Expression of downstream targets of mTOR in response to a model of maternal undernutrition and hypoxia.

Honors Thesis
Presented to the College of Agriculture and Life Sciences, Nutritional Sciences
Of Cornell University
in Partial Fulfillment of the Requirements for the
Research Honors Program

by
Jessica L. Groth
May 2012

Faculty Mentor: Patsy Brannon, Ph.D., R.D.
Division of Nutritional Sciences

ABSTRACT

The mammalian target of rapamycin (mTOR) is a key player in a signaling pathway that regulates cell growth in response to nutrients, hypoxia, and other upstream regulators. To examine the effects of nutrient restriction, hypoxia, and their possible interactive effects on placental mTOR, a series of experiments were carried out on immortalized human placental trophoblasts. To assess the temporal response, trophoblasts grown in nutrient replete (complete; C) and nutrient restricted (NR) media were harvested at 0 to 96h; phosphorylated s6 ribosomal protein (s6rp) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) protein levels were measured using western analysis. To assess the independent and interactive effects of NR and hypoxia (Hx) on mTOR, trophoblasts were grown in C and normoxia (20% oxygen;Nx), C media and Hx (1% oxygen), NR -Nx, NR -Hx and harvested at72h; total and phosphorylated s6rp protein levels were measured using western analysis. NR had a negative temporal effect on phosphorylation of s6rp and 4E-BP1. Maximal change occurred at 48h for p-s6rp and at 72h for p-4E-BP1. NR alone had no effect on total protein expression of s6rp under Nx or Hx, whereas Hx decreased total protein expression of s6rp in C media samples at 72h. NR alone decreased p-s6rp but Hx alone had no effect on p-s6rp at 72h. NR and Hx interacted to affect p-s6rp such that Hx reduced p-s6rp in C media but slightly increased it in NR media. The interactive effect of Hx and NR suggest that the mechanisms by which nutrients and Hx affect mTOR signaling have not been fully elucidated, and may shed light on how these two conditions contribute to placental complications such as intrauterine growth restriction and preeclampsia.

TABLE OF CONTENTS

INTRODUCTION AND LITERATURE REVIEW. 3

How the placenta develops under normal conditions. 3

Intrauterine growth restriction, preeclampsia, and the placental response to stress. 4

mTOR function and its downstream targets. 6

Upstream control of mTORC1. 8

mTOR-mediated signaling in the placenta. 10

Immortalized human placental trophoblast: HTR8/SVneo cells as a model. 10

Research objectives. 11

MATERIALS AND METHODS. 13

Materials. 13

Experimental design and methods. 14

Preliminary experiment. 14

Cell culture procedure and 0h timepoint collection. 15

Cell harvesting procedure. 16

Protein quantification via western blot analysis. 16

2x2 factorial design. 17

Cell culture procedure. 18

Cell harvesting procedure. 18

Protein quantification via western blot analysis. 18

Statistics. 19

RESULTS. 20

Preliminary experiment on temporal response. 20

Independent and interactive effects of NR and Hx: 2x2-factorial experiment. 22

DISCUSSION. 24

CONCLUSIONS. 29

REFERENCES. 30

INTRODUCTION AND LITERATURE REVIEW

How the placenta develops under normal conditions.

The human placenta is the highly specialized organ of pregnancy through which the fetus receives a necessary supply of nutrients and gases to support its normal growth and development. The placental unit is composed of both fetal tissue derived from the chorionic sac and maternal tissue derived from the endometrium, termed the chorionic plate and basal plate, respectively (1). The main functional units of the placenta, the extensively branched villous structures containing the fetal blood vessels, are located between these two plates in the intervillous space. Maternal blood enters this space via spiral endometrial arteries and bathes the villi to facilitate maternal-fetal exchange.

The placenta undergoes growth and development through three distinct trimesters to be able to support this maternal-fetal exchange. During the first trimester, functional cells called trophoblasts form and start to differentiate, contributing to placental development. This process occurs in a relatively hypoxic environment due to the presence of endovascular trophoblast plugs that occlude the tips of spiral arteries in the uterus and limit uterine blood flow to the conceptus (2). Several studies indicate that this low oxygen environment arrests cytotrophoblast differentiation at the initial proliferative, non-invasive stage (3, 4), such that the cytotrophoblast cells proliferate, differentiate and fuse to form the multinucleate syncytiotrophoblast layer and the extravillous trophoblast (EVT) cell columns that serve to anchor the embryo to the uterine wall. The EVT cells then begin to invade the uterine wall, remodeling the uterine arteries so as to direct an adequate supply of maternal blood to the placenta and fetus, and instigate a transition to a normoxic environment by the end of the first trimester. The invasive activity of the EVT cells peaks at 10-12 weeks gestation and declines thereafter (4).

In the second and third trimester, the growing fetal metabolic demands call for increased blood flow and growth of the placenta. During the second trimester, this demand is met by through branching angiogenesis, the formation of new branches from preexisting vessels, resulting in increased capillary density. Non-branching angiogenesis takes over around the beginning of the third trimester (24-26 weeks) (1). This type of angiogenesis creates terminal villi that dramatically increase the surface area:volume ratio of the placenta, allowing for increased gas and nutrient exchange between mother and fetus.

Intrauterine growth restriction, preeclampsia, and the placental response to stress.

Intrauterine growth restriction (IUGR) and preeclampsia represent two main complications of pregnancy. In IUGR, a reduction of fetal growth in utero leads to a small-for-gestational age baby at birth (weight below the 10th percentile for gestational age) and an increased risk of the baby developing handicaps in later childhood and chronic diseases in adult life (5). Preeclampsia is universally defined as maternal hypertension and significant proteinuria developed at or after 20 weeks of pregnancy in an otherwise normotensive woman (6). Maternal malnutrition and preeclampsia are both causes of IUGR, however, a woman can have preeclampsia without giving birth to an IUGR baby.

These adverse pregnancy outcomes are hypothesized to result from impaired invasion of EVT cells into the maternal decidua. In a normal, uncomplicated pregnancy, maternal and fetal blood flow is proportional and leads to an even circulation with efficient exchange of oxygen and nutrients. However, with insufficient invasion, the spiral arteries regulating maternal-fetal exchange are under-transformed, leading to an uneven circulation and inefficient oxygen and nutrient exchange (5). Thus, this altered exchange may cause adverse outcomes for both the placenta and fetus. Longitudinal studies on placental development support this possibility; they

have revealed that placental development follows different trajectories in normal and complicated pregnancies and that these differences are established at the end of the first trimester (2), after the majority of EVT invasion has taken place. In cases of IUGR the placenta is smaller than normal at 12 weeks but grows at the same rate thereafter. If the IUGR is accompanied by preeclampsia, the subsequent rate of growth is slowed (2).

On a molecular level, this fluctuating delivery of oxygen and nutrients puts stress on the preeclamptic and IUGR placenta. Normal protein synthesis in the placenta demands approximately 30% of human placental oxygen consumption (2). Thus, when the supply of oxygen and nutrients is reduced, as in cases of IUGR and preeclampsia, the placenta conserves energy by shutting down its protein synthetic machinery thereby reducing its rate of cell proliferation. This regulation of protein synthesis occurs primarily at the translational level and the central regulator of this translational control is the mammalian target of rapamycin (mTOR) pathway (Figure 1).

Figure 1: Overview diagram of how various stressors can reduce the rate of cell proliferation through inhibition of protein synthesis (reprinted with permission, Int J Dev Biol; 2).

mTOR function and its downstream targets.

