CHARACTERIZING THE EXPRESSION AND REGULATION OF FABP4 IN RESPONSE TO GROWTH ARREST AND HYPOXIA IN CHICKEN EMBRYO FIBROBLASTS

Characterizing the expression and regulation of FABP4 in response to growth arrest and hypoxia in Chicken Embryo Fibroblasts

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Abstract:

The process of reversible growth arrest, otherwise known as cellular quiescence or the G_0 phase denoted by withdrawal from the cell cycle, is a poorly characterized state. Subsets of growth arrest-specific (GAS) genes are upregulated during quiescence, however, these subsets are specific to/dependent on the limiting factor or circumstance inducing growth arrest. Here I characterize the expression and regulation of the lipid trafficking GAS gene Fatty Acid-Binding Protein 4 in the quiescence-inducing conditions of contact inhibition and oxygen limitation (hypoxia). Chicken Embryo Fibroblasts (CEF) were cultured to high density or subjected to hypoxia, in which oxygen is the limiting factor inducing growth arrest, or serum starvation, in which nutrients is the limiting factor inducing growth arrest. Contact inhibition and hypoxia induced FABP4 expression, whereas cycling control CEF and serum depleted CEF did not. At higher, though still hypoxic, oxygen levels that did not robustly induce FABP4, proliferation assays showed a slight reduction in CEF proliferation. The GAS gene p20k lipocalin has been shown to exhibit similar expression patterns to FABP4, with its regulation determined by the presence of the transcription factor C/EBP-β. CEF overexpressing C/EBP-β also showed strong FABP4 induction. Furthermore, chromatin immunoprecipitation (ChIP) assays revealed that C/EBP-β bound directly to the FABP4 promoter in both normoxic and hypoxic cells, although only the latter condition induced FABP4 protein expression. In summary, these results suggest that FABP4 is induced during growth arrest specifically when oxygen is the limiting factor, as induction was not seen during growth arrest mediated by starvation-induced endoplasmic reticulum (ER) stress, where nutrients was the limiting factor. The induction of these hypoxia-

responsive genes suggests that oxygen availability regulates the expression of a sub-class of growth arrest specific genes. Additionally, FABP4 was shown to be associated with growth arrest and the promotion of cell survival and proliferation, as depicted by proliferation assays. Lastly, C/EBP-β not only strongly induced FABP4 expression, but directly bound to the FABP4 promoter. This suggests that C/EBP-β is a regulator of FABP4, although there may be other interacting factors acting as activators or repressors as this FABP4-C/EBP-β interaction was observed in conditions permissive and non-permissive to FABP4 expression.

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List of Abbreviations:

AP1	Activator Protein 1
ALI	Acute Lung Injury
antimiR	Antisense Oligonucleotide
BLT1R	LTB_4 Receptor 1
bZIP	Basic Leucine Zipper
C/EBP-β	CCAAT-Enhancer Binding Protein Beta
CEF	Chicken Embryo Fibroblasts
CDK	Cyclin Dependent Kinase
ChIP	Chromatin Immunoprecipitation
CHOP	C/EBP Homologous Protein
CI	Contact Inhibition
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
ER	Endoplasmic Reticulum
ERK	Extracellular Signal-Regulated Kinase
ETC	Electron Transport Chain
EX-FABP	Extracellular Fatty Acid-Binding Protein
FA	Fatty Acid
FABP4	Fatty Acid Binding Protein 4
FFA	Free Fatty Acid
FOX	Forkhead Box
Fra2	Fos-related Antigen 2
GAS	Growth Arrest Specific
G_0	Quiescence

G1	Gap phase 1
G2	Gap phase 2
HK-2	Human Renal Proximal Tubule
IgG	Immunoglobulin
IL	Interleukin
INK	Inhibitors of Kinase
I/R	Ischemia Reperfusion
LAP	Liver Activating Protein
LCN2	Lipocalin-2
LDL	Low-Density Lipoprotein
LIP	Liver Inhibiting Protein
LPS	Lipopolysaccharides
M	Mitosis
MAPK	Mitogen Activated Protein Kinase
MCP1	Monocyte Chemoattractant Protein 1
MEK	MAPK/ERK Kinase
miRNA	microRNA
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NFM	Nuclear Factor Myeloid
O_2^-	Superoxide
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reactions
PDGFαR	Platelet-Derived Growth Factor Alpha-Receptor
PI(3)K	Phosphatidylinositol-3-OH Kinase
PKB	Protein Kinase B
PPARy	Peroxisome Proliferator-Activated Receptor Gamma
pRb	Retinoblastoma Protein
PTEN	Phosphatase and Tensin Homolog

PUFA	Polyunsaturated Fatty Acid
QRU	Quiescence Responsive Unit
RBP	Retinol Binding Protein
RCAS(B)	Replication Competent ALV LTR With a Splice Acceptor Bryan Polymerase
RNA	Ribonucleic Acid
RNAi	RNA Interference
ROS	Reactive Oxygen Species
SARP1	Secreted Apoptosis-Related Protein 1
SDS	Sodium Dodecyl Sulphate
shRNA	Short Hairpin RNA
TBS	Tris Buffered Saline
TNF	Tumour Necrosis Factor
T2DM	Type 2 Diabetes
UPR	Unfolded Protein Response
UTR	Untranslated Region

Literature Review

1. The Cell Cycle

i) An Overview of the Cell Cycle

In eukaryotes, the cell cycle consists of four phases which govern the cell's progression through growth and division. These phases include the gap 1 phase (G1), synthesis (S), the gap 2 phase (G2) and mitosis (M). The phases G1, S, and G2 are collectively referred to as interphase as they define the time period of growth and preparation before entering the final phase of mitosis. The progression through cell cycle phases is regulated by cyclins and cyclin-dependent kinases (CDKs). CDKs are serine/threonine protein kinases that are activated at specific points of the cycle via their stimulatory and inhibitory phosphorylation sites (83). In addition, their activation is also dependent on the binding of cyclins. Cyclins allow for the translocation of CDKs to the nucleus as CDKs lack nuclear localization domains (20) Unlike CDKs, cyclin expression varies throughout the cell cycle, thus regulating the activation of CDKs and subsequent progression through the cycle (19, 78).

The first stage of interphase, known as G1, is characterized by cell growth and the synthesis of mRNA and proteins (65). It is in this phase that the restriction point occurs, dictating whether the cell will proceed through the cell cycle or exit into the G_0 phase of growth arrest (34). Progression past the restriction point is largely dependent on the retinoblastoma protein (pRb). Cyclin D1 and CDK4/6 form complexes that are then dephosphorylated on the CDK site and go on to phosphorylate pRb. pRb releases the inhibition on E2F proteins thus promoting the expression of cyclin A and E, which are required to enter S phase. As the G1/S phase cyclin levels change, cyclin E associates

with CDK2, but is then replaced by cyclin A's association with CDK2. This governs the transition through S phase in which DNA is replicated. It is important that DNA replication is done accurately as errors would lead to significant genetic faults and thus cellular complications such as apoptosis. Following S phase is G2, another growth phase in which the cell increases protein levels in order to prepare for mitosis (1). The G2 phase also contains a checkpoint during which DNA damage is assessed before proceeding into mitosis. Completion of the G2 phase is governed by the dephosphorylation of the CDK1/cyclin B complex, thereby activating CDK1 to allow mitotic progression (83). Mitosis consists of prophase, metaphase, anaphase, and telophase. During prophase, the duplicated chromatin condenses into chromosomes and the nuclear envelope breaks down. Metaphase occurs next, in which the chromosomes align along the equilateral plate. Sister chromatids then separate as they are pulled to opposite spindle poles during anaphase. Finally, telophase occurs in which the cytoplasm divides and the chromatin expands, resulting in the formation of two separate daughter cells and the completion of a successful cell cycle (2).

ii) Cell Cycle Arrest

When cells experience unfavourable growing conditions, they may enter a state of growth arrest, otherwise known as cellular quiescence or the G_0 phase. Such conditions include contact inhibition (CI), lack of oxygen (hypoxia), or lack of nutrients, as each cause the exit from the cell cycle. This exit from the cell cycle is a reversible, nondividing state that occurs due to a lack of mitogenic stimulation and is characterized by the upregulation of growth arrest specific (GAS) genes. Lack of mitogenic stimulation results in the failure of activation of the Ras/Raf/MEK/ERK signaling pathway and consequent loss of Cyclin D1 expression (61). Therefore, no complex can be formed between cyclin D1 and CDK4/6, causing Retinoblastoma (pRb) proteins to remain unphosphorylated and E2F transcription factors repressed (61). As a result, the cell fails to express proteins necessary for progression passed the G1 restriction point and thus exits into G_0. This exit is largely governed by p16 and p21 family members (61, 83). Members of the p16 family bind and inactivate CDK4 and CDK6, preventing the formation of G1 progression-promoting cyclin/CDK complexes. Similarly, members of the p21 family (specifically p27) bind to cyclins to inhibit cyclin/CDK complexes (16, 48). p27 levels are therefore upregulated during quiescence, however, the opposite is the case for p21 itself. In conditions of mitotic stimulation, cells exhibit increased expression of p21 and downregulation of p27. Therefore, although from the same family, p21 and p27 play opposing roles in cell cycle progression (83).

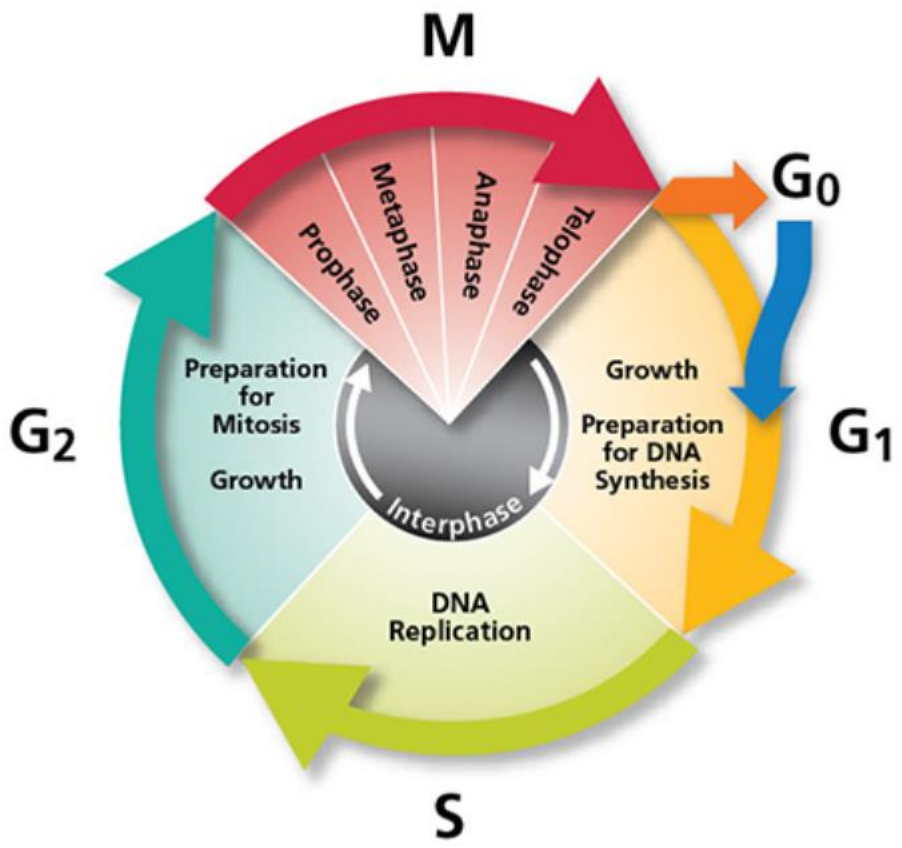


Figure 1. Schematic representation of the cell cycle and exit into G_0 (growth arrest). (Figure obtained from www.bdbiosciences.com/in/research/apoptosis/analysis/index.jsp)

2. Growth Arrest Specific (GAS) Genes

i) An Overview of GAS genes

Growth arrest is a complex process not studied nearly as extensively as cell cycle progression. While oncogenes and mitosis promoting factors strongly induce proliferation, GAS genes mediate the opposite by aiding the cell in growth arrest. GAS genes help the cell remain and survive in the growth arrested state when enduring unfavourable conditions as opposed to simply becoming apoptotic. One of the more well-known GAS genes, GAS1, mediates growth suppression by inhibiting DNA synthesis (22). Secreted apoptosis-related protein 1 (SARP1) is expressed exclusively in quiescent cells. This gene provides the cell with increased resistance to apoptosis by interfering with the Wnt-frizzled pathway through the modification of β-catenin expression (72). There also exist GAS gene products that prepare the cell to re-enter the cell cycle, such as platelet-derived growth factor a-receptor (PDGFaR) (62). This is quite interesting as it suggests that GAS genes not only have a role in suppressing cell growth so to remain in the quiescent state, but also have a role in priming and preparing the cell for re-entry into the growth cycle. It has been established that GAS genes are critical to cell cycle arrest, however, their role in varying growth arrest inducing conditions is not entirely clear.

ii) FABP4

FABP4 is a GAS gene belonging to a family of cytoplasmic fatty-acid-binding proteins with molecular weights of ~15 kDa. This multi-gene family is composed of nine family members, with a range of 20-70% amino acid sequence similarity (39). Each member has a tertiary fold that forms a β-Barrel, creating an internal, water-filled cavity which fatty acids bind to (49). Interestingly, FABP4 was recently found via microarray to be upregulated at high cell density, a condition in which cells are growth arrested, by ~30 fold (30). Taken together, FABP4 is thought to have a role in growth arrest through the regulation of fatty acids and lipid homeostasis, although its role in quiescence is not well characterized. FABP4 is also classified as a lipocalin, a family of proteins characterized by low sequence similarity and highly conserved crystal structures. Lipocalins are known to play an important role in many human diseases including cancer and various metabolic diseases (33).

The nine FABP members are expressed differentially throughout the body, each being tissue-specific to select organs. FABP4 is mainly expressed in adipose tissue and macrophages, although recent studies have uncovered its expression in additional tissues. Its expression in additional tissues tends to be in response to injury or various stressors. Studies have detected FABP4 expression in tubular cells in patients with stage IV lupus nephritis as well as in tubular epithelial cells following renal ischemia reperfusion (I/R) injury, suggesting FABP4 is associated not with regular cells, but cells subjected to injury involving hypoxia (91, 96). Furthermore, hypoxia followed by re-oxygenation induced FABP4 expression in the human renal proximal tubule cells (HK-2) *in vitro* (96). FABP4 is also found in the bloodstream, as studies investigating blood

components reported that subjects with elevated circulating FABP4 levels had increased risk for developing metabolic syndrome and type 2 diabetes (T2DM) (100, 110). Studies have shown that it is also expressed in the placenta of humans (89) and mice (67). Furthermore, these studies suggested that the protein may be involved in foeto-placental growth and placental lipid accumulation (67, 89).

Although expressed in various tissues, most studies have examined the lipid-maintenance role of FABP4 in macrophages. As such, FABP4 is known to have a large role in lipid transport as adipocytes of FABP4-/- mice exhibited reduced efficiency of lipid transport (35). Despite the numerous lipid-related functional studies on FABP4, its role in growth arrest has not been studied extensively. However, the protein has been proposed to have several lipid regulating roles including lipid trafficking, facilitation and regulation of fatty acid influx across the cell membrane by binding and preventing efflux, determination of compartmentalization and storage, modulation of enzymes involved in fatty acid metabolism, protecting membrane components from fatty acid-induced damaged, promoting inflammation, and regulating ROS production and leukotriene secretion (49, 50).

