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Effects of Hypoxic Conditions on Skeletal Myoblasts

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EFFECTS OF HYPOXIC CONDITIONS ON SKELETAL MYOBLASTS

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ABSTRACT

Hypoxic injury in skeletal muscle occurs in response to musculoskeletal traumas, diseases and following reconstructive and transplantation surgeries. However, the molecular mechanisms responsible for hypoxic injury in skeletal myoblasts have yet to be fully investigated. Understanding of these molecular mechanisms would identify potential therapeutic targets. Herein, we have determined that treatment with cobalt chloride ($CoCl_2$, a hypoxia mimicking agent) first leads to an inhibition of proliferation followed by a decrease in the number of viable myoblasts over time. FACS analysis revealed a marked increase in G2/M arrest after 24 hours of treatment. We next assessed PARP cleavage, a well-known hallmark of apoptosis, and detected cleavage after 48 hours, suggesting apoptosis as a mechanism contributing to myoblast death. Since we have recently determined that PUMA (p53 up-regulated modulator of apoptosis) plays a critical role in the apoptosis associated with skeletal myoblast differentiation, we assessed the level of PUMA expression in response to $CoCl_2$ treatment and documented an increase in PUMA protein and mRNA. We did find an increase in p53 expression suggesting that this transcription factor is responsible for the increased expression of PUMA. Moreover, this increase in PUMA expression occurs under condition of eIF2α phosphorylation, which implies the possibility of IRES (Internal Ribosome Entry Site)-mediated regulation of PUMA expression under hypoxic conditions. Indeed, actinomycin D induced abrogation of the increase in PUMA mRNA did not prevent the increase in

PUMA protein. In skeletal myoblasts, the processes of differentiation and apoptosis are coordinately regulated during myogenesis and muscle regeneration and this coordinated regulation is mediated by the transcription factor MyoD. Thus, we next assessed the effect of $CoCl_2$ treatment on MyoD expression and documented decreased levels of MyoD protein and mRNA. Although we have also determined that the decrease in mRNA was not a consequence of a shortened half-life suggesting an effect on SRF, the transcription factor controlling MyoD expression, we were unable to detect a change in SRF expression as a consequence of $CoCl_2$ treatment. Future experiments will assess the activation of SRF.

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CHAPTER I

INTODUCTION

The Skeletal myoblast model system

Skeletal muscle has long been known as a useful model system to study signal transduction pathways that control cell lineage determination and differentiation. The myogenic regulatory factors (MRFs) are crucial for the determination and terminal differentiation of skeletal muscle. The identification of the first member of MRFs, MyoD, was a milestone in understanding these processes, and soon its relatives Myf5, myogenin and MRF4 were discovered and widely studied. The MRFs are a part of a family of basic helix-loop-helix (bHLH) transcription factors which contain two domains to allow protein dimerization and facilitate DNA-binding. The HLH domain regulates dimerization with other HLH-containing factors known as E-proteins and the DNA-binding domain allows the MRF protein to bind the E-Box which is a DNA motif containing CANNTG sequence (Rudnicki et al, 1995). This DNA motif is present in the promoters and enhancers of many skeletal-muscle-specific genes. Other HLH protein

have been identified as myogenic antagonists such as Twist, which inhibits myogenic differentiation by binding to the basic region in MRFs and preventing MRFs from interacting with E-Boxes in muscle specific promoters (Spicer et al., 1996; Hamamori et al., 1997). Like Twist, Id (Inhibitor of differentiation), which lacks a basic DNA binding domain, is also a HLH protein and negatively regulates the process of myogenic differentiation by heterodimerizing with E-proteins and sequestering them away from MyoD (Walsh and Perlman, 1997). The expression of Id is promoted under high-serum conditions, and a high level of Id protein inhibits MRF activity because Id can heterodimerize with E-proteins efficiently and prevent their interactions with MRFs (Benezra et al., 1990). However, Id levels decrease when myoblast cultures are deprived of growth factors, allowing the interaction between the MRFs and their DNA targets, resulting in increased levels of the cyclin-dependent kinase (cdk) inhibitor p21 facilitating with cell cycle withdrawal, followed by the induction of muscle-specific contractile proteins such as myosin heavy chain (MHC), and culminating in the fusion of myoblasts into multinucleated myotubes.

To better understand the mechanism of skeletal myogenesis, in vitro murine myoblast cell lines have been widely used. We are using 23A2 myoblasts, which are derived from 10T1/2 fibroblasts and commit to the myogenic pathway through the stable expression of MyoD. 23A2 myoblasts can be induced to differentiate when switched from culture in growth medium (GM) to differentiation medium (DM) that contains no serum or by allowing cultures to grow to confluence in GM (Dechant et al., 2002). The Weyman lab and others have previously reported that, due to deprivation of growth factors, about 70% of myoblasts undergo differentiation and 30% undergo apoptosis, thus documenting that

programmed cell death is coordinately regulated with differentiation during skeletal myogenesis.

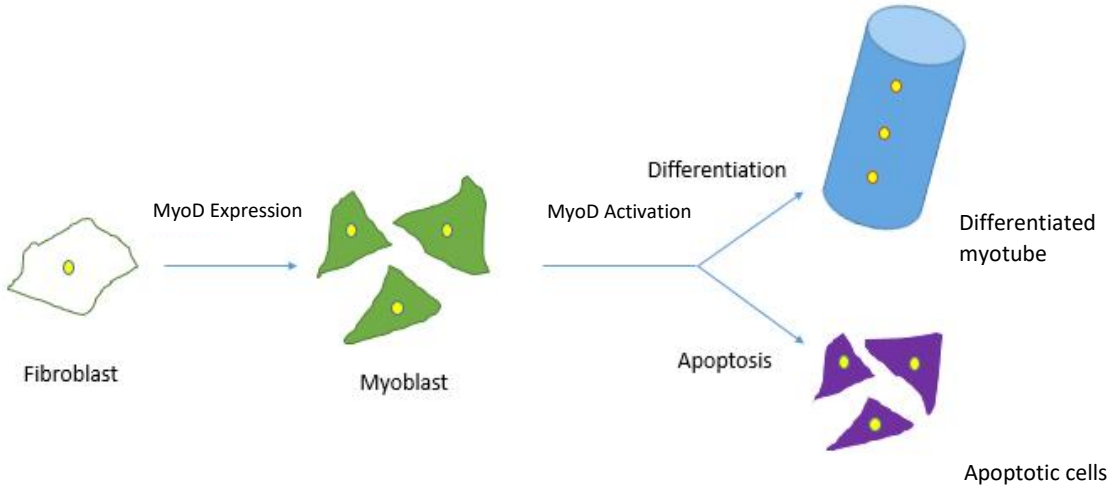


Figure 1. Skeletal myoblast model system. The myogenic program requires a group of transcription factors belonging to the muscle regulatory transcription factor (MRF) family consisting of Myf-5, MyoD, myogenin and MRF4. Expression of MyoD and/or Myf-5 commit progenitors to the myoblast lineage. Maintenance in growth media allows proliferation while maintenance in differentiation media (serum removal) results in roughly 70% of cells undergoing differentiation and about 30% of cells undergoing apoptosis.

Overview of Apoptosis

The process of programmed cell death, or apoptosis, is characterized by sensing and eliminating developmentally excess or damaged cells. It has been shown that apoptosis is an evolutionarily conserved process which is highly regulated and essential for the development of multicellular organisms (Horvitz, 1999). Therefore, deregulation in apoptosis is an important issue in the pathogenesis of a wide array of diseases such as cancer, heart disease, neurodegeneration, and other disorders.

The mechanisms of apoptosis are extremely complex and involve a cascade of molecular events. Thus far, it has been widely recognized there are two major apoptotic pathways: the intrinsic pathway and the extrinsic pathway. Briefly, the extrinsic pathway involves transmembrane receptor-mediated interactions. For example, the binding of Fas death ligand and Fas death receptor gives rise to the binding of the adaptor protein FADD which then associate with procaspase 8 leading to trans-cleavage and activation of caspase 8 (Ashkenazi and Dixit, 1998; Hsu et al., 1995; Kischkel et al., 1995). Once caspase 8 is activated, the execution phase of the apoptotic process is initiated. The intrinsic pathway involves non-receptor-mediated stimuli such as leading to an opening of the mitochondrial permeability transition pore and the release of proteins such as cytochrome c into the cytosol (Cai et al., 1998; Saelens et al., 2004). Cytochrome c activates Apaf-1 which then associates with procaspase 9 leading to trans-cleavage and activation of caspase 9 (Hill et al., 2004).

Proteins of Bcl-2 family play pivotal roles in the regulation of these apoptotic mitochondrial events (Cory and Adams, 2002). The family members have classically been divided into three groups. They are grouped based on the presence of conserved

Bcl-2 homology (BH) regions. One group inhibits apoptosis including Bcl-2, Bcl-XL, Bcl-W, Mcl1 and A1 and they all have four BH regions, the second group promotes apoptosis including BAX, BAK and BOK which contain BH 1-3 regions, while the third group is considered as BH-3 only proteins that can bind and regulate anti-apoptotic proteins to promote apoptosis. BAD, BIK, BID, BIM, NOXA and PUMA all belong to this group. A few mechanisms of how Bcl-2 family proteins play roles have been studied. Bid is cleaved by caspase 8 thus providing a link between the extrinsic and intrinsic pathway (Li et al., 1998). When Bad is phosphorylated, it is sequestered in the cytosol, while it is dephosphorylated, it will translocate to the mitochondria to promote the release of cytochrome c (Zha et al., 1996); additionally, PUMA has been found to play a role in p53-mediated apoptosis (Jeffers et al., 2003).

The extrinsic and intrinsic pathways ultimately meet on the same execution pathway, where the effector caspase such as caspase 3 is activated by the initiator caspases, and caspase 3 can cleave Poly ADP ribose polymerase (PARP), and other cytoskeletal and nuclear proteins, cause formation of apoptotic bodies and result in the biochemical and morphological changes in apoptotic cells (Slee et al., 2001).

