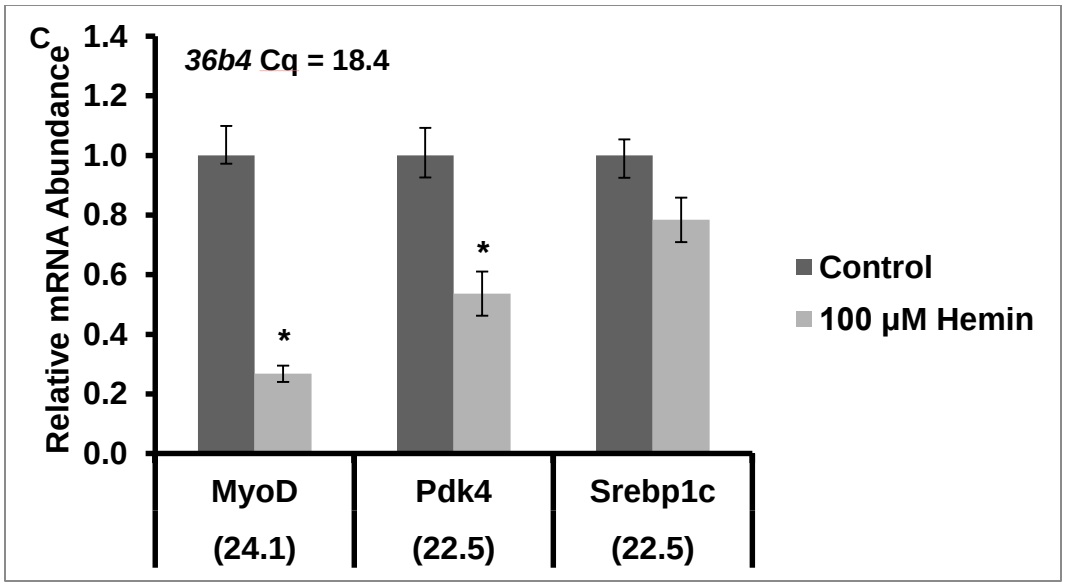


Figure 10: Metabolic gene expression in C2C12 synchronized myoblast treated with (A) 100 µM DFO (B) 100 µM FAC (C) 100 µM hemin for 18h post serum shock. Gene expression was measured by qPCR and normalized to 36b4. Data is representative of three independent experiments conducted in duplicate and are expressed as fold changes ± SEM. Significant different was determined using student's *t*-test; *P < 0.05 when compared to untreated control at same time point.