The target of rapamycin (TOR) is a highly conserved protein kinase in species ranging from yeast to man, and is aptly named for its inhibition by the drug rapamycin (7). In humans this protein is referred to as mTOR and functions as a serine/threonine kinase and part of the phosphoinositide 3-kinase related kinase (PIKK) family. It regulates a vast range of cellular activities including cell growth, transcription, translation, and survival in response to nutrient, metabolic, and hormonal signals. However, the best-described and most well-understood function of mTOR is the mechanism by which it regulates translation.

In the cytoplasm, mTOR is found in two cellular complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Knockdown of mTORC2 prevents actin polymerization and cell spreading, indicating that mTORC2 plays a role in actin cytoskeleton organization (8). The mTORC2 is generally described as being rapamycin-insensitive, but it is now becoming apparent that longer-term treatment leads to dissociation of mTORC2 (9). Little else is definitively known about mTORC2, thus for the purpose of this paper, mTORC1 will be the primary focus.

In contrast to mTORC2, mTORC1 has been extensively studied and is rapamycin-sensitive. The mTORC1 consists of the three proteins mTOR, mLST8, and Raptor (*r*egulatory *a*ssociated *protein* of m*TOR*) (9). This complex largely controls translation and cell growth through its interaction with downstream key translational regulators eukaryotic initiation factor 4E binding protein 1 (4E-BP1) and p70 ribosomal S6 kinase (S6K). The mTOR component Raptor binds mTOR and 4E-BP1 or S6K to facilitate mTOR phosphorylation of these molecules (7). In its unphosphorylated state, 4E-BP1 functions as a translation repressor, sitting on translation initiation factor eIF4E to prevent its interaction with translation initiation factor

eIF4G, thereby inhibiting ribosome binding to the 5' end of an mRNA strand (Figure 2B) (10). When mTOR phosphorylates 4E-BP1, it dissociates from eIF4E. Thus, through repression of 4E-BP1, mTOR drives cell growth and proliferation by enhancing eIF4E-mediated translation of mRNA (9).

S6K1, mTORC1's other major substrate, is in its active state when phosphorylated by mTOR. Activated S6K1 promotes translation through several downstream targets, including SKAR, eIF4B, and eEF2K (9). SKAR enhances translation efficiency through recruiting S6K1 to the exon-junction to facilitate splicing. When phosphorylated, eIF4B increases its association with eIF3 to maintain functional assembly of the mammalian ribosome initiation complex (Figure 2A). Phosphorylation of eEF2K promotes the elongation phase of protein synthesis. The functional purpose of a fourth downstream target, ribosomal protein s6 (s6rp), is still unresolved; however, phosphorylation of s6rp by S6K1 is generally a good readout for S6K1 activity (10).

Thus, through a number of downstream targets, 4E-BP1 and S6K1 influence the level of protein synthesis in cells.

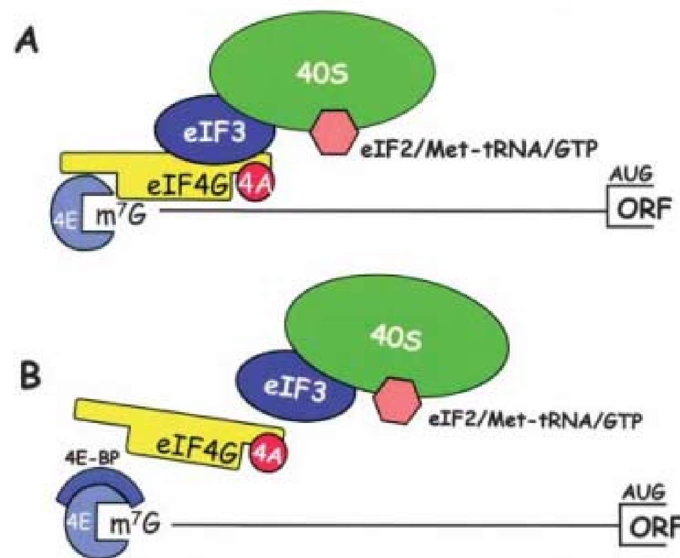

Figure 2: (A) Assembly of the mammalian ribosome intitation complex at the 5' end of an mRNA. (B) 4E-BP1 binds to surface of eIF4E to prevent its interaction with eIF4G, thereby inhibiting ribosome binding (reprinted with permission, Genes Dev; 10).

Upstream control of mTORC1.

Amongst others, nutrients, growth factors, energy metabolism, and hypoxia all are upstream regulators of mTOR activity. Growth factor-induced activation of mTOR is mediated by the phosphatidylinositol 3-kinase (PI3K) pathway (10). Insulin, as well as numerous other growth factors, binding to its receptor activates the PI3K pathway. This leads to the phosphorylation and inhibition of tumor sclerosis complex 2 (TSC2) of the TSC1/2 complex. TSC1/2 acts as an important negative regulator of mTORC1 through its role as a guanosine triphosphatase (GTPase)-activating protein (GAP) for Rheb-GTP, which binds to and activates mTORC1 directly (Figure 3).

However, the ability of insulin to activate mTOR is impaired upon a reduction in cellular ATP levels. This suggests that long-term energy deprivation, either through reduced glucose availability or the inhibition of mitochondrial respiration, also feeds into the PI3K pathway. Under severe oxygen depletion or glucose deprivation, the energy levels of the cell are dramatically reduced and the ratio of AMP:ATP increases. Such conditions activate the 5' AMP-activated protein kinase (AMPK) pathway to down-regulate mTOR activity as an attempt to conserve energy. TSC2 has been shown to be phosphorylated by AMPK at two sites, both in vitro and in vivo, suggesting that the AMPK pathway converges on the PI3K pathway at TSC2 to inhibit mTOR activity (11) (Figure 3). Thus, protein synthesis is tightly coupled with energy metabolism.

Stress signaling via hypoxia suppresses mTORC1 through multiple pathways, indicating the importance of this adaptive response for cells (12). One major pathway that has been elucidated converges downstream of the PI3K pathway at TSC1/TSC2. Hypoxia causes phosphorylation of the hypoxia-inducible transcription factor (HIF)-1α protein, inducing

transcription of the *Redd1* gene. REDD1 protein then functions to inhibit mTORC1 by stabilizing heterodimer interaction between TSC1 and TSC2. In addition to REDD1, there are reports of additional pathways through which hypoxia suppresses mTORC1, including the AMPK pathway and mTORC1. However, these require further elucidation (12). The above data indicates that numerous signaling pathways converge on TSC2, which places the TSC1/TSC2 heterodimer as a central coordinator of mTORC1 signal transduction.

Insufficient nutrients also cause a down-regulation of mTOR-mediated activity. Recently discovered, Ras-related GTPase (Rag) proteins mediate amino acid signaling to mTORC1 (Figure 3) (13). Sancak and colleagues propose a model whereby amino acid stimulation induces Rag proteins to initiate a localization change of mTORC1, taking it to Rab-containing vesicles, where Rheb is thought to reside (14). Rheb's interaction with mTORC1 then leads to activation of mTORC1.