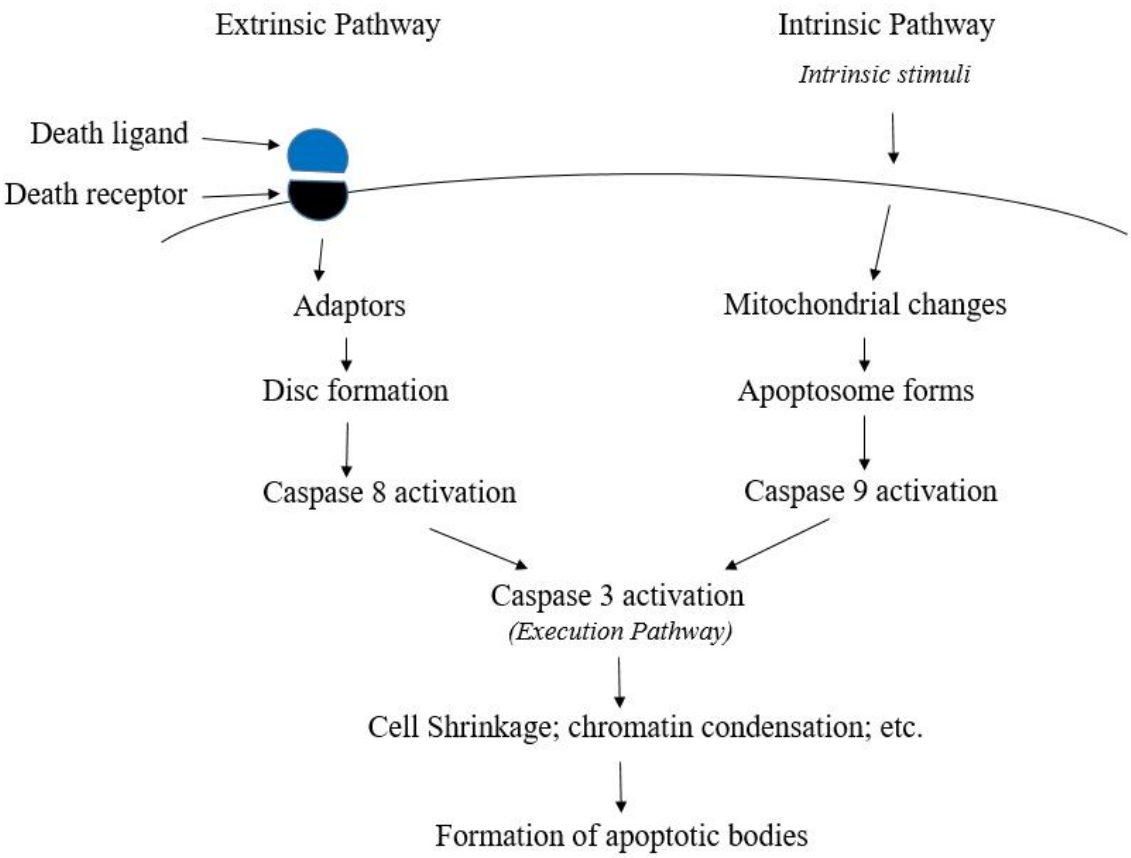


Figure 2. Extrinsic and intrinsic apoptotic pathways. The extrinsic pathway leading to apoptosis involves transmembrane death receptors which can bind to extrinsic ligands and transduce intracellular signals resulting in the activation of its initiator caspases; the intrinsic pathway involves non-receptor-mediated intracellular signals inducing activities in the mitochondria and cause the activation of its initiator caspases. Both pathways converge on the same execution pathway where executioner caspases are activated and cause cell shrinkage, chromatin condensation, apoptotic bodies, and finally phagocytosis of the apoptotic bodies.

Overview of Hypoxia

Cells of most organisms require oxygen to produce enough APT for metabolic activities. Hypoxia, or oxygen deprivation, which is a common phenomenon where the tissues are not oxygenated properly. Hypoxia reduces the ability of a cell to maintain its energy level. Hypoxia can affect gene expression, apoptosis, energy metabolism, and hormone secretion. Severe hypoxia may cause permanent damage to cells and tissues (Semenza, 2000) Although hypoxia is harmful to both normal cells and cancer cells, genetic and adaptive changes of cancer cells allow them be more adapted to hypoxic conditions. Adaption to hypoxia in cells and tissues causes the transcription of a variety of genes participating in glucose metabolism, cell proliferation/death and angiogenesis.

The key regulator of the hypoxic response is the hypoxia inducible factor 1 (HIF-1). HIF-1 has a complex role, and it has been reported that HIF-1 is involved in tumor growth, apoptosis (Carmeliet et al., 1998; Halterman et al., 1999) and embryonic development (Iyer et al., 1998; Kotch et al., 1999; Ryan et al., 1998).

Over 25 years ago, the identification of a hypoxia response element in the 3' enhancer of the gene encoding erythropoietin (EPO) helped with the discovery of HIF-1 (Goldberg et al., 1988). Subsequent studies have revealed that HIF-1 is a heterodimer composed of a hypoxically inducible factor, HIF-1α, and a constitutively expressed factor HIF-1β (Wang et al., 1995). HIF-1β is also known as the aryl hydrocarbon nuclear translocator (ARNT) (Reyes, et al., 1992), and both HIF-1α and HIF-1β belong to the basic helix-loop-helix (bHLH)-PAS (PER-ARNT-SIM) protein family (Wang et al., 1995; Semenza, 2001). The bHLH and PAS motifs are necessary for the binding between HIF-1α and HIF-1β (Crews, 1998). Later studies revealed the HIF-1α subunit contains two

transactivation domains which are N-Terminal (N-TAD) and C-Terminal (C-TAD) (Ruas et al., 2002). C-TAD was later reported to play a role in the interaction between coactivators like CBP/p300 to activate gene transcription (Lando et al., 2002). It has also been shown that the HIF-1α contains an oxygen-dependent degradation domain (ODDD) (Pugh et al., 1997).

In normoxia (normal level of oxygen), HIF-1α is hydroxylated by a prolyl hydroxylase (PHD) which requires oxygen and iron for its activity. Hydroxylated HIF-1α is rapidly degraded by proteasomes results from the ubiquitinylation by pVHL, which causes no detectable HIF-1α (Wang et al., 1995). In hypoxia, HIF-1α evades hydroxylation and translocates from the cytoplasm to the nucleus where it dimerizes with the constitutively expressed HIF-1β and becomes stable and transcriptionally active (Huang et al., 1996; Kallio et al., 1997). The activated HIF-1 complex then binds to the hypoxia response elements (HREs) in the promoters of hypoxia response genes and drives their transcription. These genes are basically critical for adaptation under hypoxic conditions, including those for glycolytic enzymes, vascular endothelial growth factor (VEGF), tyrosine hydroxylase, etc. (Semenza, 2002; Acker and Plate, 2003). However, it has been shown that HIF-1 is also involved in apoptosis in the presence of different environmental factors (Semenza, 2002).

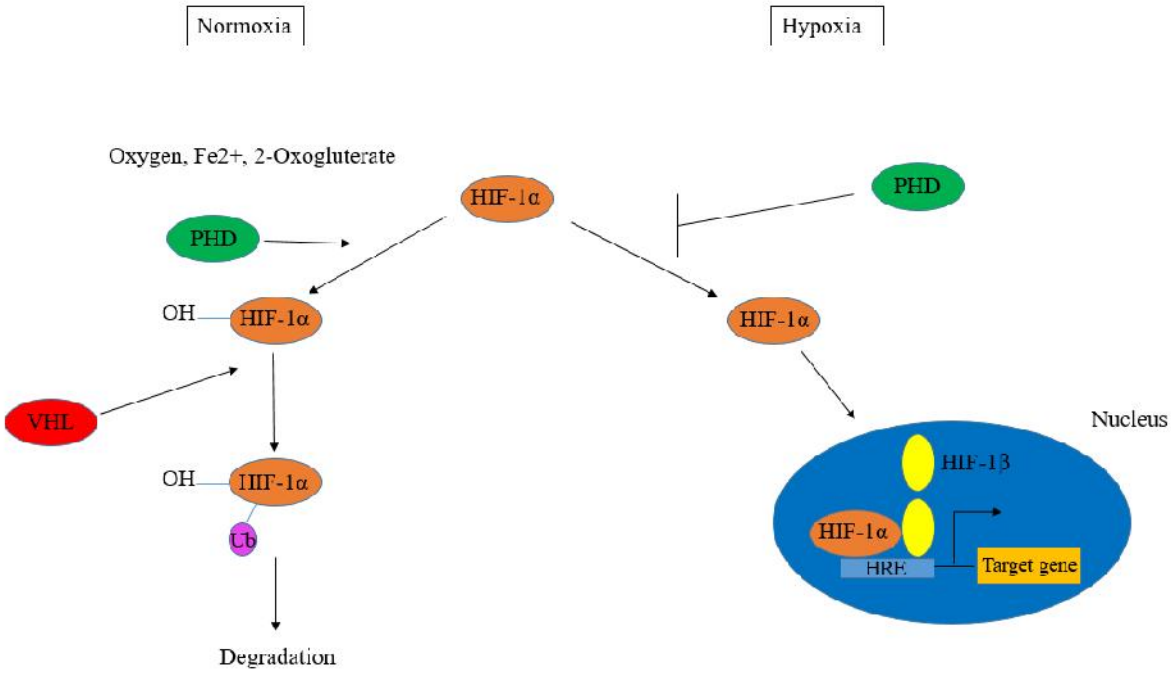
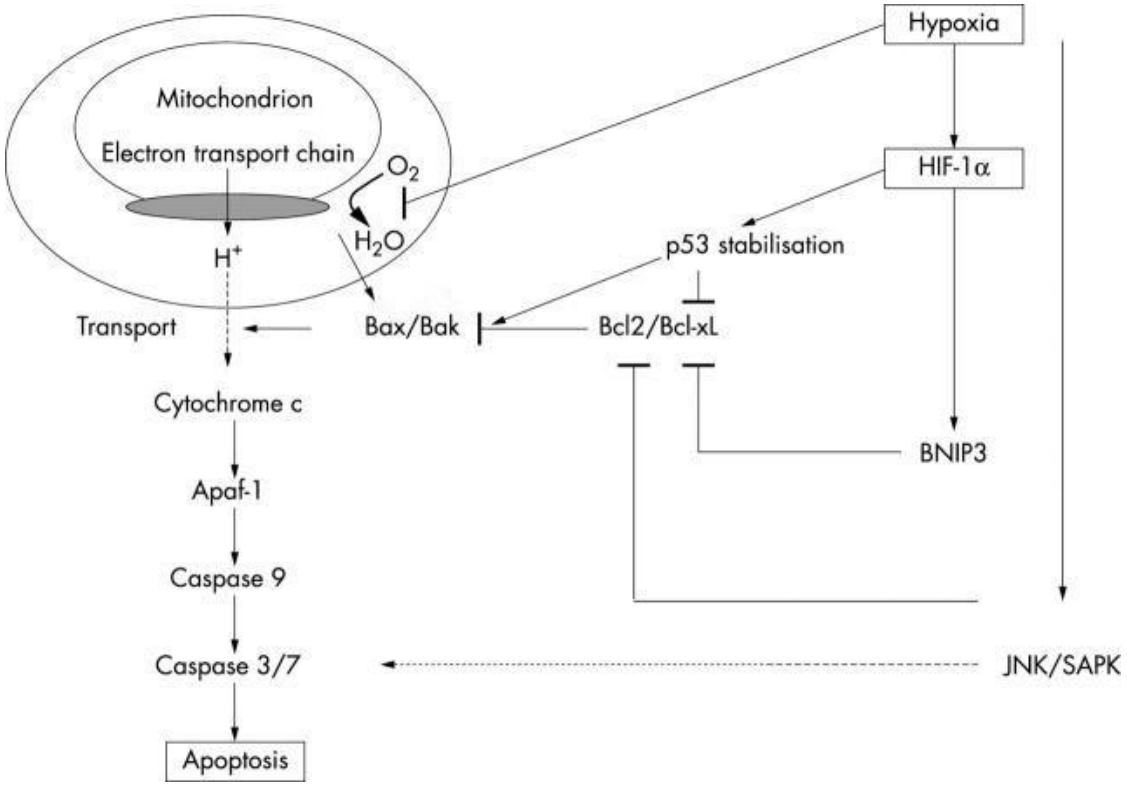


Figure 3. HIF-1α signaling pathway. In normoxia, HIF-1α is hydroxylated by PHD enzyme, and hydroxylated HIF-1α interacts with pVHL and get ubiquitylated. The ubquitylated HIF-1α is then directed to proteasome where it is degraded; however, in hypoxia, PHD enzyme lose its activity due to lack of oxygen, therefore, there is no pVHL binding and HIF-1α is not degraded, HIF-1α is then translocated to nucleus and dimerizes with HIF-1β subunit and become stabilized. The stabilized HIF-1 complex recognizes the HRE in the promoter of target genes and activate their transcription.

