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The Role of Hypoxia on Pyruvate Kinase M2, mammalian Target of Rapamycin, Mitochondrial Function, and Cell Invasion in the Trophoblast

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A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

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March 2016

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ABSTRACT

The Role of Hypoxia on Pyruvate Kinase M2, mammalian Target of Rapamycin, Mitochondrial Function, and Cell Invasion in the Trophoblast.

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Master of Science

This thesis will be organized into two chapters discussing the role of hypoxia in the human placenta. The goal of this thesis is to characterize pyruvate kinase M2, mammalian target of rapamycin, mitochondrial function, and cell invasion in hypoxic conditions in the trophoblast. Understanding the mechanisms of placental metabolism can lead to further treatments for placental diseases.

Chapter one covers the background of intrauterine growth restriction, hypoxia, placental metabolism, and pyruvate kinase M2 (PKM2). Little is currently understood about the role of the mitochondria in placental diseases. Expression of PKM2, trophoblast cell invasion, and mitochondrial function is shown to be inhibited by hypoxia. PKM2 inhibition decreases trophoblast cell invasion and nuclear expression of PKM2, but increases mitochondrial function. Studying how hypoxia affects the placenta during placental diseases can help clarify the mechanisms by which these diseases occur.

Chapter two further characterizes the background of intrauterine growth restriction and hypoxia. It also covers the background of mammalian target of rapamycin. The objective of this chapter was to assess activated mTOR in the trophoblast in hypoxia. Decreased placental and fetal weights, as well as trophoblast cell invasion were observed in hypoxia. A decrease in the activation of mTOR was also found in the hypoxic placenta. This study could provide insight into the physiological relevance of the pathways and could be targeted to help alleviate placental diseases.

Keywords: placenta, pyruvate kinase M2 (PKM2), intrauterine growth restriction (IUGR), metabolism, mammalian target of rapamycin (mTOR)

ACKNOWLEDGEMENTS

Thank you to Dr. Juan Arroyo for being my graduate chair/advisor and the guidance you have given me to help me finish my research. I would also like to thank Dr. Benjamin Bikman and Dr. Brian Poole for being on my graduate committee. Thank you to the Arroyo, Reynolds, and Bikman labs and all the undergraduate students who helped me with the research.

Thank you to my parents for the encouragement and support you have given me. Thank you to my husband, Stephen, for your love, patience, and support.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1: The Role of Hypoxia on Pyruvate Kinase M2, Mitochondrial Function, and Cell Invasion on Trophoblast Cells

Abstract

Introduction

Intrauterine growth restriction (IUGR) is a disease that affects many pregnant women and their growing fetuses. Hypoxia and shallow invasion are key characteristics of IUGR. A lack of oxygen has been shown to affect the mitochondria, which produces the energy in the cell. Pyruvate Kinase M2 (PKM2) is a metabolic enzyme known to be involved during hypoxia. Our objective was to determine the role of hypoxia in mitochondrial function, trophoblast cell invasion, and PKM2.

Methods

Human placenta tissues were collected at term to characterize the mitochondria in the cells. Human trophoblast cells were used to determine PKM2 expression. Treatment was with hypoxia, Shikonin, or Rapamycin to observe mitochondrial function and invasion characteristics of the trophoblast cells. The cells were lysed after treatment and western blots were performed to view PKM2 expression.

Results

Hypoxic treated cells showed a decrease in mitochondrial function, invasion, and nuclear and cytostolic PKM2. Shikonin treated cells showed a decrease in invasion and nuclear PKM2, and an increase in mitochondrial function.

Discussion and Conclusion

We conclude that PKM2, invasion, and mitochondrial function are all decreased in hypoxia. This could be part of the mechanism of the placental insufficiency observed during IUGR.

Introduction

Intrauterine Growth Restriction (IUGR)

Intrauterine Growth Restriction (IUGR) is a condition during pregnancy that leads to an increased risk of morbidity for the fetus. This disease is characterized by a fetus that does not grow according to its genetic growth potential and is below the 10% in birth weight (Bahr et al.; Cohen et al., 2016). This can also be called small for gestational age. IUGR can affect up to 10% of pregnancies (Bahr et al.). IUGR complications include persistent pulmonary hypertension for the newborn, perinatal hypoxia and asphyxia, and neurological delays (Brar and Rutherford, 1988; Gray et al., 1999; Pollack and Divon, 1992). It has also been linked to the adult onset of diabetes, hypertension, stroke, and death from coronary vascular diseases (Arroyo et al., 2008). Up to 44% of preterm birth (PTB) is connected with this disease (Arroyo et al., 2008). It is associated with up to 70% of neonatal deaths and leads to increased incidence of cerebral palsy, neurological defects, and pulmonary disorders in the neonate (Challis et al., 2001). Placental insufficiency is the most common cause of IUGR. IUGR placentas are pathologically characterized by decreased trophoblast invasion, reduced syncytiotrophoblast surface area, and increased placental trophoblast apoptosis (Ishihara et al., 2002; Krebs et al., 1996; Mayhew et al., 2003).

Hypoxia, IUGR, and Placental Metabolism

In order to have a successful pregnancy, proper oxygenation of the placenta and fetus is essential (Huppertz, 2014). Local hypoxia has been thought to play an important role during IUGR. It has been shown that induced transient uteroplacental hypoxia causes significant IUGR, confirming an important role for hypoxia during this disease (Tanaka et al., 1994). Studies have shown that when a fetus experiences hypoxic stress, its cardiac output is redistributed to the heart and the brain at the expense of other organs. This leads to metabolic and cardiovascular disturbances in adulthood (Bourque et al., 2012; Giussani and Davidge, 2013; Iqbal and Ciriello, 2013; Jang et al., 2015; Matheson et al., 2015; Myatt, 2006; Rueda-Clausen et al., 2014). Other studies have shown that hypoxia can affect the differentiation and growth of trophoblast cells. This suggests a role for hypoxia in the function and behavior of trophoblast cells (Caniggia and Winter, 2002; Genbacev et al., 1997; Jiang et al., 2000; Nelson et al., 1999).

Mitochondria are the powerhouses of the cell, producing the majority of energy needed for regular cellular functions. Mitochondria need oxygen to produce ATP through the electron transport chain and oxidative phosphorylation (Mandò et al., 2014). Dysfunctions of the mitochondria lead to many different disorders. In the placenta, proper metabolism is important for a successful pregnancy. During pregnancy, the metabolic activity is sustained throughout gestation by increasing mitochondrial activity (Leduc et al., 2010). Placenta metabolism is associated with oxygen availability and uses almost 40% of the uteroplacental oxygen uptake (Bahr et al.). Problems in this process could contribute to many placental pathologies, including IUGR. In previous studies, it has been shown that proper mitochondrial function is required for normal development. Impaired mitochondrial function affects the fetal and placental growth in

the embryo (Wakefield et al., 2011). This paper will investigate the effects of hypoxia on mitochondrial function in placental cells.

An important enzyme involved in metabolism is Pyruvate Kinase M2 (PKM2). PKM2 is an enzyme that catalyzes the last and rate-limiting reaction in the glycolytic pathway. This kinase is mainly found in the developing embryo and cancer cells (Wong et al., 2013). The role of PKM2 and mitochondrial function in cancer cells has been well established. Previous studies have shown that PKM2 can be inhibited by oxidative stress in cancer cells (Abate et al., 1990). The cell redirects carbon from oxidation to the anabolic pathways when PKM2 is inhibited. PKM2 is active when oxidative stress is low (Wallace, 2012). Placental cells are reminiscent of cancer cells in the way they invade the uterus during implantation. Recent experiments have shown PKM2 expression in normal, preeclamptic (PE), and IUGR pregnancies with higher levels present in preeclamptic human placentas (Bahr et al.). Further studies need to investigate the role of PKM2 in the invasive trophoblast.

An important regulator of cell growth is the mammalian target of rapamycin (mTOR) protein. It responds to the availability of growth factors and nutrients (Jansson et al., 2006; Wullschleger et al., 2006). Previous studies have shown a direct correlation between mTOR activation and trophoblast invasion (Knuth et al., 2015). Our laboratory has recently shown that activated placental mTOR is decreased near term during hypoxia-induced IUGR in the rat (Kimball et al., 2015). The exact metabolic role of mTOR in the placenta is still unknown. Investigating the mTOR pathway in relation to mitochondrial function will provide valuable insight into IUGR pathology.

Materials and Methods

Human Placental Tissues

Paraffin-embedded placental tissue slides for Control, Preeclampsia, and IUGR placenta were purchased from the Research Center for Women's and Infant's Health BioBank, Ontario, Canada. The placentas were collected shortly after delivery either by C-section or vaginally. For each group, ten samples were analyzed. IUGR samples were confirmed by ultrasound with a uterine Doppler and absent end diastolic flow. IUGR samples showed placental insufficiency and came from patients with an estimated fetal weight below the 10^{th} percentile. Preeclampsia was confirmed by increased maternal blood pressure and proteinuria.

Immunofluorescence

Immunofluorescence (IF) was performed on paraffin-embedded placental samples. In summary, slides were de-waxed, washed in a 1× Tris buffer solution (TBS), and blocked with Background Sniper (Biocare Medical, Concord, CA, USA) for 1 hr. This was followed by incubation overnight with a primary COX IV antibody (Cell Signaling Technology, Danvers, MA, USA), or Cytokeratin 7 (Dako, Carpinteria, CA, USA). Slides were then incubated for 1 hr with donkey anti-rabbit TR or donkey anti-mouse TR (Biocare Medical). 4′, 6-diamidino-2-phenylindole, dihydrochloride (DAPI) was used for counterstaining prior to mounting with glass coverslips. Slides were viewed using a Texas Red excitation and emission filter.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on trophoblast cells as previously shown in our lab (Arroyo et al., 2010a). In summary, slides were de-waxed, washed in a 1x Tris buffer solution (TBS), and blocked with Sniper for 1 hr. This was followed by incubation for 1 hr with

a primary antibody (PKM2-Cell Signaling Technology, Danvers, MA, rabbit #4053, IgG negative control-Biocare Medical, Concord, CA). Slides were then incubated with Mach 2 universal stain polymer, followed by color development with diaminobenzidine (DAB). Hematoxylin was used for nuclear counterstain. Slides were mounted using Permount media.

Cell Culture Protocol

A first trimester human trophoblast Swan 71 cell line was used in this study. This cell line was a generous gift from Dr. Gil Mor at Yale University. Cells were maintained in a 37°C incubator in RPMI media supplemented with 10% fetal bovine serum (FBS) and penicillin and streptomycin (P/S). For hypoxia treatments, cells were maintained in a 37°C incubator in 2% oxygen for 24 hr before used in invasions, mitochondrial function tests, or lysed. For treatments with Shikonin, Shikonin was purchased from Sigma-Aldrich and dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO). The cells were treated with a final concentration of 1.0 μmol Shikonin for 24 hr before used in invasions, mitochondrial function tests, or lysed.

Cytoplasmic and Nuclear Extraction

After treatment, trophoblast cells were separated into cytoplasmic and nuclear extracts following the protocol accompanying the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Scientific, Rockford, IL #78835). Cells were combined in a microcentrifuge tube with ice cold CER I. Microcentrifuge tubes were vortexed at the highest setting for 15 s, and incubated on ice for 10 min. Next, ice cold CER II was added to the tube and vortexed at its highest setting for 5 s, incubated on ice for 5 min, and vortexed again for 5 s. Tubes were centrifuged at 16,000 x g and 4°C for 5 min. Supernatants were collected into clean, chilled, pre-labeled microcentrifuge tubes and kept on ice. These were the cytoplasmic extracts. Remaining pellets in the old microcentrifuge tubes were re-suspended with ice cold NER, placed on a vortex

at the highest setting for 15 s, and incubated in ice for 10 min. This was repeated 3 times for a total incubation time of 40 min. Microcentrifuge tubes were again centrifuged at 16,000 x g and 4°C for 10 min. Supernatants were collected into clean, chilled, pre-labeled microcentrifuge tubes and kept on ice. These were the nuclear extracts.

Invasion Assay Protocol

To determine real-time invasion of trophoblast cells, an xCELLigence RTCA DP (Real-Time Cell Analysis Dual Plate) instrument from ACEA Biosciences Inc., San Diego, CA, USA was utilized. For this, cell invasion was performed using the 16-well CIM-plate. These plates are composed of an upper and lower chamber, each containing 16 wells. The top wells were coated in a 1:40 matrigel concentration and incubated for 4 hr. Treated and untreated trophoblast cells were plated in the top chamber at a concentration of 20,000 cells/well in 2% FBS RPMI in a total volume of 100 lL. The bottom chamber wells were filled with 160 lL of 10% FBS RPMI. The cells will then be placed in the RTCA DP instrument, and invasion readings will be taken every 15 min for the next 24–50 hr.

Mitochondrial Respiration Protocol

Mitochondrial function was tested as previously shown (Tippetts et al., 2014). High-resolution O_2 consumption was determined at 37°C in permeabilized cells (Sw71) using the Oroboros O2K Oxygraph with MiR05 respiration buffer. First, a baseline respiration was determined. Then, the sample was added into the respiration chambers. The chambers were then hyperoxygenated to ~350 nmol/ml. The substrate-uncoupler-inhibitor-titration (SUIT) protocol (Smith et al., 2013) was followed to determine respiration by parts or all of the following: electron flow through complex I was supported by glutamate + malate (10 and 2 mM, respectively) to determine basal oxygen consumption (GM_B). After stabilization, ADP (2.5 mM)

was added to determine oxidative phosphorylation capacity (GM_P). Succinate was then added (GMS_P) for complex I + II electron flow into the Q-junction. The chemical uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) was added (0.05 μM, followed by 0.025 μM steps until maximal O_2 flux was reached). This determined full electron transport system (F) capacity over oxidative phosphorylation. The Complex II-supported ETS was then measured by inhibiting complex I with rotenone (Rot; 0.5 μM). Adding cytochrome c (10 μM; GMc_P) allowed us to test mitochondrial membrane integrity. Lastly, residual oxygen consumption was measured by adding antimycin A (2.5 μM) to block complex III action. This stopped any electron flow and provided a baseline rate of respiration.