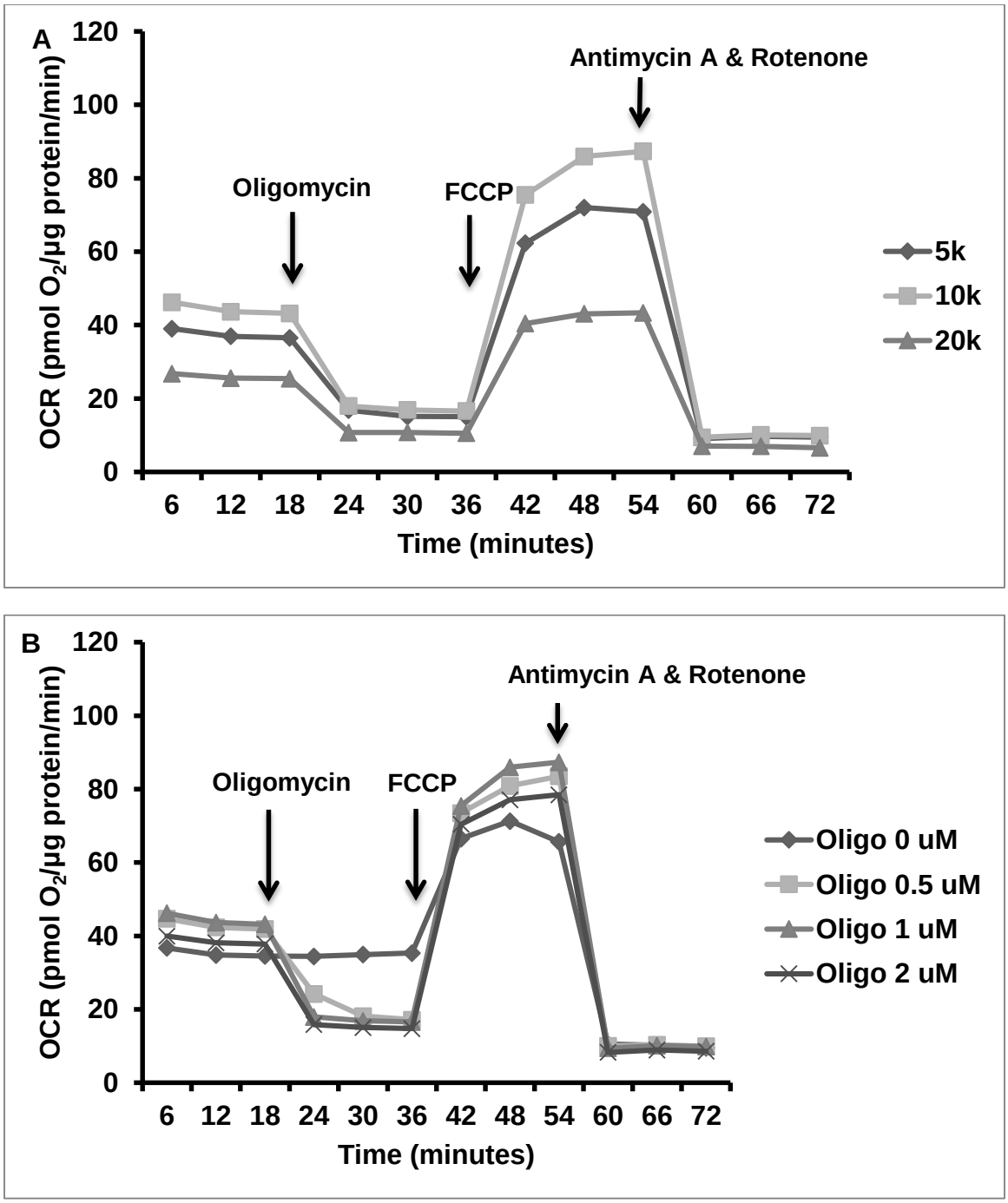


Figure 11: (A) Cell density and (B) oligomycin concentration optimization. Oxygen consumption rate (OCR) in C2C12 myoblasts was measured using Extracellular flux analyzer XF96 Seahorse in response to variation of Oligomycin concentration (0 µM, 0.5 µM, 1, µM, and 2 µM) , FCCP (3 µM), Antimycin A and Rotenone (1 µM). Cells were plated at density of 5k/well, 10k/well and 20k/well. *n*=1.