Hypoxia and apoptosis

Hypoxia can induce apoptosis by different HIF-1 dependent and independent pathways. The most direct induction of apoptosis induced by hypoxia, which is HIF-1 independent, involves the inhibition of the electron transport chain at the inner membrane of the mitochondria, resulting in the release of cytochrome c into the cytosol and initiating the cascade leading to apoptosis (reviewed in Greijer, 2004). Additionally, it has been reported that in melanoma cells, dominant negative mutants of c-Jun NH2-terminal kinase (JNK) inhibited hypoxia induced apoptosis, which suggests JNK is involved in apoptotic process.

To date, it has been well accepted there are two mechanisms inducing apoptosis via HIF-1α signaling pathway. First, stabilized HIF-1 can bind to the HRE in the promoter of the pro-apoptotic protein BNIP and induce its expression. Overexpression of BNIP3 induces apoptosis by binding and inhibiting the anti-apoptotic proteins Bcl-2 and Bcl-XL, and this mechanism is independent of cytochrome c release, APAF and caspases activation (Boyd, 1994). Second, HIF-1 can promote p53-dependent apoptosis. It was indicated that HIF-1α accumulation was necessary for the concomitant stabilization-dependent accumulation of p53 (An et al., 1998). Later, it has been reported that during hypoxia-induced apoptosis, in human breast carcinoma MCF-7 cells, both phosphorylated and dephosphorylated HIF-1α were induced. Phosphorylated HIF-1α forms complex with HIF-1β while dephosphorylated HIF-1α binds to p53. Apoptosis induced by p53 was suppressed by inhibition of dephosphorylated HIF-1α. This result suggests that phosphorylation status of HIF-1α plays a role in regulating apoptosis under hypoxic conditions (Suzuki et al., 2001).



(Greijer and van der Wall, 2004)

Figure 4. Apoptosis induced by hypoxia signaling pathway. Hypoxia can directly induce apoptosis either by inhibiting the electron transport chain at the inner membrane of the mitochondria or activating c-Jun NH2-terminal kinase; HIF-1α is also involved in hypoxia induced apoptosis. HIF-1α can induce apoptosis either by inducing overexpression of the pro-apoptotic protein BNIP3 or stabilizing the tumor suppressor gene p53. The solid lines indicate a direct impact while the dash lines indicate an indirect impact.

Hypoxia and differentiation

Hypoxia can regulate cellular differentiation as well. Hypoxia is highly correlated with the undifferentiated phenotype in breast cancer and cervical cancer (Helczynska et al., 2003; Azuma et al., 2003). Hypoxia has also been shown to inhibit the differentiation of embryonal stem cells adipocytes and skeletal myoblasts (Yun et al., 2002; Yun et al., 2005). In C2C12 skeletal myoblasts, hypoxic conditions have been reported to inhibit differentiation by decreasing the level of MyoD. One report documented that this decreased level of MyoD was due to decreased stability of the MyoD protein (Di Carlo et al., 2004) while another report documented decreased acetylation of the MyoD promoter (Yun et al., 2005). These results suggest the pivotal role played by hypoxia in the preservation of the undifferentiated stem cell phenotype.

Although HIF1α is a primary mediator of the hypoxic phenotype, its role in differentiation is not always consistent with the effect of hypoxia on differentiation. In adipocytes, hypoxia induced inhibition of differentiation is mediated by HIF1α (Yun et al., 2005). However, HIF1α has been reported to be essential for the differentiation of neuronal cells (Tomita et al., 2003), trophoblasts (Canigga et al., 2000), the mammary epithelium (Seagroves et al., 2003) and skeletal myoblasts (Ono et al., 2006).

Hypoxia and Translation

Hypoxia inhibits cap-dependent translation. It is well known that the translation of majority of mRNAs in the cell is through the cap-dependent pathway, which means translation initiation takes place at the 5' end of an mRNA molecule. Rather than during elongation or termination, the initiation stage is where protein synthesis is principally regulated. Briefly, translation initiation comprises three steps: 1) binding of the ternary complex containing the initiator methionyl tRNA, GTP and eIF2 to the small ribosomal subunit; 2) binding of the resulting pre-initiation complex to the mRNA promoted by eIF4F complex comprising eIF4E (cap-binding protein), eIF4A and eIF4G (DEAD-box RNA helicase) and locating of the initiation codon; 3) joining of the large ribosomal subunit and assembling the fully functional translation-competent ribosome (Gebauer and Hentze, 2004; Richard et al., 2010).

eIF2 plays a pivotal role during the first step of translation initiation. As a eukaryotic initiation factor, eIF2 was identified about 40 years ago. It binds GTP and initiator methionyl tRNA (Met-tRNAi), regulating the association of Met-tRNAi to the 40S ribosomal subunit (Pain, 1996). GTP is hydrolyzed to GDP and GDP-eIF2 complex leaves the ribosome at the end of the initiation. GDP bound to eIF2 must be exchanged back to GTP for eIF2 to promote another round of translation initiation and such exchange is regulated by another initiation factor eIF2B. However, in respone to hypoxia, the smallest subunit which is α subunit of eIF2 can be phosphorylated by endoplasmic reticulum kinase PERK (Koumenis et al., 2002). This phosphorylation stabilizes the eIF2-GDP-eIF2B complex while preventing the formation of the eIF2-GTP-Met-tRNAi ternary complex and inhibits cap-dependent translation (Gerlitz et al., 2002). It has also

been reported that phosphorylation of eIF2α has the potential to mediate expression of cap-independent translation initiation which is also known as IRES-mediated translation in cells under an array of physiological circumstances (Gerlitz et al., 2002).

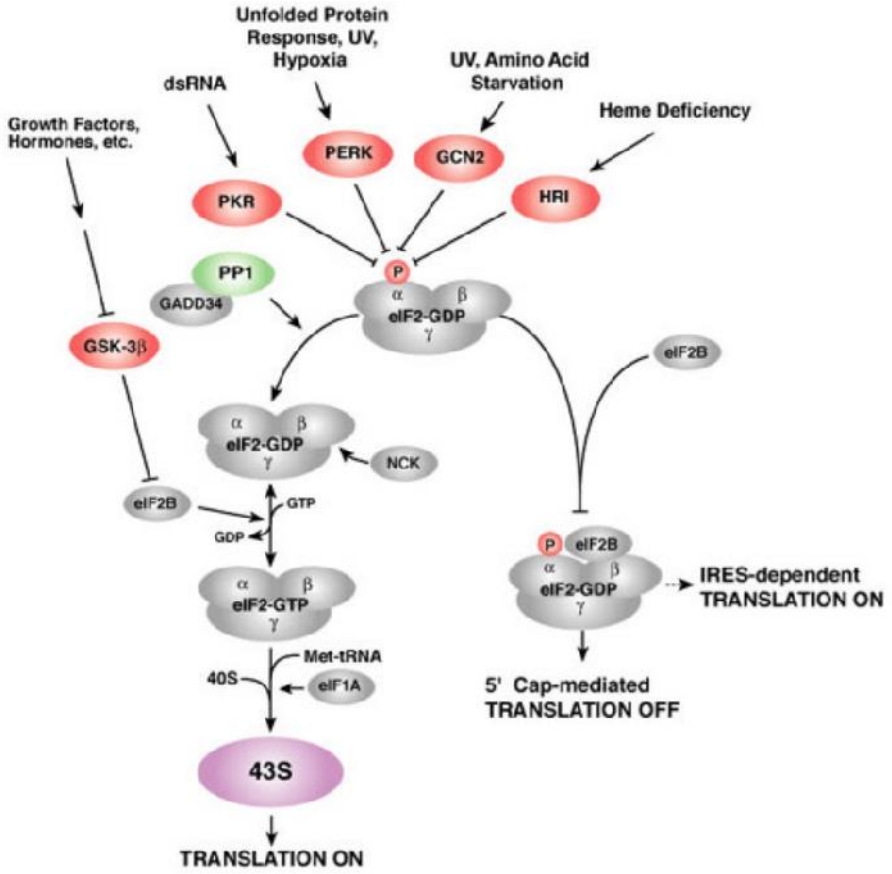


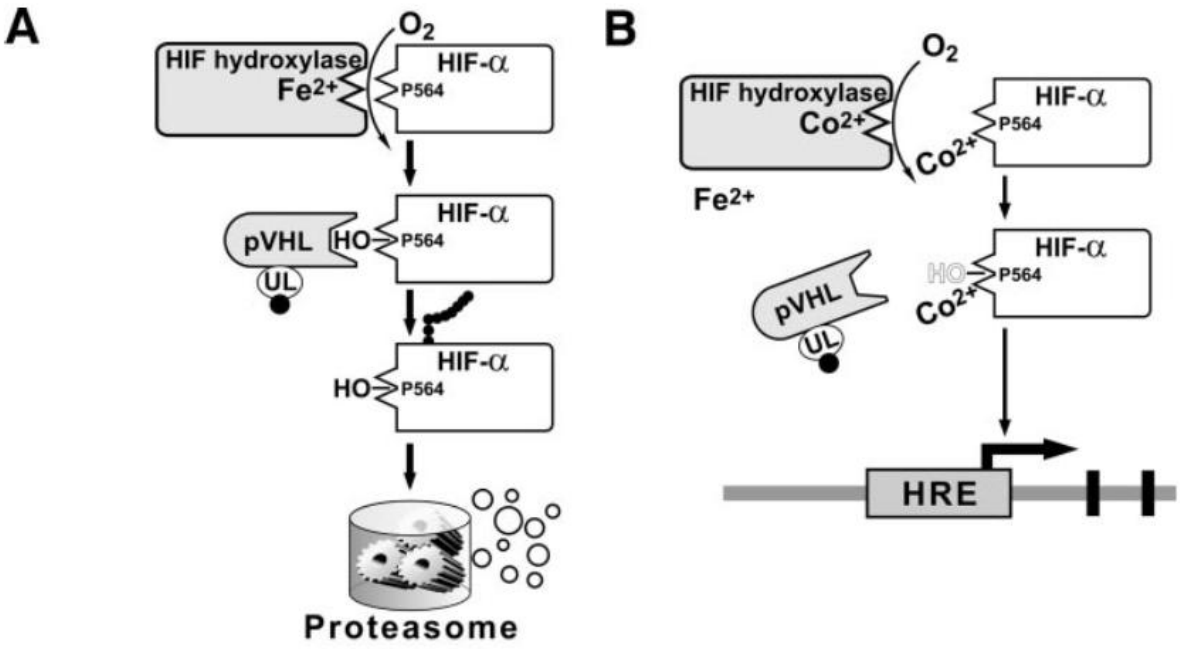
Figure 5. The role of eIF2 in the initiation of cap-dependent translation. At the beginning of translation initiation, eIF2 binds GTP and Met-tRNAi to form a ternary complex which then binds to 40S ribosome subunit resulting in the formation of 43S pre-initiation complex. At the end of initiation, GTP is hydrolyzed to GDP and eIF2B catalyze the exchange of GDP to GTP to promote another round of initiation. Under some stresses, the α subunit of eIF2 is phosphorylated and convert eIF2 from a substrate of eIF2B into a competitive inhibitor, which ultimately leads to the inhibition of global protein synthesis.