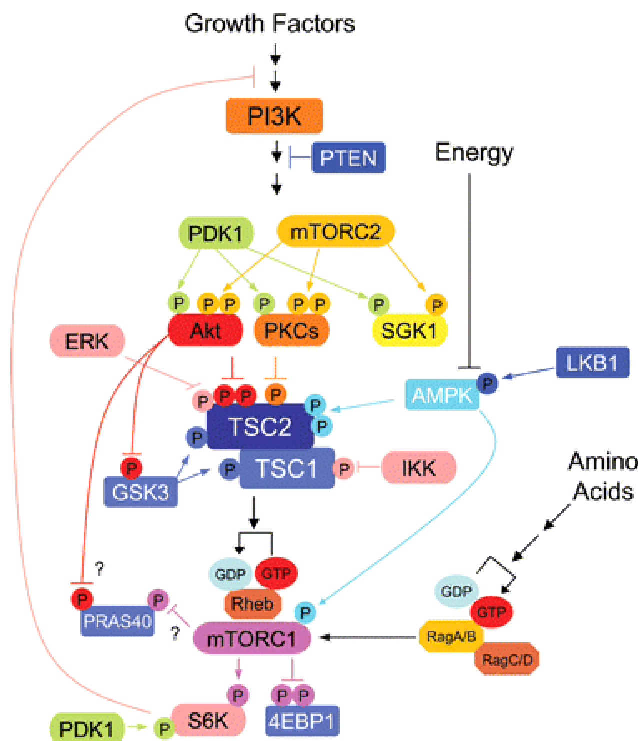

Figure 3: mTOR within the PI3K pathway (reprinted with permission, Hum Mol Genet; 15).

mTOR-mediated signaling in the placenta.

Very little is known regarding the role of mTOR-mediated signaling in trophoblast cell proliferation and differentiation in placental development. A few studies have shown that its presence in the placenta is essential, as disruption of the mTOR gene is lethal (16). Several studies have looked at mTOR's role in nutrient signaling through the placenta. Roos and co-workers have done extensive work looking specifically at amino acid transport through the placenta (16). They suggest that reduced levels of glucose and growth factors upstream of placental mTOR cause decreased mTOR activity, further leading to the down-regulation of placental amino acid transport across the syncytiotrophoblast. Further evidence of glucose and growth factors stimulating trophoblast proliferation through the mTOR pathway has been presented (17). Glucose was found to regulate mTOR in a glucosamine-dependent manner. Another in vitro study has demonstrated that angiogenic factors necessary for placental development are activated through the mTOR signaling pathway under hypoxic conditions in the trophoblast-derived BeWo cell line (18). Given that (1) hypoxia and nutrient availability both play a role in placental complications, such as IUGR and preeclampsia, and (2) the above data suggest that these two conditions are regulated through mTOR, determining the effects of both nutrient and oxygen signaling on the mTOR pathway in a human trophoblast cell line would advance our understanding of mTOR in regulating placental response to these stressors. However, no known cell culture study to date has investigated this area.

Immortalized human placental trophoblast: HTR8/SVneo cells as a model.

Such a study using primary trophoblast cultures would not be suitable for several reasons (19). First, growing up the quantity of cells necessary for such an experiment would demand long-term culturing with many passages, a requirement that primary trophoblast cultures could

not meet. Second, primary cultured trophoblasts are heterogeneous; using them presents the difficulty of not only identifying the cell type responsible for specific experimental outcomes but also ensuring the reproducibility of the results, because it is hard to match placental tissue samples. Choriocarcinoma cell lines, such as BeWo cells, are also limited in their use for study of normal trophoblast cell function because their malignant phenotype affects the way that their invasion and proliferation are regulated.

In this experiment, immortalized first trimester human placental trophoblast HTR-8/SVneo cells were used as a model system of the placenta. The parental extravillous trophoblast cell line (HTR-8), obtained from first trimester placental tissues of terminated pregnancies, was transfected with a plasmid encoding the gene for simian virus 40 large T antigen. Transfection with this antigen allows the antigen to form complexes with the tumor suppressor p53 protein resulting in a cell line with a prolonged lifespan in culture (19) in addition to other effects on cell cycle that are not fully understood. Whereas the parental HTR-8 cell line can be passaged up to fourteen times, the transfected HTR-8/SVneo cell line can be passaged at least thirty-two times. Furthermore, the transfected line retains its invasive properties, is not tumorogenic, and has similar phenotypic characteristics to the parental line. Thus, this immortalized cell line is a very useful tool for studying placental function and placental gene expression.

Research objectives.

Previous work using the HTR8/SVneo cell line has shown that these cells respond to nutrient signals to regulate cell growth (17). Our preliminary data in the Brannon lab support this finding, and furthermore show that these cells also respond to variations in oxygen availability (20, 21). When cells were grown under four conditions – a normoxic environment (Nx) with complete nutrient replete (C) media, a hypoxic environment (Hx) with C media, Nx with nutrient

restricted (NR) media, and Hx-NR media – proliferation of cells was decreased by Hx and NR alone, and further decreased by both conditions together, suggesting an interactive effect on cell number (Figure 4) and the proliferation biomarker Ki67 (data not shown). The expression of mTOR mRNA was also affected by Hx and NR (Figure 5). These preliminary data and the current gaps in the literature have led me to use our in vitro model to determine whether maternal undernutrition (NR) and Hx both act through mTOR to decrease cell growth. Through the use of a 2x2 factorial design, I will determine whether NR and Hx have independent and interactive or additive effects on mTOR downstream functioning.

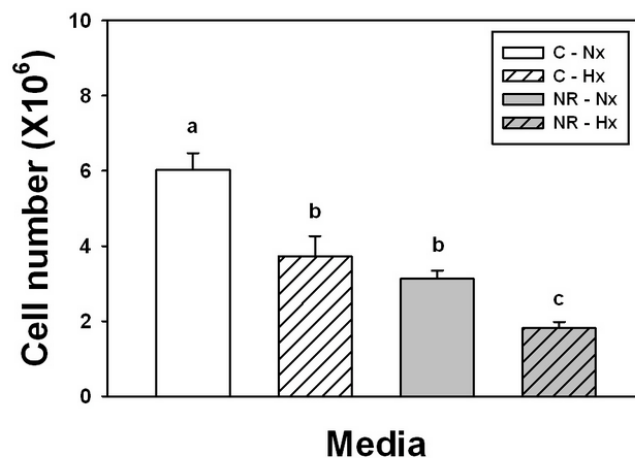

Figure 4: Effects of nutrient restriction (NR) and hypoxia (Hx) on HTR-8/SVneo human trophoblast cell growth in cells cultured 72 h in complete (nutrient replete) media and normoxic 20% oxygen levels (C – Nx); C- hypoxic (Hx) 1% oxygen levels (C – Hx); NR-Nx; and NR – Hx) (20). Values represent mean ± SE of triplicate samples from three replicate experiments. Values not sharing a superscript differed significantly ($p<0.05$) by 2-way ANOVA and post hoc comparison.

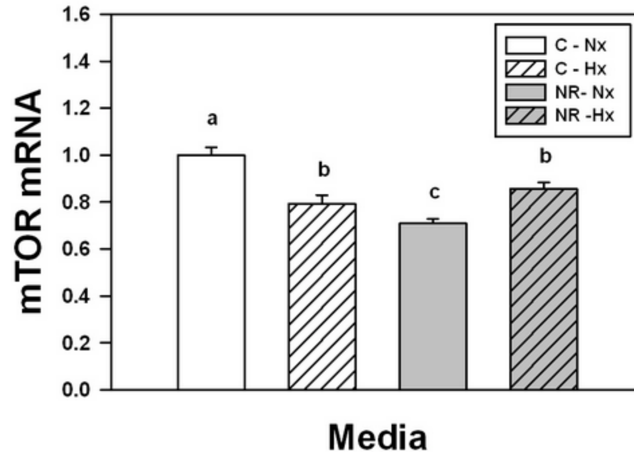

Figure 5: Effects of nutrient restriction (NR) and hypoxia (Hx) on HTR-8/SVneo human trophoblast mTOR mRNA in cells cultured 72 h in complete (nutrient replete) media and normoxic 20% oxygen levels (C – Nx); C- hypoxic (Hx) 1% oxygen levels (C – Hx); NR-Nx; and NR – Hx) (20). Values represent mean ± SE of triplicate samples from three replicate experiments. Values not sharing a superscript differed significantly ($p<0.05$) by 2-way ANOVA and post hoc comparison.