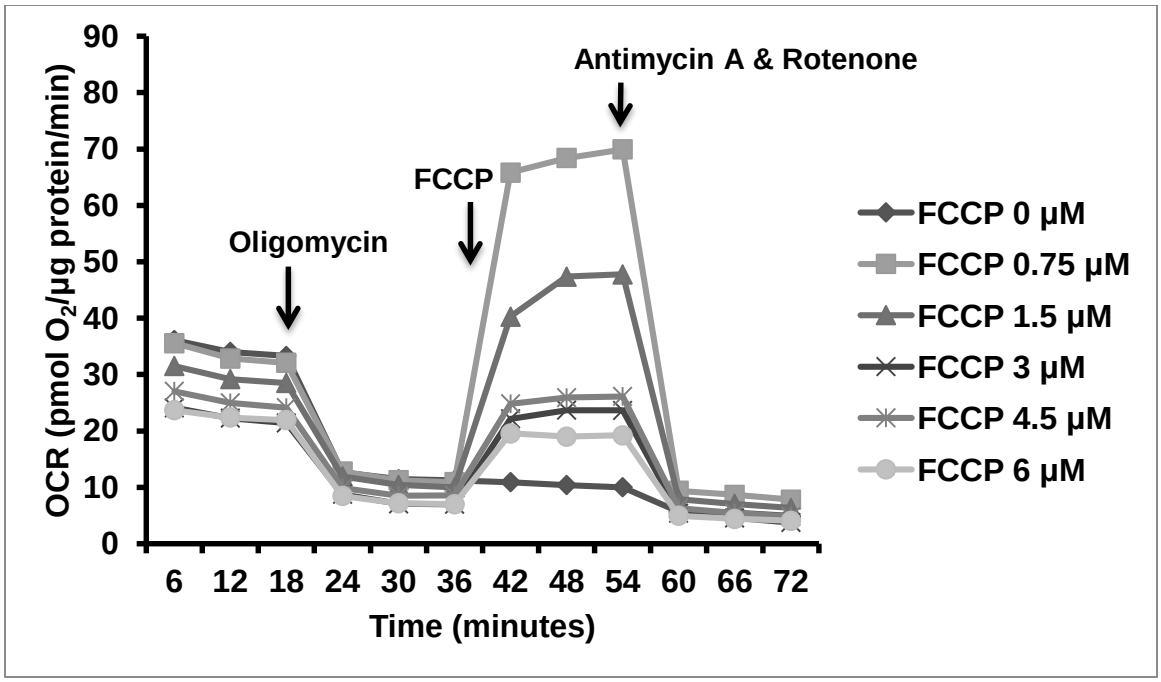
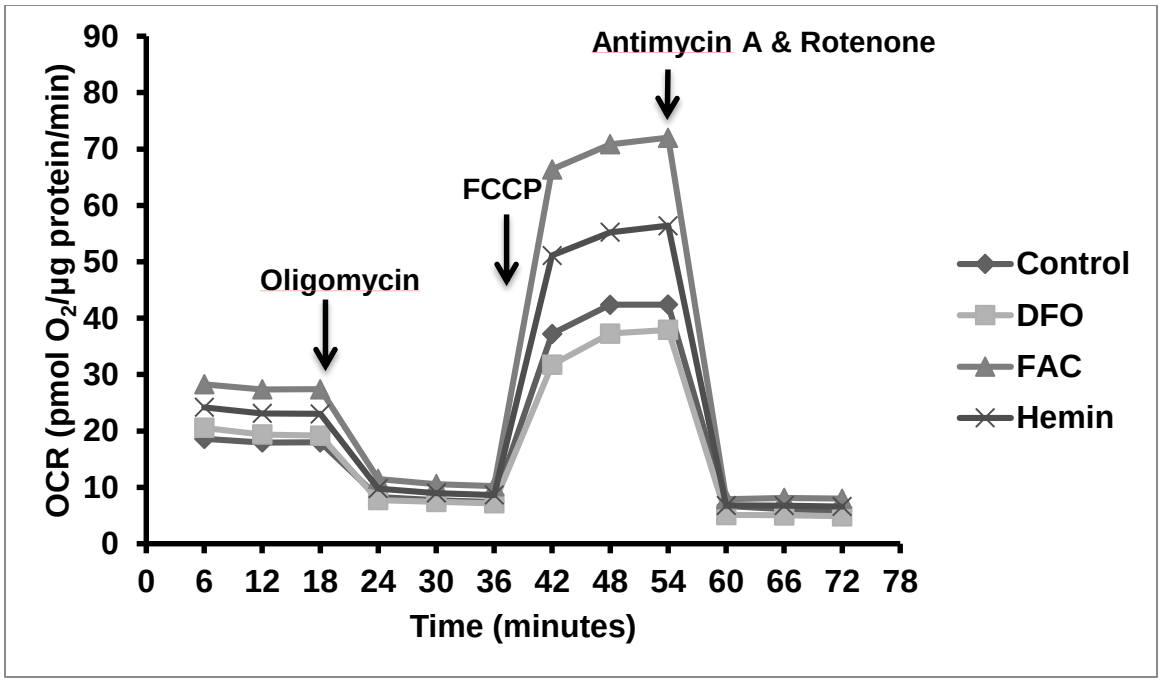


Figure 12: FCCP concentration optimization. Oxygen consumption rate (OCR) in C2C12 myoblasts was carried out using Extracellular flux analyzer XF96 Seahorse in response to the addition of Oligomycin (1 µM), FCCP (0 µM, 0.75 µM, 1.5 µM, 3 µM, 4.5 µM and 6 µM), Antimycin A and Rotenone (1 µM). Cells were plated at density of 5k/well, 10k/well and 20k/well. *n*=1.



