Characterizing the Role of the Growth Arrest Specific p20K Lipocalin in Chicken Embryo Fibroblasts

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Abstract

Cell cycle progression is regulated by complex processes which determine whether cells can continue dividing or enter a nonproliferative state. Cells exit the cell cycle and are able to enter a reversible state of growth arrest called quiescence. A subset of genes involved in mediating cellular quiescence are referred to as growth arrest specific (Gas) genes, however their mechanisms and regulatory characteristics require further analyses. The p20K lipocalin, a Gas gene, is involved in lipid transport is induced upon conditions, such as contact inhibition and hypoxia, in chicken embryo fibroblasts (CEF). The purpose of this study is to characterize the role of the p20K lipocalin upon quiescence in CEF. P20K expression was downregulated in CEF, and the cells were cultured under conditions of contact inhibition and hypoxia. There was a decrease in cell accumulation and an increase in apoptosis in CEF which downregulated p20K when placed in hypoxic conditions. Additionally, there were increased levels of reactive oxygen species (ROS) in hypoxic CEF with p20K downregulation. These results suggest that p20K regulates cellular stress by promoting cell survival and mitigating oxidative stress, however the mechanisms by which these functions are carried out remain unknown. The study also focused on characterizing the relationship between the lipocalins p20K and fatty acid binding protein 4 (FABP4). FABP4 is a Gas gene that is expressed in contact inhibited and hypoxic conditions of quiescence. FABP4 expression was analyzed in CEF with p20K downregulation in conditions of quiescence. The mis-expression of p20K impaired FABP4 expression in contact inhibited and hypoxic CEF, and the ectopic up-regulation of p20K impaired FABP4 expression in contact inhibited

CEF. These results suggest that p20K and FABP4 are co-regulated in conditions of contact inhibition and hypoxia. Overall, the p20K lipocalin is involved in cell survival and mitigating oxidative stress in quiescence which can help explain its role in lipid metabolism and the novel “Membrane Stress Response”.

Acknowledgements

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List of Abbreviations

AP-1	Activator Protein 1
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
bZIP	Basic Region-Leucine Zipper
C/EBP	CCAAT-Enhancer Binding Protein
CDK	Cyclin-Dependent Kinase
CEF	Chicken Embryo Fibroblast
CHM	Chicken Heart Mesenchymal
ChIP	Chromatin Immunoprecipitation
CHOP	C/EBP Homologous Protein
CIP/KIP	CDK/Kinase Interacting Protein
CKI	Cyclin-Dependent Kinase Inhibitor
DCFDA	2',7' –Dichlorofluorescin Diacetate
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetraacetic Acid
ER	Endoplasmic Reticulum
ERK	Extracellular Signal Regulated Kinase
Ex-FABP	Extracellular Fatty Acid Binding Protein
FABP4	Fatty Acid Binding Protein 4
FFA	Free Fatty Acid
G_0	Growth Arrest/Quiescence

G_1	Gap 1 Phase
G_2	Gap 2 Phase
GAS	Growth Arrest Specific
GDNF	Glial-Cell Line-Derived Neurotrophic Factor
HIF	Hypoxia-inducible Factor
HNE	Hydroxynonenal
HRE	Hypoxia Response Element
HRP	Horseradish Peroxidase
IgG	Immunoglobin
IL	Interleukin
INK4	Inhibitors of Kinase
JNK	c-Jun N-Terminal Kinase
LAP	Liver-Enriched Activating Protein
LIP	Liver-Enriched Inhibitory Protein
MAPK	Mitogen-Activated Protein Kinase
MAPKK	Mitogen-Activated Protein Kinase Kinase
MAPKKK	Mitogen-Activated Protein Kinase Kinase Kinase
MDA	Malondialdehyde
MEK	MAPK/ERK Kinase
mRNA	Messenger Ribonucleic Acid
NGAL	Neutrophil Gelatinase Associated Lipocalin
NRL	Neu-related Lipocalin

PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate-Buffered Saline
PDGF	Platelet-Derived Growth Factor
PDGFαR	Platelet-Derived Growth Factor α-receptor
PUFA	Polyunsaturated Fatty Acid
QRU	Quiescence Responsive Unit
R	Restriction Point
RCASBP	Replication Competent ALV LTR With a Splice Acceptor Bryan Polymerase
RNA	Ribonucleic Acid
RNAi	RNA Interference
ROS	Reactive Oxygen Species
RPM	Rotations Per Minute
RTK	Receptor-Tyrosine Kinase
S	Synthesis
SARP	Secreted Apoptosis-Related Protein
Scn	Siderocalin/ Lipocalin 2
SDS	Sodium Dodecyl Sulfate
shRNA	Short Hairpin RNA
STE	Sodium-Chloride-Tris-EDTA
TBS-T	Tris-Buffered Saline Tween
TBT	Tris-Buffered Saline

TdT	Terminal Deoxynucleotidyl Transferase
TNFα	Tumour Necrosis Factor α
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling

Literature Review

1. The Cell Cycle

i. Overview

The cell cycle is a complex process that is involved in the development of multicellular organisms, DNA replication and various diseases (Schafer, 1998). This regulated cycle consists of interphase and mitosis, which is then completed by the process of cytokinesis, resulting in two identical daughter cells (Nigg, 1996). Interphase permits DNA synthesis and is comprised of the gap 1 (G_1), synthesis (S) and gap 2 (G_2) phases. Somatic cells enter the gap 1 phase (G_1), which prepares the cell for DNA replication in the S phase. Following the S phase, the cells enter the gap 2 phase (G_2) to allow growth prior to mitosis (Schafer, 1998). The segregation of chromosomal pairs into identical nuclei occurs upon the completion of mitosis and is comprised of prophase, metaphase, anaphase and telophase. The duplicated sister chromatids attach to the mitotic spindle during prophase followed by the extension of microtubules and assembly of sister chromatids at the equatorial plane in metaphase. In anaphase, the sister chromatids are separated, and telophase completes the process of mitosis by bringing the chromatids to opposite poles of the cell (Morgan, 2007).

ii. Cell Cycle Regulators

The control mechanisms that enable cell cycle progression are regulated in an ordered and time-dependent manner. Biochemical checkpoints ensure that a cell cycle event occurs upon the correct completion of prior events. Cyclin and cyclin-dependent kinases (CDKs) form complexes that are activated in the advancement through the stages of the cell cycle. Mitogenic growth factors enable the regulatory subunit, cyclin, to bind to its respective catalytic subunit, CDK, to form a kinase holoenzyme (Graña & Reddy, 1995). Cyclin binding results in the phosphorylation of a conserved threonine residue to ensure CDK activation (Morgan, 1995).

Cell cycle regulation consists of cyclins and CDKs that are specific to the different stages of the cycle. During G_1, the D-type cyclins bind to CDK4 and CDK6 to prepare for DNA synthesis, and inhibition of this phase is controlled primarily by the INK4 family inhibitors (p15, p16, p18, p19) (Malumbres & Barbacid, 2009). When the cell has sufficient growth factors during G_1, it passes through a "Restriction Point" (R) and is committed to completion of one round of the cell cycle (Blagosklonny & Pardee, 2002). The binding of E-type cyclins to CDK2 regulates progression through the G_1/S transition. As cells prepare for mitosis in G_2, A-type cyclins first bind to CDK2 and then CDK1 at the completion of interphase. When the nuclear envelope is broken down, A-type cyclins degrade and enable B-type cyclins to bind to CDK1 and promote mitosis (Malumbres & Barbacid, 2009). The Cip/Kip family proteins function as stoichiometric inhibitors that interfere with the binding of cyclins to CDK2 and CDK1 (Vidal & Koff, 2000). Cip/Kip inhibitors (p21, p27, p57) are essential in regulating the cell cycle, as they

are upregulated in quiescence and required during embryogenesis (Besson *et al.*, 2008). See figure 1 for a schematic of the phases of the cell cycle and the respective cyclin-CDK binding events.

iii. Mitogenic Signaling

Mitogen-activated protein kinase (MAPK) signaling pathways are involved in mitogenic stimulation. MAPK pathways consist of triple kinase cascades which are activated by Thr and Tyr phosphorylation events. The four MAPK groups include extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 and extracellular signal regulated kinase-5 (ERK5) (Yang *et al.*, 2003).

The ERK1/2 cascade is a MAPK pathway that is involved in cell cycle progression and control. In this pathway, growth factors, polypeptide hormones, neurotransmitters, and chemokines activate receptor-tyrosine kinases (RTKs) (McKay & Morrison, 2007). RTKs then recruit and activate the cell surface receptor Ras, which is a GTPase. Ras activation leads to Raf kinase activation, which phosphorylates and activates the second protein kinase MAPK/ERK kinase (MEK) (McCain, 2013). As a result of MEK activation, MAP kinases ERK1/2 are phosphorylated at Thr and Tyr residues in their TEY activation loop, causing the upregulation of immediate-early gene c-*fos*. C-*fos* regulates cyclin D, thus sustained ERK 1/2 activation throughout late G_1 is required for cell cycle progression (Yamamoto *et al.*, 2006). Cyclin D_1, which is activated by Ras in various cell types, is also activated by the ERK 1/2 pathway (Albenese *et al.*, 1995). There is a functional activator protein-1 (AP-1) binding site on the promoter region of cyclin D_1 which may be stabilized by ERK 1/2 to induce cyclin D_1 transcription (Albenese *et al.*, 1995; Lavoie *et al.*, 1996). Another proposed mechanism by which ERK 1/2 signaling may regulate cyclin D_1 involves the antiproliferative gene Tob. Upon mitogenic stimulation, Tob is phosphorylated by ERK 1/2 and can no longer recruit

histone deacetylase activity to the cyclin D_1 promoter, therefore ERK 1/2 prevents Tob from suppressing cyclin D_1 expression (Yoshida *et al.*, 2003; Suzuki *et al.*, 2002). The ERK 5 cascade shares similarities with that of ERK 1/2, as it also has the TEY activation motif. ERK 5 is activated by the MAP kinase kinase (MAPKK/MEK) MEK5 and drives cyclin D_1 expression, however this process requires the cAMP response element whereas ERK 1/2 mediated cyclin D_1 expression does not (Mulloy *et al.*, 2003).

The JNK pathway is activated by cytokines and environmental stressors, such as UV radiation, lack of growth factors and DNA damaging agents (Weston & Davis, 2002). The activation of the JNK cascade begins with the activation of MAP kinase kinase kinases (MAPKKKs) which then phosphorylate and activate the MAPKK isoforms MEK4 and MEK7. These isoforms phosphorylate Tyr and Thr residues at a TPY motif, which activates JNK and enables it to translocate from the cytoplasm to the nucleus (Davis, 2000; Weston & Davis, 2002). Like the ERK 1/2 pathway, AP-1 is also a key target of the JNK signaling pathway, as its transcriptional activity increases when JNK phosphorylates c-Jun (Whitmarsh & Davis, 1996). JNK exhibits a complex role in inducing various forms of cell death, particularly in mediating apoptosis. Sustained JNK activation causes TNF-α induced apoptosis, however cells are able to survive when JNK is activated temporarily at earlier time points (Chang *et al.*, 2006; Ventura *et al.*, 2006). Furthermore, JNK phosphorylates the histone H2A variant H2AX which causes caspase-activated DNase to induce nucleosomal fragmentation in chromosomal DNA and apoptosis (Lu *et al.*, 2006).