Western Blot Analysis

Western blot analysis was used to determine expression of PKM2, phospho PKM2, and Beta-Catenin in trophoblast cells as previously shown (Arroyo et al., 2009). Cell lysates (50 μg) were separated on 4–12% Bis-Tris gel SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with antibodies against phospho PKM2, total PKM2, and Beta-Catenin (all from Cell Signaling Technology, Danvers, MA). Membranes were then incubated with a secondary horseradish peroxidase (HRP)-conjugated antibody for 1 h at room temperature. The membranes were incubated with ECL substrate, and the emission of light was detected using x-ray film. To determine loading consistencies in cytostolic lysates, each membrane was stripped and reprobed with an antibody against mouse β actin (Sigma Aldrich, St. Louis, MO). To determine loading consistencies in nuclear lysates, each membrane was stripped and reprobed with an antibody against rabbit Lamin B (Sigma Aldrich, St. Louis, MO). Expression levels of the proteins were then quantified by densitometry normalized to β actin

expression or Lamin B and changes in expression compared to the untreated controls were reported.

Statistical Analysis

Results were checked for normality and data are shown as means ± SE. Wilcoxon rank-sums test was used to compare RNA and protein differences between groups, and $P < 0.05$ is considered significant.

Results

Mitochondrial Function in Normal and Complicated Pregnancies

First, we wanted to identify mitochondrial content in normal and complicated pregnancies. We investigated the placental pathologies, PE and IUGR. We performed immunofluorescence on human placental tissues from PE, IUGR, and normal pregnancies staining with Cox IV. We report decreased Cox IV expression in both PE and IUGR tissues compared to the control tissue (Figure 1.1). This supports our hypothesis that mitochondrial function is affected during placental diseases.

Mitochondrial Function in Invasive Trophoblast During Hypoxia

Since mitochondrial function was affected in the placenta during complicated pregnancies, we next wanted to investigate mitochondrial function in vitro in human trophoblast cells. Trophoblast cells were treated in an incubator with 24 hr of hypoxia to induce IUGR conditions. We observed a decrease ($p<0.02$) in mitochondrial respiration in cells treated with hypoxia compared to the control cells in normoxic conditions (Figure 1.2). To further characterize the effects of hypoxia and decreased mitochondrial function on trophoblast cells, we next studied the affect hypoxia has on trophoblast invasion. We found that trophoblast cell

invasion was significantly decreased ($p<0.05$) in those that were treated with hypoxia (Figure 1.3A). In previous studies in our lab, we have shown that mTOR is reduced in hypoxia. The mTOR family of proteins are involved in the metabolism of many cells. To explore the relationship between mTOR and mitochondrial function in trophoblast cells, we first significantly inhibited ($p<0.05$) mTOR expression with Rapamycin (Figure 1.3B). This inhibition results in a significant decrease of trophoblast invasion ($p<0.05$) in trophoblast cells treated with Rapamycin compared to control cells (Figure 1.3C). To further identify the role of metabolism, hypoxia, and invasion of trophoblast cells we next investigated the expression of PKM2 during mTOR inhibition. When mTOR activation was reduced, we observed a significant decrease ($p<0.05$) in PKM2 expression (Figure 1.3D). After observing the connection between mTOR, hypoxia, and PKM2, we wanted to characterize PKM2 expression in trophoblast cells. We performed IHC on trophoblast cells to determine cellular localization of PKM2 in these cells. We observed an overall expression of PKM2 localized to the nucleus of the invasive trophoblast cells (Figure 1.4A). We next investigated the effect hypoxia has directly on PKM2 in trophoblast cells. We found a significant decrease in cytostolic total PKM2 ($p<0.003$) and phospho PKM2 ($p<0.0006$) when cells were treated in hypoxic conditions (Figure 1.4B and 1.4C). Our results also indicated a significant decrease in nuclear total PKM2 ($p<0.003$) and phospho PKM2 ($p<0.0002$) in cells treated with hypoxia (Figure 1.4D and 1.4E). These data supported a role for hypoxia in mitochondrial function, invasion, and the expression of PKM2 in trophoblast cells.

The Specific Role of PKM2 in Mitochondrial Function and Trophoblast Invasion

To investigate the effects of inhibiting PKM2 in trophoblast cells, we used Shikonin, a compound known to inhibit PKM2. We first looked at the effect PKM2 inhibition has on trophoblast invasion. After treating trophoblast cells with Shikonin for 24 hr, we observed a

significant decrease ($p<0.02$) in cell invasion. (Figure 1.5A). We next explored mitochondrial respiration in trophoblast cells where PKM2 was inhibited. Interestingly, we found an increase ($p<0.02$) in mitochondrial respiration in cells treated with Shikonin compared to the control cells, except in baseline respiration (Figure 1.5B). In order to understand more the mechanism by which Shikonin works, we lysed cells treated with Shikonin and looked at PKM2 expression. We observed a significant decrease in nuclear PKM2 ($p<0.03$) and phospho PKM2 ($p<0.04$) compared to control cells (Figure 1.6C and 1.6D). We found no significant changes, however, in cytostolic PKM2 and phospho PKM2 in cells treated with Shikonin compared to control cells (Figure 1.6A and 1.6B).

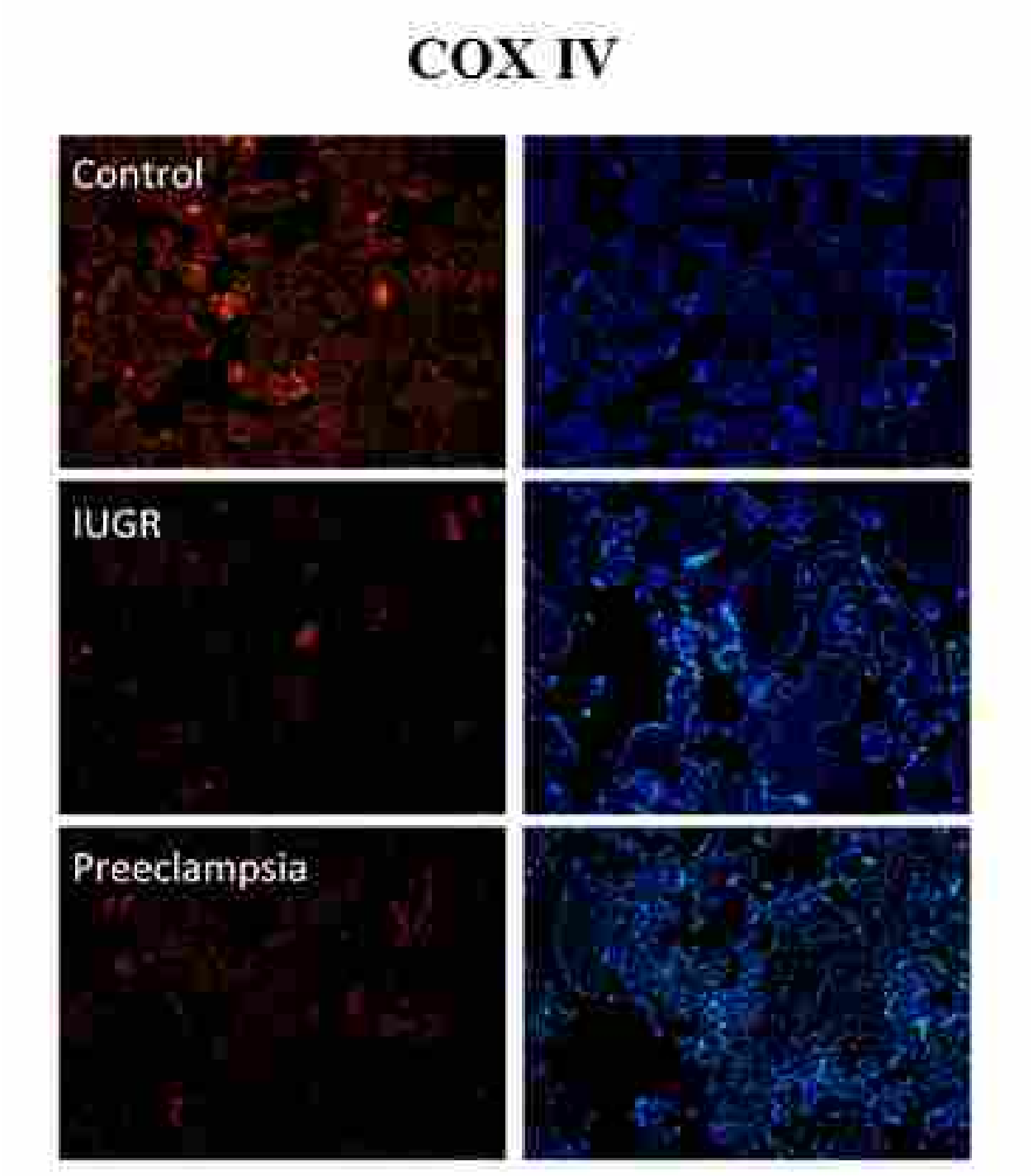

Figure 1.1: Cox IV Expression in Control, IUGR, and Preeclampsia Human Placental Samples. Cox IV staining is significantly decreased in IUGR and Preeclampsia.

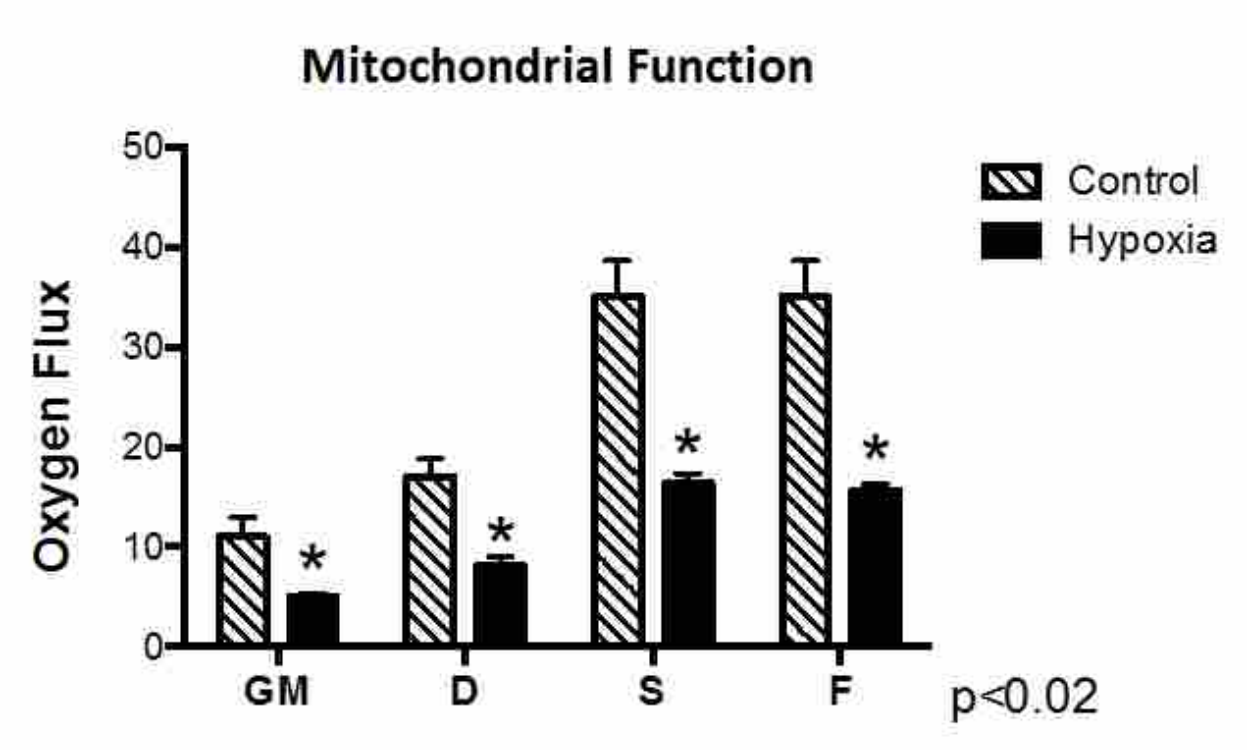

Figure 1.2: Mitochondrial Function in Trophoblast Cells in Hypoxia and Normoxia. Trophoblast cells were treated for 24 hr in the presence or absence of hypoxia. Following treatment, the rate of oxygen consumption was determined using SUIT1 (see the materials and methods section for details). Cells treated with hypoxia had decreased mitochondrial function.