Hypoxia facilitates IRES-mediated translation. Other than binding of the ribosomal subunit to the 5' cap of mRNA in cap-dependent translation initiation, IRES-mediated translation involves binding of 40S ribosomal subunits to an internal portion of mRNA promoted by an RNA structure which is termed as internal ribosome entry site (IRES) to underline the process is free of 5' cap recognition (reviewed in Merrick, 2004). IRES was firstly identified about 30 years ago in picornaviruses, following its identification, two other types of IRES in hepatitis C virus (HCV) and the dicistroviruses were discovered (Pelletier and Sonenberg, 1988; Jang and Wimmer, 1990). Soon after the first IRES element in virus was discovered, a cellular mRNA which encodes the immunoglobulin heavy chain binding protein (BiP) was found to be translated in poliovirus-infected cells at a time when the majority of host cell mRNA translation was inhibited, which suggested that translation initiation by an internal-ribosome binding mechanism can be employed by cellular mRNA (Macejak and Sarnow, 1991). Since then, IRES elements have been found in many cellular mRNAs in various species.

It is well accepted that when cap-dependent translation is impaired under some conditions, cellular IRES will start its translation process. The decrease in the amount of eIF4E associated with eIF4F complex favors IRES-mediated translation (Svitkin et al., 2005). Also, it has been found that IRES-mediated translation is favored while eIF2α is phosphorylated under stress conditions such as UV irradiation, hypoxia, ER stress and virus infection (Fernandez et al., 2002).

Hypoxia mimicking agent: Cobalt Chloride

We used Cobalt Chloride ($CoCl_2$) to chemically induce stabilization of HIF-1α. $CoCl_2$ has long been recognized as a hypoxia mimicking agent. Earlier studies reported that iron is important for the activity of the prolyl hydroxylase since the enzyme contains an iron-binding site, and it is proposed that $Cocl_2$ can inhibit its hydroxylation activity by replacing iron with cobalt. Thus, HIF-1α would not be hydroxylated and would not be a target for ubiquitin-mediated degradation. Regardless of the mechanism, $CoCl_2$ treatment results in stabilization of HIF-1α (Epstein et al., 2001). Later, another study showed that cobalt binds to the PAS motif and inhibits binding of VHL to HIF-1α even when HIF-1α is hydroxylated, thus preventing the ubiquitination and degradation of HIF-1α (Yuan et al., 2003).



(Yuan et al., 2003)

Figure 6. Mechanism of Cobalt Chloride-mediated stabilization of HIF-1α. A) Under normoxia condition, the hydroxylase catalyze the hydroxylation of HIF-1α in the presence of iron, and the hydroxylated is then ubiquitylated by pVHL and then degraded by proteasome. B) Cobalt inhibits the activity of hydroxylase by replacing iron, cobalt can still bind to the hydroxylated HIF-1α, which results in the inhibition of the interaction between pVHL and hydroxylated HIF-1α, thereby preventing the degradation of HIF-1α.

CHAPTER II

MATERIALS AND METHODS

Cell lines and Cell Culture

23A2 myoblasts are a clonally selective cell line derived from 10T1/2 fibroblast cells by 5-azacytidine conversion. All cells were cultured on gelatin-coated plates and maintained in growth medium (GM), which consists of basal modified Eagle's medium (BME), 10% fetal bovine serum (FBS), and a 1% combination of 10,000 I.U./ml penicillin and 10,000 µg/ml streptomycin (1%P/S). Cells were incubated at 37°*C* in 5% CO_2. Hypoxia was chemically induced by adding 300µM $CoCl_2$ (Fisher Science). When used, actinomycin D (Sigma) was added at a final concentration of 5µg/ml.

Cell Counting

Cells were plated at $1x10^5$ or $4x10^5$on 100mm petri dishes. Next day, cells were switched to fresh GM with or without cobalt chloride treatment and cultured as indicated in figure

legends. Cell viability were determined by counting attached and floating cells separately. Briefly, cells were washed with PBS and trypsinized. 15µl of cell suspension was loaded into Hemacytometer (Fisher). Cell number was count using hand tally counter by observing cells under the electron microscope. The experiments were performed in duplicate.

FACS Analysis

23A2 Myoblasts were plated at equal density and next day were treated with cobalt chloride and cultured as indicated in each figure legend. Cell suspensions were prepared by trypsinization and $1x10^6$ cells were washed twice with PBS. The cells were re-suspended with 0.5ml PBS and fixed in 4.5ml 70% ethanol at 4°*C* overnight and next day the cells were treated with Propidium Iodide (MP Biomedicals) and incubated at 37°C for 30min prior to being sent to Flow Core at Cleveland Clinic for further analysis.

Western Blot Analysis

Cells were plated on 100mm petri dishes and the next day were treated as indicated in each figure legend. For total cell lysates, cells were rinsed in 1X phosphate buffered saline (PBS) prior to being lysed in 1X p21 lysis buffer (20mM MOPS pH7.4, 5mM magnesium chloride, 200mM sucrose, 0.1mM EDTA and 0.001% DNAse) containing phosphatase inhibitors, protease inhibitors (aprotinin, pepstatin, leupeptin and PMSF) and 5% CHAPS. The protein concentrations of all lysates were determined using Pierce™ Coomassie (Bradford) Protein Assay per manufacturer's instructions. Following protein

determination, lysates (100µg for PARP, PUMA, eIF2α, p-eIF2α, MyoD, SRF and MHC, and 50µg for p53) were denatured in 5X sample buffer (10% SDS, 50% glycerol, 10% beta-mercaptoethanol, pH 6.8 and 0.0025% bromophenol blue) and eletrophoresed through polyacrylamide gels (8% for MHC, 10% for PARP, p53, MyoD, eIF2α, p-eIF2α and SRF, and 12% for PUMA). Following SDS polyacrylamide gel electrophoresis (SDS-PAGE), samples were transferred electrophoretically for forty five minutes to one hour at 1.5 amp onto Hybond-P polyvinylidene difluoride membrane in transfer buffer containing 1 g/L SDS, 5.8 g/L Tris base, 29 g/L glycine and 20% methanol. After transfer, membranes were blocked in TBS-T buffer (136.9mM NaCl, 25µM Tris, and 1% Tween 20) with 10% newborn calf serum and 5% non-fat dry milk/bovine serum albumin. Western blot analysis was performed by incubating the blot with primary antibody overnight at 4°C using the following concentrations: PARP 1:1000 (Cell Signaling); PUMA 1:1000 (Abcam #9643); p53 1:1000 (Cell Signaling); p-eIF2α 1:500 (Cell Signaling); eIF2α 1:1000 (Cell Signaling); MyoD 1:1000 (BD Pharmingen); and SRF 1:1000 (Santa Cruz). The blots were washed with TBS-T (5 x 5 min), and then incubated with appropriate HRP-conjugated secondary antibodies diluted 1:1000 for one hour at room temperature, and washed with TBS-T (5 x 5 min) prior to the addition of chemiluminescent substrate. The blots were incubated with SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher) following manufacturer's instructions and bands were visualized using the Odyssey scanner (LI-COR Biosciences).

RT-PCR

23A2 myoblasts were plated at equal density and the next day switched to fresh GM with or without additional treatments. Cells were cultured as indicated in each figure legend. Total cellular RNA was isolated using Trizol reagent, according to the manufacturer's recommended protocol. A 20μl reaction with 1μg of total RNA was carried out with SuperScript III (Invitrogen) for reverse transcription following the manufacturer's instructions. 2μl of this reverse-transcribed product was in a 20μl quantitative PCR (50 cycles) with 300nM forward and reverse primer and *10*µl of SYBR Green qPCR SuperMix (Bio-Rad). The following primers were used for PCR: 1) PUMA, forward, 5'-GTCCGCGCCCCTTCCCGCTC-3' and reverse, 5'-GGTGGGGCCTCCTGCCAGGG-3'; 2) MyoD, forward, 5'-GACAGGACAGGACAGGGAGG-3' and reverse, 5'-GCACCGCAGTAGAGAAGTGT-3'. Reactions were performed in triplicate, amplified, and quantified using the Opticon 2 quatitative real time PCR system (MJ Research). The cycle number at which fluorescence increased linearly was calculated by the Opticon monitor in the form of C_t values and normalized as previously described (Karasarides et al., 2006).

CHAPTER III

RESULTS

Effect of hypoxic conditions on myoblast growth

We first examined the effect of hypoxic conditions on the growth of skeletal myoblasts. We found that the number of attached (viable) cells grew exponentially in the regular growth medium (GM) over time. However, when GM was supplemented with $CoCl_2$, the number of attached cells remained constant for the first 24 hours of treatment and then decreased over time (Figure 7). Given that treatment with $CoCl_2$ prevented proliferation for the first 24 hours of treatment, we hypothesized that there might be an effect on the cell cycle progression. Therefore, we performed cell cycle analysis. In GM, roughly 42% of myoblasts are in the G1 phase of the cell cycle and 42 % are in the S phase of the cell cycle while only 16% are in G2 phase of the cell cycle. However, after culture with $CoCl_2$ for 24 hours, the percentage of cells in G2 increased from 16% to roughly 37 %, with a concomitant decrease in cells in the G1 phase from 42% to roughly 35% and a decrease in cells in the S phase from 41% to roughly 28% (Figure 8). The percentage of

cells in G2 continued to increase slightly (an additional 3%) after 48 hours of culture in with $CoCl_2$. These data correlate with the inhibition of proliferation documented in Figure 7 and indicate that $CoCl_2$ treatment induces a G2 arrest in skeletal myoblasts. We next determined if the decrease in viable cells over time as a consequence of $CoCl_2$ treatment correlated with an increase in the percentage of floating cells. As expected, the percentage of floating cells after 24 hours of treatment was only 1.63 %. However, this percentage increased to roughly 7% after 36 hours, 19% after 48 hours and 45% after 72hours (Figure 9). To confirm that the floating cells were indeed not viable, we transferred the GM containing these floating cells to a new plate and assessed attachment observed 24h, 48h and 72h, respectively. No re-attached cells were detected even after 72 hours (Data is not shown).