MATERIALS AND METHODS

Materials.

HTR-8/SVneo cells were kindly provided by Dr. Charles H. Graham in the Department of Anatomy and Cell Biology at Queen's University in Ontario, Canada. Complete (C) media was comprised of RPMI 1640 (45000-404), 1.25% USDA-certified Fetal Bovine Serum (FBS)(45000-736) and 2.0 mM glutamine (45000-676), all ordered from Mediatech. Nutrient restricted media (NR) was comprised of 25% C media, and 75% of custom media manufactured by Hyclone (producing a 75% nutrient restricted media). Hyclone custom media contained no vitamins or glucose, and only selected non-essential amino acids. 0.5% (10X) Trypsin/EDTA (15400-054) was purchased from Invitrogen, and diluted to 1X with phosphate-buffered saline (PBS). Tryphan Blue 0.4% solution used for cell counting was obtained from Mediatech.

Phosphosafe extraction buffer (71296-3, Novagen) and protease inhibitor cocktail (539134, Calbiochem) were obtained for preparation of cell lysates. Folin Ciocalteu for Lowry assay was obtained from a Lowry protein assay kit (VWR PI23240) and Fraction V Bovine Serum Albumin was obtained from Sigma. Protogel 30% (EC-890) and Temed (EC-503) for SDS-PAGE came from National Diagnostics. Sample buffer mix was at 4X concentration with Beta-mercaptoethanol (Sigma, M6250). Protein standards (928-40001) and LiCor blocking buffer (NC9877369) were from LiCor Biosciences. Membranes used for western blot analysis were Immobilon-FL PVDF transfer membranes (Millipore, IPFL20200). Primary antibodies for western blot analysis were from Cell Signaling and included: S6 ribsomal protein rabbit mAb (2217), phospho-S6 ribosomal protein rabbit mAb (2215), 4E-BP1 rabbit mAb (9452), phospho-4E-BP1 rabbit mAb (9459), and beta-actin mouse mAb (3700). Secondary goat anti-rabbit (926-32211) and goat anti-mouse (926-32220) antibodies were obtained from LiCor Biosciences.

Experimental design and methods.

Preliminary experiment.

To determine the independent and interactive effects of NR and Hx on mTOR regulation in HTR-8/SVneo human EVT cells, a preliminary experiment was first performed to determine the time point at which expression of mTOR's downstream targets, s6rp and 4E-BP, exhibited maximal change. HTR-8/SVneo cells were cultured in C or NR media, and harvested at 0h, 24h, 48h, 72h, and 96h after representative photomicrograph for each time point and condition (except the 0h) was taken. The preliminary experiment was conducted once with triplicate sample replicates for each media type and collection time point. Cells were lysed and proteins of interest were quantified via western blot analysis as described below.

Cell culture procedure and 0h timepoint collection.

Human trophoblast cells (cell line HTR-8/SVneo) were maintained in 100 mm culture dishes in RPMI 1640 with 5% FBS and 2 mM glutamine and stored in a 37°C incubator at 5.0% CO_2 and 20% O_2. At passage 14 and 70-90% confluence, media was removed, and cells were rinsed twice with PBS and detached from dish with 1X trypsin/EDTA. When cells were loose, trypsin was neutralized with media, and the cell suspension from all 16 previously growing flasks were combined into two sterile 50 ml conical tubes.

The cell suspensions were then centrifuged at room temperature twice for five minutes at 800xg, first to remove media then rince with PBS. Cell suspensions were resuspended in C media. A 20 µl representative aliquot of cells was removed, stained with 20 µl Trypan Blue 0.4% solution, and counted on BioRad TC 10 automated cell counter to determine viable cell number. The calculated cell concentration was 1.82×10^6 cells/ml of solution in the conical tube. 1.392×10^6 cells were seeded for each of the 27 samples (three samples collected from C media for the 0h time point, and three samples at 24h, 48h, 72h, and 96h in both C media and N media).

Cell suspension in conical tube was thoroughly mixed and 1.392×10^6 cells were removed to each of three microfuge tubes for 0h time point collection. Tubes were centrifuged twice at 14,000rpm and 4°C for five minutes first to remove media then rinse with PBS. PBS was removed with gentle vacuum suction and pellet was resuspended in 500 µl lysis buffer. Lysis buffer consisted of PhosphoSafe extraction buffer + 1 mM EDTA + 1/100 protease inhibitor cocktail. Tubes were left on ice for five minutes, and then centrifuged again. Supernatants were removed to individual new pre-chilled 2.0 ml microfuge tubes and stored at -80°C.

To prepare for the other twenty-four samples, cell suspension was transferred to each of two new sterile 50 ml conical tubes. The new tubes were centrifuged at room temperature for five minutes at 800xg. Media was removed, the pellet was rinsed with PBS, and another centrifugation followed, as above. PBS was removed, and pellets were resuspended in 13.0 ml of C or NR media. Cell suspensions were mixed well before plating 1.392 x 10^6 cells in each of twenty-four 100 mm dishes to grow in incubator at 37°C, 5% CO_2 and 20% O_2.

Cell harvesting procedure.

At each sampling time, three dishes containing C media and three dishes containing NR media were harvested. A representative photomicrograph was taken of each time point and media type. Media was removed, and cells were rinsed twice with PBS. PBS was removed and dish was coated with 500 µl lysis buffer. After five minutes, cells were loosened via cell scraper and suspended cell/buffer solution was transferred to pre-chilled 2.0 ml microfuge tubes. All samples were centrifuged at 14000rpm and 4°C for five minutes. Supernatants were removed to new pre-chilled 2.0 ml microfuge tubes and stored at -80°C. Cell harvesting procedure was then repeated at forty-eight, seventy-two, and ninety-six hours.

Protein quantification via western blot analysis.

Cellular protein levels, detectable from 5.0 to 80.0 µg, were determined using a Lowry assay (22). SDS-PAGE and western blot protocols were adapted from Current Protocols in Molecular Biology (23). Lysate samples (15 µg) were loaded and run on 10-well 15% SDS polyacrylamide gels at 80-130V until appropriate distance of separated proteins achieved. The separated proteins were transferred from the gel onto PVDF transfer membranes at 100V for 1h. The blots were blocked in a 50/50 TBS (Tris Buffered Saline) and LiCor blocking buffer solution overnight at 4°C, followed by two 5-minute washes and two 10-minute washes in TBST

(Tris Buffered Saline with 0.1% Tween-20). Blot was then incubated with rabbit anti-phosphorylated s6rp (1:1,000 dilution), rabbit anti-phosphorylated 4EBP-1 (1:1,000 dilution), and mouse beta-actin (1:10,000 dilution) antibodies in a TBST solution containing 5% BSA w/v for 1-2h, followed by more washes in the same sequence as previously described. Finally the blot was incubated for 1h at room temperature with goat anti-rabbit (1:5,000 dilution) and goat anti-mouse (1:20,000 dilution) antibodies in a 50/50 TBST and LiCor blocking buffer solution containing 0.02% SDS, followed by the same set of washes previously described. The signal was detected by the infrared fluorescence LiCor Odyssey detection system and analyzed using Odyssey Application Software Version 3.0.

