Regulation of IGFBP-1 Phosphorylation in Hypoxia Via mTOR Signaling

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Abstract

This study provides novel evidence for a role of fetal liver mTOR signaling in regulating IGF-I bioavailability by modulating IGFBP-1 phosphorylation due to hypoxia – a key factor in the development of reduced fetal growth in utero. I utilized HepG2 cells in vitro and demonstrated a link between mTOR inhibition and hypoxia-induced IGFBP-1 phosphorylation. Using a biological assay for IGF-I receptor autophosphorylation, my data demonstrated a functional significance for hypoxia-induced IGFBP-1 phosphorylation in reducing IGF-I bioactivity in vitro. Further, I have implicated a mechanistic link to increased CK2 activity within this regulation. I demonstrate that mTOR inhibition induced IGFBP-1 phosphorylation, which was not further enhanced by hypoxia, and that mTOR activation prevented hypoxia-induced IGFBP-1 phosphorylation. Together, my work has identified a new mechanism involving mTOR inhibition during hypoxia by which IGFBP-1 phosphorylation, and thus IGF-I bioavailability, is regulated, and also implicate increased CK2 activity as an intermediate process in this mechanism.

Keywords

IGFBP-1, mTOR, hypoxia, phosphorylation, signaling mechanism, IGF-I, HepG2 cells, immunonblotting, rapamycin, siRNA
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>°C</td>
<td>Degrees centigrade</td>
</tr>
<tr>
<td>4E-BP1</td>
<td>Eukaryotic translation initiation factor 4E-binding protein 1</td>
</tr>
<tr>
<td>A</td>
<td>Amps</td>
</tr>
<tr>
<td>AAR</td>
<td>Amino acid response</td>
</tr>
<tr>
<td>ADHD</td>
<td>Attention deficit hyperactivity disorder</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>ALS</td>
<td>Acid labile subunit</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>CK2</td>
<td>Protein kinase CK2</td>
</tr>
<tr>
<td>DEPTOR</td>
<td>DEP domain-containing mTOR interacting protein</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescent reagent</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eIF4E</td>
<td>Eukaryotic translation initiation factor 4E</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGR</td>
<td>Fetal growth restriction</td>
</tr>
<tr>
<td>FKBP12</td>
<td>FK506 binding protein</td>
</tr>
<tr>
<td>GH</td>
<td>Human growth hormone</td>
</tr>
<tr>
<td>HepG2</td>
<td>Human hepatocellular carcinoma</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia-inducible factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IGF-1R</td>
<td>Insulin-like growth factor 1 receptor</td>
</tr>
<tr>
<td>IGF-IIR</td>
<td>Insulin-like growth factor 2 receptor</td>
</tr>
<tr>
<td>IGFBP</td>
<td>Insulin-like growth factor binding protein</td>
</tr>
<tr>
<td>L</td>
<td>Liter</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>mTORC1</td>
<td>Mammalian target of rapamycin complex 1</td>
</tr>
<tr>
<td>mTORC2</td>
<td>Mammalian target of rapamycin complex 2</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Dulbecco’s Phosphate Buffered Saline</td>
</tr>
<tr>
<td>raptor</td>
<td>Regulatory-associated protein of mTOR</td>
</tr>
<tr>
<td>Rheb</td>
<td>Ras homologue enriched in the brain</td>
</tr>
<tr>
<td>rictor</td>
<td>Rapamycin-insensitive companion of mTOR</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>S6K</td>
<td>P70S6 kinase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>TBB</td>
<td>4,5,6,7-tetrabromobenzotriazole</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TSC1/2</td>
<td>Tuberous sclerosis complex ½</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μl</td>
<td>Microliter</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
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</table>
1.0 Introduction

1 Regulation of fetal growth

Fetal growth is determined by the maternal and fetal genomes, as well as the placenta. However, fetal growth is responsive to and dependent on environmental factors, of which adequate supply of oxygen and nutrients to the fetus through the placenta are the most critical (1). The maternal genome influences growth of the fetus, although its contribution is difficult to quantify as the fetal genome is half shared with the mother. In early fetal life, the maternal and fetal genomes contribute largely to the regulation of growth. However nutritional, hormonal, and environmental factors become increasingly determining of growth as gestation progresses (2). It is becoming increasingly evident that the maternal and uteroplacental environment are the largest determinants of fetal growth (2-4). Varying environmental conditions, namely the adequacy in the supply of oxygen (5) and nutrients (4), largely influence the development of the fetus. The result of inadequate supplies of nutrients or oxygen to the fetus, which can occur as a result of many different factors including poor placental function, is markedly reduced fetal growth. This can have long term physiological consequences on the affected human which persist throughout life (6-8).

1.1 Fetal growth restriction

Fetal growth restriction (FGR) refers to poor development of the fetus, and is clinically defined as fetuses whose weight is below the 10th percentile for gestational age (9). FGR is a pregnancy complication associated with poor outcomes including an increased risk of perinatal hypoxia (10) and neonatal demise (11). FGR infants are susceptible increased rates of prenatal and neonatal mortality and neonatal morbidities (11). Infants who survive gestation have an increased chance of neurodevelopmental disorders, as well as developing cardiovascular disease, obesity, and diabetes in childhood and as adults (12). Inadequate supplies of nutrients to the fetus, leading to an abnormal intrauterine milieu, have long lasting effects on the fetal and postnatal development patterns. This phenomenon can lead to a limitation in the supply of hormones and substrates necessary for fetal development and can be a precursor to the development of type-2 diabetes (13-
These limitations can permanently affect development by altering gene expression, particularly in certain cell types such as adipocytes, myocytes, pancreatic beta cells, and hepatocytes (13). Epidemiological changes in gene expression regulating growth and management of energy have been noted in various historical cases (16-19). Indeed, it is well established that fetal development and responses to environmental stimuli cause changes to expression patterns of the fetal genome, which can lead to permanent physiological changes which are retained post-natally. For example, restrictions such as low protein in the maternal diet during pregnancy have been shown to result in lifelong elevation in blood pressure in rats (20-23). These findings demonstrate that fetal growth is largely determined by environmental factors, and that a proper fetal environment is essential for a correct development. Although the etiology of FGR is multifactorial, in most instances fetal growth is constrained by abnormal placental function, which results in inadequate maternal supply of oxygen and/or nutrients. The molecular mechanisms that regulate FGR caused by hypoxia and nutrient deprivation are not well understood.

### 1.2 Regulation of fetal growth in hypoxia

Along with nutrient restriction, inadequate oxygen supplies to the fetus leads to growth restriction. In fact, decreased placental perfusion and fetal hypoxia are the leading causes of FGR (24). Pregnancies that are complicated by reduced maternal oxygen-carrying capabilities, as well as fetal anemia and hypoxia, result in severe growth restriction and FGR (5), implicating a critical importance of an adequate oxygen supply during gestation. Indeed, it has been shown through the study of chicken embryos that hypoxic conditions alone are sufficient to cause FGR (25). The fetal development of numerous tissues and organ systems are affected by fetal hypoxia. Studies have shown a correlation between fetal hypoxia and reduced hippocampal volume, as well as an increased chance of developing schizophrenia (26, 27). In fact, there is a growing body of evidence implicating fetal hypoxia as a predictor of early-onset schizophrenia (28-30). Further, it is known that fetal hypoxia results in a reduced volume of fetal blood (31). Cellular damage to the central nervous system has also been associated with fetal hypoxia (32). Fetal hypoxia also effects growth on a global scale. Infants born at high altitude are of lower birth weight (33, 34), reflecting the responsiveness of fetal growth to reduced oxygen.
availability. Numerous neurological pathologies have been associated with fetal hypoxia, including cerebral palsy, ADHD, and epilepsy (35-40). This strongly suggests that healthy levels of oxygen are critical for proper neurological development. This may also explain the association between reduced oxygen and smaller birth weight, as reductions in growth occur asymmetrically with retardations in head and brain development happening last (39). Although it is known that fetal hypoxia restricts growth, the molecular mechanisms linking hypoxia to restricted fetal growth are not well understood. Evidence has been put forth that suggest that hypoxia influences fetal growth via the insulin-like growth factor (IGF) system – the predominant growth factor system which regulates and promotes fetal growth (41).

1.3 Insulin-like growth factor system and the regulation of fetal growth

The insulin-like growth factor system is a family of ligands, receptors, and binding proteins which play a crucial role in fetal and post-natal development by promoting mitogenic actions within the cell. The system involves two IGF ligands, two IGF receptors, and six IGF binding proteins which collectively function to regulate growth in a temporal, regional, and environment-responsive manner. The insulin-like growth factors (IGF-I and II) are growth peptides which share a high sequence similarity with insulin (42, 43). They share a high structural homology with proinsulin (42, 43), and have been shown to be involved in the promotion of cell proliferation and inhibition of apoptotic pathways (44-47). Concentrations of IGF-I and II in fetal circulation are reduced by undernutrition and a deficiency of nutritionally sensitive hormones, such as insulin (48, 49). Studies by Baker et al. have shown that targeting of the Igf-I gene for knockout in mice results in a 40% reduction in body weight in comparison to wild-type littermates (50). Interestingly, a decrease in placental size did not occur as a result of Igf-I knockout. Postnatal growth of these mice was also affected, with both retarded and delayed bone growth as well as infertility occurring (50).

IGF-I is intimately involved in the regulation of postnatal organ and neural development. IGF-I signaling is involved in the regulation of neurogenesis, synaptogenesis, myelination, and dendritic branching (51-53). It has further been linked with
neuroprotection following brain damage, likely through its antiapoptotic effects (54, 55). Not surprisingly then, increased IGF-I in serum has been shown to correlate with higher IQ in children aged 5, 7, and 8 (56). The utilization of IGF-I in development ranges from gestation to growth in late puberty. Indeed, a correlation has been established between serum levels of IGF-I and the connection between short height and reduced hearing capabilities. This effect was most prominent in children aged 3-5, as well as age 18 (57), demonstrating the ongoing necessity for maintained IGF-I serum levels throughout development.

1.4 Insulin-like growth factors (I and II) in regulation of fetal growth

Both IGF-I and IGF-II have critical roles in the gestational development of humans. IGF-II has a prominent role in regulating embryonic growth in early gestation (58). In embryonic and early fetal development, Igf2 gene expression is high in numerous tissues, and IGF-II levels are high in fetal serum (59). It is believed that embryonic and early fetal growth are regulated mainly in an autocrine manner, whereas growth in the second half of gestation is strongly regulated in an endocrine manner (58). IGF-II functions by binding to IGF-I receptor (IGF-1R) in order to promote its mitogenic effects on cells (58). IGF-II also binds to IGF-2 receptor (IGF-IIIR), although interestingly the function of this receptor is to sequester bioavailable IGF-II from circulation and thus attenuate the effects of IGF-II (60). Both IGF-I and IGF-II contribute to fetal growth, with deletions of the Igf1 or Igf2 genes resulting in markedly reduced fetal growth (50). Conversely, overexpression of the Igf2 gene results in fetal overgrowth (61, 62).

During mid gestation, the emphasis on growth factor regulation by IGFs gradually switches from an IGF-II bias to an IGF-I bias, where IGF-I-mediated effects are dominant in mid and late gestation as well as in postnatal life (58). Interestingly, concentrations of serum IGF-II are higher than IGF-I in late gestation (48). However, studies have shown no association between serum IGF-II concentrations and fetal weight during late gestation. Conversely, umbilical cord and fetal IGF-I serum concentrations have been shown to be correlated with fetal weight (63, 64). This is supported by the fact that fetuses displaying FGR contain reduced concentrations of IGF-I both in utero and at
birth (65, 66), whereas neonates who are born large for gestational age contain increased IGF-I concentrations (67).

IGF-I is mainly produced and secreted by the fetal and post-natal liver and functions in an endocrine manner to promote growth globally (5). It also functions in a paracrine and autocrine manner, functioning to promote growth of the liver. Human growth hormone (GH) also plays a critical role in the development of humans (5). Both IGF-I and GH are utilized in post-natal development, where IGF-I mediates growth-promoting effects of GH (68, 69). IGF-I is critical in prenatal development, whereas GH has not demonstrated a major role during this period, as GH receptors are only expressed in low levels in the fetus (49, 70). Infants with either defects in their GH-receptor gene, or a congenital GH deficiency, only display a mild attenuation in growth size at birth (71). Conversely, defective Igf1 gene expression, as seen through mouse knockout studies, demonstrate severe growth restriction at the embryonic stage as well as at birth (50). IGF-I functions by binding to the IGF-I receptor (IGF-1R) but not IGF-IIIR (72), which subsequently stimulates numerous signaling cascades including Rac, IRS-1, and Ras/Raf activation in order to promote cellular growth, proliferation, migration, and the inhibition of apoptosis (73). Together, these data demonstrate the importance of IGF-I on the growth and development of the fetus. As such, the IGF-I system and IGF-I bioavailability need to be intimately regulated in order to respond to fetal environmental changes. This is done through the actions of insulin-like growth factor binding proteins (IGFBPs).

1.5 Insulin-like growth factor binding proteins: an important component of the IGF system

Insulin-like growth factor binding proteins are also produced and secreted into tissues and circulation. Six IGFBPs exist (IGFBP-1 to -6) which bind to IGF-I and IGF-II with high affinity. They function to stabilize IGF-I and IGF-II, prolonging their half lives and either promoting or attenuating their biological activity with IGF receptors (74, 75). IGFBPs are either produced and secreted by the liver into circulation or secreted in specific cell types to function in a paracrine manner where they bind to IGFs. Once secreted, they can perform various functions such as direct the IGF:IGFBP complexes to target tissues (76)
or prevent them from leaving circulation (77). IGFBPs are a well conserved family of proteins. The IGFBP genes have a shared structural organization, with four conserved exons having been established (78). These exons range in size from 5 kb in IGFBP-1, to over 30 kb in IGFBP-2 and IGFBP-5 (78). It has been postulated that the IGFBP gene family may be part of a larger gene superfamily, as exon 1 of the IGFBP gene family is shared by other genes (79, 80). IGFBPs are secreted into circulation or tissue extracellular matrix. IGFBP precursor proteins all contain signal peptides for secretion which range between 20-39 amino acids in length and as such, the mature IGFBP peptides are found extracellularly (81). Structurally, IGFBPs are all highly conserved and share similar tertiary patterns. The IGFBPs have similar molecular weights, ranging from approximately 24 to 50 kDa, or 216 to 289 amino acids (82).

1.6 Insulin-like growth factor binding protein structure and IGF binding

All IGFBPs contain a structured N terminal and C terminal domain, with a less structured flexible linker domain. The conserved N terminal domain contains six disulfide bonds due to a GCGCC motif (83). This is present in IGFBP-1 to IGFBP-5. IGFBP-6, however, lacks the last two cysteines of this motif and only contains five disulfide bonds as a result (84). There are differences in the organization of these disulfide bonds between IGFBPs. Despite these differences, however, structural analysis has determined that the disulfide bonds in the N terminal domain of IGFBPs are all paired within the domain (83). This provides further evidence of a conserved flexibility within the IGFBPs due to the unstructured linker region being unhindered by disulfide bonds. IGFs bind to the N terminal domain of IGFBPs. This was initially predicted via nuclear magnetic resonance (NMR) imaging (85), and later confirmed using mutagenic studies (86-88). IGFBP-3 and IGFBP-5, for example, were characterized to bind IGF-I on the N terminus via recent mutagenic studies (86). Similarly, a site-directed mutagenesis study on IGFBP-1 revealed that a 60 amino acid deletion in the N terminal of IGFBP-1 abolished IGF binding. In the same study, it was found that a specific point mutation of Cys38 to tyrosine was able to prevent IGF binding, demonstrating the necessity of the N terminal of IGFBPs in the binding of IGFs (89).
The C terminal domains of IGFBPs are also conserved in structure, in which all IGFBPs are relatively cysteine rich and contain three disulfide bonds (84, 90). Like the N terminus, the disulfide bonds within the C terminus are contained within the C terminus (91). It is known that the C terminal domains of IGFBP-1, -2, -4, and -6 all adopt a thyroglobulin type 1 fold and also possess flexible regions (83). Binding to IGFs also occurs in this region. Brinkman et al. showed that a 20 amino acid deletion in the C terminus of IGFBP-1 as well as a Cys226 mutation both prevented IGF-I binding, suggesting that the C terminus is involved in binding of IGF-I in IGFBP-1 (92).

The linker, or central domain of IGFBPs, is unstructured and flexible. No conservation in structure has been identified between IGFBPs in this region (72). The linker region contains sites for post-translational modification. Sites for proteolytic cleavage have been identified within the linker region, as well as potential phosphoacceptor sites on all IGFBPs (93). Further, phosphorylation in this region has been established on IGFBP-1, IGFBP-3, and IGFBP-5 (93), although direct binding of IGFs to the linker region has not been demonstrated.