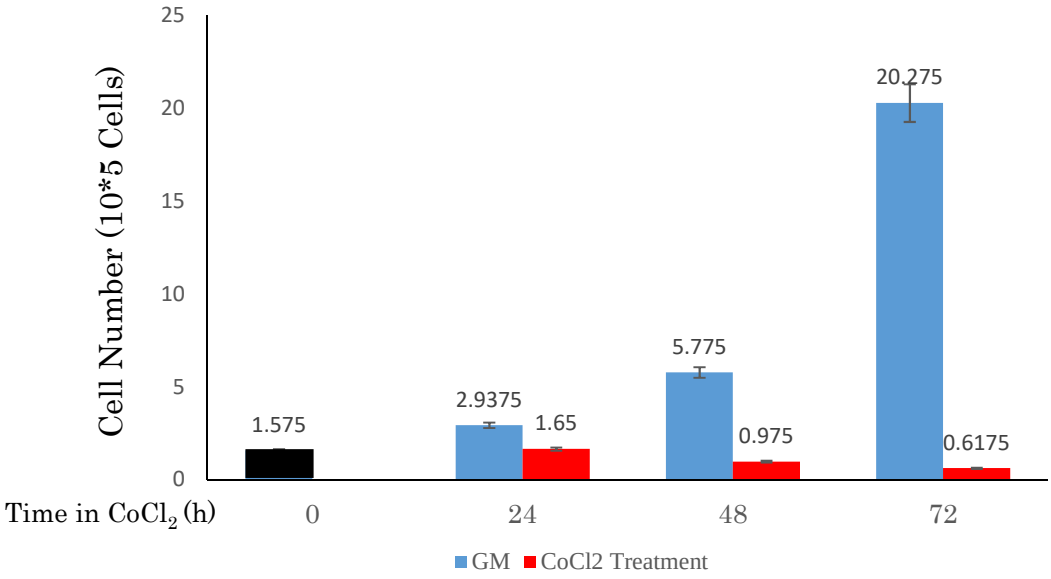
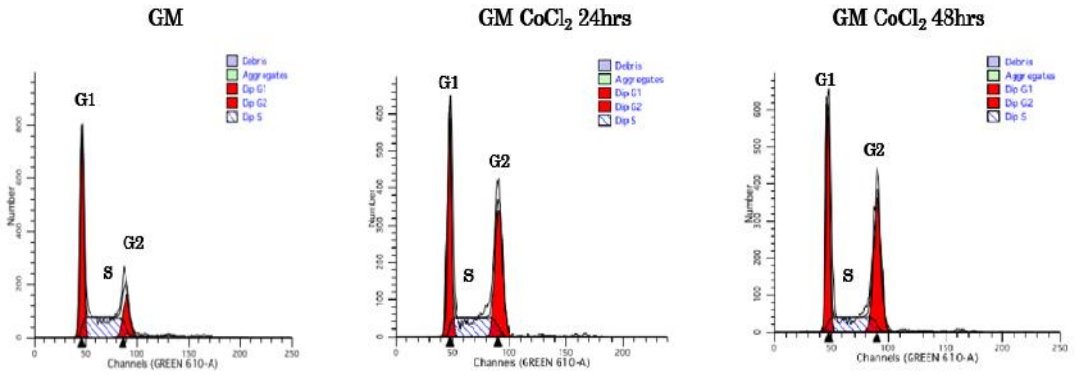


Figure 7. The effect of $CoCl_2$ treatment on skeletal myoblast growth. Equal cell numbers were plated and the next day were switched to fresh GM with or without $CoCl_2$ (300µM) and collected for the times indicated. Following PBS washing and trypsinization, cell counting was performed as described in Materials and Methods. Shown is the result of an average of two independent experiments.



	G1	S	G2
GM	41.94%	41.38%	16.68%
GM $CoCl_2$ 24hrs	34.63%	28.50%	36.86%
GM $CoCl_2$ 48hrs	36.30%	23.08%	40.62%

Figure 8. The effect of $CoCl_2$ treatment on the cell cycle. Equal cell numbers were plated and the next day were switched to fresh GM with or without $CoCl_2$ (300μM) and collected for the times indicated. Following PBS washing and trypsinization, cell counting was performed as described in Materials and Methods. Shown is the result from one experiment which is representative of two independent experiments.

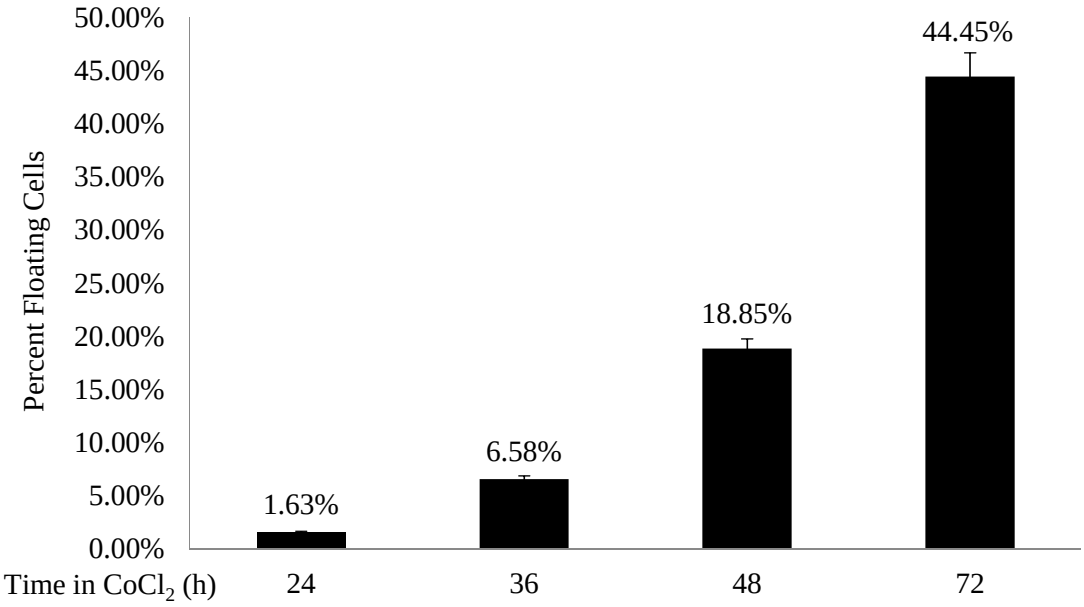


Figure 9. The percent of floating cells increases over time in response to $CoCl_2$ treatment. Equal cell numbers were plated and the next day were switched to fresh GM with $CoCl_2$ (300μM) and floating cells were collected for the times indicated. cell counting was performed as described in Materials and Methods. Shown is the result from one experiment which is representative of two independent experiments.

Apoptosis is suggested as a mechanism contributing to myoblast death

Poly ADP-ribose polymerase (PARP) is a nuclear enzyme involved in DNA stability, DNA repair and transcription regulation. The cleavage of PARP is a hallmark of apoptosis (Lazebnik et al., 1993). To examine if apoptosis is the mechanism contributing to myoblast death under hypoxic conditions, we assessed the expression of cleaved PARP in response to $CoCl_2$ treatment. Lysates were prepared from attached cells after treatment with $CoCl_2$ for 12h, 24h and 48h. Detached cells were only collected after 48 hours of treatment. Western blot analysis documented that cleaved PARP was expressed in floating cells but not attached myoblasts (Figure 10), confirming an apoptotic process.

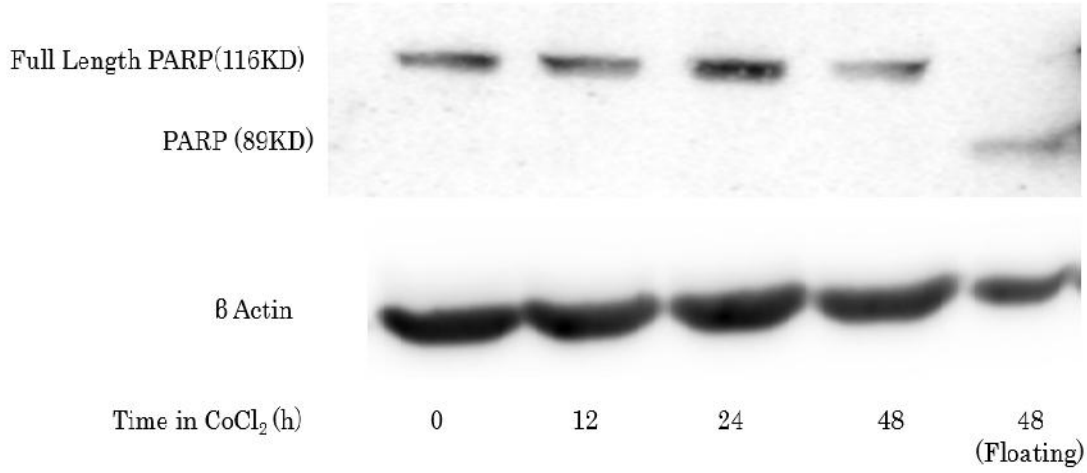


Figure 10. Cleaved PARP is expressed in floating cells. Equal cell numbers were plated and the next day were switched to fresh GM with $CoCl_2$ (300μM) for the times indicated. Whole cell lysates were prepared and subjected to SDS-PAGE. Western analysis was performed using anti-PARP or anti-beta actin. Shown is the result of one experiment which is representative of two independent experiments.

Effect of hypoxic conditions on the expression of PUMA and p53

Bcl-2 family proteins have been studied intensively as they play important roles in the regulation of apoptosis. There are three groups of Bcl-2 family proteins which are anti-apoptotic group, pro-apoptotic group and BH-3 only group. Our lab has previously reported that BH-3 only protein PUMA (p53 upregulated modulator of apoptosis) plays a critical role in the apoptosis associated with skeletal myoblast differentiation (Shaltouki et al., 2007). Therefore, we assessed the expression level of PUMA protein under hypoxic conditions. An increase in PUMA protein (roughly 2 fold) and mRNA (roughly 3-fold) was detected after 4 hours of $CoCl_2$ treatment. After 8 hours of treatment, the PUMA mRNA levels reverted to levels found in untreated myoblasts while the PUMA protein levels did not return to basal levels until 4 hours later (Figure 11A-C). As PUMA was initially suggested as a directed mediator of p53-induced cell death (Nakano and Vousden, 2001, Yu et al., 2001), we next assessed the protein expression level of the transcription factor p53. An increase in p53 expression was also detected after 4 hours of $CoCl_2$ treatment (Figure 12). Interestingly, the levels of p53 were still elevated after 24 hours of treatment while the levels of PUMA mRNA were only elevated through 6 hours of treatment, perhaps suggesting an additional regulation of the PUMA mRNA half-life.

