MECHANISM OF ACTIVATION OF THE QUIESCENCE-SPECIFIC p20K GENE

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Abstract:

Growth arrest specific (GAS) genes are highly inducible at quiescence (G_0) and repressed rapidly in response to mitogens. Aberrant disruption of quiescence can lead to abnormal development and diseases such as cancer, thus, it is important to study the signals and mechanisms responsible for expressions of quiescent specific genes. p20K, a GAS gene whose expression is highly induced in conditions of contact inhibition & hypoxia in chicken embryo fibroblasts (CEF), is studied in this thesis. Preliminary studies demonstrate that p20K activation is dependent on its Quiescence Responsive Unit (QRU), a 48bp promoter region. In addition, the binding sites of a CCAAT/enhancer binding protein (C/EBPβ) and ERK2 on the QRU of p20K promoter overlap with each other regulating the competition between activating (C/EBPβ) and inhibiting (ERK2) of the p20K gene. After culturing CEF with media rich in growth factors (10%FBS), p20K induction is delayed in hypoxia. Moreover, it is the decrease of Phospho-ERK not CHOP level that correlates with p20K inhibition in hypoxia in both 5%CCS and 10%FBS. Western blotting analysis of Hypoxia Inducible Factor 1α (HIF1α) expression indicated that this hypoxia-response factor is induced rapidly and with the same kinetics in CEF subjected to hypoxia cultured in 5%CCS or 10%FBS, indicating that they sense and respond similarly to low oxygen concentrations. These results suggest that p20K induction in hypoxia is caused by growth arrest induced by hypoxia. To further document this process, hypoxia mimicking reagent DMOG, a prolyl-hydroxylase inhibitor that can stabilize HIF in

normoxia, was used. Interestingly, p20K expression was highly induced after DMOG treatment in CEF, even if CHOP, an inhibitor of C/EBPβ, was induced in these conditions. Co-Immunoprecipitation results showed that the accumulation of CHOP-C/EBPβ heterodimers was induced during DMOG treatment. Additionally, Proliferation Assay suggested that DMOG treatment significantly inhibited CEF proliferation. Finally, Chromatin Immunoprecipitation Analysis indicated that ERK-2 did not bind to the QRU after DMOG treatment, indicating that ERK-2 dissociation correlates with p20K induction in response to DMOG in CEF. Collectively, these results demonstrate that growth arrest induced by hypoxia or DMOG treatment plays a determinant role in p20K induction. In contrast, CHOP level or CHOP-C/EBPβ heterodimer reduction did not correlate with the induction of p20K.

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List of Abbreviations:

AP-1	Activator Protein-1
ATF4	Activating Transcription Factor-4
Bcl-2	B-cell leukemia/lymphoma 2
bHLH	basic helix-loop-helix domain
BR	Basic Region
bZIP	basic Leucine Zipper Domain
CCS	"cosmic" calf serum
C/EBP	CCAAT/enhancer binding protein
CDK	Cyclin Dependent Kinase
CEF	Chicken Embryonic Fibroblast
ChIP	Chromatin Immunoprecipitation
CHO	Chinese-hamster ovary
CHOP	CCAAT/enhancer binding protein (C/EBP) homologous protein
Co-IP	Co-Immunoprecipitation
CKI	Cyclin dependent Kinase Inhibitors
DMEM	Dulbecco's modified Eagle's medium
DMOG	Dimethyloxaloylglcine
DNA	Deoxyribonucleic acid
EDTA	ethylene dinitriol tetra-acetic acid
elF2	eukaryotic initiation factor 2
elF4	eukaryotic initiation factor 4
EFA	essential fatty acids
ER	endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
Ex-FABP	Extracellular Fatty Acid Binding Protein
FBS	Fetal Bovine Serum
G_0	Reversible Growth Arrest
G1	Gap phase 1
G2	Gap phase 2
GADD	Growth arrest and DNA damage
GAS	growth-arrest specific
GATE	Gamma Activated Transcription Element
MAPK	Mitogen Activated Protein Kinase
MEF	Mouse Embryonic Fibroblast
NLS	nuclear localization signal
ODD	oxygen-dependent degradation domain
PAS	PER-ARNT-SIM domain
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PHD	prolyl-hydroxylase
qRT-PCR	quantitative reverse transcription (RT) polymerase chain reaction (PCR)
QRU	Quiescence Responsive Unit
Rb	retinoblastoma protein
RCAS	Replication Competent ALV LTR with a Splice acceptor
RCASBP	Replication Competent Avian Sarcoma Virus LTR
RD	Regulatory Domain
REFs	Rat Embryonic Fibroblasts
RNA	Ribonucleic acid
RNAi	RNA interference
RT	reverse transcriptase
SDS	sodium dodecyl-sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
sORF	small open reading frame
S-phase	DNA synthesis phase
TAD	Trans-Activation Domain
TBS	Tris buffered saline
TBS-T	Tris buffered saline 0.1% Tween-20
TNF	Tumor Necrosis Factor
ZIP	Leucine Zipper Domain

Chapter 1: Introduction

1.1 Cell Cycle and Quiescence (G_0)

Cell Cycle

Regulation of the cell cycle is fundamental for controlled cell proliferation. The survival of an organism can be compromised by disruption within the cycle. Regulation of the cell cycle provides cells with the means to respond to intrinsic and extrinsic signals in order to ensure division occurs at appropriate times. In Gap 1 (G1) phase, cells prepare themselves for DNA replication. During G1, cells are responsive to growth factors, mitogens and other extracellular factors that regulate cell cycle progression (Foster et al., 2011). Cells reach the restriction point-R during late G1 phase and mitogenic signals (growth factors) play the determinant role in the progression of G1 to restriction point while cells complete a high rate of protein synthesis during this progression. Cells will be committed to the completion of the cell cycle after R point, entering the Synthesis phase (S), in which DNA replication takes place, and will be independent of nutrient availability, mitogen and other activation external growth factors (Blagosklonny & Pardee, 2002; Bedolla et al., 2013; Schwartz & Shah, 2005). To prepare for mitosis, cells enter the secondary growth phase, Gap 2 (G2) phase. During this phase, cells will continue to grow in size (Bedolla et al., 2013). Checkpoints contained in G2 phase will allow cells to detect and repair DNA and cellular defects, for example, if DNA damage occurs, cells will not begin mitosis under the protection of checkpoints, but trigger a mechanism inducing cell cycle arrest

via activating p53 transcription factor or retinoblastoma proteins (Stark and Taylor, 2004; Reviewed by Foijer & Te Riele, 2006). As cells advance through the G2 phase, they will reach the Mitosis (M) phase and divide into two new daughter cells (Bedolla et al., 2013). During late G1 and G2/M phases, the tumor suppressor p53 can mediate cell cycle arrest (Agarwal et al., 1995). Checkpoints of cell cycle are triggered to provide time for DNA repair (Liu and Kulesz-Martin, 2001). However, factors in checkpoints such as p53 could also induce apoptosis when irreparable DNA damage occurs. However, in cancer cells activation of these cell cycle checkpoints during DNA replication often fails, and this results in uncontrolled cell proliferation.

Quiescence

Living cells divide during the cell cycle in periods of suitable conditions (Malumbres M, et al., 2001). However, if unsuitable environmental conditions occur (such as lack of nutrients, growth factors or space) cells will exit the normal cell cycle during G1 phase and enter a state called quiescence (G_0), which is a period of reversible growth arrest (Gray et al., 2004). In humans, many cells, such as glial and thyroid follicular cells or hepatocytes, remain in a state of G0 due to the fact that they do not constantly proliferate (Reviewed by Williams and Stoeber, 2012). Moreover, many of them are terminally differentiated as well. Only a small amount of cells located in self-renewing tissues such as epithelia or bone marrow are actively cycling (Potten and Loeffler, 1990; Reviewed by Williams and Stoeber, 2012).

Quiescence is a process important in homeostasis, in which cells may remain in a "resting" state, re-enter the cell cycle when favorable conditions appear or enter senescence, an irreversible growth arrest state. (Komarova & Wodarz., 2007; Malumbres M. et al., 2001) Quiescent cells have specific features because of their non-proliferating state, such as unduplicated DNA and decreased synthesis of macromolecules (Pardee et al., 1989).The key point determining whether cells enter quiescence or continue in the cell cycle is in G_1 phase (Gray et al., 2004) (Figure 1.1). It has been proven that nutrient availability and contact inhibition are two factors regulating quiescence (Gos et al., 2005). Moreover, sub-confluent cells entering quiescence by serum deprivation can re-enter the cell cycle with the addition of serum, but contact inhibited cells have to be subcultured at lower density after quiescence for them to re-enter the cell cycle (Gos et al., 2005). Although numerous studies have been performed to understand cell cycle regulation, little is known about G0.

Insensitivity to anti-proliferation signals is a hallmark of cancer cells (Hanahan &Weinberg, 2000). In cancer cells, mechanisms regulating the transition from proliferation to quiescence are disrupted, consequently, cancer cells are able to grow when low serum condition occurs or even in conditions of low growth factors (Pardee, 1989). In addition, anchorage surface is not required for cancer cell proliferation, and contact inhibition is not often observed in cancer cells as well, as cancer cells can even divide in either suspensions or under high cell density conditions (Orford et al., 1999;

Nelson & Daniel, 2002).

Since stem cells are often observed in "resting" state, it has been hypothesized that cancer stem cells (CSC) could stay in quiescence, sustaining cancer cells to become drug-resistant (Coller et al., 2006; Komarova & Wodarz., 2007; Jordan, 2006). Traditional chemo-therapy agents usually have S-phase specificity, explaining why primitive quiescent cancer cells are drug-resistant (Graham et al., 2002). Moreover, some drug therapies induce quiescence in primitive cancer cells (Komarova and Wodarz, 2007). Those quiescent cancer cells do not become apoptotic, proliferate and are less sensitive to drugs (Komarova and Wodarz, 2007). Interestingly, during single-drug treatments, there is probability that drug resistance begins at the start of treatment and is independent of quiescence, however, during two or more drug treatments, the probable generating of resistant mutants before treatment correlates with quiescence (Komarova and Wodarz, 2007). Consequently, isolating quiescent cell gene markers is of interest in cancer research (Jordan, 2006). Likewise, the pathways regulating cellular quiescence are a promising research direction.

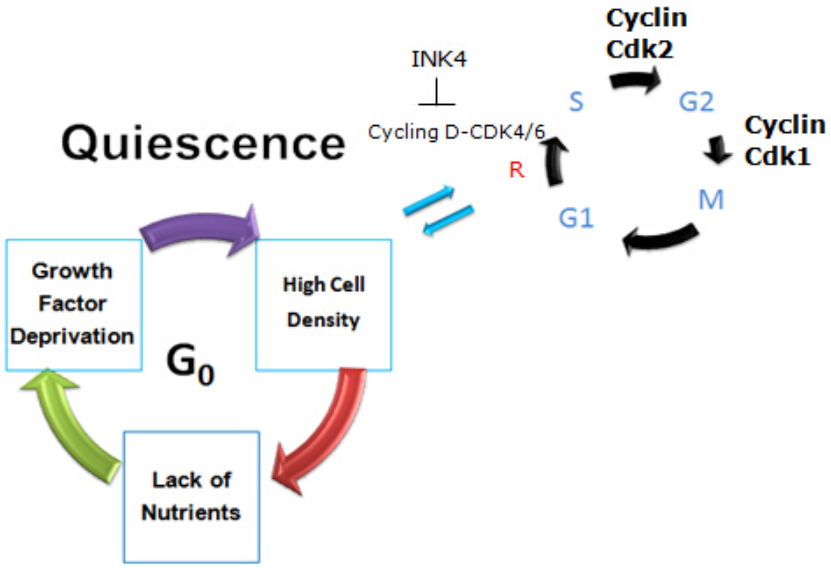


Figure 1.1 Cell cycle and quiescence.

The cell cycle consists of Interphase (G1, S and G2) and Mitosis. Cells grow in size and prepare for DNA replication in Gap phase 1 (G1), when it overcomes restriction point R, it will enter Synthesis (S) phase, where DNA replicates. The completion of replication leads cells to Gap phase 2 (G2), the secondary growth phases, to prepare cells for the Mitosis phase. During mitosis (M), chromosome segregation and cytokinesis occur, giving rise to new daughter cells. The absence of nutrients or growth factors and high cell density (contact inhibition) can lead to quiescence (G0) during G1. However G0 is a reversible cell cycle arrest, cells can return to cell cycle when favorable conditions return.

Cyclins and CDKs

A series of cyclin dependent kinases (CDK) and cyclin dependent kinase inhibitors control cell cycle progression (Schwartz & Shah, 2005). Cyclin proteins and their corresponding CDK protein partners form functional complexes regulating various steps during the cycle. For example, in early G1 phase, cell cycle progression is controlled by cyclin D-CDK4/6 (As reviewed by Black and Black, 2012). The entrance of quiescent cells into cell cycle is also controlled by the cyclin D-CDK 4/6 complex (reviewed in Black and Black, 2012). The G1 to S progression is regulated by the Cyclin E-CDK2 complex (Hwang and Clurman, 2005). Cyclin A-CDK1/2 complexes regulate the S phase and early G2 transition (Reviewed by Malumbres and Barbacid, 2005). In the progression through G2 phase, B-cyclins predominate and regulate Mitosis, while at the same time A-cyclins are degraded by ubiquitin-mediated proteolysis (Reviewed by Malumbres and Barbacid, 2005). As a result, Cyclin B creates a complex with CDK1 to regulate the transition and entrance from G2 to M (Reviewed by Malumbres and Barbacid, 2005).

Rb & related pocket proteins are targets of cyclin/CDK complexes

p107 and p130, retinoblastoma protein (pRb) and related pocket proteins, interact with different cyclin/CDK complexes in cell cycle progression (Cobrinik, 2005). S phase transition and quiescence are the two most commonly reported time points for Rb and related proteins to function in. Rb exists as two forms, hypophosphorylated (active)

and hyperphosphorylated (inactive) form and it is phosphorylated during G1 phase. In cell cycle progression, inactivated Rb and its related pocket proteins are essential because they bind to and repress E2F transcription factors (Reviewed by Foijer & Te Riele, 2006). E2F factors are crucial for G1/S transition because of their regulation on several genes, such as Cyclin E (reviewed in Foijer & Te Riele, 2006). In the absence of mitogen stimulation, Rb helps halt cell cycle progression by inhibiting E2F transcription factors to control quiescence (Chim et al., 2006). Furthermore, inactivation of Rb is needed for cell cycle to proceed. Therefore, during mitogen stimulation, Rb is initially inactivated by Cyclin D-CDK 4/6. In brief, the accumulation of Cyclin D-CDK 4/6 results in partial Rb inactivation and E2F release (Reviewed by Foijer and Te Riele, 2006). Thus, the induction of Cyclin E/Cdk 2 caused by E2F release further phosphorylates Rb to its hyperphosphorylated state, leading to the full release of E2F activity and beginning of S phase (reviewed in Foijer & Te Riele, 2006).

The cell cycle is controlled by a series of CKI

CKI, cyclin-dependent kinase inhibitors, also play important roles in cell cycle progression. CIP/KIP, Cdk-interacting protein/ kinase inhibitory protein, and INK4 are two major families of CKIs. The CIP/KIP family includes three family members, $p21^{CIP}$, $p27^{KIP1}$ and $p57^{KIP2}$. They are in charge of inhibiting Cyclin E/CDK2 and cyclin/CDK1 activity. For instance, $p27^{KIPI}$ plays a strong inhibitory role in Cyclin

E/CDK2 kinase activity when there is lack of growth factor. Furthermore, CIP/KIP family may be able to enhance Cyclin D-CDK4/6 association and activation as well (Chim et al., 2006; Reviewed by Foijer & Te Riele, 2006). Therefore, $p27^{KIP1}$ is able to prevent Rb inactivation and inhibit E2F factors (Reviewed by Foijer & Te Riele, 2006). Moreover, it has been reported that $p27^{KIP1}$ accumulates during quiescence and degrades when cells re-enter the cell cycle from the quiescent state (reviewed in Starostina and Kipreos, 2012). Another kinase inhibitor $p21^{CIP}$, which is transcriptionally regulated by p53 in response to DNA damage or cellular senescence, has been found to induce G1 and G2 cell cycle arrest in conditions of DNA damage (reviewed in Starostina and Kipreos, 2012; Dulic et al., 1998). Finally, $p57^{KIP2}$ plays a role in development, controlling cell cycle exit during embryogenesis (Matsuoka et al., 1995).

The INK4 family, including $p14^{ARF}$, $p15^{INK4B/MST2}$, $p16^{INK4A/MST1}$, and $p18^{INK4C}$, is involved in cyclin-CDKs regulation and specifically suppresses Cyclin D-cdk4/6 complexes (Grana and Reddy, 1995; Sherr and Roberts, 1995; Tvrdik et al., 2002). The loss of p16, a member of INK4 family, has been widely reported in several cancer studies, implying a pivotal point in tumor progression (Rocco and Sidransky, 2001). p16 controls cdk4 and cdk6 activity to prevent tumor progression (Rocco and Sidransky, 2001). Thus, elevated p16 levels inhibit Cyclin D-cdk4/6 complexes which are crucial for Rb phosphorylation, hence the halt of cell cycle progression.

Furthermore, cyclin D loss can increase p27 levels, contributing to Cyclin E/CDK2 inhibition and cell cycle arrest at G1.

The main difference between CIP/KIP family and INK4 is that the CIP/KIP family regulates all G1 CDK-Cyclin complexes while the INK family governs Cyclin D-cdk4/6 (Grana and Reddy, 1995; Sherr and Roberts, 1995; Tvrdik et al., 2002). Therefore, the progression order of events in cell cycle is crucial and fundamental for proper cell division, as miss expression leads to tumor formation or developmental defects.

1.2 Growth Arrest Specific (GAS) genes & p20K

Gas and Gadd genes

Two classes of genes named growth arrest specific (Gas) and growth-arrest and DNA-damage inducible (Gadd) genes are associated with cellular quiescence (Shugart et al., 1995). They are crucial in the processes of cell proliferation, gene expression, and cell death (Flemling et al., 1997). Additionally, GAS and Gadd genes are down-regulated in terminal differentiation or senescent cells, indicating that they are specific to quiescence cells, i.e. reversible growth arrest cells. (Fleming et al., 1997; Shugart et al., 1995).

By definition, growth arrest specific (GAS) genes are known to be expressed in

quiescent cells but not in cycling cells. Therefore, GAS genes are useful markers of growth arrest (Mao PL et al., 1993). To further understand the functions of gene expressed during growth arrest, 6 GAS genes (GAS 1-6) were identified in 1988 (Schneider et al., 1988). It was found that Gas genes were up-regulated in response to contact inhibition and serum starvation (Schneider et al., 1988). However, these GAS genes are not expressed during senescence, indicating that they are markers of reversible growth arrest (Cowled et al., 1994).

Of the six original GAS genes, the expression of GAS1 is regulated at the transcriptional level but others are regulated at the posttranscriptional level (Ciccarelli et al., 1990). GAS1, an integral plasma membrane protein containing two transmembrane domains, is able to induce growth arrest (Del Sal et al., 1992). Experiments performed by Del Sal et al. (1992) have proved that GAS1 expression inhibits DNA synthesis, and is able to make cells exit the cell cycle. Moreover, GAS1 caused growth arrest or apoptosis in a p53-dependent manner (Zamorano et al., 2003). These proved functions of GAS1 indicate that GAS1 is a tumour suppressor (Evdokiou et al., 1998). GAS2, a component of the microfilament system, is associated with actin (Brancolini et al., 1992). In addition, the half life of GAS2 is longer than other GAS proteins, and GAS2 is slowly degraded during G0-G1 transition (Brancolini et al., 1994). GAS3, a transmembrane glycoprotein containing three potential transmembrane domains, has been reported to be highly expressed in

myelin forming cells (Manfioletti et al., 1990; Spreyer et al., 1991). However, its expression is inhibited when cells begin to proliferate under nerve damage (Spreyer et al., 1991). In addition, overexpression of GAS3 can cause apoptosis (Fabrietti et al., 1995). Coccia et al. (1992) reported that GAS5 is expressed in G0 of Friend erytholeukemia cells, but not in cells starting to differentiate. GAS6, a ligand of Axl tyrosine kinase receptor, functions in cell proliferation (Goruppi., 1996). When it binds to Axl receptor, it is a survival signal in the serum starved NIH3T3 cell line (Goruppi, 1996). In addition, studies showed that GAS6 induced NF-κB transcription by the PI3K/Akt pathway. Thus, GAS6 induced survival is in a NF-κB dependent manner. (Goruppi et al., 1999; Demarchi et al., 2001).

DNA-damage inducible (Gadd) genes are also important in cellular quiescence regulation. The family of Gadd genes consists of five members, GADD34, GADD45α, GADD45β, GADD45γ, and GADD153, and they were first discovered in UV-treated Chinese-hamster ovary (CHO) cells (Fornace et al., 1989). These genes are induced by various cellular stress conditions such as nutrient deprivation, growth arrest and DNA damage (Carlson et al., 1993; Jackman et al., 1994; Fornace et al., 1989). Moreover, Gadd genes also regulate cell cycle arrest, apoptosis, and DNA repair (Saletta et al., 2011). Two Gadd genes, gadd153 (CHOP) and gadd45 gene, have been more intensively studied because they are expressed in almost every mammalian cell line (Zhan et al., 1994).

The expression levels of Gas and Gadd genes fluctuate in response to conditions of stress and growth arrest. The increase of CHOP mRNA levels, Gas2 and Gas5 levels is in response to the depletion of essential amino acids (Fleming et al., 1997). However, in low serum conditions, mRNA levels of Gas3 and Gas6 are induced (Fleming et al., 1997). Thus, different Gas and Gadd genes expression are induced according to different conditions of growth arrest.

p20K

p20K is a GAS protein that can be highly induced during cellular quiescence in response to hypoxic or contact inhibited conditions (Kim et al., 1999; B. Fielding, M.Sc., 2011). p20K was first discovered in chicken heart mesenchymal cells (Bédard et al., 1987). p20K is a lipid binding protein and member of the lipocalin family (Bédard et al., 1987). As a lipocalin protein, p20K has high affinity for long chain unsaturated fatty acids (Descalzi Cancedda et al., 1996) while as a quiescent specific protein, its expression is induced under conditions of high cell density (contact inhibition) and hypoxia (Fielding, MSc. Thesis, 2011; Kim et al., 1999). Although in CEF, the expression of p20K is induced in contact inhibition, it is repressed rapidly when cells are actively proliferating (Bédard et al., 1987). In addition, p20K expression is also inhibited in RSV transformed CEF, during growth factor stimulation, or addition of insulin (Bédard et al., 1980).

p20K is also named as the Extracellular Fatty Acid protein (Ex-FABP), a stress protein stimulated by inflammatory agents (Cancedda et al., 2002). When tissue remodeling actively occurs during chicken embryo development, Ex-FABP/p20K expression is induced (Cancedda et al., 1990). Studies also showed that this expression is elevated in differentiated chrondrocytes and in adult chicken cartilage of dyschondroplastic & osteoarthritic diseases (Cancedda et al., 1990; Cancedda et al., 2002). Moreover, Ex-FABP is reported to act as a survival protein, promoting cell survival in chondrocytes, myoblast, and cardiomyocytes, and its expression is fundamental to protect chondrocytes from cell death. (Gentili et al., 2005; Marco et al,. 2003). Consequently, Ex-FABP/p20K plays an important role in heart development, bone and muscle formation (Cancedda et al., 2002).