1.7 Insulin-like growth factor binding proteins and IGF-mediated growth

Each IGFBP performs a unique function, or set of functions, within humans. IGFBP-2 has been shown to demonstrate inhibitory effects on IGF actions. The inhibitory effects of IGFBP-2 seem to be greater on IGF-II, as IGFBP-2 binds to IGF-II with higher affinity than IGF-I (60). IGFBP-2 is present in fetal development. In humans, IGFBP-2 levels increase in the amniotic fluid at 9-12 weeks of gestation (94), suggesting that IGFBP-2 plays a role in the regulation of early fetal development. As IGF-II actions are potent in early fetal development, IGFBP-2 may be increased in a temporal manner to regulate these actions. IGF-II is important for embryonic development, however growth at this stage is regulated in an autocrine rather than an endocrine manner (58). It is possible that IGFBP-2 levels rise in a temporal manner to regulate IGF-II-mediated growth as growth becomes more dependent on endocrine-related regulation. Interestingly, IGFBP-2 mRNA levels were found to be 8 times and 25 times higher than IGFBP-1 in rat kidney and brain tissues, respectively, in fetal rats. This suggests that throughout fetal development,
IGFBP-2 may function in a tissue-specific biased manner (95). The bias towards IGF-I-mediated growth, which occurs during the period in pregnancy where environmental factors become increasingly determinant on the influence over fetal growth (2, 58), makes the study of IGFBP-1 a more suitable candidate for study in assessing the effects of fetal hypoxia on growth as IGFBP-1 is the primary regulator of IGF-I (96).

IGFBP-3 is the predominant and most abundant IGFBP in fetal and postnatal circulation. It is the main carrier protein of IGF-I and IGF-II, transporting >75% of the IGFs in circulation (97). The main purpose of IGFBP-3 is to prolong the half-life of IGF-I and IGF-II (98, 99). IGFBP-3 is able to form a ternary complex with IGF-I or IGF-II and the 85 kDa glycoprotein known as the acid-labile subunit (ALS) (100). Formation of the ternary complex prolongs the half-life of IGFs and prevents passage through the vascular endothelium, essentially resulting in an IGF reservoir within the circulation (101).

The predominant IGFBPs which are present in fetal circulation are IGFBP-1, IGFBP-2, and IGFBP-3 (102) of which only IGFBP-1 is shown to be increased due to fetal hypoxia (103), further demonstrating the importance of the study of IGFBP-1 for fetal hypoxia. Like IGFBP-2-3, IGFBP-4-6 do not demonstrate increased protein expression due to hypoxia (104-106). IGFBP-4 has a role in embryogenesis, where it has been shown to positively affect IGF-II-mediated actions on embryonic growth (107). Its prenatal role seems to be limited to enhancing IGF-II actions, which occur primarily at the embryonic and early fetal stages (2, 58).

IGFBP-5 is structurally similar to IGFBP-3, but unlike IGFBP-3, IGFBP-5 is produced within various tissues and is not found in fetal circulation (108). The role of IGFBP-5 in fetal growth is tissue specific, with IGFBP-5 mRNA expressed in muscle, skin, stomach, and intestinal fetal tissues, suggesting it functions in an autocrine and paracrine manner where it is able to negatively regulate IGF actions (109, 110).

IGFBP-6 has received less attention than IGFBP-1 to IGFBP-5. IGFBP-6 has been reported to bind IGF-II with higher affinity than IGF-I. The difference in binding affinity varies between studies, with 20-fold to 100-fold selective binding preference towards IGF-II being reported (111, 112). These data suggest that IGFBP-6 is primarily a
negative regulator of IGF-II action. No role for IGFBP-6 in fetal growth has been established, however, with IGFBP-6 knockout mice demonstrating no difference in size and weight compared to control mice (113). Together, these data demonstrate that the study of hypoxia-induced FGR requires the investigation of IGFBP-1 and its regulation.

1.8 IGFBP-1 and IGF-I in the regulation of fetal growth

My study focuses on IGFBP-1. IGFBP-1 is the major IGFBP responsible for the regulation of IGF-I-mediated fetal growth (96). During pregnancy, IGFBP-1 functions primarily as a negative regulator of IGF action and there is an increase in IGFBP-1 present in both the amniotic fluid as well as fetal and maternal circulation (114). It has been shown that, from as early as sixteen weeks into pregnancy, an inverse correlation exists between fetal serum IGFBP-1 levels and birth weight (102). This demonstrates the importance in circulating levels of IGFBP-1 as a regulator and determinant of fetal growth. Furthermore, fetal IGFBP-1 levels are markedly increased in FGR, particularly in cases in which uteroplacental blood flow is restricted (102). In the fetus, IGFBP-1 mRNA is found predominantly in the liver, compared to other IGFBPs which are found in other specific tissues (106). This promotes the idea of IGFBP-1 functioning as a circulatory IGFBP in fetal growth, providing regulation of growth to a large number of tissues and organ systems. IGF-I serum concentrations are thought to be primarily determined by genetic influences. Conversely, IGFBP-1 concentrations are regulated by both genetic factors and the fetal environment (106, 115). In this way, IGFBP-1 can act as a growth regulatory protein which can attenuate growth if the fetal environment is not optimal. IGFBP-1 serum concentrations can fluctuate to regulate IGF-I bioavailability in both the short term and long term, responding to potential fetal environment shortcomings such as reduced nutrient or oxygen availability (115). During pregnancy, the predominant IGFBPs present in the circulation are IGFBP-1, IGFBP-2, and IGFBP-3, of which IGFBP-2 functions in a temporal manner to regulate embryonic and early fetal development (102), and IGFBP-3 binds IGFs in complex with the ALS and maintains a reservoir of IGFs within circulation (101). Thus, IGFBP-1 is the critical regulator IGFBP which modulates IGF-mediated fetal growth in mid-to-late gestation. IGFBP-1 does have roles to play in the regulation throughout life, although its contribution to IGFBP-
mediated regulation is not predominant like in fetal development. A correlation between low weight at birth and reduced IGFBP-1 serum levels in girls with precocious pubarche has been established (116). Low birth weight has also been associated with increased IGF-I serum levels in prepubertal children, suggesting that catch-up growth occurs postnatally and that these changes are at least in part regulated through the IGFBP-1:IGF-I axis (117, 118). Interestingly, IGFBP-1 seems to affect homeostatic effects long past development. In elderly men, high levels of circulating IGFBP-1 have been associated with the pathogenesis of cardiovascular disease as well as overall mortality (119).

The C terminal domain of IGFBP-1 has been shown to play a role in promoting cell migration in Chinese hamster ovary and porcine vascular smooth muscle cells (120). It contains an Arg-Gly-Asp (RGD) domain, known as an integrin recognition sequence capable of binding the α5β1 integrin, and this interaction is implicated in the mechanism of induction for cell migration (121). It has further been shown in human trophoblasts that the IGFBP-1:α5β1 integrin interaction signals downstream to stimulate the MAPK pathway, and that this mechanism induces human trophoblast migration (122), a mechanism which may be a critical component of blastocyst implantation into the endometrial wall in the pre-embryogenesis stage of pregnancy. Whether this mechanism is cell-type specific remains to be investigated. This demonstrates the ability of IGFBP-1 to perform at least one function independent of IGF-I, although its primary function is to bind to and sequester IGF-I to reduce IGF-I bioavailability.

1.9 Regulation of IGF-I bioavailability via IGFBP-1 phosphorylation

Like other IGFBPs, IGFBP-1-mediated regulation is more complex than altering IGF-I bioavailability through IGFBP-1 serum levels. IGFBP-1 is susceptible to post translational modifications. Phosphorylation of IGFBP-1 is a well established mechanism by which IGF-I bioavailability is regulated (74, 96). To date, five IGFBP-1 phosphorylation sites have been identified (123-130). Ser95, 98, 101, and 119 are all phosphoacceptor sites which exist in the linker region of IGFBP-1 – the region of
IGFBP-1 which also contains sites for proteolysis. Furthermore, Ser169, which exists in the C terminal domain of IGFBP-1 can be phosphorylated (Figure 1A). We have previously demonstrated up to a 300-fold increase in binding affinity of IGFBP-1 to IGF-I as a result of hypoxia-induced IGFBP-1 phosphorylation at Ser98 and 169 (129). Conversely, the phosphorylation status of IGFBP-3 and IGFBP-5 (the two other IGFBPs which have been shown to be phosphorylated) does not alter their binding affinity to IGF-I (131). Structural analysis has put forth evidence that the phosphorylation sites of IGFBP-1 which exist in the linker region and C-terminal domain interact with IGF-I to enhance its binding (Figure 1B) (124). Interestingly, the phosphorylation state of IGFBP-1 may provide inverse effects on the regulation of IGF action. IGFBP-1 samples treated with alkaline phosphatase, resulting in a dephosphorylated form of IGFBP-1, have been shown to enhance IGF-I-induced DNA synthesis (132). In vivo, phosphorylated IGFBP-1 is highly predominant in circulation (131), suggesting that this effect may only occur in localized regions as necessary. This dual effect of either inhibition or enhancement of IGF-I activity due to the phosphorylation state of IGFBP-1 demonstrates the complexity and reliance of post-translational modifications on the regulation of IGF-I activity, which seems to be dependent on upregulation of both kinase and phosphatase activity depending on the cellular environment. The inhibitory effect of IGFBP-1 on IGF-I actions is a result of IGFBP-1 binding IGF-I and preventing IGF-I from interacting with its receptor IGF-1R (133). However, the mechanism by which dephosphorylated IGFBP-1 enhances IGF-I activity remains unclear. Although it is understood that hypoxia and nutrient deprivation induce IGFBP-1 phosphorylation (129), the mechanisms responsible for this induction are unknown.

Figure 1. IGFBP-1 structure

A. Linear representation of IGFBP-1. Amino acid sequence length of each domain of IGFBP-1, as well as phosphorylation sites are indicated.

B. Structural model of the IGFBP-1:IGF-I complex, based on the crystal structure of IGF-I bound to the C-terminal domain of IGFBP-1 and the N-terminal domain of IGFBP-4 (199). The N-terminal sequence of IGFBP-1 was inserted into the N-terminus of
IGFBP-4 coordinates, and the structure was resolved and optimized using the SwissModel server. Phosphosites Ser98, 101, and 169 are highlighted, demonstrating their proximity to IGF-I.
Figure 1. IGFBP-1 structure
1.10 Regulatory pathways involved in IGFBP-1 phosphorylation during hypoxia-induced FGR

The molecular mechanisms that regulate fetal growth in FGR caused by hypoxia and nutrient deprivation need to be understood. Several signaling networks are responsive to decreased oxygen and nutritional stress, such as the amino acid response (AAR) (134), the unfolded protein response (UPR) (135), the hypoxia-inducible factor (HIF) (136), and the mammalian target of rapamycin (mTOR) (137) signaling pathways. We recently demonstrated a marked inhibition of mTOR signaling linked with IGFBP-1 phosphorylation in baboon fetal liver from maternal nutrient restriction induced FGR (96). In addition, we reported that inhibition of mTOR in cultured human hepatocellular carcinoma (HepG2) cells and primary fetal baboon hepatocytes causes increased IGFBP-1 phosphorylation (96). Additionally, we previously demonstrated that hypoxia induces IGFBP-1 phosphorylation in HepG2 cells (129). Together, these suggest the involvement of mTOR inhibition in the regulation of IGFBP-1 phosphorylation which may be occurring in hypoxia.

1.11 The mammalian target of rapamycin (mTOR)

The mammalian target of rapamycin is an evolutionary conserved serine/threonine kinase which plays multiple roles in the regulation of cell growth and metabolism which are primarily mediated through modulation of protein translation (138). Figure A1 demonstrates the complexity of the mTOR signaling pathways. Furthermore, mTOR signaling is highly sensitive to environmental conditions and functions to regulate cell growth in response to stimuli such as energy levels, stress conditions, and oxygen availability (137). mTOR exists in two complexes, mTOR Complex 1 (mTORC1) and 2 (mTORC2). Each protein complex contains various proteins associated to mTOR, hence forming two distinct protein complexes (Figure 2A). mTORC1 is composed of proteins mTOR, regulatory-associated protein of mTOR (raptor) (139, 140), mammalian lethal with SEC13 protein 8 (141), PRAS40 (142), TTI1/TEL2 (143), and DEP domain-containing mTOR interacting protein (DEPTOR) (144). These proteins function in complex to facilitate and regulate the kinase activity of mTORC1. Similarly, mTORC2 is composed of proteins mTOR, rapamycin-insensitive companion of mTOR (rictor) (145),
mammalian stress-activated protein kinase interacting protein 1 (146), Protor½ (147, 148), TTl1/TEL2 (143), and DEPTOR (144). DEPTOR, a shared mTORC1 and mTORC2 protein, has been shown to play in inhibitory role in the regulation of mTOR signaling (144). Newly emerging data suggests that DEPTOR functions to basally inhibit mTOR signaling, as silencing of DEPTOR has been shown to augment the activity of both mTORC1 and mTORC2 (149, 150).

1.12 mTORC1

mTORC1 has been well established as a regulator of cell growth, with studies showing that inhibition of mTORC1 results in a decrease in cell size. mTORC1 regulates numerous processes in the cell including autophagy (151), ribosome biogenesis (152), mRNA translation (153), and nutrient metabolism (154). Although many processes are understood to function through mTORC1 signaling, much about the mechanistic pathways and their components remain unknown. The best example of this is the study of budding yeast, where numerous mTORC1-mediated processes such as autophagy and ribosomal protein synthesis are well understood, but much of the molecular pathways integrated with mTORC1 signaling remain elusive (155).

The study of mTORC1 signaling functionality is a well established practice, where two proteins directly phosphorylated by mTORC1 have been utilized in determining relative states of mTORC1 activity. As mTOR is a kinase, the relative phosphorylation levels of proteins directly phosphorylated by mTORC1 acts as an indicator of mTORC1 activity. This is useful, for example, in assessing the effectiveness of various mTOR inhibitors on mTOR activity. The two proteins directly phosphorylated by mTORC1 are S6K (156, 157) and 4E-BP1 (158) (Figure 2A), both of which have important roles in the promotion of cell growth and protein synthesis. S6K is phosphorylated by mTORC1 at Thr389 (159). Thr389 is part of a hydrophobic motif on S6K (159), and phosphorylation here has been shown to be required for activation of S6K kinase activity, where mutation of Thr389 (T389A) abolishes the endogenous kinase activity of S6K (160). Thus, mTORC1-mediated phosphorylation of S6K is absolutely required for S6K activity. S6K activation via mTORC1 results in increased mRNA biogenesis as well as cap-dependent
translation and elongation. Furthermore, S6K activation results in increased protein translation of various ribosomal proteins (137). Induction of S6K-mediated actions is a result of S6K directly phosphorylating downstream proteins, as S6K is a kinase itself. S6K is known to directly phosphorylate S6K1 aly/REF-like target (SKAR), eukaryotic elongation factor 2 (eEF2K), ribosomal protein S6, programmed cell death 4 (PDCD4), eIF4B, and CBP80 (157, 159). This is likely an incomplete list, as the complete functions and actions of S6K are not understood. Furthermore, despite the known list of proteins targeted and activated by S6K, how these proteins contribute to increased cell size remains to be determined. 4E-BP1, the other protein well established to be phosphorylated by mTORC1, functions to promote protein synthesis on a broad scale. 4E-BP1 itself is an inhibitory binding protein, which binds to the eukaryotic translation initiation factor 4E (eIF4E) and prevents its functions (158). Phosphorylation of 4E-BP1 at Thr70 (159) by mTORC1 prevents 4E-BP1 from binding eIF4E, which in turn is able to promote cap-dependent translation of numerous proteins (158). In this way, active mTORC1 signaling is able to promote cell growth through specific pathways via S6K phosphorylation, as well as broader scale changes to protein synthesis via 4E-BP1 phosphorylation.

1.13 mTORC2
mTORC2 and its functions are less well understood than mTORC1. Like mTORC1, mTORC2 functions to positively regulate cellular growth processes such as cell proliferation, survival, and metabolism (161). Interestingly, mTORC2 also seems to regulate actin cytoskeleton functions within the cell (162), demonstrating the broad diversity of potential mechanisms regulated by mTORC2 signaling. mTORC2 also functions as a kinase, but the number of proteins known to be phosphorylated by mTORC2 is notably lower than mTORC1. mTORC2 is known to directly phosphorylate protein kinase B (Akt) (163), PKCα (164), and Serum and Glucocorticoid-regulated Kinase 1 (SGK1) (165) (Figure 2A). Akt activity plays a critical role in the promotion of cell survival, proliferation, and metabolism in the cell (166). Furthermore, Akt activation requires phosphorylation at S473 by mTORC2 (167), demonstrating the necessity of
mTORC2 signaling in the regulation of Akt-mediated growth processes in the cell. mTORC2 depletion studies have shown that, in the absence of mTORC2 and thus Akt activity, phosphorylation (and thus activity) of the forkhead box protein O1 (FoxO1) and FoxO3 transcription factors are markedly reduced (168). These transcription factors control gene expression of genes involved in metabolism, stress resistance, cell-cycle arrest, and apoptosis (168). mTORC2-mediated regulation of the actin cytoskeleton has been investigated, although the mechanisms which regulate mTORC2 actin cytoskeleton regulation have not been determined (162, 169).