Stressors, such as oxidative stress, hypoxia, and cytokines, and growth factors, such as fibroblast growth factor, insulin-like growth factor 1 and PDGF, activate the p38 pathway (Zarubin & Han, 2005). MAPKKKs activate MEK3 and MEK6 which then phosphorylate the TGY motif in the p38 activation loop and cause the p38 regulated phosphorylation of Tyr and Thr residues of its substrates (Kumar *et al.*, 2003). A study by Brancho *et al.* characterized the role of p38 in tumour suppression where MEK3/6$^{-/-}$ mice that have a phenotype similar to p38$^{-/-}$ mice produced tumours while control mice did not (2003).

iv. Cell Cycle Arrest

During G_1, cells can enter a nonproliferating or growth-arrested state that is referred to as quiescence or G_0. Cells temporarily enter G_0 in conditions where cell density is high or mitogen stimulation is low (Williams & Stoeber, 2011). Quiescent cells exhibit a decrease in size, macromolecular syntheses and enzymatic activity (Pardee, 1989). Cells can only enter G_0 prior to passing through the restriction point (Zieske *et al.*, 2004). Ladha *et al.* described the intracellular processes of quiescence by placing NIH 3T3 cells in serum-starved conditions (1998). The Ras/Raf/MEK/ERK pathway remains inactivated without mitotic stimulation. Without ERK1/2 activation, cyclin D1 is not expressed and unable to form a complex with CDK4, thus causing cells to remain in the G_0 state. Additionally, high levels of the CDK inhibitor (CKI) p27 was present in starved NIH 3T3 cells, thus suggesting that CKIs are also involved in cell entering G_0 (Ladha *et al.*, 1998).

The key difference between quiescent cells and senescent cells is their ability to re-enter the cell cycle upon mitogenic stimulation and the presence of D type cyclins. Under sustained stress stimuli, cells may enter an irreversible, nonproliferative state and undergo senescence. Senescent cells exhibit hypertrophy, telomere shortening and various degenerative properties that result in cell death (Terzi *et al.*, 2016).

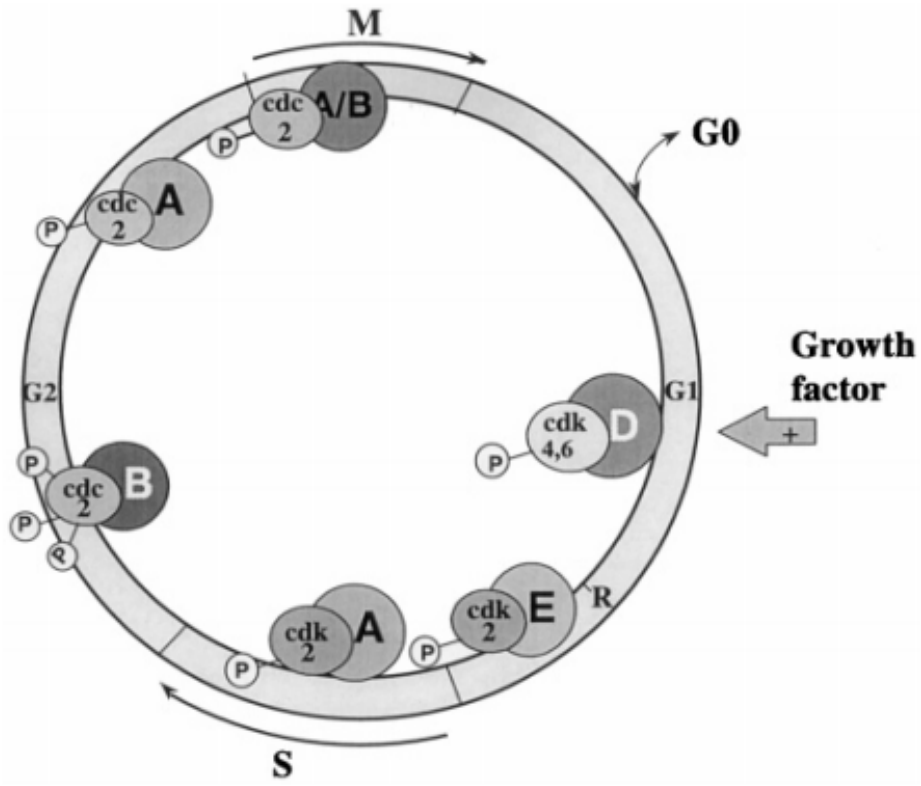


Figure 1: The Cell Cycle. Schematic representation of the phases of the cell cycle and the respective cyclin-CDK binding events. Exit from the cell cycle and entry into G_0 (quiescence) is indicated. The presence of growth factors indicates the progression of the cell cycle through the G_1, past the restriction point (R) and into the S phase. Figure was obtained from Hulleman & Boonstra, 2001

2. Growth Arrest Specific (GAS) Genes

i. Overview

The G_1 phase of the cell cycle is an important control point during cell proliferation, where cells can enter G_0 and remain growth-arrested when there is a lack of growth factors. To further understand the physiological and biochemical properties that distinguish G_0 from G_1, growth factors and gene interactions in both phases have been studied (Schneider *et al.*, 1988). When cells exit from the cell cycle, gene proliferating studies have detected an upregulation in a subset of mRNAs in nonproliferating cells. These genes are collectively called growth arrest specific (Gas) genes, and early studies conducted by Schneider *et al.* identified six Gas genes (Coccia *et al.*, 1992, Schneider *et al.*, 1988). The upregulation of Gas1 plays an integral role in inhibiting DNA synthesis and causing normal cycling cells to exit the cell cycle (Del Sal *et al.*, 1992). Gas1 inhibits the intracellular signaling of the glial-cell line-derived neurotrophic factor (GDNF), which reduces Ret autophosphorylation and AKT activation causing cellular arrest and apoptosis (Cabrera *et al.*, 2005; Dominguez-Monzon *et al.*, 2011). Additionally, Gas6 protects starved cells from undergoing apoptosis by binding to the Axl tyrosine kinase receptor (Goruppi *et al.*, 1996). Gas gene products, such as platelet-derived growth factor α-receptor (PDGFαR) proteins, bind to the three isoforms of platelet-derived growth factor (PDGF) and allow growth-arrested cells to exit from G_0 and enter G_1 (Lih *et al.*, 1996). Furthermore, quiescent cells express the secreted apoptosis related protein 1 (SARP1) and alter cellular response to apoptosis. By interfering with the Wnt-Frizzled signaling pathway, SARPs modulate intracellular β-catenin concentrations and reduce

apoptotic activity (Melkonyan *et al.*, 1997). CKIS are also Gas gene products, as p18 and KIP1/p27 were induced by quiescence in CEF (Erb *et al.*, 2016). Apart from being involved in promoting cell cycle exit, Gas genes also regulate the cellular ability to remain growth arrested in stressed conditions or re-enter the cell cycle (Schneider *et al.*, 1988).

ii. C/EBP-β and C/EBP-β Homologous Protein (CHOP)

The CCAAT/enhancer binding proteins (C/EBPs) consist of six proteins with a basic region-leucine zipper (bZIP) DNA-binding domain and the N-terminal transactivation domain. C/EBPs mitigate inflammatory responses by regulating the production and release of inflammatory cytokines, such as interleukin (IL)-1, IL-6 and TNF-α (Poli, 1998). Two widely studied C/EBP members include C/EBP-α and C/EBP-β, as these transcription factors are notably expressed in liver, adipose and lung tissue. The loss of C/EBP-α causes lipodystrophy and perinatal hypoglycemia while the loss of C/EBP-β impairs immune function, yet despite these variations all C/EBPs overall regulate differentiation (Nerlov, 2007). C/EBPs can isomerize and exhibit an array of transcriptional activities (Descombes & Schibler, 1991).

C/EBP-β, a member of the CEBP family of proteins, has many functions, one of which enables Ras-mediated senescence (Sebastian *et al.*, 2005). Epithelial tumours with cyclin D1 overexpression have also been demonstrated to correspond with elevated C/EBP-β expression (Lamb *et al.*, 2003). Furthermore, research using 3T3-L1 cell lines identified C/EBP-β as a key regulator in promoting cell differentiation (Cao *et al.*, 1991). In addition to these roles, C/EBP-β has an important role in cell proliferation due to its expression in contact inhibited chicken embryo fibroblasts (CEF) (Gagliardi *et al.*, 2003).

C/EBP homologous protein (CHOP) regulates endoplasmic reticulum (ER) stress induced by conditions of serum-deprivation/starvation or tunicamycin drug treatment and is part of the growth arrest DNA damage (GADD) protein family (Erb *et al*, 2016; Pan *et*

al., 2004). CHOP binds with C/EBP-β to form heterodimers in conditions of ER stress, and the pair induce the expression of stress responsive genes (Ubeda *et al.*, 1996). CHOP induction during ER stress is primarily regulated by the PERK/eI2Fα and ATF signaling pathways (Okada *et al.*, 2002). CHOP also regulates factors that are involved apoptosis, as it downregulates anti-apoptotic protein Bcl2 and upregulates pro-apoptotic protein DR5 (McCullough, *et al.*, 2001; Yamaguchi & Wang, 2004).

iii. Regulation of p20K by C/EBP-β

To understand the expression and function of C/EBP-β in conditions of growth arrest, it is important to identify its transcriptional activity through its binding to the quiescence-responsive unit (QRU) in target genes. The QRU is a 48-bp segment in the promoter region of the Gas gene p20K which is sufficient and necessary for p20K promoter activity in contact inhibited CEF (Mao *et al.*, 1993; Kim *et al.*, 1999). The QRU has two C/EBP binding sites that permit interaction with C/EBP-β, and this activity can induce p20K expression upon ectopic expression of C/EBP-β. By disrupting C/EBP-β function using a dominant negative mutant (Δ184-C/EBP-β), p20K induction in contact-inhibited CEF was inhibited and CEF proliferation was stimulated, suggesting that it is a negative regulator of CEF proliferation. Several mechanisms have been proposed to understand C/EBP-β regulation of proliferation, such as the expression of its various isoforms, post-translational modification by RSK, and the opposing role that it plays with AP-1 (Gagliardi *et al.*, 2003).

The dimerization event of CHOP with C/EBP-β prevents C/EBP-β from binding to the QRU, thus repressing p20K expression during ER stress events. In CEF, cycling cells express low levels of CHOP, however contact-inhibited CEF do not express CHOP thus lacking CHOP-C/EBP-β dimers. CHOP expression is therefore a feature of quiescence induced by serum deprivation, but not by contact inhibition or hypoxia when p20K is expressed (Erb *et al.*, 2016).

iv. P20K Lipocalin

The p20K lipocalin, also known as extracellular fatty acid binding protein (Ex-FABP), is a 21 kDa lipocalin that binds to and transports long chain unsaturated fatty acids (Descalzi Cancedda *et al.*, 2002). The secondary structure of lipocalins consists of an eight-stranded antiparallel β-barrel, single α- and 3_{10}-helices and a ligand binding site (Flower, 1994). The family of lipocalins have structural similarities, and p20K closely resembles the structural properties of siderocalin (Scn; also known as lipocalin 2). P20K is considered a siderocalin as it binds ferric-specific iron chelators called siderophores and is involved in iron sequestering processes. The crystal structure of p20K depicts the catechol binding sites that interact with catechol-type ferric siderophores (Fig. 2) (Correnti *et al.*, 2011). Additionally, P20K is expressed during embryonic bone and cartilage development, myogenesis, cardiogenesis (Gentili *et al.*, 1998), and in granulocytes (Dozin *et al.*, 1992). P20K mitigates various cellular stressors by regulating lipid metabolism and inflammatory responses and by promoting cell survival (Descalzi Cancedda *et al.*, 2002). The role of p20K in cellular stress is further understood through the Neu-related lipocalin (NRL), which is a potential mammalian counterpart to p20K. NRL exhibits homology to the human neutrophil gelatinase associated lipocalin (NGAL), and NRL/NGAL expression in granulocytes, inflammatory epithelial cells and hypertrophic cartilage suggests that these are stress proteins associated with the developmental remodeling of tissues and acute phase response (Descalzi Cancedda *et al.*, 2000).

