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AMP-Activated Protein Kinase Knockdown in Labyrinthine Trophoblast Cells Results in Altered Morphology and Function

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AMP-ACTIVATED PROTEIN KINASE KNOCKDOWN IN
LABYRINTHINE TROPHOBLAST CELLS RESULTS IN ALTERED
MORPHOLOGY AND FUNCTION

A thesis submitted in partial fulfillment of the
requirements for the degree of
Master of Science

By

ERICA ASHTON KAYLEIGH CAREY
B.S., Wright State University, 2011

2013
Wright State University

WRIGHT STATE UNIVERSITY
GRADUATE SCHOOL

May 28, 2013

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Erica Ashton Kayleigh Carey ENTITLED AMP-ACTIVATED PROTEIN KINASE KNOCKDOWN IN LABYRINTHINE TROPHOBLAST CELLS RESULTS IN ALTERED MORPHOLOGY AND FUNCTION BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Carey, Erica Ashton Kayleigh. M.S., Microbiology and Immunology Program, Wright State University, 2013. AMP-Activated Protein Kinase Knockdown in Labyrinthine Trophoblast Cells Results in Altered Morphology and Function.

The placenta is a transient organ that develops upon initiation of pregnancy and is essential for fetal development and survival. The rodent placenta consists of three layers with predominantly analogous cell types in the human placenta. The labyrinth layer, which lies closest to the fetus, facilitates nutrient and waste exchange between mother and baby. Abnormalities of the placenta may occur as a result of cellular stress and have been associated with the pregnancy-associated disorders, intrauterine growth restriction and placental insufficiency. AMP-activated protein kinase (AMPK) is a master sensor of cellular stress and changes in AMPK expression or activation could be detrimental to the cells ability to function. To study the role of AMPK in the placenta, we used RNA interference to knock down levels of

both AMPK isoforms, AMPK α 1 and AMPK α 2 in SM10 labyrinthine progenitor cells, and analyzed the effects on important cellular functions, including growth, differentiation, and nutrient transport.

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I. INTRODUCTION

Placenta

The placenta is a transient organ that develops upon initiation of pregnancy and is critical for the exchange of nutrients and wastes between the mother and the growing fetus. For studies involving the placenta, a rodent model can be used as they both exhibit hemochorial placentation with extensive remodeling of the spiral artery and similar development of the feto-maternal exchange surface [1]. In addition, the human and rodent placentas share numerous structural and functional aspects.

The rodent placenta consists of three distinct layers: trophoblast giant cells, spongiotrophoblasts, and labyrinthine cells. Each of these layers develops from trophoblast cells and has analogous cell types in the human placenta [2]. The trophoblast giant cells, which are closest to the mother, are responsible for invasion of the maternal blood supply and promoting increased blood flow to the developing fetus. The spongiotrophoblasts provide a

source of stem cells and act as a barrier between the giant cells and the labyrinth layer. Finally, the labyrinth layer, which lies closest to the fetus, fuse and come in contact with maternal blood. Through this connection with the blood supply, the labyrinthine cells can help transport nutrients and exchange wastes between the mother and the baby [3-4]. In humans, the labyrinth layer is functionally analogous to the floating chorionic villi [3].

Glucose transport

Glucose is the primary source of energy for the growing baby [5]. The fetus has very little gluconeogenic ability, and therefore relies on the mother for most of its energy supply [5]. Glucose transport through the placenta functions by way of facilitated glucose transporters (GLUT) [6]. A high density of glucose transporters along with the large surface area of the placental surface allows for rapid glucose uptake to the fetus [4]. GLUT isoforms 1, 3, and 4 have been isolated in the mouse and human placenta and are thought to play a role in the glucose transport ability of the organ [5-7]. GLUT1 has been found on both the maternal and fetal sides of the rodent placenta and functions to provide glucose for the metabolic needs of the placental cells [6]. GLUT 4 has been difficult to isolate

in the placenta but can be found in human syncytiotrophoblast, though its role has yet to be defined [5]. Finally, GLUT3 is located primarily on the fetal side of the placenta, the labyrinth layer in rodents, and is used for transport of glucose from the mother to the fetus [6]. GLUT 3, which is primarily expressed early in gestation, has been shown to be reduced in intrauterine growth restriction, IUGR, while the GLUT1 isoform remains unchanged [4,8]. Hypoxia and other forms of cellular stress have been shown to affect levels of glucose transporters in placental cells, though the mechanism is not yet defined [4].

Amino acid transport

Alteration of glucose transport ability in cells is correlated with altered levels of amino acid transport, due to changes in cellular net energy [4,9]. Two types of amino acid transport systems have been shown to account for the majority of transport in the placenta: System A and System L [10]. System A facilitates sodium-dependent uptake of non-essential amino acids such as alanine, serine, and glycine [4]. System L is not sodium-dependent and facilitates transport of neutral and essential amino acids [11]. The effects of IUGR on amino acid transport reported

in the literature varies by model and system of measurement used [8,12-13]. In cases of human placental growth restriction, an increase in system A amino acid transport has been reported to initially be increased, but then returns to normal levels later in pregnancy [4].

Pregnancy disorders

Abnormalities of the trophoblast cells in the placenta have been implicated in a number of pregnancy-related disorders such as IUGR, preeclampsia, and placental insufficiency [14]. These disorders not only can affect the health of the baby early in life, but also can cause lasting issues that persist into adulthood. While all cell types of the placenta play a key role in proper growth and development, alterations of the labyrinth may affect the ability of these cells to function properly to provide the fetomaternal interface [3]. Poor development of the placenta has been found in cases of IUGR, which leads to less branching and folding of the placenta and reduces the surface area needed for exchange of maternal and fetal nutrients and waste. In IUGR, this leads to small, undernourished babies [3,15]. Even minor defects in

placentation can have catastrophic effects on the pregnancy [1,15]. The ability of these cells to properly develop and differentiate is dependent upon receipt of signals that induce placental trophoblast maturation.