1.14 Rapamycin-mediated mTOR inhibition

Rapamycin is a pharmacological compound which is used as an immunosuppressant, often for kidney transplants (170). In research, rapamycin is often utilized for mechanistic studies due to its ability to inhibit mTOR signaling. mTORC2 contains associate protein rictor, which was named as such due to the traditional belief that mTORC2 is resistant to rapamycin inhibition. Rapamycin functions by forming a complex with FK506 binding protein (FKBP12), and this complex is known to inhibit mTOR function by binding to the FKBP12-rapamycin-binding (FRB) domain of mTOR and mTORC1 which subsequently prevents the activity of mTORC1. It is speculated that the rapamycin-FKBP12 complex inhibits mTORC1 function by disrupting the interaction between mTOR and raptor (171). mTORC2 does not permit binding of the rapamycin-FKBP12 complex. The reason for this in unknown, although it is speculated that rictor (or an unidentified component of mTORC2) binding to mTOR occupies the rapamycin-FKBP12 binding site or that this binding changes the structure of mTOR such that the binding site is no longer available for rapamycin-FKBP12 (161). Recently, however, new data is emerging which shows that prolonged exposure to rapamycin indeed demonstrates an inhibitory effect on mTORC2, although the mechanism responsible for this inhibition is not known (96, 163). It is possible that mTORC2 is inhibited by rapamycin due to uncomplexed mTOR proteins binding to rapamycin and preventing further mTORC2 complex assembly. This would suggest that existing mTORC2 complexes at the time of rapamycin exposure would be resistant to inhibition, but upon protein turnover complex formation of mTORC2 is inhibited by mTOR bound to the rapamycin-FKBP12 complex.
1.15 Hypoxia-mediated mTOR inhibition

It is well established that mTORC1 signaling is inhibited by hypoxia (172, 173). Many studies have reported this in numerous cell lines including 293 cells (174), MEF cells (175), and mouse primary hepatocytes (175). mTORC2 activity due to hypoxia is not as well understood, where studies have reported increases, decreases, and a lack of change in mTORC2 activity due to hypoxia. It seems that mTORC2 signaling in response to hypoxia is cell-type specific. For example, mTORC2 activity has been reported to be increased in PC12 (176), HeLa (176), and HT1080 fibrosarcoma cells (177) due to hypoxia. No change in mTORC2 activity was reported in PC-3 prostate cancer (178), as well as COS-7 and 3T3 cells following hypoxic treatment (179). Lastly, mTORC2 inhibition due to hypoxia has been demonstrated in rat myocytes (180), rat cerebrocortical cells (181), HN13 cells (182), and HepG2 cells (179). The inhibition of mTORC2 signaling due to hypoxia in HepG2 cells was reported to occur independently of serum status, with inhibition occurring in both the presence and absence of serum (179).

1.16 Mechanisms regulating mTOR inhibition in hypoxia

mTORC1 inhibition by hypoxia occurs through the tuberous sclerosis complex 1/2 (TSC1/2) complex signaling pathway (174). Hypoxic stress triggers the activation of the hypoxia-inducible factor-1α (HIF-1α), a transcription factor which subsequently induces transcription of response proteins (183). Under normal conditions, HIF-1α is hydroxylated at conserved proline residues via HIF prolyl-hydroxylases, and this hydroxylation targets HIF-1α for ubiquitination in the proteasome (184). Hypoxic conditions result in the inhibition of HIF prolyl-hydroxylases due to the fact that these proteins use oxygen as a cosubstrate, allowing for cellular accumulation of HIF-1α (185) (Figure 2B). From here, HIF-1α activity results in the activation of REDD1, which then induces TSC1/2 activity (186). The high activity state of TSC1/2 alters the ratio of Ras homologue enriched in the brain (Rheb)GDP to RhebGTP (increasing cellular RhebGDP), and this change results in the inhibition of mTORC1 signaling (187, 188). Thus, hypoxia functions on the HIF-1α signaling axis to inhibit mTORC1. The mechanisms regulating mTORC2 activity in hypoxia are unknown, although it is likely
that they differ in various cell types due to the variety of responses elicited by hypoxia which have been reported (176, 178, 189). Whether mTOR inhibition in hypoxia is responsible for IGFBP-1 phosphorylation remains to be established. Further, the kinase responsible for directly phosphorylating IGFBP-1 is not known.
Figure 2. mTOR components and mTORC1 inhibition in response to hypoxia

A. Schematic showing an incomplete list of mTORC1 and mTORC2 associated proteins. Please see text for a more complete list of mTOR-associated proteins. Also shown are the most well known downstream substrates directly phosphorylated by mTORC1 and mTORC2. Use of this schematic is permitted via open access (200).

B. Model of mTORC1 signaling in response to hypoxia. In normoxic conditions (left), prolyl hydroxylases (PHDs) readily target HIF-1α for degradation, and mTORC1 signaling is not inhibited by the HIF-1α hypoxic response, allowing for mTORC1 activity to promote cell growth and proliferation through cellular translation. Under hypoxic conditions (right), PHDs can no longer hydroxylate HIF-1α and thus facilitates the accumulation of HIF-1α which subsequently functions to inhibit mTORC1 signaling (201). Please see text for a more detailed description of this mechanism.
Figure 2. mTOR components and mTORC1 inhibition in response to hypoxia
1.17 Regulation of IGFBP-1 phosphorylation by mTOR inhibition via protein kinase CK2

Our recent work with baboon fetal liver from maternal nutrient restriction-induced FGR demonstrated increased CK2 activity along with mTOR inhibition and increased IGFBP-1 phosphorylation in FGR (96). In addition, we reported that IGFBP-1 phosphorylation via mTOR inhibition was mediated by protein kinase CK2 activity in cultured HepG2 cells and primary fetal baboon hepatocytes (96). CK2 is a conserved serine/threonine kinase which is regulated by a wide variety of upstream signals (190). It exists as a tetrameric complex, consisting of α and α’ catalytic subunits as well as two regulatory β subunits (190). We previously chose to investigate CK2 as a potential kinase for IGFBP-1 phosphorylation due to the identification of a consensus sequence within IGFBP-1 for CK2 interaction (191). Although we have recently established a link between mTOR inhibition and increased CK2 activity (96), the effect of hypoxia on CK2 activity in connection with mTOR inhibition and its role in IGFBP-1 phosphorylation during hypoxia have not been reported.

1.18 Rationale, hypothesis, objectives

Impaired oxygen and nutrient delivery to the fetus are known to result in FGR (24). It is known that mTORC1 and mTORC2 signaling in HepG2 cells are inhibited by hypoxia (189), and in the fetal liver of FGR fetuses as we have previously reported (96). IGF-I is a key regulator of fetal growth and IGF-I bioavailability is determined by IGFBP-1, which binds IGF-I and inhibits IGF signaling (72). We have previously demonstrated that hypoxia induces IGFBP-1 phosphorylation which binds IGF-I with markedly higher affinity (192). We recently also established a link between inhibition of mTOR signaling and IGFBP-1 hyperphosphorylation (96). Together, these findings provided the basis for the current mechanistic study in this dissertation and formulation of my hypothesis as follows.

Hypothesis: Regulation of IGFBP-1 phosphorylation in response to hypoxia occurs via inhibition of mTOR signaling.
Objective: The objective of the study was to elucidate the mechanistic role of mTORC1 and/or mTORC2 signaling in regulating IGFBP-1 phosphorylation, and thereby IGF-I bioavailability, in hypoxia.

The specific aims for this dissertation were as follows:

1. To investigate the role of mTOR signaling in the induction of IGFBP-1 phosphorylation.

2. A) To investigate the functional role of mTOR signaling in hypoxia-induced IGFBP-1 phosphorylation.
   B) To investigate the involvement of protein kinase CK2 as a kinase for IGFBP-1 phosphorylation induced by rapamycin and/or hypoxia.

3. To demonstrate that regulation of hypoxia-induced IGFBP-1 phosphorylation requires mTORC1 and/or mTORC2 activity.

Figure 3 illustrates functionally important mTOR-related proteins used in our mechanistic studies to explore a key link between hypoxia and increased IGFBP-1 secretion and phosphorylation.
Figure 3. Functionally important mTOR-related proteins linking mTOR to the regulation of IGFBP-1

Schematic showing a basic proposed model connecting mTOR signaling to IGFBP-1 secretion and phosphorylation. Key proteins for silencing were raptor and rictor for mTOR inhibition, and TSC2 and DEPTOR for mTOR activation. Also shown are 4E-BP1/S6K and Akt - three proteins directly phosphorylated by mTORC1 and mTORC2, respectively, which were used as functional readouts for mTORC1 and mTORC2 activity following treatments. Rapamycin and hypoxia, and their inhibitory effects on mTOR signaling are also presented.
1.19 Rationale for use of HepG2 cells in this study as a model for fetal hypoxia

Because the liver is the major source of serum IGFBP-1 \textit{in vivo}, I used liver-derived human HepG2 cells to test my hypothesis. HepG2 cells are widely used as a model for fetal hepatocytes (96, 192-195). They are human liver carcinoma cells which demonstrate biotransformation characteristics and gene expression patterns similar to primary human fetal hepatocytes (194, 195). Furthermore, HepG2 cells predominantly express fetal isoenzymes of both aldolase and pyruvate kinase, and low levels of the cell surface receptor for asialoglycoproteins – properties which are characteristic of human fetal hepatocytes (193). We recently validated the use of HepG2 cells for our studies by demonstrating a high similarity compared to baboon primary fetal hepatocytes in the response patterns of mTOR inhibition and IGFBP-1 phosphorylation in nutrient-restricted conditions (96), providing a strong justification for the use of our HepG2 cells as a model for fetal hepatocytes. Thus, as in our previous reports (96, 129), we used HepG2 cells and performed mechanistic studies on the role of mTOR in the regulation of hypoxia-induced IGFBP-1 phosphorylation.
2.0 Materials and Methods

2. Cell culture

HepG2 cells (ATCC) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with nutrient mixture F-12 (DMEM/F-12) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) at 37°C in atmospheric air (20% O₂ with 5% CO₂). HepG2 cells were grown in 10% FBS to 75% confluence in 75cm² T-75 flasks (BD Biosciences) prior to plating. Once cells reached 75% confluence, cells were plated via trypsinization (0.5% trypsin-EDTA, Life Technologies, 1:10 dilution) and re-suspension in 10% FBS. Following trypsinization but prior to plating, the cell suspension was quantified for cell count via hemocytometry. 100 µl of cell suspension was combined with 400 µl 0.4% Trypan Blue (Life Technologies), and of this 20 µl was loaded onto a 0.1 mm depth Bright-Line hemocytometer (Hausser Scientific) for cell counting. Staining with Trypan Blue allowed us to perform the Trypan Blue exclusion test of cell viability, in which we only quantified viable cells. Following hemocytometry, re-suspended cells were plated on 12-well polystyrene culture dishes (BD Biosciences) at a cell density of 1.5x10⁵ viable cells/well for 24 hours. For treatments, cells were grown to 70% confluency, then media was changed to 2% FBS overnight or serum free media for 6 hours to allow cell acclimatization.

P6 cells (mouse embryo fibroblast cells that over-express human IGF-1R) (a gift from Dr. Renato Baserga, Thomas Jefferson University) were cultured in DMEM with 4.5 g/L D-glucose and 110 mg/L sodium pyruvate using the same cell culture materials, and treatments were performed in FBS-free conditions.

2.1 Cell media and lysate preparation

Cell culture experiments were concluded with collection of both cell media and cell lysate. Cell media was collected and immediately stored at -80°C. To collect cell lysate, plated cells were first rinsed with Dulbecco’s Phosphate Buffered Saline (PBS) (BD Bioscience) to remove trace amounts of cell media, followed by addition of 175 µl of cell lysis buffer (Cell Signaling) with Protease Inhibitor Cocktail, Phosphatase Inhibitor
Cocktail 2, and Phosphatase Inhibitor Cocktail 3 (all from Sigma Aldrich, 1:100 dilution). Collected samples underwent sonification for 15 seconds on ice followed by 13000 RPM centrifugation for 30 minutes at 4°C, after which the supernatant was collected and the pellet was discarded. Determination of total protein in the cell lysate occurred via Bradford protein assay, followed by storage at -80°C.

2.2 Bradford protein assay

5 µl aliquots of cell lysis buffer were diluted with Milli-Q water (1:10 dilution) and plated in triplicate 10 µl aliquots on a Linbro® 96-well Microplate (MP Biomedicals) along with duplicate 10 µl aliquots of protein standards at concentrations of 0, 25, 125, 250, and 500 µg/ml (Thermo Scientific). 200 µl of Bradford reagent (Bio-Rad Laboratories Inc.) were added to each well followed by sealing of the wells with an adhesive cover, and the mixtures were allowed to incubate at room temperature for 5 minutes. The plate was then loaded onto a Thermo Labsystems Multiskan Ascent photometer (Thermo Scientific), and relative absorbance (representing protein concentration) was determined using light at 595 nm generating protein concentrations with units of mg/ml.

2.3 Rapamycin treatment

Plated HepG2 cells in culture had their media changed to 2% FBS overnight prior to rapamycin treatment. Rapamycin was diluted to 100 nM in DMEM/F-12 cell media supplemented with 2% FBS, followed by syringe-based (BD Biosciences) filtration (Puradisc 0.2 µm, Whatman GE Healthcare) to ensure sterilization. HepG2 cells were treated with 100 nM rapamycin for 24 hours. Control media contained 2% FBS only. Following treatments, the cell media and cell lysate were prepared and stored at -80°C.

2.4 Hypoxic treatment

HepG2 cells were incubated for overnight in 2% FBS media overnight or 6 hrs in FBS-free DMEM/F-12. Subsequently, the media was replaced with new 2% FBS or FBS-free DMEM/F-12 and cell cultures were then placed in either in an incubator with atmospheric air and 5% CO₂ (normoxia) or a hypoxia chamber (Billups–Rothenburg),
which was flushed with a 1%O₂, 5%CO₂, balanced N₂ gas mixture (BOC Canada Ltd) for 5 minutes to ensure saturation. The cells in the sealed chamber were placed in a tissue-culture incubator at 37°C on an orbital shaker at low speed to facilitate continued gas exchange between the cell environment and the surrounding air. Cells were cultured in normoxia or hypoxia for 24 hours during rapamycin and siRNA treatments. Cell media and cell lysate were collected following 24 hour exposure.

2.5 Cell viability assay

We tested the effect of our rapamycin and hypoxia treatments on cell viability via the trypan blue exclusion assay. Following rapamycin and hypoxia treatments, cells were trypsinized and resuspended in 10% FBS. Cell suspensions were diluted 1:1 with 0.4% trypan blue and counted using the Countess Automated Cell Counter (Life Technologies, Carlsbad, CA). Cell viability was determined as a measure of live/total cells.

2.6 IGF-I receptor assay

P6 cells (a BALB/c3T3 cells derivative), which are mouse fibroblast cells that over-express human IGF-1R, were plated at ~1.5×10⁶ cells per well and grown to 50% confluence in 12-well plates in DMEM with D-Glucose and sodium pyruvate supplemented with 10% FBS. P6 cells were exposed to serum free media for 6 hours prior to treatment. As a positive control, human recombinant IGF-I (Tercica Inc.) was diluted in 1 ml of P6 cell media at a concentration of 100 ng/ml in the absence of IGFBP-1. Prior to exposure on P6 cells, equal total IGFBP-1 from conditioned HepG2 cell media (approximately 1000 ng/ml) from rapamycin, hypoxia, and rapamycin+hypoxia treated HepG2 cells (as well as media from untreated HepG2 cells) was combined with P6 cell media to total 1 ml with a 100 ng/ml dilution of IGF-I, and allowed to incubate at room temperature for 2 hours. This allowed for variance in degree of IGFBP-1 phosphorylation between samples, but not variance in levels of total IGFBP-1. P6 cells were then rinsed with PBS, and then exposed to the cell media mixtures for 10 minutes. The cell media mixtures were then aspirated, and 175 ml of cell lysis buffer was added to the wells. The cell lysates were analyzed via western blotting to assess relative levels of IGF-1R phosphorylation as a result of IGF-I stimulation.
2.7 Protein kinase CK2 activity assay

CK2 activity was measured in HepG2 whole-cell lysates using the synthetic peptide substrate RRRDDDSDDD. Assays were performed for 5 min at 30 °C in a final reaction volume of 30 μl containing 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, and 0.1 mM ATP (specific activity 500–700 cpm/pmol) (PerkinElmer) and RRRDDDSDDD (0.1 mM). Reactions were initiated by addition of 9 μl of cell extract or immunocomplex and were terminated by spotting 10 μl on P81 phosphocellulose paper as described previously (96). The papers were washed four times in 1% phosphoric acid and once in 95% ethanol. Once dry, papers were immersed in scintillant and counted in a Beckman LS 5801 scintillation counter.