FABP4 itself is regulated by PPARγ, insulin, and fatty acids (25, 49, 53). As such, this protein has an important role in the development of major components of metabolic syndrome and related diseases through its actions in adipocytes and macrophages. Interestingly, inhibiting FABP4 with BMS309403, a FABP4 inhibitor that competitively inhibits the binding of fatty acids by interacting with the protein's interior fatty-acid binding pocket, caused macrophages to show a reduction in insulin, triglycerides, and cholesterol ester levels (37). This suggests an insulin-sensitizing effect via the inhibition

of FABP4, supporting the protein's key role in diabetes. Accordingly, FABP4 may be an effective therapeutic target in the treatment of diabetes. Moreover, FABP4 may be a promising target in combatting obesity as obesity markers MCP1, IL1B, IL6, and TNF were reduced following its inhibition (35).

Obese mice exhibit FABP4 dysregulation, concurrent with the findings that obese adipocytes secreted more FABP4 than control subjects (11). Also observed in obese subjects was a FABP4-linked hepatic glucose disruption. Specifically, FABP4 was secreted from adipocytes in response to fasting and proceeded to regulate hepatic glucose production (11). FABP4 not only regulates hepatic glucose production but lowers protection against metabolic abnormalities associated with diabetes (11). Furthermore, they found that the levels of FABP4 in blood were elevated in obese mice and humans, suggesting the possibility that FABP4 incorrectly signals an aspect of fasting metabolism despite nutrients and energy being available (11). Therefore, FABP4 also has a non-cytosolic role in systemic metabolic regulation via glucose production, further supporting that FABP4 be a primary target for therapeutic intervention of obesity and diabetes. What's more, FABP4 may also be an effective therapeutic target for atherosclerosis as atherosclerotic lesions, foam cell formation, and inflammatory mediators/chemoattractants (including MCP1, IL1B, IL6, and TNF) were decreased upon FABP4 inhibition (35). Additionally, its knockdown reduced the uptake of LDL into macrophages (26). This disruption to the internalization of lipids and consequent transformation of macrophages into foam cells is important as this would prevent the progression of atherosclerotic lesions and inflammatory responses.

Agreeing with the notion that obesity is considered a chronic inflammatory state (32), FABP4 is strongly associated with inflammation. It is intertwined in inflammatory cascades, for example, the inflammatory stimulator LPS was shown to upregulate the major macrophage LT receptor, LTB_4 receptor 1 (BLT1R), in a FABP4 dependent manner (50). Reactive oxygen species also had this effect, suggesting an association between FABP4 and ROS as well. Additionally, FABP4 was found to be upregulated following acute lung injury (ALI), a condition characterized by the excessive activation of the inflammatory response and intense oxidative stress causing increased ROS (63, 102), thus supporting a link between FABP4 and ROS. Given that increased ROS tends to occur during inflammation, this further supports the evidence showing that FABP4 has a key role in inflammation.

The link between FABP4 and ROS may influence cell cycle regulation as one study found that exogenous FABP4 treatment in MCF7 cells enhanced levels of FOXM1 in a ROS dependent manner (107). Interestingly, FOXM1 is a member of the forkhead box (FOX) transcription factor family and has a role in cell cycle progression. This transcription factor is also involved in human malignancies as it is overexpressed in carcinomas (107). There is therefore a potential link between FABP4, FOXM1 and ROS. Perhaps FABP4 enhances FOXM1 expression thereby assisting in cell survival in conditions of oxidative stress. This suggests an association between the GAS gene FABP4, oxidative stress, and cell cycle regulation.

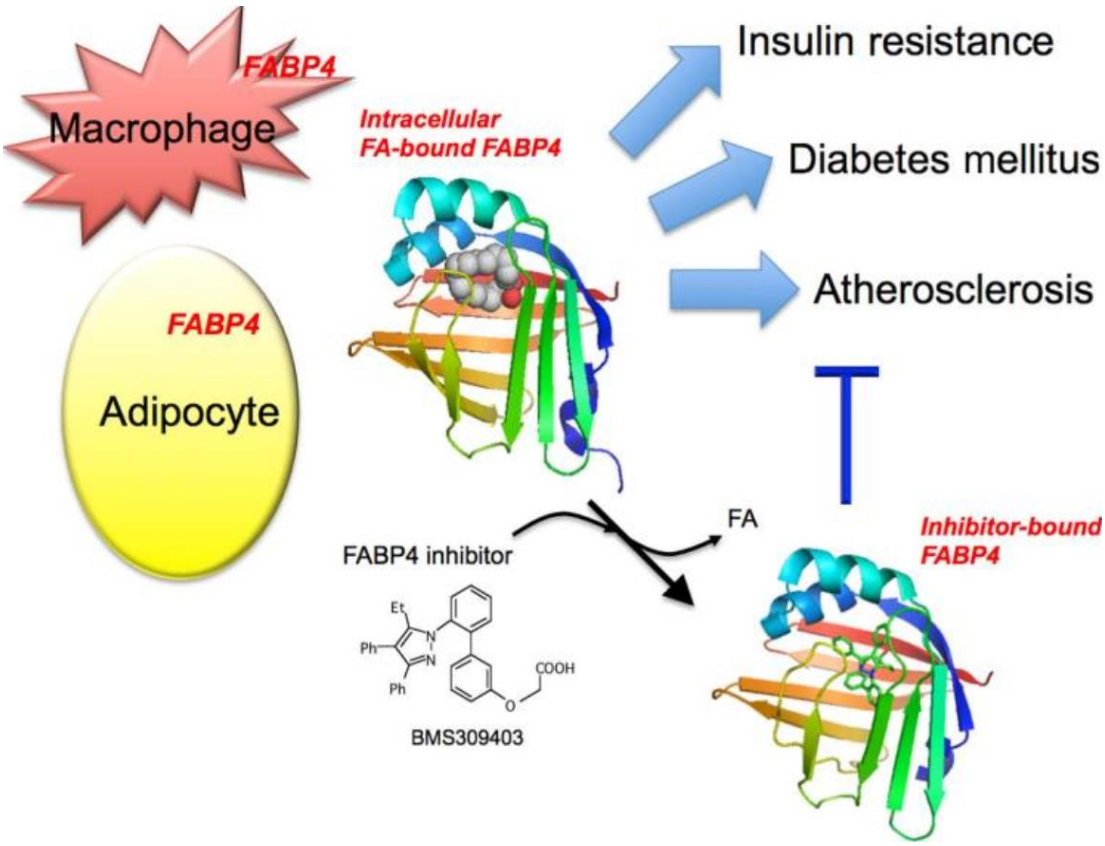


Figure 2. Association of FABP4 in adipocytes and macrophages with metabolic and cardiovascular diseases. FABP4 acts at the interface of metabolic and inflammatory pathways in adipocytes and macrophages and plays important roles in the development of insulin resistance, diabetes mellitus, and atherosclerosis. Chemical inhibition of FABP4 could be a therapeutic strategy against metabolic and cardiovascular diseases (37).

iii) p20k Lipocalin

Like FABP4, the p20K lipocalin, otherwise known as extracellular fatty acid-binding protein (EX-FABP), is mainly released from adipocytes. Being a lipocalin, p20k has a high affinity for hydrophobic molecules including long chain unsaturated fatty acids, steroid hormones, and retinoids (9, 10). Interestingly, its expression was induced in response to contact inhibition and hypoxia in chicken embryo fibroblasts (CEF) (29). It is known for binding poly-unsaturated fatty acids and is a member of the siderocalins binding iron catechol compounds (29). However, it was also found to act as a survival factor in conditions unfavourable for proliferation, including survival during saturation density in normoxia or when under limited oxygen supply (29). p20K may a have role not only in cell survival during reversible growth arrest, but in lipid homeostasis as well. This is supported by the increase in lipid oxidation, the appearance of large lipid-containing vesicles in the cytosol, and the accumulation of several lipid species in p20k inhibited CEF undergoing contact inhibition and hypoxia (29). In addition to its role in growth arrest and lipid metabolism, p20k is known to act in the processes of cell survival, inflammation, cell development, and the transport of long chain unsaturated fatty acids (9, 10, 14, 40).

With p20k's main role being in growth arrest, the transcriptional activation region of its promoter was termed the Quiescence Responsive Unit (QRU). The QRU is a 48 bp region that is required for its transcriptional activation (68) and contains two CCAAT-enhancer Binding Protein Beta (C/EBP-β) binding sites known as the A and B regions (57). Being a growth arrest specific gene, p20k lipocalin expression is repressed in cycling cells. This repression is exerted by extracellular signal-related kinase 2 (ERK2)

as this protein acts as a transcriptional repressor of the QRU (See Figure 3). ERK2 binds to repeated GAAAG sequences overlapping the C/EBP-β sites of the QRU, a recruitment which is mutually exclusive from the recruitment of C/EBP-β (30). This is the mechanism by which p20k expression is restricted to growth arrested cells, however, there are additional factors that make its growth arrest specificity even more stringent. p20k is expressed only during hypoxia-associated growth arrest, as it is not expressed during ER stress-induced growth arrest (30). In conditions of ER stress, C/EBP-β homologous protein (CHOP) is upregulated to form dimers with C/EBP-β, thereby inhibiting C/EBP-β's ability to bind to the QRU and induce p20k expression. This ER stress-induced cascade thereby restricts p20k expression to hypoxia-induced quiescence (30).

LCN2, the human equivalent of p20k in CEF, is again similar to FABP4 in that it plays an important role in inflammation and insulin resistance (107). Additionally, circulating LCN2 levels were increased in obese animals, as well as increased mRNA levels observed in ob/ob mice (110). Although there are many striking similarities between p20k and FABP4, there are also critical differences in their upstream regulating factors which may account for some of the results in our study. For example, the two proteins are regulated in opposite manners by mPPARy2 as this protein promotes FABP4 expression (97) whereas it inhibits p20k expression (57). However, both p20k and FABP4 were shown via microarray to be upregulated at growth arrest-inducing high cell density, or in other words, at contact inhibition (30).

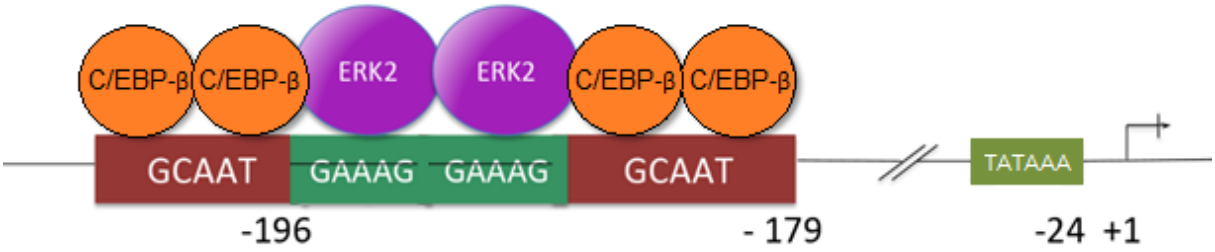


Figure 3. Schematic representation of the p20k QRU binding sites and interaction with competing homodimers of C/EBP-β (transcriptional activator) and ERK2 (transcription repressor), occurring in quiescent and proliferating CEF, respectively.

(Figure created by Dr. André Bédard)

iv) RBP7

Another member of the lipocalin family shown to be upregulated at high cell density, although only by ~7 fold, was retinol binding protein 7 (RBP7). RBPs are a family of transporters for fat-soluble retinol (50). The RBP family has multiple functions, including a role in vision, reproduction, vitamin A stability, and most importantly in metabolism and growth (48, 81). Both microarray and PCR analysis showed that RBP7 expression was predominant in adipose tissue (1). In addition to adipose tissue, RBP7 is found in endothelial cells in which it is a target of PPARy (52). Interestingly, impaired PPARy activity, which is also a FABP4 inducer, caused oxidative stress and a predisposition to hypertension (52). What's more, RBP7-deficient mice exhibited oxidative stress-induced endothelial dysfunction in response to cardiovascular stressors such as a high-fat diet (52). Additionally, RBP lipocalins have been shown to bind and protect retinol against oxidative damage during circulation (33). Although little else is known about RBP7, it is important to note that it is largely an adipose-specific gene upregulated in conditions of confluency and low oxygen availability. Additionally, RBP7 has a role in metabolism, growth, and protection against oxidative stress.

v) C/EBP-β

CCAAT/enhancer-binding protein beta (C/EBP-β) is encoded by an intronless gene and contains a leucine zipper (bZIP) domain. C/EBP-β can function as a homodimer or heterodimer with CCAAT/enhancer-binding protein alpha, delta, or gamma. It has a large role in regulating the expression of genes involved in the immune and inflammatory response, such as IL-6, IL-4, IL-5, and TNFα (21, 24, 45, 76*)*. Therefore, it is no surprise that C/EBP-β is critical for proper macrophage function (82). Additionally, C/EBP-β is activated during adipogenesis and consequently induces C/EBPα and PPARγ, giving rise to the induction of other adipocyte genes (13).

Although it is largely known for its role in inflammation, the function of C/EBP-β extends out to processes such as proliferation and growth arrest. C/EBP-β is expressed in quiescent hepatocytes and can inhibit proliferation in hepatoma cells, therefore demonstrating anti-proliferative functions. Furthermore, cells expressing a dominant negative version of C/EBP-β began proliferating and exhibited a lack of p20k protein at confluence (38). The same study found that cells null for C/EBP-β exhibited a proliferative advantage over those with one functional copy (38). Furthermore, C/EBP-β inhibition was shown to enhance the expression of 3 components of AP1 (JunD, Fra2, C-Jun), resulting in enhanced AP1 activity and a consequent increase in cyclin D1 expression (38). This promoted proliferation, showing that in this case, C/EBP-β was not only an anti-proliferative factor but played an opposing role to AP1 in GAS gene expression and proliferation (38). Additionally, C/EBP-β is expressed in mammary epithelium, a site mainly composed of quiescent cells. On the other hand, mice nullizygous for C/EBP-β exhibited impaired liver regeneration following partial

hepatectomy, showing a pro-proliferative function (23, 45). Therefore, C/EBP-β is implicated in cell growth and arrest, but this role is both cell type and context dependent (38).

There have been three proposed mechanisms to account for the variation in roles exhibited by C/EBP-β. One idea attributes these dual roles to the fact there are three different C/EBP-β isoforms, known as LAP*, LAP, and LIP (38). These three isoforms overlap in some functions, but differ in others. As such, elevated expression of one specific isoform over another may account for the discrepancies seen between different cell lines/contexts. Another proposed mechanism that may be responsible for C/EBP-β's differing roles is posttranslational modification. Phosphorylation of C/EBP-β on threonine 217 in the mouse or serine 105 in the rat has been shown to be required for hepatocyte proliferation (8) and enhanced gene expression of proliferation promoting factors. Additionally, this phosphorylation was found to create a binding inhibitory site for procaspase 1 and 8, which was essential for proliferation and lead to apoptosis when no C/EBP-β was present (8). Therefore, posttranslational modifications may result in functional differences to C/EBP-β in growth arrested versus cycling cells. The last mechanism to possibly attribute the functional differences of C/EBP-β is differentially expressed transcription factors. C/EBP-β may interact with other transcription factors that are differentially expressed in quiescent cells compared to proliferating cells. As a result, these transcription factors may be present in quiescent but not proliferating cells – or vice versa - allowing for the transcription of certain genes in only one of the two scenarios. Similarly, there may be transcriptional repressors present as is the case with ERK2 on the QRU of p20k in cycling cells.