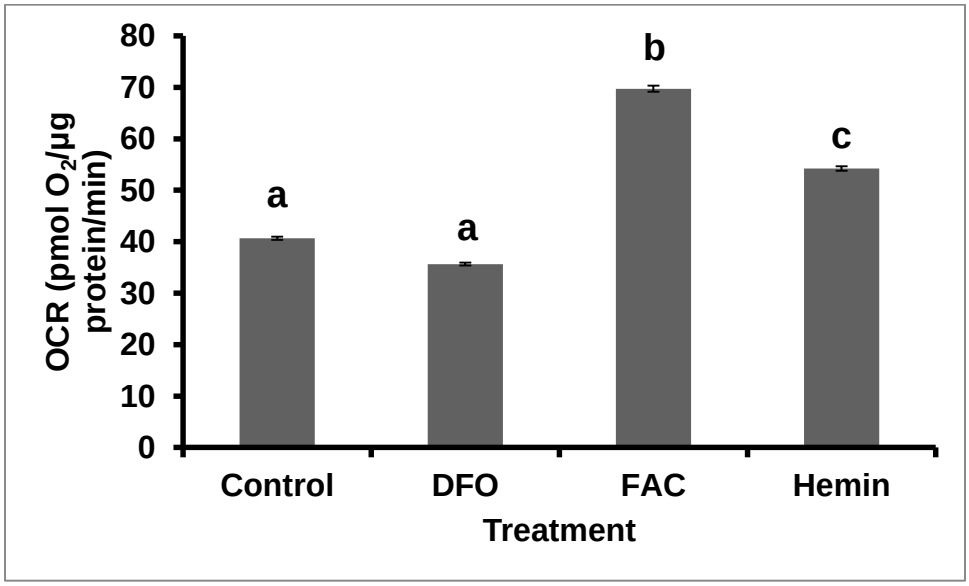
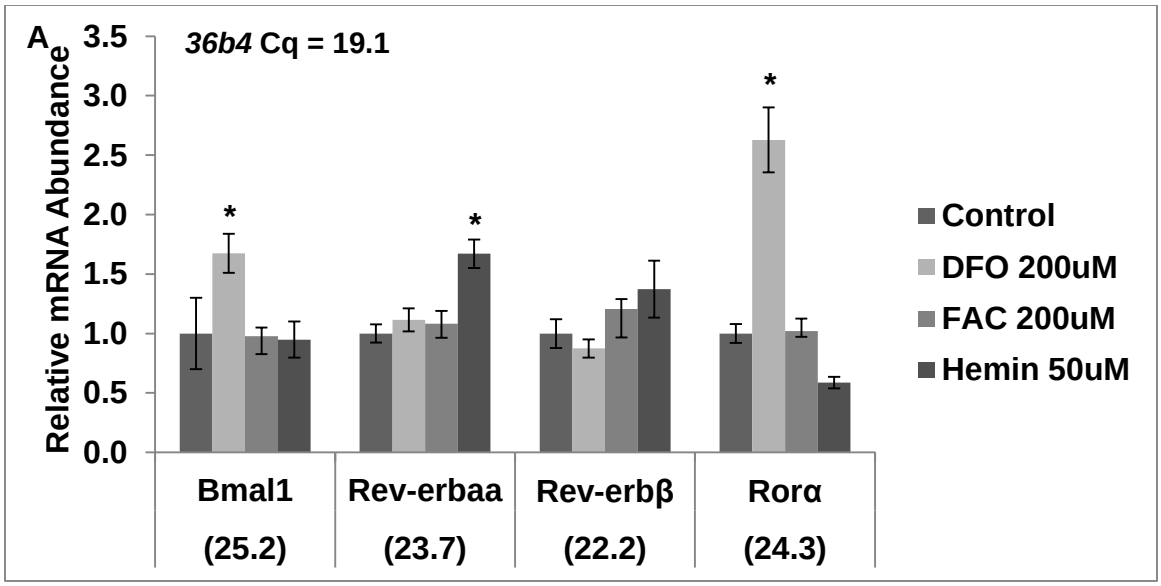
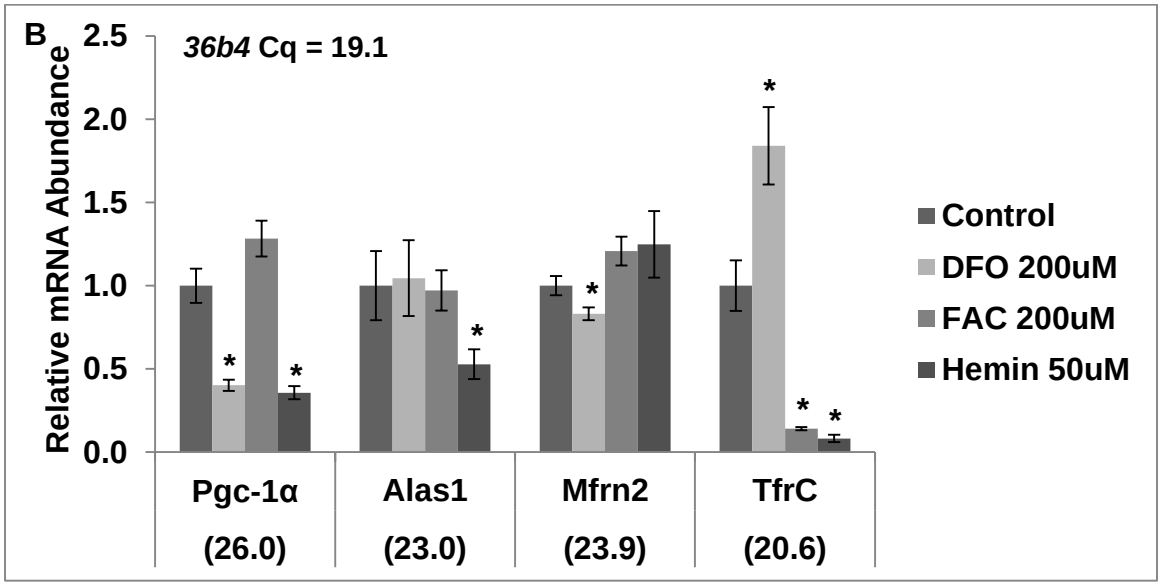