AMP-activated protein kinase

Recently, stress-activated enzymes have been reported as a possible mechanism by which placental cells are induced to mature in normal pregnancies [16-18]. Stress signaling is important in some of the first temporal events after implantation, and is important for differentiation of the trophoblast cells. One of the stress enzymes that has been studied in relation to trophoblast differentiation is AMP-activated protein kinase (AMPK). AMPK, PRKAA, or [hydroxymethylglutaryl-CoA reductase (NADPH)] kinase, is an important, evolutionarily conserved, regulator of cellular metabolism [19-24,17]. AMPK is a heterotrimeric serine/threonine kinase that is comprised of alpha, beta, and gamma subunits [19-24]. The alpha subunit of the AMPK is the catalytic subunit and exists in two isoforms depending on the cell type and intracellular localization: AMPK α 1 and AMPK α 2. The beta and gamma subunits are regulatory subunits, and the gamma subunit is responsible for the AMP or ATP binding activity of the enzyme. When the

cell is stressed, which is noted by an increase in the AMP:ATP ratio, AMPK is activated. AMPK can act at the nuclear level to turn off genes that are involved in energy-consuming anabolic processes and turns on those useful in increasing cellular ATP levels [25-26]. AMPK also functions in the cytoplasm to regulate metabolism at the enzymatic level [25-26]

AMPK has been shown to be activated in stress-inducing events, which leads to early trophoblast differentiation [27]. In the same study, application of an AMPK inhibitor (compound C) blocked maturation that would normally occur under cellular stress [27]. The stress induction of these differentiation events appears to be a normal part of post-implantation [16]. Due to the critical role of AMPK in placentation, changes in the expression levels of AMPK could cause deregulation of trophoblast maturation and lead to altered ability of the cells to function. In particular, changes in the labyrinth cell layer could lead to an inability of these cells to transport nutrients and waste between the mother and fetus.

AMPK manipulation

Because of the importance of AMPK in metabolic and stress-related regulation, certain drugs have been designed

to activate AMPK, such as AICAR (5-aminoimidazole-4-carboxamide ribonucleoside), or inhibit AMPK, in the case of compound C [22,28]. While these pharmacological compounds are effective in manipulating the levels of activated AMPK, they also have effects on other body processes, and therefore are not ideal in studying the role of the enzyme alone [29-30]. Another method of manipulating AMPK is the use of gene-ablated AMPK α 1 or AMPK α 2 mice. Though α 1 $^{-/-}$ mice and α 2 $^{-/-}$ mice can survive with only some metabolic defects, creation of a double (α 1 $^{-/-}$, α 2 $^{-/-}$) knockout mouse results in embryonic lethality at day 10.5, however the reason for lethality remains unknown [31]. In order to avoid the lethality associated with complete elimination of both AMPK isoforms, our lab has previously designed an shRNA to knock down both AMPK α 1 and AMPK α 2 levels simultaneously (Table 1, differences between sequences noted in bold print) [22]. This shRNA sequence is 100% conserved among humans, mice, and rats, and significantly reduces levels of AMPK in transduced cells [22]. This shRNA has also been shown to reduce functional activation of acetyl-CoA carboxylase (ACC), which is a direct downstream target of AMPK [22]. Using this shRNA sequence, the levels of AMPK in labyrinthine cells can be reduced in order to observe any morphological or functional effects.

Table 1. shRNA sequences

AMPK α 1&2 Loop 3 shRNA	5' - ATGATGTCAGATGGTGAATTTAAGTTCTCTAAACCT ACCATCTGACATCAT-3'
Control shRNA	5' - AC GAC CGTCAG CT GGTGC CAT GTAAGTTCTCTA CAT GC ACCAG CT GAC GT CG T -3'

SM10 cells

In this study, the mouse labyrinthine trophoblast progenitor cell line, SM10, was utilized [32-34]. These cells differentiate in the presence of physiological concentrations of transforming growth factor- β (TGF- β) [32]. Morphologically, differentiation of the labyrinth layer involves a change in cell shape and fusion of adjacent cell membranes, which occurs in the presence of TGF- β in SM10 cells [32]. For this study, the SM10 cells were transduced with lentivirus containing the AMPK shRNA sequence previously described (along with the control shRNA sequence) and stable clones were established.

Summary

The placenta is an organ that is important for the exchange of nutrients between the mother and the growing fetus during pregnancy. The ability of the trophoblast cells of the placenta to properly differentiate is important for maintaining a healthy pregnancy, and disruption of the normal differentiation pathway has been tied to numerous pregnancy-associated disorders. In this study, SM10 labyrinthine progenitor cells were transduced with lentiviral shRNA designed to simultaneously knock down levels of both AMPK alpha 1 and AMPK alpha 2. Since AMP-

activated protein kinase is an enzyme responsible for sensing the energy state of the cell and responding under situations that induce cellular stress, alterations in the expression of this enzyme might lead to changes in stress-mediated placental maturation. Malformation of the placenta has been observed in cases of IUGR and placental insufficiency. Using these transduced SM10 cells, assays such as cell growth, morphology, and nutrient transport were evaluated to determine if decreased AMPK affects these crucial labyrinth processes.

II. MATERIALS AND METHODS

Materials

Cells were cultured in RPMI-1640/L-glutamine medium (HyClone, SH30027.01) supplemented with 10% non-heat inactivated fetal bovine serum (Biowest, S01520), 1% antibiotic-antimycotic (HyClone, SV30079.01), 1mM sodium pyruvate, and 50 μ M 2 β -mercaptoethanol. TGF- β_2 was a kind gift of Dr. Steve Ledbetter (Genzyme Inc.). AMPK alpha 1 antibody was purchased from Novus Biologicals (Y365), AMPK alpha 2 antibody was purchased from GeneTex (N1C3), and Actin antibody was purchased from Seven Hills Bioreagents (LMAB-C4). Horseradish peroxidase-conjugated secondary antibodies were purchased from Promega (anti-rabbit IgG, W401B; and anti-mouse IgG, W402B). 1xRIPA buffer was purchased from Cell Signaling (#9806) and Protease Inhibitor Cocktail Tablets were purchased from Roche (#11836153001). [3 H]2-deoxyglucose, [3 H]leucine, and [3 H]methylaminoisobutyric acid was purchased from American Radiolabeled Chemicals. Hoechst dye was obtained from Sigma

(#33258) and rhodamine-conjugated phalloidin was a kind gift from Dr. Julian Gomez-Cambronero.

Cell culture and infection

The murine SM10 cell line was kindly provided by Dr. Joan Hunt (U. Kansas Medical Center) and were passaged prior to confluency and used before passage 29. Differentiation of SM10 cells was induced by the introduction of 5ng/mL transforming growth factor beta (TGF- β) for 72 hours. Creation of lentivirus containing the AMPK alpha 1 and 2 shRNA was performed using the ViraPower Lentiviral Gateway Expression Kit (Invitrogen, K4960-00). 6×10^5 293FT cells/mL were plated in 100mm culture dishes one day before transfection. Lentivirus was created following the manufacturers suggestions and SM10 cells were transduced after viral testing.

Establishment of clonal cell lines

72 hours post-infection, SM10 cells were treated with 12 ug/mL of Blasticidin (InvivoGen, ant-bl-1) for 14 days until stable pools were established. Cells from stable pools were then plated at 0.5 cells/well in a 96-well plate to generate stable clones. Cells were cultured until

resistant clones were viable under regular SM10 culture conditions and were kept in Blasticidin containing media.