3. Growth Arrest, Oxidative Stress, & Lipid Peroxidation

Hypoxia is a feature of contact inhibition as high cell density depletes oxygen availability. Ironically, hypoxia results in an intracellular increase in reactive oxygen species. Cardiomyocytes, hepatocytes, myocytes, and neural PC12 cells were all found to respond to severe hypoxia with increased production of ROS (28, 42, 51, 64). Interestingly, this increase in ROS is partially mitochondria-mediated as cells depleted of mitochondrial DNA (p^o cells) did not exhibit enhanced ROS generation during hypoxia (60). This is likely due to the fact that p^o cells lack crucial subunits of mitochondrial complexes I, III, and IV (85, 87). In normal cells possessing functional subunits, mitochondrial complexes I and III produce superoxide (O_2^-) as they are the sites of electron-transfer reactions to molecular oxygen (74). Superoxide can then be converted into hydrogen peroxide and escape the mitochondria, resulting in mitochondria-mediated ROS release (86). In addition to increased ROS levels, hypoxic episodes are characterized by elevated FFA tissue levels (73, 94, 101). Hypoxia significantly raises non-esterified free-fatty acid levels in the cell (3), and this hypoxia-induced FFA enrichment impairs mitochondrial function (31). What's more, isolated mitochondria have been found to respond to FFA with a concentration-dependent increase of ROS release. Interestingly, this phenomenon has been suggested to be derived from FFA interference with the electron transport chain (ETC) (17, 66, 85, 87, 95). Additionally, FFA have also been suggested to be potential candidates for ROS generation at plasma membrane-associated NADPH oxidases (18). Poly-unsaturated fats (PUFA) exhibit similar cellular effects, as stimulation with PUFA to PC12 cells resulted in electron transport chain (ETC) sourced ROS generation, as well as ROS-generation at

non-mitochondrial sites such as membrane-associated NADPH oxidases and enzymes (86). Therefore, hypoxia-induced elevation of fatty acids may mediate the increase in ROS seen during hypoxia by interfering with the mitochondrial ETC and plasma membrane. Overall, this lipid-associated ROS increase is supported by the observations of increased lipid oxidation, large lipid-containing vesicles, and accumulation of multiple lipid species in p20k inhibited CEF undergoing hypoxia (29), which also suggests that specific GAS genes may be implicated in the defense against and/or the response to lipid peroxidation and consequent homeostasis maintenance. Given these findings, it is possible that p20k scavenges FFA at growth arrest and hypoxia, and enhances survival by promoting mitochondrial, organelle, or membrane homeostasis.

Reactive oxygen species can cause significant cellular damage leading to cell death (5). Although ROS occurs in proliferating cells, cell death is evaded by the activity of phosphatidylinositol-3-OH kinase (PI(3)K)–PKB signalling pathway (44). In the absence of this pathway, the FOXO subfamily of Forkhead transcription factors is activated, leading to quiescence but not apoptosis despite the lack of PKB activity (58, 70). Activation of Forkheads results in the entry into the G_0 phase (58), whereas inactivation of Forkheads by activation of PKB results in re-entry into the cell cycle. This agrees with previous data describing FABP4's role in enhancing FOXM1 expression in conditions of oxidative stress, perhaps to help the cell avoid apoptosis and remain growth arrested. FABP4 may mediate this process via the maintenance of membranes susceptible to oxidatively-damaged lipids until conditions are suitable enough to re-enter the cell cycle.

Interestingly, ROS also result in lipid peroxidation, a detrimental process involving the formation and spread of lipid radicals induced by reactive oxygen species. When free lipids are attacked by hydroxyl and hydroperoxyl radicals, the generated free lipid radical can propagate free radical attacks on surrounding lipids (111). This results in a lipid peroxidation chain reaction, leading to the damage and destruction of the lipid membrane as well as production of toxic by-products. Damage to lipids consequently causes damage to the cell membrane as it is largely composed of a lipid bilayer. Therefore, it is possible that hypoxia results in membrane stress due to ROS-induced lipid damage, triggering a novel membrane stress response. As stated previously, FABP4 is a fatty-acid binding protein with a role in maintaining lipid homeostasis and has also been shown to be associated with increased ROS. Therefore, FABP4, as well as other lipid regulating GAS genes, may have a role in re-establishing lipid homeostasis following hypoxia/oxidative stress-mediated lipid damage.

4. Rationale & Proposed Mechanism

It is clear there is a link between several GAS genes and oxidative stress. This agrees with the fact that p20k was upregulated during contact inhibition, as high cell density results in significant depletion of oxygen levels (29). Furthermore, given that protein synthesis and cell proliferation are dependent on oxygen availability, hypoxia may promote growth arrest in confluent CEF monolayers, in part, via the upregulation of the GAS genes of interest. However, it should be noted that protein synthesis is controlled by the oxygen sensing REDD1 and mTOR pathway, which are likely the primary and direct regulators of growth arrest (7). The limited oxygen resulting from high cell density or hypoxia treatment yield an unfavourable environment for proliferation, resulting in increased ROS levels and entrance into quiescence (the G_0 state). Cell cycle exit induces changes in metabolic needs, such as decreased membrane biogenesis. In the absence of GAS gene products, more lipids and FFA may accumulate as less are being used for membrane biogenesis and lipid homeostasis is impaired. Lipids are a primary target for ROS, which ironically, is a key feature of hypoxia. With increased hypoxia-induced ROS, more and more lipids are damaged. This gives reason as to why lipid metabolism is likely critical in contact inhibited, hypoxic, and quiescent-promoting environments, initiating the “Membrane Stress Response”. It is at this point that these environments induce an upregulation of GAS genes including FABP4, RBP7, and p20k. Whereas RBP7 has been linked to protection against oxidative stress (52), FABP4 and p20k have a strong role in facilitating lipid transport and therefore lipid maintenance. Accordingly, it could be argued that the increase in RBP7 expression to reduce oxidative damage while FABP4 and p20k

lipocalin traffic damaged lipids with the aim of restoring lipid homeostasis. After this is achieved, the cells may re-enter the cell cycle as oxidative stress decreases and FABP4, RBP7, and p20k expression diminishes.

The extended, long-term goal of this study was to identify and characterize crucial processes involved in the novel “Membrane Stress Response”. Due to the majority of studies aiming to characterize the cell cycle in terms of proliferation and progression through, this project also aimed to determine the key processes involved in the exit from the cell cycle with the possibility of re-entry after specific cellular maintenance. The process of reversible growth arrest, denoted by the withdrawal from the cell cycle, is not a well characterized state. Although previous models on quiescence have primarily focused on *in vitro* or starvation studies, these conditions are rarely found *in vivo* and are largely non-physiological. For this reason, this study investigated the effects of contact inhibition as it is a key feature of wound healing *in vivo* and is an important aspect in cancer suppression. Contact inhibition, hypoxia, and serum-deprivation induce this exit into the G_0 state, however, through two separate pathways (See Figure 4). Both contact inhibition- and hypoxia-induced growth arrest are mediated through limiting oxygen. Limited oxygen results in lipid peroxidation, followed by membrane stress and consequent activation of GAS genes to regulate lipid homeostasis, whereas serum-depletion facilitates growth arrest through lack of nutrients leading to ER stress and the Unfolded Protein Response (UPR). The response to contact inhibition is not well defined and consequently remains to be described. Therefore, this study characterized and filled the gap in understanding key processes of

cell and lipid homeostasis through the characterization of GAS genes FABP4, p20k, C/EBP-β, and RBP7 in response to limited oxygen.

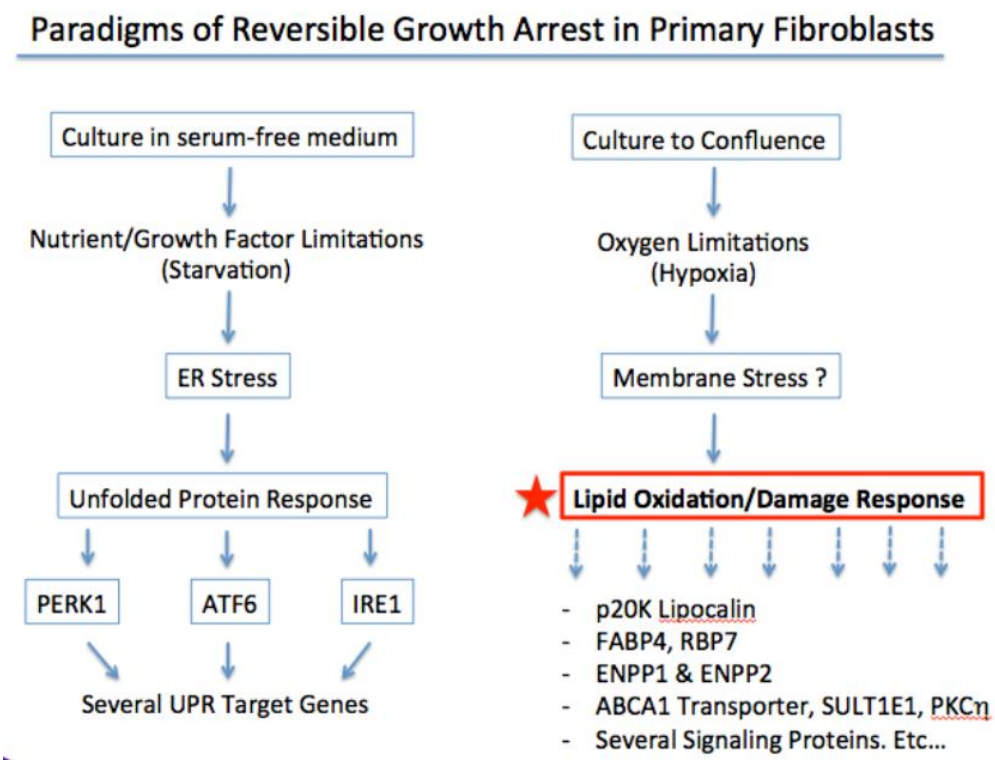


Figure 4. Distinction between pathways inducing growth arrest. ER stress induced by limiting nutrients induces the Unfolded Protein Response (UPR) which in turn upregulates UPR target genes and induces growth arrest (left). Limiting oxygen results in oxidative damage/ROS, which may cause lipid peroxidation and membrane stress. Consequently, the membrane stress response upregulates growth arrest specific genes as the cell becomes quiescent (right).

(Figure created by Dr. André Bédard)

Materials & Methods

1. Chicken Embryo Fibroblast Cell Culture.

1.1 Culture Conditions:

Early passages (n<12) of CEFs were cultured at 41.5ºC in 21% O_2 and 5% CO_2 in “complete medium” consisting of DMEM supplemented with 5% heat-inactivated (at 57ºC for 30 minutes) “Cosmic” calf serum, 2 mM L-glutamine, 0.2 mg/mL streptomycin and 0.2 U/mL penicillin. Cells were split using Trypsin-EDTA. In normoxia conditions, cells were grown at 41.5ºC in 5% CO_2 and 21% O_2. Hypoxia was induced by incubating CEF for 6-32 hrs in 1-2% O_2 as described in the text. For confluent, contact-inhibited conditions, cells were grown to 100% confluence, at which point the medium was refreshed and they were let to grow for an additional 24 hours.

1.2 Viral Infections:

For cell transformations with C/EBP-β, CEF were split in the morning and allowed 4-5 hours to adhere to the 100 mm plates. After cells were adhered, media was removed such that only 4 mL remained in the plate. 0.5 mL of viral stock was then added into the plate which was incubated overnight at 41.5ºC. Media was aspirated completely the next morning and replaced with fresh media. After growing to confluence, the cells were passaged as needed.

2. SDS-PAGE and Western Blotting.

2.1 Protein Sample Preparation:

After treatment, cells were washed three times with cold 1xPBS pH 7.4 (137 mM NaCl, 2.7 mM KCL, 4.3 mM Na2HPO4, 1.47 mM KH2PO4). Any remaining PBS was aspirated off and cells were scraped using a rubber scraper and collected in 1 mL of cold 1xPBS. Cell samples were centrifuged at 7000 RPM for 3.5 minutes. All 1xPBS was then removed and the cell pellet was resuspended in 1x Sodium Dodecyl Sulfate (SDS) sample buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol, 60 mM Tris-HCL pH 6.8) containing 1x Halt protease and Phosphatase Inhibitor Cocktail (Thermo Fisher). Samples were then vortexed briefly, boiled for 2 minutes, and spun down at 13 000 RPM for 5 minutes. The supernatant was transferred to a new microcentrifuge tube and stored in -80 ºC.

2.2 SDS-Page and Western Blotting:

Total protein extracts (50-75 µg) were subjected to SDS-polyacrylamide gel electrophoresis with a gel concentration of 12-14% depending on size of protein to be resolved. Once resolved, gels were blotted onto a nitrocellulose membrane and then blocked in a 5% solution of skim milk powder dissolved in 1xTBS (20 mM Tris pH 7.6, 140 mM NaCl) for 40 min at room temperature. Blots were incubated with primary antibodies (See Table 1 for primary antibody concentrations) in 5% milk solution overnight at 4ºC.

Table 1: Primary Antibody Dilutions

Antibody	Application	Dilution
FABP4 (AB #1) – Polyclonal – Rabbit - made with peptide antigen	Western Blotting	1:400
FABP4 (AB #1)	Immunofluorescence	1:100
FABP4 (AB #2) – Polyclonal – Rabbit - made with peptide antigen	Western Blotting	1:100
p20K (601-Y – Bédard Lab)	Western Blotting	1:400
C/EBP-β (8582 - Bédard Lab)	Western Blotting	1:100
RBP7	Western Blotting	1:400
ERK2	Western Blotting	1:1000

Table 2: Secondary Antibody Solutions

Antibody	Application	Dilution
Anti-Rabbit IgG, HRP-Linked Antibody	Western Blotting	1:25000
Anti-Mouse IgG, HRP-Linked Antibody	Western Blotting	1:25000
Anti-Rabbit IgG FITC	Immunofluorescence	1:200

The next day, blots were washed twice in 1xTBS, twice in 1xTBS-T containing 0.1% Tween, and once again in 1xTBS. Following these washes, the blots were incubated with a 1:25,000 dilution of a secondary anti-rabbit or anti-mouse antibody (See Table 2 for secondary antibody information) conjugated with horseradish

peroxidase (HRP) for two hours at room temperature in 5% milk TBS. The blots were again washed twice with 1xTBS, twice with 1xTBS-T, and once more with 1xTBS. Chemiluminescent signals were visualized by incubation with the HRP substrate Luminata Forte according to the protocol provided by the manufacturer, and hyperfilm.

3. Proliferation Assays.

Confluent CEF were split into 6 well plates and incubated in normoxia and hypoxia for the time duration indicated. Cells were treated with 1mL trypsin and diluted in 9 mL of ISOTON® II Diluent (Beckman Coulter 8546719). Cells were counted in quadruplicate samples for statistical significance utilizing a Beckman Coulter model Z2 (Coulter Corporation, Miami, FL) Coulter counter.

4. Immunofluorescence.

Cell cultures were first seeded onto glass cover slips in 60 mm plates and incubated in their respective conditions. The cover slips were washed twice in 1xPBS and subsequently fixed using 3.7% formaldehyde in 1xPBS for 10 minutes at room temperature. Cells were then permeabilized using 0.1% Triton X-100 in 1xPBS on ice for 5 min. Cells were then blocked in 5% fetal bovine serum in 1xPBS for 1 hour and subsequently incubated with the desired primary antibody (primary AB dilution 1:100) overnight at 4ºC. The following morning, cells were washed 3 times with 1xPBS and incubated with the respective secondary antibody for 1.5 hours at room temperature in the dark (secondary AB dilution 1:200). Following secondary incubation, cells were incubated with Hoechst as a nuclear stain at a concentration of 1.2 mg/mL diluted 1:300

for 3 minutes in the dark. Cells were finally washed twice with 1xPBS and twice with ddH2O, then fixed on coverslips with Aqua-Mount and imaged.

5. ChIP Assays.

5.1 Sample Preparation:

Cells were fixed with 1% formaldehyde in 1xPBS for 10 minutes. The remaining formaldehyde was quenched using 1.25 M glycine for 5 minutes. Cells were washed and harvested in 1xPBS and centrifuged at 7000 RPM for 3 minutes. Cell pellets were resuspended in 1 mL of SDS ChIP Lysis Buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.1, 1xHalt protease phosphate inhibitor cocktail (Pierce, USA)) and stored at -80ºC.