Figure 13: XF96 Seahorse mitochondria stress test in C2C12 myoblasts. Oxygen consumption rate (OCR) in C2C12 myoblasts was carried out using Extracellular flux analyzer XF96 Seahorse in response to the addition of Oligomycin (1 µM), FCCP (1 µM), Antimycin A and Rotenone (1 µM). Cells were plated at 10k/well. Significant difference was determined using one-way ANOVA; Significance is set at $P < 0.05$ and the letters represent significance different between group. *n*=3.


A
36b4 Cq = 19.1
Relative mRNA Abundance
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
DFO 200uM
FAC 200uM
Hemin 50uM
Bmal1
(25.2)
Rev-erbaa
(23.7)
Rev-erbβ
(22.2)
Rorα
(24.3)


B
36b4 Cq = 19.1
Relative mRNA Abundance
2.5
2.0
1.5
1.0
0.5
0.0
Control
DFO 200uM
FAC 200uM
Hemin 50uM
Pgc-1α
(26.0)
Alas1
(23.0)
Mfrn2
(23.9)
TfrC
(20.6)

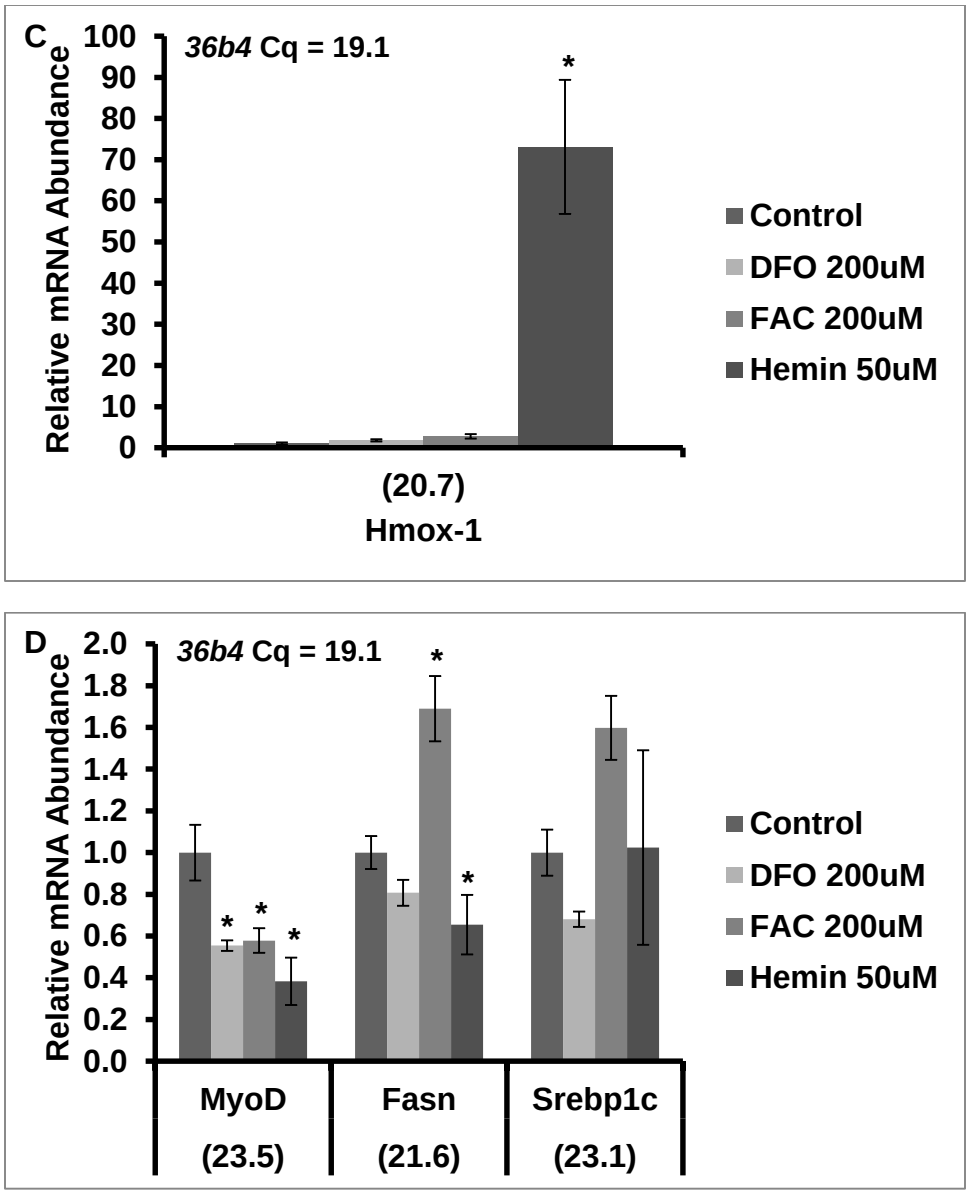


Figure 14: Circadian and metabolic gene expression in C2C12 synchronized myotubes treated with 200 μM DFO, 200 μM FAC, and 50 μM hemin. (A) Circadian (B) energy and iron metabolism, (C) *Hmox-1* and metabolic genes were analyzed. Cells were treated at ZT6 and harvest at ZT24 after 18 hours of treatment. Gene expression was measured by qPCR and normalized to 36b4. Data is representative of three independent experiments conducted in duplicate and are expressed as fold changes ± SEM. Significant different was determined using student's *t*-test; *P < 0.05 when compared to untreated control at same time point.

CHAPTER V

DISCUSSION