Using the chicken heart mesenchymal (CHM) and CEF cell models, the activation of the p20K lipocalin in quiescent cells has been characterized. By studying CHM and CEF treated with hydroxyurea, under serum starvation or at contact inhibition, cells enter growth arrest (Bédard et al. 1987). The findings from these studies confirmed the induction of p20K in conditions that induce quiescence, however it is absent in CEF and CHM in normoxia, thus characterizing it as a Gas gene (Bédard et al. 1987; Mao et al. 1993).The transcriptional activation of p20K in quiescent cells requires the binding of C/EBP-β to the QRU, while it is repressed when extracellular signal-related kinase 2 (ERK2) binds to the GAAAG sequences of the QRU that overlap C/EBP-β binding sites (Fig. 3) (Gagliardi *et al.*, 2003; Erb *et al.*, 2016).

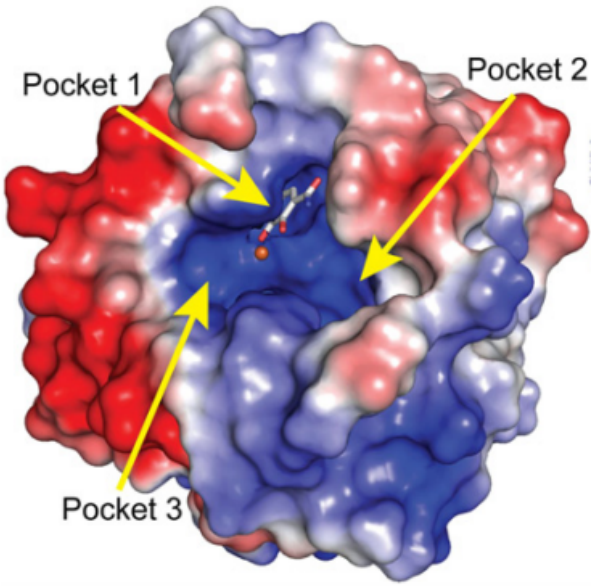


Figure 2: Crystal structure of p20K. Molecular surface representation of the crystal structure of p20K. Ligand binding pockets are indicated by the arrows, and electrostatic potential is depicted by the coloured regions. The siderophore is associated in pocket 1. Figure was obtained from Correnti *et al.*, 2011.

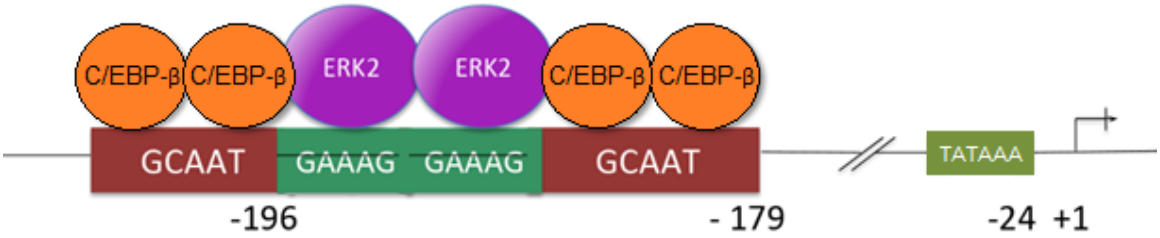


Figure 3: The transcriptional regulation of p20K. Schematic representation of the p20K QRU binding sites, where the binding of C/EBP-β to the QRU enables transcriptional activation and the binding of ERK2 to the QRU represses transcription. While all factors are represented here, the binding of C/EBP-β and ERK2 is mutually exclusive. Figure was created by Dr. André Bédard.

v. Fatty Acid Binding Protein 4 (FABP4)

FABP4, also referred to as aP2, is one of nine intracellular fatty acid-binding proteins (FABPs) involved in binding hydrophobic ligands non-covalently and reversibly. The functions of FABPs are diverse, and they participate in a variety of activities including the facilitation of fatty acid flux across the plasma membrane, intracellular fatty acid transport, storage compartmentalization and enzyme regulation in fatty acid metabolism. Each FABP is characterized by tissue-specificity, and FABP4 is expressed mainly in adipose tissue and macrophages. FABP4 interacts with the hormone sensitive lipase and is involved in trafficking fatty acids away from triglycerides after hydrolysis (Hertzel & Bernlohr 2000). Furthermore, FABP4 plays a role in the metabolic processes of diseases, such as diabetes, obesity and atherosclerosis. In aP2-deficient mice, there is reduced insulin resistance and decreased lipid transport efficiency. In macrophages, aP2-deficiency protects against early and advanced atherosclerosis in mice lacking apolipoprotein E. For these reasons, FABP4 can be a key target in pharmacologic therapy and drug development for the relevant metabolic syndromes (Furuhashi et al. 2007).

In CEF, FABP4 was strongly induced in conditions of contact inhibition and hypoxia similar to that of p20K. The interaction of C/EBP-β on the promoter region of p20K and FABP4 suggests that these Gas genes share similar regulatory mechanisms. CEF that overexpressed C/EBP-β also had an increased induction of FABP4, thus suggesting that C/EBP-β is an upstream regulator of FABP4 and p20K. Additionally, a chromatin immunoprecipitation (ChIP) assay conducted with CEF showed that C/EBP-β interacted with the FABP4 promoter region (Peragine, 2018). Lipocalins, such as p20K

and FABP4, require further characterization to understand their roles in mediating cellular stress responses.

3. Growth Arrest, Oxidative Stress, Lipid Peroxidation

Cells in vitro in favourable conditions are in the cell cycle and are proliferating. When the cellular environment is not optimal for proliferation and cells stop dividing, quiescent cells can be reactivated and enter the cell cycle once again. The balance between quiescent and proliferative cells is important because it protects cells against stress and ensures that cells meet the requirements of cell cycle checkpoints (Cheung & Rando, 2013).

Although quiescence has been understood as a passive or dormant state in the past, recent studies have identified transcriptional activity, gene regulation and CKI regulation in quiescent cells (Coller *et al.*, 2006). The growth arrest specific/Gas genes are able to prevent cells from undergoing terminal differentiation and apoptosis, while fibroblasts are even able to maintain relatively optimal metabolic rates in conditions of stress (Lemons *et al.*, 2010). The heterogeneity of quiescence suggests that specific initiating signals are able to induce distinct genes that share similarities in gene expression and regulation. Some widely studied conditions of quiescence include serum starvation, cell contact inhibition in high density populations and hypoxia (Yao, 2014).

Serum starved conditions include, but are not limited to, media with reduced growth factors or reduced hormones, and a possible reduction in basal cellular activity (Pirkmajer & Chibalin, 2011). Although fibroblasts placed in serum starved conditions for longer periods indicated a slower rate of exit from quiescence, they were still able to re-enter the cell cycle and actively divide (Yao, 2014). Cells that grow to high densities eventually stop proliferating and undergo contact inhibition. A study conducted by

Lemons et al. concluded that contact inhibited fibroblasts retain a high metabolic rate, particularly in processes such as glycolysis, the pentose phosphate pathway and the citric acid cycle. The study suggests that contact inhibited fibroblasts may degrade proteins and fatty acids at a higher rate than cycling fibroblasts. High levels of NADPH-generating enzymes (G6PD, PGD and IDH1) are also present in serum starved and contact inhibited fibroblasts, which may be required for providing glutathione and thioredoxin to lower free radicals. (Lemons *et al.*, 2010). Furthermore, extracellular matrix proteins in CI14 fibroblasts, such as fibronectin, collagen 21A1 and laminin alpha 2, were upregulated in conditions of contact inhibition, suggesting that these molecules may be involved in the final stages of wound healing or maintaining quiescent tissue (Lemons *et al.*, 2010; Senger *et al.*, 1983).

Low levels of oxygen can induce cellular growth arrest until cells recover from hypoxic conditions and re-enter the cell cycle. Cells respond to these environmental changes by utilizing anaerobic or glycolytic metabolic processes (Semenza, 2000a). Transcriptional activity induced by hypoxia can be further understood by analyzing the hypoxia response element (HRE) of the human erythropoietin gene. The hypoxia-inducible factor 1 (HIF-1) binds to HREs and is a key transcription factor that is present in hypoxic cells and absent in non-hypoxic cells (Semenza & Wang, 1992). This heterodimer is composed of the hypoxic response factor HIF-1α and the aryl hydrocarbon receptor nuclear translocator (ARNT/ HIF-1β) (Wang *et al.*, 1995). In oxygen-depleted conditions, HIF-1α translocates to the nucleus, interacts with cofactors ARNT and CBP/p300 and binds to HREs to activate transcription of hypoxia response genes

(Yamashita *et al.*, 2001). When oxygen is sufficient, HIF-1α binds to the tumour suppressor Von Hippel-Lindau (VHL) protein, is ubiquitylated, and finally degraded by the proteasome (Cockman *et al.*, 2000). HIF-1 plays a crucial role in cell cycle regulation, as it activates hypoxia-inducible genes controlling cell proliferation, apoptosis and growth arrest. HIF-1α is phosphorylated by p42/p44 MAPKs to activate other target genes that induce cell proliferation (Adrian, 2002; Conrad *et al.*, 1999). The phosphorylation of HIF-1α by p42/p44 MAPKs regulates the fate of cells in hypoxia, because phosphorylated HIF-1α binds to ARNT and activates cell survival genes while dephosphorylated HIF-1α binds to p53 and induces apoptosis (Darren *et al.*, 1999; Suzuki *et al.*, 2001). Furthermore, the WAF1/p21 and KIP1/p27 CKIs regulated cell cycle re-entry after hypoxic stress (Susannah *et al.*, 2001). The regulation of hypoxia-response genes is crucial in cell survival and tumour progression under hypoxic conditions, making hypoxia a key feature of tumorigenesis and conditions of high cell density.

A balance in oxygen levels regulates the generation of various factors involved in energy metabolism, vascular processes, cell signaling, and cell damage. Oxidative stress occurs when there is an imbalance of free radicals and reactive metabolites called reactive oxygen species (ROS) (Duracková, 2010). While normal metabolic processes produce ROS, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\bullet$) and organic peroxides, an overproduction of ROS in response to stressors in the cellular environment can damage cell structure and function (Fridovich, 1978; Fang *et al.*, 2009). A study conducted by Scortegagna *et al.* suggested that hypoxia-response genes may regulate oxidative stress processes, as mice with a HIF-2α deletion had multiple-organ

pathology and produced high levels of ROS (2003). Furthermore, mitochondrial ROS generation in cardiomyocytes increases in hypoxic conditions (Duranteau *et al.*, 1998).

The survival properties of tumorigenic cells can be linked to ROS production, as ROS can either increase cell proliferation and DNA damage or cause cell death depending on the type of cell or tissue in which they are localized and its concentration in these environments (Reuter *et al.*, 2010). For example, the nicotinamide adenine dinucleotide phosphate oxidase (Nox) enzyme family produces ROS and induces tumour cell survival in pancreatic and lung cancers (Kamata, 2009). On the other hand, various treatments use ROS, such as H_2O_2 or lipid peroxides, to induce apoptosis or programmed cell death (Jacobson, 1996).