The transcriptional activation of p20K is regulated by a 48-bp promoter region referred to as the Quiescence Responsive Unit (QRU) (Gagliard et al., 2003). The transcription factor CCAAT enhancer binding protein β (C/EBPβ) has also been reported as an essential p20K regulator (Kim et al., 1999). The activation of QRU is in a C/EBPβ-dependent manner, occurring via the interaction of C/EBPβ with two specific regions in the QRU known as A and B (C/EBPβ binding sites). (Kim et al., 1999) (Figure 1.2).

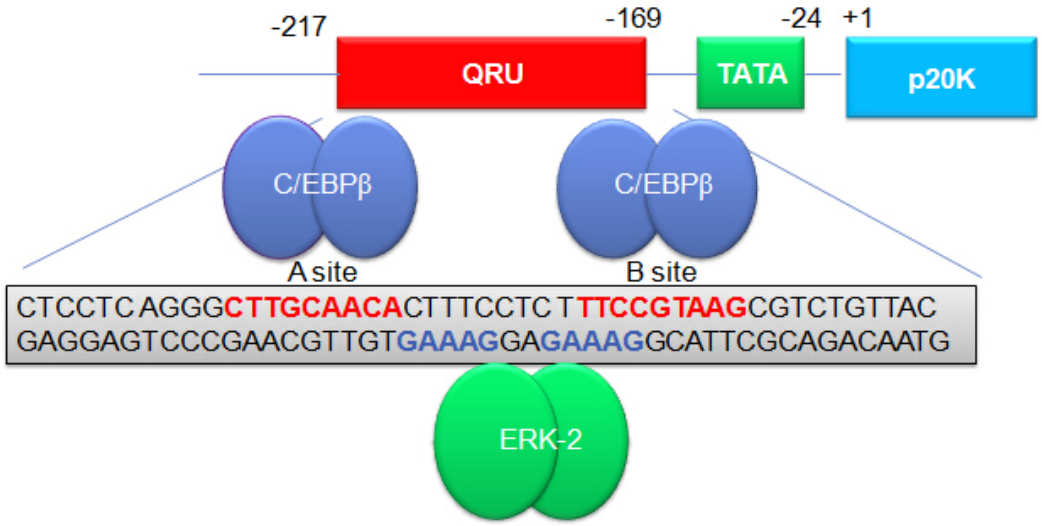


Figure 1.2 ERK-2 acts as a transcriptional repressor of p20K.

When C/EBPβ binds to the QRU of p20K, p20K gets expressed. Since the ERK-2 binding site (EBS, GAAAG) overlaps with C/EBPβ binding sites (A&B sites), the binding of ERK2 to the QRU in proliferative cells represses p20K expression in cycling CEF, however this binding wasn't observed in either growth arrested or hypoxic conditions. Thus, ERK2 acts as a transcriptional repressor of QRU, skewing C/EBPβ networks in proliferating cells (*Adapted from Athar, MSc. Thesis, 2011*).

1.3 C/EBP family and regulation of p20K by C/EBPβ

Structure & organization of C/EBPβ family

C/EBPβ is a member of the CCAAT enhancer binding protein (C/EBP) family (Grimm &Rosen, 2003). The transcriptional factor family C/EBP consists of 6 members termed C/EBP-α, -β, -γ, -δ, -ε, -ζ. The basic C/EBP family protein structure contains a transactivation domain (TAD), regulatory domain (RD), basic DNA binding domain (BDB) and a bZIP domain. The formation of dimers by one family member or with other family members via the bZIP domain determines the ability of C/EBP family to bind to DNA in a symmetrical DNA sequence of 5'-T T/G N N G N A A T/G-3', to induce transcription (Agre et al., 1989; Ryden et al., 1989; Osada et al., 1996).

C/EBPβ has three isoforms LAP*, LAP and LIP (Figure 1.3). They are produced by a leaky ribosome scanning mechanism, where alternative translational start sites are used (Sears & Sealy, 1994). Different isoforms occur by the alternative translational initiation at multiple AUG start sites (Xiong et al., 2001). The C/EBPβ mRNA contains four in-frame AUGs and an internal out-of frame AUG associated with a small open reading frame (sORF) which functions in the regulation of choice of initiation site (Xiong et al., 2001). For instance, sORFs may serve as a start site for the synthesis of oligopeptides which causes the leaky scanning of ribosomes, based on mediating some of the ribosomes to skip the first downstream AUG codon and

proceed to the next initiation site (Xiong et al., 2001). LAP*, so called LAP1 or C/EBPβ-1, liver enriching activating protein, is a 38 kDa protein and the full length isoform of C/EBPβ, while LAP (LAP2 or C/EBPβ-2) is a 35 kDa truncated version of the LAP* and differs from LAP* by 23 N-terminal amino acids. LIP, liver enriched inhibitory protein, is a 20 kDa polypeptide lacking a transactivation domain and acting as a dominant negative mutant (Descombes et al., 1990; Eaton et al., 2001). It can act as an inhibitor of LAP* and LAP. LAP* (or LAP1) is predominantly expressed in all normal cells as the additional N-terminal residues mediate the recruitment of chromation remodeling complexes (Kowenz-Leutz, E., & Leutz, A., 1999). While LAP* & LAP act as stimulators of C/EBP activity, LIP is a competitive inhibitor. Different expression profiles of LAP* and LAP demonstrate their different roles in gene activation and cell proliferation (Eaton et al., 2001; Kowenz-Leutz and Leutz, 1999). Previous studies have reported that only LAP was able to activate Cyclin D1 promoter, demonstrating that it is an important factor in cell growth promoter in mammary epithelial cells. It is also interesting to know that LAP* was the most distinctively expressed isoform in normal mammary epithelial cells while LAP expression was observed in breast cancer cell lines (Eaton et al., 2001). The expression ratio of LIP and LAP is controlled by translation initiation factors elF-2 and elF-4 (Calkhoven et al., 2000). When levels of elF-2 and elF-4 are high, LIP and LAP are predominantly expressed, however when elF-2 and elF-4 levels are low, LAP* are highly generated (Calkhoven et al., 2000). To sum up, preliminary studies

suggest that C/EBPβ isoforms have different functions and are regulated in different mechanisms.

Functions of C/EBPβ in differentiation

C/EBPβ regulates cellular differentiation. Former studies performed by Cortes-Centelli et al. (2002) showed that C/EBPβ promoted neuronal differentiation via activating C/EBPα gene. In addition, loss of C/EBPβ results in defects in mammary gland lobuloalveolar development (Grimm & Rosen, 2003). Finally, in tissues of adipocyte, keratinocyte, epidermal and ovarian origin, C/EBPβ is also associated with cell differentiation and its deletion can cause aberrant developmental effects (Cao et al., 1991; Zhu et al., 1999; Maytin & Habener, 1998; Sterneck et al., 1997).

Role of C/EBPβ in cell survival

C/EBPβ regulates cell proliferation in multiple tissues, hence its important function in cell fates. Studies performed by Gagliardi et al. (2003) reported that C/EBPβ could inhibit cellular proliferation. In CEF, over expression of a dominant negative C/EBPβ mutant could eliminate p20K induction under contact inhibition and stimulate cell proliferation (Gagliardi et al., 2003). Higher levels of Activator Protein -1 (AP-1) and AP-1 transcriptional activity were also observed in dominant negative C/EBPβ mutant cells compared to control cells (Gagliardi et al., 2003). Moreover, when

over-expressing wt C/EBPβ, levels of AP-1 was decreased, indicating that C/EBPβ negatively regulates AP-1 (Gagliardi et al., 2003). Interestingly, their studies found that when c-Jun, a component of AP-1, was over-expressed, cell proliferation was promoted even in dense cell conditions while p20K expression was inhibited (Gagliardi et al., 2003). To sum up, their findings show that C/EBPβ and AP-1 produce opposite effects on Gas gene regulation and cell proliferation (Gagliardi et al., 2003).

C/EBPβ also regulates apoptosis in multiple tissues. In fibroblasts, C/EBPβ protects cells from cell death caused by tumor necrosis factor-alpha (Ranjam & Boss, 2006). In mouse keratinocytes, C/EBPβ inhibits p53 protein levels to promote cell survival under carcinogenic stress (Yoon et al., 2007). Moreover, in C/EBPβ null uterine epithelial cells, increased apoptosis was observed, indicating that C/EBPβ promotes cell survival (Ramanthal et al., 2010). However, in neuronal cells, C/EBPβ promotes apoptosis via p53 polypeptide activation (Cortes-Canteli, 2002). Thus, C/EBPβ does not always promote survival indicating that its action is context specific. In CEF, the over expression of a dominant negative mutant of C/EBPβ enhanced survival and blocks the expression of the pro-apoptotic CHOP protein (Maynard et al., in preparation).

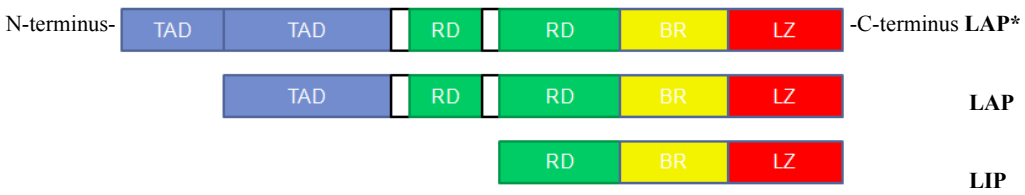


Figure 1.3 Schematic representation of C/EBPβ functional domains

C/EBP family members share a common bZIP structure. The full-length C/EBPβ (38kDa) LAP-1 structure contains the Transactivation Domain (TAD), Regulatory Domain (RD), Basic Region (BR) (DNA Binding Domain), and Leucine Zipper (LZ) (Dimerization Domain). Structures of the other two isoforms, LAP-2 & LIP, are also shown: Liver-enriched Activating Protein, LAP-2 (34-kDa) & Liver-enriched Inhibitory Protein, LIP (21-kDa). LAP-2 is a transcriptional activator containing an N-terminal truncated version of LAP-1. LIP is a transcriptional repressor lacking the Transactivation Domain. (*Adapted from Wethmar et al., 2010*).

1.4 Regulation of CHOP and its function on p20K induction

CHOP

C/EBPζ also known as CHOP10 or GADD153, plays a major role in p20K induction. Due to the fact that CHOP10 contains a glycine and proline residue substitution in its basic DBD, altering the α-helical structure of the basic DBD to with other family members, it is unable to form homodimers and must heterodimerize with other C/EBP proteins, specifically C/EBPβ (Sok et al., 1999) (Figure 1.4). This mutation also allows CHOP to bind to a modified C/EBPβ element (Ron and Habener, 1992; Ubeda et al., 1996). However, CHOP is identified as a C/EBP inhibitor due to the fact that its expression blocks C/EBP family member binding abilities to their consensus DNA target sequences (Ubeda & Habener, 2003).

CHOP is a transcription factor that is highly expressed in conditions of cellular stress (Zinszner et al., 1998). Under severe ER stress, CHOP is highly induced and promotes programmed cell death (Wang et al., 1996; Oyadomari & Mori, 2004). Genotoxic agents and UVA/UVB stress are also able to transcriptionally activate CHOP and promote its expression (Luethy & Holbrook, 1992). In addition, CHOP also plays an important role in apoptosis (Maytin et al., 2001). CHOP has a basic low expression level in normal cell proliferating, however the mechanism of its expression in normal cycling cells has not been well studied (Fawcett et al., 1996).

Previous studies on CHOP demonstrated that its repression in hypoxia allows C/EBPβ homodimer formation, enhancing DNA binding of C/EBPβ and induction of p20K (Fielding, M.Sc., 2011). Furthermore, the down-regulation of CHOP stimulates induced p20K expression level and increases cell proliferation under either hypoxic or contact inhibited condition, but has no effect in cycling cells (Camacho., M.Sc.,2013). Since CHOP is reported to be highly expressed in cellular stress condition (Zinszner et al., 1998), its participation in growth arrest or apoptosis induction in fibroblast cells during prolonged periods of Endoplasmic Reticulum (ER) stress or starvation has also been observed (Friedman., 1996). Although a low, basal CHOP expression level was observed in cycling CEF, this level reduced when cells became contact inhibited or treated in hypoxia (Camacho. MSc., 2013). ER stress inducer drugs Thapsigarin and Tunicamycin induced CHOP expression and inhibited p20K expression in contact inhibited CEF and hypoxic CEF, but not in cycling CEF where p20K was not expressed (Fawcett et al., 1996; Camacho., M.Sc., 2013). Furthermore, Co-Immunoprecipitation data showed that C/EBPβ-CHOP heterodimers accumulate under high ER stress, inhibiting p20K expression, and confirmed the basal level of C/EBPβ-CHOP heterodimers in cycling cells, suggesting that C/EBPβ-CHOP heterodimer accumulation represses p20K induction (Camacho., M.Sc., 2013). Finally, RT-qPCR shows the attenuation of CHOP mRNA expression in CEF in either hypoxic or contact inhibited condition, confirming CHOP's important role in p20K expression (Camacho., M.Sc., 2013).

Figure 1.4 Schematic representation of CHOP functional domains

CHOP (25kDa) is displayed with the Transactivation Domain (TAD), Regulatory Domain (RD), Basic Region (BR) (DNA Binding Domain), and Leucine Zipper (LZ) (Dimerization Domain). CHOP contains a conserved proline residue in its basic DNA binding domain which prevents it from homodimerizing. Thus, CHOP can only heterodimerize with other C/EBPβ family members. In addition, chicken CHOP has five leucine residues aligned on the same side of α-helix in its LZ domain, forming a typical structure of other leucine zipper transcription factors (*Adapted from Ghosh, MSc. Thesis. 2009*).

1.5 ERK family and its role as transcriptional repressor of p20K

ERK1/2 are activated by mitogenesis

Extracellular signal-regulated protein kinase 1/2 (ERK1/2) are mitogen-activated protein kinases (MAPK) involved in mediating cellular activities in response to extracellular signaling. ERK1 (a 44 kDa protein) and ERK2 (a 42 kDa protein) are homologous, sharing approximately 83% identity in amino acid sequence (Chen et al., 2001; Cargnello & Rous 2011). Under stimulation of growth factor, a mitogen-activated protein kinase kinase kinase (MAPKKK) Raf is activated, which subsequently phosphorylates and activates MEK1/2, a mitogen-activated protein kinase kinase (MAPKK) (Cargnello & Rous 2011). MEK1/2 then phosphorylates and activates ERK1/2 by phosphorylating a ThrGlu-Tyr motif within the ERK1/2 activation loop (Kolch, 2000; Cargnello & Rous 2011). Therefore MEK1/2 are dual specificity kinases.

In quiescence cells, ERK1 and ERK2 are localized at cytoplasm and associated with the cytoskeleton (Reviewed in Yu, 2012). However, after extracellular stimulation ERK1/2 will become activated, translocating to the nucleus and targeting many proteins, for example, the two components of activator protein 1 (AP-1), c-Fos and JunD, and the transcription factor Elk-1 (Chen et al., 1992; Gille et al., 1995). Elk-1 stimulates immediate early gene expression, including c-Fos. The phosphorylation of c-Fos by ERK1/2 can stabilize c-Fos and lead to its interaction with c-Jun, hence the

production of active AP-1 complexes (Gille et al., 1995; Whitmarsh &Davis, 1996). ERK1/2 activation via phosphorylation of threonine and tyrosine residues has an effect on the growth factor signaling cascades involving Ras, Raf, MEK and ERK1/2 activation and has functions in cell survival and proliferation (Hetman and Gozdz, 2004).

C/EBPβ is a target of activated ERK1/2. ERK1/2 regulates C/EBPβ activity via phosphorylation of a conserved threonine 189 site, located within the regulatory domain of murine C/EBPβ (Roy et al., 2005; Roy et al., 2002). C/EBPβ target genes could not be activated in C/EBPβ mutants that lacked an ERK1/2 phosphorylation site (Hu et al., 2011). ERK1/2 is acting as an important regulator of cell proliferation, and its activation is necessary for cell cycle progression and transit from G1 to S phase.

Functional differences between ERK1 and ERK2

ERK1 and ERK2 have specific functions in cell processes and brain function during development. ERK2 knockout is embryonically lethal, while ERK1 null mice are viable (Yao et al., 2003; Selcher et al., 2001), suggesting that ERK2 is essential for the physiologic roles during development. In addition, ERK2 plays essential roles in cell proliferation, while ERK1 functions as a negative regulator by antagonizing ERK2 during cell proliferation (Vantaggiato et al., 2006). ERK2 deficiency in humans correlates with microcephaly, impaired cognition, and delay of development (Samuels

et al., 2008). Although the mechanisms mediating ERK1/2 nuclear accumulation are unclear, a novel nuclear translocation sequence (NTS) located within the kinase insert domain was recently identified as a new mechanism for ERK1/2 nuclear translocation (Cargnelloaand Roux, 2011). More recently, the N-Terminal domain of ERK1 was reported to be responsible for the functional differences with ERK2 in cell proliferation (Marchi et al., 2008). It is suggested that the slower rate of ERK1 compared to ERK2 during shuttling between the cytoplasm and nucleus was caused by the N-terminal domain of ERK1, and this reduced rate of nuclear shuttling lead to a strong reduction of ERK1 ability in carrying proliferative signals to the nucleus (Marchi et al., 2008).

ERK-2 as a Transcriptional Repressor of p20K

Studies have demonstrated that ERK2 functions as a transcriptional repressor via directly binding to a DNA consensus sequence (G/CAAAG/C) (Hu et al., 2009). Roy et al. (2000) indicated that C/EBPβ correlated with a GATE element in the proximal promoters of IRF9, and activated its transcription via interferon gamma (IFNγ). Hu et al. (2009) showed that ERK2 is also involved in the regulation of interferon gamma-induced genes upon IFN-γ signaling response. Interestingly, ERK2 acts as a transcriptional repressor of GATE, binding to an overlapping site within the GATE element (Hu et al., 2009). Stimulation by IFNγ removes ERK-2 allowing C/EBPβ to bind to and activate genes harboring a GATE sequence. Consequently, activation of

the GATE-driven genes is regulated by the competitive binding of C/EBPβ & ERK-2 to GATE elements (Hu et al., 2009).

Previous studies performed by Athar (M.Sc., 2010) demonstrated that ERK2 acts as a transcriptional repressor of p20K. The p20K promoter region QRU has a similar consensus ERK2 binding sequence (GAAAGGAGAAAG). The ERK2 DNA binding sites (EBS) overlap the two C/EBPβ binding sites in the QRU. Thus, it is hypothesized that activation of p20K is regulated by the competitive binding of C/EBPβ & ERK-2 to the QRU promoter (Athar, MSc. Thesis, 2011). Former student Athar M. (M.Sc., 2010) observed that the binding of ERK2 to the QRU in proliferative cells represses p20K expression, however the binding wasn't observed in growth arrested conditions. Likewise, the loss of ERK2 binding to the QRU was correlated with p20K expression in hypoxia. Moreover, this interaction is specific to ERK2 and is not observed with the ERK1 kinase (Athar M.Sc., 2010). Thus, ERK2 acts as a transcriptional repressor of QRU, regulating the specificity of genes controlled by C/EBPβ in proliferating cells (Athar M.Sc., 2010).

1.6 Hypoxia & Quiescence

Quiescence induced by hypoxia

Cells can decrease their proliferation rate in response to the environmental stress caused by hypoxia. G1 arrest caused by hypoxia was due to the induction of a CDK

inhibitor - p27 in primary murine embryo fibroblasts (Gardner et al., 2001). Moreover, the hypophosphorylation of retinoblastoma protein plays a functional role in triggering hypoxia induced quiescence (Gardner et al., 2001). It is also proven that in tumor cells, hypoxia induces quiescence in order to compensate and increase survival in the stress environment caused by hypoxia (Gao et al., 2011). In addition, in cancer cells, quiescence caused by hypoxia functions helps cancer cells to evade the deleterious effects of radiotherapy and chemotherapy (Brown and Wilson., 2004). Previous study has shown that in non-transformed primary rat embryonic fibroblast cells (REFs), hypoxia could trigger quiescence while in oncogene-transformed REFs it would contribute to apoptosis as a result of enhanced acidosis. Consequently, not all transformed cells are able to survive in hypoxia (Schmaltz et al., 1998).

The quiescent state of stem cells can also be caused by hypoxia. For instance, Satellite cells, those skeletal muscle stem cells that typically in quiescence, only differentiate when muscle growth, exercise, or injury occurs (Hawke and Garry, 2011). In addition, hypoxia could block myogenic differentiation via activating Notch or P13K pathways (Liu et al., 2012; Gustafsson et al., 2005; Majmundar et al., 2012). In conditions of nutrient deprivation, hypoxia contributes to the quiescence of myoblasts (Liu et al., 2012). These studies suggest that there is a strong correlation between hypoxia and quiescence in maintaining the stem cell properties and increasing cell survival.

Functions and structures of HIF family

Hypoxia caused cellular response can be categorized into Hypoxia Inducible Factor (HIF) response and HIF independent response. For instance, the inhibition of kinase mammalian target of rapamycin (mTOR) pathways is in a HIF1 independent manner (Wouters and Koritzinsky, 2008). This inhibition in hypoxia relys on activating tuberous sclerosis protein TSC1-TSC2 complex in a AMP-activated protein kinase (AMPK) dependent method (Wouters and Koritzinsky, 2008). Moreover, the activation of unfolded protein response (UPR) pathway in hypoxia is via PKR-like ER kinase (PERK) (an ER stress sensor mechanism) activating, a HIF-independent manner (Wouters and Koritzinsky., 2008).