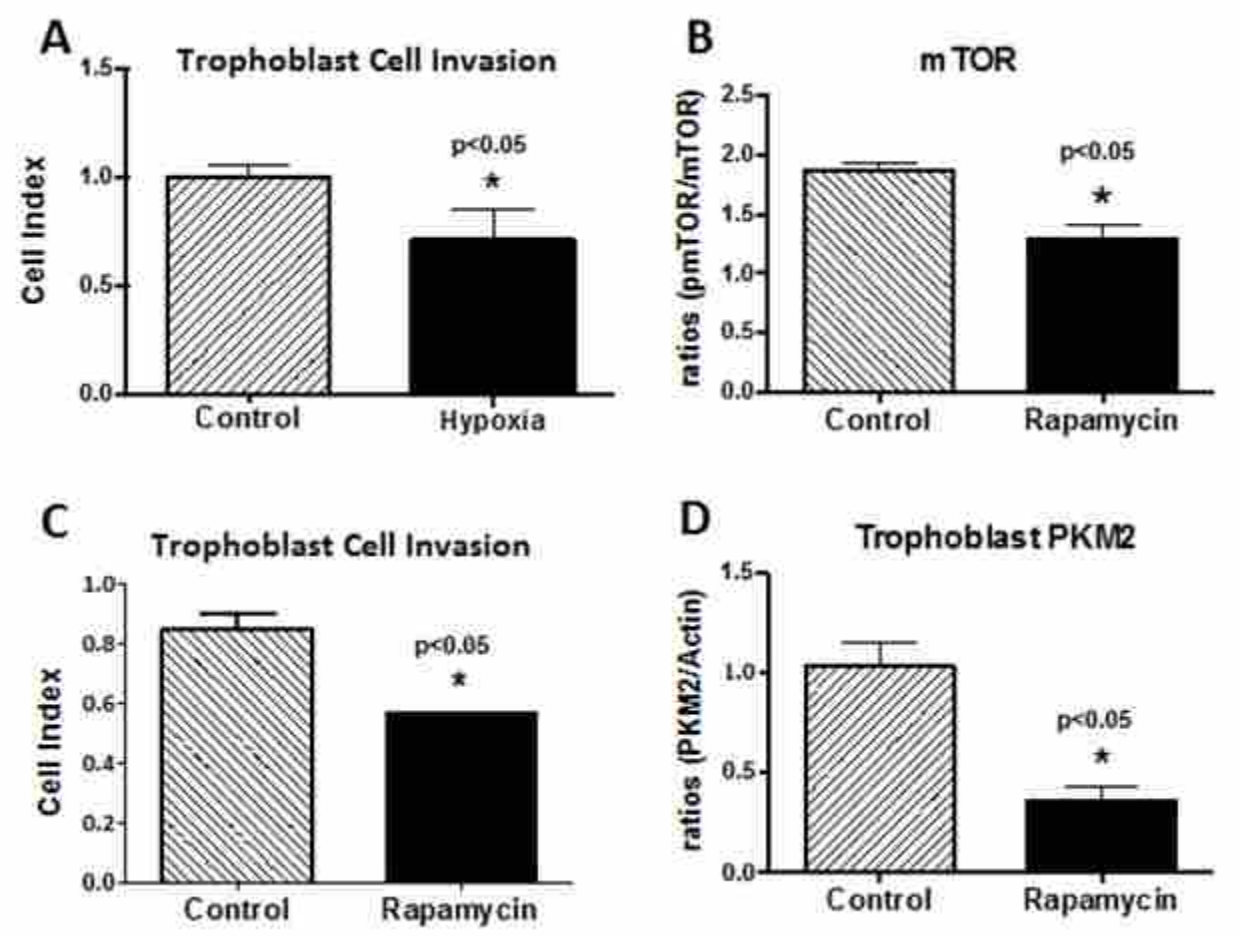

Figure 1.3: Trophoblast Cell Invasion, mTOR, and PKM2. Trophoblast cells show a decreased invasion after treatment with hypoxia (A). mTOR activation was reduced with treatment with Rapamycin (B). This reduction in mTOR correlated with impaired cell invasion in trophoblast cells (C) and a decrease in PKM2 (D).

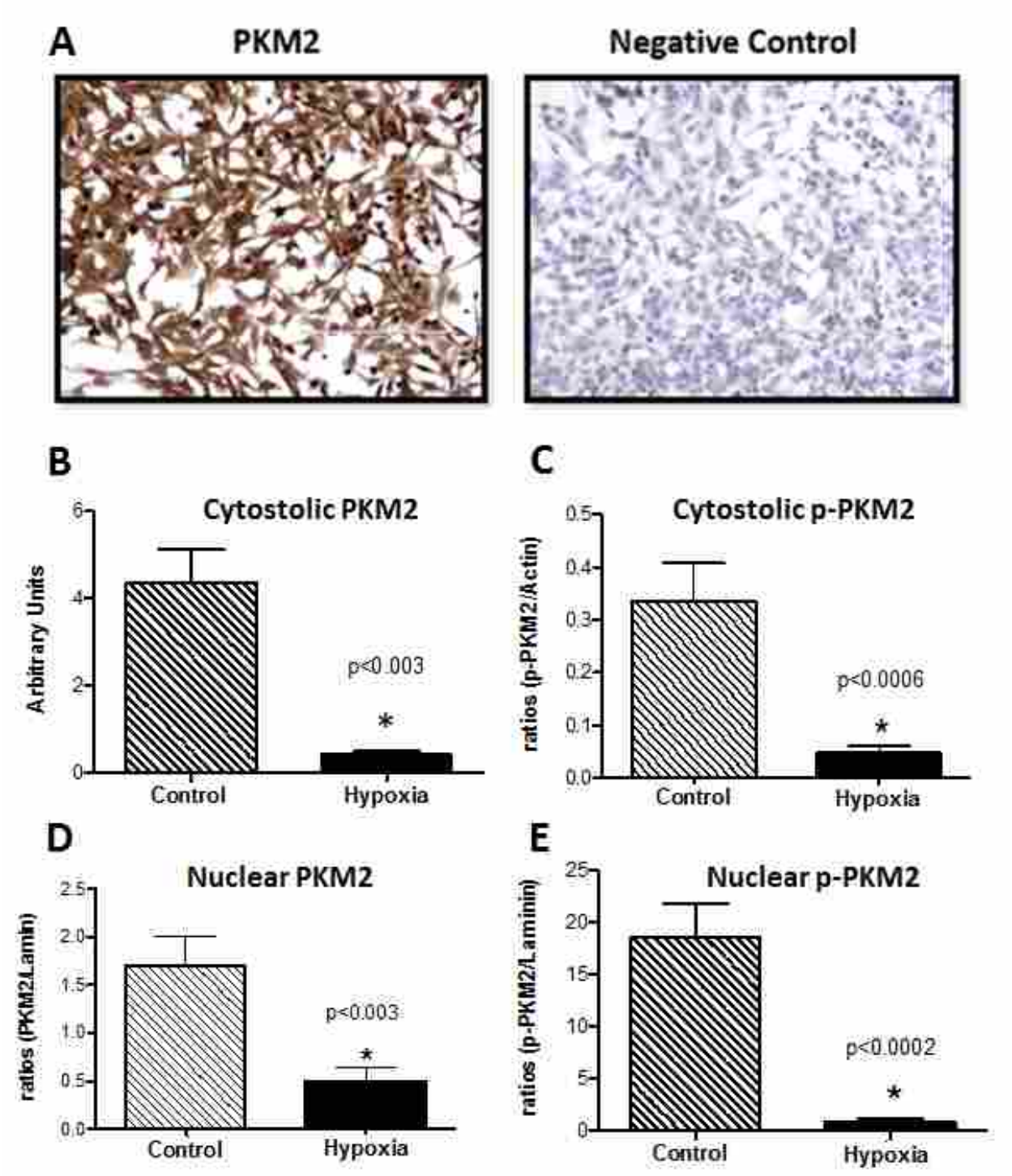

Figure 1.4: Characterization of PKM2 Expression in Trophoblast Cells. IHC showed mostly nuclear expression of PKM2 in the invasive trophoblast cells (A). Western blots showed a decrease in nuclear and cytostolic PKM2 and p-PKM2 expression in the invasive trophoblast cells treated with 24 hr of hypoxia (B-E).

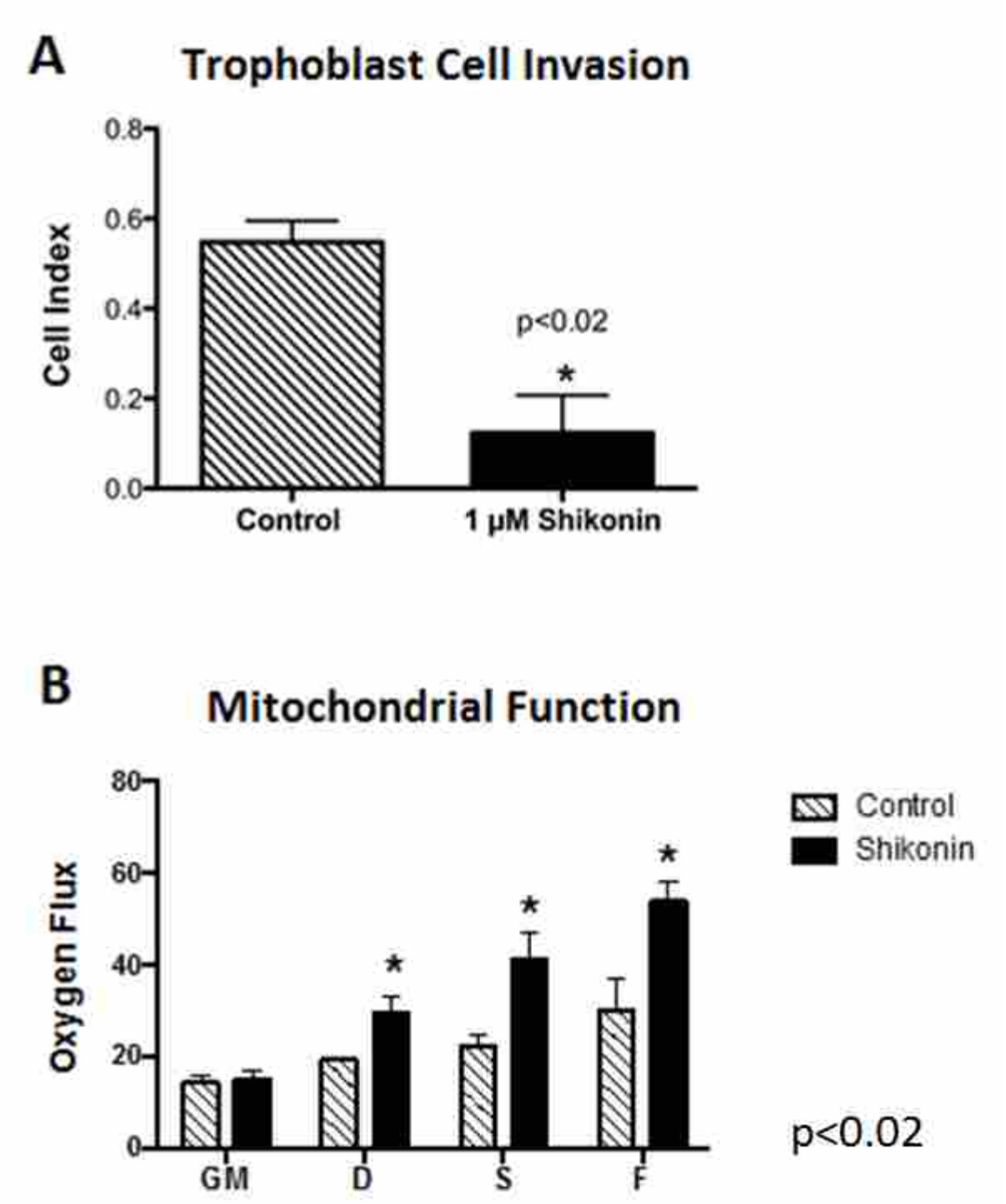

Figure 1.5: Mitochondrial Function and Cell Invasion After Treatment with Shikonin. Trophoblast cells were treated with 24 hr of Shikonin to inhibit PKM2. Following treatment, the rate of oxygen consumption was determined using SUIT1 (see the materials and methods section for details). Cells treated with Shikonin had increased mitochondrial function (A). Trophoblast cell invasion was inhibited with treatment with Shikonin (B).