Hypoxic conditions are further responsible for increased cellular non-esterified free fatty acids (FFAs) (Gardiner *et al.*, 1981). Under oxidative stress, ROS and free radicals can damage lipids with carbon-carbon double bonds in a process called lipid peroxidation. This process consists of the formation of a carbon-centered lipid radical that reacts with oxygen in forming a peroxy radical, which in turn makes a new lipid radical and hydroperoxide. This chain of events continues to take place until an antioxidant donates a hydrogen to the peroxy radical and forms other nonradical products. Polyunsaturated fatty acids (PUFAs), glycolipids, phospholipids and cholesterol are common targets of lipid peroxidation (Ayala *et al.*, 2014). Some products of this process include lipid hydroperoxides and aldehydes, such as malondialdehyde (MDA), propanal, hexanal and 4-hydroxynonenal (4-HNE) (Esterbauer *et al.*, 1991). Overall, it is essential to mitigate lipid peroxidation in biological membranes, as it results in diminished fluidity,

decreased membrane potential and the possibility for membrane rupture (Gutteridge, 1995).

Rationale & Objectives

Cells experience a variety of environmental stressors and conditions that assist in determining their proliferative fate. Quiescence is a particularly significant aspect of the cell cycle, as it allows the cell to survive potentially detrimental cellular stress and re-enter the cell cycle once conditions for growth become favourable. This process is remarkably coordinated and carried out by factors, such as Gas genes (Collet *et al.*, 2006; Schneider *et al.*, 1988). Conditions of growth arrest have been studied in CHM and CEF, where contact inhibition, hypoxia and serum starvation induce overlapping yet distinct gene regulation patterns. While Gas genes have been studied in environments of oxidative stress and lipid homeostasis, the mechanisms pertaining to their regulatory roles requires further analyses. Understanding the functions of Gas genes in conditions of reversible growth arrest helps to elucidate the molecular pathways involved in mitigating cellular stress responses.

The p20K lipocalin has been characterized as a Gas gene based on its expression in quiescent CEF. Upon incubating the cells in 1-1.8% oxygen to establish a hypoxic condition, cells adapted to the absence of aerobic metabolism by releasing hypoxia HIFs. Preliminary studies in the Bédard lab used proliferation assays, lipid peroxidation assays and TUNEL assays to study cell viability and apoptosis upon p20K downregulation and upregulation in CEF. These studies suggested that cell viability decreases when p20K is downregulated in oxygen-depleted conditions in comparison to control CEF. As p20K is a lipocalin that is involved in lipid transport, Oil Red-O lipid staining was also used in CEF exhibiting a downregulation of p20K during hypoxia to study lipid accumulation.

The findings suggested that p20K is required to transport lipids out of the cell in hypoxic conditions, as there was a greater accumulation of lipophilic dye in large lipid vesicles in the p20K-deficient cells in comparison to the control group when incubated in hypoxia (Erb, 2016). Furthermore, as a result of low oxygen, lipid peroxidation and the formation of reactive oxygen species (ROS) can be identified as possible outcomes of p20K downregulation in CEF. While some work pertaining to lipid peroxidation has been conducted in CEF with p20K downregulation, the quantification of ROS and its associated products would be valuable in the characterization of Gas genes regulating lipid homeostasis (Erb, 2016).

Both p20K and FABP4 are Gas genes that have demonstrated analogous expression patterns in both actively dividing and in growth arrest conditions. While these lipocalins are not present in actively dividing or serum starved CEF, they are upregulated at contact inhibition and in hypoxic conditions. Furthermore, if C/EBP-β functions as a regulator of both p20K and FABP4, their regulatory mechanisms may be similar and they may be involved in co-regulating the response to cellular stress. Studies should thus explore the individual roles of the p20K lipocalin and FABP4 in quiescence as well as a potential relationship which may exist between the two Gas genes as part of a larger lipid membrane homeostasis response.

Given the preliminary analyses of lipocalins in quiescence conditions, the primary focus of this study is to characterize the growth arrest specific p20K lipocalin in CEF under hypoxia and contact inhibition. Chapter 1 of this study will validate the pro-survival characteristics of p20K in quiescent CEF by analyzing cellular proliferation,

apoptosis and the response to oxidative stress. Chapter 2 of this study will then focus on characterizing the relationship between p20K and other lipocalins, such as FABP4. Based on the induction of the Gas genes p20K and FABP4 in response to cellular dysfunction and the proposed role of these factors in lipid shuttling, their regulation in lipid homeostasis is of key interest. Therefore, the findings from this study will contribute towards understanding the functionality of Gas genes in the novel "Membrane Stress Response" using CEF as a cellular model.

Materials & Methods

1. Chicken Embryo Fibroblasts Cell Culture and Culture Conditions

CEF were propagated in a complete medium containing Dulbecco's modified eagle medium (DMEM) high glucose 1x (Sigma D5546), 5% heat-inactivated cosmic calf serum (Hyclone AUA33984), 5% tryptose phosphate broth (Sigma T8782), 2mM L-Glutamine (Gibco 25030), 500 units of Penicillin and 500 μg of streptomycin (Gibco 15140). During cell passaging, complete media was removed from the cells using sodium chloride-tris-EDTA **(**STE) buffer (150 mM NaCl, 50 mM Tris, 1mM EDTA pH 7.2). Cells were resuspended in trypsin-EDTA (Sigma 59417C) to ensure that cells were dissociated. Cells that were used to prepare lysate were grown in 100 mm dishes, cells that were used for microscopic analysis were grown in 60 mm dishes on coverslips, cells that were used in proliferation assays were grown in 24-well dishes and cells that were used in absorbance assays were grown in 96-well microplates.

Cells were grown and harvested in conditions of cycling (normoxia), hypoxia, contact inhibition and serum depletion. Cycling CEF in normoxia were cultured at 41.5° C, 21% oxygen (O_2), 5% carbon dioxide (CO_2) and harvested at 40-60% confluence. Cells in hypoxia were grown in conditions at 41.5° C, 1.8% O_2, and 5% CO_2 for 24-30 hours. Contact-inhibited cells were grown to 100% confluence in 41.5° C, 21% O_2, and 5% CO_2, then replenished with fresh complete medium and harvested 24 hours later. Serum depleted samples were grown to 40-60% confluence in 41.5° C, 21% O_2, and 5% CO_2, then replenished with serum-free media (DMEM, 2mM L-Glutamine, 500 units of Penicillin, 500 μg of streptomycin) and harvested 48 hours later.

2. SDS-PAGE and Western Blotting

2.1 Protein Sample Preparation

1X phosphate-buffered saline (PBS) pH 7.4 was made with 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.47 mM KH_2PO_4 and then chilled. Upon the completion of the cell culture treatments, media was removed and the cells were washed with cold 1X PBS three times. The remaining PBS was aspirated, cells were scraped using a rubber scraper and collected in 1 mL of 1X PBS in a microcentrifuge tube. The cell suspensions were centrifuged at 7000 RPM for 3.5 minutes, and the supernatant was then removed. The cell pellet was resuspended in an appropriate volume of 1X sodium dodecyl sulfate (SDS) sample buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol, 60 mM Tris-HCl pH 6.8) with 1X Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher 78441) to ensure complete lysis. The samples were then vortexed for 15 seconds, boiled for 2 minutes and centrifuged at 13000 RPM for 5 minutes. The supernatant was then transferred to another microcentrifuge tube and stored in -80°C until required for a Bradford Assay or for SDS-Page/Western Blotting.

2.2 SDS-Page and Western Blot

Total cell protein extracts (50-75 µg) were subjected to SDS- polyacrylamide gel electrophoresis using gel concentrations of 12-14% depending on the size of the protein to be resolved. Following this, the samples were blotted to a nitrocellulose membrane (Schleicher and Schuell, BA85) and then blocked in 5% skim milk powder dissolved in 1X tris-buffered saline (TBS) (20 mM Tris pH 7.6, 140 mM NaCl) at room temperature for 1 hour. The membranes were subsequently incubated in a primary antibody made in a

5% skim milk and 1X TBS solution at 4°C overnight (see Table 1 for primary antibody concentrations).

Table 1: Primary Antibody Dilutions

Antibody	**Dilution**
p20K (601-Y - Bédard Lab)	1:500
FABP4 (LifeTein, South Plainfield, NJ, USA)	1:250
CHOP (Tulip Bleed 2 - Bédard Lab)	1:1500
ERK2 (Millipore #05-057)	1:2000

The following day, the blots were washed twice in 1X TBS, twice in 1X TBS-T (20 mM Tris pH 7.6, 140 mM NaCl, 0.1% Tween) and once in 1X TBS each for 5 minutes. The membranes were incubated in a secondary antibody conjugated with horseradish peroxidase (HRP) made in a 5% skim milk and 1X TBS solution at room temperature for 2 hours (see Table 2 for secondary antibody concentrations). The membranes were subsequently washed using the cycle described previously. The chemiluminescent signals were detected by incubating the blots in Luminata Forte Western HRP Substrate (Millipore WBLUF0100). The signal was revealed using hyperfilm (GE Healthcare 28906839) as per the manufacturer's protocols.

Table 2: Secondary Antibody Dilutions

Antibody	**Dilution**
Anti-Rabbit IgG, HRP-Linked Antibody (Cell Signaling #7074S)	1:25000
Anti-Mouse IgG, HRP-Linked Antibody (Cell Signaling #7076)	1:25000

3. shRNAi and Overexpression Vector Transfection

Prior to the vector transfection, 10 µg of the plasmid construct, 20 µg of salmon sperm DNA and 0.2M NaCl were combined along with twice that volume of 100% ethanol for overnight DNA precipitation. The following day, the $CaPO_4$ transfection protocol described by Graham & Eb was used (Graham FL & van der Eb AJ, 1973). Subconfluent CEF was replenished with fresh complete media 4 hours prior to the transfection. The DNA precipitate solution was centrifuged at 13000 RPM for 15 minutes. Upon aspirating the supernatant, the DNA precipitate was washed with 70% ethanol, vacuum dried and resuspended in 200 µL of sterile ddH_2O. 62 µL of 2M $CaCl_2$ and 238 µL of sterile ddH_2O were added to the solution. The sample was added dropwise to 500 µL of 2X HBSP pH 7.12 buffer (15 mM Na2HPO4, 10 mM KCl, 280 mM NaCl, 12 mM Glucose, 50 mM HEPES) and vortexed. The DNA mixture was incubated for 20 minutes and then added dropwise to cells in a 100 mm culture dish for 5 hours. After the incubation, the media was aspirated and the cells were shocked with 4 mL of a glycerol solution (15% sterile glycerol, 1X HBSP) for 1 minute. The glycerol solution was aspirated, the cells were washed twice with complete media and subsequently incubated

in normoxic cell culture conditions overnight. The cells were split and cultured at 90% confluency for a minimum of 2 passages prior to analyses.

4. Proliferation Assays

CEF from confluent 100 mm dishes were passaged and seeded into 24-well microtiter dishes. Upon cell adhesion and proliferation, cells were trypsinized and resuspended in 1 mL of Trypsin- EDTA for single-cell dissociation. The cell suspension was transferred to a counting cup and diluted with 9 mL of ISOTON® II Diluent (Beckman Coulter 8546719). Using a Coulter counter (Beckman Coulter), cells were counted in quadruplicate after incubation for 24 hours, 48 hours and 72 hours in the specified culture conditions.

5. TUNEL Assay

CEF from confluent 100 mm dishes were passaged and seeded onto coverslips in 60 mm dishes and cultured in conditions of normoxia and hypoxia for 30 hours. Upon the completion of the incubation, the cells were washed three times with sterile, cold 1X PBS and fixed in 4% paraformaldehyde in 1X PBS for 30 minutes in room temperature. The cells were washed three times with sterile, cold 1X PBS again followed by placing the cells on ice and adding 3 mL of the permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate) to each dish. Upon subsequent washes with 1X PBS, the cells were incubated with terminal deoxynucleotidyl transferase (TdT) and TMR red-fluorescent labelled dUTP (Roche 12156792910) for 1 hour in 37°C. The cells were washed with 1X PBS and stained with Hoechst 33342 (Thermo Fisher H1399) for 2 minutes, followed by washes in 1X PBS and then ddH_2O. The coverslips were mounted on a drop of 1X PBS

and imaged in quadruplicate, ensuring that 100 cells per sample were counted. The number of TUNEL positive cells were counted per 100 cells, and the average of the quadruplicates was used to calculate the percentage of TUNEL positive cells.