2x2 factorial design.

To examine the independent and interactive effects of NR and Hx, a 2x2 factorial design was used as shown in the Latin square below (Figure 6). C media and NR media were prepared as described in preliminary experiment. 1% O_2 was selected as Hx because it resulted in maximal growth inhibition, whereas 20% O_2 was selected as Nx because it resulted in maximal growth stimulation in previous lab experiments (20).

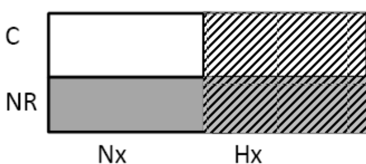

Figure 6: Latin square representing 2x2-factorial conditions used in final experiment (20).

HTR-8/Svneo human EVT cells were grown under conditions of C-Nx, C-Hx, NR-Nx and NR-Hx and harvested at 72h with representative photomicrograph taken of each condition. The final experiment was conducted three times (replicates 1-3) with triplicate sample replicates for each

of the four conditions in each replicate. Cells were lysed and proteins of interest were quantified via western blot analysis as described below.

Cell culture procedure.

Human trophoblast cells (cell like HTR-8/Svneo) were grown in RPMI 1640, 2 mM glutamine, and 5% FBS until 70-90% confluence in 100 mm dishes. The passage number for each experimental replicate are as follows: passage 14 for replicate 1, passage 4 for replicate 2, and passage 5 for replicate 3. Cells were harvested, and cell suspensions were combined, centrifuged, and resuspended in C media according to same procedure described in preliminary experiment. An aliquot was removed for manual cell counting, trypan blue exclusion, and % viability. An aliquot of 9,744,021 cells was added to each of two new 15ml conical tubes. The 15ml tubes were centrifuged at room temperature twice for five minutes at 800xg, first to remove media then rince with PBS. Cells of one tube were resuspended in 7ml of complete media + 1.25% FBS, and cells of the other tube were resuspended in 7ml 75% NR media. For each media condition, 1.392×10^6 cells were seeded in each of six 100 mm culture dishes already containing 12ml of respective media. For each group, three dishes were placed in 37°C incubator at 1% O_2 and 5% CO_2 and other three dishes were placed in 37°C incubator at 20% O_2 and 5% CO_2. Dishes were incubated for 72h.

Cell harvesting procedure.

After seventy-two hours, all dishes were harvested and stored as described in the preliminary experiment.

Protein quantification via western blot analysis.

Lowry assay, SDS-page, and western blot analysis were carried out according to protocols previously described in preliminary experiment. Lysate samples (15 µg) were loaded

and run on 15-well 10% SDS polyacrylamide gels at 80-130V until appropriate distance of separated proteins achieved. Gels were transferred, and blots were blocked and washed as previously described in preliminary experiment. Blot was then incubated with rabbit anti-phosphorylated s6rp (1:1,000 dilution), rabbit anti-total s6rp (1:1,000 dilution), and mouse beta-actin (1:10,000 dilution) antibodies in solution as previously described in preliminary experiment, followed by same washes. Finally, secondary antibody incubation as previously described, followed by infrared fluorescence detection on LiCor Odyssey system.

Statistics.

Integrated intensity of proteins of interest was normalized against beta-actin fluorescence on LiCor Odyssey software. A filter control cell lysate sample was included on all westerns so that samples could also be normalized to the cell lysate control in order to be able to compare integrated intensities across all samples within and between blots. Data presented represent integrated intensity values that have been normalized both to beta-actin and the control cell lysate, and are expressed as mean $\pm$ S.E. Data were analyzed for statistical significance using SigmaPlot version 11.0 software. Statistical significance was determined by one-way ANOVA for the preliminary experiment and two-way ANOVA for the final 2x2 factorial experiment, followed by Holm-Sidak all pairwise multiple comparison procedures (24). A value of $p < 0.05$ was interpreted to mean that observed experimental differences were statistically significant. The 2x2 factorial values were log-transformed to achieve normality and equal variance, and analyzed by two-way ANOVA.

RESULTS

Preliminary experiment on temporal response.

NR had a negative temporal effect on phosphorylation of downstream targets of mTOR, p-4E-BP1 and p-s6rp (Figure 7 and 8; Table 1). Phosphorylation of both proteins significantly decreased after 24h. p-s6rp decreased maximally 86% at 48h under NR (Figure 8). p-4E-BP1, however, maximally decreased maximally at 72h under NR by 92% (Table 1). Although p-s6rp exhibited maximal change at 48h, the near-absence of p-4E-BP1 at72h under NR (Table 1) suggested that 72h would be the most appropriate time point to examine the effects of NR and Hx on mTOR downstream activity. Of note, samples from the 96h collection could not be analyzed because of the extremely low cell number (3-5% confluence) under NR and insufficient protein in the cell lysate for western analysis.

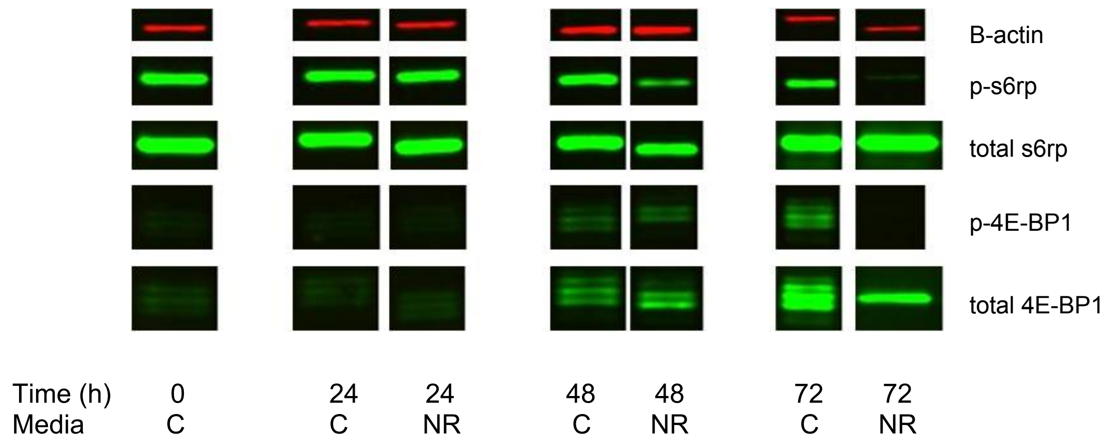

Figure 7: Representative blots of temporal response of phosphorylated (p) s6rp and p-4E-BP1 to nutrient restriction (NR). One sample is displayed from each condition (nutrient replete culture (C) and NR) and time point.