2.8 RNA interference-mediated silencing

Silencing raptor and/or rictor individually or combined, DEPTOR, and TSC2 in HepG2 cells (~1.5×10⁶ cells per well grown to 70% confluence in 12-well plate) was achieved using transfection with 100 nM siRNA (Sigma-Aldrich) and Dharmafect transfection reagent 4 (Thermo Scientific). HepG2 cells were switched to serum free DMEM/F-12 cell media for 6 hours prior to transfection to allow for cell acclimatization. During acclimatization, siRNA cell media was prepared. siRNAs were incubated in 100 µl/well serum free media for 5 minutes. Separately, 5 µl/well Dharmafect transfection reagent 2 was incubated in 100 µl/well serum free media for 5 minutes to allow for emulsification. Following 5 minute incubations, siRNAs and transfection reagent were combined (total 200 µl/well) and allowed to incubate for 20 minutes for further emulsification. All incubations were performed at room temperature. Following serum free cell acclimatization and siRNA:transfection reagent emulsification, HepG2 cells were rinsed with PBS and exposed to the siRNA:transfection reagent mixture (200 µl/well) for 2.5 hours. 800 µl serum free cell media was added after 2.5 hours to yield a total of 1 ml cell media/well. siRNA treatment occurred for 72 hours. Immediately following transfection, cells were cultured for 48 hours in normoxia (20% O₂), followed by an additional 24 hours either in normoxia or in hypoxia (1% O₂). The efficiency of target silencing was determined at the protein and functional levels using western blot analysis.
2.9 SDS-PAGE and western blots

Equal amounts of cell lysate protein (20-50 µg) were used to determine phosphorylation and total expression of p70S6K at Thr389, 4E-BP1 at Thr70, Akt at Ser473, IGF-1R at Tyr1135, as well as total expression levels of CK2 (α, α’, and β), albumin, and siRNA target proteins. IGFBP-1 secretion (8 µl) and phosphorylation (40 µl) at Ser101, 119 and 169 by HepG2 cells were determined using equal volume of cell media. As in our previous study, IGFBP-1 secretion and phosphorylation in cell media samples were normalized via equal loading of sample, as there are no valid loading controls available for secreted proteins. For immunoblot analysis, either 5% skim milk or 5% BSA in Tris-buffered saline (TBS) plus 0.1% Tween-20 or 0.5% gelatin in PBS (albumin blotting) were used for blocking. All primary antibodies were obtained from Cell Signaling Technologies (Beverly, MA) with the exception of the monoclonal antihuman IGFBP-1 (mAb 6303) (Medix Biochemica) and a IGFBP-1 polyclonal antibody (a gift from Dr. R. Baxter, Kolling Institute of Medical Research), and polyclonal albumin antibody (Bethyl Laboratories). Custom IGFBP-1 polyclonal antibodies targeting Ser101, Ser119, and Ser169 were generated at YenZyme Antibodies LLC and were used for detection of phosphorylation at the three respective sites. Primary antibodies were used at a dilution of 1:1000 except for total IGFBP-1 antibody that was 1:10000 and β-actin 1:5000. Peroxidase-labeled goat-anti mouse or goat-anti rabbit antibodies (1:10000, Bio-Rad) were used as secondary antibodies.

To monitor the progress of electrophoresis and electrophoretic transfer, and to visualize protein marker bands directly on our western blots, 8 µl of BenchMark™ Pre-Stained Protein Ladder and 2 µl of MagicMark™ XP Western Protein Standard (both from Life Technologies), respectively, were loaded into one of the lanes of the gel. Protein separations were conducted on 1.5 mm SDS polyacrylamide gels at concentrations of 8%, 10%, 12%, and 15% (Bio Rad). Gels were run for 30 minutes at 80 V, followed by approximately 1.5 hours at 120 V. Electrophoretic transfer was performed at 0.8 A for 1 hour in a Bio-Rad Trans-Blot® Cell apparatus onto nitrocellulose membranes (Pall Life Sciences), followed by blocking for 1 hour at room temperature on a shaker with previously mentioned solutions. Incubation in primary antibodies occurred overnight at
4°C on a shaker to ensure complete membrane antibody exposure. The membranes were then washed with TBS + 0.1% Tween (BioShop) 3 times for 10 minutes each on a shaker at room temperature, followed by a 1 hour incubation in secondary antibody on a shaker at room temperature. In order to wash away excess antibody, the membranes were washed 3 more times for 10 minutes each on a shaker at room temperature. The membranes were then exposed to 4 ml of enhanced chemiluminescence (ECL) reagents and imaged on a VersaDoc Imager (Bio-Rad). Band intensities were determined using densitometry and Image Lab (Beta 3) software (Bio-Rad).

2.10 Two-dimensional immunoblot of IGFBP-1

Two-D immunoblot analysis with HepG2 cell media (~150 μl) using polyclonal IGFBP-1 antibody was performed as previously described (96, 130, 192, 196, 197). Briefly, Equal volumes (100 μl) of cell media were desalted and concentrated 10-fold using 10-kDa M, cutoff (MWCO) Centricon tubes (Pall Life Sciences). Desalted samples were reconstituted with rehydration buffer {8 M urea, 2% (3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS) (Bio-Rad), 50 mM dithiothreitol, 0.2% Biolyte (Bio-Rad), (pH 3–10 ampholyte), and 0.001% bromophenol blue} and transferred onto a polyvinylidene fluoride membrane by wet transfer (31). Membranes were blocked in 4% nonfat dry milk and then incubated overnight with IGFBP-1 polyclonal antibody (1:10,000 dilution). The goat anti-rabbit HRP-conjugated antibody (1:8000 dilution) was used as a secondary antibody, and proteins were visualized using the ECL Plus system.

2.11 Data presentation and statistics

Statistics were performed using GraphPad Prism 5 (Graph Pad Software Inc.). For each protein quantified, the mean density of the control sample bands was assigned an arbitrary value of 100. All individual densitometry values were expressed relative to this mean. To compare means, Student t-test and ANOVA were used and the results were expressed as Mean ± SEM. Significance was accepted at \( P < 0.05 \).
3.0 Results

3 Results divided into three aims

Aim 1: To investigate the role of mTOR signaling in the induction of IGFBP-1 phosphorylation

3.1 Rapamycin induces IGFBP-1 secretion and phosphorylation in a dose dependent manner

We first determined the effect of rapamycin on IGFBP-1 secretion and phosphorylation in a dose dependent manner. We tested the effects of 3 concentrations of 24 hour rapamycin treatment on HepG2 cells – 50, 75, and 100 nM. IGFBP-1 secretion was significantly increased by both 75 and 100 nM of rapamycin (Figure 4A). We next assessed IGFBP-1 phosphorylation, and again found that a significant increase in IGFBP-1 phosphorylation was detected at both 75 and 100 nM. Phospho-sites Ser101 (Figure 4B) and Ser169 (Figure 4D) had high levels of induced phosphorylation due to 75 and 100 nM treatment, demonstrating a plateau effect in the degree of phosphorylation seen. Interestingly, phosphorylation at Ser119 (Figure 4C) was notably higher at 100 nM than at 75 nM rapamycin treatment. Due to the highest induction in Ser119 phosphorylation seen at 100 nM and the plateau effect seen by phosphorylation at Ser101 and Ser169, as well as total secreted IGFBP-1, we performed subsequent rapamycin treatments at 100 nM as in the literature (163, 169, 198) and our previous study (96).
Figure 4. The effect of dose dependent rapamycin treatment on IGFBP-1 secretion and phosphorylation in HepG2 cells

HepG2 cells were cultured for 24 hours with 0, 50, 75, and 100 nM rapamycin (n=3 each).

A. A representative western blot of IGFBP-1 conditioned media of control and rapamycin treated HepG2 cells (n=3 each) using equal aliquots of conditioned media. Rapamycin treatment of HepG2 cells significantly increased IGFBP-1 secretion. A strong induction of IGFBP-1 secretion was seen at 75 and 100 nM rapamycin treatment. Values are displayed as mean + SEM. *p< 0.05, **p= 0.001-0.05, ***p < 0.0001 versus control; One-way analysis of variance; Dunnet’s Multiple Comparison Test; n=3.

B-D. Representative western blots of phosphorylated IGFBP-1 at Ser101, 119 and 169 in conditioned media of control and rapamycin treated HepG2 cells (n=3 each) using equal aliquots of conditioned media. Rapamycin treatment of HepG2 cells significantly increased IGFBP-1 phosphorylation at all three serine sites. A strong induction of IGFBP-1 phosphorylation was seen at all three sites with 100 nM rapamycin treatment. Values are displayed as mean + SEM. *p< 0.05, **p= 0.001-0.05, ***p < 0.0001 versus control; One-way analysis of variance; Dunnet’s Multiple Comparison Test; n=3.
Figure 4. The effect of dose dependent rapamycin treatment on IGFBP-1 secretion and phosphorylation in HepG2 cells
3.2 Rapamycin inhibits mTORC1 and mTORC2 signaling in a time dependent manner

In order to assess the effects of rapamycin on mTORC1 and mTORC2 signaling in HepG2 cells, we treated HepG2 cells with 100 nM rapamycin for 0, 1, 2, 3, 6, and 12 hours. mTORC1 signaling, as assessed by S6K phosphorylation (Thr389), was significantly inhibited by rapamycin within the first hour of treatment (Figure 5A). mTORC2 signaling, as assessed by Akt phosphorylation (Ser473), was significantly inhibited by rapamycin between 3 and 6 hours into the treatment (Figure 5B). This demonstrated that a prolonged exposure to 100 nM rapamycin was sufficient to inhibit both mTORC1 and mTORC2 signaling in HepG2 cells.
Figure 5. The effect of time dependent rapamycin treatment on mTORC1 and mTORC2 signaling in HepG2 cells.

HepG2 cells were cultured with and without rapamycin (100 nM) (n=3 each) for 0, 1, 2, 3, 6, or 12 hours.

A. A representative western blot of S6K (Thr389) phosphorylation in HepG2 cell lysate following time dependent rapamycin treatment (n=3). Equal loading (35 µg) was performed. Rapamycin inhibited mTORC1 signaling after 1 hour of rapamycin treatment.

B. A representative western blot of Akt (Ser473) phosphorylation in HepG2 cell lysate following time dependent rapamycin treatment (n=3). Equal loading (30 µg) was performed. Rapamycin inhibited mTORC2 signaling after 6 hours of rapamycin treatment. Values are displayed as mean + SEM. *p < 0.05, **p = 0.001-0.05, ***p < 0.0001 versus control; One-way analysis of variance; Dunnet’s Multiple Comparison Test; n=3.
Figure 5. The effect of time dependent rapamycin treatment on mTORC1 and mTORC2 signaling in HepG2 cells.
3.3 Rapamycin inhibits mTOR signaling and induces IGFBP-1 secretion and phosphorylation

Based on the effectiveness of rapamycin at inhibiting both mTORC1 and mTORC2 signaling within 12 hours, and the induction of IGFBP-1 seen by 100 nM of rapamycin at 24 hours, we validated our findings by treating HepG2 cells with 100 nM of rapamycin for 24 hours and determined the effects of this treatment on mTORC1 and C2 signaling, as well as IGFBP-1 secretion and phosphorylation. Indeed, our 24 hour treatment significantly inhibited mTORC1 and mTORC2 signaling (Figure 6A-B). The treatment also significantly increased IGFBP-1 secretion (Figure 6C) and phosphorylation at Ser101, 119, and 169 (Figure 6D-F), validating our subsequent use of 100 nM rapamycin treatment for 24 hours.
Figure 6. The effect of rapamycin treatment on mTORC1 and mTORC2 signaling and IGFBP-1 secretion and phosphorylation in HepG2 cells.

HepG2 cells were cultured for 24 h with and without rapamycin (100 nM) (n=3 each).

A. A representative western blot of S6K (Thr389) phosphorylation in HepG2 cell lysate. Equal loading (35μg) was performed. Rapamycin treatment inhibited mTORC1 signaling. B. A representative western blot of Akt (Ser473) phosphorylation in HepG2 cell lysate. Equal loading (20μg) was performed. Rapamycin treatment inhibited mTORC2 signaling. C. A representative western blot of IGFBP-1 in conditioned media of HepG2 cells using equal aliquots of conditioned media. Rapamycin treatment increased IGFBP-1 secretion. D-F. Representative western blots of phosphorylated IGFBP-1 at Ser101, 119, and 169 in conditioned media of HepG2 cells using equal aliquots of conditioned media. Rapamycin treatment increased IGFBP-1 phosphorylation at all three sites. Values are given as means + SEM. P < 0.05 versus control; unpaired Student’s t test.
Figure 6. The effect of rapamycin treatment on mTORC1 and mTORC2 signaling and IGFBP-1 secretion and phosphorylation in HepG2 cells.
Aim 2A: To investigate the functional role of mTOR signaling in the hypoxia-induced IGFBP-1 phosphorylation

3.4 Rapamycin and/or hypoxia inhibit mTOR signaling

We determined the phosphorylation of mTORC1 and C2 downstream targets as functional readouts in response to rapamycin and/or hypoxia. As shown in Figure 7A-B, rapamycin caused a profound decrease in the phosphorylation of 4E-BP1 at Thr70 (-44%) and Akt at Ser473 (-74%) demonstrating that 100 nM rapamycin significantly inhibits both mTORC1 and C2 signaling in HepG2 cells, in agreement with our recent report (96). Further, data in Figure 7A shows that phosphorylation of 4E-BP1 (Thr70) was significantly reduced in response to hypoxia, or in hypoxia combined with rapamycin. The degrees to which mTORC1 activity were inhibited in response to rapamycin, hypoxia or rapamycin combined with hypoxia were not significantly different from each other. Similarly, phosphorylation of Akt at Ser473 (mTORC2 functional readout) was markedly inhibited by rapamycin, hypoxia, and rapamycin combined with hypoxia (Figure 7B). These data demonstrate that both mTORC1 and C2 signaling are inhibited to similar degrees in response to rapamycin and/or hypoxia (Figure 7A-B).

3.5 mTOR inhibition by rapamycin and/or hypoxia increase IGFBP-1 phosphorylation

To establish that mTOR signaling constitutes a key molecular link between altered oxygen levels and changes in IGFBP-1 secretion and phosphorylation, we examined the effects of rapamycin and hypoxia on IGFBP-1 secretion and phosphorylation in HepG2 cells. Using equal volumes of HepG2 cell media, we demonstrated that IGFBP-1 secretion was significantly increased by both rapamycin (+167%), or hypoxia (+162%), however these effects were not additive when rapamycin and hypoxia were combined (Figure 7C). To assess IGFBP-1 phosphorylation qualitatively, we resolved IGFBP-1 phosphoisoforms in cell media by 2-D immunoblotting (Figure 7D, top left panel), where an increased number of spots shifting to the left (positive) represented increased phosphorylation. mTOR inhibition using rapamycin caused a pronounced
hyperphosphorylation of IGFBP-1 (Figure 7D, top right panel) and hypoxia had a similar effects (Figure 7D, bottom left panel). Interestingly, in the presence of rapamycin, hypoxia did not cause any additional phosphorylation of IGFBP-1 (Figure 7D, bottom right panel). These results support the hypothesis that hypoxia induces IGFBP-1 hyperphosphorylation mediated by mTOR inhibition. Using phosphosite specific antibodies, we further demonstrated a pronounced increase in phosphorylation at the three specific serine sites Ser101, 119 and 169 (Figure 7E-G) in response to rapamycin, hypoxia or combined rapamycin+hypoxia, with no significant differences between these treatments (Figure 7E-G). These findings suggest that induction of IGFBP-1 secretion and phosphorylation due to hypoxia is mediated via inhibition of mTOR signaling. We also validated the use of equal loading for cell media in our western blots by assessing albumin secretion. Rapamycin, hypoxia, as well as combined rapamycin+hypoxia all moderately yet significantly reduced albumin secretion (Figure A2). As phospho-IGFBP-1 levels were increased in conditions where we were also able to demonstrate a reduction in albumin secretion, we concluded that equal loading of cell media for western blotting is a suitable loading control.
Figure 7. The effect of rapamycin and/or hypoxia treatment on IGFBP-1 secretion and phosphorylation

HepG2 cells were cultured for 24 hours in normoxia (20% pO2) or in low oxygen (1% pO2, hypoxia) with and without rapamycin (100 nM) (n=3 each).

A. A representative western blot of 4E-BP1 (Thr70) phosphorylation in HepG2 cell lysate. Equal loading (20µg) was performed. Both rapamycin and hypoxia treatments separately inhibited mTORC1 signaling. Rapamycin+hypoxia treatment combined inhibited 4E-BP1 (Thr70) phosphorylation, but not to a further degree than either treatment alone. B. A representative western blot of Akt (Ser473) phosphorylation in HepG2 cell lysate. Equal loading (30µg) was performed. Both rapamycin and hypoxia treatments separately inhibited mTORC2 signaling. Rapamycin+hypoxia treatment combined inhibited Akt (Ser473) phosphorylation, to a similar degree as either treatment alone. C. A representative western blot of total IGFBP-1 in cell media of control, rapamycin, hypoxia and rapamycin+hypoxia treated HepG2 cells (n=3 each) using equal aliquots of cell media. Separate rapamycin and hypoxia treatment of HepG2 cells for 24 hrs significantly increased IGFBP-1 secretion. A combined treatment of rapamycin and hypoxia also induced IGFBP-1 secretion, but with no additional increase than either treatment alone, suggesting that induction of IGFBP-1 secretion in hypoxia is mediated by mTOR inhibition. D. A representative western blot of IGFBP-1 phosphoisoforms in cell media resolved by 2-D immunoblotting (top left panel), where an increased number of spots shifting to the left (positive) represent increased phosphorylation. Equal loading (volume) was performed. Both rapamycin (top right panel) and hypoxia (bottom left panel) treatments separately induced IGFBP-1 phosphorylation. In the presence of rapamycin, hypoxia did not cause any additional changes in phosphorylation of IGFBP-1 (bottom right panel) compared to either treatment alone. E-G. Representative western blots of phosphorylated IGFBP-1 at Ser101, 119 and 169 in cell media of control, rapamycin, hypoxia and rapamycin+hypoxia treated HepG2 cells (n=3 each) using equal aliquots of cell media. Rapamycin and hypoxia treatment of HepG2 cells individually increased IGFBP-1 phosphorylation significantly at all three serine sites. Combinatorial treatment (rapamycin +hypoxia) did not increase IGFBP-1 phosphorylation more than
either treatment alone, suggesting that hypoxic effects on IGFBP-1 phosphorylation (Ser101, 119, and 169) are mediated through mTOR inhibition. Values are displayed as mean + SEM. *p < 0.05, **p = 0.001-0.05, ***p < 0.0001 versus control; One-way analysis of variance; Dunnet’s Multiple Comparison Test; n=3.
Figure 7. The effect of rapamycin and/or hypoxia treatment on IGFBP-1 secretion and phosphorylation.
3.6 Rapamycin and/or hypoxia treatments do not alter cell viability

We tested cell viability as a result of our rapamycin, hypoxia, and combined rapamycin+hypoxia treatments using the Trypan Blue exclusion assay using the Countess Automated Cell Counter. This was done to ensure that relative fold inductions of IGFBP-1 observed were reflective of the treatments and not altered due to cell death. Cell viability between treatments was remarkably similar (thus no detectable error bars), demonstrating that our treatments were not causing cell death and that the inductions of IGFBP-1 observed were accurate (Figure A3).