However, HIF-dependent response of cellular hypoxia is a common status during normal cell development and cancer, which induces HIF accumulation (Chi et al., 2006; Ercan et al., 2012). HIF functions as a regulator in tissue homeostasis during conditions of hypoxia (Ercan et al., 2012). Hypoxia inducible factor (HIF) family includes HIF1,2,3α and HIF1β. HIF1 is a heterodimer consisting of HIF1α and HIF1β (Semenza et al., 2010). The complex of HIF-1,2α and HIF1β is responsible for activating gene expression under hypoxia via binding to Hypoxic Responsive Element (HRE), the promoter region of their target genes (Pietras et al., 2011). In hypoxia, HIF family members regulate genes functioning in angiogenesis, cell proliferation and apoptosis (Rankin et al., 2008). HIF1α contains five domains, including a basic

helix-loop-helix domain (bHLH), PAS (PER-ARNT-SIM) domain, oxygen-dependent degradation domain (ODD), and two transactivation domains (Rankin et al., 2008; Figure 1.5). It is a prominent transcriptional regulator of hypoxia (Semenza., 2010). In normoxia, HIF1α is ubiquitously degraded. HIF is regulated by post-translational modification via hydroxylation of specific prolyl and asparaginyl residues in HIF-α subunits (Elvidge et al., 2006). Members of the 2-oxoglutarate (2-OG) dioxygenase family consisting of PHD family members (PHD1, PHD2 and PHD3) and FIH-1 target their residues (Elvidge et al., 2006). Under high oxygen conditions, HIF post-translational modification mechanisms are activated, mediating HIF binding to the Von Hippel-Lindau (VHL) E3 ubiquitin ligase complex and promoting its proteasomal degradation (Frais et al., 2009).

Control mechanisms of HIFα

The stability of HIFα plays an important role in its function. The two substrates, oxygen and α-ketoglutarate, are present and activate the enzyme prolylhydroxylase (PHD) in conditions of normoxia (Kaelin and Ratcliffe., 2008). The activation of PHD contributes to the hydroxylation of the two prolines, P402 and P564, in the ODD domain (Chowdhury et al., 2008). The von-Hippel-Lindau tumor suppressor (VHL)/E2 ubiquitin ligase complex then binds to the HIFα subunit via the hydroxylated sites (Kaelin and Ratcliffe., 2008). At this point the HIFα subunit is ubiquitinylated for degradation by the 26S proteasome (Chowdhury et al., 2008).

However, when the oxygen level is below 1.8%, the PHD will be inactivated and HIF1α is no longer hydroxylated, thus it is stabilized. In addition, mitochondria could produce a by-product of anaerobic respiration- the reactive oxygen species (ROS) in conditions of hypoxia. Previous studies have indicated that ROS can inhibit PHD activity, hence another mechanism of HIFα stabilization (Majmundar et al., 2010). Moreover, Patel and Simon (2008) also suggested that HIF1α was acutely stabilized while HIF2α was chronically stabilized during hypoxia. Another control mechanism of HIF1α activity is provided by Factor inhibiting HIF1α (FIH1) which contributes to HIF1α transcriptional activation (Lando et al., 2002). In normoxia, FIH1 can hydroxylate N803 in the C-TAD which inhibits the HIF1α from interacting with the co-activators p300 & CBP (Mahon et al., 2001).

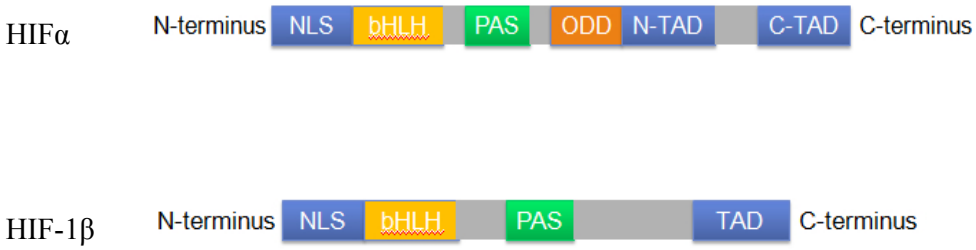


Figure 1.5 Protein structures of HIF family members.

The protein structure of HIFα members contains a nuclear localization signal (NLS), a basic helix-loop-helix (bHLH) domain, a PER-ARNT-SIM (PAS) domain, an oxygen-dependent degradation (ODD) domain and two transactivation domains (TAD). Dimerization occurs through the bHLH domain. HIFα hydroxylation by PHD occurs in the ODD domain. The protein structure of HIF-1β contains a NLS, a bHLH domain, a PAS domain and a TAD (*Adapted from Fielding, MSc. Thesis. 2011*).

1.7 p20K is induced in Hypoxia

Former microarray analyses verified that HIF1α was upregulated in growth arrest cells containing a hypoxic signature. Thus, HIF1α is the main factor of the HIF family that is in charge of hypoxic response (Ghosh, M.Sc., 2009). Preliminary data suggests that p20K expression is induced in condition of hypoxia (Fielding. MSc., 2011). In addition, recent data showed that hypoxia could trigger the decrease of CEF proliferation rate (Fox-chen. BSc., 2012). Moreover, studies performed by Fielding B, a previous M.Sc student in the lab, proved that the expression of HIF1α was not associated with the QRU induction of p20K, since the kinetics of HIF1α expression did not correlate with p20K expression either in hypoxia or in contact inhibition. Dimethyloxalylglycine (DMOG) is a prolyl-hydroxylase inhibitor that can mimick a hypoxia response via stabilizing Hypoxia Inducible Factor (HIF) under normoxia (Barnucz et al., 2013). DMOG can inhibit the PHDs in normoxia, leading to HIF stabilization and induction of hypoxia responsive genes (Barnucz et al., 2013). Previous studies by Ghosh (M.Sc., 2009) and Fielding (M.Sc., 2011) revealed that p20K is strongly induced by DMOG. These data demonstrate that hypoxia contributes to Gas genes induction, such as p20K, in quiescence.

1.8 Research Rationale:

Hypoxia may control the expression of p20K directly by acting on factors binding to the QRU or indirectly by promoting growth arrest. The objective of this thesis is to

investigate the mechanisms underlying the induction of p20K by low-oxygen concentrations and hypoxia-mimetic such as DMOG. The link between growth arrest and hypoxia or DMOG will be examined thoroughly. Particular attention will be devoted to the role of CHOP and ERK-2, two potential inhibitors in the control of p20K by hypoxia and DMOG.

Title of Thesis

Mechanism of activation of the quiescence-specific p20K gene

1.9 Hypothesis:

Growth arrest caused by hypoxia or hypoxia-mimetic such as DMOG promotes the activation of the growth-arrest specific p20K gene.

1.10 Main Objectives:

The main objective of my thesis is to study the mechanism responsible for the quiescence specific gene p20K induction in conditions of low-oxygen or hypoxia mimetic treatment.

In order to verify if p20K induction in hypoxia is caused by growth arrest induced by hypoxia, 10% Fetal Bovine Serum (10% FBS) was used to provide tissue culture media rich in growth factors for CEF. Western blotting was performed to further

determine effects of growth factors on p20K induction in conditions of contact inhibition and hypoxia, as well as the serum effects on kinetics of HIF-1α in CEF. In addition, p20K expression kinetics in CEF cultured in 5%CCS and 10%FBS subjected to hypoxia were further analyzed.

To further document the process of p20K expression in hypoxia, hypoxia-mimetic DMOG was used to study the mechanism of DMOG-caused p20K induction. Regulation of CHOP, Phospho-ERK and p20K and the interaction of CHOP and C/EBPβ in response to DMOG treatment were investigated. In addition, Chromatin Immunoprecipitation assays were employed to detect the association between ERK-2 and the QRU of p20K promoter in DMOG-treated CEF.

Chapter 2: Materials and Methods.

2.1 Cell Culture

Chicken Embryo Fibroblasts (CEFs) were cultured in Dulbecco's Modified Eagle Medium (DMEM) High Glucose (Gibco #11995). Only early passages (n<11) were used in experiments. Either 5% heat inactivated "cosmic" calf serum (Thermo Scientific HyClone Cat#30087.03) with 5% tryptose phosphate broth (Sigma, #T9157), or 10% heat inactivated Fetal Bovine Serum (Thermo Scientific HyClone Cat#30088.03IR) was used to supplement media along with 6 ml 1% L-Glutamine (Gibco Cat #25030), and 6 ml 1% Penicillin/Streptomycin (Gibco Cat#15140). CEFs were cultured at 41.5^{o}C, 5% CO_2 atmosphere with proper humidity. Cells were split 1:3 every 2-3 days with 0.05% Trypsin-EDTA (Gibco #25300), and seeded in 100 mm culture dishes (BD Falcon #353003).

2.2 Conditions of Cell Culture

2.2.1 Hypoxia & Contact Inhibition

For hypoxia treatment with cycling CEF, confluent cells were split 1:3 and incubated for 10 hours to allow re-attachment to the plate. In normoxia – the control group, CEF was incubated in standard incubator with 21% oxygen. In the hypoxia group, a hypoxia chamber was used and set to 1.8% O_2 for treatment. After 24 hours, cell lysates were collected. For contact inhibited cells, CEF reaching confluence and undergoing contact inhibition were fed the day before to limit the possibility that

quiescence would result from depletion of nutrients, after that lysates were collected. Cycling cells kept in a standard incubator with 21% oxygen were subjected to parallel conditions.

2.2.2 DMOG Treatment

A concentration of 1 mM Dimethyloxaloylglycine (DMOG) (0.01% DMOG) (Sigma #053M4756V) was added to the medium of cycling CEF, 0.01% Ethanol (ETOH) (P006EAAN Lot#15338) was used as parallel condition. CEF was then incubated in standard incubator with 21% oxygen. Lysates from ETOH & DMOG treated cells were collected 12, 18, 24, 30 and 36 hours after treatment. Prior to ETOH or DMOG treatment, CEF was split 1:3 and left for 8 hours to allow recovery.

2.3 Proliferation Assays

Cells, seeded at a concentration of 10,000 cells per well in quadruplicate in 24-well dishes (BD Falcon #353047), were cultured in condition of normoxia or hypoxia (1.8% O_2) and counted in IsoFlow^{TM} counting solution every 24 hours for 8 consecutive days using a Beckman Coulter Particle Counter.

Alternatively, cell counting was made in triplicate every time lysates were collected. Cells were seeded in 60 mm tissue culture plates (BD Falcon #353002), 24 hours later cells were trypsinized in 2 ml of 0.05% Trypsin. A mixture aliquot combined 500 µl

trypsin with floating cells was transferred and resuspended in 9.5 ml of IsoFlow™ Sheath Fluid counting solution (Fisher Scientific). Cell counting was made at different time-points using Beckman Coulter Particle Counter.

2.4 Western Blotting Analysis

2.4.1 Cell Lysate Collection

Cells were washed twice with 1X PBS pH7.4 (137 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 4.3 mM Na_2HPO_4). Lysates were collected in 1 mL of 1X PBS with a scraper and were centrifuged at 6500 rpm for 3 minutes at 4°C. The PBS was aspirated and discarded and CEFs were lysed with SDS sample buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol, 60 mM Tris pH6.8) with Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific # OG191369). Lysates were boiled for 3 minutes and centrifuged at 13000 rpm for 15 minutes at 4°C. Finally, supernatants were transferred to a new eppendorf tube and samples were stored at -80°C before further treatment. Bradford assay was performed to determine protein concentrations.

2.4.2 SDS-PAGE gel and Western Blotting

Western blotting analysis used 80 μg of protein lysates per lane. Lysates were run on a 12% SDS-polyacrylamide gel (SDS-PAGE) overnight (16 hours). Next day, proteins on the gel were transferred to a nitrocellulose membrane (Schleicher and Schuell, Bioscience) at 4°C for 3hours at 60 volts in 1X transfer buffer (300 ml 10X transfer

buffer, 600 ml methanol and 2100 ml ddH_2O, 4L 10X transferring buffer was made by 121.2 g Tris, 576.4 g Glycine solved in ddH_2O). After transfer, blocking buffer (1X Tris Buffered Saline (TBS) with 5% non-fat dry milk and 0.02% Na Azide) was used to block the membrane for one hour on a rotator at room temperature. The membrane was then incubated with primary antibody overnight at 4^oC with gentle rotation. Antibodies were diluted in 1X TBS with 5% non-fat milk powder. p44/42 MAPK (ERK1/2) was diluted in 1X TBS containing 5% BSA (Sgima #SLBH2718V) instead of milk powder. Specific dilutions of antibodies used are listed in Table 2.1. On the following day the membrane was washed 5 times after primary antibody incubation, once in TBS, twice in TBS-T (TBS + 0.1% Tween-20) and then twice again in TBS, each wash was for 5 minutes. The membrane was then incubated in secondary antibody for 2-2.5 hours with gentle shaking at room temperature. The secondary antibody (Anti-rabbit IgG HRP linked (Cell signaling Cat# 7074)) was diluted in 1x TBS to 1:25000 with 5% non-fat milk powder or BSA without Na Azide. Then 5 washes (once in 1x TBS, twice in TBS-T, twice in TBS) were repeated to get rid of unbound secondary antibodies. The immune-protein complex was visualized via chemiluminescent reaction (Luminata Forte Western HRP Substrate (Millipore # WBLUF0100)) and exposed to Amersham Hyperfilm ECL (GE Healthcare). Digital image was obtained via film scanning and ImageJ was used to quantify signal intensity. ERK-1 was used as control and all signals were corrected for loading.

Table 2.1- Western Blotting Antibodies

Primary Antibodies	Dilutions
ERK-1 (Santa Cruz Biotechnology Cat # sc-94)	1:2000
p20K (601-Y) (Bédard et al., 1987)	1:1000
CHOP (AB-3 Tulip)	1:1500
C/EBPβ (AB3-B5) (Gagliardi et al., 2001)	1:1500
Phospho-p44/42 MAPK (ERK1/2) (Cell Signaling Cat# 4370)	1:2000
HIF-1α (Fielding. MSc. 2011)	1:500
Secondary Antibody	
Anti-rabbit IgG HRP linked (Cell Signaling Cat# 7074)	1:25,000

2.5 Co-Immunoprecipitation

Cell medium was aspirated and cells were washed 3 times with 1X PBS. Cells were collected using 1 mL 1X PBS and pelleted by centrifugation at 6000 rpm at 4^{o}C for 3 minutes. Cells were resuspended and lysed in 1 mL TNE buffer (50 mM Tris HCl pH7.6, 150 mM NaCl, 1% Nonident P-40, 2 mM EDTA pH8, EGTA pH8.5, Halt^{TM} Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific # OG191369)). To ensure full lysis, lysates were placed on ice for 15 minutes before being centrifuged for 10 minutes at 13000rpm at 4^{o}C to remove insoluble debris. Bradford Assay was performed to determine protein concentration. 500 µg of protein aliquot was used per co-immunoprecipitation (IP). Either 2 µg of rabbit AB3 Pre-immune serum (control) or 2 µg of C/EBPβ AB3 serum was added to each IP. Protein samples were left overnight with gentle rotation at 4^{o}C. Protein G Sepharose Beads (GE Healthcare 17-0618-01) were washed and blocked overnight at 4^{o}C on a rotator. Next day, 60 µl of washed and blocked bead slurry (made in TNE buffer) was added into each sample

and incubated on a rotator at 4°C for 3 hours. Samples were pulse centrifuged for 25 seconds then the supernatant was carefully removed. 1 ml TNE buffer was used to wash the bead-antibody mixture for 5 times. Samples were vortexed for 15 seconds every time after TNE buffer was added to decrease unspecific binding. Then samples were pulse centrifuged for 25 seconds and the supernatant was aspirated. 60 µl of SDS sample buffer was used to resuspend beads. Beads were then quickly vortexed, heated for 3 minutes at 100°C and centrifuged at 13000 rpm for 15minutes at 4°C. Protein samples were stored at -80°C before Western blotting analysis. For Western blotting, a dilution of 1:500 of Clean blot IP detection reagent (Thermo Scientific #21230) in 1X TBS with 5% non-fat milk powder was used instead of a standard secondary antibody.

2.6 DNA Precipitation and Transfection

2.6.1 DNA precipitation

For each DNA precipitation, 10 µg of DNA construct supplemented with 20 µg of Salmon sperm carrier DNA was used for transfection of a 100 mm plate. After that, DNA was ethanol precipitated at -20°C. The following day, the sample was centrifuged at 13,000 rpm for 10 minutes at 4°C in order to recover precipitated DNA. Supernatant was aspirated and DNA pellet was rinsed with 70% ethanol, then the tube was centrifuged at 13,000rpm at 4°C for 5 minutes. After discarding the supernatant, DNA pellet was set 3 minutes on ice to be air dried. Then 200 µl of ddH_2O and 62 ul 2M $CaCl_2$ was used to resuspend DNA.

2.6.2 Calcium Phosphate Transfection

Cells were split 1:3 16 hours before transfection. The next day, the medium was aspirated and 6 ml of complete new medium was added per 100 mm tissue culture plate 4 hours before transfection. CEFs were about 50-70% confluent at the time of transfection. After DNA was precipitated and resuspended as described above, 500 µl 2x HBSP pH 7.12 (1.5 mM Na_2HPO_4, 10 mM KCl, 280 mM NaCl, 12 mM Glucose, and 50 mM Hepes) was slowly added to the DNA while vortexing. The DNA precipitate mix solution was incubated at room temperature for 20 minutes before adding to culture dishes. Cells were incubated with the DNA precipitate for 5 hours. Then the medium was gently discarded and cells were glycerol shocked (15% glycerol in HBSP buffer) for 90 seconds. Cells were washed twice with serum-free medium after incubation before adding 8 ml of complete medium.

RCAS vectors used for transfection experiments are RCAS(B) (empty vector, used as control) and RCASBP Δ184-C/EBPβ (C/EBPβ dominant negative mutant (Gagliardi et al., 2001)). RCAS is an acronym for Replication Competent ALV LTR with a Splice acceptor (Hughes, 2004). It is an avian RSV derived replication-competent retrovirus lacking a v-src gene (Hughes, 2004). RCASBP Δ184-C/EBPβ vector encodes a dominant negative mutant of C/EBPβ. The RCASBP vectors generate replication-competent viruses capable of infecting the entire cell population.

2.7 Chromatin Immuno-precipitation

2.7.1 Sample preparation for ChIP

CEFs were fixed in 1% formaldehyde for 10 min and 10x glycine (1.25M glycine) was used to quench excess formaldehyde. Then cells were washed twice with cold 1x PBS, and then collected via scraping in 1ml PBS per plate. Samples were centrifuged at 6500 rpm for 3 minutes at 4^{o}C and lysed in SDS lysis Buffer (1%SDS, 10 mM EDTA, 50mM Tris pH8.1, Halt protease and phosphatase inhibitor cocktail) Samples were stored at -80^{o}C before sonication.

2.7.2 Sonication, immunoprecipitation and DNA Purification

Samples were thawed on ice before being aliquoted for sonication. 400 µl of original lysates were used per immunoprecipitation (IP). The sonication conditions are as follows: 50% output, 20 second pulses of sonication/rest for 6 minutes per lysate on an inverted cup horn sonicator. After that, lysates were centrifuged at 13000 rpm for 3 minutes to remove insoluble debris. Then 60 µl of blocked Protein A beads (Millipore #16-757) was used to pre-clear each IP sample. Afterwards the beads were discarded and lysates were diluted to 1 ml using ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH8.1, 167 mM NaCl, Halt protease and phosphatase inhibitor cocktail). After this step 10 µl pre-immune 'input sample' was taken and stored at -20^{o}C untill next day. The remainder of lysate was divided into two immunoprecipitations: an IgG IP control and an experimental IP sample (500 µl for

each one). IgG for mouse serum (4ug, Sigma, Lot # SLBC6078V) and Mouse α-ERK2 (4ug, Millipore #05-157 clone 1B3B9) were used for precipitation. Then the IP's were diluted via ChIP dilution buffer to a final volume of 1 mL and incubated with antibodies overnight at 4^{o}C on a rotator. The following day 100 µl of blocked Protein A beads was added to each IP sample and incubated for 1 hour to pull down antibody complexes. Before the incubation, 2 µg of rabbit α-mouse IgG (Sigma, Lot # 013M4808V) was added to IP's to increase mouse antibody affinity for Protein A beads. After the incubation, samples were centrifuged to pull down the bound antibody-protein-DNA complexes. Then five washes, once in low salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), once in high salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH8.1, 500 mM NaCl), once in LiCl immune complex wash buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholic acid (sodium salt), 1 mM EDTA, 10 mM Tris-HCl pH8.1), twice in TE buffer (10 mM Tris-HCl, 1 mM EDTA pH8.1) were performed. Each wash was done for 5 minutes at 4^{o}C and 1 ml wash solution was used each time. Between washes, samples were centrifuged to pellet beads in order to proceed with the next wash. After washes, 100 µl elution buffer (0.1 M $NaHCO_3$, 0.005% SDS) was added to each IP to elute bound complexes at room temperature for 15 minutes. During this time, 200 µl of elution buffer was added to 'Input Samples' and incubated at room temperature as well. Then beads were centrifugated to be pelleted and the supernatant was transferred to a new eppendorf

tube. The elution step was performed twice and supernatant fractions were combined afterwards. These eluted samples and 'Input Samples' were de-crosslinked in dilution buffer overnight at 65^{o}C in a waterbath. The next day RNase A and Proteinase K was used to digest RNA and protein respectively. Then DNA was ethanol precipitated, pelleted and resuspended in 20 µl 1x TE buffer.

2.7.3 PCR Amplification

PCR reactions were performed to assess the immunoprecipitation of the QRU. ChIP purified DNA was used as template. The QRU Primers against the p20K promoter region were used to amplify any QRU genomic DNA pulled down in ChIP purified DNA. Additionally, p20K intron primers were conducted in parallel to test the purity of QRU specificity. Sequences of oligos are listed in Table 2.2. Two samples were made for each primer set: an IgG control, IgG for mouse serum, and an experimental IP, Mouse α-ERK2, both of them were done in duplicate, one for the p20K intron and the other for the QRU. GoTaq Green PCR mix (Promega #M712B) was used for PCR reactions. Every PCR reaction was composed of 12.5µl of 2x GoTaq Green PCR Mix, 2µl ChIP purified template DNA, and 1mM forward and reverse primers. Autoclaved ddH_2O was added as the last step to make a final volume of 25µl PCR reaction samples. Samples were subjected to amplification with the following PCR program: 5 min at 94^{o}C (30 sec at 94^{o}C, 30 sec at 55^{o}C, 35 sec at 72^{o}C) x15 cycles, the elongation time increases by 5 sec every cycle after the first 15 cycles to make a total of 40 cycles,

then 10 min at 72°C. Samples were cooled at 4°C after these steps until taken out of the PCR machine. Agarose gel electrophoresis was then performed and UV transilluminator was used for visualizing the PCR products.