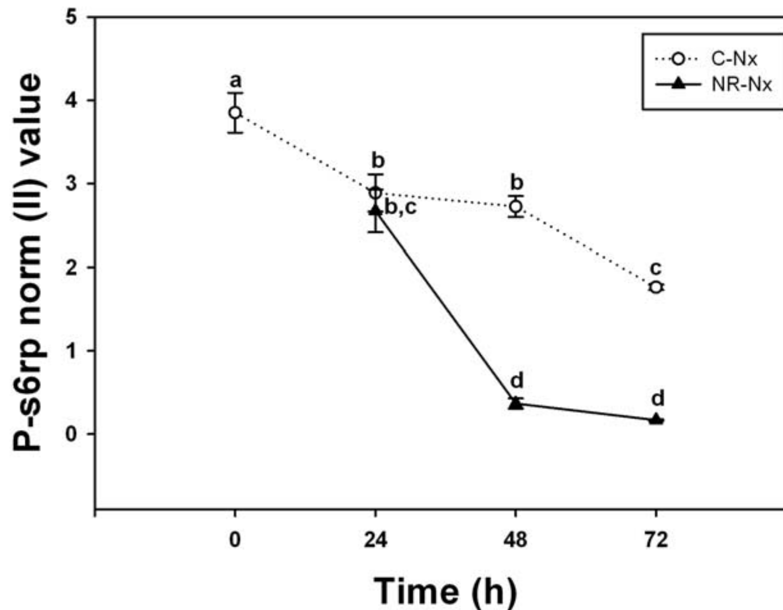

Figure 8: Effects of NR on p-s6rp protein in human trophoblasts cultured with C media (RPMI 1640 + 1.25% FBS + 2 mM glutamine) or NR media (25% C media + 75% nutrient restricted custom media from Hyclone) at 0, 24, 48, and 72h. Values represent mean ± SE of triplicate samples from one experiment. [a-d]Values not sharing a superscript differed significantly ($p<0.001$) by ANOVA and by Holm-Sidak (24) all pairwise multiple comparisons.

Even in C media, however, p-s6rp decreased significantly over 72h (Figure 8) by 54%. Nonetheless, the magnitude of decrease with NR was far greater than the more modest reduction with time.

Table 1: Effects of nutrient restriction on protein expression of phosphorylated 4E-BP1 in human trophoblasts cultured at different time points[1].

Time point	Integrated Intensity of phosphorylated 4E-BP1[2]	
	Complete Media	Nutrient Restricted Media
0h	0.93 ± 0.11[a,b]	--
24h	0.77 ± 0.04[a]	0.69 ± 0.05[a,c]
48h	1.11 ± 0.03[b]	0.73 ± 0.08[a]
72h	1.80 ± 0.11[d]	0.05 ± 0.00[e]

[1]Cells were cultured with C media (RPMI 1640 + 1.25% FBS + 2 mM glutamine) or NR media (25% C media + 75% nutrient restricted custom media from Hyclone) for 0, 24, 48, or 72h.
[2]Values represent mean ± SE for triplicate samples from one experiment. [a-e]Values not sharing a superscript differed significantly ($p<0.001$) by ANOVA and by Holm-Sidak (24) all pairwise multiple comparisons.

In contrast to p-s6rp, there was a significant increase in p-4E-BP1 (Table 1) by 48% over 72h in C media.

Independent and interactive effects of NR and Hx: 2x2-factorial experiment.

NR alone had no effect on total protein expression of s6rp under normoxic conditions or hypoxic conditions (Figure 9 and 10), whereas Hx decreased total protein expression of s6rp in cells that had grown in C media ($p<0.05$).

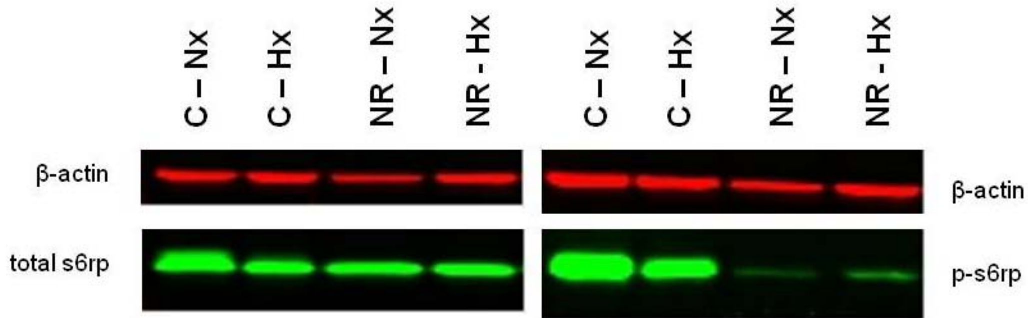

Figure 9: Representative blots of phosphorylated s6rp and total s6rp. P-s6rp and total s6rp were normalized against β-actin and a lysate control sample to account for differences between respective blots.

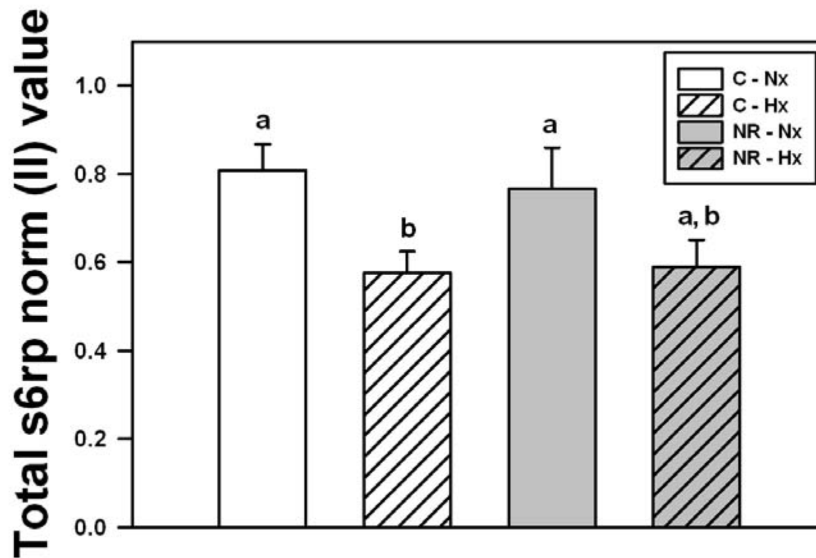

Figure 10: Effects of NR and Hx on total s6rp protein expression in cultured human trophoblast cells at 72h. Values represent mean ± SE of triplicate samples from three experiments. [a-b]Values not sharing a superscript differ significantly ($p<0.05$) by two-way ANOVA and by Holm-Sidak (24) all pairwise multiple comparisons.

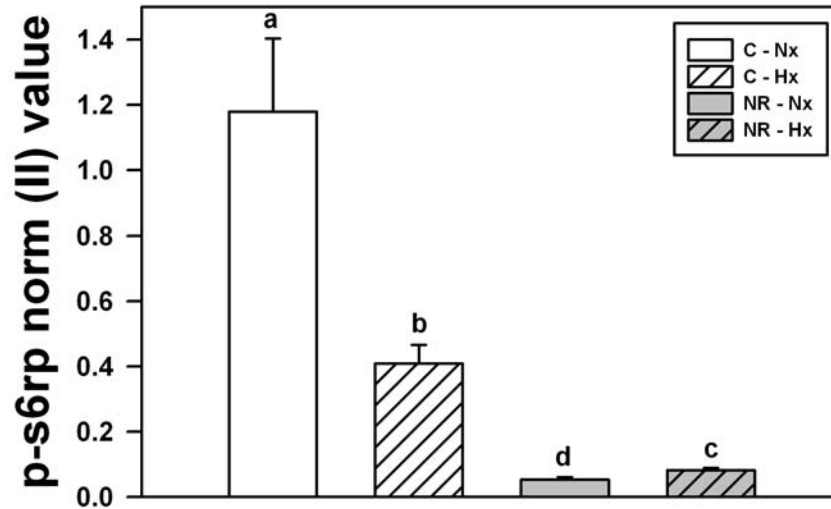

Figure 11: Effects of NR and Hx on phosphorylated s6rp protein expression in cultured human trophoblast cells at 72h. Values represent mean ± SE of triplicate samples from three experiments. Values not sharing a superscript differ significantly ($p<0.001$ for all comparisons, except c and d, $p=0.035$) by two-way ANOVA and by Holm-Sidak (24) all pairwise multiple comparisons.