3.7 IGFBP-1 phosphorylation in response to rapamycin and/or hypoxia inhibit IGF-I function

We utilized our IGF-I receptor β (IGF-1Rβ) autophosphorylation assay in P6 cells (96, 197) to assess the functional effects of IGFBP-1 phosphorylation. P6 cells incubated with IGF-I but without HepG2 cell media and thus no IGFBP-1 (positive control) demonstrated a marked increase in IGF-1R phosphorylation (+2495%) compared to P6 cells without IGF-I or HepG2 cell media (negative control) (Figure 8). This demonstrated the ability of IGF-I to stimulate IGF-1R autophosphorylation in P6 cells. When P6 cells were incubated with IGFBP-1 in untreated (basal) HepG2 cell media+IGF-I (control), a significant reduction in IGF-1R autophosphorylation was observed (-72%) compared to P6 cells incubated with IGF-I only. This suggested that basal levels of IGFBP-1 secreted by HepG2 cells (control) were able to sequester bioavailable IGF-I and subsequently reduce IGF-1R signaling. A further reduction (-58% to -64%) in IGF-1R autophosphorylation was observed when P6 cells were incubated with HepG2 cell media from either rapamycin, hypoxia, or rapamycin+hypoxia combined + IGF-I, compared to untreated (basal) HepG2 cell media (Figure 8). As equal concentration of total IGFBP-1 from HepG2 cell media was used to activate IGF-1R in P6 cells, this data suggest that inhibition in IGF-1R autophosphorylation was caused mainly by increased phosphorylation of IGFBP-1. Collectively, these data provide evidence for a key role of mTOR inhibition in linking hypoxia to increased IGFBP-1 phosphorylation and reduced IGF-I bioavailability in cells.
Figure 8. The effect of IGFBP-1 phosphorylation due to rapamycin, hypoxia, and combined rapamycin+hypoxia in HepG2 cell media on IGF-1R autophosphorylation in P6 cells

A representative western blot of P6 cell IGF-1R phosphorylation. HepG2 cells were treated with or without rapamycin (Rapa) for 24 hours in normoxia (20% pO2) or in low oxygen (1% pO2, hypoxia) (n=3 each), followed by HepG2 cell media collection. Equal concentrations of IGFBP-1 were mixed in P6 serum free cell media with human recombinant IGF-I (100 ng/ml) for 2 hours to allow IGFBP-1-mediated IGF-I sequestration, followed by 10 minute exposure to P6 cells to induce P6 cell IGF-I-mediated IGF-1R autophosphorylation. Equal loading of P6 cell lysate (30μg) was performed. Increased IGFBP-1 phosphorylation due to rapamycin, hypoxia, or combined rapamycin+hypoxia (R+H) resulted in significantly decreased IGF-1R activation. Values are displayed as mean ± SEM. *p< 0.05, **p= 0.001-0.05, ***p < 0.0001 versus control; One-way analysis of variance; Dunnet’s Multiple Comparison Test; n=3.
Figure 8. The effect of IGFBP-1 phosphorylation due to rapamycin, hypoxia, and combined rapamycin+hypoxia in HepG2 cell media on IGF-1R autophosphorylation in P6 cells
Aim 2B: To investigate the functional role of mTOR signaling in the hypoxia-induced IGFBP-1 phosphorylation

3.8 mTOR inhibition and hypoxia increase CK2 activity but not expression

As we previously demonstrated a link between increased CK2 expression and activity and FGR in baboon fetal liver as well as a mechanistic link between mTOR inhibition and increased CK2 activity in HepG2 cells, we investigated the effects of rapamycin and hypoxia on CK2 expression and activity. In agreement with our previous findings (96), rapamycin did not alter the expression levels of all three CK2 subunits (α, α’, and β). Furthermore, hypoxia did not significantly alter the expression levels of all three subunits. CK2α and CK2β levels were slightly decreased by hypoxia and rapamycin+hypoxia, but not to a significant degree (Figure 9A-C). The lack of increase in CK2 subunit expression during rapamycin and hypoxia was consistent when rapamycin and hypoxia treatments were combined. This suggests that both mTOR inhibition and hypoxia do not regulate CK2 at the transcriptional or translational levels in HepG2 cells, which is in agreement with our HepG2 cell rapamycin treatment study (96).

We measured CK2 activity using our previously established CK2 $^{32}$P phosphorylation assay (96, 126) to investigate the effect of mTOR inhibition and hypoxia on CK2 activity in HepG2 cells. CK2 activity was significantly increased (+145%) to similar levels in rapamycin, hypoxia, and combined rapamycin+hypoxia treatments, with no additive effect seen when treatments were combined (Figure 9D). This data suggests that HepG2 cell CK2 activity in hypoxia is regulated through mTOR inhibition, which is in alignment with our data on hypoxia-induced IGFBP-1 in HepG2 cells.
Figure 9. The effect of rapamycin and hypoxia on CK2 protein expression and activity in HepG2 cells.

A-C. Representative blots of CK2α, CK2α’, and CK2β expression in lysates of HepG2 cells after 24 hours of rapamycin, hypoxia, or rapamycin+hypoxia treatments. Equal protein loading (30 µg) was performed. Rapamycin, hypoxia, and combined rapamycin+hypoxia did not significantly alter the expression of the three CK2 subunit proteins. D. Summary of CK2 activity in HepG2 cell lysate after 24 hours of rapamycin, hypoxia, or rapamycin+hypoxia treatments. The kinase activity assays were performed using equal amounts of protein from whole-cell lysates. Rapamycin, hypoxia, and rapamycin+hypoxia treatments significantly increased CK2 activity to similar levels. Values are displayed as mean + SEM. *p < 0.05, **p = 0.001-0.05, ***p < 0.0001 versus control; One-way analysis of variance; Dunnet’s Multiple Comparison Test; n=3.
Figure 9. The effect of rapamycin and hypoxia on CK2 protein expression and activity in HepG2 cells.
Aim 3: To demonstrate that regulation of hypoxia-induced IGFBP-1 phosphorylation requires mTORC1 and/or mTORC2 activity

3.9 mTOR inhibition by raptor and rictor silencing induces IGFBP-1 secretion and phosphorylation

We tested the hypothesis that mTOR inhibition mechanistically links hypoxia to IGFBP-1 hyperphosphorylation using an RNA interference strategy to silence raptor or rictor individually in presence and absence of hypoxia. We first confirmed that our RNA interference approach efficiently silenced the mTORC1 and mTORC2 signalling pathways individually or in combination with a significant reduction in raptor and rictor protein expression (Figure 10A-B).

Moreover, as shown in Figure 10C, mTORC1 activity was reduced both due to raptor silencing (-40%) as well as combined raptor+rictor silencing (-48%), as assessed by a reduced phosphorylation of 4E-BP1 (Thr70). Similarly, mTORC2 activity was reduced by rictor (-52%) and combined raptor+rictor silencing (-60%) as indicated by a reduction in phosphorylation of Akt (Ser473) (Figure 10D). Thus we demonstrated the ability of raptor and rictor silencing to functionally inhibit mTORC1 and mTORC2 signaling, respectively.

Next, we assessed the effects of raptor and/or rictor silencing on IGFBP-1 secretion and phosphorylation. IGFBP-1 secretion increased following siRNA targeting raptor, rictor and combined raptor+rictor silencing (Fig 10E). In addition, we demonstrated that IGFBP-1 phosphorylation was increased by selective mTORC1 or mTORC2 inhibition, as well as combined mTORC1+mTORC2 inhibition (Figure 10F-H). Although significant increases in phosphorylation occurred at all three specific sites (Ser101 +181%, 119 +198%, and 169 +245%), phosphorylation at Ser169 was most prominently effected. Inhibition of mTORC1, mTORC2, or combined mTORC1+mTORC2 all
increased phosphorylation to similar levels (Figure 10F-H). These data indicate that both mTORC1 and mTORC2 regulate IGFBP-1 secretion and phosphorylation.
Figure 10. The effect of raptor and rictor silencing on IGFBP-1 secretion and phosphorylation

HepG2 cells were cultured for 72 hours with scrambled, raptor, rictor, or combined raptor+rictor siRNA (100 nM) (n=3 each).

A. A representative western blot raptor in HepG2 cell lysate. Equal loading (50µg) was performed. Raptor and combined raptor+rictor siRNA reduced total raptor protein expression to similar levels. B. A representative western blot rictor in HepG2 cell lysate. Equal loading (50µg) was performed. Rictor and combined raptor+rictor siRNA reduced total rictor protein expression to similar levels. C. A representative western blot of 4E-BP1 (Thr70) phosphorylation in HepG2 cell lysate. Equal loading (20µg) was performed. Raptor and combined raptor+rictor silencing inhibited 4E-BP phosphorylation. D. A representative western blot of Akt (Ser473) phosphorylation in HepG2 cell lysate. Equal loading (30µg) was performed. Rictor as well as combined raptor+rictor silencing both inhibited Akt phosphorylation to similar degrees. E. A representative western blot of IGFBP-1 in cell media of scrambled, raptor, rictor, and combined raptor+rictor siRNA treated HepG2 cells (n=3) using equal aliquots of cell media. Raptor, rictor, and combined raptor+rictor siRNA all significantly increased IGFBP-1 secretion, with the greatest induction occurring due to combined raptor+rictor silencing. F-H. Representative western blots of phosphorylated IGFBP-1 at Ser101, 119 and 169 in cell media of scrambled, raptor, rictor, and combined raptor+rictor siRNA treated HepG2 cells (n=3 each) using equal aliquots of cell media. Raptor, rictor, and combined raptor+rictor siRNA all significantly increased IGFBP-1 phosphorylation at all three sites, suggesting inhibition of either complex is sufficient to induce IGFBP-1 phosphorylation. Values are displayed as mean + SEM. *p< 0.05, **p= 0.001-0.05, ***p < 0.0001 versus control; One-way analysis of variance; Dunnet’s Multiple Comparison Test; n=3.
A

Scrambled + - - -
Raptor - + - +
Rictor - - + +

150 kDa [Raptor]
42 kDa [β-actin]

Relative Raptor Expression
(Arbitrary units)

B

Scrambled + - - -
Raptor - + - +
Rictor - - + +

192 kDa [Rictor]
42 kDa [β-actin]

Relative Rictor Expression
(Arbitrary units)

C

Scrambled + - - -
Raptor - + - +
Rictor - - + +

20 kDa [p-4EBP1 (Thr70)]
20 kDa [4EBP1]
42 kDa [β-actin]

Relative 4EBP1 phosphorylation
(Arbitrary units)

D

Scrambled + - - -
Raptor - + - +
Rictor - - + +

56 kDa [p-4EBP1 (Thr70)]
56 kDa [p-Akt (Ser473)]
42 kDa [Akt]
42 kDa [β-actin]

Relative Akt phosphorylation
(Arbitrary units)
Figure 10. The effect of raptor and rictor silencing on IGFBP-1 secretion and phosphorylation
3.10 Activation of mTORC1 and mTORC2 by DEPTOR silencing prevents hypoxia-induced IGFBP-1 secretion and phosphorylation

To confirm that mTORC1 and/or mTORC2 mediate the increase in IGFBP-1 secretion and phosphorylation in response to hypoxia, we determined the effect of hypoxia on IGFBP-1 secretion and phosphorylation in cells in which mTORC1 and C2 were activated. To activate mTOR we silenced DEPTOR, an endogenous mTORC1 and mTORC2 inhibitor.

We first validated that DEPTOR silencing reduced protein expression of target protein (-52%) in normoxia (Figure 11A). As shown in Figure 11B-C, phosphorylation of 4E-BP1 (Thr70) (+92%) and Akt (Ser473) (+74%) were significantly increased following DEPTOR silencing. Treatment of cells with DEPTOR siRNA resulted in a significant decrease in IGFBP-1 secretion (Figure 11D). Furthermore, DEPTOR silencing caused a pronounced decrease in phosphorylation at the three serine sites Ser101, 119 and 169 (Figure 11E-G) with the decrease at Ser169 being slightly more prominent (Figure 11G). Collectively, this data demonstrates that activation of mTOR signaling reduces both IGFBP-1 secretion and phosphorylation in HepG2 cells.
Figure 11. The effect of mTORC1 and mTORC2 activation via DEPTOR silencing on IGFBP-1 secretion and phosphorylation

HepG2 cells were treated with scrambled or DEPTOR siRNA 72 hours (n=3 each).

A. A representative western blot of DEPTOR in HepG2 cell lysate from cells transfected with scrambled or DEPTOR siRNA. Equal loading (30 µg protein) was performed. DEPTOR siRNA silencing significantly decreased total DEPTOR protein expression. B. A representative western blot of 4E-BP1 phosphorylation (Thr70) in HepG2 cell lysate from cells transfected with scrambled or DEPTOR siRNA. Equal loading (20µg protein) was performed. DEPTOR silencing significantly increased 4E-BP1 (Thr70) phosphorylation. C. A representative western blot of Akt phosphorylation (Ser473) in HepG2 cell lysate from cells transfected with scrambled or DEPTOR siRNA. Equal loading (30µg protein) was performed. DEPTOR silencing significantly increased Akt phosphorylation (Ser473). D. A representative western blot of IGFBP-1 secretion in HepG2 cell media from cells transfected with scrambled or DEPTOR siRNA. Equal loading (aliquots of cell media) was performed. DEPTOR silencing caused a significant decrease in IGFBP-1 secretion. E-G. Representative western blots of phosphorylated IGFBP-1 at Ser101, 119, and 169 in HepG2 cell media from cells transfected with scrambled or DEPTOR siRNA. Equal loading (aliquots of cell media) was performed. DEPTOR silencing significantly decreased IGFBP-1 phosphorylation at Ser101, 119, and 169. Values are given as means + SEM. $P <0.05$ versus control; unpaired Student’s $t$ test.
Figure 11. The effect of mTORC1 and mTORC2 activation via DEPTOR silencing on IGFBP-1 secretion and phosphorylation
To further investigate the role of mTOR in inducing IGFBP-1 secretion and phosphorylation in response to hypoxia, we silenced DEPTOR or raptor+rictor combined in HepG2 cells. As determined by the protein expression of the targets (DEPTOR, raptor or rictor), silencing efficiency was high in both normoxia and hypoxia (Figure 12A-C). As shown in Figure 12D-E, in normoxic cells DEPTOR silencing activated both mTORC1 and mTORC2 activity and raptor+rictor silencing caused a pronounced decrease in mTORC1 and mTORC2 activity. Hypoxia alone markedly inhibited the activity of mTORC1 and also of mTORC2 to similar levels. Importantly, activation of mTOR in DEPTOR silenced cells prevented mTORC1 and C2 inhibition in response to hypoxia. Furthermore, mTORC1 and C2 activity were reduced to a similar extent in raptor+rictor silenced and in hypoxic cells, respectively.

As expected, hypoxia alone induced IGFBP-1 secretion. However, hypoxia failed to induce IGFBP-1 secretion in DEPTOR silenced cells (Figure 12F). Moreover, in raptor+rictor silenced cells hypoxia did not induce further secretion of IGFBP-1 than in normoxic raptor+rictor silenced cells (+353%) (Figure 12F). Similarly, DEPTOR silencing prevented the increase in IGFBP-1 phosphorylation at Ser101, 119 and 169 in response to hypoxia (Figure 12G-I). Furthermore, hypoxia did not induce further IGFBP-1 phosphorylation in cells with raptor+rictor silencing (+421%) (Figure 12G-I). Together, these data provide evidence that IGFBP-1 secretion and phosphorylation in response to hypoxia are mediated by inhibition of the mTORC1 and/or mTORC2 signaling pathway.
Figure 12. The effect of mTORC1+mTORC2 inhibition and/or activation with and without hypoxia on IGFBP-1 secretion and phosphorylation

HepG2 cells were treated with DEPTOR siRNA (to activate mTORC1+C2) or raptor+rictor siRNA (to inhibit mTORC1+C2) for 48 hours, and then cells were additionally cultured for 24 hours in normoxia (20% pO2) or in low oxygen (1% pO2, hypoxia) (n=3 each).