Table 2.2 Primers used in PCR reaction

Region amplified	Forward Primer (5'-3')	Reverse Primer (5'-3')
QRU	CAT CCC CTC TTC ATTCTC CA	CAC TGC TAT TGT TGG CAT GG
p20K intron	TGG GGG GAA ATG AAT GCTA	AAA TTA CTC TGG GGG CTGA

Chapter 3: Results

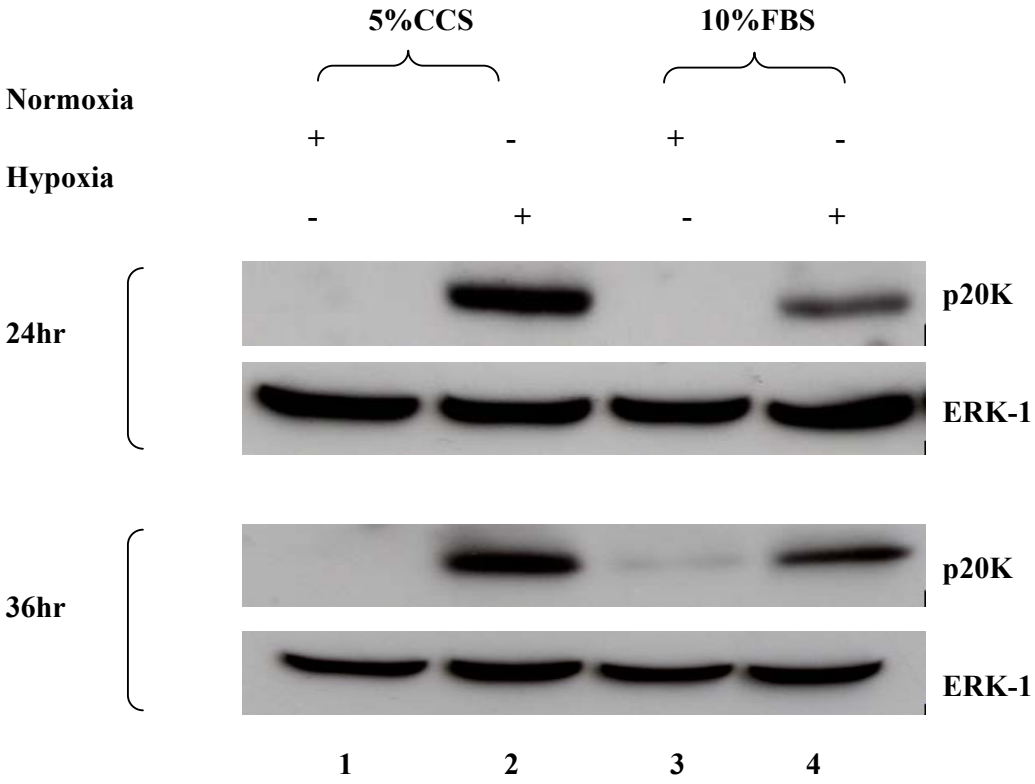
3.1 In hypoxic condition, p20K induction is determined by growth arrest caused by hypoxia

Previous research indicated that either contact inhibition or hypoxia could induce p20K expression (Fielding, MSc. Thesis, 2011; Kim et al., 1999). Severe hypoxic conditions (1% O_2) can cause cell cycle arrest (Goda et al., 2003). Moreover, CEF proliferation can be reduced markedly by mild hypoxia (1.8% O_2) (Garnett, BSc., 2012; Fox-chen, BSc., 2013). It was therefore important to determine whether p20K induction is caused by hypoxia directly or by the growth arrest caused by hypoxia. Since FBS contains more growth factors than CCS, it was used to create a condition that could delay cells reaching growth arrest by providing enhanced mitogenic stimulation.

3.1.1 Effects of growth factor on p20K induction in hypoxia

Cycling CEF cultured in 5%CCS or 10%FBS were incubated in normoxia (21% oxygen) or hypoxia (2% oxygen) for either a 24 hour or a 36 hour period. At the end of the incubation periods, CEF samples were collected, quantified with Bradford Assay, and analyzed by Western blot. The results (Figure 3.1A) indicate that in hypoxia, p20K induction is delayed when CEF cells are cultured in 10%FBS, compared with CEF cultured in 5%CCS, the induction level of p20K in CEF cultured in 10%FBS is much less after either a 24h period or a 36h period in hypoxia. Thus, these results suggest that growth factors delay p20K induction.

A.


5%CCS
10%FBS
Normoxia
+
-
+
-
Hypoxia
-
+
-
+
24hr
p20K
ERK-1
36hr
p20K
ERK-1
1
2
3
4

B.

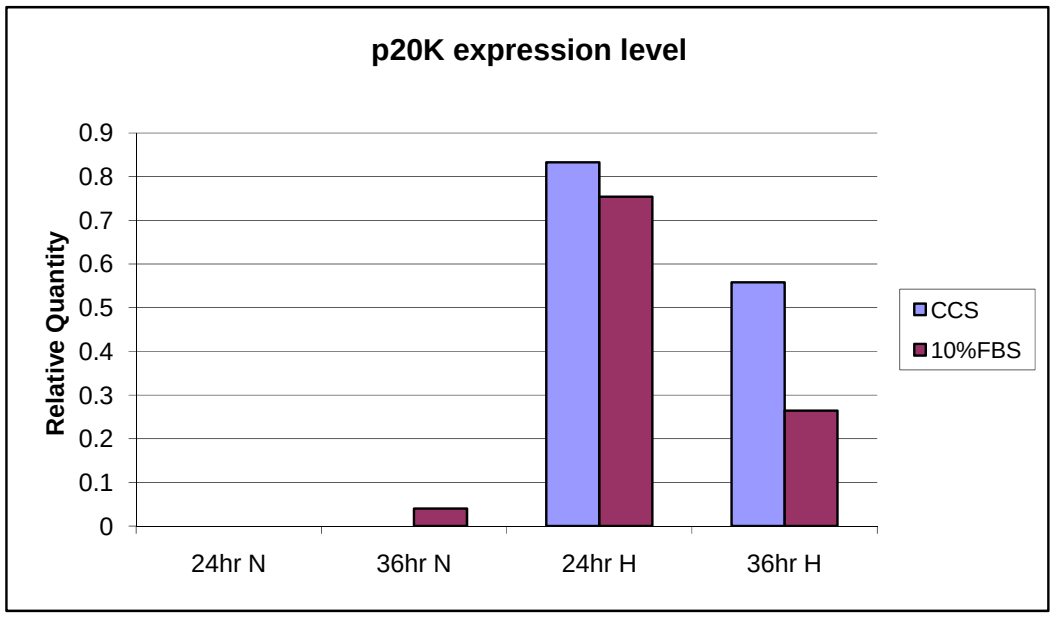

p20K expression level
Relative Quantity
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
24hr N
36hr N
24hr H
36hr H
CCS
10%FBS

Figure 3.1: Delay of p20K induction caused by 10%FBS in hypoxia.

Cycling CEF were cultured in either 5%CCS or 10%FBS and confluent CEF were split 12 hours before being transferred to hypoxia chamber. Lysates of CEF placed in either normoxic (21% O_2) or hypoxic (1.8% O_2) conditions were collected after 24 hours and 36 hours. Then SDS-Page gel and Western blotting were used to analyze those samples, ERK-1 was probed as loading control. This experiment was performed 3 times with same results.

A. Expression of p20K from CEF cultured 5%CCS or 10%FBS after 24 hour or 36 hour incubation in hypoxic or normoxic conditions. In hypoxia, P20K expression is less in 10%FBS group compared with 5%CCS group. After 36 hour incubation in hypoxia, p20K expression in 10%FBS group is induced compared with 24 hour group.

B. P20K expression is quantified relative to ERK-1 levels using ImageJ.

3.1.2 Effects of serum on p20K induction in conditions of contact inhibition & hypoxia

Hypoxia

The induction of p20K could be induced by hypoxic condition or contact inhibition (Fielding, MSc.,2011). Figure 3.1 suggests that growth factors contained in 10%FBS serum can delay p20K induction in hypoxia. Here further experiments are performed to determine the effects of variable concentration of serum on p20K induction.

Since Figure 3.1 shows that p20K induction in 10%FBS is more apparent compared with it in 5%CCS after a 36 hours incubation in hypoxia, CEF were cultured in 5%CCS, 2.5%, 5% and 10%FBS and treated by low-oxygen concentration (1.8% O_2) for 36 hours. p20K expression was corrected for protein loading using ERK-1 as control. In 10%FBS group, the p20K expression level is markedly lower than 5%CCS group (Figure 3.2), consistent with the idea that growth factors can delay p20K induction in hypoxia conditions.

Interestingly, this effect is not dose-dependent. In 5%FBS group, p20K has higher expression level than it in 2.5%FBS and 10% FBS groups. Studies have proved that p20K has high affinity for polyunsaturated fatty acids (such as linoleic acid) and promotes their uptake (Cancedda, et al., 1996; Kim et al., 1999). Kim et al has also demonstrated that p20K expression is controlled by an essential fatty acid (EFA),

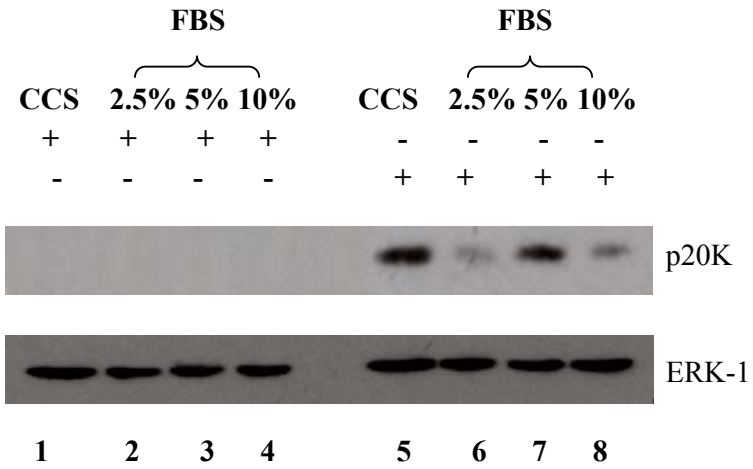
linoleic acid, via its binding to p20K. Thus, Figure 3.2 indicates that the components of serum, such as growth factor, essential fatty acids, and other possible factors, inhibit p20K induction.

Contact inhibition (C.I.)

Previous studies have indicated that GAS gene p20K expression can be induced by contact inhibition (Bédard, et al. 1987). Thus, effects of serum on p20K induction in condition of contact inhibition were further studied.

Cells were cultured in 2.5%FBS, 5%FBS, 10%FBS and 5%CCS as described before, lysates were collected one day after CEF became confluent to ensure contact inhibition condition in all the groups. Then SDS-PAGE gel and Western blotting were performed. The results from this experiment (Figure 3.3) indicate that in C.I. condition, p20K was approximately equally induced under 5%CCS, 5% and 10%FBS groups, while in 2.5%FBS group, p20K expression showed the lowest level, which may indicate that in 2.5%FBS group cells did not reach the same density and contact inhibition when growing in more limiting amounts of serum and essential fatty acids. To conclude, growth factors in 10% FBS could not overcome contact inhibition in confluent CEF, and when CEF become contact inhibited, p20K will be induced.

A.



B.

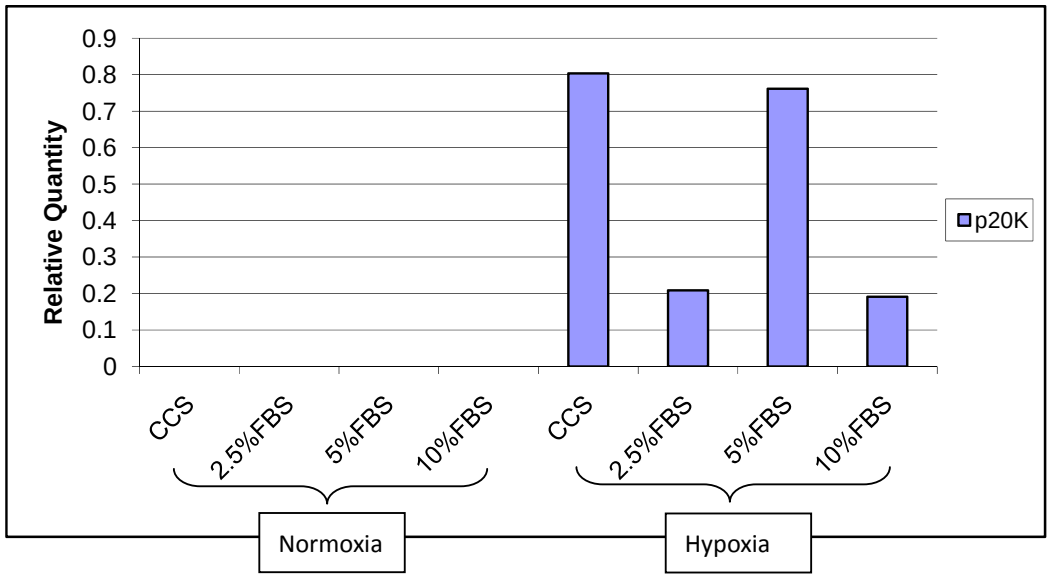


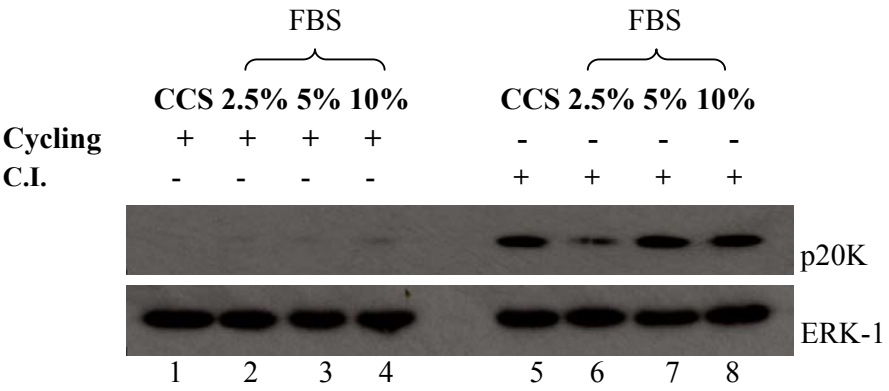
Figure 3.2: Expression of p20K under different concentration of growth factor in hypoxia.

Cycling CEF were cultured in 2.5%FBS, 5%FBS and 10%FBS and CCS was used as a control. Then CEF were placed in either normoxic (21% O_2) or hypoxic (1.8% O_2) conditions and lysate was collected after 36 hours.

A. Western blot analysis shows p20K expression level in CCS and FBS groups.

B. Quantification of p20K expression from western blot (Figure 3.1.2A). Levels of p20K were corrected to the level of ERK-1.

A.



B.

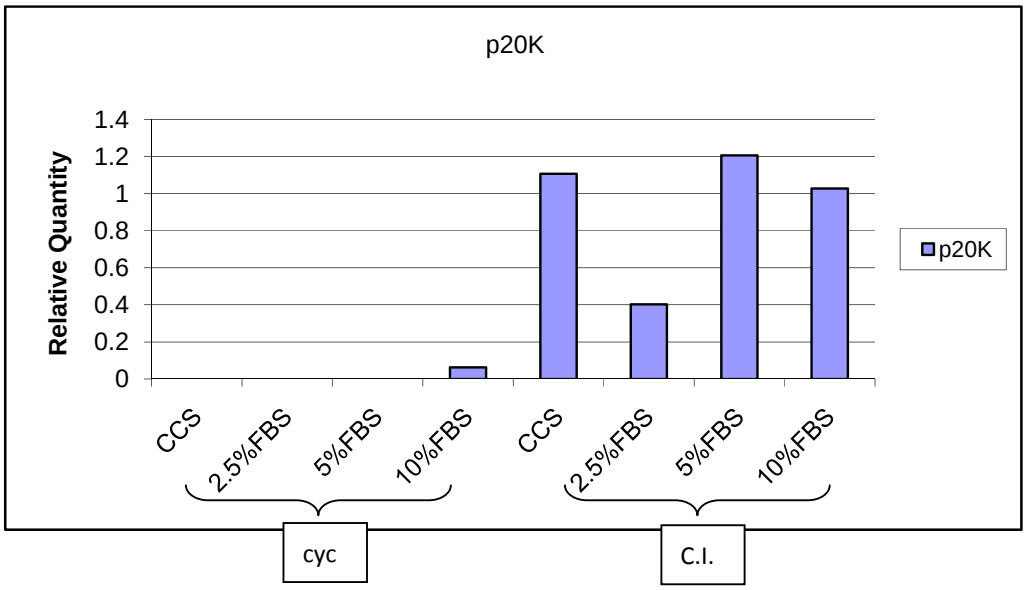


Figure 3.3: Expression of p20K under different concentration of growth factor in contact inhibition.

CEF were cultured in 2.5%FBS, 5%FBS and 10%FBS and CCS was used as a control. For each group, lysates from cycling and contact inhibited (C.I.) cells were collected.

A. Western blot analysis was performed to determine p20K expression level. Results show that except 2.5%FBS group, p20K induction in CEF reached approximately the same level after contact inhibition.

B. p20K expressions were quantified relative to ERK-1 levels through ImageJ.

3.1.3 Effects of serum on the kinetics of p20K expression in hypoxia

Since experiments described above show that growth factor can delay p20K induction under hypoxic condition, it was of interest to determine whether growth factor could change p20K expression kinetics. In order to provide an environment rich in grow factors, 10%FBS was used in these experiments. Also, previous studies by Athar (M.Sc., 2011) demonstrated that ERK2 acts as a transcriptional repressor of the p20K QRU. Results revealed that ERK2 is recruited to the QRU and represses p20K in cycling cells, but not in growth arrest conditions or hypoxia (Athar, M.Sc., 2011). Thus, phospho-ERK level was investigated in the following experiments in order to further analyse possible mechanism of p20K induction.

CHOP repression in hypoxia allows C/EBPβ homodimer formation, promoting the binding of C/EBPβ on the QRU, thus p20K expression is induced (Fielding, M.Sc., 2011). In conditions of either hypoxia or contact inhibition, when CHOP is down-regulated, the induction of p20K expression is stimulated to be super-induced and cell proliferation is increased (Camacho., M.Sc.,2013). In addition, the accumulation of C/EBPβ-CHOP heterodimers represses p20K expression during starvation and normoxia cycling CEF (Camacho, M.Sc., 2013). Moreover, according to the results of qRT-PCR, CHOP mRNA expression is attenuated in CEF in either hypoxic or contact inhibited condition (Camacho., M.Sc., 2013). According to these results, it is of interest to further study the function of CHOP on the kinetics of p20K

expression.

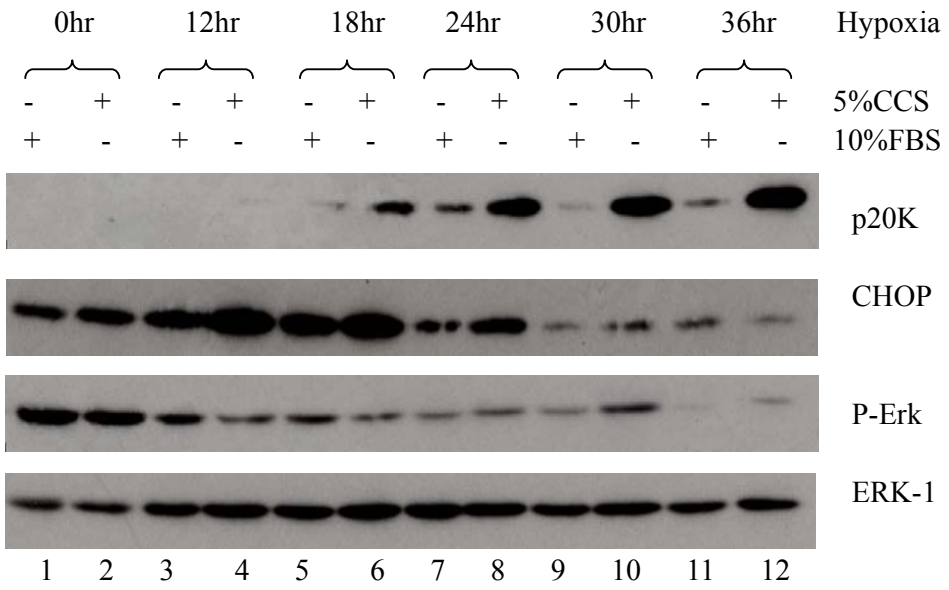
Cycling CEF (cultured in 5%CCS or 10%FBS) were placed in hypoxia (1.8% oxygen). Cell lysates were collected from hypoxic cells after 0, 12, 18, 24, 30 and 36 hours and cell number was counted each time a cell lysate was made. Triplicate cell counts were made for every sample. Lysate samples were run on a SDS-Page gel and analyzed via Western blotting.

As shown in Figure 3.4, p20K was induced after 18hr in hypoxia in CCS while in FBS, its expression was delayed and began to show after 24hr post-treatment. Meanwhile, p-ERK level was markedly decreased after 12hr treatment in hypoxia and could barely be observed at 36hr post-treatment in both CCS and FBS, while CHOP expression remained at a high level until 24hr post-treatment. These results suggest that the induction of p20K correlates best with the decrease in p-ERK level but not the decrease in CHOP levels in both FBS and CCS. This conclusion is consistent with the preliminary data showing that when CHOP was inhibited by shRNAi, p20K induction was increased only after 24hr hypoxia treatment, there was no p20K expression observed when the cells were in normoxia or early hypoxia while when CHOP was over-expressed, p20K induction was inhibited in ER stress and hypoxia, but no p20K expression was observed in cycling CEF in normoxia condition (Camacho, MSc., 2013). Interestingly, there was a significant difference in p20K expression level

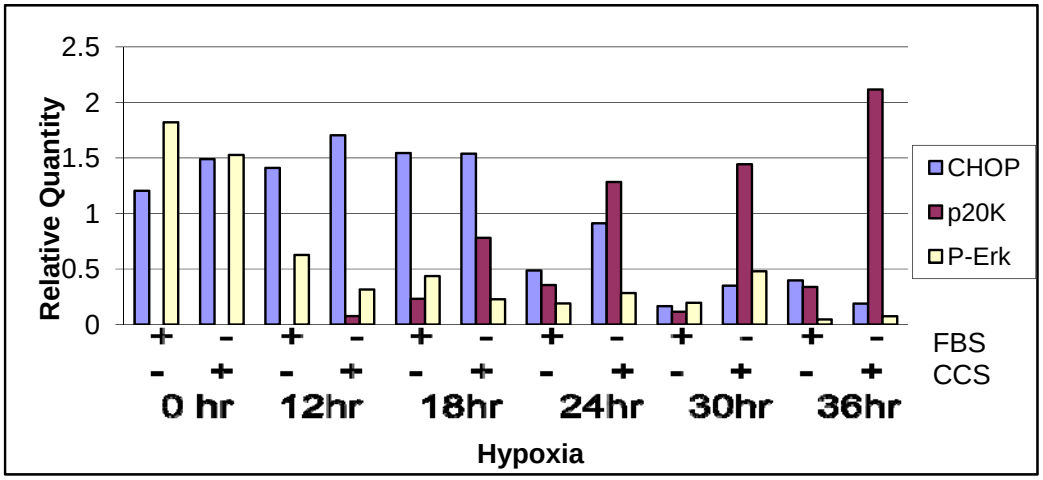
between CCS group and FBS group, this difference began from 24hr and increased with the time of hypoxia treatment on CEF. This result confirms that FBS significantly inhibits p20K induction in hypoxia.