5.2 Immunoprecipitation:

Aliquots of 500 µL were sonicated utilizing a Sonifier Cell Disruptor 350 at 50% output for 6 minutes at 20 second pulses. Samples were pre-cleared with salmon sperm DNA blocked Protein A beads and diluted to a final volume of 1 mL ChIP Dilution Buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCL pH 8.1, 167 mM NaCl, and Halt inhibitor cocktail (Pierce, USA)). A 10 µL sample was extracted, and the remaining chromatin solution was immunoprecipitated using 2 µg of C/EBP-β and pre-immune C/EBP-β antibodies overnight at 4ºC. After overnight incubation, antibody complexes were precipitated using salmon sperm DNA blocked Protein A beads for 1 hour. Samples were then centrifuged and subjected to several washes: one wash with 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), one wash with High Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCL pH 8.1, 500 mM

NaCl), once with LiCl Immune Complex Wash Buffer (0.25M LiCl, 1% NP-40, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris- HCL pH 8.1) and twice with TE Buffer (10 mM Tris-HCL, 1 mM EDTA pH 8.0). Next, bound complexes were eluted using 200 µL of Elution Buffer (0.1 M NaHCO3, 0.005% SDS). Samples were then de-crosslinked at 65ºC. RNase A and Proteinase K (2 µg) were used to digest RNA and protein, respectively. The DNA was ethanol precipitated by adding 3x the amount of 95-100% ethanol, shaken/mixed, then put into -20ºC overnight. The next morning, samples were centrifuged at 13 000 RPM for 25 min. The ethanol was then removed and the pellet gently washed with 70% ethanol. The 70% ethanol was removed and the pellet left to air dry for 20 min. The pellet was then resuspended in 20 µL of 1xTE buffer and stored at -20ºC

5.3 PCR Amplification:

PCR amplifications for immunoprecipitated chromatin were done on the FABP4 promoter. The following primers were used for amplification:

Forward: TGT GCA GCA ATG AAA TGG GG

Reverse: TGT GTT GGT CCA CAT ATT TTG ATA T

PCR reactions were carried out in 25 µL reaction consisting of 12.5 µL 2X GoTaq PCR Mix, 1 µL of template DNA, and 1mM forward and reverse primers. The PCR program consisted of: 94ºC for 5 minutes, 55 cycles of 94ºC for 30 seconds, 55ºC for 30 seconds and 72ºC for 35 seconds. The program finished with a 10 minute incubation at 72ºC. Following amplification, PCR products were prepared consisting of 25 µL PCR samples, 2 µL marker, and 3 µL of 10X TAE. PCR products were resolved on a 1.2% agarose gel (1.88 g agarose in 125 mL of 1X TAE buff, heated in microwave to dissolve,

followed by addition of 2.5 µL ethidium bromide) for 45-60 min and visualized in a UV transilluminator.

Results

Part 1: Identification and characterization of FABP4 as a hypoxia and growth arrest specific gene in CEF.

FABP4 and RBP7 expression is induced by hypoxia and contact inhibition, but not starvation.

As both FABP4 and RBP7 were shown via gene profiling to be upregulated at high cell density (30), we analyzed the expression of these proteins in contact inhibited CEF. These proteins were also analyzed in hypoxic conditions as oxygen deprivation has been shown to be a feature of confluence (29). Additionally, a recent study showed that HK2 cells exhibited increased expression of the FABP4 protein as a result of hypoxia and reoxygenation (31). Our results supported these findings as FABP4 was induced by hypoxia and contact inhibition (Figure 5). This was also true for RBP7 (Figure 6). FABP4 and RBP7 expression are therefore a feature of hypoxia and contact inhibition, however, FABP4 was not induced by serum starvation. This is likely due to the fact that serum starvation results in ER stress and consequently induces the ER stress response/UPR. The absence of FABP4 expression in serum-depleted conditions confirmed that FABP4 has a separate pathway of induction, being the hypoxia/ROS stress response, from ER stress response.

FABP4 re-localizes in response to hypoxia.

Little is known about the intracellular localization of FABP4, which led us to characterize its localization in response to CI and hypoxia. In cycling CEF, low levels of FABP4 were detected in the ER-golgi system and focal adhesion plaques (Figure 7).

This agrees with the results from Figure 5 in which very slight FABP4 expression is seen in the western blot analysis of cycling CEF. Following hypoxia (1% O_2) incubation for 24 hours, expression of FABP4 was strongly induced throughout the cell cytoplasm and organelles such as the endoplasmic reticulum (ER) and golgi (Figure 7). Notably, this hypoxia-induced localization in the endo-membrane system is similar to that of p20k (6).

FABP4 and p20k expression are induced by hypoxia, with p20k having a stricter range of inducible O_2 concentrations.

To determine whether hypoxia-induced FABP4 was oxygen concentration specific, a western blot analysis of proteins expressed at varying oxygen concentrations was performed. The results showed that FABP4 was induced by 1% and 2% O_2, while concentrations above 2% were not sufficient for strong induction (Figure 8). Similar results were previously reported for p20k lipocalin (29), however, our results showed that p20k exhibited a stricter cut off for oxygen concentrations sufficient to induce expression. Although 1% O_2 strongly induced p20k, 2% and above was not sufficient (Figure 8). Therefore, although both p20k and FABP4 are inducible by limiting oxygen, FABP4 is more sensitive than p20k to higher concentrations, or a wider range of hypoxic concentrations. Interestingly, the relative induction of p20k at 1% O_2 was almost 2-fold that of FABP4 induction, suggesting the expression of p20k is more strongly induced in very severely hypoxic environments.

Hypoxia-induced expression of FABP4 and p20k are time-dependent, with more rapid upregulation of p20k.

Given the varying sensitivity to oxygen concentrations, we wanted to determine if the kinetics and time-dependent inducibility would vary as well. Through western blot analysis of lysate harvested from CEF incubated in hypoxic conditions for varying time periods, we found that p20k was induced as quickly as 6 hours into hypoxia incubation, whereas it took 12-24 hours for FABP4 to show strong induction (Figure 9). Therefore, although FABP4 expression is more sensitive than p20k when it comes to varying oxygen concentrations, it exhibits a less rapid onset. Notably, contact inhibited cells exhibited more than a 2-fold FABP4 relative induction compared to p20k, suggesting FABP4 presence is more important at high confluency than p20k.

It should be noted that Western blots sometimes showed a lower FABP4 band at ~12 kDa (data not shown). We hypothesized this to be another FABP4 isoform or that the protein was being cleaved the longer CEF were incubated in hypoxia, further warranting a time-dependent analysis. However, when a new FABP4 antibody was obtained, this second band disappeared. The second band was therefore likely due to non-specific binding of an older antibody.

FABP4 is associated with growth arrest and possibly survival.

Given that only extremely low hypoxia concentrations (≤2% O_2) were sufficient to strongly induce FABP4, we questioned whether its presence was correlated with cell survival in such harsh conditions. Proliferation assays showed growth arrest at 1.5%, 3%, and 4% oxygen (Figure 10A,B,C). Western blot analysis of lysate from CEF in

these treatments confirmed FABP4 in 1.5% O_2 and minimal FABP4 in 3% O_2 (Figure 10D). Presence of FABP4 may support low levels of proliferation, as CEF exhibited slight growth up to 48 hours in 1.5% O_2 (Figure 10A). However, CEF growth arrested completely at 3% O_2 (Figure 10B), and even began to die off at 4% O_2 (Figure 10C). This could be due to a complete lack of FABP4 at 4% O_2, as low signal was detected in 3% O_2 (Figure 10D). This small amount of FABP4 at 3% O_2 may have aided in at least growth arrest/minimal survival rather than the slight continued proliferation observed at 1.5% O_2 when it was strongly expressed (Figure 10A). Therefore, lack of FABP4 expression may alter CEF proliferation/survival in various oxygen concentrations. Furthermore, FABP4 may be a feature strictly of ≤2% O_2 hypoxia and not directly of growth arrest, because although growth arrest occurred at 1.5% and 3% O_2, FABP4 expression was observed in 1.5% as well as 3% O_2, although only minimally in the latter. Additionally, CEF incubated for 24 hours in normoxia became 95% percent confluent as they continued to grow without arrest. This caused them to reach high density and thus experience low O_2 concentrations (hypoxia), resulting in FABP4 induction.

These results highlight the importance of identifying the signals controlling FABP4 and p20k expression in various conditions of growth arrest and hypoxia. Clearly, FABP4 and p20k induction can be distinguished by controlling the degree of confluence and/or hypoxia.

Part 2: FABP4 as a transcriptional target of *C/EBP-β*

FABP4 expression is inducible by C/EBP-β.

C/EBP-β is a transcription factor that has been shown to regulate a number of pro-survival genes (90). However, previous studies conducted in the Bédard lab elucidated C/EBP-β's ability to regulate the growth arrest specific p20k lipocalin gene in CEF (30). Given that FABP4 has similar inducibility to p20k, it was of interest to determine if both genes were under this same key regulator. Western blot analysis showed C/EBP-β strongly induced FABP4 in cycling CEF overexpressing C/EBP-β (Figure 11). These results suggest that C/EBP-β is an upstream regulator of both FABP4 and p20k.

C/EBP-β protein interacts with the FABP4 promoter.

To determine whether C/EBP-β was directly responsible for the upregulation of FABP4 in C/EBP-β overexpressing cells, a ChIP assay was performed. Indeed, C/EBP-β directly bound and interacted with the FABP4 promoter (Figure 12). Although the results for contact inhibited CEF were inconclusive, this interaction was found in both cycling and hypoxic CEF. This suggested that there must be other factors/regulators at work, as FABP4 protein expression is present only in hypoxic and not cycling CEF (Figure 5).

Discussion

Part 1: Identification and characterization of FABP4 as a hypoxia and growth arrest specific gene in CEF.

FABP4 and RBP7 expression and activation are mediated by the hypoxia stress response pathway but not the ER stress response pathway.

FABP4 and RBP7 are two GAS genes that were shown via micro-array to be upregulated at high cell density by approximately 30- and 7-fold, respectively (30). This led us to explore the expression of these proteins in contact inhibition as well as in hypoxia as oxygen deprivation has been shown to be a feature of confluence (29). FABP4 was recently shown to be upregulated following hypoxia and re-oxygenation (31). What's more, intermittent hypoxia has been shown to aggravate adipose tissue dysfunction and metabolic disorders (79, 80), both of which FABP4 has been associated with. Indeed, our results supported these findings as we established that FABP4 was induced by hypoxia and contact inhibition (Figure 5). This pattern was also demonstrated by the GAS gene RBP7 (Figure 6). Therefore, FABP4 and RBP7 expression is a feature of hypoxia and contact inhibition. Although serum starvation is a condition similar to hypoxia and contact inhibition in that starvation induces brief growth arrest, this process is mediated by a different pathway, known as the ER stress response/Unfolded Protein Response (UPR). The lack of FABP4 expression in serum-depleted samples therefore allowed us to differentiate between FABP4's role in growth arrest associated with hypoxia/ROS or the UPR – the latter being a result of strictly ER stress. It is important to note that this agrees with the notion that both are growth arrest

specific genes, as cells undergo quiescence during contact inhibition as well as in hypoxia. In addition, the induction of these hypoxia-responsive genes suggests that oxygen availability regulates the expression of growth arrest specific genes including FABP4 and RBP7, and that this is a separate pathway of induction from ER stress-induced growth arrest in which nutrients is the limiting factor (88).

With clearly inducible expression in hypoxic conditions, we questioned how this condition would affect the localization of FABP4. Although its expression is specific primarily to adipose tissue and macrophages (35), little is known about the intracellular localization of FABP4. In cycling CEF, what little FABP4 expressed was concentrated at what seemed to match the location of focal adhesion plaques and endo-membrane system of the ER-Golgi (Figure 7). This agreed with the western blot analysis as very little FABP4 expression was seen in cycling CEF (Figure 5). However, co-localization studies using ER and focal adhesion plaque markers would be required to confirm this localization. Focal adhesions are congregations of molecules on the plasma membrane which transmit signals between the cell and the extracellular matrix (ECM) (15). The composition and dynamics of the ECM are important for correct cellular functions, including proper lipid metabolism in adipocytes. During adipogenesis, the ECM develops from a fibrillar to a laminar structure as cells undergo commitment and differentiation, a process which is characterized by the storage of triglycerides (69). As preadipocytes develop into adipocytes, the cell shape changes, which is largely dependent on changes in the ECM (15). Interestingly, many adipocytes in obese subjects are hypertrophic, and it has been proposed that hypertrophy of adipocytes prevents proper oxygen supply to the cell, creating a state of hypoxia (99). It is possible

that FABP4 contributes to focal adhesion plaque-mediated signaling between the cell and ECM, where it is mobilized/activated in response to hypoxia. Additionally, unsaturation of fatty acids and lysophospholipids were observed in higher abundance in samples with increased FABP4 expression (41). This is consistent with our findings that FABP4 associates with cell adhesion plaques as studies have suggested that unsaturated fatty acids play a crucial role in the pathway leading to tumour progression, another process which FABP4 has been associated with, by increasing cancer cell adhesion (54). However, several future western blots exhibited zero or very little expression in cycling CEF. Therefore, the fluorescence seen in cycling cells may simply be due to non-specific binding; this remains to be investigated using polypeptide competition or additional antibodies for FABP4. After being incubated in hypoxia (1% O_2) for 24 hours, expression of FABP4 was strongly induced throughout the cell cytoplasm and organelles including the ER and golgi (Figure 7). This not only confirmed its inducibility in response to hypoxia, but that it may take on a new role in this condition. This expression pattern is similar to that of p20k lipocalin (29, 30), suggesting that both proteins function similarly in response to hypoxia. These results support the idea that FABP4 functions to transport lipids damaged by ROS in hypoxic conditions to help maintain lipid homeostasis.

It should be noted that similar patterns were seen in contact-inhibited CEF, although the cells were so tightly compact that precise intra-cellular localization was difficult to determine in these conditions.

FABP4 and p20k exhibit differing kinetics and sensitivity to hypoxia.

Having confirmed FABP4's expression, intra-cellular localization, and therefore activation in hypoxia, it was of interest to determine how sensitive this induction was to specific oxygen concentrations. Our results showed that FABP4 was induced by 1% and 2% O_2, while concentrations above 2% were not sufficient (Figure 8), similar to the pattern of p20k induction previously reported (29). However, our analysis of p20k showed more stringent regulation as only 1% O_2 could induce its expression (Figure 8). Taken together, both p20k and FABP4 are inducible by limiting oxygen, with p20k exhibiting a stricter inducible range. Therefore, FABP4 may respond to higher O_2 concentrations than p20k. Interestingly, a recent study showed intermittent hypoxia stimulated the upregulation of FABP4 *in vitro* (104). This further supports the idea that oxygen availability regulates the expression of growth arrest specific genes such as FABP4 and p20k, and additionally that there are varying oxygen thresholds depending on the protein. The increased O_2% sensitivity of FABP4 may be due to the fact that p20k does not get induced until extremely low O_2 concentrations. The cell may induce FABP4 pre-p20k induction as means to aid in survival in more modest conditions of hypoxia. As stated previously, p20k expression is inhibited by the binding of ERK2 as this prevents C/EBP-β from interacting with the Quiescence Responsive Unit (QRU) (30). Perhaps ERK2 does not fall off of the QRU until extremely harsh hypoxia occurs (≤1% O_2), up until which FABP4 is induced in order to help the cell survive, or at the very least, enter a state of growth arrest. Alternatively, mPPARy2 may be responsible for the discrepancy in FABP4 versus p20k expression. mPPARy2 promotes FABP4 expression (97), whereas it inhibits p20K expression (57). Since the two proteins are

regulated differentially, perhaps PPAR mediates some sort of feedback loop to control the cell from making too much of both p20K and FABP4 unnecessarily.