6. DCFDA Cellular ROS Detection Assay

CEF from confluent 100 mm dishes were passaged, and 2.5 x 10^4 cells were seeded in quadruplicate onto a 96-well microtitre plate for a 30 hour incubation in normoxia or hypoxia. CEF treated with a 55μM tert-butyl hydrogen peroxide (TBHP) and incubated for 1 hour in normoxia were utilized as a positive control. Cells were then washed with 1X Buffer and stained with 25 μM DCFDA in 1X Buffer for a 45 minute incubation in the conditions of normoxia or hypoxia. Cells were washed again with 1X Buffer and the fluorescence intensity was measured at Ex/Em = 485/535 nm using a cytation plate reader. The reagents and adapted protocol were provided in the DCFDA Cellular ROS Detection Assay Kit (Abcam ab113851).

Results

Chapter 1: The p20K Lipocalin Exhibits Pro-Survival Characteristics and is Involved in Mediating Oxidative Stress in Hypoxic Chicken Embryo Fibroblasts

P20K expression is induced by hypoxia but down-regulated in cells transfected with the RCASBP(A) - p20K RNAi vector. Hypoxia is a feature of contact inhibition in CEF and it induces the expression of p20K in CEF placed in conditions where O_2 levels are less than 2% (Erb *et al.*, 2016). The mis-expression of p20K allows for the analyses of the p20K lipocalin functionality in chicken embryo fibroblasts in conditions of growth arrest, such as hypoxia, contact inhibition and serum deprivation. CEF transfected with a group A retroviral vector expressing a control shRNA for GFP (RCASBP (A) - GFP RNAi) or a shRNA for p20K down-regulation (RCASBP (A) – p20K RNAi) were incubated in normoxic (21% O_2) and hypoxic (1.8% O_2) conditions for 24 hours. The western blot analysis of the respective samples demonstrates that p20K is not expressed during conditions of normoxia in either cell type. In contrast, p20K is expressed in the control hypoxia sample but is down-regulated in the p20K shRNA sample when incubated in conditions of low oxygen (Fig. 4 A,B). This confirms that the CEF transfected with the RCASBP(A) - p20K RNAi vector in subsequent experiments down-regulates p20K.

Cell accumulation is impaired by the shRNA-mediated down-regulation of p20K in chicken embryo fibroblasts. As hypoxia and saturation density can impair cell proliferation, cell survival and proliferation of CEF with p20K mis-expression in conditions of growth arrest conditions were studied. Control cells transfected with the

RCASBP(A) - GFP RNAi vector continued to proliferate for three days as depicted by increasing cell numbers (Fig. 5). Upon measuring the cell proliferation of the p20K shRNA samples in normoxia, cell proliferation continued, but at a lower rate than the control samples. All samples incubated in conditions of hypoxia (1.8% O_2) showed lower rates of cell proliferation in comparison to CEF incubated in conditions of normoxia, however the p20K shRNA samples proliferated at a significantly lower rate when exposed to low oxygen in comparison to the control samples in hypoxic conditions as measured at all three time points (Fig. 5). These results indicate that p20K is necessary for normal cell proliferation or survival in conditions of hypoxia and as cells become contact inhibited.

Increased apoptosis is induced by the shRNA-mediated down-regulation of p20K in chicken embryo fibroblasts in conditions of hypoxia. A TUNEL assay analysis of sub-confluent control and p20K shRNA CEF in conditions of normoxia (21% O_2; 24 hours) revealed negligible levels of apoptosis in both groups (Fig. 6A). In conditions of hypoxia (1.8% O_2; 30 hours), control CEF had low levels of apoptosis, however p20K shRNA CEF had an increased level of apoptosis by an approximate factor of five (Fig. 6 B,C). The TUNEL-positive cells that were indicative of apoptosis also showed chromatin condensation and nuclear fragmentation when visualized with the Hoechst 33342 fluorescent stain.

Increased cellular reactive oxygen species are released by the shRNA-mediated down-regulation of p20K in chicken embryo fibroblasts in conditions of hypoxia. Using the DCFDA reactive oxygen species assay to quantify the release of ROS, a significant

increase in cellular ROS in both the control and p20K shRNA samples in hypoxia (1.8% O_2; 30 hours) was noted in comparison to the samples grown in normoxia (21% O_2; 24 hours) (Fig. 7A). When comparing the cellular ROS levels between the control and p20K shRNA samples in hypoxia, CEF with p20K mis-expression exhibit slightly greater cellular ROS release than the control sample (Fig. 7A). Increased cellular ROS levels in response to p20K down-regulation suggest that p20K is required to alleviate the effects of lipid oxidation in conditions of hypoxia.

Chapter 2: The Mis-expression of the p20K Lipocalin Alters the Expression of FABP4 in Chicken Embryo Fibroblasts in Various Conditions of Growth Arrest

FABP4 expression is impaired in chicken embryo fibroblasts with shRNA-mediated down-regulation of p20K in conditions of contact inhibition and hypoxia. Similar to p20K, the expression of FABP4 is a feature of growth arrest conditions, such as hypoxia and contact inhibition, but not of sub-confluent, normoxic cells. To understand the relationship between p20K and FABP4 in a regulatory pathway regulated by the transcription factor C/EBP-β, control and p20K shRNA samples were incubated in conditions of normoxia (21% O_2; 24 hours), hypoxia (1.8% O_2; 24 hours) and contact inhibition and protein samples were analyzed using western blots. In conditions of normoxia, there was minimal to no FABP4 expression in the control and p20K shRNA samples (Fig. 8, 9). As for p20K, FABP4 expression was induced by hypoxia and contact inhibition in control cells (Fig. 8, 9). Surprisingly, when p20K shRNA samples were incubated in hypoxia, both p20K and FABP4 expression were impaired (Fig. 9). Similarly, contact-inhibited p20K shRNA samples indicated significantly reduced levels of p20K and FABP4 expression (Fig. 8).

FABP4 expression is impaired by the ectopic up-regulation of p20K in chicken embryo fibroblasts in conditions of contact inhibition. CEF transfected with a group B retroviral vector control (RCASBP (B)) or a vector expressing the p20K cDNA (RCASBP (B) – p20K) were incubated in normoxic (21% O_2, cycling) and hypoxic (1.8% O_2) conditions for 24 hours. In conditions of normoxia, there was minimal or no FABP4 expression in the control sample (Fig. 10). Western blot analyses indicated that

the p20K overexpression sample showed increased levels of p20K but low levels of FABP4 expression in normoxia (Fig. 10). However, the upregulation of both p20K and FABP4 was observed at contact inhibition (Fig. 10). In these conditions, higher levels of p20K were only observed at contact inhibition while FABP4 levels were actually reduced upon ectopic expression of p20K (Fig. 10). Since FABP4 expression in the p20K overexpression sample incubated to saturation density was slightly less than FABP4 levels in the confluent control sample, it cannot be concluded that FABP4 and p20K expression are positively correlated in p20K overexpression samples grown to contact inhibition. FABP4 expression in the contact inhibited p20K overexpression sample may simply be as a result of the growth arrest condition rather than as a result of enhanced p20K expression.

Discussion

Control of P20K gas gene by shRNA expression in quiescence. In understanding the fates of cell-cycle arrest, quiescence is unique in that it allows cells to re-enter the cell cycle upon the restoration of nutrients, mitogens and growth factors (Terzi *et al.*, 2016). By identifying Gas genes, the dynamic processes involved in allowing cells to remain in a reversible and nondividing state can be defined. The induction of the p20K lipocalin expressed under specific conditions of quiescence in CHM and CEF can be attributed to regulatory elements of the QRU that promote its activation (Mao *et al.*, 1992). P20K, which can therefore be considered as a Gas gene, is a gene of interest with regards to understanding the cellular stress response particularly in CEF. By generating a retroviral shRNAi RCASB(P) vector that mis-expresses p20K and transfecting it in CEF (Erb, 2016), an effective cellular model was used to study the effects of p20K in growth arrested conditions. In accordance with previous studies that reported a repression of p20K expression in actively dividing CEF (Bédard *et al.*, 1987), proliferating control (RCASBP (A) – GFP RNAi) and p20K shRNAi samples expressed negligible levels of p20K (Fig. 4). Additionally, hypoxic quiescence induced p20K expression in the control sample, but its expression was repressed in the p20K shRNAi transfectant (Fig. 4). This western blot analysis validates that p20K was downregulated by the p20K shRNAi transfectant in quiescent CEF in subsequent experiments.

Role of p20K in cell survival. Growth arrested cells exhibit a unique response to stressful conditions that differs from cellular senescence. Senescent cells undergo a degenerative, irreversible process that may result in cell death. Replicative senescence

occurs as a result of irregular telomerase activity when the DNA damage response persists (Masutomi *et al.*, 2003). Physiological senescence or irreversible growth arrest is observed in differentiated cells undergoing "permanent quiescence" due to the presence of CKIs (Blagosklonny, 2013). Another irreversible mechanism that halts cell division is observed in apoptosis or programmed cell death (Horvitz, 1999). Apoptosis can be both an anti-tumorigenic defense mechanism that occurs when cells may exhibit ectopic proliferation as well as a homeostatic mechanism to regulate cell population and damage (Norbury and Hickson, 2001). As this study focuses on the role of p20K in hypoxia, it is important to note that apoptosis can occur when cells are placed in severe and sustained hypoxic conditions. It has been proposed that HIF-1α can induce apoptosis by either binding to p53 and facilitating a cascade of events or through its involvement in producing the proapoptotic protein BNIP3 (Chen *et al.*, 2003; Bruick, 2003). The results from the TUNEL assay completed in this study verified these findings, because there were more apoptic cells in the samples placed in hypoxia (1.8% O_2) for 30 hours in comparison to actively dividing cells in normoxia (21% O_2) (Fig. 6A). Significantly, cells lacking p20K displayed a five-fold increase in apoptosis in comparison to control cells both in hypoxia (Fig. 6B). Additionally, the cell proliferation assay indicated that differences in cell numbers or cell accumulation were greater between control cells and cells with p20K downregulation when cells were hypoxic or approaching cell confluence at normoxia (Fig. 5). In both experimental conditions, CEF that downregulate p20K proliferated at lower rates in comparison to the control, however p20K should not be expressed in cycling cells unless some regions of the dish showed uneven seeding (Fig.

5). Given that these cells are incubated in the respective conditions for 72 hours, it could be that the cells are undergoing contact inhibition at later time points, which is another quiescence condition. Previous studies have validated p20K expression in both hypoxia and contact inhibition, therefore the downregulation of p20K in contact inhibited cells may contribute towards the decrease in cell number. When assessing the results from the TUNEL and proliferation assays, it is evident that p20K is required for cells to adapt to the conditions of quiescence, at best as determined by hypoxia.