Western blotting

Whole cell lysates were collected when plates were ~90% confluent in 1xRIPA buffer (Cell Signaling, #9806) with the addition of Protease Inhibitor Cocktail Tablets (Roche, #11836153001). Lysates were sonicated on ice for 15 seconds and protein concentrations were determined using the Bradford method [22,35]. Lysates were then boiled in Laemmli sample buffer [36] and 150ug of protein from each sample was run on a 10% SDS polyacrylamide gel. Proteins were then transferred to an Immobilon-P Transfer Membrane (Millipore, IPVH00010) and blocked for one hour in Tris-buffered saline (pH 7.4) containing 0.1% Tween-20 and 5% fat-free dry milk. Both AMPK alpha 1 and 2 primary antibodies were incubated overnight at 4°C at a 1:2,000 dilution in Tris-buffered saline with 0.1% Tween-20 and 5% fat-free dry milk. The actin antibody was used at a dilution of 1:20,000 for one hour at room temperature. Horseradish peroxidase-conjugated secondary antibodies were diluted to 1:25,000, and incubated for one hour at room temperature. Membranes were developed using the Super-Signal West Pico Chemiluminescent Substrate kit (Thermo

Scientific, 34080) and exposed to x-ray film (Thermo Scientific, #34091).

RT-PCR

Cells were plated at 2×10^5 cells/p60 and allowed to grow for 24 hours. 5ng/mL TGF- β was added and cells were treated for 24-72 hours to induce differentiation. RNA was collected by harvesting the cells, trypsinization, and centrifugation at 2,000 rpm for 5 minutes at room temperature. Media was removed and RNA was extracted using the RNeasy Kit (Qiagen) following the manufacturer's directions, including the addition of RNase-free DNase I for 20 minutes at room temperature. Reverse transcription was completed using 1 μ g of RNA and the Accuscript Reverse Transcriptase Kit according to the manufacturers instructions. PCR was then completed using Taq Polymerase (Fisher). Briefly, PCR was performed using 2.5 units of Taq polymerase, 1 x PCR buffer without MgCl₂, 1.8mM MgCl₂, 0.2mM dNTPs, 200nM of gene-specific forward and reverse primers, and 40nM of β -actin forward and reverse primers were coamplified in the same reaction as an internal control. Denaturation on the DNA was performed for 3 minutes at 94°C, followed by 35 cycles at 94°C for 30 seconds, primer annealing at correct melting temperature (T_m) for 30 seconds (Table 2), and elongation

Table 2. Primer sequences

Gene name:	Primer sequences:	Tm:
GLUT1	forward: 5'-GTCCTATCTGAGCATCGTGG-3' reverse: 5'-CAAGGTGAAGACTACAGTGTG-3'	55°C
GLUT3	forward: 5'-CCTTCTTTGAGATTGGACC-3' reverse: 5'-CATTGGCGATCTGGTCA-3'	53°C
GLUT4	forward: 5'-CCCACAGAAGGTGATTGAAC-3' reverse: 5'-TGAAGACCGTATTGACCACAC-3'	52°C
B-actin	forward: 5'-ATCGTGGGCCGCCCTAGGCA-3' reverse: 5'-TGGCCTTAGGGTTCAGAGGGG-3'	52-65°C

at 68°C for 45 seconds. Elongation was continued for 10 minutes at 68°C. The products were then evaluated by gel electrophoresis with DNA detection by ethidium bromide.

Glucose and amino acid transport

Glucose and system-L amino acid uptake were analyzed as previously described [33,35]. Briefly, cells were plated at 1×10^4 cells in a 24-well plate for use the following day. After overnight growth, media was removed and each well was washed 3 times with 1xPBS at 37°C. 1mL of transport buffer containing 25mM HEPES, 0.8mM $MgSO_4$, 140mM NaCl, and 1.8mM $CaCl_2$ was warmed to 37°C for each well to be tested. For glucose transport, 1 μ Ci/mL of [3 H]2-deoxyglucose was added to the prewarmed transfer buffer and for system-L amino acid transport, 1 μ Ci/mL of [3 H]leucine was added. Cells were incubated at 37°C for 10 minutes for glucose and 20 minutes for leucine. The transport buffer was then removed and each well was washed with ice-cold transport buffer 3 times. Cells were suspended using 600 μ L of 0.03% SDS and 400 μ L of the suspension was added to 4mL of Scintiverse II solution (Fisher). For system-A amino acid transport, uptake was evaluated as previously reported with modifications from glucose and leucine transport as follows [38-40]. The transport buffer used contained 137mM NaCl, 2.7mM KCl,

10.6mM Na₂HPO₄, 1.5mM KH₂PO₄, 0.49mM MgCl₂, and 0.9mM CaCl₂. Cells were incubated with 1μCi/mL of α-[³H]methylaminoisobutyric acid (MeAIB) for 20 minutes at 37°C. Samples were then counted on the tritium channel (Perkin Elmer Tricarb 2810TR) for 1 minute and counts were normalized to protein concentration.

Phalloidin staining

SM10 cells were plated at 2x10⁵ cells/p60 and allowed to grow for 24 hours. The following day the cells were treated with 5ng/mL TGF-β for 72 hours to induce differentiation. Cells were rinsed and fixed with a 4% paraformaldehyde solution in 1xPBS. Cells were then rinsed thoroughly with 1xPBS and permeabilized with a solution containing 3% goat serum, 250mM KCL, 20mM HEPES, 0.1% glycine, and 0.5% Triton X-100 in 1xPBS for 30 minutes. Cells were then incubated with 6.6μM rhodamine-conjugated phalloidin in 1xPBS for 1 hour, rinsed in 1xPBS, and incubated with 1μg/mL of Hoechst 33528 dye for 2 minutes to stain the nuclei. Coverslips were mounted on the plates and images were taken by fluorescence microscopy with MetaMorph software.

Statistical analysis

Experiments were performed a minimum of 3 independent times. Cell growth, glucose, and amino acid uptake experiments were analyzed by one-way ANOVA with Tukey-Kramer post-hoc testing, and statistical significance was determined as $p \leq 0.05$.