Figure 3.4C suggests that compared with CEF cultured in CCS, cells in FBS began to show better growth after 12hr incubation in hypoxia. Thus, FBS treated cells has more proliferation and less p20K expression levels than CCS group in hypoxic condition. Moreover, the cell proliferation rate in FBS was greater than in CCS. CEF proliferation rate in CCS almost reached a plateau after 24hr hypoxia treatment while cell proliferation rate in FBS continued to increase. This suggests that mitogenic factors contained in FBS can promote cell proliferation rate and inhibit growth arrest in hypoxia. In addition, the cell proliferation rate decreased with time in both CCS and FBS. Compared with Figure 3.4A, decreased activation of ERK1/2 is associated with the decrease in cell proliferation rate, which is consistent with the fact that ERK is an important regulator of cell proliferation and its activation is required for G1 to S phase transition (Cargnello & Rous, 2011).

A.


0hr
12hr
18hr
24hr
30hr
36hr
Hypoxia
5%CCS
10%FBS
p20K
CHOP
P-Erk
ERK-1
1 2 3 4 5 6 7 8 9 10 11 12

B.


Relative Quantity
2.5
2
1.5
1
0.5
0
CHOP
p20K
P-Erk
FBS
CCS
0 hr
12hr
18hr
24hr
30hr
36hr
Hypoxia

C.

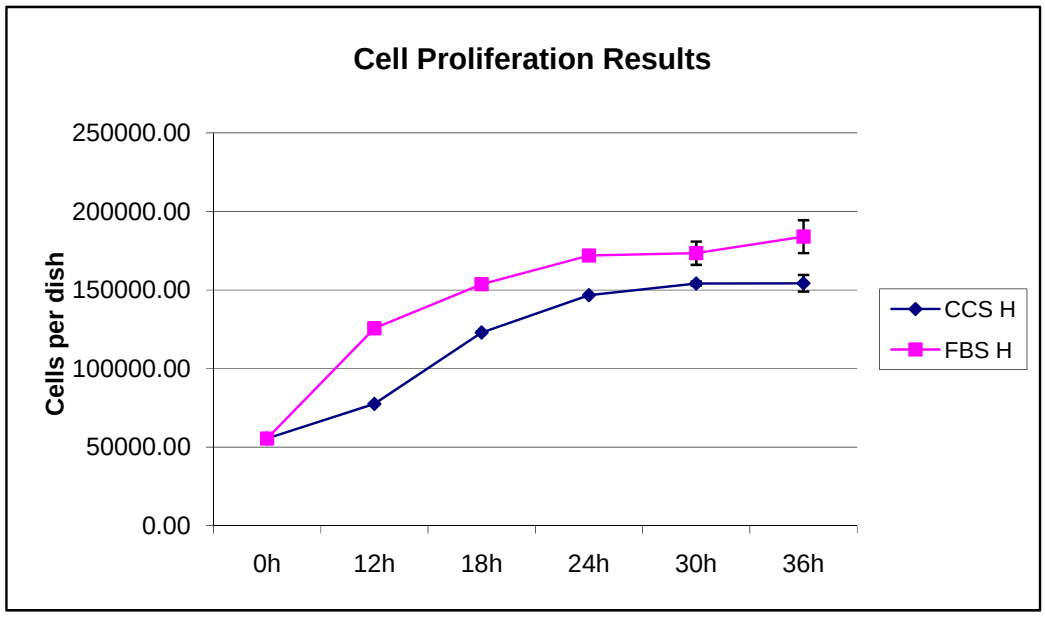


Figure 3.4: Kinetics of p20K expression in hypoxia under two kinds of conditions: CCS and FBS.

CEF were cultured in 5%CCS until confluent, cells were then trypsinized and centrifuged at 1000rpm for 5 minutes. Half of the pelleted CEF were resuspended in 5%CCS while the other part of cells were resuspended in 10%FBS and seeded into 60 mm tissue culture plates, incubated for 18 hours to recover. Cycling CEF (cultured in 5%CCS or 10%FBS) were placed in hypoxia (1.8% oxygen). Cell lysates were collected after 0, 12, 18, 24, 30 and 36 hours. Cell numbers were recorded at every time point when lysate was collected. Three counts were made for every sample. Lysates were run on a SDS-Page and analyzed via Western blotting.

A. Expression of p20K, CHOP, P-Erk and ERK-1 in hypoxia.

B. p20K, CHOP and P-Erk levels quantified relative to ERK-1 levels using ImageJ.

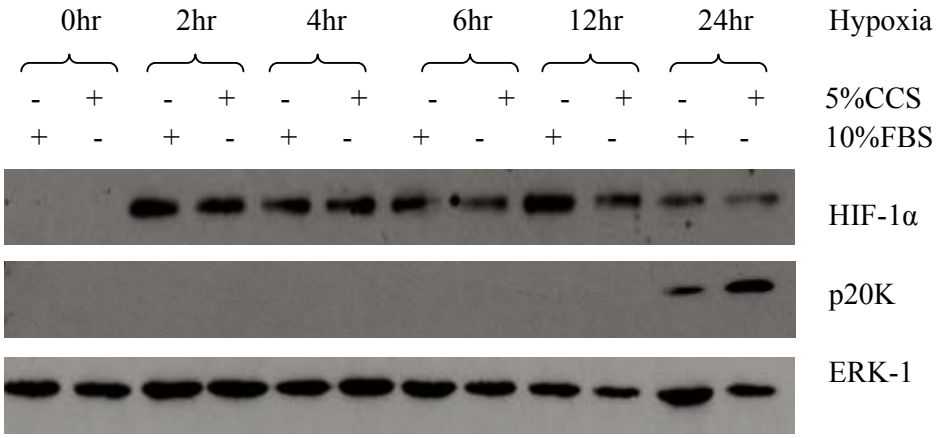
C. Proliferation assay of CEF in hypoxia under either 5%CCS or 10%FBS condition. The cell counts differences between the two groups are statistically significant at 12, 18, 24, 30, 36 hour time-points in an unpaired t-test ($p<0.05$).

3.1.4 Serum effects on kinetics of HIFα in CEF

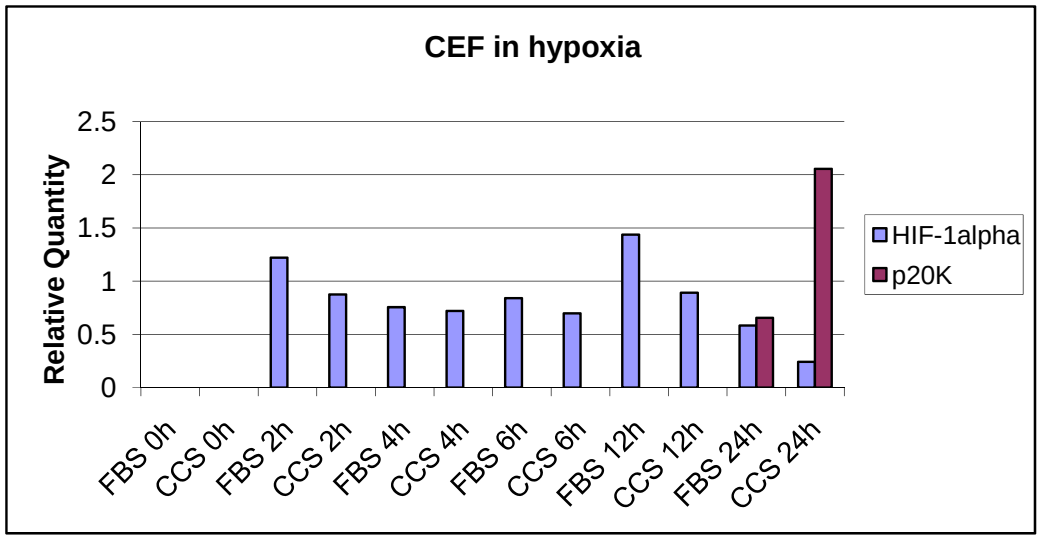
To determine if the response of CEF to hypoxia is modulated by serum factors, we also looked at the accumulation of HIF-1α, a well established early marker of hypoxia (Semenza et al., 2010; Fielding. MSc., 2011). CEF were cultured and treated as described before. As shown in Figure 3.5, HIF-1α accumulated rapidly (within 2 hours) at a comparable level in CEF cultured in CCS & FBS, indicating that serum factors did not alter the response to hypoxia.

As shown in Figure 3.5, HIF-1α was highly induced within 2 hours in hypoxia but diminished overtime. Its expression was observed in both CCS and FBS group from 2hr to 24hr post-treatment. p20K induction only appeared after 24hr in both 5%CCS and 10%FBS group, with a markedly lower level in FBS group. These results suggest that FBS does not change the response of CEF to hypoxia when it inhibits p20K induction. Interestingly, the FBS treated cells began to show higher HIF-1α level after 6hr hypoxic treatment compared with the CCS treated cells, but less p20K induction. As observed before, proliferation assay showed that although cell growth was repressed by hypoxia, FBS treated cells proliferated more than CCS treated cells, indicating that FBS reduced growth arrest and promoted proliferation. Accordingly, in hypoxic condition, p20K expression is not induced by hypoxia but by growth arrest caused by hypoxia.

A.


0hr
2hr
4hr
6hr
12hr
24hr
Hypoxia
- + - + - + - + - + - +
5%CCS
+ - + - + - + - + - + -
10%FBS
HIF-1α
p20K
ERK-1

B.


CEF in hypoxia
Relative Quantity
2.5
2
1.5
1
0.5
0
FBS 0h
CCS 0h
FBS 2h
CCS 2h
FBS 4h
CCS 4h
FBS 6h
CCS 6h
FBS 12h
CCS 12h
FBS 24h
CCS 24h
HIF-1alpha
p20K

C.

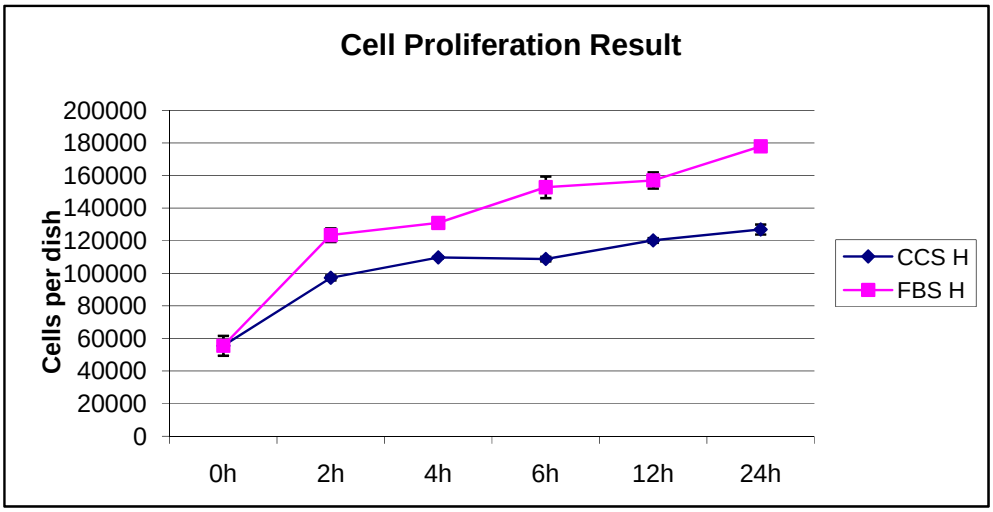


Figure 3.5: Expression of HIF-1α in hypoxia under CCS and FBS conditions.

CEF were cultured, trypsinized, resuspened, seeded and treated as described in Figure 3.4. Cell lysates were collected from CEF after 0, 2, 4, 6, 12 and 24 hours hypoxic treatment. Cell numbers were recorded at every time point when lysate was collected. Three counts were made for every sample. Samples were run on a SDS-Page and analyzed through Western blotting. This experiment was performed twice with same results.

A. Expression of HIF-1α, p20K, CHOP and ERK-1in CCS and FBS group.
B. HIF-1α, p20K and CHOP levels quantified relative to ERK-1 levels using ImageJ.
C. Proliferation assay of CEF in either 5%CCS or 10%FBS condition.

3.2 The mechanism of DMOG induced p20K induction

Previous studies performed by MSc. Students R. Ghosh (2009) and B. Fielding (2011) showed that p20K expression can also be induced by dimethyloxalylglycine (DMOG), which is a prolyl-hydroxylase inhibitor that mimics hypoxia response (Barnucz et al., 2013). Recent studies proved that p20K induction by DMOG is C/EBPβ dependent (Camacho, M.Sc., 2013). Since DMOG mimics hypoxic condition, it is of interest to verify the mechanisms of p20K regulation in DMOG treated CEF.

3.2.1 Regulation of CHOP, phospho-ERK and p20K in response to DMOG

Previous research by Fielding B. (MSc., 2011) demonstrated that over-expression of CHOP leads to the repression of p20K, and Camacho D. (M.Sc., 2013) showed that down-regulation of CHOP enhances p20K expression at contact inhibition, confirming the role of CHOP as a regulator of p20K. ERK-2 also functions as a transcriptional repressor of p20K via binding to the QRU during proliferative states directly, but this binding was not observed during growth arrest or hypoxic condition, where p20K is induced (Athar, M.Sc., 2011). Studies in 3.1 further confirm that hypoxia induced p20K expression by causing growth arrest. Therefore, it is of interest to verify whether DMOG induced p20K expression by the same mechanism as hypoxia.

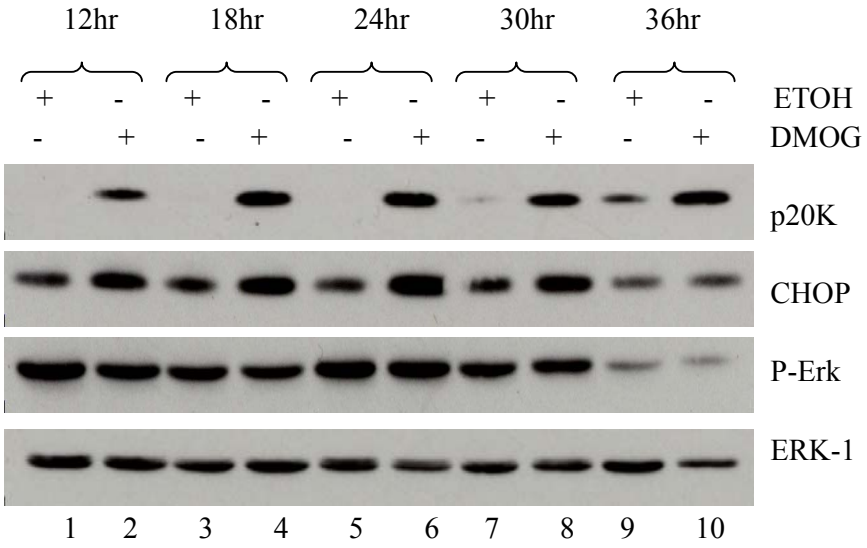
A concentration of 0.01% of Dimethyloxaloylglycine (DMOG) was added to the media of cycling CEF, and 0.01% of ethanol (ETOH, the solvent for DMOG) was

used as control. Lysates from ETOH and DMOG treated cells were collected at 12hr, 18hr, 24hr, 30hr and 36hr post-treatment. Levels of p20K, CHOP, phospho-ERK and ERK-1 were analyzed via Western blot. Cells were counted each time the lysates were collected. We found that the inhibition of prolyl-hydroxylases by DMOG induced p20K expression markedly at 12hr, 18hr, 24hr and 30hr when compared to cycling cells treated with ETOH (Figure 3.6A). CEF treated with ETOH began to express p20K at 30hr post-treatment. At 36hr, ETOH and DMOG treated CEF were more than confluent and reached contact inhibition, thus the expression of p20K in ETOH treated cells and a robust induction of p20K expression in DMOG treated cells. Interestingly, CHOP levels increased during DMOG treatment from 12hr to 30hr, in contrast to the effect observed in CEF placed in hypoxia (Figure 3.4). At the time point of 36hr, CHOP levels in ETOH group decreased, as well as the P-Erk levels, indicating that cells reached growth arrest. In the DMOG group, at 36hr time point CHOP level decreased by 50% compared to it at 30hr, and P-Erk level was barely detected. Cell counting showed that DMOG treatment reduced proliferation (Figure 3.6C). These results indicate that CHOP does not have determinant function in DMOG induced p20K expression. Since CHOP level increased in DMOG group (Figure 3.6A), it is of interest to verify whether the accumulation of CHOP-C/EBPβ heterodimer is caused by DMOG. Moreover, the competition of C/EBPβ and ERK-2 binding on the QRU controls p20K expression in normoxia and hypoxia, therefore it is important to further study whether the factor ERK-2 plays a major role in p20K induction when

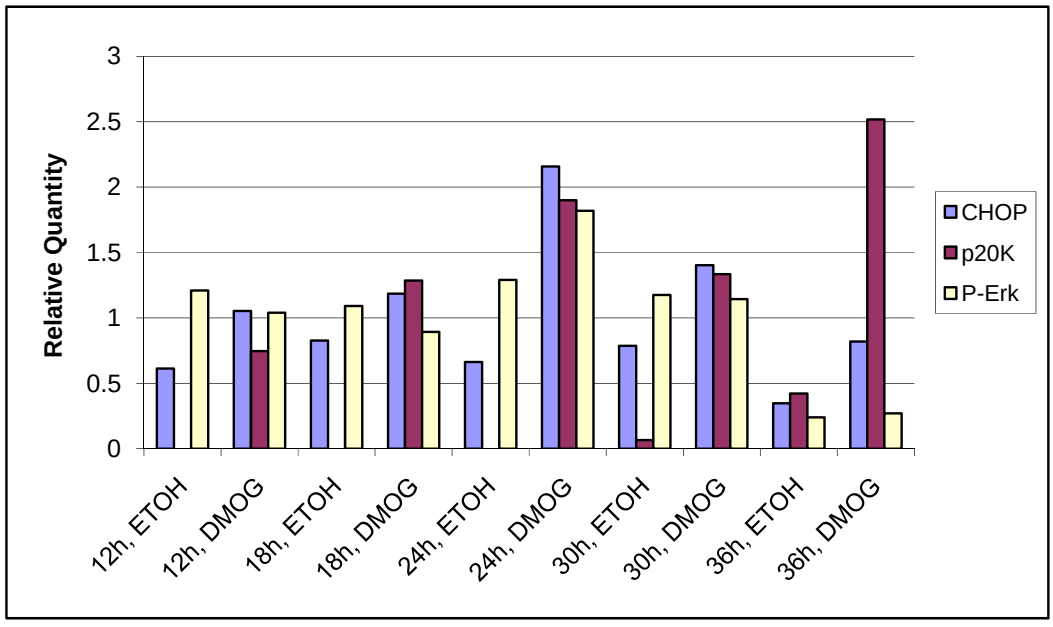
treating CEF with DMOG (Athar, MSc., 2011).

Cell counting in Figure 3.6C suggests that DMOG inhibits CEF proliferation. To further confirm this result, CEF were seeded at 10,000 cells per well in 24 well dishes. After 24 hours, CEF were treated with either 0.01% ETOH or 0.01% DMOG. CEF were then counted every 24 hours for six days in a Beckman Coulter Counter. Four replicates were counted for each sample. As shown in Figure 3.7, cell proliferation was impaired by treatment with DMOG. These results confirm that the prolyl-hydroxylase inhibitor DMOG markedly inhibits cell proliferation.

A.


12hr
18hr
24hr
30hr
36hr
+ - + - + - + - + -
ETOH
- + - + - + - + - +
DMOG
p20K
CHOP
P-Erk
ERK-1
1 2 3 4 5 6 7 8 9 10

B.


Relative Quantity
3
2.5
2
1.5
1
0.5
0
12h, ETOH
12h, DMOG
18h, ETOH
18h, DMOG
24h, ETOH
24h, DMOG
30h, ETOH
30h, DMOG
36h, ETOH
36h, DMOG
CHOP
p20K
P-Erk

C.

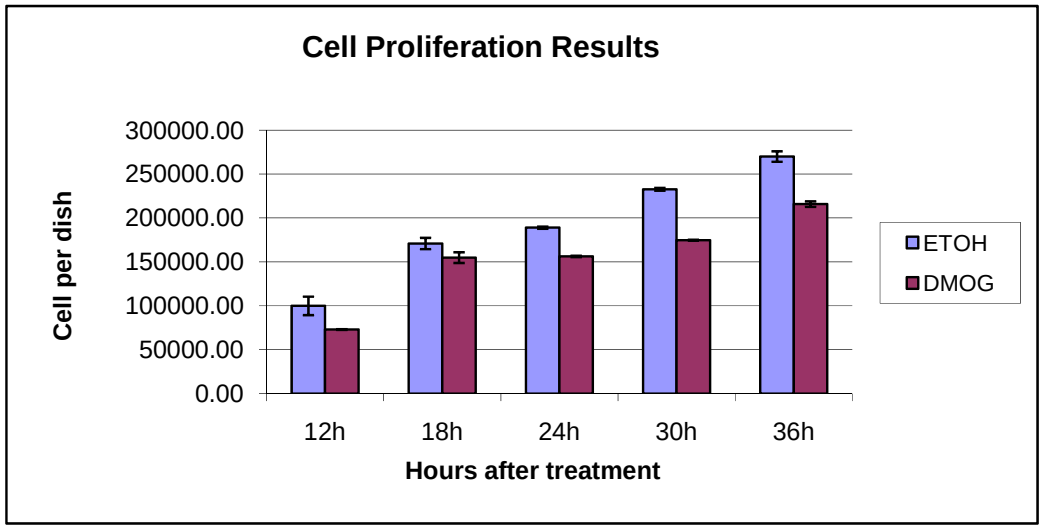


Figure 3.6: Expression Kinetics of p20K, CHOP and Phospho-specific ERK under dimethyloxaloglycine (DMOG) treatment.