Studies previously conducted in the Bédard lab reported time dependent inducibility and demonstrated the kinetics of p20k lipocalin in response to contact inhibition. p20k was induced in contact inhibited cells for up to 9 days, after which its expression subsided as CHOP expression began to increase (30). We questioned whether the kinetics and time-dependent inducibility of FABP4 would resemble or diverge from that of p20k, considering they exhibited differing responses to various oxygen concentrations (Figure 8). The results showed that p20k was induced as quickly as 6 hours into hypoxia incubation, whereas it took 12 to 24 hours for FABP4 expression to become apparent (Figure 9). Therefore, FABP4 expression is more sensitive than p20k when it comes to varying oxygen concentrations, but has a slower onset of induction. This slow onset may be due to additional factors interacting with the FABP4 promoter. It is possible that other transcriptional regulators of FABP4 are recruited in a slower manner, thus resulting in less rapid induction. If these regulators can be identified, their inhibition may be useful as therapeutic targets in metabolic diseases. These factors would be more realistic therapeutic targets as their slow recruitment/activation would allow for more time for targeting and inhibition. Another possible mechanism behind the slower onset is post-transcriptional regulation. While the post-transcriptional regulation of p20k has not been investigated, FABP4 has been shown to be post-transcriptionally regulated by microRNA (miRNA). miRNA is a small, non-coding RNA molecule that silences genes by binding to RNA transcripts and preventing their translation or promoting their degradation. An inverse association

between FABP4 and miR-409-3p expression was observed as ovarian cancer cell lines showed low expression of miR-409-3p and high FABP4 expression, with the opposite occurring in control cells (41). Furthermore, HeyA8 MDR cells transfected with a miR-409-3p mimic led to a significant decrease in FABP4 expression (41). FABP4 was not only confirmed a target of miR-409-3p, but its target sequence established as well. Control cells stably expressing a FABP4 construct lacking the 3' UTR of FABP4 were no longer sensitive to injections of a miR-409-3p mimic, as their ability to metastasize was not decreased as seen in cancer cells expressing regular FABP4 (41). Interestingly, the same study found that hypoxia could downregulate miR-409-3p, therefore removing its inhibitory effect on FABP4 and resulting in increased FABP4 levels (41). Perhaps this miRNA is not expressed in ≤2% O_2, thus relieving FABP4 mRNA of inhibition, resulting in an increase in expression occurring over a rate of time dependent on the dissociation of the miRNA.

FABP4 is induced by contact inhibition and hypoxia defined strictly at ≤2% O_2, and is associated with growth arrest and possibly cell survival.

After finding little FABP4 expression in 3% oxygen, we wanted to determine if this correlated with CEF proliferation or survival in comparison to oxygen concentrations that did induce high expression. The proliferation assays showed growth arrest at 1.5%, 3%, and 4% oxygen (Figure 10A,B,C), although western blot analysis confirmed strong FABP4 expression in only 1.5% O_2, and minimal in 3% O_2. (Figure 10D). This suggests that, as oxygen concentrations dwindle, FABP4 is upregulated and may assist in growth arresting the cell. Additionally, FABP4 may aid in slight cell proliferation/survival once expressed robustly enough as seen at 1.5% O_2 (Figure 10A). Furthermore, this

suggests FABP4 is a feature of hypoxia, specifically ≤2% O_2, which then promotes quiescence. On the other hand, CEF began to die off at 4% O_2 (Figure 10C), which may have been due to a complete lack of FABP4 at 4%. As stated before, higher amounts of unsaturated fatty acids and lysophospholipids were observed in samples with increased FABP4 expression (41). Additionally, studies have suggested that unsaturated fatty acids play a key role in pathways leading to tumour progression (proliferation) by activating the β-catenin pathway or downregulating PTEN in the PTEN tumour suppressor pathway (56, 103). What's more, the knock down of FABP4 led to a significant reduction in migration and invasion of ovarian cancer cells (41). The same study also discovered that treatment with tamoxifen decreased FABP4 expression by 50%, which resulted in the impairment of free fatty acid uptake as well as a significant decrease in the migratory abilities of cancer cells (41). Furthermore, previous studies have shown that FABP4 presence in the stromal cells can lead to cancer progression by providing a source of energy (lipids) to cancer cells or by increasing angiogenesis (47, 77). Taken altogether with our results, we postulated that FABP4 may interact with or effect, via fatty acid regulation, pathways regulating CEF proliferation/survival in conditions of hypoxia (defined at ≤2% O_2).

Furthermore, FABP4 may be a feature strictly of ≤2% O_2 hypoxia and not directly of growth arrest, because although growth arrest occurred at 1.5% and 3% O_2, FABP4 expression was only robustly seen at 1.5% (and minimally at 3% O_2) (Figure 10A,B,D). Additionally, the expression of FABP4 observed in CEF incubated for 24 hours in normoxia further confirmed its inducibility by contact inhibition and hypoxia as these cells grew to confluency after 24 hours in normoxia. At this point, the cells became

dense, resulting in contact inhibition and low oxygen supply (hypoxia). As time was limited, we could not determine whether the oxygen concentrations in these samples were specifically 2% or less. It would be of interest in the future to measure the oxygen concentration in contact inhibited samples as this would determine whether ≤2% is indeed required for FABP4 induction. This would allow insight into if specific oxygen supplementation to cells could minimize FABP4 induction and therefore potentially combat obesity and metabolic diseases in which FABP4 is associated with.

Part 2: FABP4 as a transcriptional target of C/EBP-β.

FABP4 exhibits complex regulation at the transcriptional level.

Given that FABP4 exhibited similar inducibility to p20k, it was of interest to determine if both genes were under the same key regulator. C/EBP-β is a transcription factor that has been shown to regulate several pro-survival genes (90), however, this protein has also been shown to act in an anti-proliferative manner. In CEF, C/EBP-β inhibition was shown to enhance the expression of the 3 components of AP1 - JunD, Fra2, and C-Jun (38). This then enhanced AP1 activity, consequently increasing cyclin D1 expression (38). Cyclin D1 promotes proliferation, showing that in this case, C/EBP-β has a clear upstream anti-proliferative role as its inhibition promoted proliferation. In addition to this, C/EBP-β regulates the growth arrest specific p20k lipocalin gene in CEF (30). C/EBP-β acts as a transcriptional activator of p20k by binding to the QRU, however, ERK2 competes with C/EBP-β for this site on p20k to function as a transcriptional repressor of the gene (30). Although ERK2 was not investigated in relation to FABP4, C/EBP-β strongly induced FABP4 in cycling CEF overexpressing C/EBP-β (Figure 11). These results agree with the fact that C/EBP-β is required for proper functioning macrophages (82), a cell type in which FABP4 is commonly expressed. These results suggest that C/EBP-β is an upstream regulator of FABP4, although it is unclear if C/EBP-β is necessary for FABP4 induction in response to hypoxia or contact inhibition. Future experiments should be conducted to determine whether C/EBP-β is necessary for FABP4 induction when in hypoxic conditions or at contact inhibition.

To further elucidate the regulation of FABP4 by C/EBP-β, we wanted to determine whether C/EBP-β acted directly on the FABP4 promoter. A ChIP analysis showed that C/EBP-β directly bound and interacted with the FABP4 promoter in both cycling and hypoxic CEF (Figure 12). Interestingly, cycling CEF do not express FABP4 at the protein level (Figure 5). Therefore, there may be other factors interacting with the FABP4 promoter to suppress its expression in cycling CEF when C/EBP-β is bound. Furthermore, these additional factors may be recruited at a slower rate (as we showed a slower rate of induction compared to p20k) that work in tandem with C/EBP-β in FABP4 induction during growth arrest.

As stated previously, FABP4 is a target for silencing by miR-409-3p on its 3’ UTR (41). What’s more, the same study found that hypoxia could downregulate miR-409-3p, thereby relieving FABP4 of inhibition (41). This provides evidence of the possibility that post-transcriptional regulation of FABP4 occurs in CEF so to upregulate FABP4 when oxygen is limited. The mechanism behind this may be that, following the hypoxia-mediated downregulation of miR-409-3p, C/EBP-β is able to produce and accumulate FABP4 transcript which is then translated and expressed. It should be noted that this FABP4 accumulation would only occur when the miRNA is down-regulated in growth arrested, hypoxic cells. Considering the 3' UTR is known to contain regulatory regions that influence gene expression via post-transcriptional modifications, this may indeed be what occurs in hypoxic CEF.

Alternatively, perhaps C/EBP-β has an inhibitory role on FABP4 when cells are cycling, but is then post-translationally modified upon hypoxia exposure which causes it to relieve FABP4 of inhibition, consequently activating FABP4 in response to hypoxia.

C/EBP-β activity is known to be controlled by conserved negative regulatory domains adjacent to and interacting with the trans-activating region of the protein (57). One proposed model of its post-translational modification describes how the de-repression of the concealed transactivating region is influenced by the phosphorylation of specific residues of the regulatory domains or association with trans-acting factors (59, 106). These processes would then result in the unfolding of C/EBP-β (59, 106). Furthermore, many studies have demonstrated C/EBP-β's phosphorylation-mediated activation (75, 98, 105), supporting the possibility that post-translational modification of C/EBP-β influences FABP4 expression through the activation or inhibition of C/EBP-β.

Conclusion & Future Directions

Several conclusions can be drawn from our results. Similar to the characterization of p20k in the Bédard Lab, we concluded that both FABP4 and RBP7 are induced by contact inhibition and hypoxia, but not by starvation. We attribute this to the differential induction of stress response pathways. With hypoxia being a feature of contact inhibition, both conditions induce a growth arrest-mediated lipid stress response, where oxygen is a limiting factor, resulting in the upregulation of GAS genes. However, these GAS genes - FABP4, RBP7, and p20k – are not induced by serum starvation as this facilitates ER stress and the UPR, conditions which have previously been shown to antagonize p20k expression (30). The induction of these hypoxia-responsive genes suggests that oxygen availability regulates the expression of a sub-class of growth arrest specific genes.

We concluded that hypoxia not only induces the production of FABP4, but also possibly its localization to organelles such as the ER and golgi. We observed a strong increase in fluorescence at these locations in hypoxic CEF compared to the minimal fluorescence seen at focal adhesion plaques in cycling CEF. It is possible that FABP4 re-localized from plaques to move intrinsically throughout the cell when oxygen became limited. These results not only confirmed FABP4's upregulation in response to hypoxia, but also suggest that it "spreads" throughout the cells to perhaps facilitate the trafficking of lipids damaged by ROS, as ROS levels are increased during hypoxia (29). This agrees with the hypothesis that FABP4 has a role in lipid homeostasis. To determine whether the little FABP4 present in cycling cells does indeed localize to focal adhesion

plaques, a future experiment consisting of a co-stain of FABP4 and a cell adhesion plaque marker such as FAC or SRC should be performed.

FABP4 induction by hypoxia was confirmed to be oxygen concentration specific, as FABP4 was only present at oxygen concentrations of ≤2% O_2. p20k oxygen requirements were even more stringent, as it was only inducible predominantly at 1% O_2. Furthermore, this inducibility was time dependent and varied between proteins as p20k exhibited a more rapid onset than FABP4 upon hypoxia exposure. Again, this supports the idea that oxygen availability regulates the expression of GAS genes, and additionally, that there are protein-specific oxygen concentration thresholds and kinetics depending on the sensitivity of the protein.

Growth arrest occurred at 1.5%, 3%, and 4% O_2, while FABP4 was only expressed strongly in the lysate of 1.5% O_2 and minimally at 3% O_2. This suggested that FABP4 may be a feature of hypoxia defined specifically at ≤2% O_2. This agrees with the fact it was expressed in contact inhibited samples, as high confluency results in low oxygen concentrations, although we did not measure if the oxygen levels were ≤2% in these samples. Additionally, the results from the proliferation assays suggested that FABP4 is associated with growth arrest and may promote survival, as slight proliferation was observed at 1.5% O_2 when it was strongly expressed, whereas cells began to arrest and die off at oxygen concentrations in which FABP4 expression was low (3-4% O_2) (Figure 10). Future experiments should investigate the O_2% in contact inhibited CEF to confirm if ≤2% O_2 truly is the threshold for FABP4 induction or if it is a separate feature of growth arrest. However, as implied by the 3% O_2 proliferation assay in which growth arrest occurred with little FABP4 present, it is unlikely that FABP4 is a feature of

growth arrest by itself. A future experiment should be conducted to assess whether mild oxidants induce FABP4, thus confirming it strictly as a feature of oxygen limitation. Future experiments should also consist of western blot analysis of 4% O_2, a condition in which cells began to die off, in order to confirm no expression of FABP4 in this condition.

Similar to p20k lipocalin, the results showed that FABP4 is inducible by forced expression C/EBP-β (Figure 11). Furthermore, using ChIP assays, we found that C/EBP-β interacts directly with the FABP4 promoter (Figure 12). Therefore, it would be of interest to determine if C/EBP-β is required for FABP4 activation in hypoxic and contact inhibited CEF. This could be done by either the down-regulation of C/EBP-β by shRNA/RNAi, or by transfecting CEF with a dominant negative version of C/EBP-β, resulting in non-functional C/EBP-β. This additional analysis would form a more complete picture of the pathway of induction of FABP4.

Other future directions include further elucidating the regulation of FABP4 in relation to C/EBP-β. It is possible that FABP4 induction depends on an additional factor that is more slowly induced or recruited (as we demonstrate slower induction than p20k) and cooperates with C/EBP-β in FABP4 induction during growth arrest. An alternative interpretation of FABP4 regulation by C/EBP-β is that C/EBP-β itself acts as a repressor on the FABP4 promoter. C/EBP-β may sit on the promoter until post-translationally modified upon hypoxia or ROS exposure, becoming a potent activator driving FABP4 transcription. Both of these interpretations would be possible avenues to explore in the future. A ChIP analysis using antibodies of candidate proteins with FABP4 primers would be ideal for the testing of candidate regulatory factors. An additional future

direction would be to conduct a luciferase assay to analyze the activity of C/EBP-β on the FABP4 promoter in cycling and hypoxic CEF.

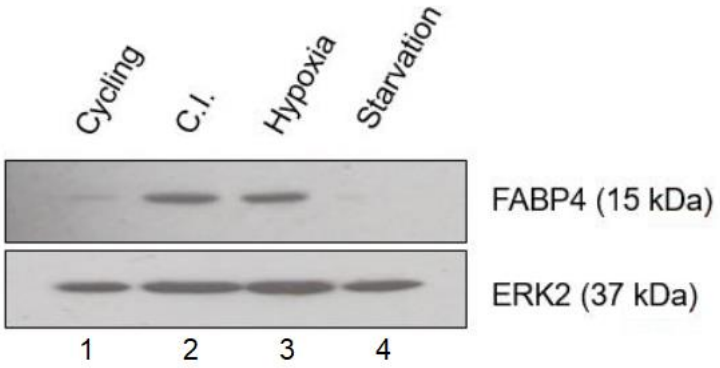
Lastly, post-transcriptional regulation may be another potential mechanism influencing FABP4 expression. miRNA-mediated inhibition may silence FABP4 expression even when C/EBP-β is bound to FABP4 DNA in cycling cells. However, once hypoxic conditions minimize the amount of FABP4 mRNA-targeting miRNA, FABP4 transcripts will be produced and thus will accumulate, resulting in protein expression. A western blot experiment employing the use of antisense oligonucleotides (antimiRs) to inhibit miR-409-3p in cycling CEF should be performed. As this would increase FABP4 transcript levels, it could be determined whether miRNA is required for the repression of FABP4 in cycling CEF.

The long-term goal of this study was to identify and characterize crucial processes involved in a novel “Membrane Stress Response”, as proposed by Bédard and co-investigators (Erb et al, unpublished). Due to the majority of studies aiming to characterize the cell cycle in terms of proliferation and progression through, this project aimed to determine the key processes involved in the exit from the cell cycle as well as the ability to re-enter after specific cellular maintenance. Contact inhibition, hypoxia, and serum-deprivation induce this exit into the G_0 state, however, through two separate pathways. Both contact inhibition- and hypoxia-induced growth arrest are mediated at least in part by limiting oxygen (29). When oxygen is limited, blocking p20k expression, and possibly other GAS genes, exacerbates lipid peroxidation (29). Oxygen sensitive GAS genes likely have a large role in regulating oxidized lipids to maintain lipid homeostasis, whereas serum-depletion facilitates growth arrest through ER stress and

the UPR. This project characterized the response to contact inhibition and hypoxia, the expression and regulation of FABP4 in these conditions, and how this may relate to cell survival and cell cycle exit. Although this study characterized and filled a gap in understanding key processes of cell homeostasis in relation to GAS genes, there remains many questions and future directions. This includes a more extensive investigation of the transcriptional regulators of FABP4, especially C/EBP-β, as elucidating this pathway could provide means of combatting diabetes, obesity, atherosclerosis, among many other metabolic diseases that FABP4 has been associated with.