III. RESULTS

Generation of AMPK knockdown clones

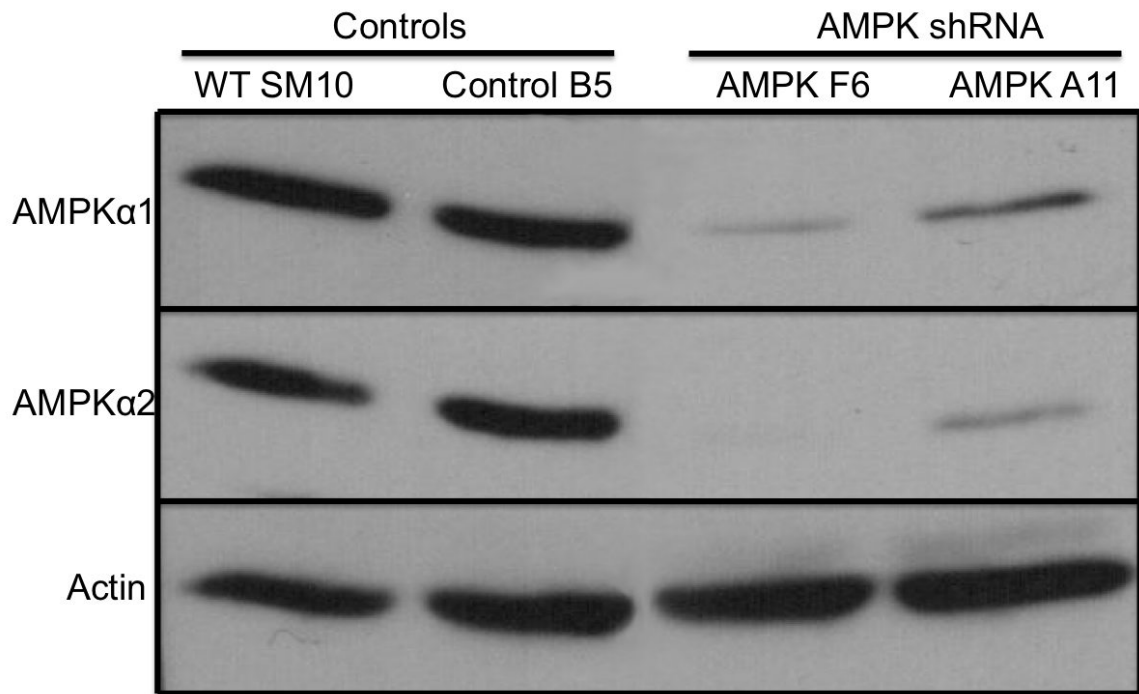
To study the effects of AMPK knockdown on the function of labyrinthine cells, SM10 mouse labyrinthine progenitor cells were transduced with lentivirus containing AMPK shRNA or a control shRNA sequence. The transduced cells were then selected and stable clones were generated. Multiple control shRNA clones were isolated, and control clone B5 was chosen for further experimentation. Two viable clones containing the AMPK shRNA were isolated. To determine the levels of AMPK α 1 and AMPK α 2 in both the control and knockdown clones, whole cell lysate was collected and SDS-PAGE performed. Western blotting was performed with AMPK α 1, AMPK α 2, and actin antibodies, with a representative blot shown in Figure 1. In the first column, wild type SM10 cells were tested for AMPK levels as a base line for comparison of control B5 as well as the AMPK knockdown clones. Control clone B5 is shown in the second column, with AMPK α 1 and AMPK α 2 levels similar to the wild type cells, confirming that the control shRNA sequence does not affect AMPK

Figure 1. AMPK knockdown in SM10 clones

Western blot showing knockdown of AMPK alpha 1 and alpha 2 subunits with actin loading control. The first column shows wild-type SM10 cells, the second shows cells infected with the control shRNA sequence clone B5, the third and fourth columns show cells infected with the AMPK alpha 1 and 2 shRNA sequence clones F6 and A11 respectively.

Representative blot shown.

Figure 1



levels. In column three, AMPK knockdown clone F6 shows dramatically reduced levels of AMPK α 1 and nearly eliminated expression of AMPK α 2. Finally, in the fourth column, AMPK knockdown clone A11 exhibits a reduction of both AMPK α 1 and AMPK α 2 levels, though not as much as clone F6. AMPK knockdown clone F6 was chosen for further experiments as it showed the highest level of AMPK knockdown.

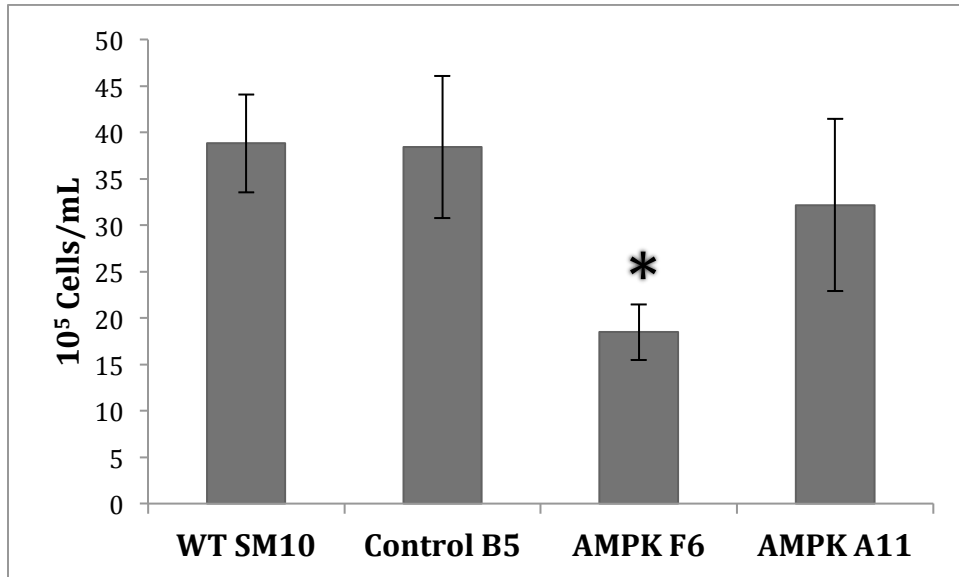
AMPK knockdown inhibits placental cell growth

To determine if AMPK reduction affects cell growth, wild type, control B5, and AMPK knockdown F6 were plated at equal cell number. After 72 hours of growth, cell number was determined as shown in Figure 2. The control clone B5 showed cell growth levels similar to the wild type cells, confirming that the presence of control shRNA does not affect the cells proliferative ability. The AMPK knockdown clone F6 had significantly less cell growth after the 72-hour period, with a 50% reduction when compared to the control clone B5. AMPK knockdown clone A11 was tested and some reduction in growth ability was examined, though not statistically significant.

Figure 2. Cell growth in AMPK knockdown clones

Viable cell number of SM10 cells. The first column represents wild-type SM10 cells, the second shows cells infected with the control shRNA sequence clone B5, the third and fourth columns show cells infected with the AMPK alpha 1 and 2 shRNA sequence clones F6 and A11 respectively. Statistical significance is denoted by (*) and has a p value of ≤ 0.05 .