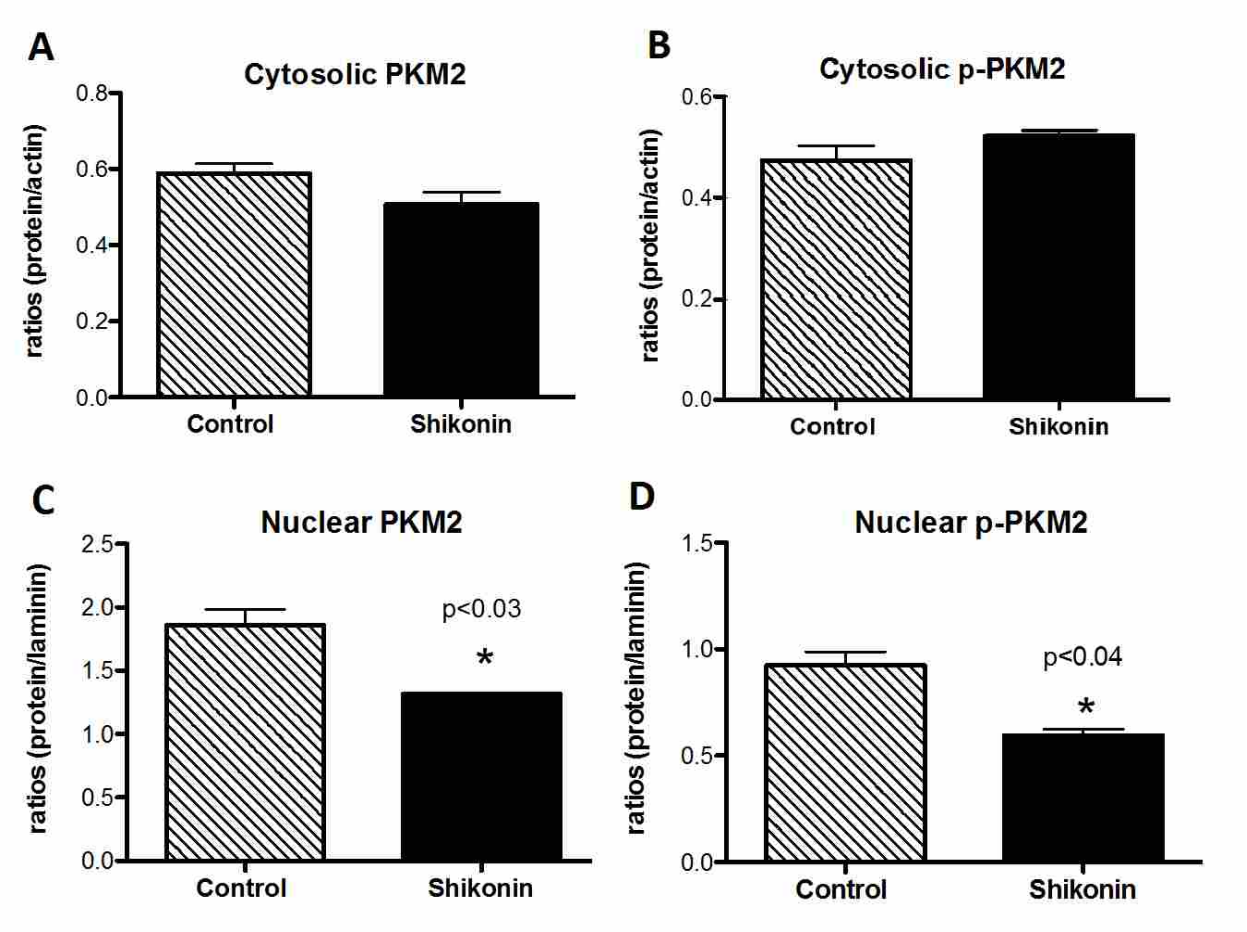

Figure 1.6: Characterization of PKM2 After Treatment with Shikonin. Trophoblast cells were treated with 24 of Shikonin. Treatment had no significant effect on cytostolic PKM2 and p-PKM2 (A and B). Nuclear PKM2 and p-PKM2 were decreased with treatment (C and D).

Discussion

A key characteristic of IUGR is a lack of oxygen getting to the fetus from the placenta (Arroyo et al., 2009). In order to study this disease, we used a hypoxia model similar to previous studies to induce IUGR in trophoblast cells (Kimball et al., 2015). In the cell, oxygen is vital to proper to mitochondria function. It allows the mitochondria to go through oxidative phosphorylation and produce the maximum amount of ATP (Mandò et al., 2014). Knowing this information, our lab wanted to investigate the role of hypoxia in mitochondrial function in the placenta. We first wanted to characterize the mitochondria in human placental tissue in complicated pregnancies. By staining with Cox IV we were able to show a decrease in mitochondrial functioning in PE and IUGR as compared to term control placentas. This confirmed our hypothesis that mitochondria are affected in placental diseases related to a lack of oxygen. This data is also supported by other studies that have shown a decrease in mitochondrial DNA in IUGR placentas (Diaz et al., 2014). To further characterize the affect hypoxia has on mitochondrial functioning and invasion of trophoblast cells, mitochondrial respiration was determined during normoxia or hypoxia treated cells. Our results showed a decrease in respiration in the hypoxia treated cells in every stage of the protocol. This indicates that a lack of oxygen not only decreases mitochondrial content, but also decreases its respiration.

Trophoblast cell invasion in the uterus requires a great deal of energy (Knuth et al., 2015). Because the mitochondria provide the energy for the cell, we wanted to look at the relationship between mitochondrial function and trophoblast invasion. We performed an invasion assay comparing the invasiveness of trophoblast cells treated with hypoxia to those that were treated with normoxia. Our results showed a significant decrease in invasion in hypoxic cells. This established a relationship between a decrease in mitochondrial function and a decrease in

trophoblast invasion. Our laboratory recently published a paper showing evidence that hypoxia decreases the cell growth regulator, mTOR (Kimball et al., 2015). This led us to research the interaction between mTOR and the mitochondria. After inhibiting mTOR, we found a decrease in PKM2, a metabolic enzyme key to glycolysis. We also observed a decrease in trophoblast invasion when mTOR was inhibited. This led us to further investigate PKM2 in trophoblast cells. Through IHC, we were able to see that PKM2 is expressed mainly in the nucleus of trophoblast cells. Next, we wanted to characterize PKM2 in hypoxic conditions. After treating the cells in hypoxia and lysing them, we saw a depletion of both nuclear and cytostolic PKM2 and p-PKM2. This leads us to believe that hypoxia not only inhibits mTOR, but PKM2 as well. These data gave us further evidence connecting hypoxia, trophoblast invasion, and mitochondrial function.

Knowing that PKM2 is expressed in trophoblast cells and that it is reduced in hypoxia, we wanted to explore the effects of inhibiting it in trophoblast cells. We first investigated the effect it has on trophoblast cell invasion. We found that after treating the cells with a known PKM2 inhibitor, Shikonin, cell invasion was decreased. This data gives evidence that PKM2 is a key part of trophoblast cell invasion. It is important to note that recent studies have reported that Shikonin inhibits migration and invasion in glioblastoma cells through inhibiting matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) (Zhang et al., 2015). This data along with our laboratory's data gives us new insight in how Shikonin inhibits invasion. Next, we looked at the effect inhibiting PKM2 had on mitochondrial function. Our results showed that mitochondrial function was actually increased after treating the cells with Shikonin. The mechanism by which Shikonin inhibits PKM2 is unknown. To better characterize this, we treated the cells with Shikonin and lysed them. We found a decrease in nuclear PKM2 and p-PKM2. Cytostolic PKM2 and p-PKM2, however, were not significantly affected. This

gives evidence that Shikonin inhibits PKM2 through the nucleus. Interestingly, cytostolic p-PKM2 was increased with Shikonin, though not significantly. This could provide more insight on why Shikonin increases mitochondrial function. These unexpected results need to be further studied to explain how Shikonin relates to mitochondrial function.

Our results could suggest possible targets to investigate in order to alleviate IUGR. They could provide insight into the pathogenesis of this disease. Additional research of PKM2 and mitochondrial function in the hypoxic trophoblast could provide evidence for a therapeutic treatment for IUGR fetuses.

CHAPTER 2: Hypoxia Reduces Placental mTOR Activation in a Hypoxia-Induced Model of Intrauterine Growth Restriction

Abstract

mTOR is a protein that regulates cell growth in response to altered nutrient and growth factor availability. Our objective was to assess activated mTOR and its intracellular intermediates p70, and 4EBP1 in placental and invasive trophoblast cells in a hypoxia-induced model of IUGR in rats. Rats were treated with hypoxia (9%) for four days. Placental and fetal weights, as well as conceptus numbers were recorded at the time of necropsy. Immunohistochemistry was used to determine the level of trophoblast invasion and apoptosis. Western blots were used to determine the activation of mTOR, p70, and 4EBP1 in the placenta and the uterine mesometrial compartment. We observed: 1) decreased placental (21%) and fetal (24%) weights ($p<0.05$) 2) decreased trophoblast invasion 3) significantly increased active 4EBP1 (28%; $p<0.05$) in invasive trophoblast cells yet no changes in the activation of mTOR and p70 proteins, and 4) a significant decrease in the activation of mTOR (48%; $p<0.05$) with no differences in p70 or 4EBP1 activation in the placenta. We conclude that the development of IUGR is correlated with decreased activation of the mTOR protein in the placenta and increased 4EBP1 activity in the invading trophoblast. These results provide important insight into the physiological relevance of these pathways. Furthermore, modification of these and other related targets during gestation may alleviate IUGR severity.

Introduction

Intrauterine growth restriction (IUGR) is an obstetric complication linked to an increased risk of morbidity and mortality for the fetus. This disease is characterized by low birth weight (below the 10th percentile) and it affects up to 10% of all pregnancies (Bahr et al.). IUGR

complications include perinatal hypoxia and asphyxia, neurological delays, and persistent pulmonary hypertension for the newborn (Brar and Rutherford, 1988; Gray et al., 1999; Pollack and Divon, 1992). This complication has also been linked to the adult onset of diabetes, hypertension, stroke, death from coronary vascular diseases, and can lead to Preterm birth (Arroyo et al., 2008). Placental insufficiency is the most common cause of IUGR. Placentae in growth-restricted pregnancies are pathologically characterized by reduced syncytiotrophoblast surface area, decreased trophoblast invasion, and increased placental trophoblast apoptosis (Ishihara et al., 2002; Krebs et al., 1996; Matheson et al., 2015). Hypoxia has been thought to play an important role during IUGR. It has been shown that induced transient uteroplacental hypoxia causes significant IUGR suggesting a role localized hypoxia during IUGR (Tanaka et al., 1994). More recently, a study had shown that exposing rodents to hypoxia induced fetal weight reduction and IUGR, and induces metabolic and cardiovascular disturbances in adulthood, confirming an important role for hypoxia during this disease (Bourque et al., 2012; Giussani and Davidge, 2013; Iqbal and Ciriello, 2013; Jang et al., 2015; Matheson et al., 2015; Myatt, 2006; Rueda-Clausen et al., 2014). In-vitro studies have demonstrated that hypoxia can affect the growth and differentiation of trophoblast cells, suggesting a role for hypoxia in trophoblast functioning and behavior (Caniggia and Winter, 2002; Genbacev et al., 1997; Jiang et al., 2000; Nelson et al., 1999). To better study the molecular pathobiochemistry of IUGR, we chose to use a maternal hypoxia-induced IUGR model in the rat. This model provides an essential tool to study trophoblast interactions in the uterine compartment, mTOR signaling, and apoptosis during hemochorial placentation. The rat uterine mesometrial compartment is the part of the uterus in which the blood vessel enters and models both endovascular and interstitial

trophoblast invasion suggesting that, similar to humans, there is a specific function for trophoblast invasion during pregnancy in the rat (Soares et al., 2012).

The mammalian target of rapamycin (mTOR) protein regulates cell growth in response to the availability of nutrients and growth factors (Jansson et al., 2006; Wullschleger et al., 2006). Signaling intermediates of the mTOR pathway include the 70-kDa ribosomal protein S6kinase 1 (p70S6K) protein and the eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) proteins. Activation of these proteins regulates transcriptional control of target genes and protein synthesis necessary in the control of diverse response pathways (Arroyo et al., 2009). Studies have showed that the mTOR protein is increased during IUGR, whereas placental phospho (p)-p70S6K protein is downregulated (Arroyo et al., 2009; Roos et al., 2007). Such differential responses suggest alternative mechanisms for these proteins in the placenta during IUGR that has not yet been adequately defined. Our laboratory has shown a direct correlation between mTOR protein activation and trophoblast invasion, suggesting a central role for this pathway in the regulation of trophoblast invasion during IUGR (Knuth et al., 2015). We hypothesized that maternal hypoxia will induce IUGR by regulating proteins associated with the mTOR pathway. In the present study, we characterized the family of signaling proteins regulated by mTOR in the placenta and in the uterine mesometrial compartment during a hypoxia-induced IUGR.

Materials and Methods

Animals and Tissue Preparation

A total of fourteen weight-matched (~400g) Holtzman Sprague-Dawley rats (HSD) were used for this study, which was approved by the Brigham Young University Animal Care and Use Committee (IACUC). To obtain timed pregnancies, females were caged with HSD males overnight. The presence of sperm in a vaginal smear was designated as day 0.5 of pregnancy.

Placentae and uterine mesometrial compartments were dissected from pregnant rats at the time of necropsy (18.5 days of gestation; dGA). Dams, placentae and fetuses were weighed (for each an average of 5 per litter) and tissues were snap frozen in liquid Nitrogen for RNA and protein analysis. For immunohistochemistry (IHC) analysis, whole concepti were frozen in dry ice-cooled heptane. All tissue samples were stored at -80°C until used.

Environmental Chamber and Hypoxia Exposure

To induce IUGR, pregnant rats were placed in a hypoxic chamber (n=7) for four days (14.5 dGA to 17.5 dGA). The treatment consisted of animal treated with hypoxic conditions of 9% O_2. Exposed animals were labeled HX (n=7; maternal weight 426g ±12) and control (n=7; maternal weight 393g ±7) were maintained at 21% O_2. Animals were euthanized (CO_2 asphyxiation and cervical dislocation) at day 18 dGA when necropsies were performed.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on frozen whole conceptus sections as previously performed in our laboratory (Arroyo et al., 2009). In summary, slides were blocked with Sniper (Biocare Medical, Concord, CA) and incubated for one hour with a rabbit polyclonal primary antibody against Cytokeratin 7 (CK7; Dako, Carpinteria, CA) for trophoblast localization, anti- cleaved (active) caspase 3 (rabbit, Cell Signaling, Danvers, MA) antibody to assess apoptosis, anti phospho mTOR (Abcam, Cambridge, MA), anti phospho 4EBP1 (Abcam, Cambridge, MA) or with a universal IgG negative control (Biocare Medical; Concord, CA). Slides were incubated with Mach 2 universal stain polymer (Biocare Medical, Concord, CA) followed by color development with diaminobenzidine (DAB; brown color). Hematoxylin was used for nuclear counterstaining.

RNA Isolation

RNA was isolated using the Tri-reagent method (Sigma, Saint Louis, MO) as suggested by the manufacturer. Briefly, placenta and uterine mesometrial compartment frozen tissues (100 mg) were homogenized in 1 ml of Tri-reagent, and choloroform was added. The supernatant was transferred to a clean tube prior to the addition of cold isopropanol. Pellets were visualized and washed in 75% DEPC/EtOH solution and allowed to air dry for 10 min. Pellets were then resuspended in 50 µl of DEPC water and RNA was quantified using a Nanodrop.