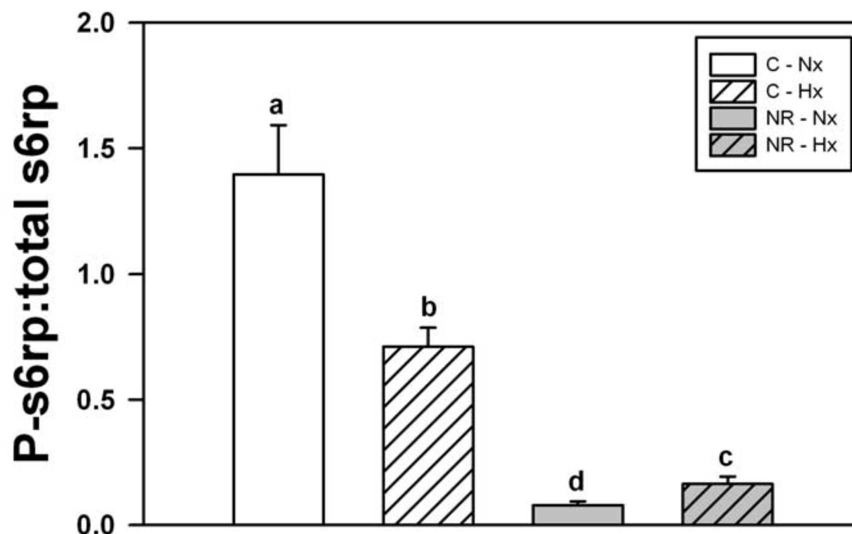

Figure 12: Effects of NR and Hx on phosphorylated s6rp protein expression relative to total s6rp protein expression in cultured human trophoblast cells at 72h. Values represent mean ± SE of triplicate samples from three experiments. Values not sharing a superscript differ significantly ($p<0.001$ for all comparisons, except a and b, p 0.020, and c and d, $p=0.004$) by two-way ANOVA and by Holm-Sidak (24) all pairwise multiple comparisons.

NR alone decreased p-s6rp under both normoxic and hypoxic conditions ($p<0.001$) (Figure 9 and 11). Hx alone did not affect p-s6rp. NR and Hx interacted to affect p-s6rp such that Hx reduced by 65% phosphorylation of s6rp in C media ($p<0.001$), but slightly increased phosphorylation of s6rp in NR by 36% ($p=0.035$).

When examining the ratio of p-s6rp expression to total s6rp expression, similar results were seen. NR alone decreased p-s6rp under both oxygen conditions ($p<0.001$), while Hx alone did not affect p-s6rp expression in relation to total s6rp expression. The interactive effect of NR and Hx was such that Hx decreased s6rp phosphorylation relative to total s6rp in C media by 49% ($p=0.020$), and increased p-s6rp relative to total s6rp in NR by 51% ($p=0.004$).

Thus NR independently decreased downstream mTOR activity, as evidenced by decreased phosphorylation of s6rp, but Hx interacts with nutrients to either up-regulate or down-regulate mTOR activity based on their presence in the trophoblast cell (Figures 11 and 12). These results suggest that NR alone has slightly more severe effects on s6rp phosphorylation, and therefore mTOR activity, than does Hx alone.

DISCUSSION

The temporal response in the preliminary experiment allowed the chance to examine the effects of long-term nutrient deprivation on mTOR downstream functioning. The decrease in p-s6rp and p-4E-BP1 (and thus mTOR activity) under NR seen over time in the preliminary experiment agrees with what is known about upstream control of mTORC1: as the cells are deprived of nutrients, the energy levels of the cell decreases causing subsequent down-regulation of mTOR activity in attempts to conserve energy and survive until conditions improve.

The significant decrease in p-s6rp and significant increase in p-4E-BP1 in C media over time, however, was more puzzling. Had phosphorylation of both proteins been decreased, one could propose that the decrease was due to contact interference of the cells growing in culture, such that the HTR8/SVneo cells stopped growing when they reached a high enough confluence. The answer to this discrepancy between the temporal effects on the two proteins might possibly be hidden in s6rp's unknown function. One possible explanation is that s6rp is part of a further downstream pathway; perhaps once s6rp gets activated via phosphorylation, it activates a downstream target and subsequently gets degraded or unphosphorylated, thus causing a decrease in its presence over time.

It is important to note that the preliminary experiment was only carried out once due to time constraints, a major limitation of the preliminary experiment. It is possible that after repeating the experiment two more times, the significant differences seen in the C media condition over time would no longer exist. However, for the purposes of this study, carrying out the preliminary experiment once did not hinder progress towards deciding upon an appropriate time point for the final 2x2-factorial experiment. The large decrease of both p-s6rp and p-4E-BP1 seen at seventy-two hours in comparison with the other three time points was a compelling reason to choose this time point for the final experiment.

Measurement of 4EBP-1 was not continued in the final 2x2 factorial experiment due to uncertainty with measuring protein expression. In the preliminary experiment, p-4E-BP1 showed up as multiple bands (Figure 7), which were all quantitated as p-4E-BP1, even though verifying each of the multiple bands could not be definitively ascertained from the literature. Given that phosphorylation of s6rp and 4E-BP1 are both evidence of mTOR activity, it was decided that measuring only one of the two would suffice for the final experiment. There is a chance that

mTOR may differentially phosphorylate its downstream targets under NR and Hx, such that decreased s6rp phosphorylation is not an absolute indicator of decreased mTOR activity. However, this is quite unlikely, for mTOR only has two known downstream targets, and NR affected the phosphorylation of both.

NR alone decreased p-s6rp in the HTR-8/SVneo cell line. p-s6rp was significantly decreased within C media and within NR media when subjected to Hx. However, total s6rp expression was not affected by NR, and was only affected by hypoxia under complete media conditions. This decrease in total s6rp was only about one-fourth the decrease in phosphorylated s6rp, suggesting that although both total and p-s6rp were decreased under hypoxic conditions in complete media, Hx had a greater overall effect on s6rp phosphorylation.

Interestingly, the effect of NR was greater than the effect of Hx on phosphorylation of s6rp. Because the HTR-8/SVneo cell line came from a first trimester placental sample, and first trimester placental cells develop under Hx until proper angiogenesis occurs, it is possible that the cell line is better able to continue growth in the absence of oxygen than it is in the absence of glucose, amino acids, and other nutrients.

Our 2x2-factorial experimental model examines how a deficiency of oxygen and/or nutrients affect trophoblast growth and machinery, and thus placental development, in order to gain a better understanding of possible role players in the development of IUGR and preeclampsia. Roos et al. have proposed that placental mTOR constitutes a mechanistic link between maternal nutrient availability and fetal growth, such that mTOR integrates nutrient and other upstream signals (such as hypoxia) to control nutrient transport from mother to fetus (16). Because the inefficient transfer of nutrients and oxygen are so integrally tied under IUGR and preeclamptic insults, it was an important goal of this study to examine the interactive effects of

these two variables on mTOR functioning. The interaction found in this study is the first known time an interactive effect between NR and Hx on mTOR activity has been revealed.

The interaction between Hx and NR was such that Hx reduced mTOR downstream functioning under nutrient repletion and increased it under nutrient deprivation. Previous evidence describes hypoxia's inhibitory role on mTOR signaling (12). Given this evidence, one would suggest that Hx would have an additive affect, such that under conditions of both NR and Hx, p-s6rp would be reduced even further. While this evidence corresponds with my results on the effects of Hx in C media, it does not explain how Hx interacted with low nutrient levels to *upregulate* mTOR.