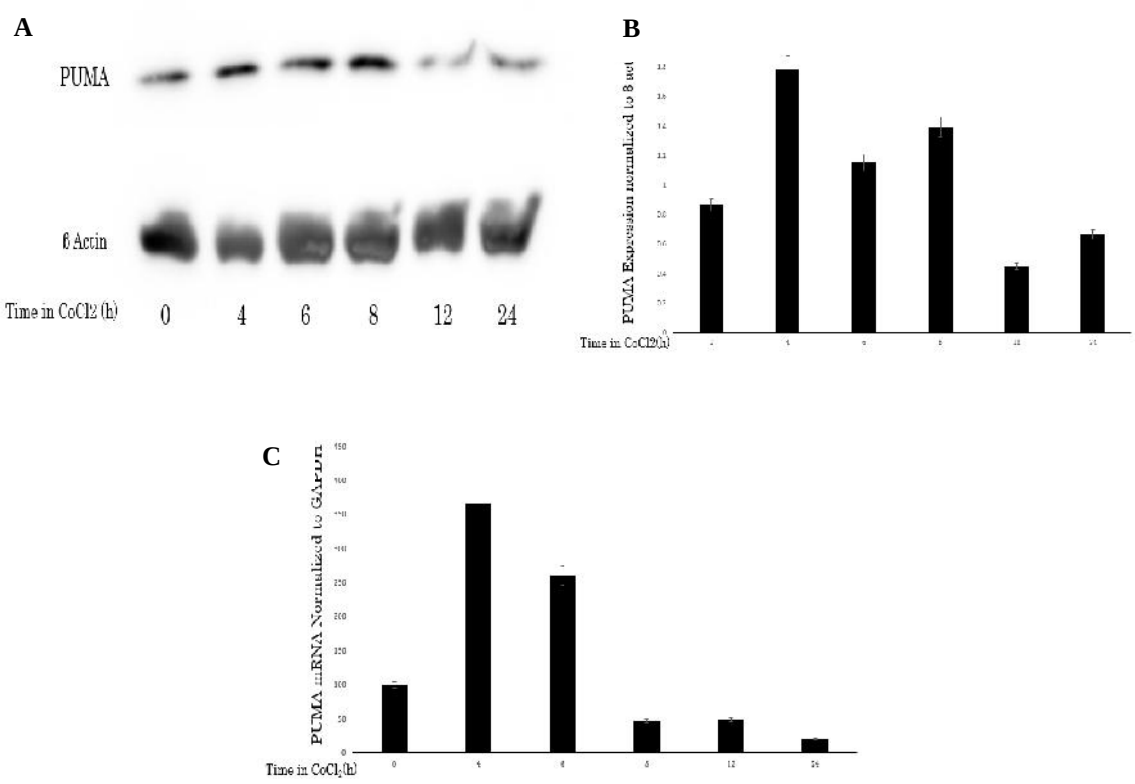


Figure 11. PUMA protein and mRNA expression increase at early time points in response to $CoCl_2$ treatment. Equal cell numbers were plated and the next day were switched to fresh GM with or without $CoCl_2$ (300μM) for the times indicated. In (A), Whole cell lysates were prepared and subjected to SDS-PAGE. Western analysis was performed using anti-PUMA or anti-beta actin. In (B), PUMA protein expression is normalized with that of loading control beta actin. In (C), quantitative PCR was performed as described in Materials and methods, GAPDH was measured for normalization. Shown are the results of one expreiment for each that is average of three independent experiments.

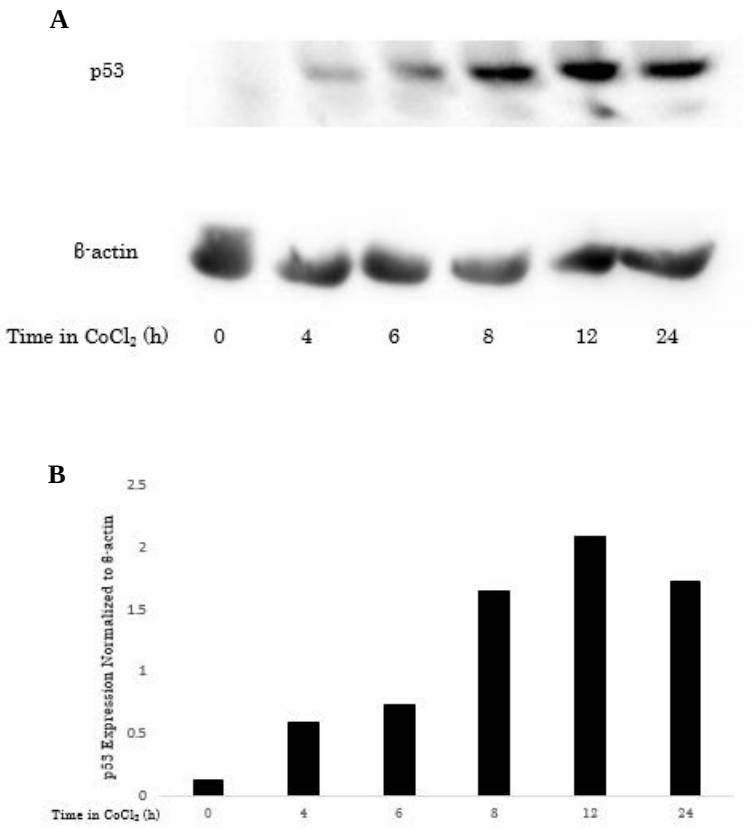


Figure 12. p53 protein expression increases in response to $CoCl_2$ treatment. Equal cell numbers were plated and the next day were switched to fresh GM with or without $CoCl_2$ (300μM) for the times indicated. In (A), Whole cell lysates were prepared and subjected to SDS-PAGE. Western analysis was performed using anti-p53 or anti-beta actin. In (B), p53 protein expression is normalized with that of loading control beta actin. Shown is the result that is the representative of two independent experiments.

Translational regulation of PUMA under hypoxic conditions

We have previously reported that the increase in PUMA protein in response to culturing skeletal myoblasts in differentiation media still occurs even if the increase in PUMA mRNA is blocked (Shaltouki et al., 2013). Thus, we next explored whether this would be the case in response to $CoCl_2$ treatment. We determined that under conditions where treatment with Actinomycin D was sufficient to block the increase in PUMA mRNA, and in fact to lower PUMA mRNA levels below the level found in untreated myoblasts (Figure 13A), the increase in PUMA protein levels could still be detected (Figure 13B and C). This result suggests post-transcriptional regulation of PUMA, either at the level of translation or alteration of PUMA protein half-life. Since we have previously reported IRES-mediated translation of PUMA in response to culture in differentiation media, we explored whether $CoCl_2$ treatment could induce conditions that favor IRES-mediated translation. Specifically, we assessed the phosphorylation status of eIF2α in response to $CoCl_2$ treatment. Consistent with an increase in PUMA protein after only 4 hours of $CoCl_2$ treatment, we detected an increase in the level of phosphorylated eIF2α after only 4 hours of $CoCl_2$ treatment (Figure 14A and B). Since this increase in the level of phosphorylated eIF2α occurred in the absence of an increase in total eIF2α (Figure 14C and D), we can confirm that this represents an increase in the percentage of phosphorylated eIF2α (Figure 14E). Interestingly, this increased level of phosphorylated eIF2α is maintained for at least 24 hours even though increased PUMA protein level are only maintained for 8 hours, thus suggesting possible additional regulation at the level of inhibited translation or a shortened protein half-life. These results are consistent with the

possibility that PUMA expression is IRES mediated under hypoxic conditions but validation of this awaits further experimentation.

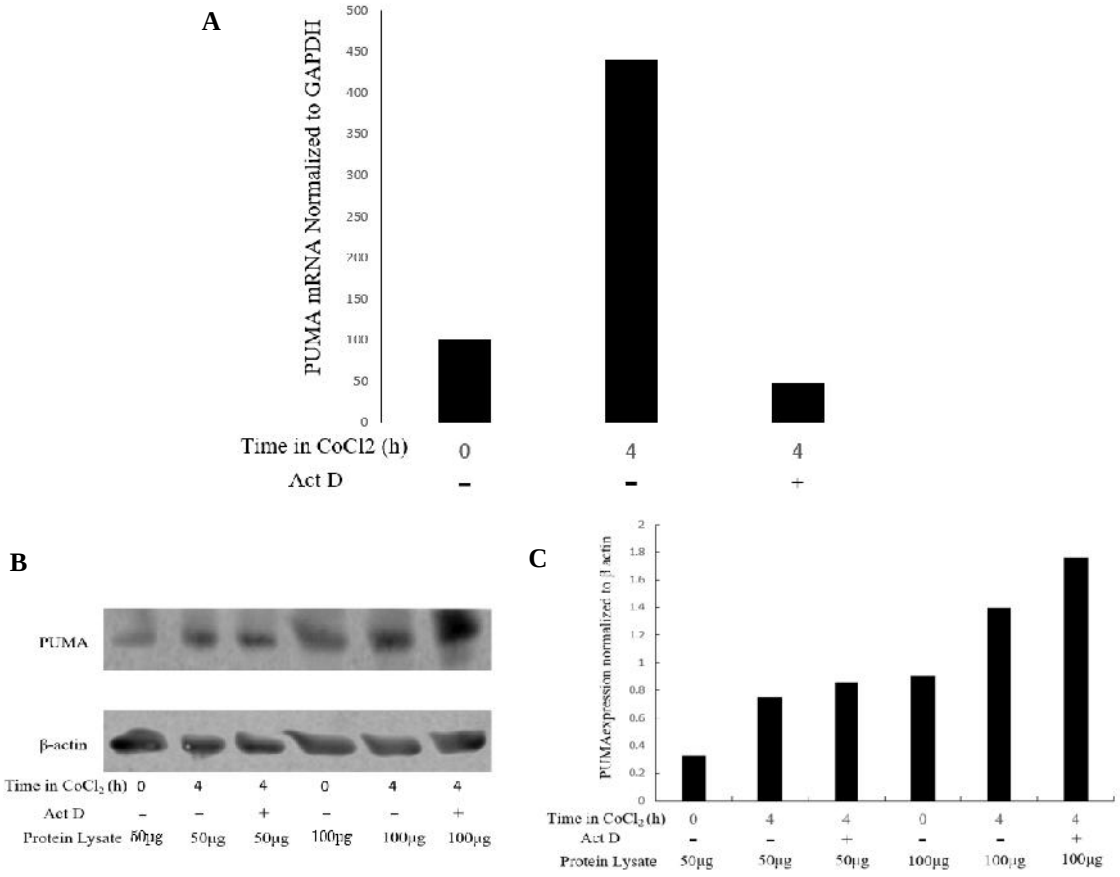


Figure 13. Actinomycin D treatment blocks the increase in PUMA mRNA but not protein in response to $CoCl_2$ treatment. Equal cell numbers were plated and the next day were switched to fresh GM with or without $CoCl_2$ (300µM) for the times indicated. 5ug/ml actinomycin D were used. In (A), quantitative PCR was performed as described in Materials and methods, GAPDH was measured for normalization. In (B), Whole cell lysates were prepared and subjected to SDS-PAGE. Western analysis was performed using anti-PUMA or anti-beta actin. In (C), PUMA protein expression is normalized with that of loading control beta actin. Shown are the results of one expreiment for each that is representative of two independent experiments.