Figure 2



Altered morphology of AMPK knockdown clones

In order to examine the effect of AMPK knockdown on cell morphology and fusion, wild type, control clone B5, and both AMPK knockdown clones were plated, and TGF- β was introduced to the media after 24 hours to induce differentiation (Figure 3). As has been previously shown [32-33], untreated progenitor SM10 cells are small, singular cells. Treatment with TGF- β induces differentiation and dramatic cell island formation. In the first row, wild type SM10 cells are shown in the progenitor state in the first column. In the second column, the differentiated cells are forming placental islands that are characteristic of labyrinthine trophoblast cells. The control B5 clone is shown in the second row with similar morphology to the wild type cells as expected. The third row shows the AMPK knockdown clone F6 with or without the presence of TGF- β . In the first column, the AMPK clone F6 progenitor cells appear to have the same morphology as both the wild type and control B5 cells. But with the addition of TGF- β , the cells do not exhibit the same island formation as the control cells, though they do not appear to remain in a pure progenitor state based on morphology alone, as the cells appear larger. The AMPK knockdown clone A11 shows an

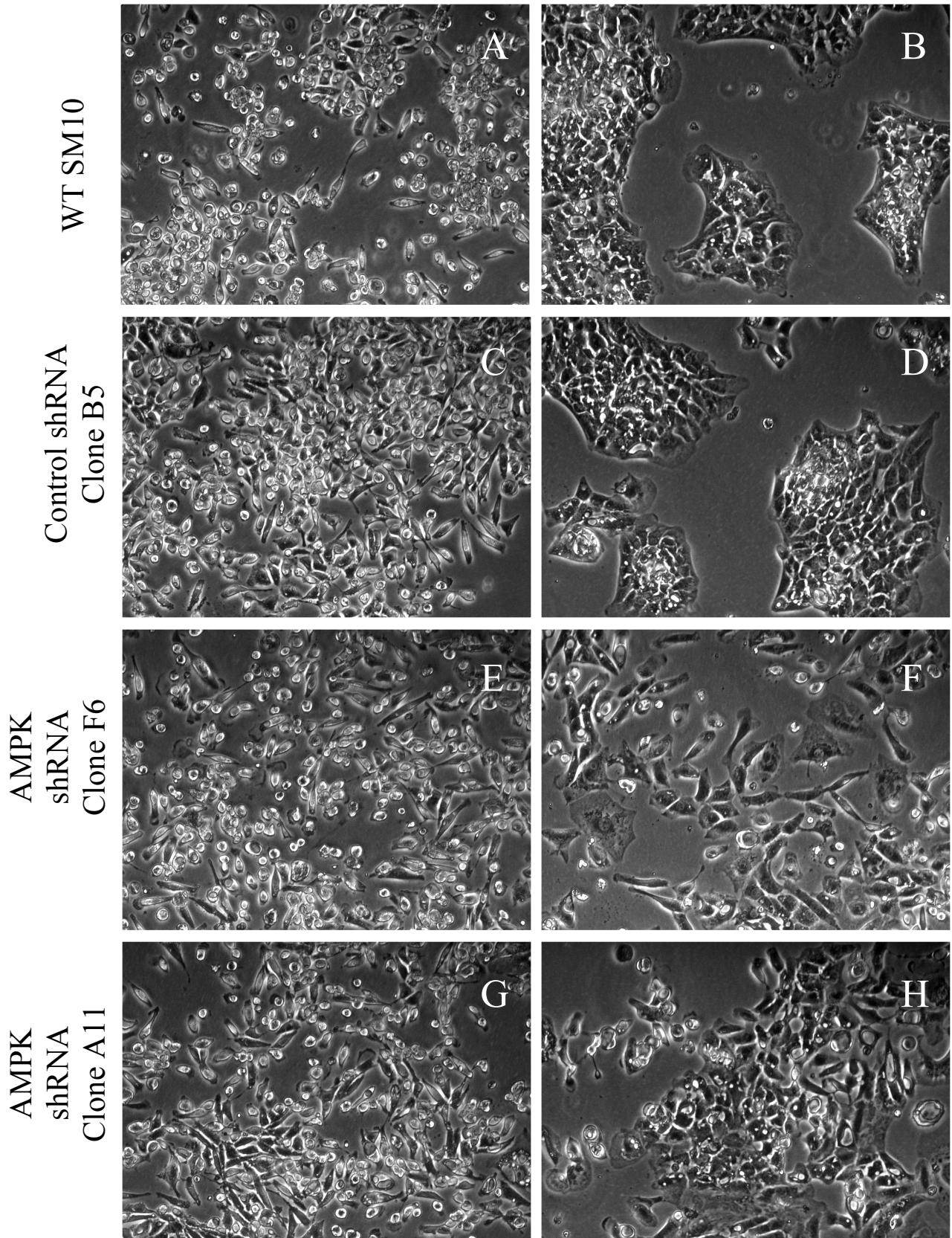
Figure 3. Morphology of AMPK knockdown clones (brightfield)

Representative images of SM10 clones with and without the addition of TGF- β after 72 hours. Wild-type SM10 cells without TGF- β (A) and with TGF- β (B), control shRNA clone B5 without TGF- β (C) and with TGF- β (D), AMPK alpha 1 and 2 shRNA sequence clones F6 without TGF- β (E) and with TGF- β (F), AMPK alpha 1 and 2 shRNA sequence clones A11 without TGF- β (G) and with TGF- β (H).

Figure 3

(-)TGF- β

(+)TGF- β



intermediate differentiated morphology, with some cell fusion, though not as much as the controls. Figure 4 utilizes rhodamine-conjugated phalloidin staining to highlight the lack of cell fusion and island formation between the differentiated control B5 and AMPK F6 cells, as well as the differences between the AMPK F6 clone in the progenitor and differentiated states.

Impaired glucose transport

To determine how AMPK knockdown affects the ability of the labyrinthine cells to transport nutrients, tritiated glucose transport assays were performed in both control B5 and AMPK knockdown F6 cells. The amount of glucose uptake was determined by liquid scintillation counting and was normalized to protein concentration. In Figure 5, in both the progenitor and differentiated states, AMPK knockdown cells have significantly less glucose transport when compared to controls. This decrease in glucose transport was more pronounced in the progenitor cells than the differentiated cells. To further look at the reason behind the impaired glucose transporter ability, levels of glucose transporter expression were analyzed via RT-PCR. In figure 6A, levels of GLUT1 were equal between the control B5 cells and the AMPK knockdown F6 cells. GLUT4 was not found to be

Figure 4. Morphology of AMPK knockdown clones (phalloidin)

Representative images of SM10 clones stained with rhodamine-conjugated phalloidin and Hoechst dye. SM10 control clone B5 cell images are in the first column and SM10 AMPK clone F6 images are in the second column. The first row shows images of cells without TGF- β and the second row shows cells in the presence of TGF- β .