The existence of circadian clock in metabolic tissues such as liver, pancreas, adipose and skeletal muscle indicates the important role circadian rhythm plays in metabolic regulation. The purpose of this study is to (I) determine the effect of iron status on circadian rhythm and metabolic gene expression in C2C12 myoblasts and myotubes and (II) examine the oxidative capacity of mitochondrial in synchronized C2C12 myoblasts. The hypothesis of this study was that with disrupted iron homeostasis, circadian rhythm and circadian controlled metabolic gene expression will be altered.

In this study, the effect of iron chelation and iron treatment in response to circadian rhythm and metabolism was analyzed by utilizing synchronized C2C12 myoblast model. C2C12 myoblasts have the potential to differentiate into myotubes and form contractile muscle fibers, making the model physiological relevance. Low serum (2% horse serum DMEM) media is used for differentiating proliferating myoblasts to myotubes [135]. Synchronization of C2C12 cells is important in circadian rhythm study and serum shock technique has been practice over the past decades and common in circadian study.

To examine the extent to which iron chelation and iron loading alters changes in iron labile pool, IRP1 and IRP2 spontaneous and total RNA binding activity is the optimal measurement for iron deficiency and iron overloading in cells. The spontaneous binding activity for IRP1 and IRP2 in DFO treated cells was increased by 2.1-fold while FAC and hemin treated cells showed a decreased of 0.25- and 0.2-fold, respectively (data not shown; n = 1). Total RNA binding activity of IRP1 was also measured to ensure that the spontaneous RNA binding activity of IRP1 is due to a shift in [4Fe-4S] containing cytosolic-aconitase losing [4Fe-4S] cluster, leaving IRP1 in its active binding form.