A. A representative western blot of DEPTOR in HepG2 cell lysate from cells transfected with scramble (normoxia), scramble (hypoxia), DEPTOR (normoxia), DEPTOR (hypoxia), raptor+rictor (normoxia) and rictor +rictor (hypoxia) siRNA. Equal loading (50 µg protein) was performed. DEPTOR siRNA significantly decreased total DEPTOR protein expression, regardless of hypoxic status. B. A representative western blot of raptor in HepG2 cell lysate from cells transfected with scramble (normoxia), scramble (hypoxia), DEPTOR (normoxia), DEPTOR (hypoxia), raptor+rictor (normoxia) and rictor+rictor (hypoxia) siRNA. Equal loading (50 µg protein) was performed. Raptor siRNA silencing in cells decreased total raptor protein expression regardless of hypoxic status. C. A representative western blot of rictor in HepG2 cell lysate from cells transfected with scramble (normoxia), scramble (hypoxia), DEPTOR (normoxia), DEPTOR (hypoxia), raptor+rictor (normoxia) and rictor+rictor (hypoxia) siRNA. Equal loading (50 µg protein) was performed. Rictor siRNA silencing decreased total rictor protein expression regardless of hypoxic status. D. A representative western blot of 4E-BP1 phosphorylation (Thr70) phosphorylation in HepG2 cell lysate from cells transfected with scramble (normoxia), scramble (hypoxia), DEPTOR (normoxia), DEPTOR (hypoxia), raptor+rictor (normoxia) and rictor+rictor (hypoxia). Equal loading (20µg protein) was performed. Hypoxia alone significantly inhibited 4E-BP1 (Thr70) phosphorylation. When treated with DEPTOR siRNA, 4E-BP1 (Thr70) phosphorylation was significantly increased regardless of hypoxic status, suggesting that DEPTOR knock down prevented mTORC1 inhibition due to hypoxia alone. When treated with raptor+rictor siRNA, 4E-BP1 phosphorylation was significantly inhibited regardless of hypoxic status. E. A representative western blot of Akt phosphorylation (Ser473) in HepG2 cell lysate from cells transfected with scramble (normoxia), scramble (hypoxia),
DEPTOR (normoxia), DEPTOR (hypoxia) rictor+raptor (normoxia) and rictor+raptor (hypoxia). Equal loading (30µg protein) was performed. Hypoxia alone significantly inhibited Akt phosphorylation (Ser473). When treated with DEPTOR siRNA, Akt phosphorylation (Ser473) was significantly increased compared to the control regardless of hypoxic status, suggesting that DEPTOR knock down prevented mTORC2 inhibition due to hypoxia alone. When treated with raptor+rictor siRNA, Akt phosphorylation (Ser473) was significantly inhibited regardless of hypoxic status. F. A representative western blot of IGFBP-1 secreted by HepG2 cells transfected with scramble (normoxia), scramble (hypoxia), DEPTOR (normoxia), DEPTOR (hypoxia), raptor+rictor (normoxia) and rictor+rictor (hypoxia) (n=3) using equal aliquots of cell media. Hypoxia alone significantly increased IGFBP-1 secretion. When treated with DEPTOR siRNA, IGFBP-1 secretion was not significantly different from control levels regardless of hypoxic status, suggesting that constitutively activated mTOR signaling (due to DEPTOR silencing) prevented the induction of IGFBP-1 secretion due to hypoxia. Conversely, when treated with raptor+rictor siRNA, IGFBP-1 secretion was significantly increased to levels similar to hypoxia alone, suggesting that hypoxia exerts its effects on IGFBP-1 secretion via mTORC1+C2 signaling. G-I. Representative western blots of IGFBP-1 phosphorylation at Ser101, 119, and 169 in cell media of cells transfected with scramble (normoxia), scramble (hypoxia), DEPTOR (normoxia), DEPTOR (hypoxia), raptor+rictor (normoxia) and rictor+rictor (hypoxia) (n=3) using equal aliquots of cell media. Hypoxia alone significantly increased IGFBP-1 phosphorylation at Ser101, 119 and 169. When treated with DEPTOR siRNA, IGFBP-1 phosphorylation was significantly reduced regardless of hypoxic status, suggesting that constitutively activated mTOR signaling (due to DEPTOR siRNA silencing) prevented IGFBP-1 phosphorylation as well as the induction of IGFBP-1 phosphorylation caused by hypoxia alone. When treated with raptor+rictor siRNA, IGFBP-1 phosphorylation was significantly increased to levels similar to hypoxia alone, suggesting that inhibition of mTOR during hypoxia is responsible for the regulation of IGFBP-1 phosphorylation. Values are displayed as mean ± SEM. *p < 0.05, **p = 0.001-0.05, ***p < 0.0001 versus control; One-way analysis of variance; Dunnet’s Multiple Comparison Test; n=3.
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**p4E-BP1 (Thr70)**

20 kDa

20 kDa

42 kDa

**Relative 4E-BP1 phosphorylation (arbitrary units)**

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**pAkt (Ser473)**

56 kDa

56 kDa

42 kDa

**Relative Akt phosphorylation (arbitrary units)**

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**Relative IGFBP-1 secretion (arbitrary units)**

- **p<0.001**

**Relative IGFBP-1 phosphorylation (S101) (arbitrary units)**

- **p<0.001**
Figure 12. The effect of mTORC1+mTORC2 inhibition and/or activation with and without hypoxia on IGFBP-1 secretion and phosphorylation.
3.11 Activation of mTORC1 signaling decreases IGFBP-1 secretion and phosphorylation

In order to investigate whether mTORC1 signaling specifically is involved in mediating the effects of hypoxia on IGFBP-1, we next silenced the endogenous mTORC1 inhibitor TSC2 to activate primarily mTORC1 in HepG2 cells. We validated silencing efficiency of TSC2, and as shown in Figure 13A, total TSC2 protein expression was reduced (-67%). Silencing of TSC2 led to significant increases in mTORC1 signaling alone as determined by phosphorylation of 4E-BP1 at (Thr70) (+107%) (Figure 13B), while mTORC2 signaling was not affected as assessed by the lack of change in phosphorylation of Akt at (Ser473) (Figure 13C). Therefore these data confirmed that TSC2 silencing results in increased mTORC1 activity without effecting mTORC2 activity in cells.

We then determined changes in IGFBP-1 secretion and phosphorylation as a result of mTORC1 activation. TSC2 silencing resulted in a modest yet significant reduction in IGFBP-1 secretion (-32%) (Figure 13D). Importantly, IGFBP-1 phosphorylation was significantly reduced at all three serine residues (Ser119, 169, and 101) and the degree of reduction was much more marked, (-70%) (Figure 13E-G) than the decreases in IGFBP-1 secretion. These data suggest that mTORC1 signaling is an important regulator of IGFBP-1 phosphorylation.
Figure 13. The effect of mTORC1 activation via TSC2 siRNA on IGFBP-1 secretion and phosphorylation

HepG2 cells were treated with scrambled or TSC2 siRNA 72 hours (n=3 each).

A representative western blot of TSC2 in HepG2 cell lysate from cells transfected with scrambled or TSC2 siRNA. Equal loading (30 µg protein) was performed. TSC2 siRNA silencing of HepG2 cells significantly decreased total TSC2 protein expression. B. A representative western blot of 4E-BP1 phosphorylation (Thr70) phosphorylation in HepG2 cell lysate from cells transfected with scrambled or TSC2 siRNA. Equal loading (20µg protein) was performed. TSC2 silencing significantly increased 4E-BP1 (Thr70) phosphorylation. C. A representative western blot of Akt phosphorylation (Ser473) in HepG2 cell lysate from cells transfected with scrambled or TSC2 siRNA. Equal loading (30µg protein) was performed. TSC2 silencing did not affect Akt (Ser473) phosphorylation. D. A representative western blot of IGFBP-1 secretion in HepG2 cell media from cells transfected with scrambled or TSC2 siRNA. Equal aliquots of cell media were loaded. TSC2 silencing caused a significant yet modest decrease in total IGFBP-1 secretion. E-G. Representative western blots of IGFBP-1 phosphorylation at Ser101, 119, and 169 in HepG2 cell media from cells transfected with scrambled or TSC2 siRNA. Equal aliquots of cell media were loaded. TSC2 silencing caused a significant decrease in IGFBP-1 phosphorylation at Ser101, 119, and 169. Values are given as means + SEM. P <0.05 versus control; unpaired Student’s t test.
Figure 13. The effect of mTORC1 activation via TSC2 siRNA on IGFBP-1 secretion and phosphorylation
3.12 mTORC1 activation prevents the induction of IGFBP-1 secretion and phosphorylation in response to hypoxia

To further explore the role of mTORC1 in mediating the effect of hypoxia on IGFBP-1, we studied the effects of hypoxia in cells with mTORC1 activation by TSC2 silencing. In parallel we silenced raptor or rictor to inhibit mTORC1 and mTORC2 individually. As shown in 14A-C, protein expression of TSC2, raptor and rictor were decreased following corresponding siRNA silencing and the silencing efficiency was similar in hypoxic cells.

As expected, hypoxia inhibited mTORC1 and mTORC2 activity as evidenced by a marked decrease in 4E-BP1 (Thr70) and Akt phosphorylation (Ser473) (Figure 14D-E) and induced IGFBP-1 secretion (+180%) and phosphorylation at all three specific sites (Ser101 +184%, 119 +188%, and 169 +235%), of which Ser 169 was most prominently effected (Figure 14F-I). TSC2 silencing prevented mTORC1 inhibition caused by hypoxia. mTORC2 on the other hand, was not affected by TSC2 or raptor siRNA but was significantly inhibited due to hypoxia or rictor siRNA treatment (-75%). TSC2 silencing prevented the increase in IGFBP-1 secretion in response to hypoxia (Figure 14F). Similarly, mTORC1 activation decreased IGFBP-1 phosphorylation at all three sites and prevented hypoxia-induced increases in phosphorylation (Figure 14G-I), indicating the involvement of mTORC1 signaling. These data demonstrate that mTORC1 signaling specifically regulates IGFBP-1 secretion and phosphorylation in response to hypoxia. Furthermore, although phosphorylation at all three sites was altered, the Ser169 site was more specific to mTORC1 in regulation of IGFBP-1 phosphorylation. Interestingly, rictor silencing induced IGFBP-1 secretion, albeit to a relatively lesser extent, suggesting that inhibition of either mTORC1 or C2 are able to drive IGFBP-1 secretion and phosphorylation.
Figure 14. The effect of individual mTORC1 activation and individual mTORC1 and mTORC2 inhibition with and without hypoxia on IGFBP-1 secretion and phosphorylation

HepG2 cells were treated with TSC2 (to activate mTORC1), raptor (to inhibit mTORC1), and rictor (to inhibit mTORC2) siRNA for 48 hours, and then cells were additionally cultured for 24 hours in normoxia (20% pO2) or in low oxygen (1% pO2, hypoxia) (n=3 each).

A. A representative western blot of TSC2 in HepG2 cell lysate from cells transfected with scramble (normoxia), scramble (hypoxia), TSC2 (normoxia), TSC2 (hypoxia), raptor (normoxia), raptor (hypoxia), rictor (normoxia) and rictor (hypoxia) siRNA. Equal loading (30 µg protein) was performed. TSC2 siRNA significantly decreased total TSC2 protein expression, regardless of hypoxic status. B. A representative western blot of raptor in HepG2 cell lysate from cells transfected with scramble (normoxia), scramble (hypoxia), TSC2 (normoxia), TSC2 (hypoxia), raptor (normoxia), raptor (hypoxia), rictor (normoxia) and rictor (hypoxia) siRNA. Equal loading (50 µg protein) was performed. Raptor siRNA significantly decreased total raptor protein expression, regardless of hypoxic status. C. A representative western blot of rictor in HepG2 cell lysate from cells transfected with scramble (normoxia), scramble (hypoxia), TSC2 (normoxia), TSC2 (hypoxia), raptor (normoxia), raptor (hypoxia), rictor (normoxia) and rictor (hypoxia) siRNA. Equal loading (50 µg protein) was performed. Rictor siRNA significantly decreased total rictor protein expression, regardless of hypoxic status. D. A representative western blot of 4E-BP1 phosphorylation (Thr70) in HepG2 cell lysate from cells transfected with scramble (normoxia), scramble (hypoxia), TSC2 (normoxia), TSC2 (hypoxia), raptor (normoxia), raptor (hypoxia), rictor (normoxia), and rictor (hypoxia) siRNA (n=3). Equal loading (20 µg protein) was performed. Hypoxia alone significantly inhibited 4E-BP1 (Thr70) phosphorylation. When treated with TSC2 siRNA, 4E-BP1 phosphorylation was significantly increased from control levels regardless of hypoxic status. Raptor siRNA significantly inhibited 4E-BP1 phosphorylation regardless of hypoxic status. Rictor siRNA did not affect 4E-BP1 phosphorylation, but 4E-BP1 phosphorylation was significantly reduced in rictor siRNA+hypoxia treatment. E. A
representative western blot of Akt phosphorylation (Ser473) in HepG2 cell lysate from cells transfected with scramble (normoxia), scramble (hypoxia), TSC2 (normoxia), TSC2 (hypoxia), raptor (normoxia), raptor (hypoxia), rictor (normoxia), and rictor siRNA (hypoxia) (n=3). Equal loading (30µg protein) was performed. Hypoxia alone significantly inhibited Akt (Ser473) phosphorylation. TSC2 siRNA did not affect Akt phosphorylation, although Akt phosphorylation was significantly reduced as a result of hypoxia during TSC2 siRNA treatment. Similarly, raptor siRNA alone did not affect Akt phosphorylation, but raptor siRNA+hypoxia treatment significantly reduced Akt phosphorylation. Rictor siRNA significantly reduced Akt phosphorylation regardless of hypoxic status. F. A representative western blot of IGFBP-1 secreted by HepG2 cells transfected with scramble (normoxia), scramble (hypoxia), TSC2 (normoxia), TSC2 (hypoxia), raptor (normoxia), raptor (hypoxia), rictor (normoxia), and rictor siRNA (hypoxia) (n=3) using equal aliquots of cell media. Hypoxia alone significantly increased IGFBP-1 secretion. When treated with TSC2 siRNA, IGFBP-1 secretion was significantly reduced from control levels regardless of hypoxic status, suggesting that constitutively activated mTORC1 signaling (due to TSC2 silencing) prevented the induction of IGFBP-1 secretion due to hypoxia. Conversely, when treated with raptor or rictor siRNA, IGFBP-1 secretion was significantly increased to levels similar to hypoxia alone, suggesting that hypoxia exerts it effects on IGFBP-1 secretion via mTORC1 or combined mTORC1+C2 inhibition. G-I. Representative western blots of phosphorylated IGFBP-1 (Ser101, 119, and 169) secreted by HepG2 cells transfected with scramble (normoxia), scramble (hypoxia), TSC2 (normoxia), TSC2 (hypoxia), raptor (normoxia), raptor (hypoxia), rictor (normoxia), and rictor siRNA (hypoxia) (n=3) using equal aliquots of cell media. Hypoxia alone significantly increased IGFBP-1 phosphorylation. When treated with TSC2 siRNA, IGFBP-1 phosphorylation was significantly reduced from control levels regardless of hypoxic status at Ser101 and 169, and was also reduced at Ser119 albeit not significantly, suggesting that constitutively activated mTORC1 signaling (due to TSC2 silencing) prevented the induction of IGFBP-1 phosphorylation due to hypoxia. Conversely, when treated with raptor or rictor siRNA, IGFBP-1 phosphorylation was significantly increased to levels similar to hypoxia alone, suggesting that hypoxia exerts it effects on IGFBP-1 phosphorylation via mTORC1 or combined
mTORC1+C2 signaling. Values are displayed as mean + SEM. *$p< 0.05$, **$p= 0.001-0.05$, ***$p < 0.0001$ versus control; One-way analysis of variance; Dunnet’s Multiple Comparison Test; n=3.
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**p4E-BP1**

- 20 kDa
- 20 kDa
- 42 kDa

**4E-BP1**

**β-actin**

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**pAkt**

- 56 kDa
- 56 kDa
- 42 kDa

**Akt**

**β-actin**

### Graphs

**D**

**Graph 1:** Relative 4E-BP1 phosphorylation (arbitrary units).

**Graph 2:** Relative Akt phosphorylation (arbitrary units).
Figure 14. The effect of individual mTORC1 activation and individual mTORC1 and mTORC2 inhibition with and without hypoxia on IGFBP-1 secretion and phosphorylation.
4.0 Discussion

4 Key findings

Using HepG2 cells as a model for human fetal hepatocytes (96, 192-195) we have identified a novel molecular mechanism by which mTOR regulates IGFBP-1 secretion and phosphorylation in response to hypoxia. We demonstrate that mTORC1 or C2 inhibition increases whereas mTORC1 or C2 activation decreases IGFBP-1 secretion and phosphorylation. Further, hypoxia failed to induce additional IGFBP-1 secretion in cells with mTORC1 or C2 inhibition. Importantly, activation of mTORC1 or C2 prevented IGFBP-1 secretion and phosphorylation in response to hypoxia. These findings suggest that both mTORC1 and C2 signaling regulate IGFBP-1 secretion and phosphorylation in a coordinated manner and that inhibition of either complex is sufficient to drive these coordinated functional effects in hypoxia. We provide evidence that mTOR-mediated IGFBP-1 phosphorylation in hypoxia reduces IGF-1R signaling and draw a link between hypoxia-mediated mTOR inhibition and increased CK2 activity. Furthermore, we demonstrate that IGFBP-1 phosphorylation was significantly increased at three serine residues (Ser101, 119 and 169) in our treatments, of which phosphorylation at Ser169 was most prominent. Together this work proposes that increased IGFBP-1 secretion and site-specific phosphorylation mediated by mTOR inhibition may contribute to restricted fetal growth in response to hypoxia.