Figure 4

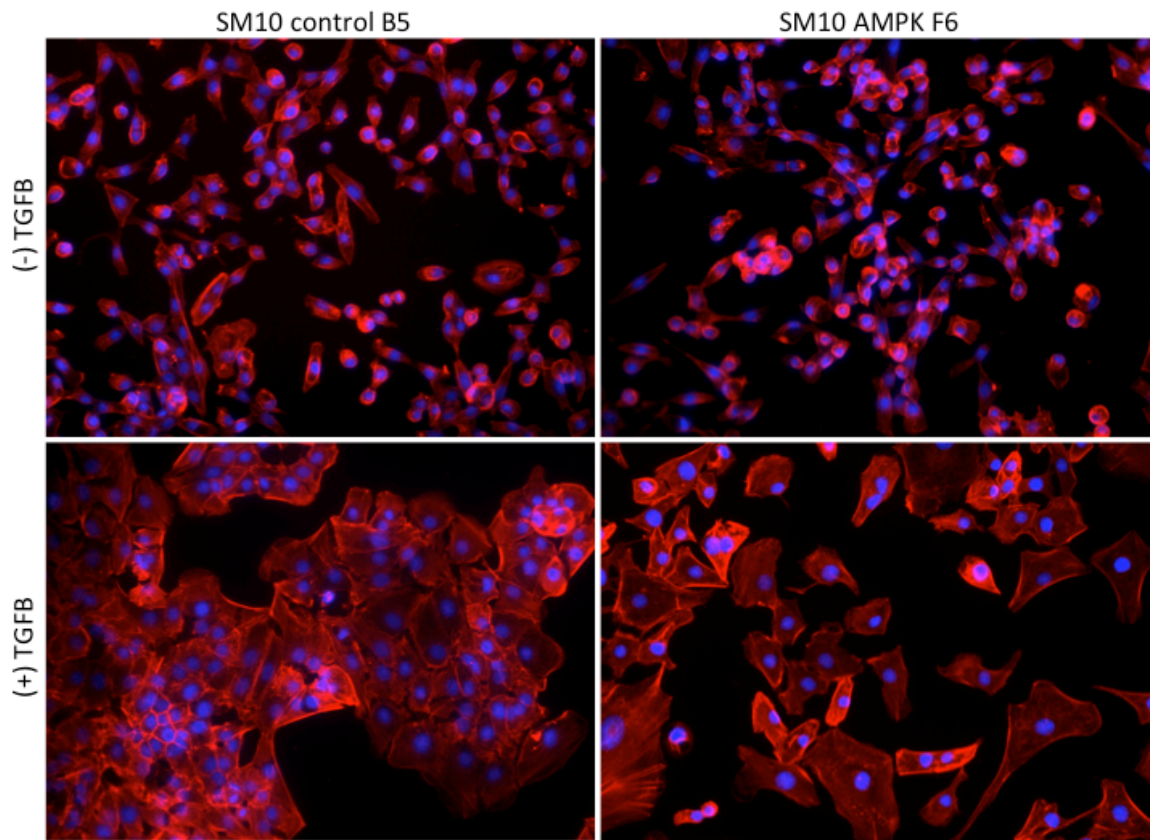


Figure 5. Glucose transport

The first two columns show SM10 control clone B5 and SM10 AMPK clone F6 without TGF- β and the second two columns show these cells in the presence of TGF- β . Glucose uptake was reported as pmol/min/ μ g and statistical significance (*) was determined by a p value ≤ 0.05 .

Figure 5

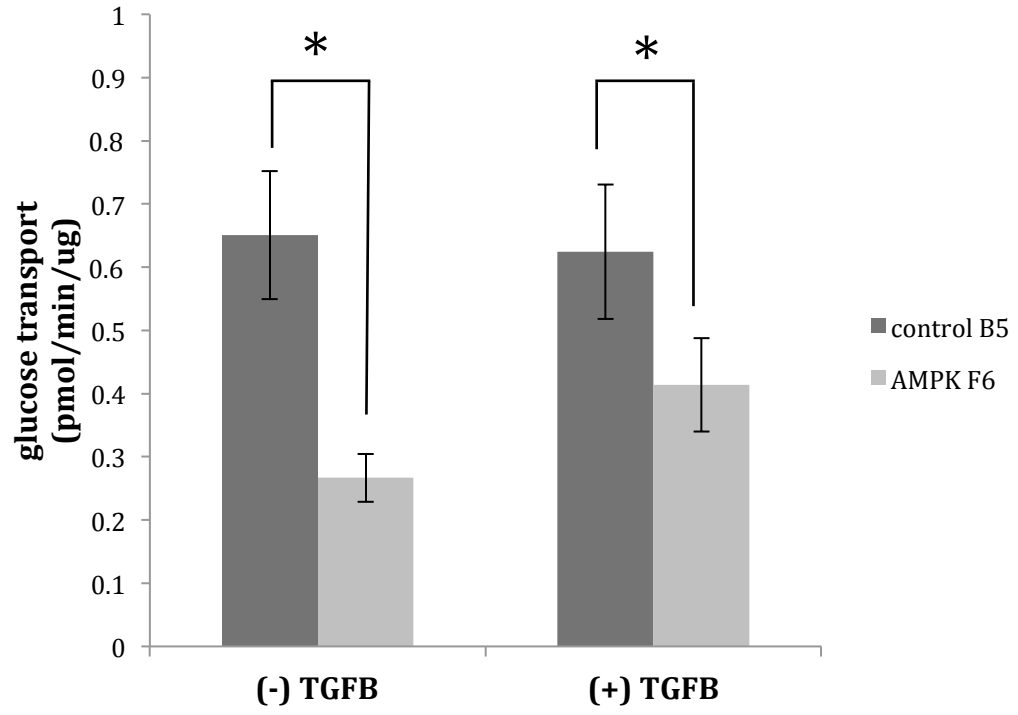
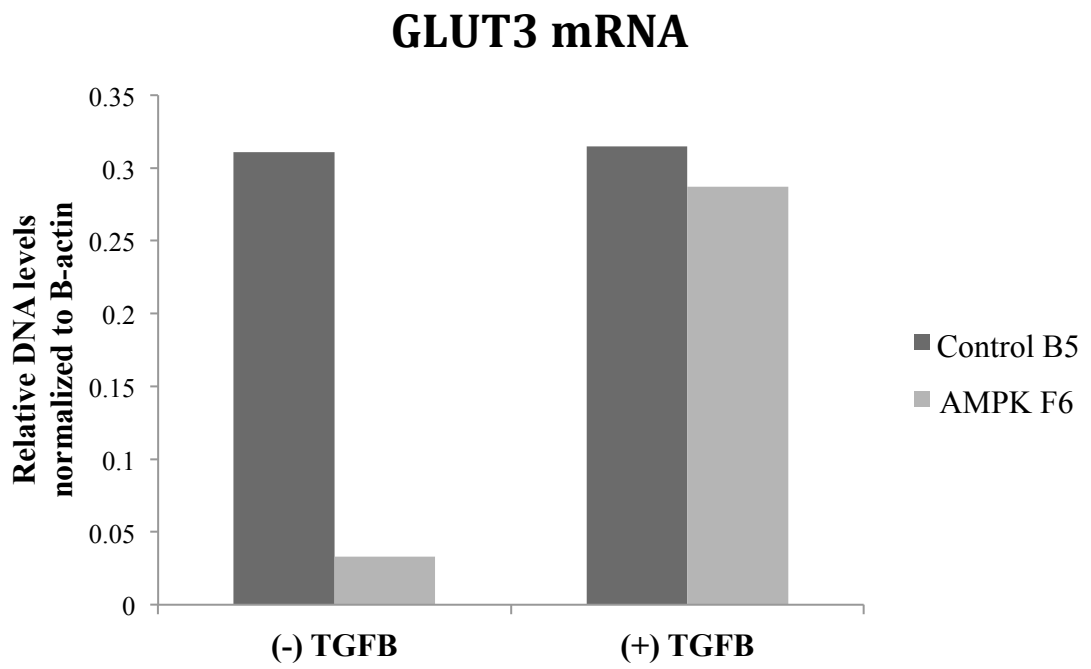
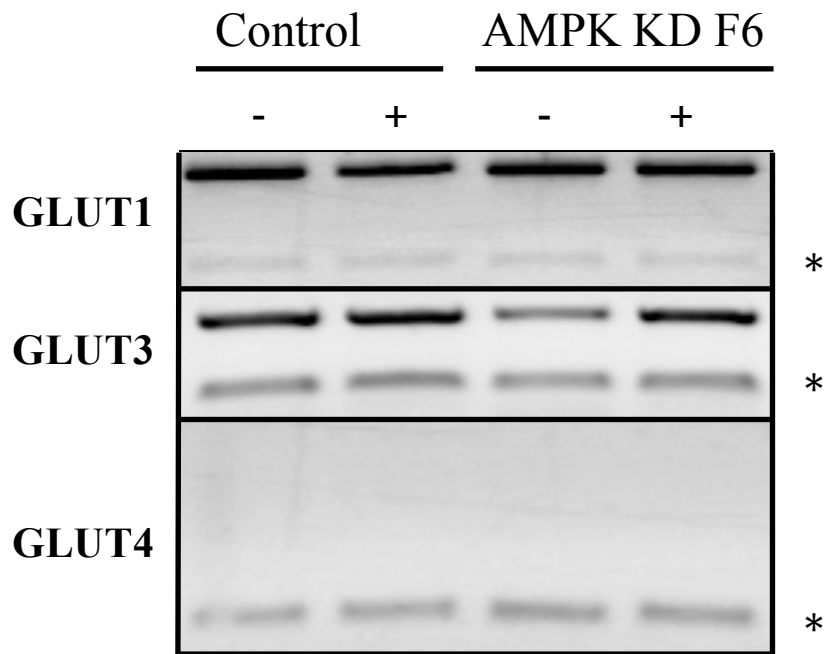