Real Time PCR (RT-PCR)

Real time PCR was performed to determine the activation of the mTOR and mTOR signaling associated genes. cDNA was synthesized using Oligo (dT) and SuperScript II Reverse Transcriptase (both from Invitrogen by Life Technologies, Carlsbad, CA) by following the protocol suggested by the manufacturer. RT-PCR was performed using SsoFast EvaGreen Supermix (Bio-rad Laboratories, Hercules, CA), which contains a cocktail of all necessary components excluding primers and templates. Sso7d-fusion polymerase was used as the enzyme. Primers for rat mTOR (Fwd.-ACCAATTATACTCGCTCCCTG, Rev.-GTCATAGCAACCTCAAAGCA), p70 (Fwd.-CAGAGCGGAATATTCTGGAG, Rev. -CATAAATAGTTCTCCTCCACTGAG), and 4EBP1 (FWD.- GATGAGCCTCCCATGCAG, Rev.- CCATCTCAAACTGTGACTCTTCA) were utilized with 18S primers (Fwd.-GGGAGGTAGTGACGAAAAATAACAAT, REV.-CCCTCCAATGGATCCTCGTT) as a baseline control for the various experiments. Results (ΔΔ CT) were tested for significance against control animal tissues. Cycling conditions were as follows: 95°C for 30 seconds; 95°C for 5 seconds; 60°C for 30 seconds; melt curve, 65°C for 2 seconds and 95°C for 5 seconds. An

mTOR PCR array (PARN-098Z; Qiagen, Valencia, CA) was performed to identify other genes related to mTOR signaling potentially affected by hypoxia.

Western Blot Analysis

Western blot analysis was used to determine expression of the mTOR family of proteins in the placenta and uterine mesometrial compartment of control and treated animals as previously shown (Arroyo et al., 2009). Cell lysates (50 μg) were separated on 4–12% Bis-Tris gel SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with antibodies against phospho-mTOR (Ser2448), total mTOR, phospho- p70 S6 kinase (SK6) (Thr389), total $p70^{SK6}$, phospho-4EBP1 (Thr37/46), and total 4EBP1 (all from Cell Signaling Technology, Danvers, MA, excluding total p70 from Epitomics, Burlingame, CA). Membranes were then incubated with a secondary horseradish peroxidase (HRP)-conjugated antibody for one hour at room temperature. The membranes were incubated with ECL substrate, and the emission of light was detected using x-ray film. To determine loading consistencies, each membrane was stripped and reprobed with an antibody against mouse beta-actin (Sigma Aldrich, St. Louis, MO). Expression levels of the proteins were quantified by densitometry normalized to β actin expression and changes in expression compared to the untreated controls were reported.

Statistical Analysis

Results were checked for normality and data are shown as means ± SE. Wilcoxon rank-sums test was used to compare RNA and proteins differences between groups and $p < 0.05$ was considered significant.

Results

Fetal and Placental Weights

IUGR is characterized by decreased fetal and placental weight; therefore, we first investigated the effects of maternal hypoxia treatment on placental and fetal weights during pregnancy. Studies were performed exposing pregnant animals from a rage of 8 to 10% O2 conditions (data not shown). Exposing animals to 9% O2 was chosen as this was the lowest oxygen level treatment with no significant effects in viable concepti numbers as compared to controls (Figure 2.1A). We found a 1.3-fold reduction in fetal weight ($p<0.003$) with a 1.2-fold reduction in placental weight ($p<0.002$) in rats exposed to hypoxia at the time of necropsy (Figure 2.1B). These data supported a role for maternal hypoxia in fetal and placental weight deviations in this model of IUGR.

Trophoblast Invasion and Apoptosis

Shallow invasion of the trophoblast and increased placental apoptosis are hallmarks of IUGR. We accordingly investigated trophoblast invasion and apoptosis in the placenta during maternal -induced IUGR. Cytokeratin 7 (CK7) was used to identify the localization of trophoblast cells in the placental villi. CK7 IHC showed decreased invasion of trophoblast cells into the uterine mesometrial compartment in hypoxia-exposed animals compared to controls (Figure 2.2A top panels). We next investigated whether apoptosis of the invading trophoblast cells was affected by hypoxia exposure. To achieve this, we immunostained for cleaved (active) caspase 3, a protein implicated in apoptosis. Hypoxia treatment showed an increased active caspase 3 staining in the invading trophoblast cells compared to controls (Figure 2.2A bottom panels). Immunoblotting for active Caspase 3 was performed in the placenta to semi quantitatively determine Caspase 3-mediated apoptosis. We observed a 1.5-fold ($p<0.05$)

increase in placental active Caspase 3 in treated animals when compared to controls (Figure 2.2B). Our results suggested that hypoxia is likely involved in decreased trophoblast invasion and increased apoptosis observed in IUGR.

mTOR Family of Proteins in the Placenta and Uterine Mesometrial Compartment

To precisely clarify mTOR gene expression patterns during hypoxia-induced IUGR, we performed real time PCR using RNA isolated from the uterine mesometrial compartment. We observed a significant increase (2.2-fold; $p<0.05$) in the expression of active mTOR, p70, and 4EBP1 (Figure 2.3A) in the hypoxia group when compared to controls. An evaluation of mTOR related genes led to the finding that vascular endothelial growth factor A (VEGF-A) was decreased (1.7-fold; $p<0.05$) while significant increases were observed for the mRNA of the protein phosphatase, regulatory subunit B (Ppp2r2b; 4.0-fold, $p<0.05$) and the protein kinase, AMP-activated, gamma 3 non-catalytic subunit (Prkag3; 1.8-fold, $p<0.05$) in the uterine mesometrial compartment of hypoxia-exposed rats (Figure 2.3A). We next investigated placental mTOR family gene expression. We observed increased mTOR mRNA (1.8%; $p<0.05$) in exposed animals compared to controls (Figure 2.3B). There were no significant changes in the expression of active p70 or 4EBP1 in exposed animals compared to controls (Figure 2.3B). PCR array of the mTOR related genes revealed increased insulin receptor substrate 1 (Irs1; 3.7-fold, $p<0.05$), phosphoinositide-3-kinase, regulatory subunit 1 (alpha) (Pik3r1; 1.3-fold, $p<0.05$), protein phosphatase regulatory subunit B (Ppp2r2b; 2.0-fold, $p<0.05$), protein kinase, AMP-activated, gamma 3 non-catalytic subunit (Prkag3; 2.6-fold, $p<0.05$), ribosomal protein S6 kinase polypeptide 2 (Rps6ka2; 1.5-fold, $p<0.05$) and the vascular endothelial growth factor C (VEGF-C; 1.8-fold, $p<0.05$) genes (Figure 2.3B). Only the protein kinase, AMP-activated, gamma 2 non-catalytic subunit (Prkag2) gene was decreased (1.8-fold, $p<0.05$) in the placenta during

maternal hypoxia treatment (Figure 2.3B). To determine the protein changes of the mTOR related proteins western blot was performed. Figure 2.4 shows a characteristic western blot for the phospho and total mTOR, p70 and 4EBP1 in the uterine mesometrial compartment (Figure 2.4A) and the placenta (Figure 2.4B) of treated animals as compared to control. We first investigated the protein levels of mTOR, p70, and 4EBP1 in the uterine mesometrial compartment. Immunoblotting demonstrated a significant increase in 4EBP1 activation (1.8-fold; $p<0.05$) in treated animals compared to controls (Figure 2.5C). IHC was performed to confirm that 4EBP1 was spatially expressed in the invading trophoblast of the uterine mesometrial compartment. Phospho 4EBP1 IHC was increased (Figure 2.5D, top panels) in the uterine mesometrial compartment of treated animals when compared to controls. This activation was present in invasive trophoblast cells (Figure 2.5D, bottom panels). There were no significant differences in the expression of active mTOR or p70 between treated and control animals (Figures 2.5A, and 2.5B). No changes in the expression of these markers suggested plausible posttranscriptional regulation of mTOR and p70 in the uterine mesometrial compartment in hypoxia-induced models of IUGR.

In the placenta, expression of active mTOR ptotein was significantly decreased (1.9-fold; $p<0.05$) in exposed animals compared to controls (Figure 2.6A). However, there was no difference in the expression of p70 and 4EBP1 proteins (Figure 2.6B and 2.6C) between the groups. Phospho mTOR IHC was performed in the placenta to determine if decreased active mTOR localized to trophoblast cells. Phospho mTOR staining showed that decreased mTOR protein expression was significantly localized to the Junctional Zone (JZ) of the placenta where robust endocrine cells are located. The JZ is also where invasive trophoblast cells are suggested to originate (Figure 2.6D) (Ain et al., 2005). Ck7 IHC was performed to confirm the identity of

the trophoblast cells present in the JZ. These compelling data suggested a function for placental mTOR activation in the development of IUGR during hypoxia.

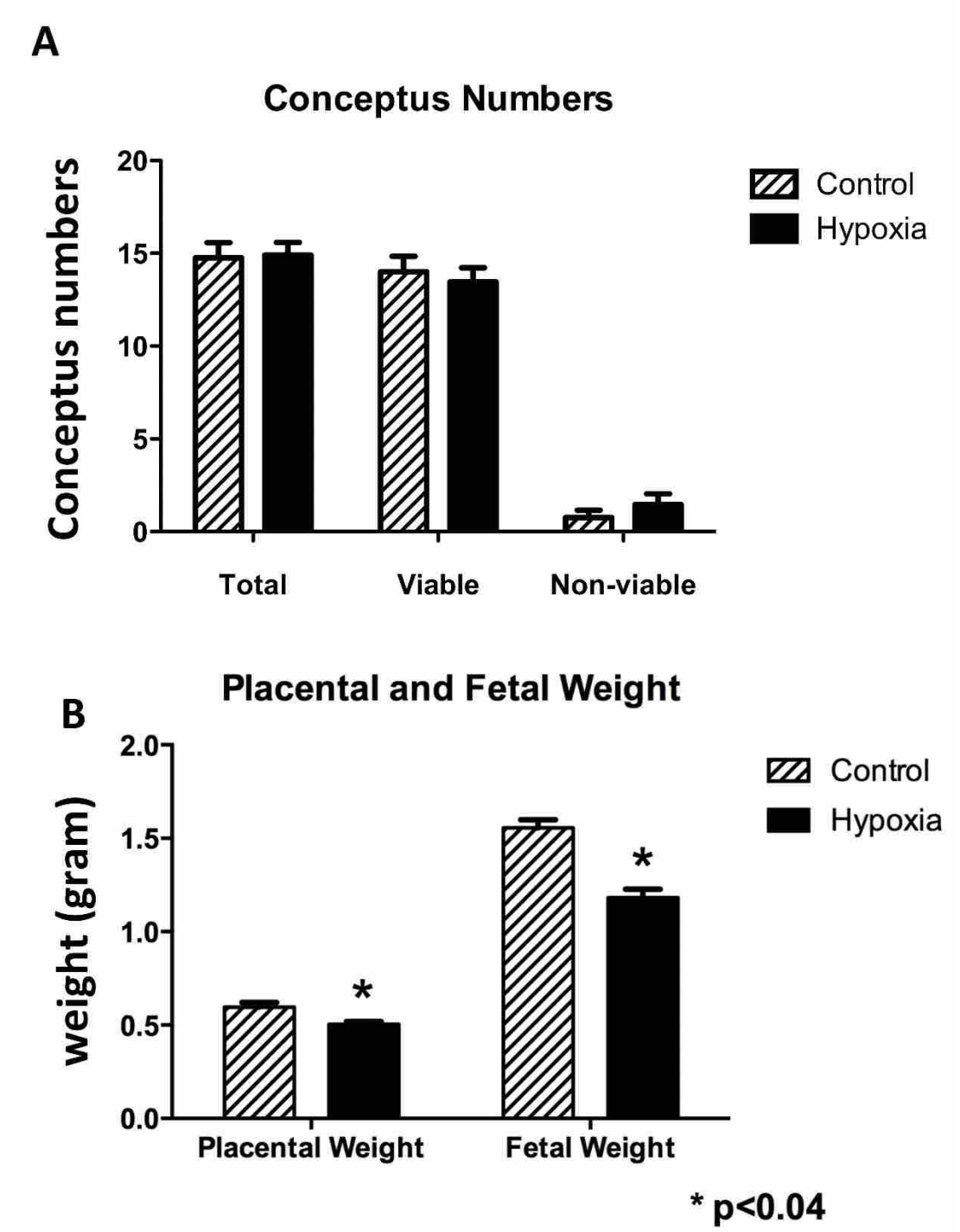

Figure 2.1: Placental and Fetal Weight Differences During Maternal Hypoxia Treatment in the Rat. A significant decrease in placental (1.2-fold; $p< 0.002$) and fetal weights (1.3-fold; $p< 0.003$) was observed in hypoxia (9% 0_2) treated animals as compared to controls (21%O_2) (A). There were no significant differences in viable and non-viable fetuses between treated and control animals (B).