4.1 Mimicking fetal hypoxia in vitro

Phosphorylation increases the affinity of IGFBP-1 for IGF-I (202) and it is likely that induction of IGFBP-1 phosphorylation in hypoxia is a powerful mechanism for the regulation IGF bioavailability in the modulation of fetal growth. This assumption was supported by our previous in vitro data demonstrating that hypoxia resulted in increased phosphorylation of IGFBP-1 which increased its binding affinity to IGF-I 300-fold and markedly inhibited IGF-I-stimulated cell growth (192). The use of 1% O₂ in vitro to represent the in vivo hypoxic state has been well established (172, 192, 203). Low oxygen tension, which is usually defined as hypoxia in vitro, has been established in many tissues with an average of 3% O₂ (204-206), and has been shown to reach as low as 1-2% in the
descending aorta within hypoxic fetal lambs (207) compared to healthy oxygen levels which range from 4% O\textsubscript{2} in muscles to 12.1% O\textsubscript{2} in the kidney (208). 1% O\textsubscript{2} due to low oxygen tension has further been detected in bone marrow, thymus, and the kidney medulla (209). Using atmospheric air (20% O\textsubscript{2}) in vitro to represent normoxia in vivo is also commonly used (106, 172, 192, 203), and is usually referred to as normoxia although it may be a state of hyperoxia. Subsequently, we used 1% O\textsubscript{2} (hypoxia) and 20% O\textsubscript{2} (normoxia) in our in vitro studies in this dissertation as in our previous work (192, 203).

Studies on the role of IGFBP-1 phosphorylation and the mechanisms regulating hypoxia-induced IGFBP-1 phosphorylation in the development of FGR are limited. However, we recently provided evidence that IGFBP-1 is hyperphosphorylated in human FGR (96). We also established a causative link between IGFBP-1 hyperphosphorylation and mTOR inhibition in a baboon model of FGR in vivo (96). Furthermore, we conducted mechanistic studies using HepG2 cells and validated the use of our HepG2 cell culture model using primary fetal hepatocytes (96). Using this as the basis, the mechanistic data in this current study was generated using HepG2 cells. HepG2 cells are human liver carcinoma cells that demonstrate biotransformation characteristics and the gene expression patterns similar to primary human fetal hepatocytes (194, 195). Thereby using HepG2 cells in this study we have now established a novel mTOR mediated mechanism linking hypoxia to increased IGFBP-1 phosphorylation that also inhibited IGF-I function.

### 4.2 Regulation of IGFBP-1 in hypoxia

Elevated IGFBP-1, resulting in decreased bioavailability of IGF-I, has been proposed to be an important mechanism restricting fetal growth in both human FGR and in animal models of chronic intrauterine hypoxia (104, 210-213). Hypoxia up-regulates IGFBP-1 mRNA and protein expression in HepG2 cells and human fetal hepatocytes in vitro (106), as well in zebra fish in vivo (104). Previous studies on the role of IGFBP-1 in the regulation of fetal growth have mainly focused on the mechanisms that determine induction of IGFBP-1 gene transcription and expression. Regulation of gene transcription by hypoxia involves binding of the transcription factor HIF-1\textalpha to a HRE which has been identified in the IGFBP-1 gene (106). mTORC1 promotes the expression of HIF-1\textalpha by
regulating the translation of the α subunit of HIF which activates the transcription of several genes responsive to hypoxia such as Glut1, Pfkp, and Pdk1 (214). Furthermore, a role for HIF1 has been implicated in IGFBP-1 gene expression (106). Thus, there is an indirect indication for a role of mTOR signaling in linking hypoxia to the expression of IGFBP-1 in fetal hepatocytes. The mechanisms modulating IGFBP-1 phosphorylation and implications of mTOR signaling in changes to IGFBP-1 phosphorylation in response to hypoxia have received much less attention. Thus, the post-translational effects of IGFBP-1 phosphorylation due to hypoxia were investigated in this study.

4.3 The functional significance of IGFBP-1 phosphorylation on IGF-I signaling

The ability of hypoxia-induced IGFBP-1 phosphorylation to modulate IGF function due to mTOR inhibition was tested through our IGF-I induced IGF-1R autophosphorylation assay. The bioassay utilizing P6 cells (IGF-1R overexpressing BALB/c3T3 derivative) (126) in our current study was a direct adaptation of the previously established assay system using NIH-3T3 cells (215, 216). Here, we demonstrate the ability of recombinant human IGF-I to stimulate human IGF-1R expressed by P6 cells. Unstimulated BALB/3T3 cells contain approximately 8000 IGF-1 receptors per cell, whereas P6 cells transfected with human IGF-1R cDNA contain upwards of 43000 receptors, making them highly sensitive to induction by IGF-I (217).

IGF-1R is a transmembrane receptor tyrosine kinase which is responsible for the mediation of IGF-I action. It has a high binding affinity towards IGF-I and IGF-II, and thus functions to facilitate IGF signaling (72). Upon ligand binding to IGF-1R, the receptor kinase is activated and IGF-1R tyrosine autophosphorylation, as well as downstream substrate tyrosine phosphorylation occur (60). This process leads to enhanced cellular proliferation and protein synthesis as well as the inhibition of apoptosis (218). IGF-1R is a tertameric protein which consists of 2 extracellular α-subunits that are disulphide-bound to each other and to 2 β-subunits which span the cell membrane and also contain a cytoplasmic portion (219). The β-subunits contain the kinase activity of IGF-1R, thus providing a rationale for our analysis of IGF-1Rβ phosphorylation only (220). The assessment of Tyr1135 phosphorylation as a representation of IGF-1R
activation has been well established (96, 126, 221, 222). The activation loop of IGF-1R is a flexible portion of the C-lobe of the kinase domain (223). In the unactivated state of IGF-1R, the activation loop forms an autoinhibitory conformation in which Tyr1135 is bound to the active site. This prevents Tyr1135 phosphorylation in the absence of IGF-1R ligand binding (223). IGF-I binding to IGF-1Rα induces IGF-1Rβ autophosphorylation which occurs in trans (one IGF-1Rβ kinase domain phosphorylating the other), and three tyrosine residues of IGF-1Rβ are phosphorylated by the process – Tyr1131, Tyr1135, and Tyr1136 (224). Although Tyr1131 and Tyr1136 are also phosphorylated by the event, studies have demonstrated that Tyr1135 is the first residue to be phosphorylated and in general is the most predominant phospho-site involved in IGF-1R activation (223). Furthermore, only Tyr1135 has been characterized to be bound in the active site during the autoinhibitory conformation (223). These data support our use of assessing Tyr1135 phosphorylation as a means to determine IGF-1R activation due to IGF-I ligand binding. Our data demonstrates that IGF-I-mediated IGF-1R activation is reduced in the presence of HepG2 cell media, suggesting that IGFBP-1 was able to reduce interactions between IGF-I and IGF-1R.

Through densitometric analysis of IGFBP-1 secretion from our rapamycin, hypoxia, and rapamycin+hypoxia treatment, we were able to quantify levels of total IGFBP-1 present in the conditioned medias. This allowed us to use aliquots of conditioned HepG2 cell media which were normalized to contain equal total IGFBP-1. As the induction of IGFBP-1 phosphorylation was proportionately higher than secretion in treated HepG2 cell media, our resultant media aliquots contained equal total IGFBP-1, but differing degrees of phosphorylated IGFBP-1 - namely increased phosphorylation in the treated HepG2 cell media aliquots. Importantly, mixtures of treated HepG2 cell media containing a proportionately higher quantity of phosphorylated IGFBP-1 (from treated HepG2 cells) along with human recombinant IGF-I were able to reduce the degree of IGF-1R autophosphorylation to a greater extent compared to the untreated HepG2 cell media, reinforcing the notion that rapamycin and/or hypoxia-induced phosphorylated IGFBP-1 from HepG2 cells was able to yield a greater inhibitory effect on IGF-I-mediated IGF-1R signaling. It is possible that secreted IGFBP-3 may interfere with IGF-I sequestration.
However, the use of equal concentration of total IGFBP-1 exposed to free IGF-I in control and treated samples suggests that increased interactions between IGFBP-1 and IGF-I were driven by IGFBP-1 phosphorylation, functioning to increase IGF-I sequestration and decrease IGF-1R signaling. This is supported by evidence showing that HepG2 cells secrete and IGFBP-3 in negligible quantities compared to the overabundance of IGFBP-1 secretion (203), and our previous studies showed that mutating key IGFBP-1 phospho-sites to alanine strongly reduced the binding affinity of IGFBP-1 to IGF-I (126). Here, we furthered these findings by showing that both mTOR inhibition by rapamycin and hypoxia functionally altered the degree of IGF-1R signaling observed, and that combined treatment did not further inhibit IGF-1R signaling, reinforcing the concept that hypoxia-induced IGFBP-1 phosphorylation is mediated through mTOR inhibition.

4.4 HepG2 cell viability is unaltered by rapamycin and hypoxic treatments

Although our rapamycin and hypoxia treatments resulted in increased IGFBP-1 secretion and phosphorylation, we tested the viability of cells post-treatment to ensure that the degree of induction seen was valid and not altered due to increased cell death via the Trypan Blue exclusion assay. Trypan blue is a diazo dye which is commonly used in microscopy for cell counting and tissue viability (225). It can allow for effective distinguishing of live versus dead cells by exploiting the high selectivity of live cell membranes. As cells must be highly selective regarding which compounds can pass through the cell membrane, foreign compounds such as trypan blue can be used to stain cells. Live cells will exclude the absorption of trypan blue, whereas dead cells will be stained blue throughout (225). We were able to utilize this technique using an automated cell counter to yield high-throughput results and effectively quantify a large number of cells for viability. Our results show that none of the rapamycin, hypoxia, or combined rapamycin+hypoxia treatments altered cell viability, demonstrating that the induction of IGFBP-1 secretion and phosphorylation quantified due to our treatments was accurate and not affected by cell death.
4.5 The involvement of CK2 in hypoxia-induced IGFBP-1 phosphorylation

The molecular mechanisms upstream or downstream of mTOR which ultimately induce IGFBP-1 secretion and phosphorylation are unknown. Although in our recent study we have provided clear evidence for a key role of CK2 in IGFBP-1 phosphorylation (96), whether CK2 directly phosphorylates IGFBP-1 is currently unknown. CK2 is a well established serine/threonine protein kinase which is classified to function in a messenger-independent manner, as its activity is not dependent on small molecules which are typically involved in second messenger kinase regulation (226). CK2 functions in a tetrameric complex composed of CK2α and CK2α’ catalytic subunits as well as two CK2β regulatory subunits (227, 228). Variance in the subunits has been characterized between different organisms. The presence of both CK2α and CK2α’ catalytic subunits are well documented in humans (227, 228). However, various mammalian systems have been shown to contain either two CK2α or two CK2α’ catalytic subunits (229). Further, in humans, only one CK2β regulatory subunit has been identified, whereas in *Saccharomyces cerevisiae* multiple forms of CK2β have been established (230). We previously chose to investigate CK2 as a potential kinase for IGFBP-1 due to the identification of a consensus sequence within IGFBP-1 for phosphorylation by CK2 (191).

The regulation of CK2 under various conditions of cellular stress seems to occur in a cell- and tissue-type specific manner (96). We have previously demonstrated that CK2 subunit expression is increased in an *in vivo* maternal nutrient restriction model using baboon fetal liver (96). In these cells, CK2 activity was also increased. Interestingly, mTOR inhibition via rapamycin treatment increased CK2 activity in HepG2 cells, but not expression of the CK2 subunits (96). This suggests that mTOR inhibition regulates CK2 activity, but not at the transcriptional or translational levels in HepG2 cells. Further, silencing of CK2 via siRNA resulted in significantly decreased IGFBP-1 phosphorylation in HepG2 cells, reinforcing the involvement of CK2 in IGFBP-1 phosphorylation (96). Here, we were able to replicate these results with rapamycin treatment on HepG2 cells,
where CK2 subunit expression was not altered but activity was increased. Interestingly, we have now demonstrated very similar results with hypoxic treatment as well as combined rapamycin+hypoxia treatment. In these treatments, CK2 subunit protein expression was not significantly different from control cells, although a trend of reduction in CK2α and CK2β subunit expression due to hypoxia was observed. However, these treatments did significantly induce CK2 activity to very similar levels as rapamycin treatment, further demonstrating mTOR inhibition as a mediatory step in hypoxia-induced IGFBP-1 phosphorylation which involves the activity of CK2. It seems likely that inhibition of mTOR in hypoxia activates CK2 which in turn phosphorylates IGFBP-1 either directly or through intermediate mechanisms which currently remain unknown. As mTOR is a kinase, we postulate that mTOR inhibition activates CK2 through inhibition of phosphatase activity, in which mTOR inhibition results in the downregulation of an unidentified phosphatase which otherwise prevents phosphorylation-mediated CK2 activation (190). In this way, inhibition of a kinase (mTOR) can plausibly result in the activation of another kinase. Identification of this interaction remains to be investigated.

IGFBP-1 is readily secreted out of the cell due to a signal peptide sequence for secretion in the IGFBP-1 precursor peptide (81), suggesting that protein maturation and phosphorylation occur in a localized manner near the cell membrane. Interestingly, numerous studies have reported localized CK2 activity at the cell membrane (231-234), providing indirect evidence for CK2-mediated IGFBP-1 phosphorylation.

4.6 The roles of TSC2 and DEPTOR in mTOR regulation during hypoxia

DEPTOR is a naturally occurring inhibitor of mTOR signaling and directly binds to mTORC1 and mTORC2 and inhibits mTOR activity (235). Reduced DEPTOR expression has been shown to increase 4E-BP1 (Thr70) (149) and S6K (Thr389), as well as Akt (Ser473) phosphorylation (236) – phospho-sites directly phosphorylated by mTORC1 and mTORC2, respectively, and thus constitutively activates the functions of both mTORC1 and mTORC2 (236). Indeed, our data demonstrates that there is a basal DEPTOR-mediated inhibition of both mTORC1 and mTORC2 signaling in HepG2 cells. Similarly, activation of mTORC1 individually has been achieved by silencing TSC2, a
negative regulator of mTORC1 (237) and by silencing TSC2 alone we were able to activate mTORC1 in our current studies.

mTORC1 inhibition by TSC2 occurs upstream of mTOR, and the effects are indirect. The TSC1/2 complex acts as a negative regulator of mTORC1 by activating the GTPase activity of protein Rheb (187, 188). TSC2 specifically contains a GTPase activating protein (GAP) domain which, when stabilized by TSC1, facilitates the conversion of RhebGTP to RhebGDP (188). The presence of RhebGDP does not have an effect on mTORC1 signaling. However, cellular accumulation of RhebGTP is a potent activator of mTORC1 activity (187, 188). We demonstrate in this study that siRNA-mediated silencing of TSC2 was able to activate mTORC1 signaling with no effect on mTORC2 signaling, which presumably occurred through the accumulation of RhebGTP. Furthermore, it is known that hypoxia functions to reduce mTORC1 signaling through this pathway. Hypoxic conditions result in the activation of the activation of HIF-1α, activating protein REDD1 which then induces TSC1/2 activity (186). Thus, silencing of TSC2 was able to prevent the effects of hypoxia on mTORC1 signaling.

Interestingly, hypoxic effects on mTORC1 and mTORC2 signaling were also attenuated through DEPTOR silencing. It has been shown that DEPTOR binds directly to both mTORC1 and mTORC2 (144), and that silencing of DEPTOR results in the activation of both mTORC1 and mTORC2 signaling (150). This direct interaction between DEPTOR and both mTOR complexes has been implicated as a form of basal inhibition for mTOR signaling (150). Here we have demonstrated that alleviation of this interaction was able to increase both mTORC1 and mTORC2 activity in hypoxia. This reinforces the concept that mTOR signaling can be altered through multiple pathways, and that hypoxia-mediated TSC1/2 activation was not able to effect mTOR signaling as the alleviation of direct DEPTOR:mTOR interaction resulted in increased mTOR signaling regardless of oxygen status. DEPTOR silencing activated mTORC1 and mTORC2, which reduced IGFBP-1 secretion/phosphorylation and prevented the IGFBP-1 response to hypoxia. These findings are consistent with the possibility that both mTORC1 and mTORC2 are involved in mediating the effect of hypoxia on IGFBP-1 secretion and phosphorylation. Using silencing of TSC2, which specifically activates mTORC1, we confirmed that
mTORC1 activation is sufficient to reduce IGFBP-1 secretion and phosphorylation and prevent the effects of hypoxia on IGFBP-1.

4.7 mTOR signaling and hypoxia-induced IGFBP-1 phosphorylation

It is well-established that mTORC1 signaling is inhibited by hypoxia (172, 173), whereas the effects of hypoxia on mTORC2 have not been well characterized. Previous studies have generated inconsistent results, reporting both increased and decreased mTORC2 activity in response to hypoxia in different cells (189). In agreement with previous literature, our data shows that mTORC1 and mTORC2 activity are decreased due to hypoxia in HepG2 cells (189). To determine the specific roles of the two mTOR complexes in regulating IGFBP-1 secretion and phosphorylation during hypoxia, we utilized systematic mTOR inhibition and activation strategies in combination with hypoxia.