Figure 6. GLUT mRNA isoform expression

GLUT1, GLUT3, and GLUT4 glucose transporter gene expression with coamplified B-actin (-) without TGF- β and (+) with TGF- β . Relative levels of GLUT3 expression normalized to B-actin (noted by *). Data was analyzed with GIMP image software.

Figure 6



present in the SM10 cell line in either control B5 or AMPK F6 (Figure 6). Finally, GLUT3 expression was dramatically decreased in the AMPK knockdown progenitor cells when compared to the control SM10 cells (Figure 6).

System L and System A amino acid transport

Because of the importance of amino acid transport in labyrinthine cells, system L amino acid transport was determined using tritiated leucine (Figure 7) and system A transport using tritiated methylaminoisobutyric acid (Figure 8). In both the control cells and AMPK knockdown cells, addition of TGF- β does not alter system L transport. In the undifferentiated and differentiated state there is not a statistically significant difference in system L transport ability between the control B5 cells and AMPK knockdown F6 cells. In both control and AMPK knockdown cells, addition of TGF- β increases system A amino acid transport ability. AMPK knockdown cells have significantly more system A transport activity in both the undifferentiated and differentiated state when compared to controls.

Figure 7. System L transport

The first two columns show SM10 control clone B5 and SM10 AMPK clone F6 without TGF- β and the second two columns show these cells in the presence of TGF- β . System L uptake was reported as pmol/min/ μ g and statistical significance (*) was determined by a p value ≤ 0.05 .

Figure 7

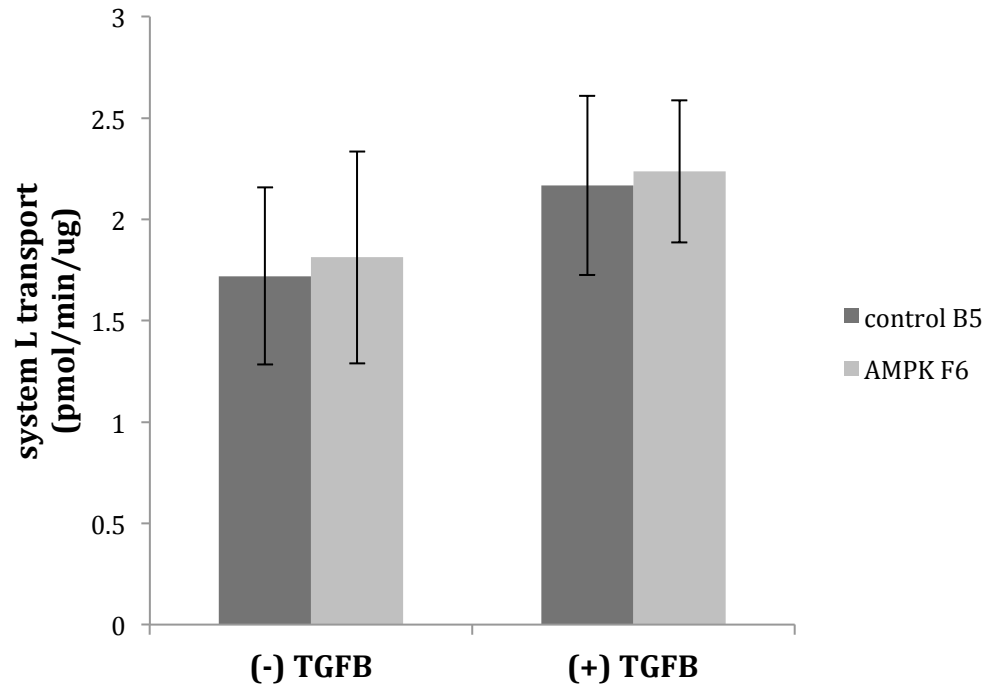
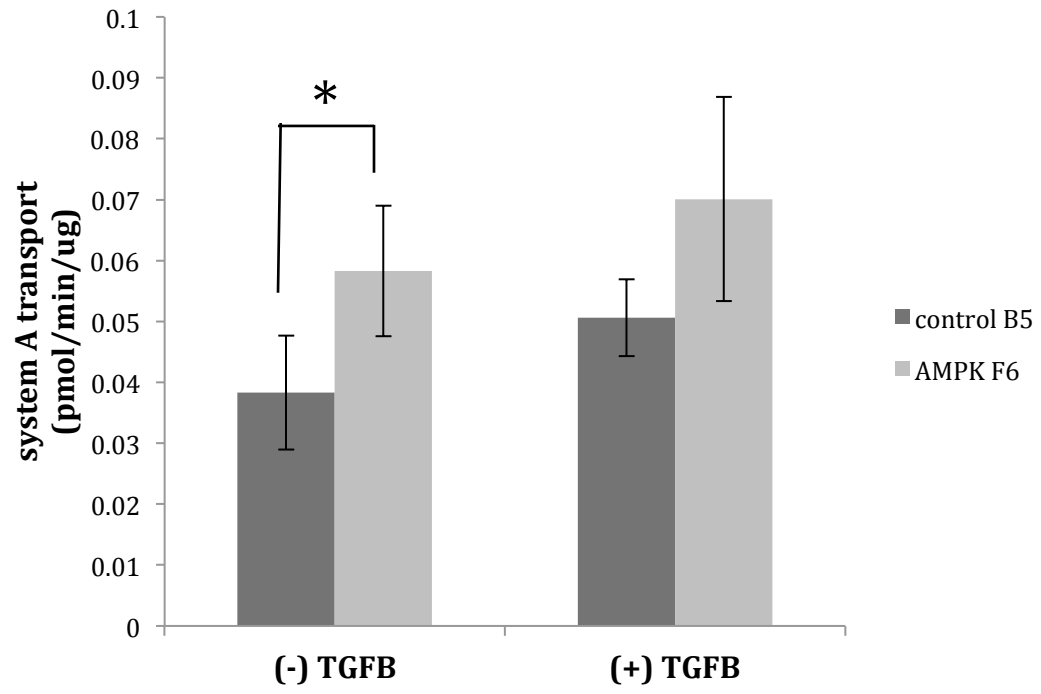