Confluent CEF were split and incubated for 10 hours before treatment to allow recovery. Then they were treated with either 0.01% DMOG or 0.01% Ethanol (ETOH). Cell lysate was collected after 12, 18, 24, 30 and 36 hours treatment. Cell counting was performed each time lysates were collected. Three counts were made for each sample. Lysate samples were run on a SDS-Page gel and analyzed via Western blotting. This experiment was performed 3 times with same results.

A. Expression levels of p20K, CHOP and Phospho-specific ERK in CEF cells after being treated with ETOH or DMOG. CHOP and P-Erk expression increased after DMOG treatment, while p20K expression decreased.

B. Quantification of protein expression from Western blot Figure 3.2.1 A. Levels of p20K, Phospho-specific ERK and CHOP were calculated relative to the level of ERK-1 which was used as a loading control using ImageJ.

C. Proliferation assay of CEF in either ETOH or DMOG condition.

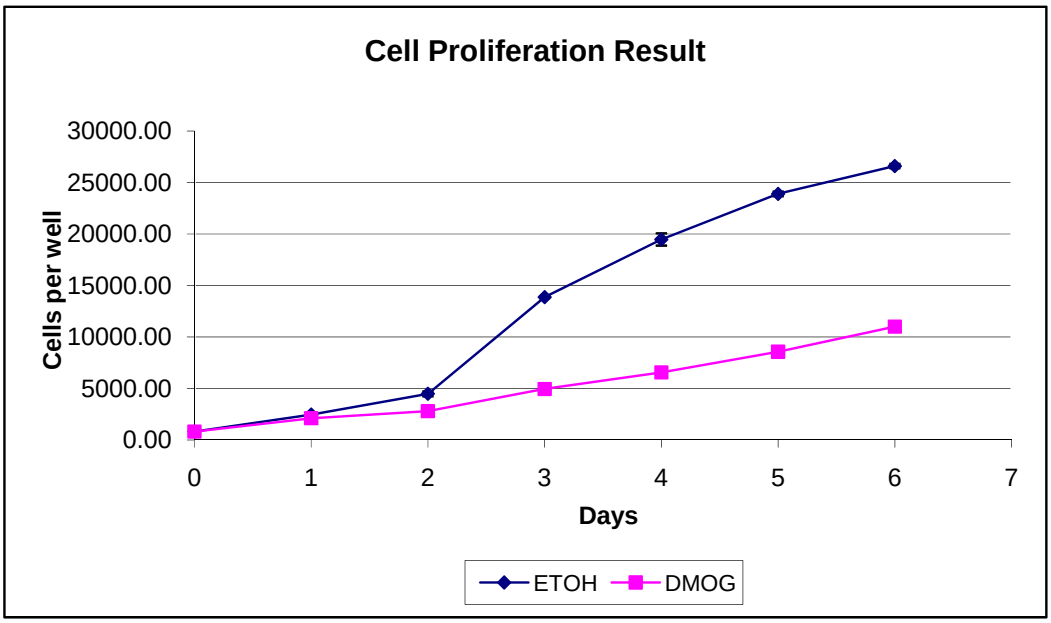


Figure 3.7: Proliferation Assay of CEF in ETOH and DMOG.

CEF were seeded 10,000 per well into 24 well dishes. Either 0.01% ETOH or 0.01% DMOG was added to cell media 24 hours later on Day 0. Cell counting was performed every 24 hours for 6 days and four counts were made for each sample. Results indicate that CEF in ETOH group show a greater proliferation than CEF in DMOG group and a marked difference of proliferation rate began to be observed 3 days post-treatment.

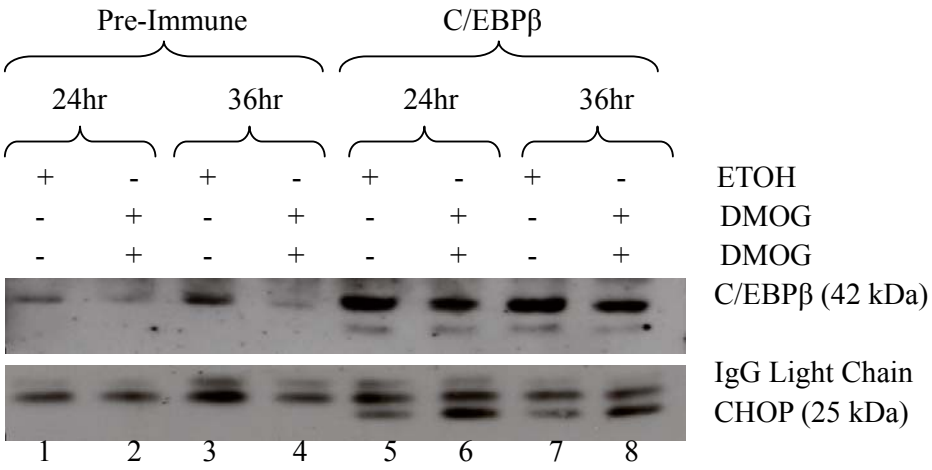
3.2.2 Interaction of CHOP and C/EBPβ in p20K induction during DMOG treatment

The induction of p20K by contact inhibition and hypoxia depends on the interaction of C/EBPβ with two separate elements of a promoter region known as the Quiescence Responsive Unit or QRU (Fielding, M.Sc Thesis; Kim et al., 1999). The induction of p20K is also characterized by a reduction in the expression of CHOP and its interaction with C/EBPβ when overexpressing CHOP functions as an inhibitor of p20K (Fielding MSc. 2012; Camacho, MSc. 2013). The following experiment seeked to determine whether DMOG inhibits the formation of CHOP-C/EBPβ dimers due to the fact that CHOP levels are not reduced until late time points after the addition time of DMOG treatment and the induction of p20K (Figure 3.6A). Accordingly, a series of co-immunoprecipitation assays were performed to examine the interaction of CHOP with C/EBPβ in DMOG-treated CEF.

Since CHOP was highly inducible after 24-hour DMOG treatment, CHOP levels were monitored after adding either 0.01% ETOH (control) or 0.01% DMOG to CEF media one day after passaging. Cell lysates were collected at 24-hour post treatment and 36-hour post treatment. A Bradford assay was performed to measure protein concentration and then 500 μg of protein was used for IPs. Once all cell lysates were made and immunoprecipitated, the IP samples were further analyzed via Western blot and probed for CHOP. CHOP expression levels were quantified relative to the C/EBPβ

bands in order to exclude loading errors. We found that when C/EBPβ was immunoprecipitated, the level of the CHOP band presenting the immuno-complex was greater in the DMOG group, indicating that DMOG treatment enhanced the accumulation of C/EBPβ-CHOP heterodimers (Figure 3.8). These results suggest that DMOG does not promote the formation of C/EBPβ homodimers by reducing the amount of CHOP interacting with this factor. This suggests that DMOG induces p20K expression independently of CHOP.

A.


Pre-Immune
C/EBPβ
24hr
36hr
24hr
36hr
+ - + - + - + -
- + - + - + - +
- + - + - + - +
ETOH
DMOG
DMOG
C/EBPβ (42 kDa)
IgG Light Chain
CHOP (25 kDa)
1 2 3 4 5 6 7 8

B.

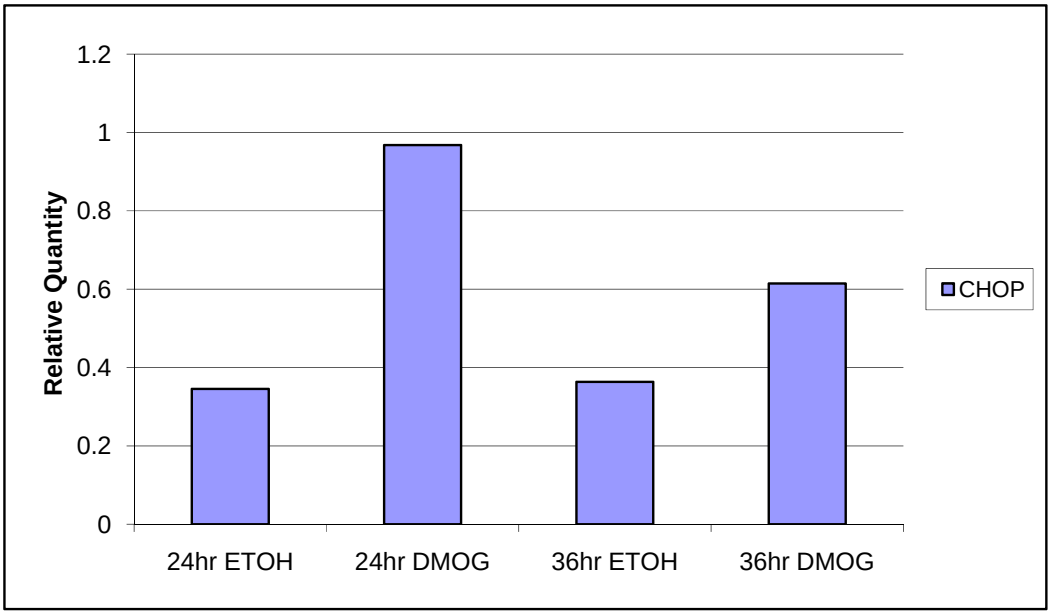

1.2
1
0.8
0.6
0.4
0.2
0
Relative Quantity
24hr ETOH
24hr DMOG
36hr ETOH
36hr DMOG
CHOP

Figure 3.8: Analysis of C/EBPβ CHOP Association in DMOG treatment by Co-Immunoprecipitation

Cycling CEFs were treated with either 0.01% ETOH or 0.01% DMOG. Lysates were collected after 24 hours and 36 hours and were analyzed with a CO-IP. Then samples were run on a SDS-Page gel and analyzed by Western Blot. CHOP level was quantified to the level of C/EBPβ via ImageJ.

A. Western Blotting shows that DMOG treatment group has more accumulation of C/EBPβ-CHOP heterodimers than ETOH group and this accumulation increased with time.

B. CHOP expression is quantified relative to C/EBPβ levels using ImageJ.

3.2.3 Association between ERK-2 and the QRU of p20K promoter in DMOG treated CEF

In this study, we examined the interaction of the ERK-2 transcriptional repressor on the p20K promoter after DMOG treatment. Chromatin Immunoprecipitation (ChIP) Analysis is a sensitive method for detecting the recruitment of transcription factors on promoters or DNA binding sites *in vivo*. Previous ChIP analysis has shown that ERK2 interacts with the QRU and acts as a transcriptional repressor when cells are in a proliferative state, but not when cells are growth arrested or in hypoxia condition. Moreover, this interaction is not observed with the related ERK1 kinase, indicating that it is specific to ERK2 (Athar., M.Sc., 2011). Since CHOP does not appear to be the target of DMOG (Figure 3.6), we examined the recruitment of ERK-2 by ChIP in response to the addition of this potent inducer of p20K. CEF was treated with either 0.01% ETOH (control) or 0.01% DMOG and ChIP was performed with IgG for mouse serum (IP negative control) and Mouse α-ERK2 antibody. Purified DNA samples were used as template DNA to be amplified using PCR reaction. Primers specific for the QRU were used to amplify the region of interest. Primers against a p20K intron sequence were used for parallel PCR reactions to assess QRU specificity and ensure that DNA was properly sonicated. These series of ChIP & PCR analyses helped us to assay the interactions between ERK-2 and the QRU after treating CEF with DMOG. The results are shown in Figure 3.9.

Figure 3.9 suggests that ERK2 immunoprecipitates with the QRU in ETOH-treated, control CEF for 12hr and 24hr, while it does not immunoprecipitate with the QRU when the cells were treated with DMOG. These results indicate that DMOG treatment blocks the recruitment of ERK-2 with the QRU, which explains the observation that although there is high level of phospho-ERK in DMOG treated CEF (Figure 3.6A), p20K is induced in response to DMOG. Whether or not activated phospho-ERK2 is localized in the nucleus in DMOG-treated CEF remains to be investigated.

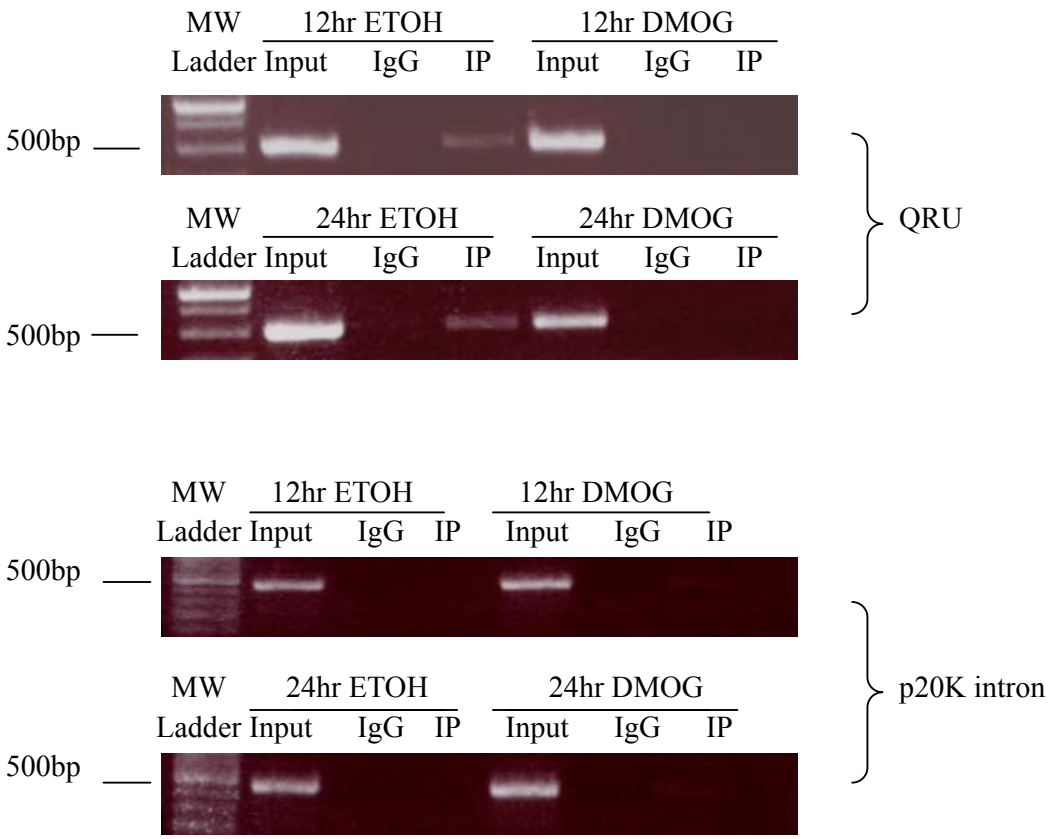


Figure 3.9: ERK2 binds to the QRU in ETOH but not DMOG treated CEF.

ChIP analysis of ETOH and DMOG treated CEFs. α-ERK2 monoclonal antibody was used to immunoprecipitate sample lysates. IgG was used as an IP negative control. Precipitated DNA was used in PCR reactions containing primers which amplify the QRU. Parallel PCR reactions with primers against a p20K intron were employed to ensure that the QRU was being specifically precipitated.

Chapter 4: Discussion

4.1 Hypoxia causes growth arrest & induces p20K expression: Modulation by serum factors.

p20K expression can be induced by either contact inhibition or hypoxia (Fielding, MSc. Thesis, 2011). Ghosh et al. (2009) performed microarray analysis of RNA from serum starved and contact inhibited CEF, revealing a hypoxia signature in contact inhibited but not starved CEF, which suggests that hypoxia could stimulate cells to exit the cell cycle and enter quiescence. Preliminary results confirmed that hypoxia inhibits CEF proliferation (Fox-chen. BSc., 2012). Therefore, it is important to determine whether p20K induction is caused by hypoxia or by the growth arrest caused by hypoxia. 10% FBS was used in this experiment to provide a rich source of growth factors in tissue culture media.

Figures 3.1A and 3.1B demonstrate that after treating CEF in hypoxia for 24hrs and 36hrs, the p20K level in 10% FBS group is markedly lower than it is in the 5% CCS group, almost 60% less after 24hrs and 30% less after 36hrs. This trend suggests that high concentrations of growth factors delay p20K expression in hypoxia. To further confirm this result, different concentrations of FBS were used to verify its function on p20K expression in hypoxia. In Figure 3.2, although the expression of p20K in hypoxia is not linear, p20K levels in 10% FBS group is lower than the CCS group after 36hrs at 1.8% oxygen. Figure 3.2B shows that the 10%FBS group has the lowest

p20K level. Moreover, CEF cultured in 10%FBS has a lower p20K level than in 5%CCS group at contact inhibition, indicating that the concentration of growth factors contained in 10%FBS is enough to attenuate p20K induction in either hypoxia or at contact inhibition (Figures 3.2B & 3.3B).

Further experiments were performed to document the function of growth factors on the kinetics of p20K expression. In Figure 3.4B, a striking reduction of p-Erk level is observed after 12hr treatment in hypoxia in both CCS and FBS group while little p20K induction was observed after 18hr in CCS and 24hr in FBS group. However the level of CHOP remains unchanged until 24hr of hypoxia treatment in both CCS and FBS group. These results suggest that p-Erk may be associated with p20K induction in hypoxia. Cell proliferation results (Figure 3.4C.) show that the proliferation rate of CEF in FBS group is greater than CCS in hypoxia, suggesting that growth factors in FBS increase cell proliferation in hypoxia. This may explain why p20K expression is attenuated in FBS.

Furthermore, in order to verify whether CEF in CCS and FBS respond differently to hypoxia, we examined HIF-1α expression in response to hypoxia in the two treatment conditions at various time-points. As shown in Figure 3.5A, HIF-1α expression is evident or detected after 2hr-hypoxia treatment in both CCF and FBS treated-group. After quantifying HIF-1α level for loading using ERK-1 as a control, Figure 3.5B

indicates that CEF in FBS group express more HIF-1α than in CCS group. p20K starts to show induction after 24hr hypoxia in both FBS and CCS group while its level is higher in CCS group, confirming the results in Figure 3.4. Proliferation assays in Figure 3.5C prove that CEF in FBS group have higher proliferation rate compared with CCS group, as observed in Figure 3.4C. Thus, CEF cultured in 5%CCS and 10%FBS have the same response to hypoxia. Since HIF-1α expression does not correlate with p20K induction (Fielding, MSc., 2011), the induction of p20K in hypoxia is not caused by hypoxia itself, but by growth arrest caused by hypoxia.

4.2 ERK-2 correlates with p20K induction in response to DMOG.

In order to document the relationship between hypoxia, growth arrest and p20K expression, the hypoxia-mimetic DMOG was used to assess the proliferation of CEF & expression of p20K when CEF were cultured in a conventional incubator containing 21% O_2. Preliminary studies performed by MSc. Students R. Ghosh (2009) and B. Fielding (2011) showed that p20K expression can be induced by DMOG, a prolyl-hydroxylase inhibitor that induces a hypoxia response, i.e. HIF stabilization, macrophages and NF-κB pathway activation (Barnucz et al., 2013; Hams et al., 2012). The PHD family, PHD1, PHD2 and PHD3, and FIH-1 are involved in regulating HIF-1α proteasomal degradation at high oxygen levels (Elvidge et al., 2006). DMOG is responsible for inhibiting PHD family members in normoxia (Koditz et al., 2007). Thus, DMOG stabilizes HIF-1α, and inhibits its degradation in order to mimick a

hypoxia response in normoxic conditions. Recent studies proved that p20K induction by DMOG is C/EBPβ dependent (Camacho, M.Sc., 2013). Since DMOG mimics hypoxia, it is interesting to determine whether the mechanisms of p20K regulation in DMOG treatment are the same as in hypoxia. Moreover, previous results shown in Figure 3.4 suggest that the inactivation of ERK functions as a factor in p20K induction in hypoxia, therefore it is of interest to examine the levels of activated ERK (indicated by phospho-ERK) in response to DMOG.

Hu et al. (2009) first described the role of ERK-2 as a transcriptional repressor in genes controlled by GATE-elements. Binding of ERK-2 to a consensus DNA sequence inhibited GATE-driven gene transcriptional activities (Hu et al., 2009). Studies performed by M. Athar (MSc. Thesis, 2011) showed that ERK-2 acted as a transcriptional repressor of p20K in cycling CEF, when binding to a consensus DNA sequence GAAAGGAGAAAG of the QRU overlapping with the two C/EBPβ binding sites. Thus, ERK-2 binding to QRU blocks p20K expression. ERK1/2 phosphorylation is necessary for its activation and nuclear translocation, consequently, a phospho-site specific ERK antibody was used to detect ERK activation in CEF (Lenormand et al., 1998).

Figure 3.6 shows that after treating CEF with 1 mM of DMOG, p20K induction begins at 12hr and peaks at 36hr post treatment. Interestingly, DMOG modestly

up-regulated CHOP levels, starting at 12hr and reaching a maximum level at 24hr (Figure 3.6B). However, the p-ERK levels do not show obvious changes after treating CEF with DMOG, especially at 24hr, although with robust p-ERK level, p20K is highly induced compared to the ETOH group. Therefore, proliferation assays and Co-Immunoprecipitation analysis were performed to characterize the response of CEF to DMOG treatment. At 36hr post treatment, CEF reached a high cell density and p20K expression was detected in ETOH treated cells. At the same time, P-Erk and CHOP levels decreased significantly compared to 30hr time-point in both ETOH and DMOG treated cells. These results suggest that CEF reached contact inhibition after 36hr treatment in ETOH and DMOG groups, which indicate that CHOP expression can remain at a relatively high level in DMOG induced p20K expression, but this phenomenon is restricted to cycling CEF.

CO-IP analysis demonstrated that DMOG induces the formation of CHOP-C/EBPβ heterodimer, in agreement with the increase in CHOP levels (Figure 3.6A). Previous data reported that in condition of ER stress, CHOP played a role in p20K expression (Camacho. MSc., 2012). These results with DMOG further confirm that the decrease of CHOP observed in hypoxia or contact inhibition is not a determinant factor of p20K induction in these conditions. In addition, the inhibition of CEF proliferation in DMOG (Figure 3.6C) quite possibly indicates that the removal of P-ERK from the QRU is the likely factor in p20K expression regulation.