Experimental Figures

A.



B.

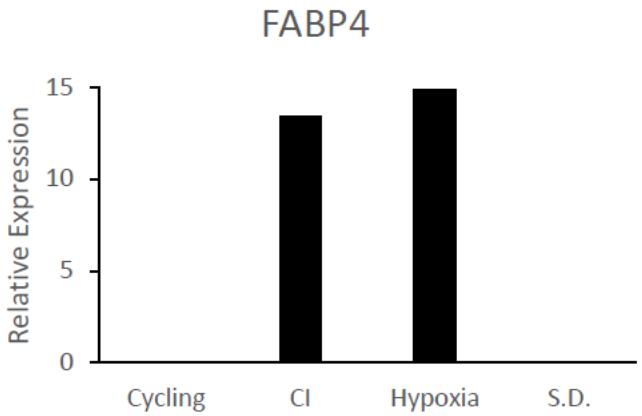
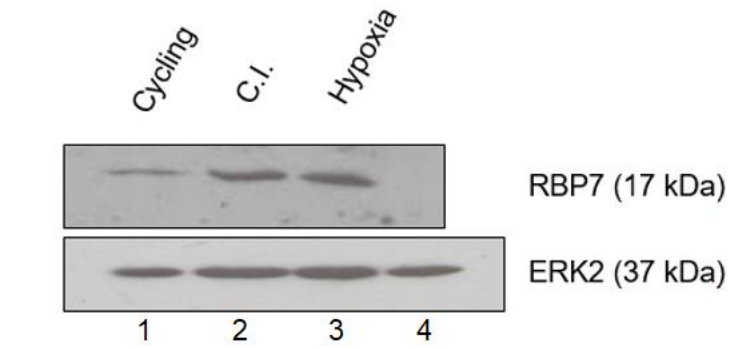
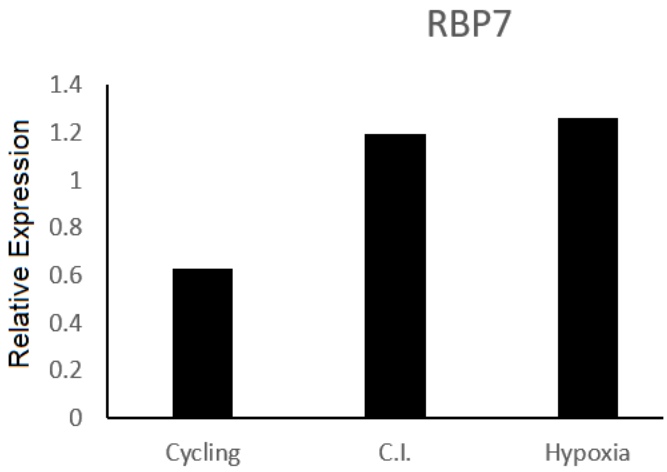


Figure 5. Western blot analysis of FABP4 in CEF incubated in hypoxia (1% O_2), undergoing contact inhibition (CI), or incubated in serum-free medium (serum depletion/starvation) (SD). Protein loading was examined by probing the blot for ERK2. B. Quantification of western blot.



A.



B.

Figure 6. A. Western blot analysis of RBP7 in CEF incubated in hypoxia (1% O_2) or undergoing contact inhibition. Protein loading was examined by probing the blot for ERK2. B. Quantification of western blot.

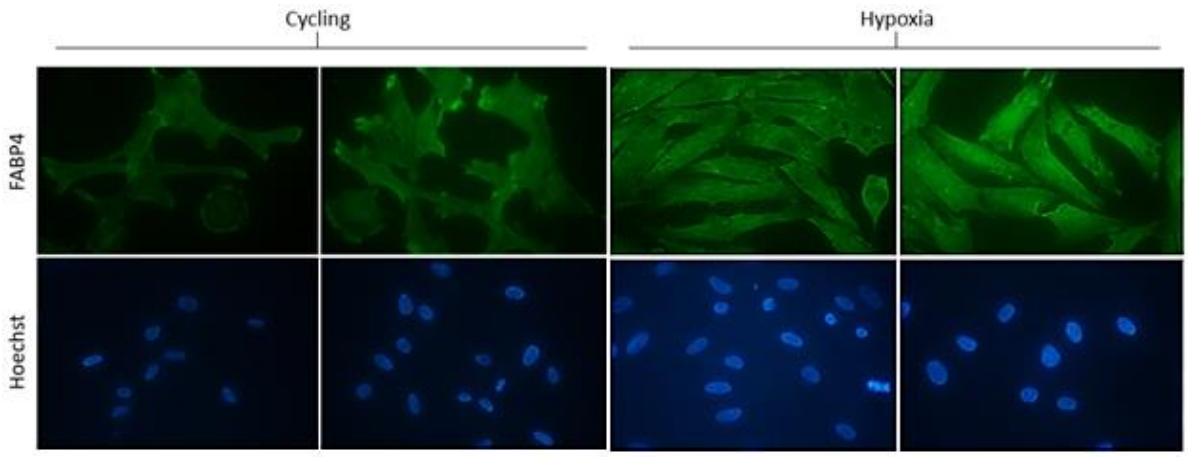
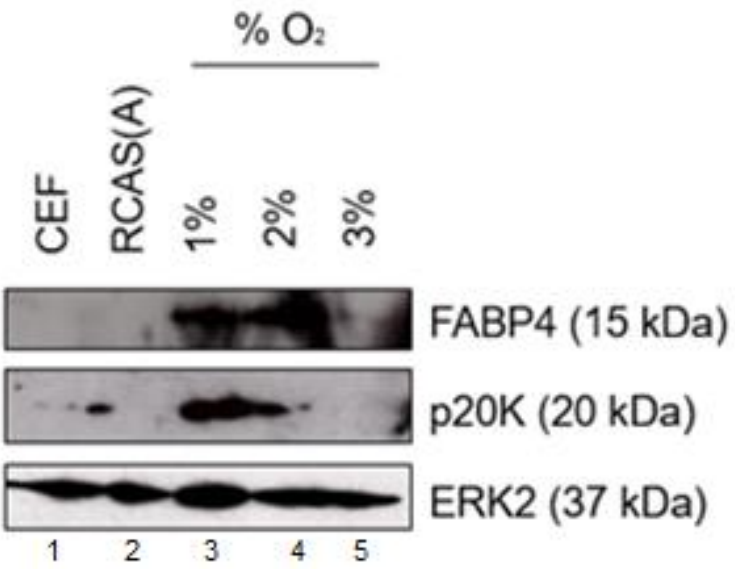
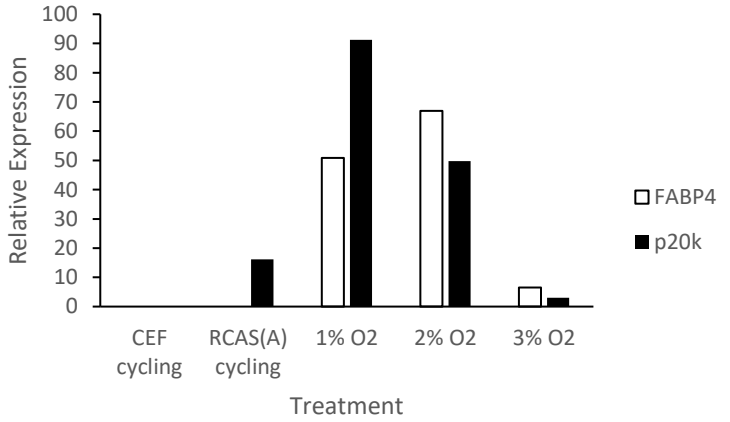


Figure 7. Immunofluorescence analysis of FABP4 protein localization in cycling CEF and CEF undergoing hypoxia. Hoechst stain was used to visualize nuclei in comparison to protein localization.

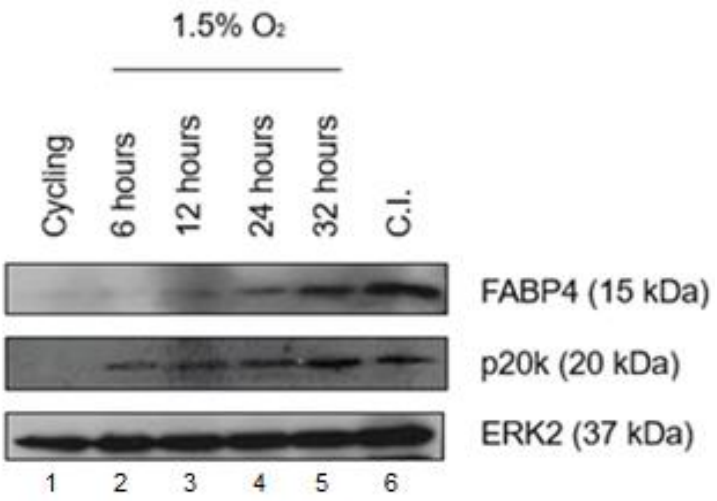


A.

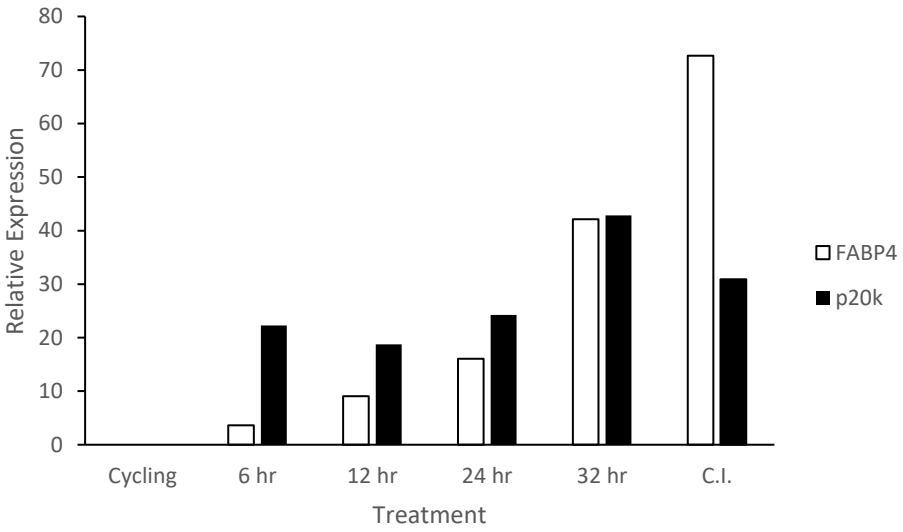


B.

Figure 8. A. Western blot analysis of FABP4 and p20K in CEF incubated in varying oxygen concentrations (1%, 2%, 3% O_2). Protein loading was examined by probing the blot for ERK2. B. Quantification of western blot.

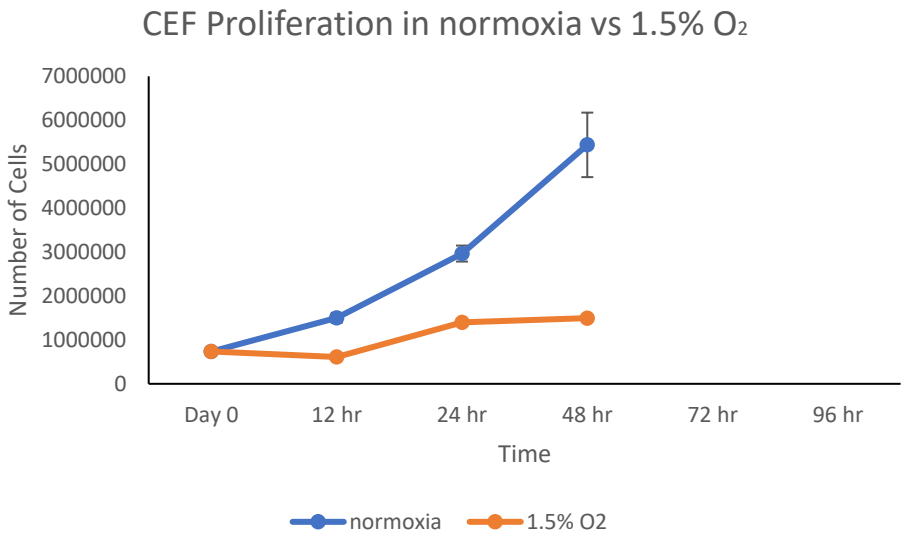


A.

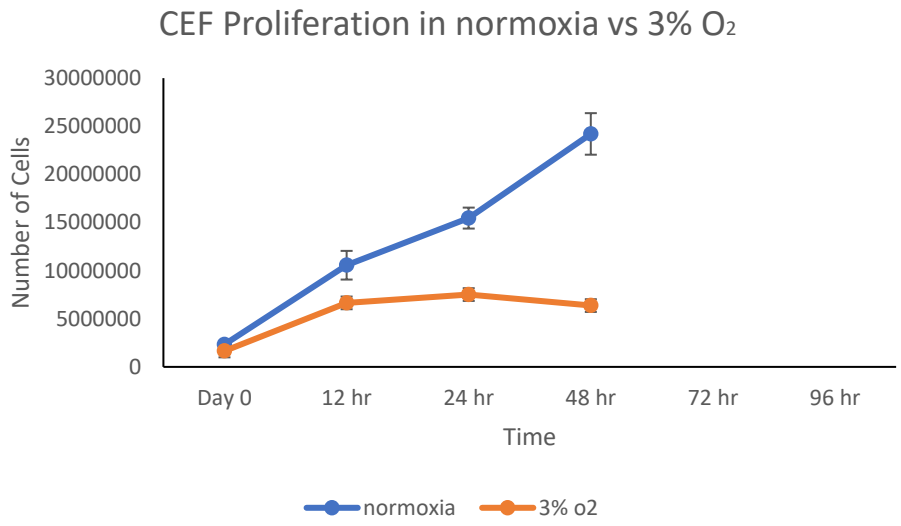


B.

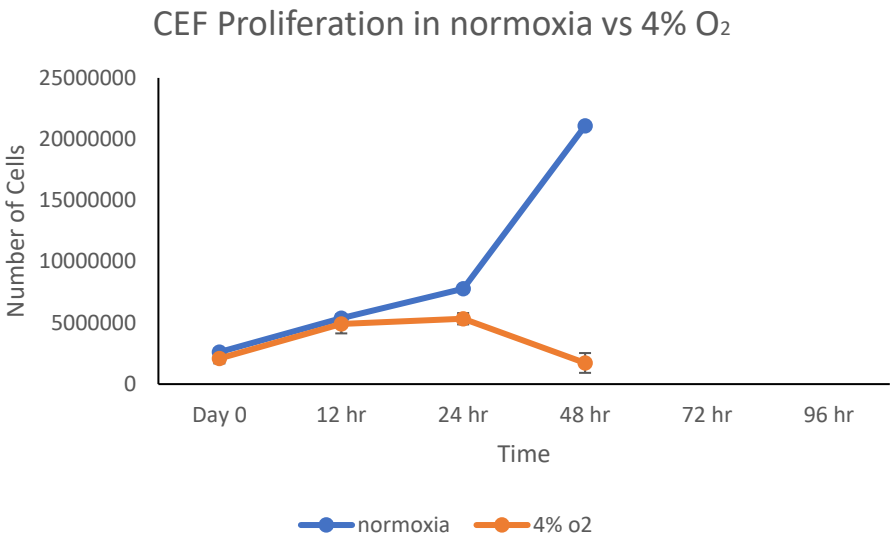
Figure 9. A. Western blot analysis of FABP4 in CEF incubated in hypoxia for various time periods (6, 12, 24, 32 hours) or undergoing contact inhibition. Protein loading was examined by probing the blot for ERK2. B. Quantification of western blot.