It is possible that this interactive effect provides evidence of an existing undiscovered protective mechanism in the trophoblast cell, such that Hx and NR act together to increase mTOR functioning. Fujita et al discovered *in vitro* that activation of mTOR is crucial for the production of angiogenic factors, VEGF and Endoglin, under Hx. While the *in vitro* model does not necessary demonstrate actual events occurring during early placentation or the pathogenesis of preeclampsia, their findings likely demonstrate that mTOR activity plays an important role in uterine vascular modeling under hypoxic conditions. This same reasoning may provide a possible explanation for the increased mTOR activity seen under Hx and NR. It is possible that under low nutrient conditions, hypoxia increases mTOR activation to induce activity of angiogenic factors, increase vascular remodeling and thus increase nutrient supply to the trophoblast cells. While this hypothesis is conceivable, much future research would have to be done to investigate its validity.

At the least, the interaction between NR and Hx provides further evidence that the pathways by which oxygen and nutrient availability regulate mTOR through the placenta have

yet to be fully determined. Indeed, current literature on signaling through mTOR leaves many gaps, even when not localized to placental studies. It has been shown that hypoxia signals through multiple mechanisms to regulate mTOR, some of which require further follow up (12). In an *in vivo* study on my same cell line, Wen et al. recently demonstrated that glucose signals through mTOR via the PI3K-independent GFAT pathway (17), through a yet-to-be-discovered mechanism. Given the amount that is unknown, it is not unlikely that, under the right stress conditions, hypoxia may feed into this nutrient signaling pathway.

It is important to be able to replicate the 2x2-factorial results seen here in this study, to provide evidence that these results are replicable beyond s6rp phosphorylation in the HTR8/SVneo cell line. First, the experiment should be replicated using 4E-BP1 to confirm the effects of NR and/or Hx on mTOR activity in our cell line, and see if the results seen with s6rp are obtained. Thus, an appropriate way to measure 4E-BP1 via western analysis will need to be determined. Replicating this experiment on p-4E-BP1 would be beneficial because the results may be even more dramatic, given that the maximal change of p-4E-BP1 over 72h was much greater than the maximal change of p-s6rp at 48h. The 2x2 factorial results should then be replicated on cell lines that have been deemed appropriate for modeling human placental cells *in vivo*. Obtaining the same interactive effect of NR and Hx will be further evidence for a possible protective cellular mechanism during times of decreased nutrient and oxygen availability.

Future studies should also be designed to investigate possible explanations for the interactive effects seen between NR and Hx. One possible way of designing such an experiment would be to investigate the outcome of knocking down certain proteins that are integral in known hypoxia and nutrient signaling pathways. One such experiment may be to determine the effects of a knockdown of HIF-1α, the protein responsible for inducing REDD1 expression. If, under

HIF-1α knockdown, the interactive effect of HR and Hx is still present, the mechanism by which these two conditions interactively affect mTOR activity is likely not via the HIF-1α-REDD1 Hx signaling pathway.

CONCLUSIONS

The independent effects of NR caused a more severe down-regulation of mTOR functioning than did the effects of Hx alone, as measured by phosphorylation of downstream targets s6rp and 4E-BP1. There was an interactive effect of Hx and nutrient availability, such that Hx decreased mTOR functioning in conditions of adequate nutrients and slightly increased mTOR functioning under nutrient deprivation. The interactive effect of Hx and NR suggests the need for future research to further examine unknown mechanisms by which nutrient and oxygen signaling may affect mTOR signaling. These findings, along with future research, may provide insight on how nutrient and oxygen availability contribute to placental complications such as IUGR and preeclampsia.

REFERENCES

1. Gude NM, Roberts CT, Kalionis B, King RG. Growth and function of the normal human placenta. Thromb Res 2004; 114: 397-407.
2. Burton GJ, Jauniaux E, Charnock-Jones, DS. The influence of the intrauterine environment on human placental development. Int J Dev Biol 2010; 54: 303-311.
3. Genbacev O, Zhou Y, Ludlow JW, Fisher SJ. Regulation of human placental development of oxygen tension. Science 1997; 277(5332): 1669-72.
4. Caniggia I, Winter J, Lye SJ, Post M. Oxygen and placental development during the first trimester: implications for the pathophysiology of pre-eclampsia. Placenta 2000; 21: S25-30.
5. Pardi G, Marconi AM, Cetin I. Placental-fetal interrelationship in IUGR fetuses – a review. Placenta 2002; 23, Supplement A, Trophoblast Research, 16: S136-S141.
6. Shamshirsaz AA, Paidas M, Graciela K. Preeclampsia, hypoxia, thrombosis, and inflammation. J Pregnancy 2012; 2012: 374047.
7. Corradetti MN, Guan KL. Upstream of the mammalian target of rapamycin: do all roads pass through mTOR? Oncogene 2006; 25: 6347-6360.
8. Jacinto E, Loewith R, Schmidt A, Lin S, Rüegg MA, Hall A, Hall MN. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat Cell Biol 2004; 11: 1122-1128.
9. Dunlop EA, Tee AR. Mammalian target of rapamycin complex 1: Signalling inputs, substrates and feedback mechanisms. Cell Signal 2009; 21: 827-835.
10. Hay N, Sonenberg N. Upstream and downstream of mTOR. Genes Dev 2004; 18: 1926-1945.
11. Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. Cell 2003; 115(5):577-90.
12. Cam H, Houghton, PJ. Regulation of mammalian target of rapamycin complex 1 (mTORC1) by hypoxia: causes and consequences. Targ Oncol 2011; 6:95-102.
13. Sancak Y, Peterson TR, Shaul YD et al. The rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science 2008; 320: 1496-1501.

14. Sancak Y, Sabatini DM. Rag proteins regulate amino-acid-induced mTORC1 signalling. Biochem Soc Trans 2009; 37: 289-290.

15. Inoki K, Guan K. Tuberous sclerosis complex, implication from a rare genetic disease to common cancer treatment. Hum Mol Genet 2009; 18: R95.

16. Roos S, Powell TL, Jansson T. Placental mTOR links maternal nutrient availability to fetal growth. Biochem Soc Trans 2009; 37: 295-298.

17. Wen HY, Abbasi S, Kellems RE, Xia Y. mTOR: a placental growth signaling sensor. Placenta 2005; 26 Suppl A: S63-S69.

18. Fujita D, Tanabe A, Sekijima T, et al. Role of extracellular signal-regulated kinase and AKT cascades in regulating hypoxia-induced angiogenic factors produced by a trophoblast-derived cell line. J Endocrinol 2010; 206: 131-140.

19. Graham CH, Hawley TS, Hawly RG, et al. Establishment and characterization of first trimester human trophoblast cells with extended lifespan. Exper Cell Res 1993; 206: 204-211.

20. Brannon PM, Jones S. Nutrient restriction and hypoxia independently decrease placental cell proliferation and differentially regulate Igf2, Mtd and Phlda 2 gene expression in vitro. FASEB J 2010; 24:544.2

21. Brannon PM. Personal communication, 2012.

22. Lowry, OH, Rosebrough, NJ, Farr, AL, and Randall, RJ 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

23. Gallagher, S.R. 2006. One-dimensional SDS gel electrophoresis of proteins. Curr. Protoc. Mol. Biol. 75:10.2.1-10.2A.37.

24. SigmaPlot for Windows Version 11.0 Build 11.2.0.5. Copyright 2008 Systat Software, Inc.