Role of p20K in environments with increased oxidative stress. Although several conditions of quiescence have been studied, the role of p20K in hypoxia can further elucidate its involvement as a lipocalin in mitigating the effects of lipid peroxidation. Previous findings from the Bédard lab began to identify the presence of ROS and markers of lipid peroxidation in p20K shRNAi CEF placed in hypoxia. While the C11-BODIPY$^{581/591}$ fluorescent probe provided a visual analysis of the localization of lipid peroxidation and the MDA colorimetric assay measured cellular MDA levels, these experiments showed evidence for increased lipid peroxidation in hypoxic CEF with p20K downregulation (Erb *et al.*, submitted). These conditions also displayed increased levels of 4-HNE, which is another byproduct of lipid peroxidation and mediator of apoptosis under oxidative stress (Erb *et al.,* submitted; Sharma *et al.*, 2008). To quantify the presence of ROS, such as hydroxyl and peroxyl, the control and p20K mis-expressing CEF were placed in normoxia or hypoxia and probed with the DCFDA/H_2DCFDA fluorogenic dye (Fig. 7). Although all samples placed in hypoxia produced high levels of ROS, the samples with p20K downregulation had undergone increased oxidative stress as

indicated by higher levels of ROS production. Based on these findings, it is clear that p20K plays a role in lipid homeostasis and in an adaptive stress response mechanism, as p20K is necessary to minimize effects of cell death and lipid peroxidation.

Expression of the FABP4 lipocalin upon p20K mis-expression. As another member of the family of lipocalins, FABP4 exhibits similar patterns of expression as p20K under specific conditions of quiescence and is also regulated by C/EBP-β (Peragine, 2018). To further explore whether one gene regulates the other or if both are co-regulated under conditions of quiescence, the control and p20K shRNAi CEF in normoxia, hypoxia and contact inhibition were compared. More specifically, FABP4 was compared to p20K in the specified conditions to investigate how FABP4 expression was affected by the mis-expression of p20K. While cells in normoxia did not express p20K or FABP4, contact inhibited and hypoxic control CEF expressed both p20K and FABP4 as expected (Fig. 8, 9). The key finding from the western blot analysis shows that when p20K is downregulated in both conditions of quiescence, FABP4 expression is also negligible (Fig. 8, 9). CEF that overexpressed p20K were also analyzed under contact inhibited conditions, however the overexpression of p20K did not increase FABP4 expression (Fig. 10). The FABP4 expression in contact inhibited samples that overexpressed p20K may alternatively be attributed to the cellular growth arrest condition. Preliminary results did not show an evidence of CHOP upon p20K downregulation (our unpublished results). As both p20K and FABP4 are controlled by C/EBP- β, an increased level of CHOP would provide an explanation for the co-

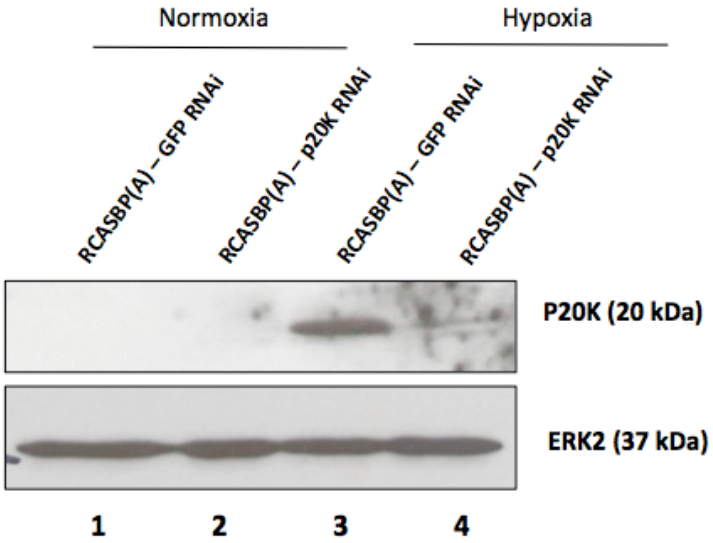
regulation of p20K and FABP4 by the p20K shRNAi. To this date, the mechanism of the co-regulation remains unknown.

Conclusion & Future Directions

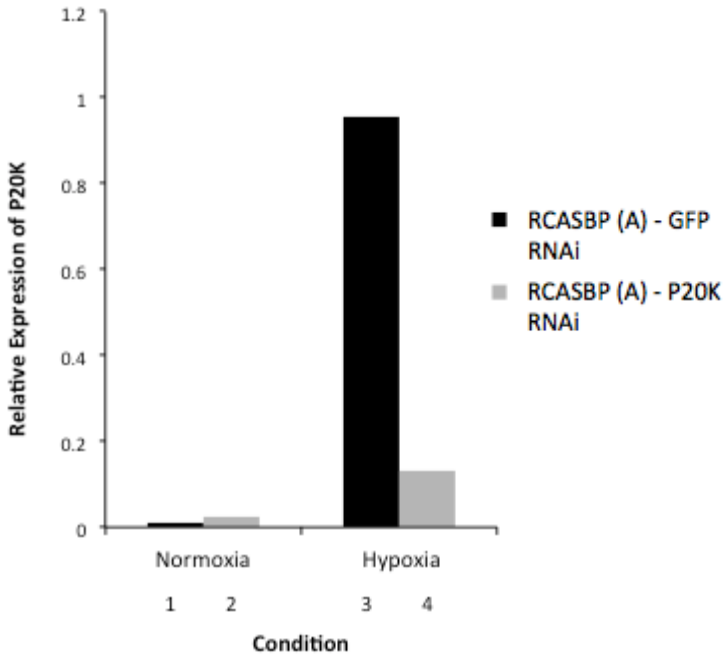
The cellular response to stressors that cause cells to enter the G_0 phase can be understood by studying Gas genes, such as p20K and FABP4. Under conditions of hypoxia, the regulation of both lipocalins in mitigating cellular stress can provide insight into understanding the "Membrane Stress Response" (Erb *et al.*, submitted). The findings from this study provide further evidence for the role of the p20K lipocalin in pro-survival mechanisms and in minimizing cellular damage resulting from oxidative stress. Furthermore, it is evident that p20K mis-expression also alters the expression of FABP4 in hypoxic and contact inhibited CEF by a mechanism that remains to be characterized.

The p20K and FABP4 lipocalin genes have been classified as Gas genes, however future studies may involve further analysis of FABP4 and its regulation in relation to p20K. Upon validating a FABP4 shRNA, the FABP4 gene can be further analyzed in similar conditions of quiescence. More specifically, the role of FABP4 in survival and lipid peroxidation can be assessed utilizing the same experiments conducted in this study, and these findings can be used to determine whether its cellular activity resembles that of p20K. Additionally, FABP4 mis-expression experiments can be used to detect p20K expression in conditions of quiescence to elucidate the regulatory relationship between both genes. Overall, the characterization of various Gas genes provides for a better understanding of the complex and dynamic processes involved in cellular quiescence.

Experimental Figures



A.



B.

Figure 4. A) Western blot analysis of p20K in CEF transfected with a group A retroviral vector expressing a control shRNA for GFP (RCASBP (A) - GFP RNAi) or a shRNA for p20K (RCASBP (A) – p20K RNAi) incubated in normoxic (21% O_2) and hypoxic (1.8% O_2) conditions for 24 hours. The blot was probed for ERK2 (loading control). B) The relative expression of p20K was quantified by correcting for protein loading using ERK2.

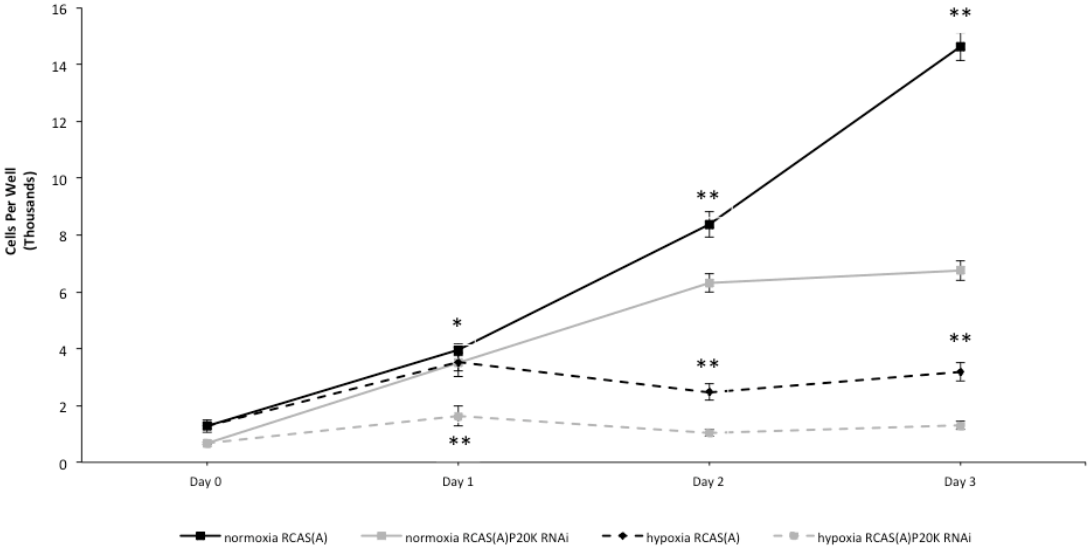
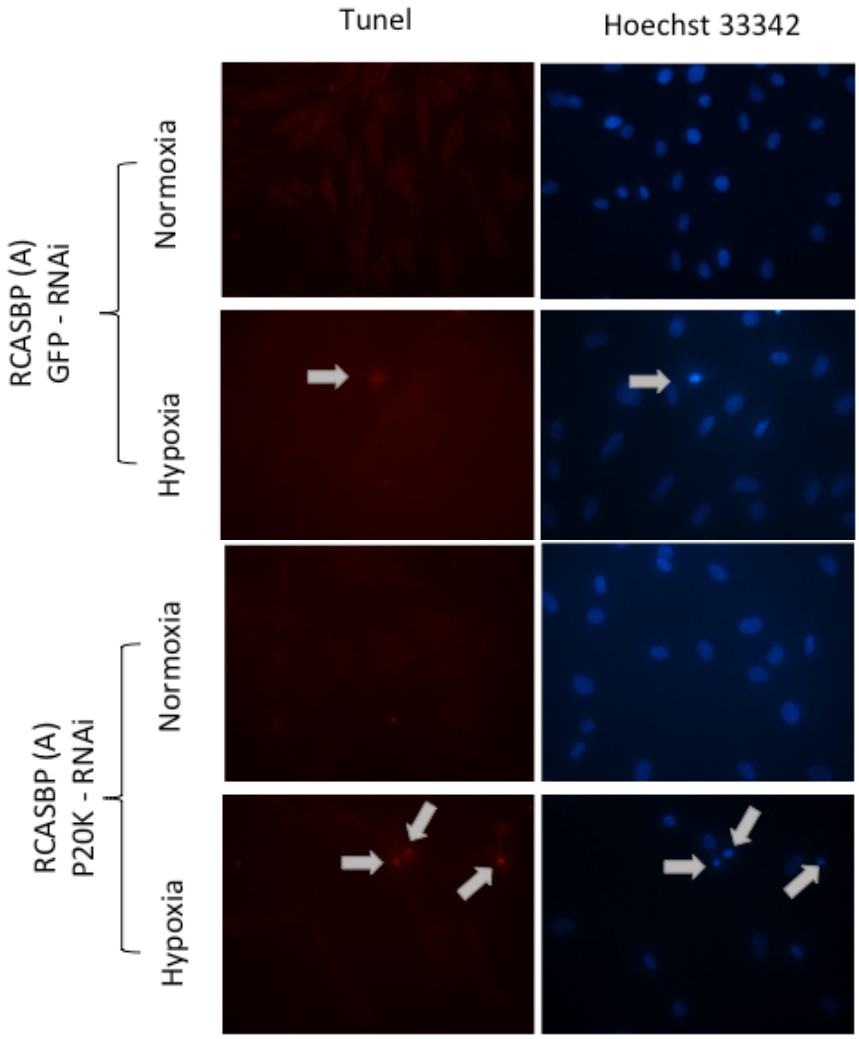
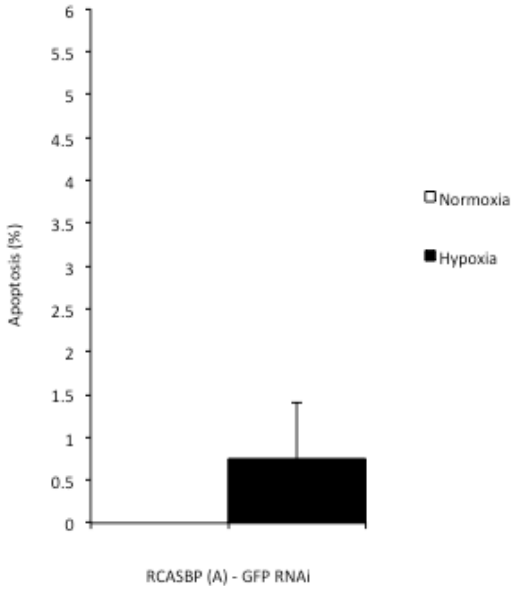