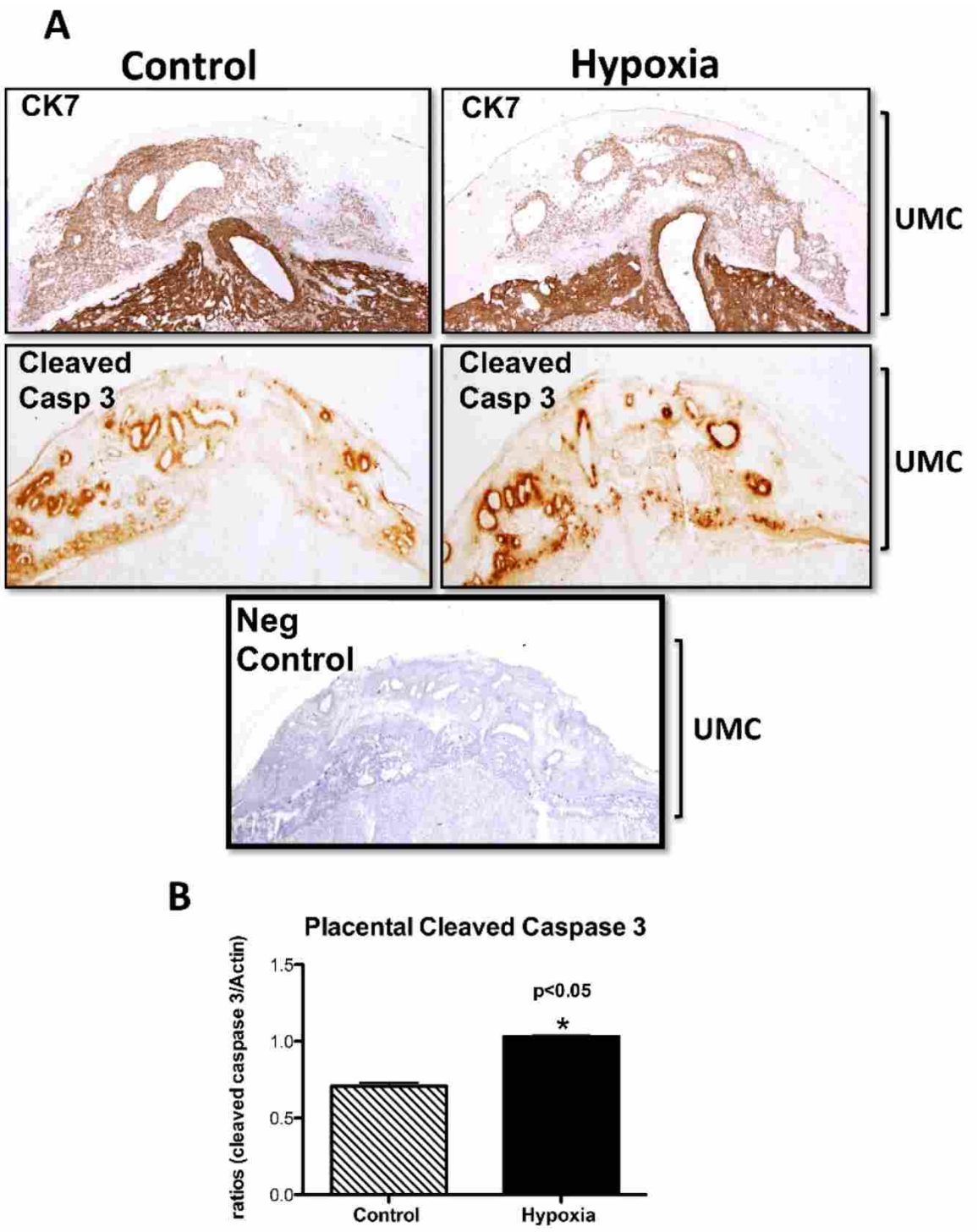

Figure 2.2: Trophoblast Invasion and Apoptosis During Hypoxia Treatment in the Rat. CK7 IHC showed decreased trophoblast invasion into the uterine mesometrial compartment (UMC) of treated animals as compared controls. Active caspase 3 IHC demonstrated increased apoptosis in invasive trophoblasts in the UMC of the treated animals when compared to controls (A). Active caspase 3 was increased with maternal hypoxia in the placenta of treated animals as compared to controls (B). * identifies $p \leq 0.05$.

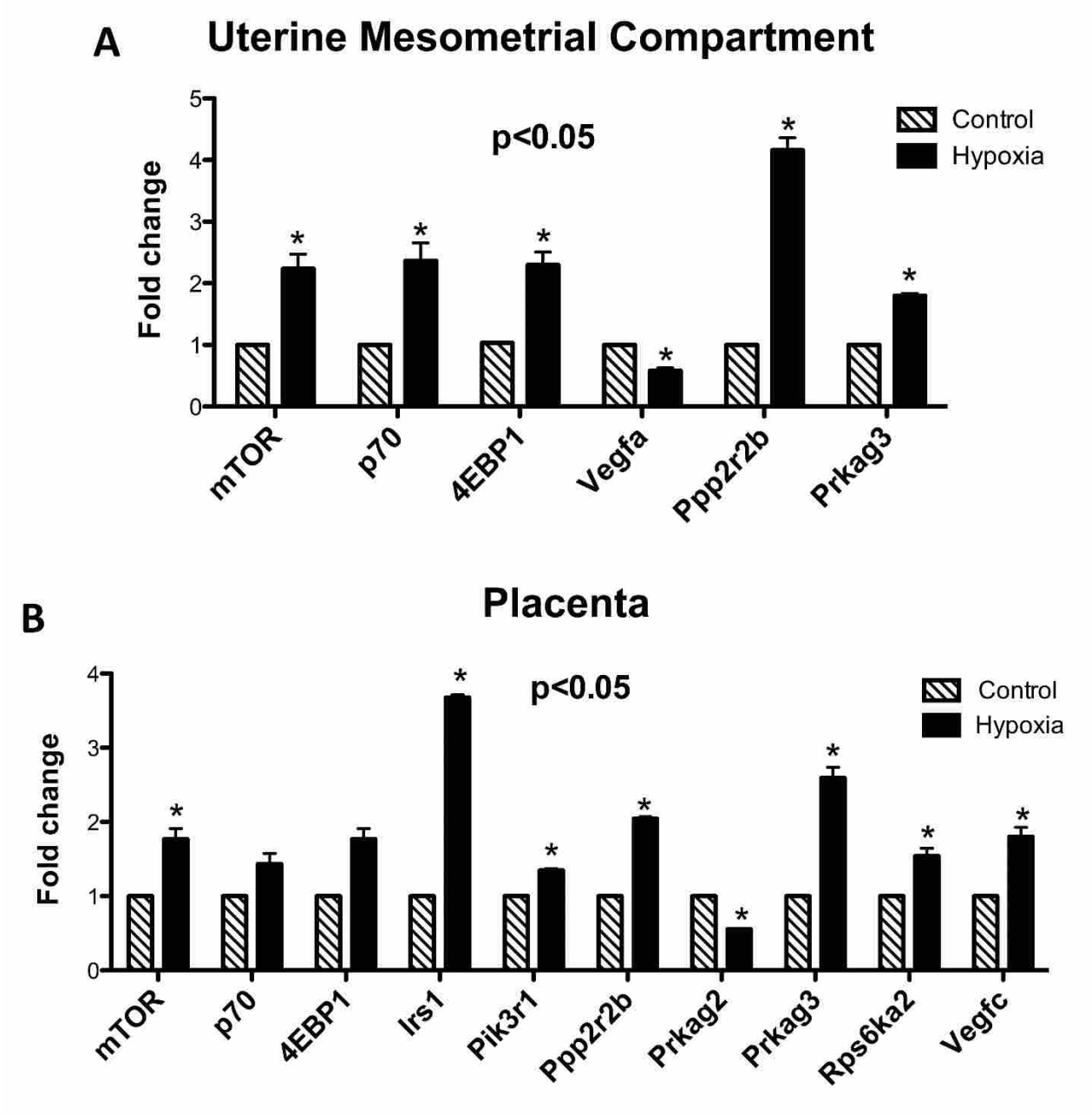

Figure 2.3: Uterine Mesometrial Compartment and Placental mTOR Gene Activation During Hypoxia in the Rat. mTOR, p70, 4EBP1, Ppp2r2b and Prkag3 genes were significantly induced in the uterine mesometrial compartment of the hypoxia treated animals when compared to controls (A). In the placenta, hypoxia induced a significant increase of mTOR, Irs1, Pik3r1, Ppp2r2b, Prkag3, Rps6ka2 and VEGF-C and a significant decrease of Prkag2 in the treated animals when compared to controls (B).

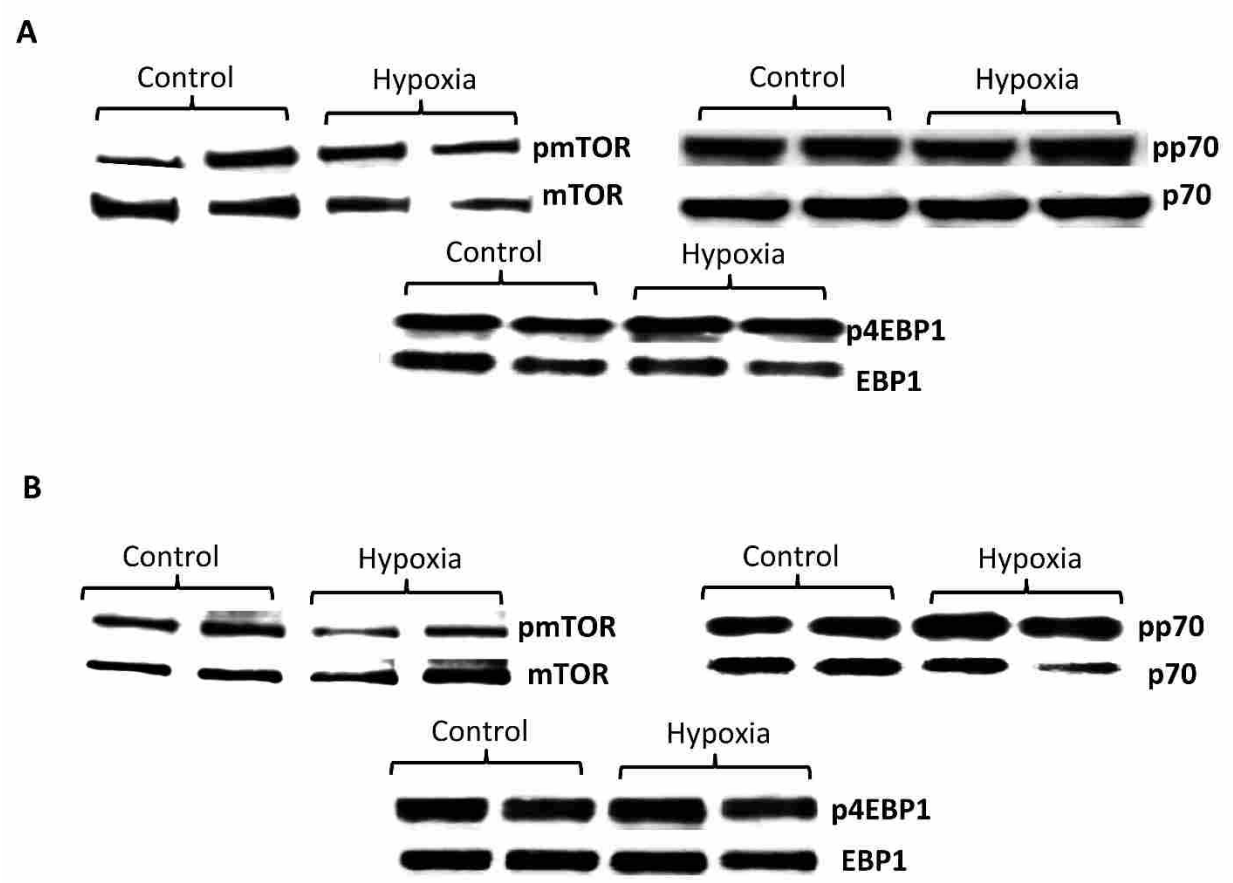

Figure 2.4: Characteristic Western Blot. mTOR, p70 and 4EBP1 proteins in the Uterine mesometrial compartment (A) and Placental (B) of control and treated animals.

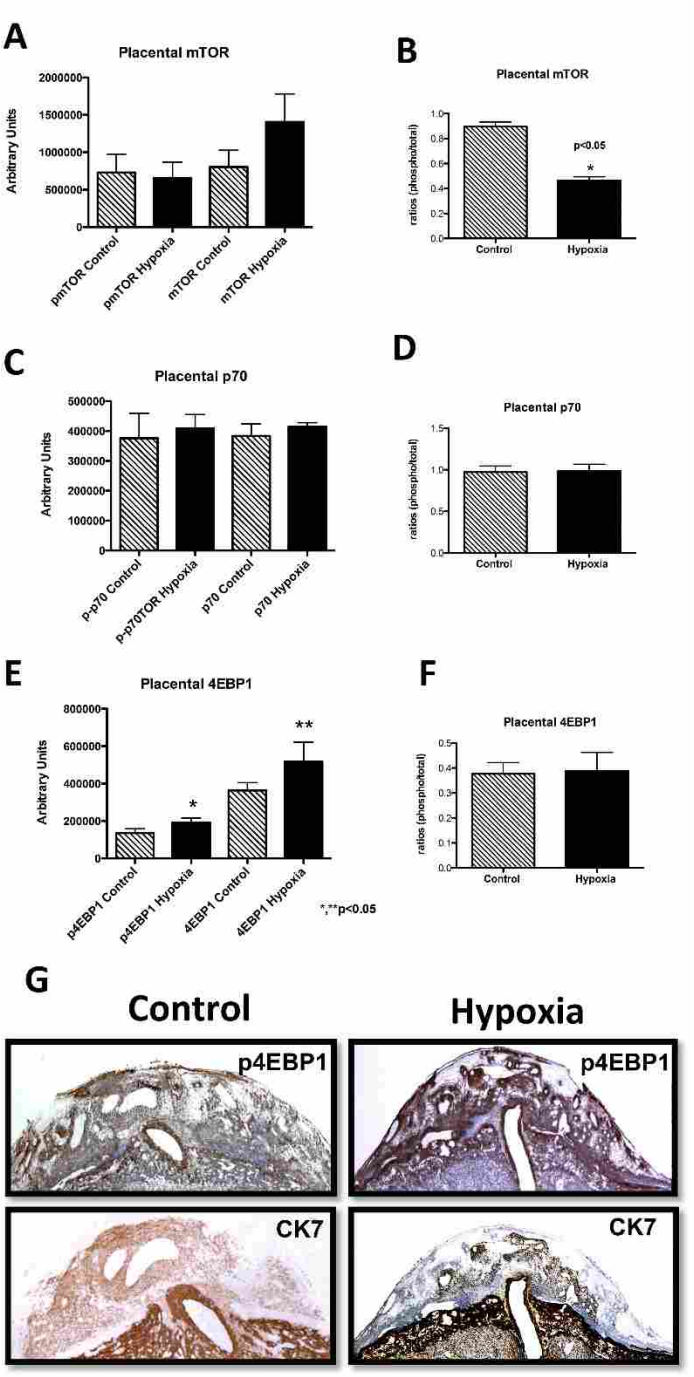

Figure 2.5: Activation of mTOR, p70 and 4EBP1 Proteins in the Uterine Mesometrial Compartment During Hypoxia Treatment in the Rat. There were no differences in mTOR (A) or p70 (B) activation in the uterine mesometrial compartment during maternal hypoxia treatment. In contrast, there was a significant increase in 4EBP1 activation (1.8-fold; $p<0.05$) in the uterine mesometrial compartment of treated rats when compared to controls. (C) 4EBP1 activation was localized to the invasive trophoblast of the uterine mesometrial compartment (UCM) of the hypoxia treated animals.