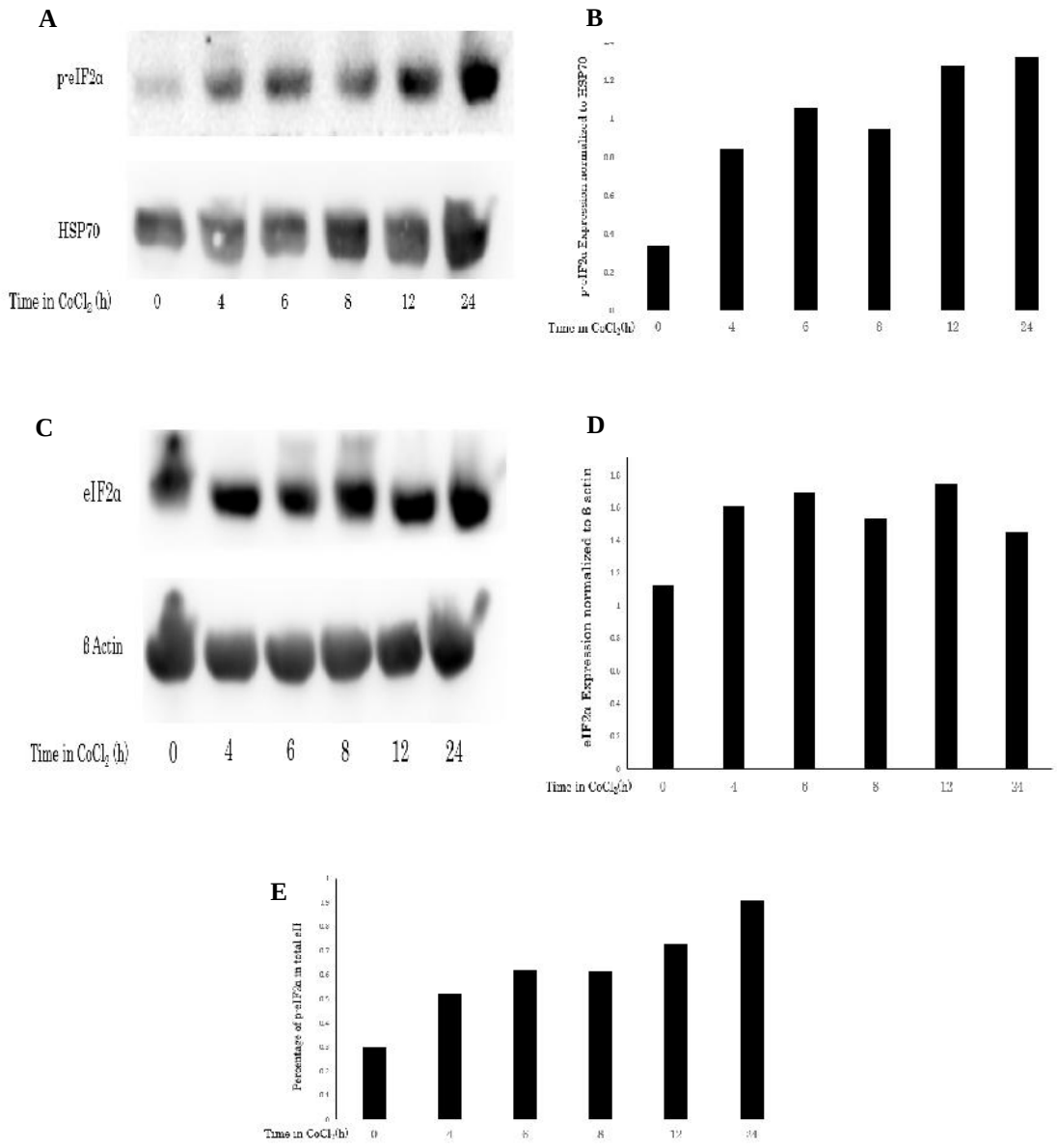


Figure 14. The percent of p-eIF2α in total eIF2α increases in response to $CoCl_2$ treatment. Equal cell numbers were plated and the next day were switched to fresh GM with or without $CoCl_2$ (300μM) for the times indicated. In (A), whole cell lysates were prepared and subjected to SDS-PAGE. Western analysis was performed using anti-p-eIF2α or anti-HSP70. In (B), p-eIF2α protein expression is normalized with that of loading control HSP70. In (C), whole cell lysates were prepared and subjected to SDS-PAGE. Western analysis was performed using anti-eIF2α or anti-beta actin. (D), eIF2α protein expression is normalized with that of loading control beta actin. In (E), expression of normalized p-eIF2α is quantified with that of normalized total eIF2α. Shown are the results of one expreiment for each that is representative of two independent experiments.

Hypoxic conditions and skeletal myoblast differentiation

In addition to investigating the apoptotic process under hypoxic conditions, we also examined how the differentiation process is regulated under hypoxic conditions. The muscle regulatory transcription factor MyoD is one of the earliest markers of myogenic commitment and once activated, it induces the withdrawal of myoblasts from the cell cycle and differentiation of myoblasts into myotubes. Thus, we assessed the expression level of MyoD in myoblasts treated with $CoCl_2$. A reduction of both MyoD protein and mRNA levels could be detected after 4 hours of $CoCl_2$ treatment and this reduction was maintained for at least 24 hours (Figure 15A-C). The decreased level of MyoD protein is most likely attributed to the decreased level of mRNA. The decreased level of MyoD mRNA can be caused by either the accelerated decay or decreased transcription. Thus, we firstly compared the half-life of MyoD mRNA under normal conditions and hypoxic conditions. We determined that the half-live of MyoD mRNA was roughly 30 minutes under both conditions (Figure 16) suggesting that the decreased level of MyoD mRNA as a consequence of $CoCl_2$ treatment is a result of decreased transcription.

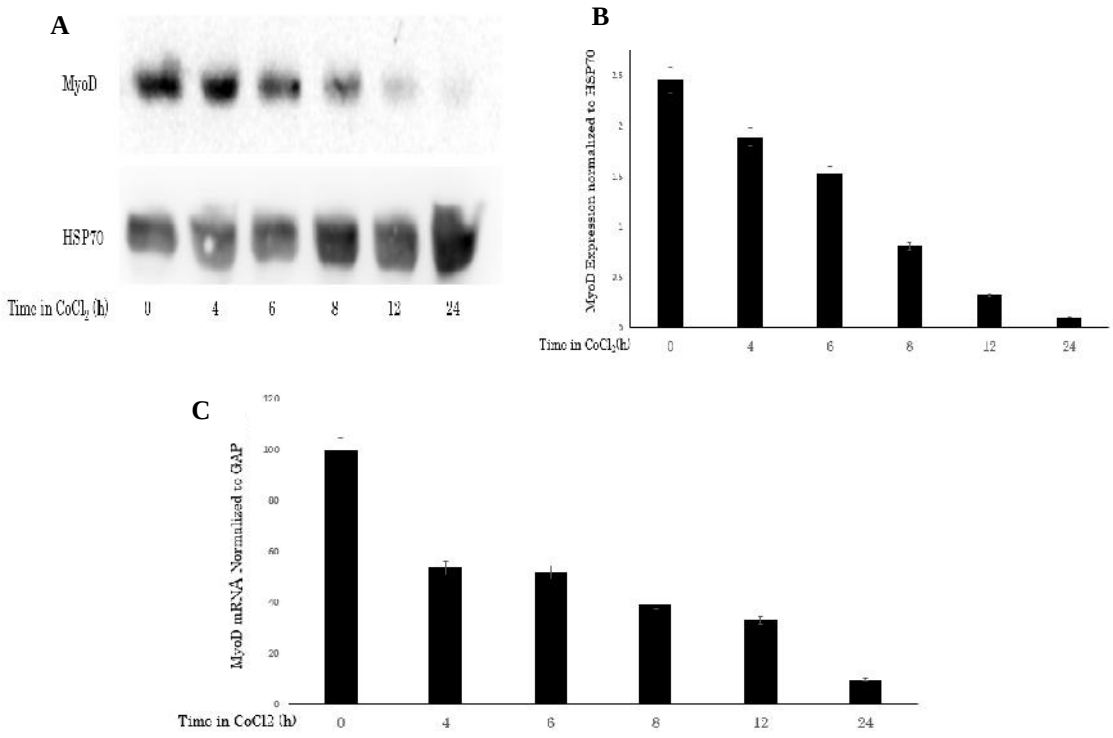


Figure 15. MyoD expression decreases in response to $CoCl_2$ treatment. Equal cell numbers were plated and the next day were switched to fresh GM with or without $CoCl_2$ (300μM) for the times indicated. In (A), Whole cell lysates were prepared and subjected to SDS-PAGE. Western analysis was performed using anti-MyoD or anti-HSP70. In (B), MyoD protein expression is quantified with that of loading control HSP70. In (C), quantitative PCR was performed as described in Materials and methods, GAPDH was measured for normalization. Shown are the results of one expreiment for each that is representative of two independent experiments.

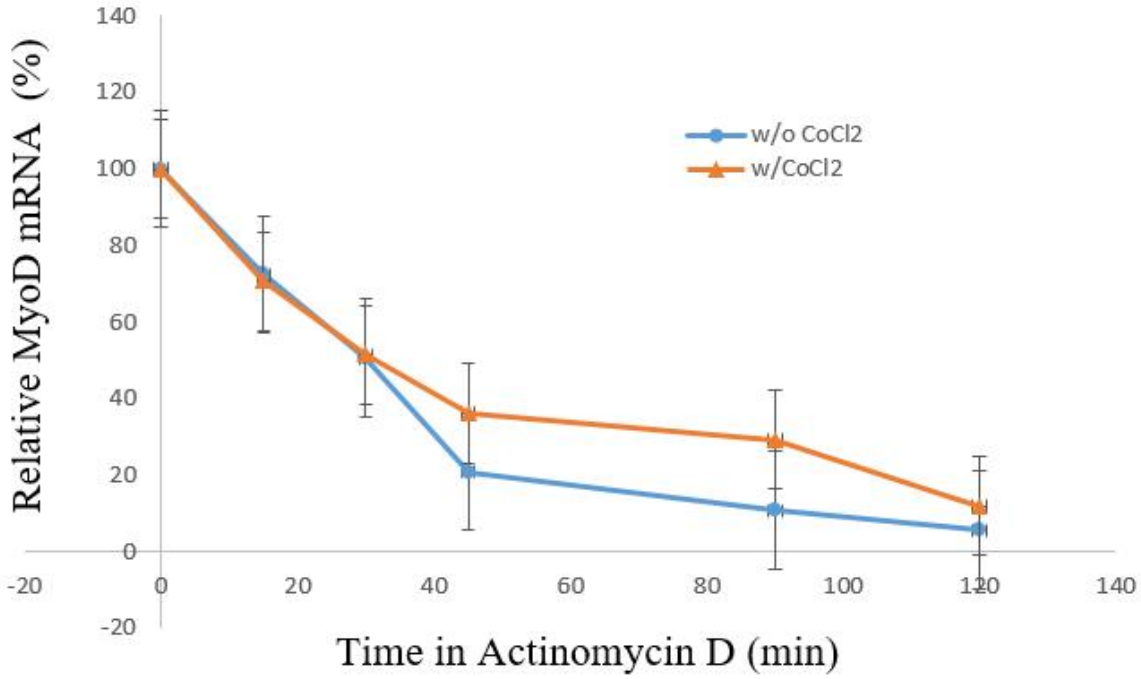


Figure 16. $CoCl_2$ treatment does not affect the half-life of MyoD mRNA. Equal cell numbers were plated and the next day were switched to fresh GM with or without $CoCl_2$ (300μM). Total RNA samples were collected at indicated time points and quantitative PCR was performed as described in Materials and methods, GAPDH was measured for normalization. Shown is the result that is representative of two independent experiments.

The transcription factor – SRF (serum response factor) is responsible for maintaining MyoD expression (L'honore et al., 2003). We, therefore, next assessed the expression of both the activated phospho-SRF and total SRF in response to $CoCl_2$ treatment. The Western analysis results showed that there is no marked effect on the expression of either phospho-SRF and total SRF under hypoxic conditions (Figure 17A-D). Further experimentation is necessary to determine if $CoCl_2$ treatment affects the binding of phospho-SRF to the promoter of the MyoD gene.