CEF Proliferation in normoxia vs 1.5% O2
Number of Cells
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
Day 0
12 hr
24 hr
48 hr
72 hr
96 hr
Time
normoxia
1.5% O2

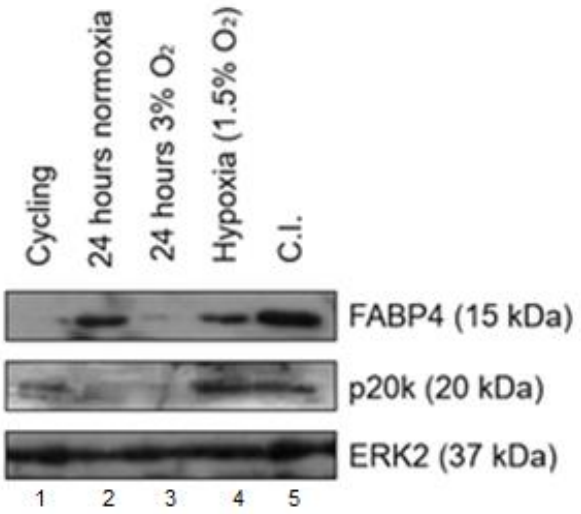
A.


CEF Proliferation in normoxia vs 3% O2
Number of Cells
30000000
25000000
20000000
15000000
10000000
5000000
0
Day 0
12 hr
24 hr
48 hr
72 hr
96 hr
Time
normoxia
3% o2

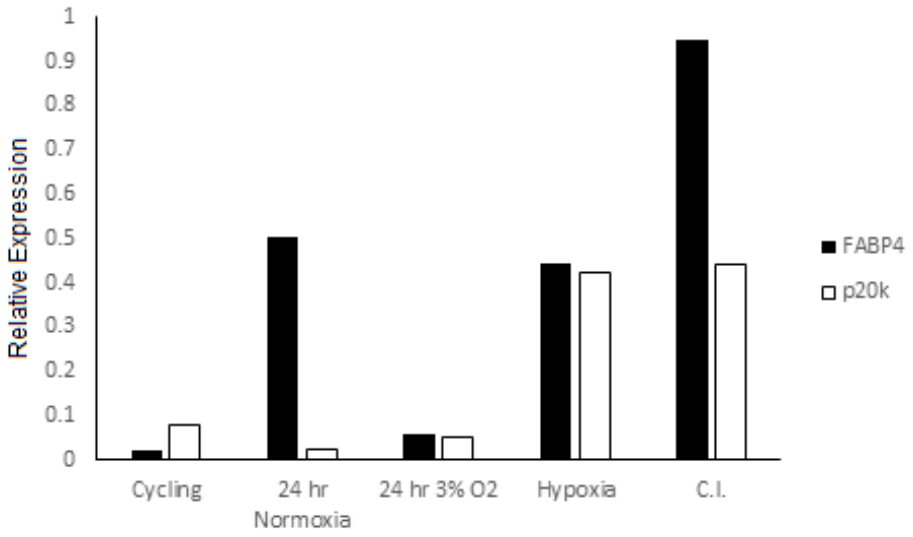
B.


CEF Proliferation in normoxia vs 4% O2
Number of Cells
25000000
20000000
15000000
10000000
5000000
0
Day 0
12 hr
24 hr
48 hr
72 hr
96 hr
Time
normoxia
4% o2

C.


Cycling
24 hours normoxia
24 hours 3% O2
Hypoxia (1.5% O2)
C.I.
FABP4 (15 kDa)
p20k (20 kDa)
ERK2 (37 kDa)
1
2
3
4
5

D.



E.

Figure 10. A. Proliferation assay at 1.5% oxygen. B. Proliferation assay at 3% oxygen. C. Proliferation assay at 4% oxygen. D. Western blot corresponding to proliferation assay conditions. D. Western blot probing for FABP4 and p20k in cycling, 24 hour normoxia (confluent), 24 hours 3% 24 hours 1.5% O_2, and contact inhibition. Protein loading was examined by probing the blot for ERK2. E. Quantification of western blot.

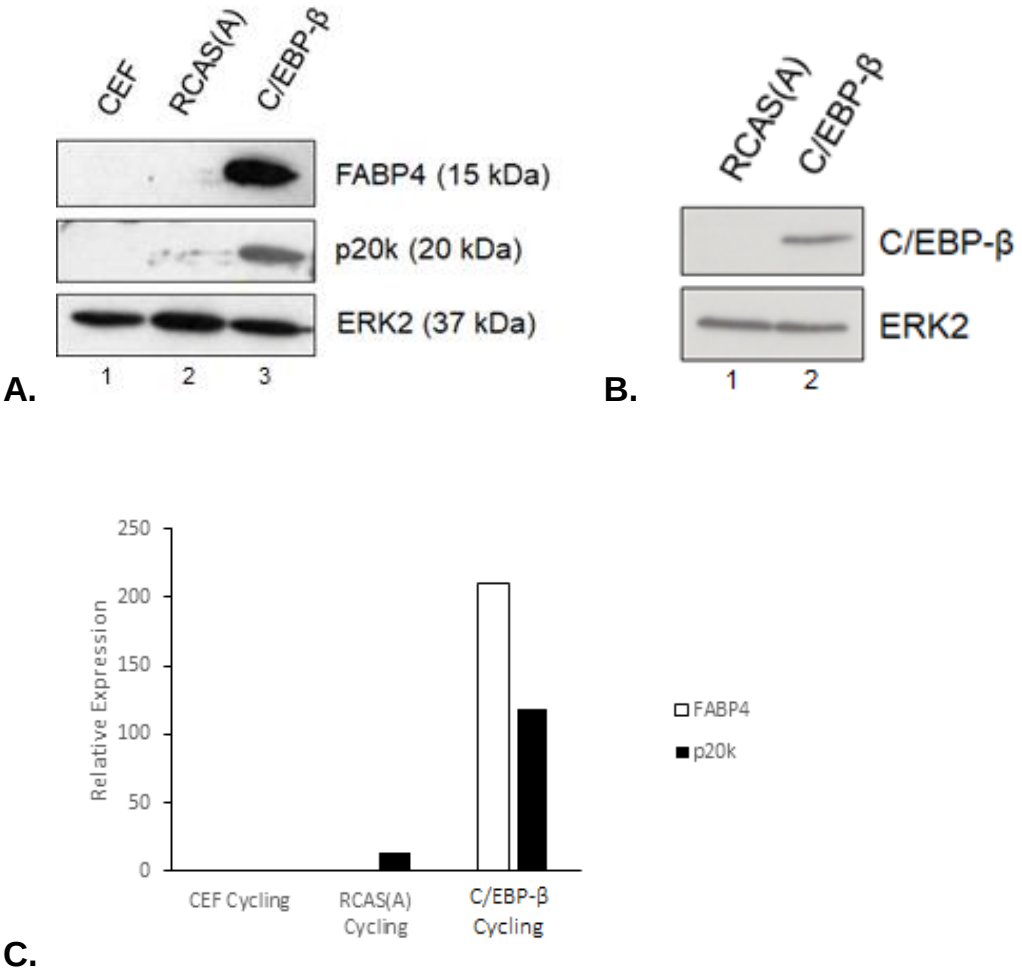


Figure 11. A. Western blot analysis of FABP4 and p20K in CEF, RCAS(A), and C/EBP-β overexpressing cells. Protein loading was examined by probing the blot for ERK2. B. Western blot analysis confirming overexpression of C/EBP-β in cells infected with NFM-A vector. Protein loading was examined by probing the blot for ERK2. C. Quantification of western blot. All cells were sub-confluent and cultured in conditions of normoxia in these experiments.

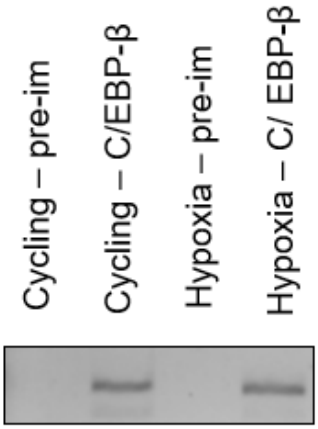


Figure 12. ChIP assay of cycling and hypoxic (1.5% O_2) CEF probed with C/EBP-β antibody and amplified with FABP4 primers. Probing with pre-immune antibody was used as a control. Bands correspond to 500 bp.

Reference List

1. Ahn, J. *et al.* Identification of the Avian RBP7 Gene as a New Adipose-Specific Gene and RBP7 Promoter-Driven GFP Expression in Adipose Tissue of Transgenic Quail. *PLOS ONE* **10,** e0124768 (2015).
2. Alberts, B. *et al. Molecular Biology of the Cell*. (Garland Science, 2002).
3. Bazán, N. G. Effects of ischemia and electroconvulsive shock on free fatty acid pool in the brain. *Biochim. Biophys. Acta* **218,** 1–10 (1970).
4. BD Biosciences Research - Apoptosis, Cell Cycle, and Cell Proliferation - Analysis - Cell Cycle. Available at: http://www.bdbiosciences.com/in/research/apoptosis/analysis/index.jsp. (Accessed: 7th August 2018)
5. Beckman, K. B. & Ames, B. N. The free radical theory of aging matures. *Physiol. Rev.* **78,** 547–581 (1998).
6. Bédard, P. A., Balk, S. D., Gunther, H. S., Morisi, A. & Erikson, R. L. Repression of quiescence-specific polypeptides in chicken heart mesenchymal cells transformed by Rous sarcoma virus. *Mol Cell Biol* **7,** 1450–1458 (1987).
7. Brugarolas, J. *et al.* Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. *Genes Dev* **18,** 2893–2904 (2004).
8. Buck, M., Poli, V., van der Geer, P., Chojkier, M. & Hunter, T. Phosphorylation of rat serine 105 or mouse threonine 217 in C/EBP beta is required for hepatocyte proliferation induced by TGF alpha. *Mol. Cell* **4,** 1087–1092 (1999).
9. Cancedda, F. D. *et al.* The Ch21 protein, developmentally regulated in chick embryo, belongs to the superfamily of lipophilic molecule carrier proteins. *International Journal of STD and AIDS, The Journal of biological chemistry, The Journal of biological chemistry.* **265,** 19060–19064 (1990).

10. Cancedda, F. D. *et al.* The developmentally regulated avian Ch21 lipocalin is an extracellular fatty acid-binding protein. *J. Biol. Chem.* **271,** 20163–20169 (1996).
11. Cao, H. et al. Adipocyte lipid chaperone aP2 is a secreted adipokine regulating hepatic glucose production. *Cell Metab.* **17,** 768–778 (2013).
12. Carlevaro, M. F., Cermelli, S., Cancedda, R. & Descalzi Cancedda, F. Vascular endothelial growth factor (VEGF) in cartilage neovascularization and chondrocyte differentiation: auto-paracrine role during endochondral bone formation. *J. Cell. Sci.* **113 (Pt 1),** 59–69 (2000).
13. CEBPB Gene - GeneCards | CEBPB Protein | CEBPB Antibody. Available at: https://www.genecards.org/cgi-bin/carddisp.pl?gene=CEBPB. (Accessed: 21st August 2018)
14. Cermelli, s *et al.* Extracellular fatty acid binding protein (Ex-FABP) modulation by inflammatory agents: 'Physiological' acute phase response in endochondral bone formation. *European journal of cell biology* **79,** 155–64 (2000).
15. Chen, C. S., Alonso, J. L., Ostuni, E., Whitesides, G. M. & Ingber, D. E. Cell shape provides global control of focal adhesion assembly. *Biochem. Biophys. Res. Commun.* **307,** 355–361 (2003).
16. Chen, J., Saha, P., Kornbluth, S., Dynlacht, B. D. & Dutta, A. Cyclin-binding motifs are essential for the function of p21CIP1. *Mol. Cell. Biol.* **16,** 4673–4682 (1996).
17. Cocco, T., Di Paola, M., Papa, S. & Lorusso, M. Arachidonic acid interaction with the mitochondrial electron transport chain promotes reactive oxygen species generation. *Free Radic. Biol. Med.* **27,** 51–59 (1999).
18. Corey, S. J. & Rosoff, P. M. Unsaturated fatty acids and lipoxygenase products regulate phagocytic NADPH oxidase activity by a nondetergent mechanism. *J Lab Clin Med* **118,** 343–351 (1991).
19. Darzynkiewicz, Z., Gong, J., Juan, G., Ardelt, B. & Traganos, F. Cytometry of cyclin proteins. *Cytometry* **25,** 1–13 (1996).

20. David-Pfeuty, T. & Nouvian-Dooghe, Y. Human cyclin B1 is targeted to the nucleus in G1 phase prior to its accumulation in the cytoplasm. *Oncogene* **13,** 1447–1460 (1996).
21. Davydov, I. V., Krammer, P. H. & Li-Weber, M. Nuclear factor-IL6 activates the human IL-4 promoter in T cells. *J. Immunol.* **155,** 5273–5279 (1995).
22. Del Sal, G., Ruaro, M. E., Philipson, L. & Schneider, C. The growth arrest-specific gene, gas1, is involved in growth suppression. *Cell* **70,** 595–607 (1992).
23. Descombes, P. & Schibler, U. A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. *Cell* **67,** 569–579 (1991).
24. Dijk, T. B. van *et al.* A Composite C/EBP Binding Site Is Essential for the Activity of the Promoter of the IL-3/IL-5/Granulocyte-Macrophage Colony-Stimulating Factor Receptor βc Gene. *The Journal of Immunology* **163,** 2674–2680 (1999).
25. Distel, R. J., Robinson, G. S. & Spiegelman, B. M. Fatty acid regulation of gene expression. Transcriptional and post-transcriptional mechanisms. *J. Biol. Chem.* **267,** 5937–5941 (1992).
26. Domschke, G. *et al.* Systematic RNA-interference in primary human monocyte-derived macrophages: A high-throughput platform to study foam cell formation. *Scientific Reports* **8,** 10516 (2018).
27. Du, Z.-P. *et al.* A systematic analysis of human lipocalin family and its expression in esophageal carcinoma. *Sci Rep* **5,** (2015).
28. Duranteau, J., Chandel, N. S., Kulisz, A., Shao, Z. & Schumacker, P. T. Intracellular signaling by reactive oxygen species during hypoxia in cardiomyocytes. *J. Biol. Chem.* **273,** 11619–11624 (1998).
29. Erb, M. J. Characterizing The Transcriptional Regulation And Role Of The Growth Arrest Specific P20K Lipocalin In Chicken Embryo Fibroblasts. Masters Thesis. *McMaster University.* Available from: http://hdl.handle.net/11375/20435 (2016).