Cells Per Well (Thousands)
16
14
12
10
8
6
4
2
0
Day 0
Day 1
Day 2
Day 3
*
**
**
**
**
**
normoxia RCAS(A)
normoxia RCAS(A)P20K RNAi
hypoxia RCAS(A)
hypoxia RCAS(A)P20K RNAi

Figure 5. Proliferation of CEF transfected with a group A retroviral vector expressing a control shRNA for GFP (RCASBP (A) - GFP RNAi) or a shRNA for p20K (RCASBP (A) – p20K RNAi) were incubated in normoxic (21% O_2) and hypoxic (1.8% O_2) conditions. Day 0 represents the cell count taken prior to the hypoxia incubation. Day 1, 2 and 3 represent the subsequent cell counts taken at intervals of 24 hours after incubation in normoxia and hypoxia. Error bars represent the mean standard deviation of cell counts measured in quadruplicate. The statistical analysis for this assay was conducted using the two-tailed t-test. Asterisks represent significance between samples within the same conditions, where * indicates $P \leq 0.05$ and ** indicates $P \leq 0.01$.

A.


Tunel
Hoechst 33342
RCASBP (A)
GFP - RNAi
Normoxia
Hypoxia
RCASBP (A)
P20K - RNAi
Normoxia
Hypoxia

B.


Apoptosis (%)
6
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Normoxia
Hypoxia
RCASBP (A) - GFP RNAi

C.

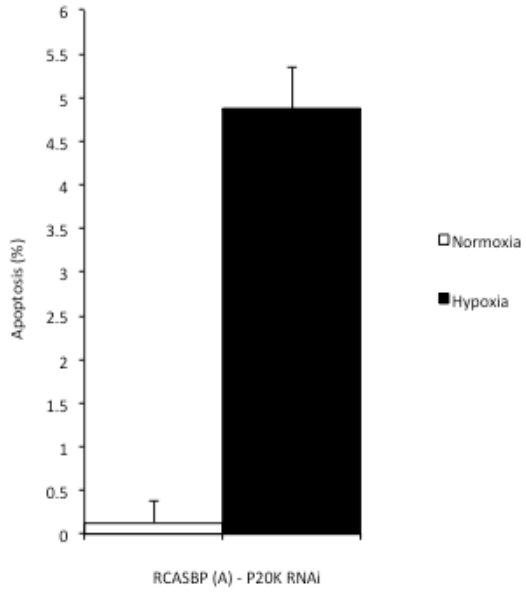
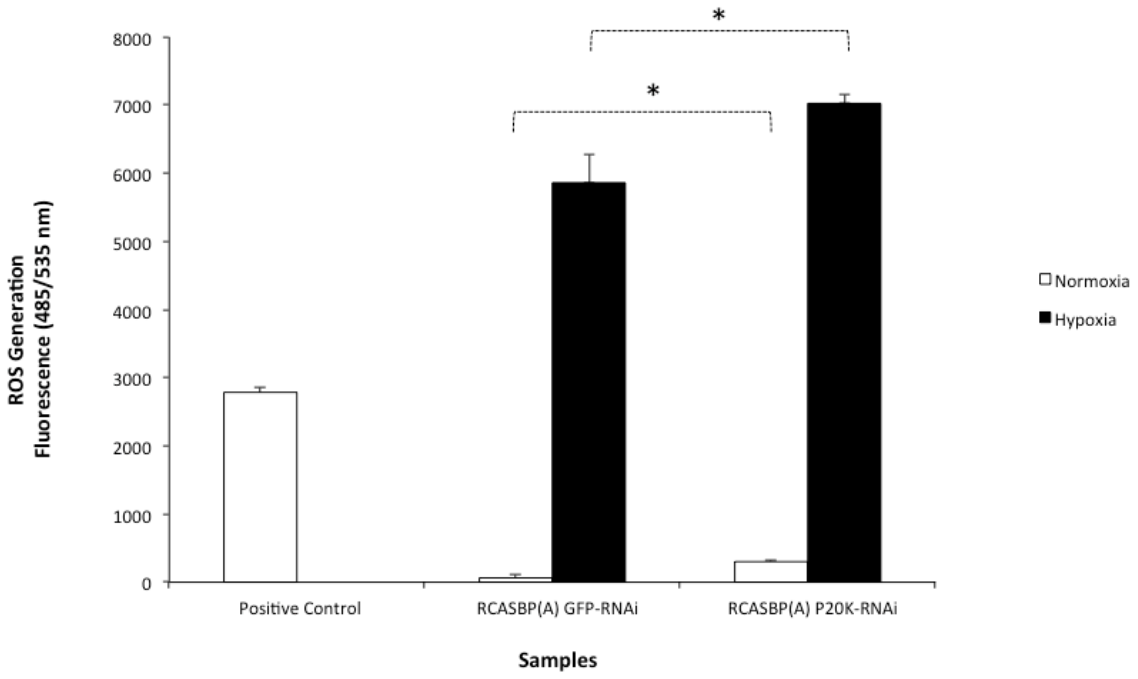
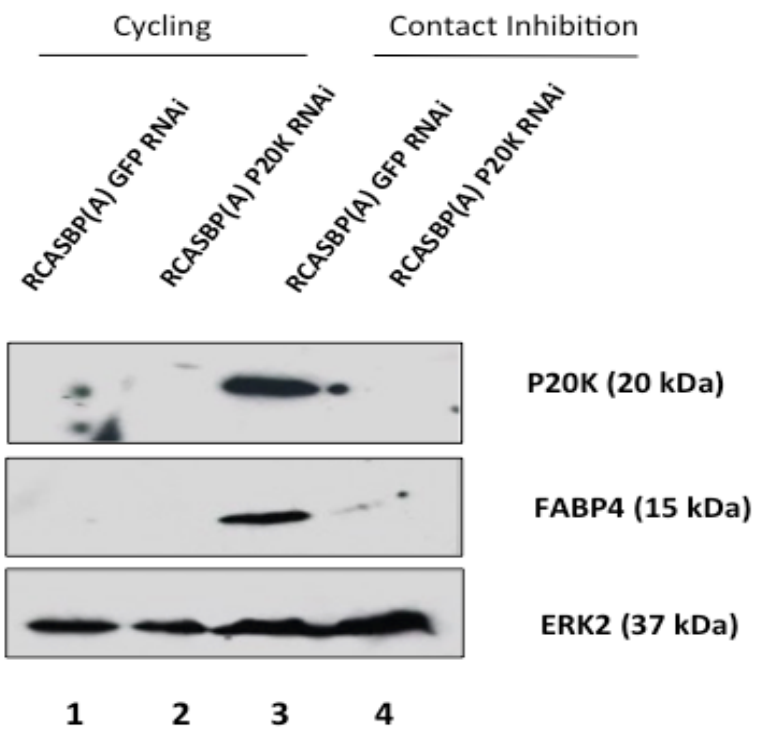

Apoptosis (%)
6
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Normoxia
Hypoxia
RCASBP (A) - P20K RNAi

Figure 6. A) Fluorescent staining with TUNEL assay. CEF transfected with a group A retroviral vector expressing a control shRNA for GFP (RCASBP (A) - GFP RNAi) or a shRNA for p20K (RCASBP (A) – p20K RNAi) were incubated in normoxic (21% O_2) and hypoxic (1.8% O_2) conditions for 30 hours. The left panel represents cells stained with TdT and TMR red-fluorescent labelled dUTP. The right panel represents cells stained with Hoechst 33342. Arrows on the left panel point to TUNEL-positive (apoptotic) cells, and arrows on the right panel point to cells undergoing chromatin condensation and nuclear fragmentation. B) The levels of apoptosis in RCASBP (A) GFP - RNAi and C) RCASBP (A) p20K - RNAi were quantified. Error bars represent the mean standard deviation of the levels of apoptosis per 200 cells measured in quadruplicate.

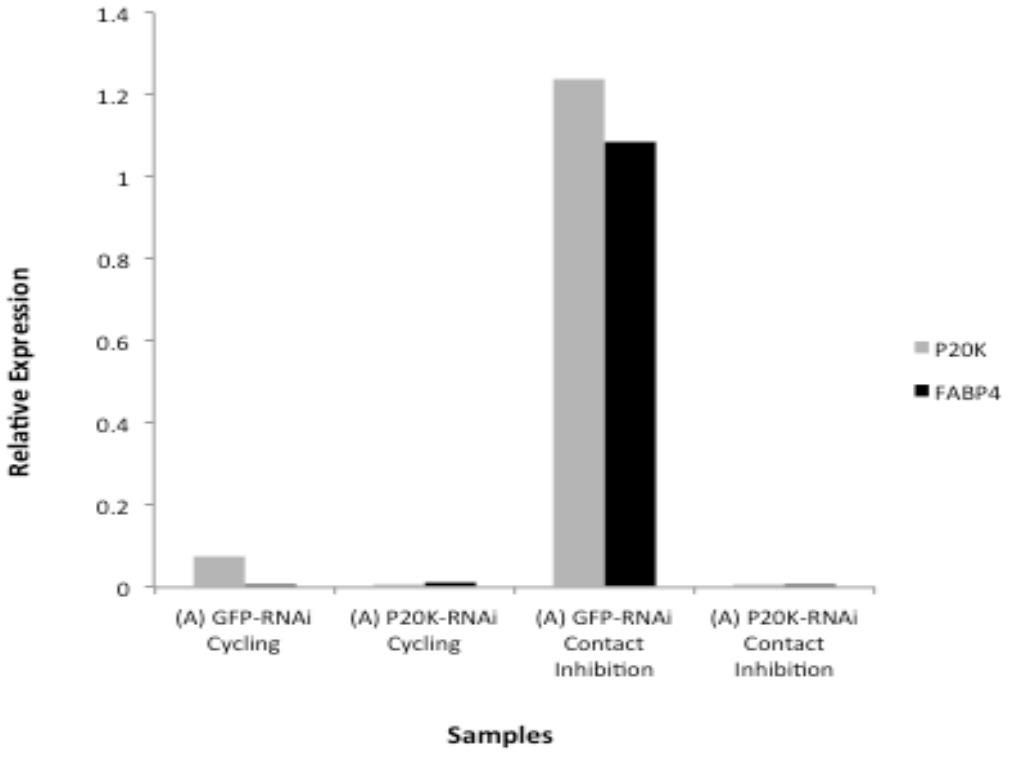

*
*
8000
7000
6000
5000
4000
3000
2000
1000
0
ROS Generation
Fluorescence (485/535 nm)
Positive Control
RCASBP(A) GFP-RNAi
RCASBP(A) P20K-RNAi
Samples
Normoxia
Hypoxia

A.