Alteration of circadian and metabolic genes over 24 hour period

With validation of the C2C12 low iron and high iron model by IRPs RNA binding activity. We examine the extent to which iron status alters circadian rhythmicity in C2C12 myoblast cells by collecting cells sample every 6 h for a period of 24 h after treatment. We hypothesized that with iron chelation by DFO, *Tfrc* gene expression would be up-regulated due to *Tfrc* mRNA stabilization by IRPs. Moreover, with decrease in labile iron pool, heme biosynthesis is hypothesized to be down regulated as well as *Bmal1* gene expression which is regulated by heme-mediated REV-ERBα. *Tfrc* is not a circadian gene and does not regulate in a circadian manner, however, its transcription is affected by iron availability in the cells. At ZT18, *Tfrc* gene expression peaked by 2.5-fold ($p = 0.016$). From ZT18 to ZT 24, *Bmal1* gene expression peaked from 2.1-fold to 5.1-fold ($p = 0.040$). *Rev-erbα* gene expression started to show significant increase at ZT18 (2.0-fold; $p = 0.013$) and continue to be up-regulated at ZT24 (2.7-fold; $p = 0.033$). The circadian genes *Alas1, Bmal1* and *Rev-erbα* showed circadian rhythmicity, ensuring that synchronization occurs within the cells. In DFO treated cells, *Alas1* gene expression peaked 2.6-fold ($p =0.007$) at ZT12 indication an increased in the conversion of acetyl-CoA and glycine to ALA (aminolevuleneic acid). The up-regulation of *Tfrc* further validated low iron C2C12 model. These results demonstrate that low iron status in the C2C12 cells potentially lead to up-

regulation of *Alas1* which drives heme biosynthesis. Despite an increase in *Alas1* gene expression, the final product of heme production requires incorporation of ferrous iron by the action of ferrochelatase which is down-regulated due to low iron availability and possibly less production of ferrochelatase. With less heme production, *Bmal1* gene expression is up-regulated due to the de-repression of REV-ERBα and competitive binding of RORα on *Bmal1* promoter. Increased gene expression of *Rev-erbα* suggested that there is an increased production of BMAL1 resulted in increased in CLOCK:BMAL1 heterodimers for regulation of *Rev-erbα*. This confirmed our hypothesis that changes in iron status (low iron) lead to alteration in circadian gene expression in C2C12 myoblasts.

FAC and hemin were used as iron loading sources. In FAC treated cells, up-regulation of *Alas1* was observed and it is hypothesized to be regulated by PGC-1α due to production of ROS from free iron [3]. Despite an increased in *Alas1* regulation, the availability of iron source for heme biosynthesis increased resulting in more heme availability. Heme binds to REV-ERBα which repressed gene expression of *Bmal1*. Less CLOCK:BMAL1 heterodimers are formed and thus causing a delay in the downstream *Rev-erbα* gene expression by 6 h. *Tfrc* in FAC treated cells were not significantly repressed, therefore, the changes observed in circadian and metabolic gene expression may occur with minimal increase in iron.

In hemin treated cells, down regulation of *Alas1* was expected as heme is a direct inhibitor of *Alas1* gene expression. *Bmal1* mRNA level was lowered from ZT0 to ZT24 but values were not significant. The slightly lowered *Bmal1* gene expression may correlates with the slight change in trough and peaked of *Rev-erbα* gene expression despite no change in oscillation was observed. In hemin treated cells, *Tfrc* gene expression was significantly lowered at ZT6 and ZT24, making it a high iron C2C12 myoblast model.

In conclusion, changes in iron status in C2C12 proliferating myoblasts exhibit variation in diurnal expression of circadian (*Alas1, Bmal1* and *Rev-erbα*) and iron metabolic gene expression (*Tfrc*). Alteration in heme biosynthesis rate-limiting enzyme gene expression *Alas1* may potentially play a role in regulation of *Bmal1* by heme-mediated REV-ERBα.

Alteration of circadian and metabolic genes at ZT18

In DFO treated cells, *Rorα* and *Rev-erbα* were significantly up-regulated which may indicate increased in regulation by CLOCK:BMAL1 heterodimer. In agreement with previous literatures, *Pdk4* gene expression increased may be due to increase in *Pgc-1α, Hif1α, ERRα* and/or *PPAR* gene expression due to DFO treatment [48], [109], [147], [148]. Interestingly, ID rat's skeletal muscle displayed a decreased in *Pdk4* gene expression [53]. The explanation for these different could be due to the global regulation of rat's skeletal muscle, however, cell culture regulation of *Pdk4* maybe specific to skeletal muscle.

In FAC treated cells, *Rev-erbβ* was down-regulated as well as other metabolic gene expression such as *MyoD, Pdk4,* and *Srebp-1c*. Despite an increased in *Pgc-1α* gene expression, *Pdk4* gene expression is down regulated indicating other modulator (i.e. HIF1α) of *Pdk4* gene expression is involved [56]. *Alas1* gene expression is regulated by its coactivator *Pgc-1α,* leading to up-regulation in heme biosynthesis. Repression of *MyoD* and *Srebp-1c* is expected with increased in heme-mediated REV-ERBs activity. REV-ERBs repressed *Bmal1* gene expression resulting in decreased *Rev-erbβ* gene expression.