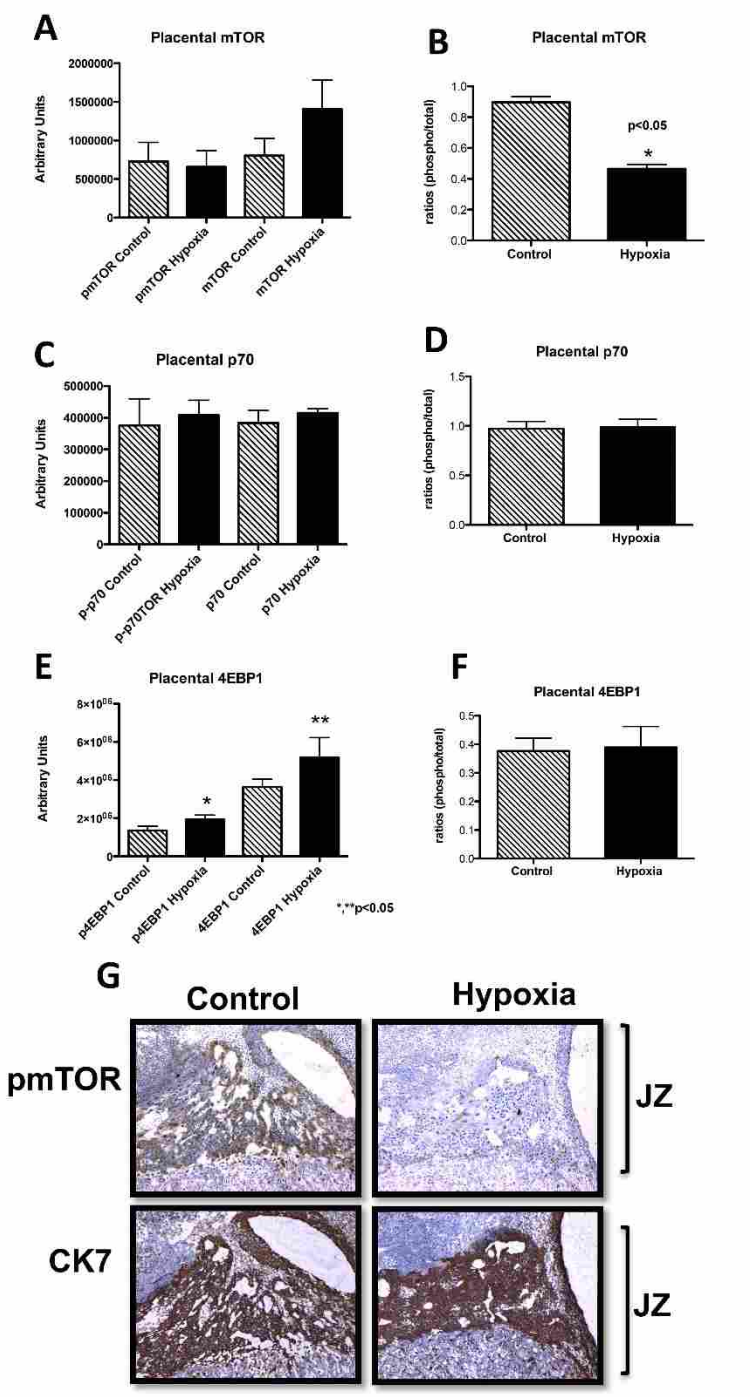

Figure 2.6: Activation of mTOR, p70 and 4EBP1 Proteins in Placenta During Hypoxia Treatment in the Rat. There was a significant hypoxia-induced decrease in mTOR protein activation (1.9-fold; $p<0.05$) (A). In contrast, there were no significant differences in p70 (B) or 4EBP1 (C) in the placenta of treated rats when compared to controls. Decreased mTOR activation was localized to the trophoblast in the Junctional Zone (JZ) of the placenta of the hypoxia treated animals when compared to controls.

Discussion

Hypoxia is known to be associated with the development of IUGR (Tapanainen et al., 1994). We have chosen a hypoxia-induced IUGR model in the rat in order to study molecular signaling pathways potentially involved in the progression of IUGR. *In vivo* maternal hypoxia treatment induced significantly decreased fetal and placental weight, decreased trophoblast invasion, and elevated apoptosis, all characteristics of IUGR. This treatment did not affect the conceptus number in the treated animals compared to the control animals. These observations support the fact that hypoxia treatment is a factor sufficient to induce IUGR characteristics in pregnant rats during hemochorial placentation. It is important to mention that previous publications have shown a possible sex induced differences for hypoxia treated offspring (Giussani and Davidge, 2013; Matheson et al., 2015; Reyes et al., 2015; Shah et al., 2015). A limitation of our study is the fact that fetal sex was not differentiated at the time of necropsy but the decreased in fetal weights were obtained from fetuses obtained from the two uterine horns of each pregnancy.

Because IUGR pregnancies are characterized by decreased nutrients delivered to the fetus, we next investigated the mTOR family of proteins. The mTOR pathway of proteins is known to regulate cell growth in response to nutrients and growth factors (Blume-Jensen and Hunter, 2001; Jansson et al., 2006; Wullschleger et al., 2006). Previous research has identified plausible roles for mTOR in IUGR; however, no definitive studies have been performed that evaluate its family members in the low oxygen tension placenta. In the present study, we investigated the mTOR pathway in both the placenta and uterine mesometrial compartment in normal and hypoxia-induced IUGR pregnancies. The uterine mesometrial compartment showed an increase in mTOR, p70, and 4EBP1 mRNA expression suggesting IUGR from hypoxia

treated animals is mediated, at least in part, by the activation of the mTOR family of genes in the uterine mesometrial compartment. Interestingly, we also observed increased expression of the Ppp2r2b and Prkag3 genes. These are both involved in the negative regulation of mTOR (Qian et al., 2015). Ppp2r2b activation is known to be involved in the negative control of cell growth and division. It is also involved in the downregulation of mTOR activation (Qian et al., 2015). Prkag3 is a regulatory subunit of the AMP-activated protein kinase (AMPK) and it plays a role in the regulation of energy metabolism in cells (Milan et al., 2000). AMPK regulates ATP synthesis and is known to inhibit mTOR activation (Rehman et al., 2014). In contrast, we observed decreased expression of VEGF-A with hypoxia treatment. VEGF-A is involved in vasculogenesis and angiogenesis and can act as a potent vasodilator in stable vasculature (Eriksson et al., 2002). Our results convey the concept that although maternal hypoxia activates the mTOR family of proteins, it also activates negative regulators of the mTOR signaling pathway and inactivates VEGF-A, a common positive regulator of mTOR (Trinh et al., 2009). Interestingly, we only observed a significant increase in active 4EBP1 protein in the invading trophoblast and not increased mTOR or p70. Such a discrepancy in mRNA and protein suggests possible posttranscriptional regulation for mTOR and p70, as previously postulated by others (Lechuga et al., 2004; Mura et al., 2014). Such regulation may perhaps include the activation of the Ppp2r2b and Prkag3 genes. Although the mTOR mRNA was upregulated, decreased active protein expression could lead to the overall condition of a poorly developed and immature fetus. It is possible that mesometrial tissues attempt to compensate for hypoxia by activating 4EBP1 protein, but such a response fails to activate this anabolic pathway.

In the placenta, however, we observed a much different response. Only mTOR mRNA was increased in the placenta. We also observed an upregulation in the mTOR positive regulators

Irs1, Pik3r1, Rps6ka2 and VEGF-C while there was a downregulation in the mTOR negative regulator Prkg2. Consistent with the observation in the uterine mesometrial compartment; we observed an upregulation of the Prkag3 gene. Both Irs1 and Pik3r1 are involved in insulin cellular processes (Pollack and Divon, 1992; Wullschleger et al., 2006). Irs1 participates in the transduction of signaling initiated at the activation of insulin receptors and is required for cellular metabolism (Copps and White, 2012). Pik3r1 is involved in the insulin-mediated increase of glucose uptake (Miled et al., 2007). IUGR is characterized with decreased amino acids and growth factors, which can affect placental metabolism. Therefore, the activation of Irs1 and Pik3r1 is in accordance with a variety of mechanisms related to fetal metabolic adaptations. For example, increasing peripheral insulin sensitivity for glucose utilization and decreasing insulin sensitivity for protein synthesis during IUGR are plausible maternal responses in an attempt to ensure fetal survival during these low nutrients conditions (Thorn et al., 2011). Rps6ka2 and VEGF-C genes are involved in promoting cell growth motility and survival, suggesting that, similar to Irs1 and Pik3r1, induction could be due to a survival mechanism triggered by hypoxic stress (Milan et al., 2000; Roux et al., 2003). In agreement with this concept are data related to decreased placental expression of Prkag2, an mTOR negative regulator that is involved with AMP signaling (Stapleton et al., 1996).

When we studied placental activation of mTOR, p70 and 4EBP1, only mTOR protein activation was decreased in treated animals, while there were no differences in the expression of p70 and 4EBP1. IHC demonstrated that decreased active mTOR was more localized to the trophoblasts in the Junctional Zone (JZ) of the placenta, a structure suspected to primarily give rise to invasive trophoblast cells. The importance of the JZ is underscored by the previous observation that decreased trophoblast invasion is observed when mTOR activation is inhibited

(Knuth et al., 2015). We therefore conclude that IUGR is correlated with increased 4EBP1 activity in the invading trophoblast cells of the uterine mesometrial compartment, decreased activation of the mTOR protein, and decreased trophoblast invasion in the placenta during maternal hypoxia-induced IUGR.

Our results could provide needed insight into the physiological relevance of these pathways in the pathogenesis of IUGR and suggest possible targets that may assist in the alleviation of IUGR. As extensions of our findings in the low oxygen tension placenta, additional research on the mTOR pathway and the alteration of associated proteins may further elucidate mechanistic progression factors for IUGR and provide a more effective modality for the therapeutic treatment of growth restricted fetuses.

REFERENCES

Abate, C., Patel, L., Rauscher, F.J., 3rd, Curran, T., 1990. Redox regulation of fos and jun DNA-binding activity in vitro. Science (New York, N.Y.) 249, 1157-1161.

Ain, R., Canham, L.N., Soares, M.J., 2005. Dexamethasone-induced intrauterine growth restriction impacts the placental prolactin family, insulin-like growth factor-II and the Akt signaling pathway. J. Endocrinol. 185, 253-263.

Arroyo, J.A., Anthony, R.V., Galan, H.L., 2008. Decreased placental X-linked inhibitor of apoptosis protein in an ovine model of intrauterine growth restriction. Am.J.Obstet.Gynecol. 199, 80-88.

Arroyo, J.A., Brown, L.D., Galan, H.L., 2009. Placental mammalian target of rapamycin and related signaling pathways in an ovine model of intrauterine growth restriction. Am. J. Obstet. Gynecol. 201, 7.

Bahr, B.L., Price, M.D., Merrill, D., Mejia, C., Call, L., Bearss, D., Arroyo, J., Different expression of placental pyruvate kinase in normal, preeclamptic and intrauterine growth restriction pregnancies. Placenta 35, 883-890.

Blume-Jensen, P., Hunter, T., 2001. Oncogenic kinase signalling. Nature 411, 355-365.

Bourque, S.L., Dolinsky, V.W., Dyck, J.R., Davidge, S.T., 2012. Maternal resveratrol treatment during pregnancy improves adverse fetal outcomes in a rat model of severe hypoxia. Placenta 33, 449-452.

Brar, H.S., Rutherford, S.E., 1988. Classification of intrauterine growth retardation. Semin.Perinatol. 12, 2-10.

Caniggia, I., Winter, J.L., 2002. Adriana and Luisa Castellucci Award lecture 2001. Hypoxia inducible factor-1: oxygen regulation of trophoblast differentiation in normal and pre-eclamptic pregnancies--a review. Placenta 23 Suppl A, S47-57.