Figure 7. A) Quantitation of cellular reactive oxygen species (ROS) with DCFDA reactive oxygen species assay. CEF transfected with a group A retroviral vector expressing a control shRNA for GFP (RCASBP (A) - GFP RNAi) or a shRNA for p20K (RCASBP (A) – p20K RNAi) were incubated in normoxic (21% O_2) and hypoxic (1.8% O_2) conditions for 30 hours. CEF treated with 55μM TBHP served as a positive control. Error bars represent the mean standard deviation of the fluorescence intensity measured in quadruplicate. Asterisks represent significance between samples within the same conditions, where * indicates $P \leq 0.05$.

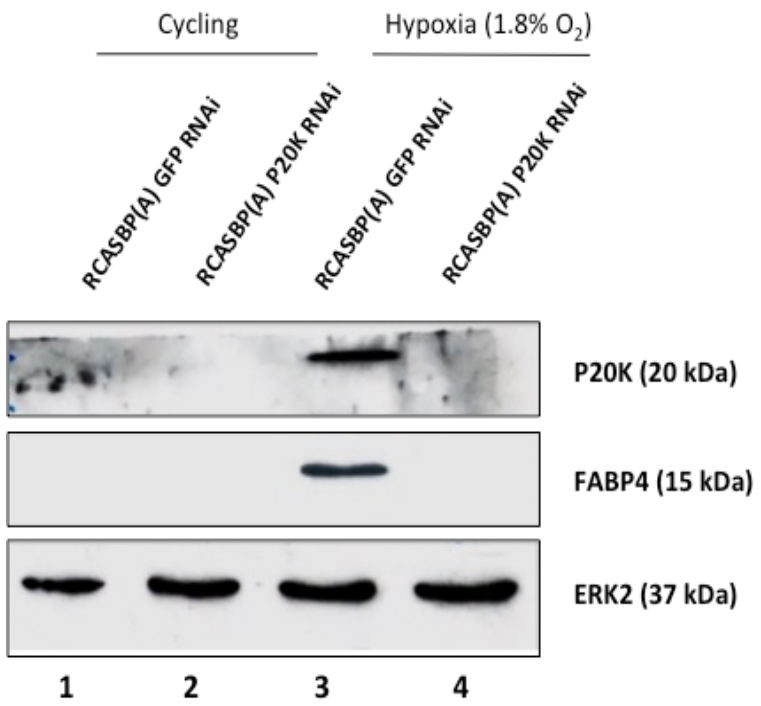

Cycling
Contact Inhibition
RCASBP(A) GFP RNAi
RCASBP(A) P20K RNAi
RCASBP(A) GFP RNAi
RCASBP(A) P20K RNAi
P20K (20 kDa)
FABP4 (15 kDa)
ERK2 (37 kDa)
1
2
3
4

A.

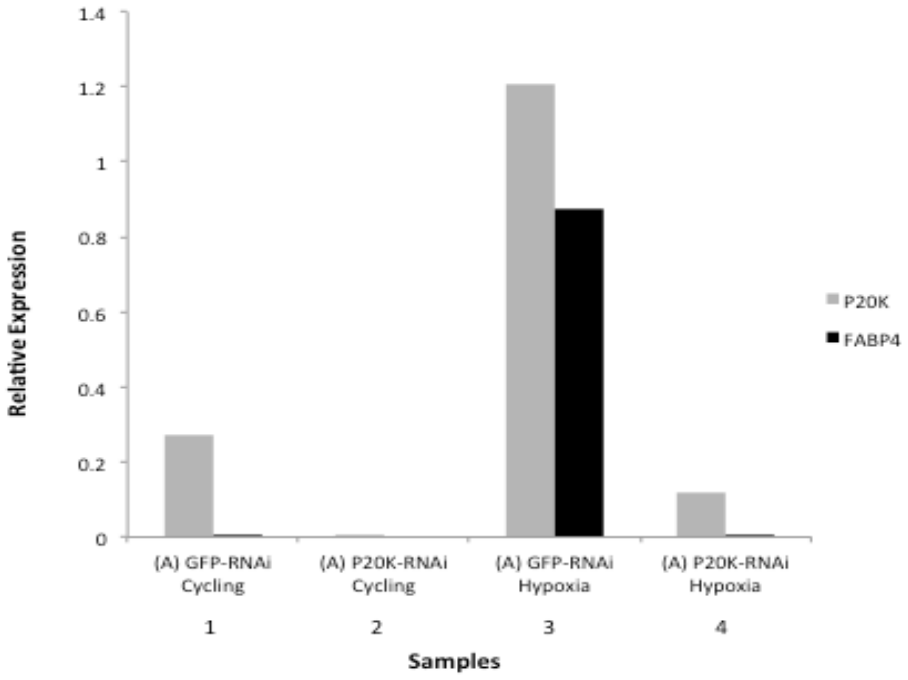

1.4
1.2
1
0.8
0.6
0.4
0.2
0
Relative Expression
P20K
FABP4
(A) GFP-RNAi Cycling
(A) P20K-RNAi Cycling
(A) GFP-RNAi Contact Inhibition
(A) P20K-RNAi Contact Inhibition
Samples

B.

Figure 8. A) Western blot analysis of p20K and FABP4 in CEF transfected with a group A retroviral vector expressing a control shRNA for GFP (RCASBP (A) - GFP RNAi) or a shRNA for p20K (RCASBP (A) – p20K RNAi) incubated in cycling, normoxic (21% O_2) and contact inhibited conditions. The blot was probed for ERK2 (loading control). B) The relative expression of p20K and FABP4 were quantified by correcting for protein loading using ERK2.

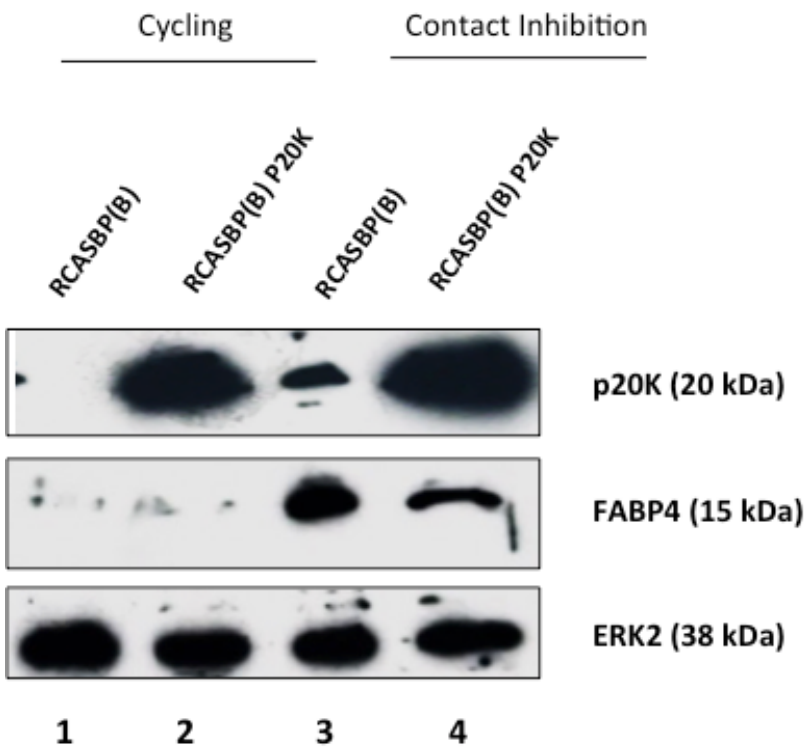

Cycling
Hypoxia (1.8% O_2)
RCASBP(A) GFP RNAi
RCASBP(A) P20K RNAi
RCASBP(A) GFP RNAi
RCASBP(A) P20K RNAi
P20K (20 kDa)
FABP4 (15 kDa)
ERK2 (37 kDa)
1
2
3
4

A.

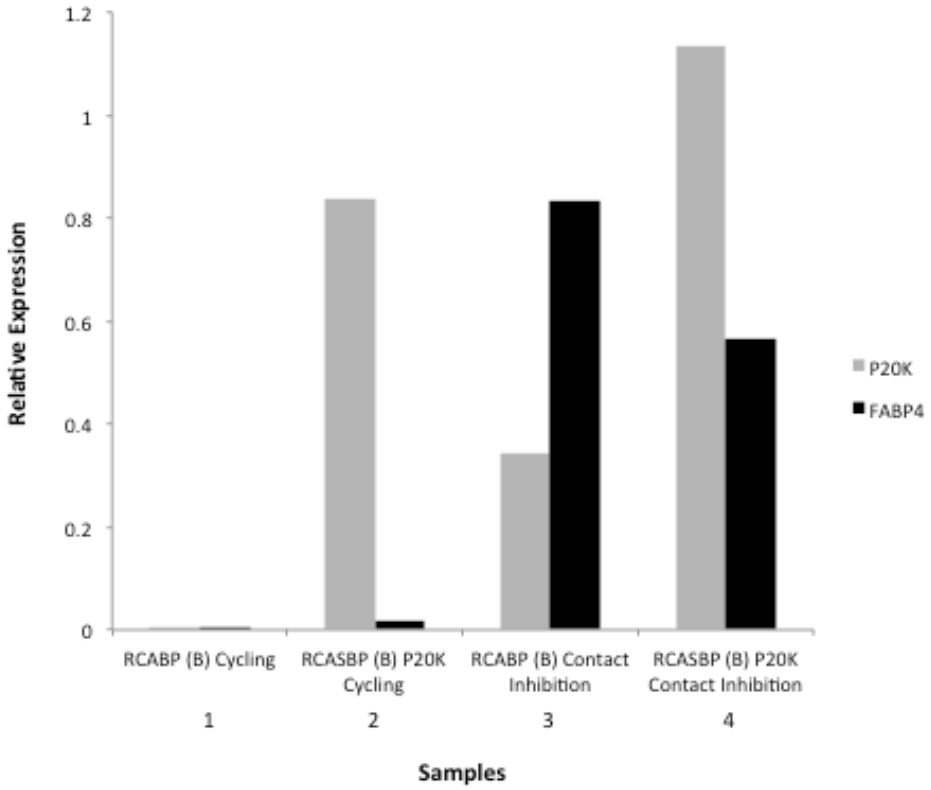

Relative Expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
P20K
FABP4
(A) GFP-RNAi Cycling
(A) P20K-RNAi Cycling
(A) GFP-RNAi Hypoxia
(A) P20K-RNAi Hypoxia
1
2
3
4
Samples

B.

Figure 9. A) Western blot analysis of p20K and FABP4 in CEF transfected with a group A retroviral vector expressing a control shRNA for GFP (RCASBP (A) - GFP RNAi) or a shRNA for p20K (RCASBP (A) – p20K RNAi) incubated in cycling, normoxic (21% O_2) and hypoxic (1.8% O_2) conditions. The blot was probed for ERK2 (loading control). B) The relative expression of p20K and FABP4 were quantified by correcting for protein loading using ERK2.


Cycling
Contact Inhibition
RCASBP(B)
RCASBP(B) P20K
RCASBP(B)
RCASBP(B) P20K
p20K (20 kDa)
FABP4 (15 kDa)
ERK2 (38 kDa)
1
2
3
4

A.


1.2
1
0.8
0.6
0.4
0.2
0
Relative Expression
P20K
FABP4
RCABP (B) Cycling
RCASBP (B) P20K Cycling
RCABP (B) Contact Inhibition
RCASBP (B) P20K Contact Inhibition
1
2
3
4
Samples

B.

Figure 10. A) Western blotting analysis of p20K and FABP4 in CEF transfected with a group B control retroviral vector (RCASBP (B)) or a vector overexpressing p20K (RCASBP (B) – p20K) incubated in cycling, normoxic (21% O_2) and contact inhibited conditions. The blot was probed for ERK2 (loading control). B) The relative expression of p20K and FABP4 were quantified by correcting for protein loading using ERK2.

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