30. Erb, M. J. *et al.* Extracellular Signal-Regulated Kinase 2 and CHOP Restrict the Expression of the Growth Arrest-Specific p20K Lipocalin Gene to G_0. *Mol. Cell. Biol.* **36,** 2890–2902 (2016).
31. Feldkamp, T., Kribben, A., Roeser, N. F., Senter, R. A. & Weinberg, J. M. Accumulation of nonesterified fatty acids causes the sustained energetic deficit in kidney proximal tubules after hypoxia-reoxygenation. *Am. J. Physiol. Renal Physiol.* **290,** F465-477 (2006).
32. Ferroni, P., Basili, S., Falco, A. & Davì, G. Inflammation, insulin resistance, and obesity. *Curr Atheroscler Rep* **6,** 424–431 (2004).
33. Flower, D. R. The lipocalin protein family: structure and function. *Biochem J* **318,** 1–14 (1996).
34. Foster, D. A., Yellen, P., Xu, L. & Saqcena, M. Regulation of G1 Cell Cycle Progression: Distinguishing the Restriction Point from a Nutrient-Sensing Cell Growth Checkpoint(s). *Genes & Cancer* **1,** 1124–1131 (2010).
35. Furuhashi, M. *et al.* Treatment of diabetes and atherosclerosis by inhibiting fatty-acid-binding protein aP2. *Nature* **447,** 959–965 (2007).
36. Furuhashi, M. & Hotamisligil, G. S. Fatty acid-binding proteins: role in metabolic diseases and potential as drug targets. *Nat Rev Drug Discov* **7,** 489–503 (2008).
37. Furuhashi, M., Saitoh, S., Shimamoto, K. & Miura, T. Fatty Acid-Binding Protein 4 (FABP4): Pathophysiological Insights and Potent Clinical Biomarker of Metabolic and Cardiovascular Diseases. *Clin Med Insights Cardiol* **8,** 23–33 (2014).
38. Gagliardi, M. *et al.* Opposing roles of C/EBPbeta and AP-1 in the control of fibroblast proliferation and growth arrest-specific gene expression. *J. Biol. Chem.* **278,** 43846–43854 (2003).
39. Gagliardi M, Maynard S, Bojovic B, Bedard PA. The constitutive activation of the CEF-4/9E3 chemokine gene depends on C/EBPβeta in v-src transformed chicken embryo fibroblasts. *Oncogene* **20**:2301-2313 (2001).
40. Gentili, C. *et al.* Expression of the Extracellular Fatty Acid Binding Protein (Ex-FABP) during Muscle Fiber Formation in Vivo and in Vitro. *Experimental Cell Research* **242,** 410–418 (1998).

41. Gharpure, K. M. *et al.* FABP4 as a key determinant of metastatic potential of ovarian cancer. *Nat Commun* **9,** (2018).
42. Gong, Y., Yi, M., Fediuk, J., Lizotte, P. P. & Dakshinamurti, S. Hypoxic neonatal pulmonary arterial myocytes are sensitized to ROS-generated 8-isoprostane. *Free Radic. Biol. Med.* **48,** 882–894 (2010).
43. Gos, M. *et al.* Cellular quiescence induced by contact inhibition or serum withdrawal in C3H10T1/2 cells. *Cell Prolif.* **38,** 107–116 (2005).
44. Gottlob, K. *et al.* Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase. *Genes Dev.* **15,** 1406–1418 (2001).
45. Greenbaum, L. E. *et al.* CCAAT enhancer- binding protein beta is required for normal hepatocyte proliferation in mice after partial hepatectomy. *J. Clin. Invest.* **102,** 996–1007 (1998).
46. Greenwel, P. *et al.* Tumor necrosis factor alpha inhibits type I collagen synthesis through repressive CCAAT/enhancer-binding proteins. *Mol. Cell. Biol.* **20,** 912–918 (2000).
47. Harjes, U. *et al.* Antiangiogenic and tumour inhibitory effects of downregulating tumour endothelial FABP4. *Oncogene* **36,** 912–921 (2017).
48. Harper, J. W. *et al.* Inhibition of cyclin-dependent kinases by p21. *Mol. Biol. Cell* **6,** 387–400 (1995).
49. Hertzel, A. V. & Bernlohr, D. A. The mammalian fatty acid-binding protein multigene family: molecular and genetic insights into function. *Trends Endocrinol. Metab.* **11,** 175–180 (2000).
50. Hertzel, A. V., Xu, H., Downey, M., Kvalheim, N. & Bernlohr, D. A. Fatty acid binding protein 4/aP2-dependent BLT1R expression and signaling. *J. Lipid Res.* **58,** 1354–1361 (2017).
51. Höhler, B. *et al.* Hypoxic upregulation of tyrosine hydroxylase gene expression is paralleled, but not induced, by increased generation of reactive oxygen species in PC12 cells. *FEBS Lett* **457,** 53–56 (1999).

52. Hu, C. *et al.* Retinol-binding protein 7 is an endothelium-specific PPARγ cofactor mediating an antioxidant response through adiponectin. *JCI Insight* **2,** (2017).
53. Hunt, C. R., Ro, J. H., Dobson, D. E., Min, H. Y. & Spiegelman, B. M. Adipocyte P2 gene: developmental expression and homology of 5'-flanking sequences among fat cell-specific genes. *Proc. Natl. Acad. Sci. U.S.A.* **83,** 3786–3790 (1986).
54. Johanning, G. L. & Lin, T. Y. Unsaturated fatty acid effects on human breast cancer cell adhesion. *Nutr Cancer* **24,** 57–66 (1995).
55. Kanai, M., Raz, A. & Goodman, D. S. Retinol-binding protein: the transport protein for vitamin A in human plasma. *J. Clin. Invest.* **47,** 2025–2044 (1968).
56. Kim, H. *et al.* Unsaturated Fatty Acids Stimulate Tumor Growth through Stabilization of β-Catenin. *Cell Rep* **13,** 495–503 (2015).
57. Kim, S., Mao, P. L., Gagliardi, M. & Bédard, P. A. C/EBPbeta (NF-M) is essential for activation of the p20K lipocalin gene in growth-arrested chicken embryo fibroblasts. *Mol. Cell. Biol.* **19,** 5718–5731 (1999).
58. Kops, G. J. P. L. *et al.* Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. *Mol. Cell. Biol.* **22,** 2025–2036 (2002).
59. Kowenz-Leutz, E., Twamley, G., Ansieau, S. & Leutz, A. Novel mechanism of C/EBP beta (NF-M) transcriptional control: activation through derepression. *Genes Dev.* **8,** 2781–2791 (1994).
60. Kwast, K. E., Burke, P. V., Staahl, B. T. & Poyton, R. O. Oxygen sensing in yeast: Evidence for the involvement of the respiratory chain in regulating the transcription of a subset of hypoxic genes. *Proc Natl Acad Sci U S A* **96,** 5446–5451 (1999).
61. Ladha, M. H., Lee, K. Y., Upton, T. M., Reed, M. F. & Ewen, M. E. Regulation of exit from quiescence by p27 and cyclin D1-CDK4. *Mol. Cell. Biol.* **18,** 6605–6615 (1998).

62. Lih, C. J., Cohen, S. N., Wang, C. & Lin-Chao, S. The platelet-derived growth factor alpha-receptor is encoded by a growth-arrest-specific (gas) gene. *Proc Natl Acad Sci U S A* **93,** 4617–4622 (1996).
63. Lin, W.-C. *et al.* Kallistatin protects against sepsis-related acute lung injury via inhibiting inflammation and apoptosis. *Sci Rep* **5,** 12463 (2015).
64. Lluis, J. M. *et al.* Critical Role of Mitochondrial Glutathione in the Survival of Hepatocytes during Hypoxia. *J. Biol. Chem.* **280,** 3224–3232 (2005).
65. Lodish, H. *et al. Molecular Cell Biology*. (W. H. Freeman, 2000).
66. Loskovich, M. V., Grivennikova, V. G., Cecchini, G. & Vinogradov, A. D. Inhibitory effect of palmitate on the mitochondrial NADH:ubiquinone oxidoreductase (complex I) as related to the active-de-active enzyme transition. *Biochem. J.* **387,** 677–683 (2005).
67. Makkar, A., Mishima, T., Chang, G., Scifres, C. & Sadovsky, Y. Fatty acid binding protein-4 is expressed in the mouse placental labyrinth, yet is dispensable for placental triglyceride accumulation and fetal growth. *Placenta* **35,** 802–807 (2014).
68. Mao, P., Beauchemin, M. & A Bédard, P. Quiescence-dependent activation of the p20K promoter in growth-arrested chicken embryo fibroblasts. *The Journal of biological chemistry* **268,** 8131–9 (1993).
69. Mariman, E. C. M. & Wang, P. Adipocyte extracellular matrix composition, dynamics and role in obesity. *Cell Mol Life Sci* **67,** 1277–1292 (2010).
70. Medema, R. H., Kops, G. J., Bos, J. L. & Burgering, B. M. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. *Nature* **404,** 782–787 (2000).
71. Melki, S. A. & Abumrad, N. A. Expression of the adipocyte fatty acid-binding protein in streptozotocin-diabetes: effects of insulin deficiency and supplementation. *J. Lipid Res.* **34,** 1527–1534 (1993).

72. Melkonyan, H. S. *et al.* SARPs: A family of secreted apoptosis-related proteins. *Proc Natl Acad Sci U S A* **94,** 13636–13641 (1997).
73. Michiels, C. *et al.* Identification of the phospholipase A(2) isoforms that contribute to arachidonic acid release in hypoxic endothelial cells: limits of phospholipase A(2) inhibitors. *Biochem. Pharmacol.* **63,** 321–332 (2002).
74. Murphy, M. P. How mitochondria produce reactive oxygen species. *Biochem. J.* **417,** 1–13 (2009).
75. Nakajima, T. *et al.* Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL6. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 2207–2211 (1993).
76. Natsuka, S. *et al.* Macrophage differentiation-specific expression of NF-IL6, a transcription factor for interleukin-6. *Blood* **79,** 460–466 (1992).
77. Nieman, K. M. *et al.* Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. *Nat. Med.* **17,** 1498–1503 (2011).
78. Pines, J. Cyclins: wheels within wheels. *Cell Growth Differ.* **2,** 305–310 (1991).
79. Polotsky, V. Y. *et al.* Intermittent Hypoxia Increases Insulin Resistance in Genetically Obese Mice. *J Physiol* **552,** 253–264 (2003).
80. Poulain, L. *et al.* Visceral white fat remodelling contributes to intermittent hypoxia-induced atherogenesis. *European Respiratory Journal* **43,** 513–522 (2014).
81. RBP7 retinol binding protein 7 [Homo sapiens (human)] - Gene - NCBI. Available at: https://www-ncbi-nlm-nih-gov.libaccess.lib.mcmaster.ca/gene/116362. (Accessed: 21st August 2018)
82. Ruffell, D. *et al.* A CREB-C/EBPβ cascade induces M2 macrophage-specific gene expression and promotes muscle injury repair. *Proc Natl Acad Sci U S A* **106,** 17475–17480 (2009).
83. Schafer, K. A. The cell cycle: a review. *Vet. Pathol.* **35,** 461–478 (1998).

84. Schneider, C., King, R. M. & Philipson, L. Genes specifically expressed at growth arrest of mammalian cells. *Cell* **54,** 787-793 (1998).
85. Schönfeld, P. & Reiser, G. Rotenone-like Action of the Branched-chain Phytanic Acid Induces Oxidative Stress in Mitochondria. *J. Biol. Chem.* **281,** 7136–7142 (2006).
86. Schönfeld, P., Schlüter, T., Fischer, K.-D. & Reiser, G. Non-esterified polyunsaturated fatty acids distinctly modulate the mitochondrial and cellular ROS production in normoxia and hypoxia. *J. Neurochem.* **118,** 69–78 (2011).
87. Schönfeld, P. & Wojtczak, L. Fatty acids decrease mitochondrial generation of reactive oxygen species at the reverse electron transport but increase it at the forward transport. *Biochim. Biophys. Acta* **1767,** 1032–1040 (2007).
88. Schröder, M. & Kaufman, R. J. ER stress and the unfolded protein response. *Mutat. Res.* **569,** 29–63 (2005).
89. Scifres, C. M., Chen, B., Nelson, D. M. & Sadovsky, Y. Fatty acid binding protein 4 regulates intracellular lipid accumulation in human trophoblasts. *J. Clin. Endocrinol. Metab.* **96,** E1083-1091 (2011).
90. Sebastian, T. & Johnson, P. F. Stop and Go: Anti-Proliferative and Mitogenic Functions of the Transcription Factor C/EBPβ. *Cell Cycle* **5,** 953–957 (2006).
91. Shi, M. *et al.* Pharmacological inhibition of fatty acid-binding protein 4 (FABP4) protects against renal ischemia-reperfusion injury. *RSC Advances* **8,** 15207–15214 (2018).
92. Sia, A. K., Allred, B. E. & Raymond, K. N. Siderocalins; Siderphore binding proteins evolved for primary pathogen host defense. *Curr. Opin. Chem. Biol.* **17,** 150-7 (2013).
93. Stenvang, J., Petri, A., Lindow, M., Obad, S. & Kauppinen, S. Inhibition of microRNA function by antimiR oligonucleotides. *Silence* **3,** 1 (2012).
94. Sun, D. & Gilboe, D. D. Ischemia-Induced Changes in Cerebral Mitochondrial Free Fatty Acids, Phospholipids, and Respiration in the Rat. *Journal of Neurochemistry* **62,** 1921–1928 (1994).
95. Takeuchi, Y., Morii, H., Tamura, M., Hayaishi, O. & Watanabe, Y. A possible mechanism of mitochondrial dysfunction during cerebral ischemia: inhibition of mitochondrial respiration activity by arachidonic acid. *Arch. Biochem. Biophys.* **289,** 33–38 (1991).

96. Tanaka, M. *et al.* Ectopic expression of fatty acid-binding protein 4 in the glomerulus is associated with proteinuria and renal dysfunction. *Nephron Clin Pract* **128,** 345–351 (2014).

97. Tontonoz, P., Hu, E. & Spiegelman, B. M. Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. *Cell* **79,** 1147–1156 (1994).

98. Trautwein, C. *et al.* Transactivation by NF-IL6/LAP is enhanced by phosphorylation of its activation domain. *Nature* **364,** 544–547 (1993).

99. Trayhurn, P., Wang, B. & Wood, I. S. Hypoxia and the endocrine and signalling role of white adipose tissue. *Arch. Physiol. Biochem.* **114,** 267–276 (2008).

100. Tso, A. W. K. *et al.* Serum adipocyte fatty acid binding protein as a new biomarker predicting the development of type 2 diabetes: a 10-year prospective study in a Chinese cohort. *Diabetes Care* **30,** 2667–2672 (2007).

101. van der Vusse, G. J., Glatz, J. F., Stam, H. C. & Reneman, R. S. Fatty acid homeostasis in the normoxic and ischemic heart. *Physiol. Rev.* **72,** 881–940 (1992).

102. Vaporidi, K., Tsatsanis, C., Georgopoulos, D. & Tsichlis, P. N. Effects of hypoxia and hypercapnia on surfactant protein expression proliferation and apoptosis in A549 alveolar epithelial cells. *Life Sci* **78,** 284–293 (2005).

103. Vinciguerra, M. *et al.* Unsaturated fatty acids promote hepatoma proliferation and progression through downregulation of the tumor suppressor PTEN. *J. Hepatol.* **50,** 1132–1141 (2009).

104. Wang, Y., Mak, J. C. W., Lee, M. Y. K., Xu, A. & Ip, M. S. M. Low-Frequency Intermittent Hypoxia Promotes Subcutaneous Adipogenic Differentiation. *Oxidative Medicine and Cellular Longevity* (2018).

105. Wegner, M., Cao, Z. & Rosenfeld, M. G. Calcium-regulated phosphorylation within the leucine zipper of C/EBP beta. *Science* **256,** 370–373 (1992).

106. Williams, S. C., Baer, M., Dillner, A. J. & Johnson, P. F. CRP2 (C/EBP beta) contains a bipartite regulatory domain that controls transcriptional activation, DNA binding and cell specificity. *EMBO J* **14,** 3170–3183 (1995).

107. Wu, G. *et al.* FABP4 induces asthmatic airway epithelial barrier dysfunction via ROS-activated FoxM1. *Biochem. Biophys. Res. Commun.* **495,** 1432–1439 (2018).

108. Wu, G. *et al.* Mechanism and clinical evidence of lipocalin-2 and adipocyte fatty acid-binding protein linking obesity and atherosclerosis. *Diabetes Metab. Res. Rev.* **30,** 447–456 (2014).

109. Xu, A. *et al.* Circulating adipocyte-fatty acid binding protein levels predict the development of the metabolic syndrome: a 5-year prospective study. *Circulation* **115,** 1537–1543 (2007).

110. Yan, Q.-W. *et al.* The adipokine lipocalin 2 is regulated by obesity and promotes insulin resistance. *Diabetes* **56,** 2533–2540 (2007).

111. Yin, H., Xu, L. & Porter, N. A. Free radical lipid peroxidation: mechanisms and analysis. *Chem. Rev.* **111,** 5944–5972 (2011).