Results shown in Figure 3.7 demonstrate that 1mM DMOG significantly reduces CEF proliferation. Upon quantitative analysis through ImageJ in Figure 3.6B, although p-ERK expression remains at a high level after DMOG treatment, CEF proliferating rate is impaired. Since ERK1/2 is responsible for regulating cell proliferation, and its binding to the QRU of p20K promoter is required for inhibiting p20K expression, it is of interest to further study the location of ERK1/2 to the QRU after treating CEF with DMOG via ChIP analysis. Preliminary ChIP performed by Athar (M.Sc., 2011) demonstrated that ERK2 was recruited to the QRU and repressed p20K in cycling cells, but not in growth arrest conditions or hypoxia (Athar, MSc., 2011). ChIP analysis in Figure 3.9 provided circumstantial evidence for ERK2 functioning as a Transcriptional repressor at the QRU only in ETOH group but not DMOG group. This is inferred from the fact that ERK2 is only detected at the QRU when DMOG was not added to the media of CEF and p20K was transcriptionally repressed. However after DMOG treatment, ERK2 cannot be detected on the QRU by ChIP and p20K expression is induced (Figures 3.6 & 3.9). These results support a model where DMOG causes p20K induction via driving ERK2 away from the QRU. This explains the results in Figure 3.6 that although p-ERK expressions remain at almost the same levels in DMOG group compared to ETOH group, ERK2 does not bind to the QRU after DMOG treatment, relieving the block to p20K transcriptional activities.

4.3 C/EBPβ as a sensor and regulator of gene expression in cell fate.

C/EBPβ is ubiquitously expressed and regulates cell proliferation, cell differentiation and apoptosis in multiple tissues. Previous data revealed that C/EBPβ and AP-1 play opposite roles in regulating Gas genes and cell proliferation (Gagliardi et al., 2003). In addition, C/EBPβ is associated with cellular differentiation in tissues of adipocyte, keratinocyte, epidermal and ovarian origin (Cao et al., 1991; Zhu et al., 1999; Maytin & Habener, 1998; Sterneck et al., 1997). In addition, in response to carcinogenic stress, C/EBPβ could reduce p53 protein levels to promote survival while in neuronal cells, it induces apoptosis in a p53 dependent manner (Yoon et al., 2007; Cortes-Canteli., 2002). Therefore, C/EBPβ acts in a context-specific manner to control cell fate.

Kim et al. (1999) indicated that C/EBPβ is an essential factor for p20K induction either in contact inhibited CEF and overexpression of C/EBPβ dominant negative Δ184 construct could inhibit p20K induction in contact inhibition conditions. Previous research performed by Ghosh R. (MSc., 2009) and Fielding B. (MSc., 2011) showed that the overexpression of C/EBPβ dominant negative construct could inhibit p20K induction in hypoxic conditions as well, indicating that p20K induction is C/EBPβ dependent. Ghosh R. (MSc., 2009) also found that Δ184-C/EBPβ mutant CEF increased cell survival during prolonged starvation. Finally, over-expression of C/EBPβ dominant negative Δ184 in CEF increased cell proliferation rate and cell survival in normoxia and hypoxia, indicating that growth arrest and p20K induction

were impaired by this mutant (Figure 3.10 in Appendix). In addition, the DMOG induced p20K expression is C/EBPβ dependent as well, the abolished p20K expression was observed after transfecting CEF with Δ184-C/EBPβ despite DMOG treatment (Camacho, MSc., 2013).

Former data shows that growth arrest is a hypoxia-responsive factor in Gas gene p20K regulation (Figure 3.6). The QRU of p20K contains two C/EBPβ binding sites (A and B) (Kim et al., 1999). A mutation to either of them is able to disrupt p20K expression in contact inhibited and serum starvation cells (Kim et al., 1999). In addition, compared to the B site, the A site of C/EBPβ has a stronger activity in quiescent cells (Kim et al., 1999). The QRU is also crucial for p20K induction in hypoxia, however further studies need to be performed to specify the region responsible for this (B. Fielding, MSc. Thesis, 2011). Additionally, preliminary data showed that in hypoxia, contact inhibition and DMOG treatment, the levels of CHOP-C/EBPβ heterodimers does not have a determinant function on p20K induction (Camacho, MSc., 2013; Figure 3.8 & 3.9). These results suggest that growth arrest (and possibly the presence of P-ERK2 on the QRU) is the key factor controlling p20K expression. Moreover, since the binding sites of C/EBPβ and ERK2 on the QRU of p20K promoter overlap with each other, the competition between C/EBPβ and ERK2 for binding to the QRU is the key mechanism in p20K expression in hypoxia, DMOG treatment and contact inhibition (Athar, MSc., 2011, Figure 3.8 & 3.9).

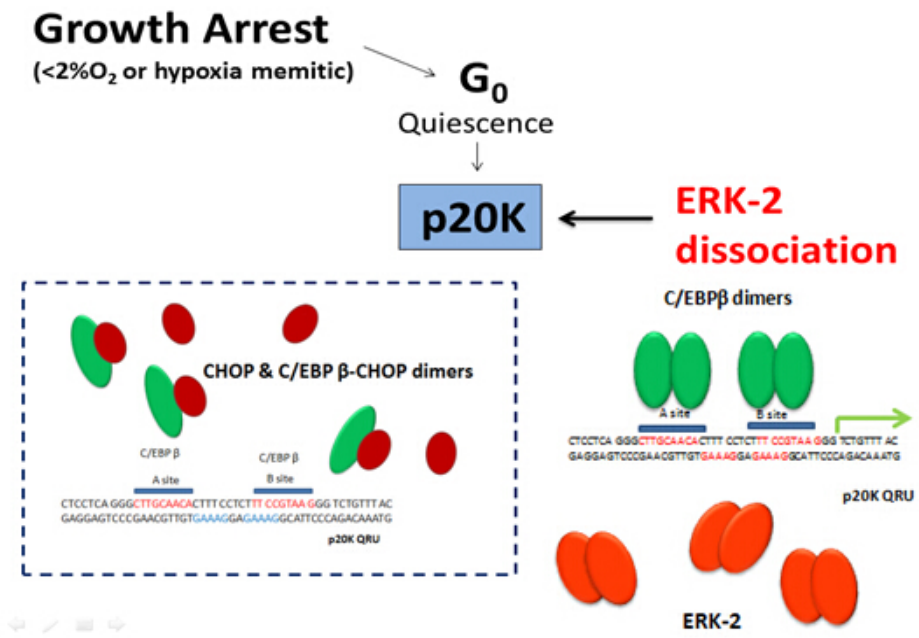


Figure 4.1: Repression of p20K by ERK-2 dissociation from the QRU.

Growth arrest caused by hypoxia (1.8% O_2) or hypoxia mimetic (such as DMOG) leads to ERK-2 dissociation from the QRU of the p20K promoter. The binding of C/EBPβ dimers to the two binding sites within the QRU activates p20K in hypoxia or contact inhibition. The accumulation or reduction of CHOP or C/EBPβ-CHOP heterodimers is not correlated with the transcription initiation of p20K. When cycling CEF is cultured in normoxia condition (21% O_2), the binding of ERK-2 to its DNA consensus site (GAAAGGAGAAAG) on the QRU represses p20K activation via hindering C/EBPβ binding to the QRU. Fig3.4C & Fig3.7 suggest that CEF proliferation is reduced in conditions of hypoxia (1.8% O_2 or DMOG treatment).

4.4 Future Experiments

4.4.1 Test the modulation of serum factors on the induction of p20K in hypoxia

Influence of growth factors on ERK2 recruitment to the QRU by ChIP

A series of ChIP analysis provided evidence for a role of ERK2 as a transcriptional repressor of the p20K QRU in cycling CEF (MSc. Thesis, M. Athar, 2011). No binding of ERK-2 to QRU was observed during hypoxia or contact inhibition conditions where p20K is predominantly expressed. Results shown in Figure 3.4 indicate that a hypoxia condition significantly inhibits p-ERK level in CEF after 12hr incubation in both CCS and FBS group, which plays an important role in inducing p20K expression. However, the density of p20K band in FBS is much lighter than it in CCS, indicating that growth factors contained in the serum may change the intracellular localization of ERK2 in hypoxia. Although the p-ERK level in FBS group in hypoxia is low, it is highly possible that all of the p-ERK existing in CEF is binding to the QRU, thus the repression of p20K induction. Thus, follow up studies requires analyzing the effect of growth factors on levels of phospho-specific ERK & ERK2 binding to the QRU via ChIP analysis. It is of important to verify and characterize the binding of ERK2 to the QRU in order to determine whether and how it inhibits p20K induction in hypoxia in CEF.

Influence of other serum factors such as EFA on p20K induction

In addition, Figure 3.1, Figure3.2 and Figure3.3 show that although FBS (growth

factors) could delay p20K induction in hypoxia, it is not dose-dependent. 5%FBS DMEM group shows more robust p20K induction compared with 2.5% FBS and 10% FBS groups. This phenomenon suggests that there contains other factors other than growth factors that regulate p20K expression. p20K expression is regulated at the transcriptional level. Previous studies have proved that transcriptional activation of C/EBPβ contributes to its accumulation in contact inhibition, thus C/EBPβ is a growth arrest-specific gene in CEF (Kim et al., 1999). Kim et al has also demonstrated that p20K expression is controlled by an essential fatty acid, linoleic acid, via its binding to p20K. In addition, linoleic acid could markedly inhibit p20K synthesis without inducing mitogenesis in contact inhibited condition (Kim et al., 1999). Transient expression assays also showed that QRU activity was repressed by linoleic acid (Kim et al., 1999). Since the binding of C/EBPβ to QRU induces QRU activity, while the overexpression of Δ184, a dominant negative mutant of C/EBPβ, markedly reduces QRU activity, Kim et al concluded that C/EBPβ implicated the essential fatty acid-linoleic acid in the regulation of the QRU. In addition, CHOP10 acts as a repressor of the QRU (Shi Yan). Since linoleic acid is an EFA, C/EBPβ may play a role in EFAs while regulating p20K expression (Cancedda, et al., 1996; Kim et al., 1999). In Type 2 diabetes, inhibiting the ERK1/2 MAP kinase pathway or reducing mitochondrial fatty acid oxidation elicits C/EBPβ expression and overexpression of C/EBPβ resulted in increasing apoptosis. Thus, C/EBPβ may be involved in free fatty acids mediation (Plaisance et al., 2009).

Additionally, a mixture of conjugated isomers of linoleic acid (CLA) has antiproliferative properties (Sehat et al., 1998; Herbel et al., 1998). Studies have proved that cytostatic concentrations of CLA induced cell cycle arrest in G1 in breast cancer MCF-7 cell line. Furthermore, CLA also reduced the expression of factors required for G1-S transition such as cyclins D1 & E. Thus, the antiproliferative properties of CLA appear to be involved in cell growth arrest regulation (Kemp et al., 2003). To sum up, it is highly possible that FBS has a higher concentration of EFAs than CCS and hence is more potent at inhibiting p20K. Finally, it may be the balance between growth factors and EFA that govern the level of cell proliferation and p20K expression.

Figure 3.4 demonstrates that p20K induction is repressed by FBS in hypoxia and Figure 2 indicating that although various concentrations of FBS could decrease p20K level in hypoxia, this effect is not linear. Future experiments examining the role of fatty acids included in the FBS media are needed. Moreover, transient expression assays will also be required to further investigate the mechanism of linoleic acid or other types of EFA in regulating p20K expression.

4.4.2 Examinate the influence of down-regulating ERK2 expression on p20K induction

Previous studies by M. Athar (MSc. Thesis, 2011) have characterized ERK-2 as an

inhibitor of p20K expression mediated through C/EBPβ. DMOG treatments on CEF drive ERK-2 away from the QRU, thus the induction of p20K expression (Figure 3.9). Additionally, down-regulating CHOP levels with a retrovirus vector shRNAi (354-CHOP-10) did not restore p20K levels at earlier time points in cycling CEF in hypoxia and the accumulation of CHOP-C/EBPβ is not associated with p20K induction in hypoxia mimetic agent DOMG (Camacho. MSc. Thesis, 2013; Figure 3.8). These results come to the hypothesis that ERK-2 is responsible for p20K activity in CEF. When treating CEF with MEK1/2 inhibitor PD184352, p20K expression was induced in cycling cells, which resulted from the decrease in ERK1/2 phosphorylation and subsequent activation in CEF (Mantella. BSc., 2014). These results support the statement that the ERK1/2 pathway is a major regulator of p20K expression, moreover it can be hypothesized that the interaction between ERK1/2 and C/EBPβ may be a mode of regulation for several more inducible pathways. In addition, preliminary studies performed by M. Erb (BSc. 2014) suggest that in hypoxia, AP-1 activity and expression was dramatically down-regulated, which resulted in part from the loss of ERK1/2 activation and subsequently led to a loss of JunD phosphorylation. Moreover, AP-1 plays a role in the basal expression level of CHOP, while CHOP repression caused by down regulating AP-1 is not sufficient to induce p20K expression in subconfluent CEF. However when AP-1 is over-expressed, p20K induction is attenuated in hypoxia (Erb. BSc., 2014). These results are in agreement with the results shown in this thesis (Figure 3.6-3.9) stating that ERK2 instead of CHOP is

responsible for p20K regulation in CEF. Thus, it is of interest to specifically knock down ERK2 by RNAi in order to figure out its function in p20K activity in CEF.

4.4.3 Elucidate the localization of ERK2 after DMOG treatment via Immunofluorescence

The QRU, a 48 base pair promoter region, has been well characterized in p20K regulation (Kim et al., 1999). The ERK2 binding motif on the QRU is nested between the two (A and B) C/EBPβ binding sites (Figure 1.2), thus the competition between C/EBPβ and ERK2 for binding to the QRU becomes the key factor in regulating p20K induction (Athar, Msc., 2011). ChIP analysis has confirmed that treatment with DMOG, a hypoxia mimetic chemical, on CEF is able to inhibit ERK2 binding to the QRU (Figure 3.9), which plays a role in p20K induction in these circumstances. Preliminary data showed that during G0 phase, ERK2 is inactivated and located in the cytoplasm while once extracellular stimulation appears and activates ERK2, it will translocate to the nucleus in order to regulate transcription (Chen et al., 1992; Gille et al., 1995). However in DMOG experiments, p-ERK levels remain at a high level until 30hr post treatment (Figure 3.6A) but without binding to the QRU (Figure 3.9). Thus one future study should be to determine by Immunofluorescence analysis whether ERK2 is still located in the nucleus after DMOG treatment. Immunofuorescence is a visual technique that would be beneficial to provide *in vivo* evidence of the location of ERK2 before and after DMOG treatment.

4.5 Conclusion

In summary, in this thesis we provide evidence that growth arrest induced by hypoxia or a hypoxia-mimetic such as DMOG, is a factor in regulating p20K expression. In contrast, reducing the level of CHOP & CHOP-C/EBPβ heterodimers is not correlated with the induction of p20K.

Chapter 5: Appendix

5.1 The overexpression of the C/EBPβ negative mutant Δ184 promotes CEF proliferation in normoxia & hypoxia

Rationale

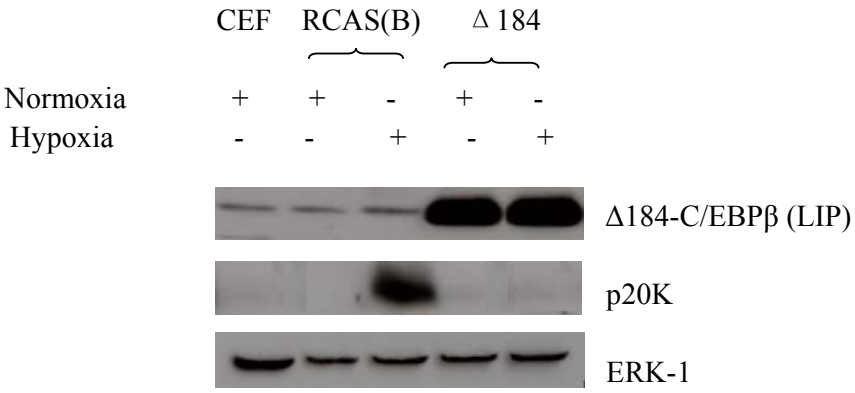
Previous studies indicate that overexpression of C/EBPβ dominant negative Δ184 inhibits p20K induction in conditions of contact inhibition or hypoxia (Kim et al., 1999; Ghosh. MSc., 2009; Fielding. MSc., 2011). Ghosh R. (MSc. Thesis, 2009) also found that the Δ184-C/EBPβ mutant CEF increased cell survival during prolonged starvation. Thus, we further studied the effects of overexpression of C/EBPβ dominant negative Δ184 construct on cell proliferation in normoxia & hypoxia.

Results

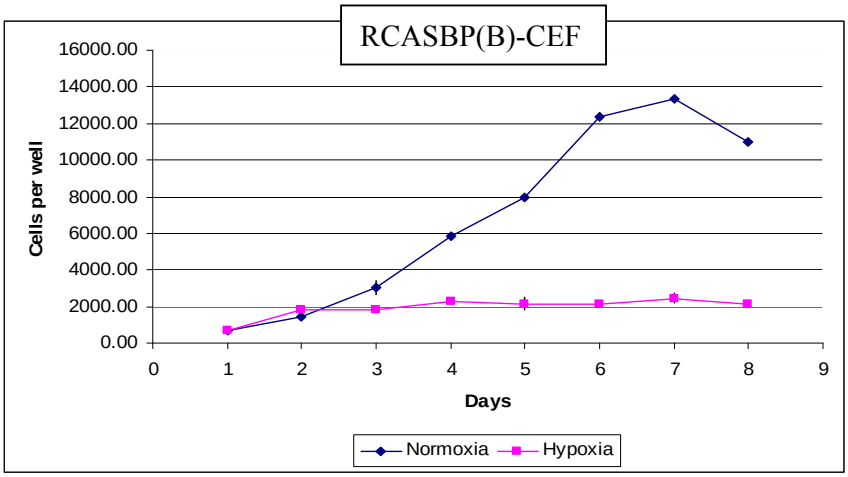
CEF were transfected with RCAS(B) or RCAS(B) Δ184 retroviral vector by the calcium phosphate method (Graham and Van Eb). Cells were then cultured for 3 passages to ensure complete infection. Cycling CEF cells were then incubated in either normoxic or hypoxic conditions for 24 hours and cell lysates were prepared. SDS-Page gels and Western blotting analysis were performed to examine the C/EBPβ-Δ184 expression level in order to ensure the successful transfection. Transfected CEF were seeded at 10,000 cells per well into 24-well dishes and left overnight to recover and attach. Cells were then placed in either normoxic (21% oxygen) or hypoxic (1.8% oxygen) condition and cell counts were obtained every 24

hours for eight days without any media replenishment. Figure 5.1A shows that CEF transfected with RCAS(B)-Δ184 has great C/EBPβ-Δ184 expression level and low p20K level, indicating that the transfection worked well. Results mentioned in 5.1B and 5.1C indicate that CEF-RCAS(B) & CEF-Δ184 proliferate better in normoxia than in hypoxia. Cell counts increased from Day 1 to Day 7 but begin to go down after Day 7. Figure 5.1D shows that compared with CEF-RCAS(B), CEF-Δ184 have a greater proliferation rate after Day 4 in normoxia. The cell count differences in normoxia from Day 6 to Day 8 are statistically significant in an unpaired t-test ($p<0.05$). Although in hypoxia the cell count results are not statistically significant, Figure 5.1D suggests that CEF-Δ184 begin to have larger cell number than CEF-RCAS(B) from Day 6 to Day 8. Therefore, the over-expression of C/EBPβ dominant negative Δ184 construct in CEF increases cell survival during prolonged normoxia, indicating that growth arrest time was postponed while p20K induction was inhibited in normoxia.

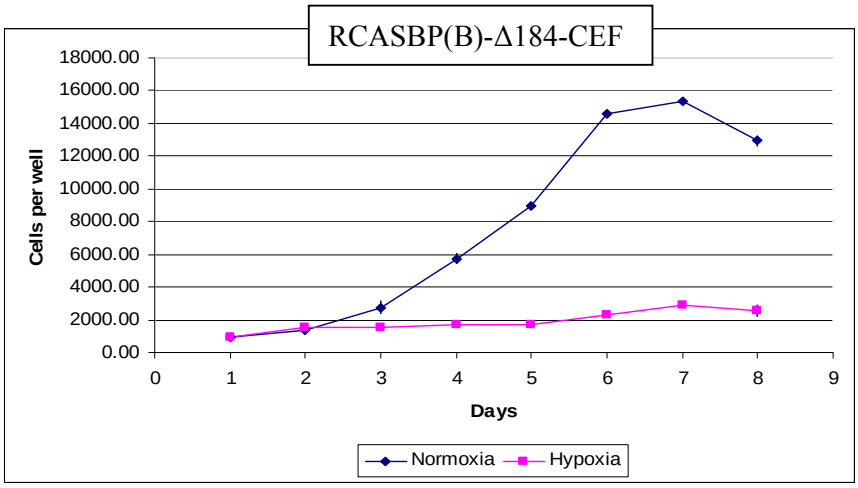
A.


CEF
RCAS(B)
Δ 184
Normoxia + + - + -
Hypoxia - - + - +
Δ184-C/EBPβ (LIP)
p20K
ERK-1

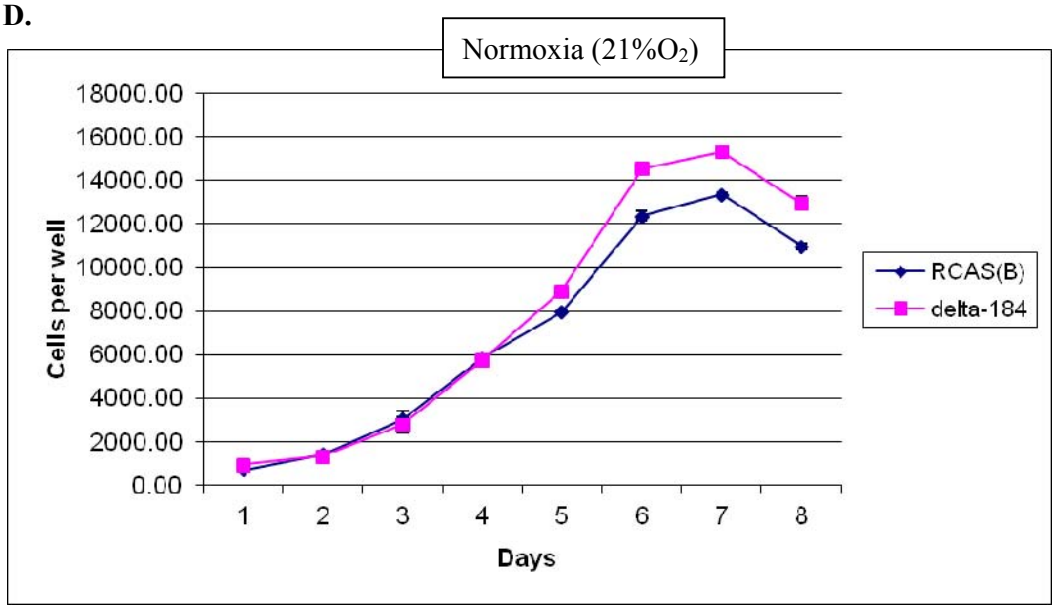
B.