Challis, J.R., Lye, S.J., Gibb, W., Whittle, W., Patel, F., Alfaidy, N., 2001. Understanding preterm labor. Annals of the New York Academy of Sciences 943, 225-234.

Cohen, E., Wong, F.Y., Horne, R.S., Yiallourou, S.R., 2016. Intrauterine growth restriction: impact on cardiovascular development and function throughout infancy. Pediatr Res.

Copps, K.D., White, M.F., 2012. Regulation of insulin sensitivity by serine/threonine phosphorylation of insulin receptor substrate proteins IRS1 and IRS2. Diabetologia 55, 2565-2582.

Diaz, M., Aragones, G., Sanchez-Infantes, D., Bassols, J., Perez-Cruz, M., de Zegher, F., Lopez-Bermejo, A., Ibanez, L., 2014. Mitochondrial DNA in placenta: associations with fetal growth and superoxide dismutase activity. Hormone research in paediatrics 82, 303-309.

Eriksson, A., Cao, R., Pawliuk, R., Berg, S.M., Tsang, M., Zhou, D., Fleet, C., Tritsaris, K., Dissing, S., Leboulch, P., Cao, Y., 2002. Placenta growth factor-1 antagonizes VEGF-induced angiogenesis and tumor growth by the formation of functionally inactive PlGF-1/VEGF heterodimers. Cancer cell 1, 99-108.

Genbacev, O., Zhou, Y., Ludlow, J.W., Fisher, S.J., 1997. Regulation of Human Placental Development by Oxygen Tension. Science (New York, N.Y.) 277, 1669-1672.

Giussani, D.A., Davidge, S.T., 2013. Developmental programming of cardiovascular disease by prenatal hypoxia. Journal of developmental origins of health and disease 4, 328-337.

Gray, P.H., O'Callaghan, M.J., Harvey, J.M., Burke, C.J., Payton, D.J., 1999. Placental pathology and neurodevelopment of the infant with intrauterine growth restriction. Dev.Med.Child Neurol. 41, 16-20.

Huppertz, B., 2014. Oxygenation of the placenta and its role in pre-eclampsia. Pregnancy hypertension 4, 244-245.

Iqbal, W., Ciriello, J., 2013. Effect of maternal chronic intermittent hypoxia during gestation on offspring growth in the rat. Am J Obstet Gynecol 209, 564.e561-569.

Ishihara, N., Matsuo, H., Murakoshi, H., Laoag-Fernandez, J.B., Samoto, T., Maruo, T., 2002. Increased apoptosis in the syncytiotrophoblast in human term placentas complicated by either preeclampsia or intrauterine growth retardation. Am.J.Obstet.Gynecol. 186, 158-166.

Jang, E.A., Longo, L.D., Goyal, R., 2015. Antenatal maternal hypoxia: criterion for fetal growth restriction in rodents. Frontiers in physiology 6, 176.

Jansson, N., Pettersson, J., Haafiz, A., Ericsson, A., Palmberg, I., Tranberg, M., Ganapathy, V., Powell, T.L., Jansson, T., 2006. Down-regulation of placental transport of amino acids precedes the development of intrauterine growth restriction in rats fed a low protein diet. J. Physiol.-London 576, 935-946.

Jiang, B., Kamat, A., Mendelson, C.R., 2000. Hypoxia prevents induction of aromatase expression in human trophoblast cells in culture: potential inhibitory role of the hypoxia-inducible transcription factor Mash-2 (mammalian achaete-scute homologous protein-2). Molecular endocrinology (Baltimore, Md.) 14, 1661-1673.

Kimball, R., Wayment, M., Merrill, D., Wahlquist, T., Reynolds, P.R., Arroyo, J.A., 2015. Hypoxia reduces placental mTOR activation in a hypoxia-induced model of intrauterine growth restriction (IUGR). Physiological reports 3.

Knuth, A., Liu, L., Nielsen, H., Merril, D., Torry, D.S., Arroyo, J.A., 2015. Placenta Growth Factor Induces Invasion and Activates p70 during Rapamycin Treatment in Trophoblast Cells. American Journal of Reproductive Immunology 73, 330-340.

Krebs, C., Macara, L.M., Leiser, R., Bowman, A.W., Greer, I.A., Kingdom, J.C., 1996. Intrauterine growth restriction with absent end-diastolic flow velocity in the umbilical

artery is associated with maldevelopment of the placental terminal villous tree. Am.J.Obstet.Gynecol. 175, 1534-1542.

Lechuga, C.G., Hernandez-Nazara, Z.H., Dominguez Rosales, J.A., Morris, E.R., Rincon, A.R., Rivas-Estilla, A.M., Esteban-Gamboa, A., Rojkind, M., 2004. TGF-beta1 modulates matrix metalloproteinase-13 expression in hepatic stellate cells by complex mechanisms involving p38MAPK, PI3-kinase, AKT, and p70S6k. American journal of physiology. Gastrointestinal and liver physiology 287, G974-987.

Leduc, L., Levy, E., Bouity-Voubou, M., Delvin, E., 2010. Fetal programming of atherosclerosis: possible role of the mitochondria. European journal of obstetrics, gynecology, and reproductive biology 149, 127-130.

Mandò, C., De Palma, C., Stampalija, T., Anelli, G.M., Figus, M., Novielli, C., Parisi, F., Clementi, E., Ferrazzi, E., Cetin, I., 2014. Placental mitochondrial content and function in intrauterine growth restriction and preeclampsia. American Journal of Physiology - Endocrinology and Metabolism 306, E404-E413.

Matheson, H., Veerbeek, J.H., Charnock-Jones, D.S., Burton, G.J., Yung, H.W., 2015. Morphological and molecular changes in the murine placenta exposed to normobaric hypoxia throughout pregnancy. The Journal of physiology.

Mayhew, T.M., Ohadike, C., Baker, P.N., Crocker, I.P., Mitchell, C., Ong, S.S., 2003. Stereological investigation of placental morphology in pregnancies complicated by pre-eclampsia with and without intrauterine growth restriction. Placenta 24, 219-226.

Milan, D., Jeon, J.T., Looft, C., Amarger, V., Robic, A., Thelander, M., Rogel-Gaillard, C., Paul, S., Iannuccelli, N., Rask, L., Ronne, H., Lundstrom, K., Reinsch, N., Gellin, J., Kalm, E., Roy, P.L., Chardon, P., Andersson, L., 2000. A mutation in PRKAG3 associated with excess glycogen content in pig skeletal muscle. Science (New York, N.Y.) 288, 1248-1251.

Miled, N., Yan, Y., Hon, W.C., Perisic, O., Zvelebil, M., Inbar, Y., Schneidman-Duhovny, D., Wolfson, H.J., Backer, J.M., Williams, R.L., 2007. Mechanism of two classes of cancer mutations in the phosphoinositide 3-kinase catalytic subunit. Science (New York, N.Y.) 317, 239-242.

Mura, M., Hopkins, T.G., Michael, T., Abd-Latip, N., Weir, J., Aboagye, E., Mauri, F., Jameson, C., Sturge, J., Gabra, H., Bushell, M., Willis, A.E., Curry, E., Blagden, S.P., 2014. LARP1 post-transcriptionally regulates mTOR and contributes to cancer progression. Oncogene.

Myatt, L., 2006. Placental adaptive responses and fetal programming. The Journal of physiology 572, 25-30.

Nelson, D.M., Johnson, R.D., Smith, S.D., Anteby, E.Y., Sadovsky, Y., 1999. Hypoxia limits differentiation and up-regulates expression and activity of prostaglandin H synthase 2 in cultured trophoblast from term human placenta. Am J Obstet Gynecol 180, 896-902.

Pollack, R.N., Divon, M.Y., 1992. Intrauterine growth retardation: definition, classification, and etiology. Clin Obstet Gynecol 35, 99-107.

Qian, X.J., Li, Y.T., Yu, Y., Yang, F., Deng, R., Ji, J., Jiao, L., Li, X., Wu, R.Y., Chen, W.D., Feng, G.K., Zhu, X.F., 2015. Inhibition of DNA methyltransferase as a novel therapeutic strategy to overcome acquired resistance to dual PI3K/mTOR inhibitors. Oncotarget 6, 5134-5146.

Rehman, G., Shehzad, A., Khan, A.L., Hamayun, M., 2014. Role of AMP-activated protein kinase in cancer therapy. Archiv der Pharmazie 347, 457-468.

Reyes, L.M., Kirschenman, R., Quon, A., Morton, J.S., Shah, A., Davidge, S.T., 2015. Aerobic exercise training reduces cardiac function in adult male offspring exposed to prenatal hypoxia. American journal of physiology. Regulatory, integrative and comparative physiology 309, R489-498.

Roos, S., Jansson, N., Palmberg, I., Saljo, K., Powell, T.L., Jansson, T., 2007. Mammalian target of rapamycin in the human placenta regulates leucine transport and is down-regulated in restricted fetal growth. The Journal of physiology 582, 449-459.

Roux, P.P., Richards, S.A., Blenis, J., 2003. Phosphorylation of p90 ribosomal S6 kinase (RSK) regulates extracellular signal-regulated kinase docking and RSK activity. Molecular and cellular biology 23, 4796-4804.

Rueda-Clausen, C.F., Stanley, J.L., Thambiraj, D.F., Poudel, R., Davidge, S.T., Baker, P.N., 2014. Effect of Prenatal Hypoxia in Transgenic Mouse Models of Preeclampsia and Fetal Growth Restriction. Reproductive Sciences 21, 492-502.

Shah, A., Reyes, L.M., Morton, J.S., Fung, D., Schneider, J., Davidge, S.T., 2015. Effect of resveratrol on metabolic and cardiovascular function in male and female adult offspring exposed to prenatal hypoxia and a high-fat diet. The Journal of physiology.

Smith, M.E., Tippetts, T.S., Brassfield, E.S., Tucker, B.J., Ockey, A., Swensen, A.C., Anthonymuthu, T.S., Washburn, T.D., Kane, D.A., Prince, J.T., Bikman, B.T., 2013. Mitochondrial fission mediates ceramide-induced metabolic disruption in skeletal muscle. The Biochemical journal 456, 427-439.

Soares, M.J., Chakraborty, D., Karim Rumi, M.A., Konno, T., Renaud, S.J., 2012. Rat placentation: an experimental model for investigating the hemochorial maternal-fetal interface. Placenta 33, 233-243.

Stapleton, D., Mitchelhill, K.I., Gao, G., Widmer, J., Michell, B.J., Teh, T., House, C.M., Fernandez, C.S., Cox, T., Witters, L.A., Kemp, B.E., 1996. Mammalian AMP-activated protein kinase subfamily. The Journal of biological chemistry 271, 611-614.

Tanaka, M., Natori, M., Ishimoto, H., Miyazaki, T., Kobayashi, T., Nozawa, S., 1994. Experimental growth retardation produced by transient period of uteroplacental ischemia in pregnant Sprague-Dawley rats. Am.J.Obstet.Gynecol. 171, 1231-1234.

Tapanainen, P.J., Bang, P., Wilson, K., Unterman, T.G., Vreman, H.J., Rosenfeld, R.G., 1994. MATERNAL HYPOXIA AS A MODEL FOR INTRAUTERINE GROWTH-RETARDATION - EFFECTS ON INSULIN-LIKE GROWTH-FACTORS AND THEIR BINDING-PROTEINS. Pediatr. Res. 36, 152-158.

Thorn, S.R., Rozance, P.J., Brown, L.D., Hay, W.W., Jr., 2011. The intrauterine growth restriction phenotype: fetal adaptations and potential implications for later life insulin resistance and diabetes. Seminars in reproductive medicine 29, 225-236.

Tippetts, T.S., Winden, D.R., Swensen, A.C., Nelson, M.B., Thatcher, M.O., Saito, R.R., Condie, T.B., Simmons, K.J., Judd, A.M., Reynolds, P.R., Bikman, B.T., 2014. Cigarette smoke increases cardiomyocyte ceramide accumulation and inhibits mitochondrial respiration. BMC cardiovascular disorders 14, 165.

Trinh, X.B., Tjalma, W.A., Vermeulen, P.B., Van den Eynden, G., Van der Auwera, I., Van Laere, S.J., Helleman, J., Berns, E.M., Dirix, L.Y., van Dam, P.A., 2009. The VEGF pathway and the AKT/mTOR/p70S6K1 signalling pathway in human epithelial ovarian cancer. British journal of cancer 100, 971-978.

Wakefield, S.L., Lane, M., Mitchell, M., 2011. Impaired Mitochondrial Function in the Preimplantation Embryo Perturbs Fetal and Placental Development in the Mouse. Biology of Reproduction 84, 572-580.

Wallace, D.C., 2012. Mitochondria and cancer. Nature reviews. Cancer 12, 685-698.

Wong, N., De Melo, J., Tang, D., 2013. PKM2, a Central Point of Regulation in Cancer Metabolism. International journal of cell biology 2013, 242513.

Wullschleger, S., Loewith, R., Hall, M.N., 2006. TOR signaling in growth and metabolism. Cell 124, 471-484.

Zhang, F.Y., Hu, Y., Que, Z.Y., Wang, P., Liu, Y.H., Wang, Z.H., Xue, Y.X., 2015. Shikonin Inhibits the Migration and Invasion of Human Glioblastoma Cells by Targeting Phosphorylated beta-Catenin and Phosphorylated PI3K/Akt: A Potential Mechanism for the Anti-Glioma Efficacy of a Traditional Chinese Herbal Medicine. International journal of molecular sciences 16, 23823-23848.

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PUBLICATIONS

Hypoxia reduces placental mTOR activation in a hypoxia-induced model of intrauterine growth restriction (IUGR). Kimball et al. Physiological Reports. 2015

POSTER PRESENTATIONS

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