Constitutive inhibition of mTORC1 and mTORC2 via raptor and rictor siRNA, respectively, induced both IGFBP-1 secretion and phosphorylation, which was not enhanced further by hypoxia. This suggests that mTOR inhibition in hypoxia is responsible for the induction of IGFBP-1. As the siRNA treatment occurred for 72 hours, with only the last 24 hours with and without hypoxia, an additive effect in IGFBP-1 induction would be expected if hypoxia was driving IGFBP-1 secretion/phosphorylation through a molecular pathway unrelated to mTOR signaling. We have shown here that constitutive mTOR inhibition is able to drive IGFBP-1 induction, and that the subsequent addition of hypoxia did not further enhance levels of IGFBP-1. Interestingly, cells exposed to scrambled siRNA with the last 24 hours of the 72 hour incubation in hypoxia resulted in levels of total and phosphorylated IGFBP-1 which were similar to cells exposed to raptor and rictor siRNA in normoxia for 72 hours. This suggests that siRNA treatment operates in a temporal manner, in which constitutive mTOR inhibition was more potent within approximately the last 24 hours of treatment. This is in agreement with the literature, in which 72 hour siRNA transfection periods in order to yield optimal effects of the treatment are common (238-240). Further, constitutive inhibition of either
complex due to individual raptor or rictor siRNA were both able to induce IGFBP-1 secretion and phosphorylation to similar levels as hypoxia alone, with raptor silencing resulting in slightly higher induction than rictor silencing. Again, these effects were not augmented by hypoxia, suggesting that constitutive inhibition of mTORC1 or mTORC2 are sufficient to drive IGFBP-1 secretion and phosphorylation. This suggests that both mTORC1 and mTORC2 may function in a coordinated manner to regulate IGFBP-1.

Conversely, constitutive mTORC1 and mTORC2 activation via DEPTOR silencing reduced both IGFBP-1 secretion and phosphorylation suggesting that basal levels of mTOR signaling regulate IGFBP-1 under untreated conditions, and that increased mTOR activity due to alleviation of endogenous basal mTOR inhibition reduces IGFBP-1 secretion and phosphorylation to below basal levels. This relief of basal inhibition resulted in increased mTOR activity which remained consistent in hypoxia, and the sustained and elevated mTOR signaling activity in hypoxia was able to prevent hypoxia-induced IGFBP-1 secretion and phosphorylation. This strongly implicates the inhibition of mTOR signaling in the regulation of hypoxia-induced IGFBP-1 secretion and phosphorylation in HepG2 cells.

We also investigated the effect of constitutive mTORC1 signaling via silencing of TSC2. TSC2 silencing alone caused a modest reduction in IGFBP-1 secretion, but a larger reduction in IGFBP-1 phosphorylation. As previously discussed, the TSC1/2 pathway is responsible for incorporating cellular responses to hypoxia with mTORC1 signaling. When TSC2 silencing was performed, resulting in constitutive mTORC1 activation in combination with hypoxia, both IGFBP-1 secretion and phosphorylation induction due to hypoxia were prevented. This data suggests that even though constitutive inhibition of mTORC1 or mTORC2 signaling were able to induce IGFBP-1 to similar levels as hypoxia alone, the inhibition of mTORC1 specifically due to hypoxia is responsible for hypoxic regulation of IGFBP-1. This is consistent the possibility that induction of IGFBP-1 and subsequent reduction in IGF-I bioavailability can be mediated by signaling events which only affect one mTOR complex, representing a wide array of possible
mechanisms by which IGFBP-1 secretion and phosphorylation, and therefore IGF-I bioavailability and fetal growth, can be regulated.

4.8 IGFBP-1 phosphorylation at specific residues due to hypoxia

It is known that IGFBP-1 is phosphorylated at multiple (five serine) sites (123, 128, 192, 196, 197, 202, 241). We have earlier demonstrated that IGFBP-1 phosphorylation at three specific serine residues (Ser101, 119 and 169) was increased in amniotic fluid (196, 241) and umbilical cord plasma of human FGR babies as well as in fetal liver and cord plasma from our baboon model of maternal nutrient restriction which results in FGR (96). In FGR, reductions in nutrient and oxygen delivery are the most common challenges to the developing fetus. Interestingly, in our previous study using HepG2 cells we have demonstrated significant induction of IGFBP-1 phosphorylation at all three sites (Ser101, 119 and 169) examined in hypoxia and leucine deprivation (192). Furthermore, using mass spectrometry (192) we earlier showed that although Ser101 was a common site, two distinct patterns of IGFBP-1 phosphorylation were detected between the two stimuli: hypoxia caused IGFBP-1 hyperphosphorylation at Ser98 and 169 while leucine deprivation at Ser119 which concomitantly led to 300- and 30-fold increases in IGF-I affinity, respectively (192). The data from the current study demonstrated a similar pattern of increases in IGFBP-1 phosphorylation at Ser101, 119 and 169. This suggests that site-specific phosphorylation of IGFBP-1 under conditions of cellular stress occurs in order to modulate the affinity of IGFBP-1 to IGF-I, potentially providing increased control over the bioavailability of IGF-I for IGF-I-mediated growth in FGR. Although Ser98 phosphorylation is considered to be highly significant in combination with Ser169 in increasing binding affinity of IGFBP-1 for IGF-I and reducing IGF-I bioavailability, as suggested by our previous study (129), it could not be tested due to a lack of available antibody. Furthermore, it is also possible that additional novel sites may be involved in phosphorylation during mTOR inhibition and/or hypoxia for which further studies would need to be conducted.
4.9 Future studies

Investigating the role of CK2 in hypoxia-induced IGFBP-1 phosphorylation

Although data from this study implicates mTOR signaling in the regulation of hypoxia-induced IGFBP-1 secretion and phosphorylation, numerous aspects of this regulatory system remain to be elucidated. Our recent data implicates protein kinase CK2 in the regulation of IGFBP-1 downstream of mTOR (96). Previous work in our lab with 4,5,6,7-tetrabromobenzotriazole (TBB), a chemical inhibitor of CK2, has shown that phosphorylation of IGFBP-1 is attenuated when TBB is combined with rapamycin (96). This demonstrates the role of CK2 in mTOR-inhibition mediated IGFBP-1 phosphorylation. Combining CK2 inhibitor TBB or CK2 siRNA with hypoxia would lead to a greater understanding of the specific role of CK2 in hypoxic regulation of IGFBP-1. It would be expected that hypoxia induces IGFBP-1 secretion and phosphorylation, and that silencing or inhibiting of CK2 in combination with hypoxia may prevent IGFBP-1 secretion and phosphorylation. Another possibility would be probe for direct CK2:IGFBP-1 interaction via the utilization of immunofluorescence microscopy to test for co-localization between CK2 and IGFBP-1, as well as co-immunoprecipitation, GST pull-down assays, or fluorescence resonance energy transfer (FRET) binding assays with recombinant CK2 and IGFBP-1. These experiments would allow us to determine if intermediary processes occur between CK2 activation and IGFBP-1 secretion and phosphorylation or if CK2 directly phosphorylates IGFBP-1.

Investigating the *in vivo* role of IGFBP-1 phosphorylation in regulation of fetal growth in hypoxia

Previously, an *in vivo* study using chick embryos demonstrated that both hypoxia and nutrient deprivation were associated with FGR; however prenatal hypoxia and undernutrition may have differential effects on fetal development (238). As previous work in the literature has demonstrated an *in vivo* link between hypoxia and FGR in chicken embryos (25), investigating the effects of hypoxia on IGFBP-1 secretion and phosphorylation in chicken embryos *in vivo* would be a logical progression of our studies.
This would allow us to effectively compare site-specific IGFBP-1 phosphorylation and IGF-I actions between our *in vitro* model and fetal hypoxia *in vivo*. Phosphorylation may be induced at the same or even different sites compared to humans, but importantly it would be expected that IGFBP-1 phosphorylation would increase its affinity to and reduce the actions of IGF-I. *In vivo* chicken embryo hypoxia +/- experiments could be followed up with detailed mechanistic studies. The use of morpholino injection to target and silence mTOR components raptor and rictor in chicken embryos with and without hypoxia would allow us to investigate the role of mTOR in the hypoxic regulation of IGFBP-1 *in vivo*. The overall effects on fetal growth could be tested via tissue-specific and entire organism wet weights measurement, in order to validate chicken embryos as a model for hypoxia-induced FGR and to investigate the effects of our treatments on fetal growth *in vivo*.

Development of therapeutic strategies

Through *in vitro* studies, it may be possible to investigate the potential development of therapeutic small molecule inhibitors aimed at increasing levels of IGF-I signaling. For example, a small molecule which could bind to Cys38 of IGFBP-1 may be able to prevent IGF-I:IGFBP-1 binding, resulting in increased fetal growth. This is supported by evidence showing that mutation of Cys38 of IGFBP-1 abolished IGF-I binding (89). Another target residue on IGFBP-1 which binding of a small molecule inhibitor may prove beneficial is Ser169, as we have shown that phosphorylation of Ser169 occurs during hypoxia, and hypoxia-induced IGFBP-1 phosphorylation binds to IGF-I with markedly higher affinity (192). Initial proof of principle studies would need to be conducted, likely through IGF-I bioavailability assays as performed in this study and IGFBP-1:IGF-I binding affinity assays.
4.10 General summary

Together we have demonstrated a novel molecular link between the mTOR and IGF signaling axes, providing compelling evidence that hypoxia-induced IGFBP-1 secretion and site-specific phosphorylation occurs through the inhibition of mTORC1 signaling. We also demonstrate that prolonged inhibition of mTORC2 signaling is able to drive IGFBP-1 secretion and phosphorylation. Further, we have established that mTOR-mediated hypoxia-induced IGFBP-1 phosphorylation is linked with increased CK2 activity, and is functionally significant because these changes caused a marked decrease in IGF-I bioavailability. Thus, our data provide a mechanistic link between fetal hypoxia and reduced IGF-I signaling via mTOR-inhibition-mediated increases in IGFBP-1 secretion and site specific phosphorylation (Figure 15). These pathways may contribute to restricted fetal growth in response to hypoxia in vivo.
Figure 15. Proposed model linking hypoxia to IGFBP-1

Inhibition of mTOR signaling (resulting in an increase in CK2 activity) is a key molecular link between hypoxia, increased IGFBP-1 secretion and phosphorylation, and reduced IGF-I bioavailability in FGR (96).
4.11 Limitations

Although we were able to determine site-specific phosphorylation of IGFBP-1 due to hypoxia, we were not able to investigate the effects of our treatments on other phosphorylation sites of IGFBP-1, namely Ser95 and Ser98, or novel phosphorylation sites. This was due to the lack of available phospho-site specific antibodies. Determining if phosphorylation at Ser95 and 98 as well as other novel sites was increased due to our treatments would have been an intriguing possibility that would have allowed us to determine if IGFBP-1 is preferentially phosphorylated in the linker and/or C terminal regions due to hypoxia. These possibilities are under investigation by the use of mass spectrometry.

Further, we speculate that IGFBP-1 phosphorylation increases its affinity towards IGF-I through synergistic interactions of its phospho-sites. This synergism likely involves novel phosphorylation sites, as well as the previously established Ser95 and 98 phospho-sites. The investigation into this potential synergism through the use of site-directed mutagenesis of various combinations of phosphorylation sites would have provided compelling evidence of this synergism.
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Appendices

Appendix A: Supplementary figures, data, and permissions to use copyrighted material

Figure A1. Broad perspective of mTOR signaling

Schematic showing a thorough list of mTORC1 and mTORC2 interactions, demonstrating the complexity of mTOR signaling and its many roles in the cell (137).
Figure A2. The effect of rapamycin and/or hypoxia treatment on albumin secretion

HepG2 cells were cultured for 24 hours in normoxia (20% pO2) or in low oxygen (1% pO2, hypoxia) with and without rapamycin (100 nM) (n=3 each).

A representative western blots of secreted albumin in cell media of control, rapamycin, hypoxia and rapamycin+hypoxia treated HepG2 cells (n=3 each) using equal aliquots of cell media. Rapamycin, hypoxia, and combined rapamycin+hypoxia all modestly yet significantly reduced albumin secretion. Values are displayed as mean + SEM. *p< 0.05, **p= 0.001-0.05, ***p < 0.0001 versus control; One-way analysis of variance; Dunnet’s Multiple Comparison Test; n=3.
Figure A3. The effect of rapamycin and hypoxia on HepG2 cell viability.

Summary of HepG2 cell viability after 24 hours of rapamycin, hypoxia, or rapamycin+hypoxia treatments. Cell viability was assessed using equal aliquots of cell suspension (10 µl) via the Trypan Blue exclusion assay. Cell viability was determined as a measure of live/total cells. Rapamycin, hypoxia, and rapamycin+hypoxia treatments all demonstrated nearly identical cell viability to control cells. Values are displayed as mean ± SEM. *p < 0.05, **p = 0.001-0.05, ***p < 0.0001 versus control; One-way analysis of variance; Dunnet’s Multiple Comparison Test; n=3.
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Figure 7. The proposed model. Inhibition of mTOR signaling and activation of protein kinase CK2 in the fetal liver constitutes a key molecular link between nutrient deprivation, increased IGFIRP-1 expression and phosphorylation, and decreased IGF-1 bioavailability in FGR.

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Liver mTOR controls IGF-1 bioavailability by regulation of protein kinase CK2 and IGFIRP-1 phosphorylation in fetal growth restriction

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Curriculum Vitae

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2nd Best Basic Science Oral Presentation
May 2014

UWO Department of Paediatrics Graduate Studentship
Value $15,000.00
2014-2015

Publications:


Damerill I, Biggar K, Abu Shehab M, Li S, Jansson T, Gupta MB. 2014. Increased IGFBP-1 phosphorylation in response to hypoxia is mediated by inhibition of mTOR signaling. (Currently preparing for submission – see following pages for title page and abstract)
Increased IGFBP-1 phosphorylation in response to hypoxia is mediated by inhibition of mTOR signaling

Ian Damerill\textsuperscript{1}, Kyle K. Biggar\textsuperscript{1}, Majida Abu Shehab\textsuperscript{1}, Shawn Shun-Cheng Li\textsuperscript{1}, Thomas Jansson\textsuperscript{2} and Madhulika B. Gupta\textsuperscript{1,3,4*}.

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\textbf{Short title:} \hspace{0.5cm} Regulation of IGFBP-1 phosphorylation in hypoxia

\textbf{Corresponding author:} \hspace{0.5cm} Madhulika B. Gupta

\textbf{Key words:} Hypoxia, HepG2 cells, IGFBP-1 phosphorylation, mTOR
Abstract

Impaired oxygen and nutrient delivery to the fetus result in fetal growth restriction (FGR). IGF-I is a key regulator of fetal growth and IGF-I bioavailability is determined by IGFBP-1, which binds IGF-I and inhibits its signaling. Hypoxia induces IGFBP-1 hyperphosphorylation resulting in decreased IGF-I bioavailability. We recently established a link between inhibition of mechanistic target of rapamycin (mTOR) and IGFBP-1 hyperphosphorylation. Here, we tested the hypothesis that IGFBP-1 hyperphosphorylation in hypoxia is mediated by mTOR inhibition. Using HepG2 cells, we inhibited mTOR signaling either by rapamycin or siRNA targeting raptor (mTORC1) and/or rictor (mTORC2) in hypoxia (1% O₂) or normoxia (20% O₂). Conversely, we activated mTORC1 or mTORC1+mTORC2 by silencing endogenous mTOR inhibitors (TSC2/DEPTOR) in hypoxia or normoxia. Western blotting was used to assess IGFBP-1 secretion/phosphorylation and the effects of IGFBP-1 phosphorylation on IGF-I bioavailability using IGF-1R autophosphorylation. Using phospho-site specific IGFBP-1 antibodies we demonstrated that hypoxia or inhibition of either mTORC1 and/or mTORC2 induced similar degrees of IGFBP-1 secretion and phosphorylation at Ser101/119, and 169, which markedly reduced IGF-1R autophosphorylation. Activation of mTORC1+C2 by DEPTOR silencing or activation of mTORC1 by silencing TSC2 reduced IGFBP-1 secretion/phosphorylation and prevented IGFBP-1 hyperphosphorylation in response to hypoxia.

Multiple Reaction Monitoring Mass Spectrometry (MRM/MS) quantitatively validated IGFBP-1 hyperphosphorylation at Ser101/119, and 169. MRM MS analysis further showed increased phosphorylation at Ser98 and at a novel residue Ser174 which was sensitive to mTOR inhibition. Structural modeling indicated that rapamycin-sensitive phospho Ser174 being in close proximity to IGF-I binding site may directly influence IGF-I affinity. Together, this study demonstrates that signaling through either the mTORC1 or mTORC2 pathway is sufficient to induce site-specific hyperphosphorylation of IGFBP-1 in response to hypoxia. These data put forward a novel mechanistic link...
between hypoxia, IGFBP-1 hyperphosphorylation and decreased IGF-I bioavailability mediated by inhibition of mTOR which may contribute to restricted fetal growth in response to hypoxia.