RCASBP(B)-CEF
Cells per well
16000.00
14000.00
12000.00
10000.00
8000.00
6000.00
4000.00
2000.00
0.00
0 1 2 3 4 5 6 7 8 9
Days
Normoxia
Hypoxia

C.


RCASBP(B)-Δ184-CEF
Cells per well
18000.00
16000.00
14000.00
12000.00
10000.00
8000.00
6000.00
4000.00
2000.00
0.00
0 1 2 3 4 5 6 7 8 9
Days
Normoxia
Hypoxia

D.


Normoxia (21%O2)
18000.00
16000.00
14000.00
12000.00
10000.00
8000.00
6000.00
4000.00
2000.00
0.00
Cells per well
1
2
3
4
5
6
7
8
Days
RCAS(B)
delta-184

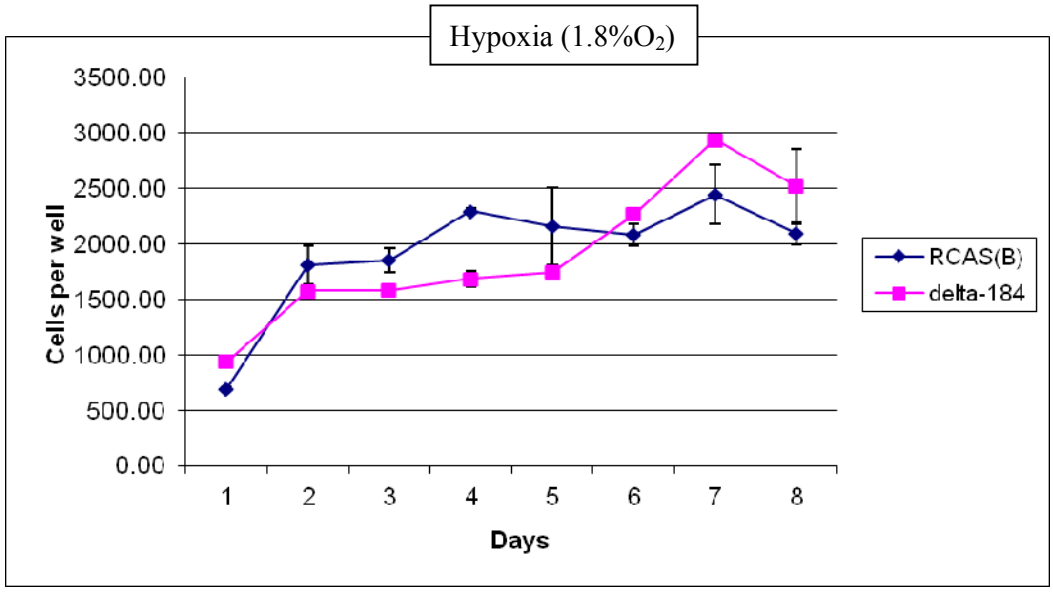

Hypoxia (1.8%O2)
3500.00
3000.00
2500.00
2000.00
1500.00
1000.00
500.00
0.00
Cells per well
1
2
3
4
5
6
7
8
Days
RCAS(B)
delta-184

Figure 5.1: Proliferation assay after transfecting CEF with RCAS(B) or Δ184 in hypoxia and normoxia.

CEF were transfected with RCAS(B) (empty vector) and Δ184-C/EBPβ to inhibit C/EBPβ expression. Error bars indicate the standard error. Transfected CEF were seeded 10,000 per well into 24 well dishes. After 24 hours, they were placed in either normoxic (21% O_2) or hypoxic (1.8% O_2) conditions. Cells were counted every 24 hours for eight days without any media replenishment. This was done in quadruplicate and the average number of CEF was used to generate the proliferation curve.

A. CEF transfected with Δ184 showed great Δ184-C/EBPβ (18kDa) expression level in comparison to those transfected with RCAS(B).

B. Proliferation of CEF transfected with RCAS(B) (CEF-RCAS(B)) is greater in normoxia than in hypoxia. Cell numbers rise from Day 1 to Day 7 and begin to go down after Day 7.

C. Cell proliferation of CEF transfected with Δ184 (CEF-Δ184) in normoxia is greater than the proliferation in hypoxia and cell counts begin to decrease after Day 7 as well.

D. Compared with CEF-RCAS(B), CEF-Δ184 have a greater proliferation rate after Day 4 in normoxia and begin to show more cell number at Day 6 in hypoxia condition.

5.2 Human lung fibroblast cell lines MRC-5 & IMR-90 have different behavior compared with CEF in hypoxia

Rationale

The GAS gene p20K was first discovered in chicken heart mesenchymal cells and further studied in chicken embryo fibroblasts (CEF) (Bédard et al., 1987). The regulation of its expression and induction in CEF has been reported in various conditions such as hypoxia, contact inhibition, DMOG (Bédard et al., 1987; Kim et al., 1999; Fielding. MSc., 2011; Camacho. MSc., 2013). Preliminary data has indicated that CEF proliferation was significantly inhibited by hypoxia, which led to the inhibition of P-Erk expression and p20K expression (Athar. MSc., 2011; Fox-chen. BSc., 2012). Moreover, p20K induction was inhibited by the dominant negative mutant Δ184-C/EBPβ in prolonged hypoxia. CEF proliferation was also enhanced by Δ184-C/EBPβ (Romita. MSc., 2009). Growth arrest caused by hypoxia induces p20K expression in CEF (Figures 3.4&3.5). Since GAS genes are markers of quiescence and ERK1/2 functions in p20K expression regulation, it is of interest to determine if the same mechanisms of growth arrest caused by hypoxia are also present in primary human fibroblast cells (Jordan., 2006; Athar.MSc., 2011).

Results

MRC-5 and IMR-90 are two primary human lung fibroblast cell types that have been well studied in tissue culture, thus they were used in these proliferation assays. The

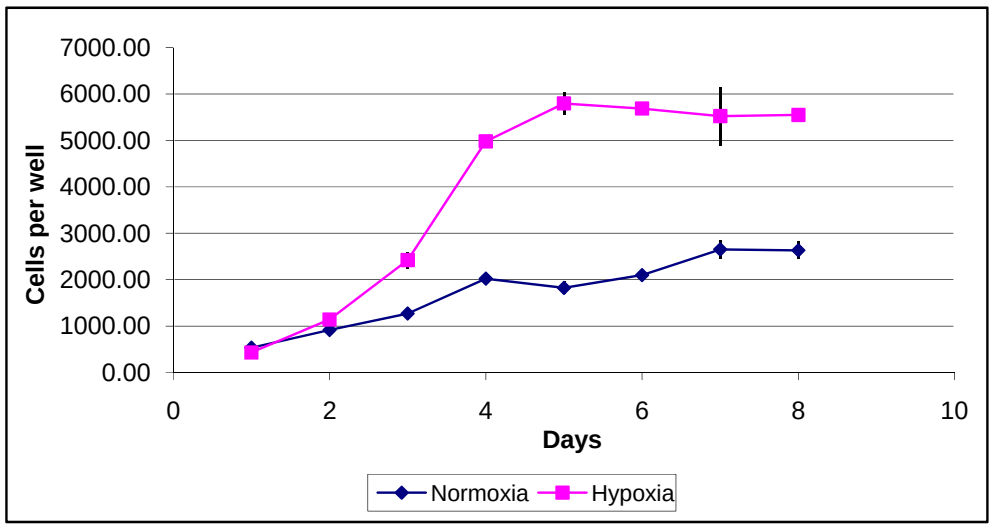
Hela cell line was derived from cervical cancer cells and was used as a control in this experiment. MRC-5, IMR-90 and Hela Cells were cultured in 10%FBS, resuspended and seeded 10,000 per well into 24 well dishes. After 24 hours, they were placed in either normoxic (21% O_2) or hypoxic (1.8% O_2) conditions. Cell counting was performed in quadruplicate for each cell line every 24 hours for 8 days without any media replenishment. The average number of CEF was used to generate the proliferation curve.

Interestingly, results in Figure 5.2 indicated that MRC-5 and IMR-90 cells had higher proliferation rate in hypoxia compared to normoxia while Hela cells grew faster in normoxia. To further confirm the response of human fibroblast cells to hypoxia, we also looked at the accumulation of HIF-1α, a well established early marker of hypoxia, in MRC-5 cell line (Semenza et al., 2010; Fielding. MSc., 2011). Cells were cultured and treated as described before. Cell lysates were collected after 1.5hr, 4hr, 12hr and 24hr post-treatment. Samples collected from cells cultured in normoxia were used as control group. Samples were run on a SDS-Page and analyzed by Western blotting. As shown in Figure 5.3, HIF-1α accumulated rapidly (within 1.5 hours) in hypoxia and reached a peak level at around 4hr post-treatment in hypoxia in MRC-5 cells, indicating that MRC-5 had similar response to hypoxia as CEF (Figure 3.5). The level of Phospho-ERK was also examined in MRC-5 cells in condition of Hypoxia. Figure 5.4B shows that the P-Erk remained at almost the same level after 0hr, 6hr and 36hr of

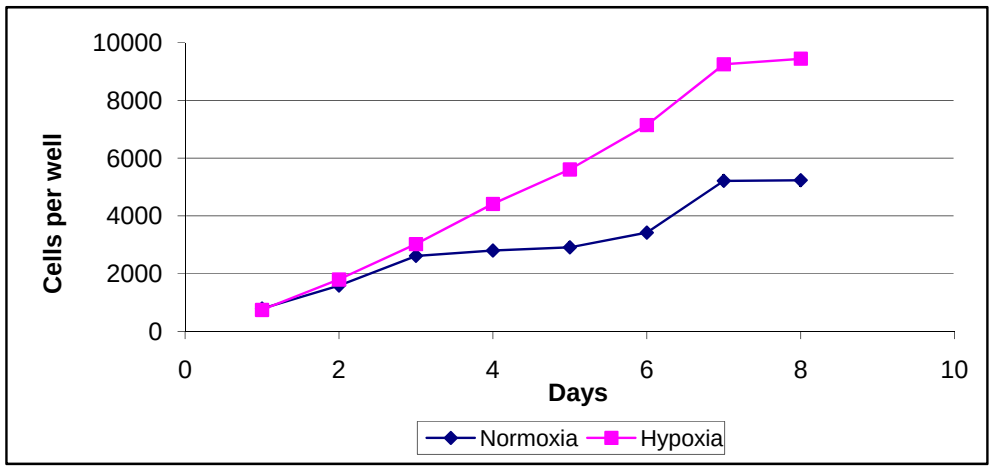
hypoxia, and even increased at 24hr post-treatment, which suggests that hypoxia does not influence the phospho-ERK level in primary human lung fibroblast MRC-5 as it does in CEF (Figure 3.4). These results indicate that human fibroblast cells have a different response in hypoxia compared to chicken fibroblast cells.

The observation that MRC-5 & IMR-90 accumulate exponentially in reduced oxygen level suggests that these primary cells are negatively affected by the conditions of normoxia (21% O_2) existing *in vitro*. Interestingly, Hela cells, a well established cell line, have the opposite behaviors and its proliferation is significantly inhibited by reduced O_2 level (1.8% O_2).

A. MRC-5 Cells



B. IMR-90 Cells



C. Hela Cells

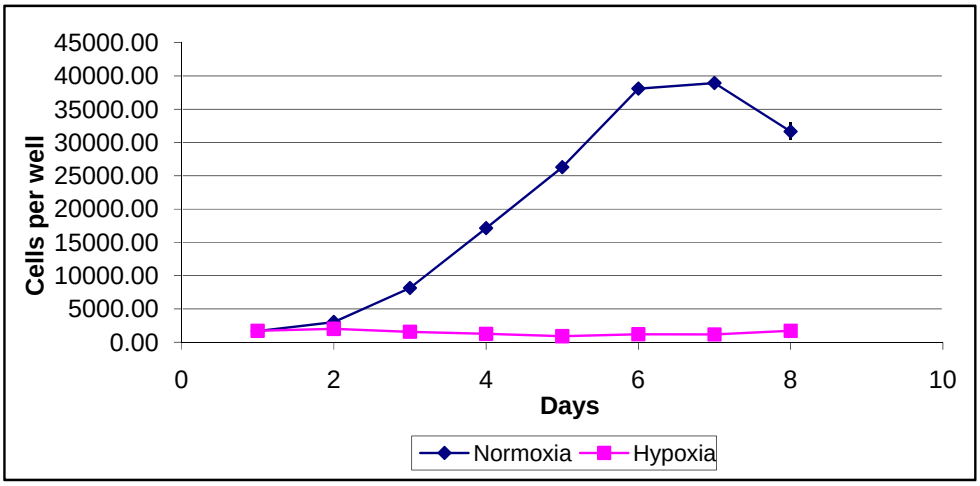
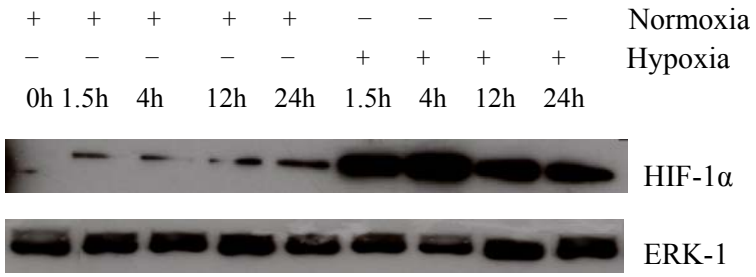


Figure 5.2: Proliferation of MRC-5, IMR-90 and Hela Cells in normoxia (21%O_2) & hypoxia (1.8% O_2).

MRC-5, IMR-90 and Hela Cells were cultured in 10%FBS before being seeded 10,000 per well into 24 well dishes. After 24 hours, they were placed in either normoxic (21% O_2) or hypoxic (1.8% O_2) conditions. Cells were counted every 24 hours for eight days without any media replenishment. This was done in quadruplicate and the average number of CEF cells was used to generate the proliferation curve. Proliferation assays of MRC-5 and IMR-90 were performed twice with same results.
A.MRC-5 proliferation result in normoxia & hypoxia.
B. IMR-90 proliferation result in normoxia & hypoxia.
C. Hela cell proliferation result in normoxia & hypoxia.

A.



B.

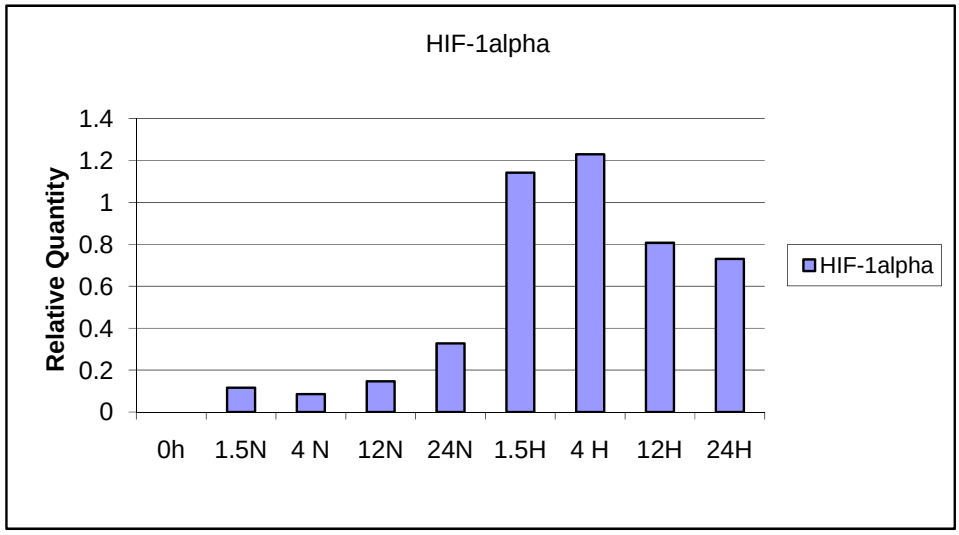
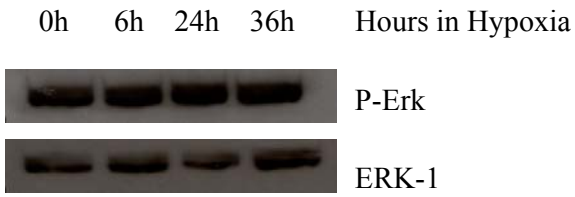


Figure 5.3: Expression of HIF-1α in MRC-5 cells under normoxia & hypoxia.

MRC-5 cells were cultured in 10%FBS and cell lysate was collected before hypoxia treatment as control (0h group). Cells were then placed in either conventional incubator (21% O_2, normoxia) or hypoxia chamber (1.8% O_2). After 1.5, 4, 12, 24 hours incubation, MRC-5 lysates from both normoxia and hypoxia were collected. Samples were run on a SDS-Page gel and analyzed by Western blotting.
A. Expression of HIF-1α and ERK-1in normoxia and hypoxia.
B. Levels of HIF-1α were quantified relative to ERK-1 levels using ImageJ.

A.



B.

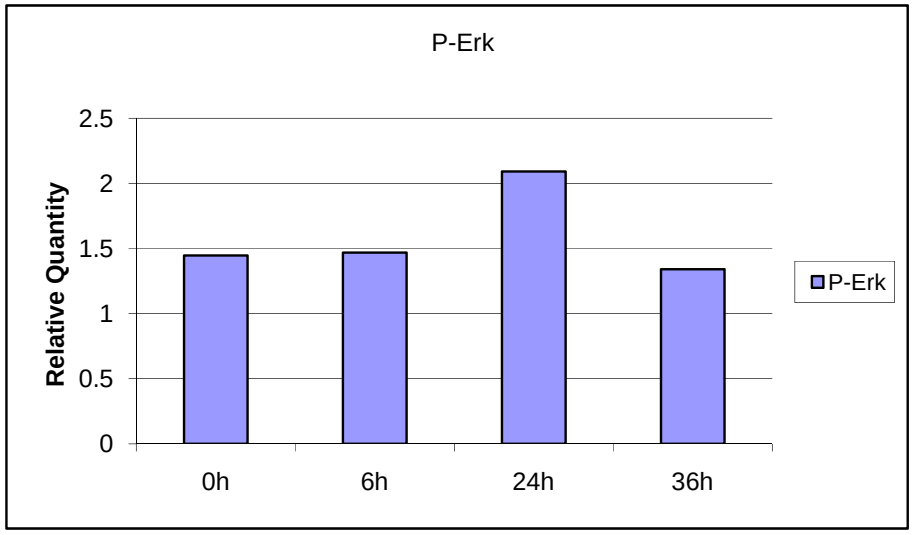


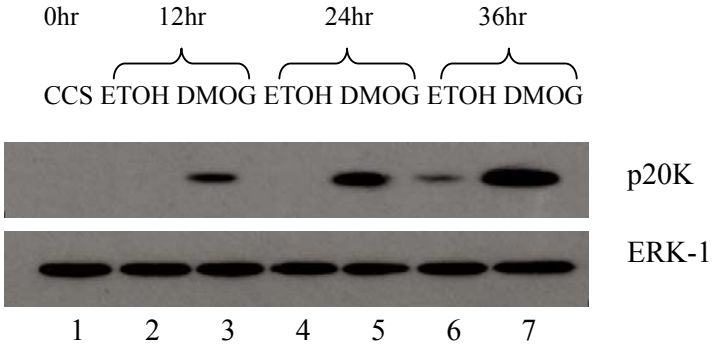
Figure 5.4: Expression of P-Erk in MRC-5 cells under hypoxia.

MRC-5 cells were cultured in 10%FBS and cell lysates were collected at 0 hr (control), 6 hr, 24 hr and 36 hr post-treatment in hypoxia (1.8% O_2). SDS-Page gel and Western blotting were hired to analyze the samples.
A. Expression of P-Erk and ERK-1in MRC-5.
B. Expression of P-Erk was quantified relative to ERK-1 through ImageJ.

5.3 ETOH does not have influences on p20K expression.

Hypoxia mimetic DMOG was used in this thesis to study the mechanism of p20K expression. Since the DMOG powder was dissolved in ETOH, 0.01% ETOH (same concentration as DMOG used in experiments) was used as a control. Further experiment was performed to assess whether 0.01%ETOH could influence p20K expression in normoxia. CEF were cultured and treated as described before in section 3.2. Results shown in Figure 5.5 indicate that 0.01% ETOH has no influence on p20K expression compared to normal condition (5%CCS) in normoxia (lane 1&2 in Figure 5.5A). Thus, it can be used as a control in DMOG treatment.

A.



B.

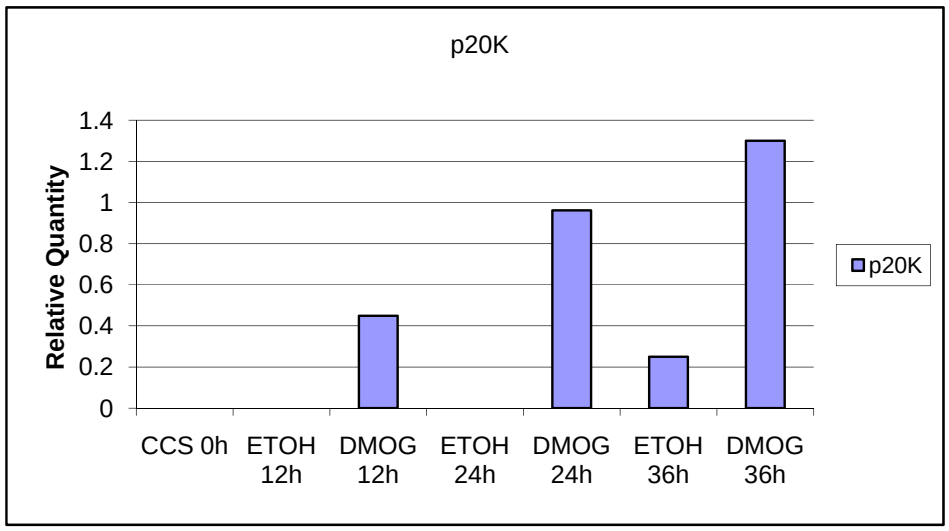


Figure 5.5 p20K induction in DMOG treatment.

Confluent CEF were split and incubated for 10 hours before treatment in order to recover and attach. Then they were treated with either 0.01% DMOG or 0.01% Ethanol (ETOH). Cell lysate was collected after 12, 24 and 36 hours treatment. Untreated CEF was used as control (Lane 1). Lysate samples were run on a SDS-Page gel and analyzed via Western blotting.

A. Expression levels of p20K and ERK-1 in CEF cells.

B. Quantification of protein expression from Western blotting Figure 5.5A. Levels of p20K are corrected to the level of ERK-1 using ImageJ.

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