In hemin treated cells, *Hmox-1* gene expression was up-regulated resulting in breakdown of heme into ferrous iron, biliverdin and CO. The increase in availability of free iron induces ROS and eventually PGC-1α production. Intriguingly, *Pgc-1β* gene expression decreased. Similar to FAC treated cells, *MyoD* and *Pdk4* are down-regulated in hemin treated cells which may potentially be regulated by increase in *Pgc-1a* gene expression. In this study, changes in iron

status in C2C12 proliferating myoblasts exhibit variation in circadian, heme biosynthesis and iron metabolic gene expression.

Mitochondria oxidative capacity

Significant increase in mitochondria oxidative capacity was observed in FAC and hemin treated cells. However, no significant change was observed despite a downward trend in oxidative capacity. The mitochondria stress test showed that iron is important in regulating mitochondria function and necessary for proliferating skeletal muscle cells. This test suggest that iron is required for mitochondria function and overloading iron may potentially increase heme biosynthesis and iron sulfur cluster for making of ISCU, hemoprotein, and mitochondria complexes.

Interesting findings

Pgc-1α is up-regulated while *Pgc-1β* is down-regulated in all treatment. This alteration in *Pgc-1* family maybe due to ROS production due to disruption in iron homeostasis [3]. *Rorα* was significantly up-regulated in DFO treated cells but no change was observed in FAC or hemin treatment. *Pdk4* was significantly up-regulated in DFO but down-regulated in FAC and Hemin. This may suggest an important role iron in the regulation of *Rora* and *Pdk4*, however, future study is needed to understand the mechanism.

Study Limitation

There are several limitations of the study on iron status and circadian rhythm. The commonly used model of circadian rhythmicity is C2C12 myotubes due to its ability to contract and produce proteins involves in differentiation muscle. However, study of iron status is very sensitive to amount of iron (transferrin) present in the serum in the media. The low serum condition is similar to low transferrin condition in media, therefore, the media affects iron

chelation and iron loading treatment in our study. Differentiation required cells to be grown in low serum media (2% horse serum) and this low serum mimics the effect of iron chelation by DFO by having induction in *Tfrc* gene expression (data not shown). Therefore, the better model to study iron status is in C2C12 myoblast where cells are grown in 10% FBS in DMEM for the entire experiment. Moreover, the effect of iron loading was greatly potentiated since the media is low in iron (transferrin). Furthermore, most common form of iron in myotubes is heme-containing protein and DFO is unable to chelate iron from hemoprotein such as myoglobin. Therefore, differentiated C2C12 cells were not affected by DFO treatment, even at high concentration (1000 μM) which may be toxic to many proliferating cells.

Tfrc gene expression is commonly used in measuring the iron status, however, it is not as sensitive as measuring the spontaneous binding activity of IRP1 and IRP2.This study is lacking sufficient data to support the validity of iron deficient or iron overloading model. Despite that, *Tfrc* gene expression is a good indicator and downstream target genes of IRPs and IRE.

The present study has shown that proliferating C2C12 myoblast display diurnal variation in response to iron chelation and iron treatment at the level of gene expression. In contrast, we have not investigate whether the observed gene expression are associated with concomitant changes in protein expression. Moreover, the present study has not determined the relationship between changes in diurnal variation in circadian gene expression and the metabolic changes as well as mitochondrial respiratory activity in C2C12 cells. Additionally, we have not defined the mechanism(s) responsible for skeletal muscle variation in metabolic gene expression (*MyoD* and *Pdk4*) in this cell model.

In conclusion, the results presented demonstrate that iron status affects circadian rhythm and metabolic gene expression in C2C12 myoblasts. To further assess the direct binding of REV-ERBα to *Bmal1* gene promoter in these cells, we plan to perform chromatin immunoprecipitation

(ChIP) assay on C2C12 purified DNA products that has been crosslinked which stabilizes protein-DNA complexes in the cell and assess in interaction with qPCR. Moreover, we plan to perform western blots on cytosolic protein extracts to analyze the changes in protein expression of PGC-1α and HIF1α to better explain the mechanism involved in *Pdk4* overexpression in DFO treated cells. Since heme is the direct regulator of REV-ERBs, we also plan to measure heme concentration in these cells via heme colorimetric assays. This may validate if heme biosynthesis was affected with changes in iron in C2C12 cells.

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