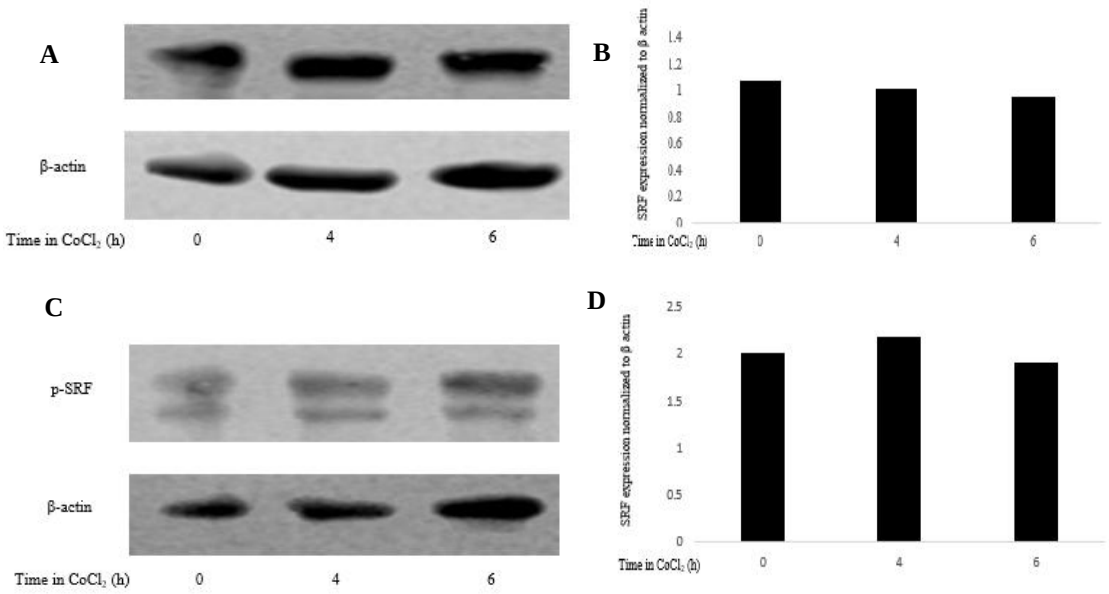


Figure 17. $CoCl_2$ treatment has no marked effect on SRF and p-SRF expression. Equal cell numbers were plated and the next day were switched to fresh GM with or without $CoCl_2$ (300μM) for the times indicated. In (A), Whole cell lysates were prepared and subjected to SDS-PAGE. Western analysis was performed using anti-SRF or anti-beta actin. In (B), SRF protein expression is quantified with that of loading control beta actin. In (C), Whole cell lysates were prepared and subjected to SDS-PAGE. Western analysis was performed using anti-p-SRF or anti-beta actin. In (D), p-SRF protein expression is quantified with that of loading control beta control. Shown are the results of one experiment for each that is representative of two independent experiments.

CHAPTER IV

DISCUSSION

The maintenance of oxygen homeostasis is important for survival and hypoxia plays a critical role in the pathogenesis of cancer, ischemia, lung diseases, and heart diseases. Thus, to understand and reveal the mechanism of hypoxia regulation is important for gaining new insights about potentially novel therapeutic approaches. Ischemic injury in skeletal muscle caused by hypoxic conditions occurs in response to vascular and musculoskeletal traumas, diseases and following reconstructive and transplantation surgeries. The molecular mechanism of ischemic injury in skeletal muscle, however, is still yet to be explored. The Weyman lab has been focusing on studying the coordinate regulation of differentiation and apoptosis in mouse skeletal myoblasts. It is well known that MyoD is a key regulator of skeletal muscle differentiation and we have previously reported that the pro-apoptotic protein PUMA plays a role in the apoptosis associated with skeletal myoblast differentiation (Shaltouki et al., 2007). Hence, we studied the

regulation of these molecules under hypoxic conditions and herein we report a general picture of how hypoxic conditions affect skeletal myoblasts.

We first examined the growth of skeletal myoblast under hypoxic conditions. A recent study has found that hypoxia can inhibit mesenchymal stem cell proliferation (Kumar and Vaidya, 2016), and in fact, hypoxia induces decreased cell proliferation for most cell types as an increased cell number requires a consequent increase in oxygen (reviewed in Hubbi and Semenza, 2015). We found in 23A2 skeletal myoblasts, $CoCl_2$-induced hypoxia leads to stabilization in the number of viable cells for the first 24 hours, indicating decreased cell proliferation under hypoxic conditions. FACS data showed that there is a marked increase in G2 fractions and thus G2/M arrest in response to $CoCl_2$ treatment. It has been reported that hypoxia can induce marked G0/G1 arrest in different cell lines (Achison and Hupp, 2003, Kumar and Vaidya, 2016), which is inconsistent with our findings. Thus, inconsistency could be cell type specific or unique to $CoCl_2$ treatment as a method of mimicking hypoxia. However, in future, it is worthy of studying the expression of molecules correlated with G2/M arrest in our skeletal myoblast model system.

After 24 hours of $CoCl_2$ treatment, the number of viable cells decreases while the number of floating cells increases over time. We also transferred the medium containing floating cells to a new plate and observe it continually for three days to see if any floating cells re-attach to the plate. No reattachment of a single cell indicates those floating cells are dead cells. To investigate if apoptosis contributes to the death of myoblasts as a consequence of $CoCl_2$ treatment, we assessed the expression of cleaved PARP, a marker for apoptosis. PARP cleavage was not detected in attached cells but was detected in floating cells

suggests that the apoptosis contributes to the cell death as a consequence of $CoCl_2$ treatment. Our lab has reported that when 23A2 myoblast cultured in DM (GM containing low or no serum), during skeletal myoblast differentiation, roughly 30% of the population undergo apoptosis (DeChant et al., 2002), while as a consequence of $CoCl_2$ treatment, the percent of dead cells continues to increase to more than 40% at 72 hours, we propose that this difference is a consequence of the fact that in DM, myoblasts can also undergo differentiation while cell death is the only fate cells can have when cultured under hypoxic conditions.

Our lab has identified the pro-apoptotic Bcl-2 family protein PUMA as a critical regulator in the skeletal myoblast apoptosis in response to culture in DM (Shaltouki et al., 2007). It has been shown that PUMA contributes to the endoplasmic reticulum (ER) stress-dependent component of cardiomyocyte apoptosis as ischemia/reperfusion (IR) is associated with ER stress (Nickson et al., 2007). Further, PUMA is involved in the apoptosis of cerebral astrocytes upon I/R injury (Chen et al., 2014). We hence looked at the effect of hypoxic conditions on PUMA expression levels. We found the expression of PUMA protein increases at early time points in response to $CoCl_2$ treatment, indicating hypoxic conditions induce PUMA expression in skeletal myoblasts. We also investigated if PUMA protein expression is regulated at the level of transcription. The RT-PCR data revealed the expression of PUMA mRNA is correlated with that of PUMA protein. As PUMA was initially discovered as a p53-induced Bcl-2 family protein (Nakano and Vousden, 2001). p53 is a transcription factor that regulates the expression of stress response genes and a well-known tumor suppressor that can kill cancer cells via the apoptotic process (Vousden and Prives, 2009). Moreover, p53-driven apoptosis in

response to hypoxia has been reported as well (An et al., 1998, Suzuki et al., 2001). We next assessed the expression of p53 under hypoxic conditions. The increase in p53 expression in response to $CoCl_2$ treatment suggests that p53 might be responsible for the increase of PUMA under hypoxic conditions. Interestingly, the increase in PUMA is transient, declining after 8 hours while the increase in p53 persists for at least 24 hours. These results suggest additional regulation, perhaps at the level of decreasing PUMA half-life. Our lab has previously documented that MyoD contributes to the skeletal myoblast apoptosis as a consequence of cultured in DM and this role is correlated with PUMA induction (Harford et al., 2010). Therefore, future experiments are needed not only to determine the mechanism by which p53 regulates the expression of PUMA under hypoxic conditions in skeletal myoblasts, but also to investigate if MyoD plays a role in the regulation of PUMA expression under hypoxic conditions.

In collaboration with Dr. Anton Komar, our lab identified an IRES element in the PUMA mRNA that facilitate cap-independent translation in murine skeletal myoblasts in response to culture in DM as well as other stimuli including etoposide (Shaltouki et al., 2013). It aroused our interest in testing the IRES activity of PUMA under hypoxic conditions. It is known that the translation initiation factor eIF2α plays a vital role in cap-dependent translation initiation and phosphorylation of eIF2α inhibits the initiation step. In addition, it has been reported that the phosphorylation of eIF2α potentially regulate IRES-mediated translation (Gerlitz et al., 2002). We, therefore, first examined the expression of both total eIF2α and phosphor-eIF2α in response to $CoCl_2$ treatment and found that the phosphorylation of eIF2α increased over time. We also demonstrated that Actinomycin D-induced abrogation of the increase in PUMA mRNA did not prevent the

increase in PUMA protein in response to $CoCl_2$ treatment suggesting post-transcriptional control. Whether the PUMA IRES is responsive to $CoCl_2$ treatment awaits further investigation.

The factors that are critical to the determination and terminal differentiation of skeletal muscle are known as myogenic regulatory factors (MRFs) which are basic helix-loop-helix transcription factors, including MyoD, Myf-5, MRF4 and myogenin. MyoD was the first myogenic regulatory gene to be identified (Davis et al., 1987), and since its discovery, MyoD has been widely recognized for the role it plays in the specification of skeletal muscle and establishment of the myogenic program. It has been reported that multinucleated myotube formation and the expression of differentiation markers could be strongly inhibited when myoblasts were exposed to hypoxia and MyoD degradation was associated with this hypoxia-dependent inhibition of differentiation, and, the marked decrease in MyoD protein was not paralleled by a decrease in the mRNA level (Di Carlo et al., 2004). We also assessed the expression of MyoD in our skeletal myoblast model system in response to $CoCl_2$ induced hypoxic conditions. We documented that the level of MyoD protein and mRNA were correlated with each other and both decreased over time. We also documented no marked change in the stability of MyoD mRNA with or without $CoCl_2$ treatment. Taken together, these results suggest that the reduction of MyoD protein could be due to the decreased MyoD mRNA levels regulated at the transcription level. It has been reported that the transcription factor SRF is required for myoblast differentiation (Vandromme et al., 1992) as well as the expression of the MyoD gene in proliferating myoblasts (Soulez et al., 1996). Later, an SRF binding CArG element was found in the MyoD gene distal regulatory region (DRR) involved in the

regulation of MyoD gene expression in mouse and human skeletal myoblasts (L'honore et al., 2003). Our lab has also shown that it is SRF that needs to be recruited to MyoD gene to drive its transcription in our skeletal myoblast model system (Ross and Weyman, manuscript in preparation). However, we could detect no marked effect on the expression of either total SRF or phosphorylated SRF in response to $CoCl_2$ treatment. Interestingly, a previous study on adaptive myogenesis under hypoxia in C2C12 mouse myoblasts showed that hypoxia can induce a transient histone deacetylation in the promoter region of MyoD and thus repression of MyoD transcription (Yun et al., 2005). Further research to demonstrate in our model system if a decrease in SRF binding to MyoD promoter is responsible for the histone deacetylation is necessary. Future experiments must determine if $CoCl_2$ treatment affects SRF binding to the DRR of the MyoD gene. Finally, elucidation of the signal transduction pathway activated by $CoCl_2$ to repress MyoD expression is warranted. A comprehensive understanding of the mechanisms responsible for the effects of hypoxia on myoblast will identify more targets for therapeutic purposes.

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