Figure 8. System A transport

The first two columns show SM10 control clone B5 and SM10 AMPK clone F6 without TGF- β and the second two columns show these cells in the presence of TGF- β . System A uptake was reported as pmol/min/ μ g and statistical significance (*) was determined by a p value ≤ 0.05 .

Figure 8



IV. DISCUSSION

Changes in growth and morphology

In this study we have demonstrated that knock down of AMPK alpha subunits in SM10 labyrinthine progenitor cells leads to dramatically altered morphology and function. This cell line is derived from the layer of the rodent placenta that is primarily involved in transport of nutrients, such as glucose and amino acids, between the mother and the growing baby [3]. Proper development of the placenta is crucial for maintaining a healthy pregnancy, and improper formation of any of the trophoblast layers could lead to reduced or altered transport of nutrients. Reduced supply of nutrients to the fetus has been implicated in pregnancy-associated disorders such as IUGR, preeclampsia, and placental insufficiency [4].

SM10 cells are proliferative placental progenitor cells and the ability to continue to grow and replicate are important for ensuring the placenta is well-formed and the flow of nutrients is uninterrupted. This study shows that

when AMPK is knocked-down in SM10 cells, there is a significant decrease in cell growth, suggesting impaired proliferative ability. Since AMPK is a critical regulator of cellular metabolism, it follows that changes in the level of active AMPK enzyme would change the cellular metabolism. AMPK is activated during cellular stress and signals for cells to stop using energy and begin producing ATP in order to replenish the cells energy stores [31,40]. In the absence or reduction of AMPK, this signaling pathway ceases and the cells continue to remain in a low-energy state. The lack of ATP to fuel processes such as growth and proliferation likely leads to a reduction of cellular growth in the AMPK knockdown SM10 cells.

SM10 cells, in the presence of physiological levels of TGF- β , differentiate into mature labyrinthine cells. During this process, neighboring cells fuse and form placental islands [3]. Our data shows that AMPK knockdown leads to a decreased ability for these cells to fuse and form placental islands. This morphological change is important because proper fusion of the trophoblast cells in the labyrinth allows for enhanced transport ability between the cells. An alteration of the morphology of these cells could lead to a decrease in the ability of these cells to

transport nutrients from the mother to the fetus.

Altered function

Our study indicates that AMPK knockdown in SM10 cells leads to a significant reduction in glucose transport ability. This reduction is found in both the progenitor and differentiated state, with the progenitor cells displaying a more dramatic difference in transport ability than the differentiated cells. Analysis of mRNA levels of the glucose transporters of these cells shows no change in GLUT1 but a large decrease in GLUT3 levels. In normal cells, when the energy of the cell is low, AMPK is known to activate genes that are responsible for increasing the glucose uptake ability of the cells to replenish the ATP pool. Since AMPK is a metabolic regulator and is known to affect transcription of genes involved in glucose transport, a reduction of AMPK that leads to a decrease in GLUT3 being produced is not unexpected.

Our study shows no change in system L transport ability with AMPK reduction, but a significant increase in system A transport. This increase in system A is consistent with the work of Ganguly et al., in which they found a decrease in GLUT3 leads to an increase in system A transport [9,41]. It is possible that this increase in

system A transport in compensating for the lack of glucose in the system because alanine, one of the amino acids transported by system A, can be converted to pyruvate via alanine transaminase in the placental cells and used to fuel the TCA cycle in order to restore energy levels of the cells [42] This increase in system A activity has been observed in placentas of pregnancies with intrauterine growth restriction [4].

For both the glucose and amino acid experiments, a larger alteration of function is seen in the progenitor state compared to the differentiated cells. Because differentiation should not cause silencing of the shRNA, as determined experimentally by the continued resistance to blasticidin, another cause must be determined. TGF- β is the growth factor that has been shown to induce differentiation in both SM10 labyrinthine cells as well as in the functioning placenta [32]. Another downstream target of TGF- β is AMPK, by which TGF- β induces AMPK activity [43]. This TGF- β -induced activation of AMPK might increase remaining AMPK levels in the SM10 knockdown cells, which would reduce the observed functional effects of the AMPK reduction. Further research into the effects of TGF- β on AMPK activation in the placenta will be important to completely understand this interaction.

Future directions

The results of this study show the morphological and functional changes that result from simultaneous AMPK α 1 and α 2 knock down in the labyrinthine cells of the mouse placenta. To determine if these changes are confined to the nutrient transport layer or all cells of the placenta, AMPK knock down should be observed in spongiotrophoblast cells and trophoblast giant cells. Rcho-1 cells are trophoblast giant cell progenitors and can be used for studying the giant cell layer of the mouse placenta [44-45]. The giant cells are responsible for invasion of the maternal spiral artery and defects have been implicated in preeclampsia [3]. Hypoxia-inducible factor-1 (HIF-1) is speculated to be involved in the remodeling of the maternal spiral artery and research into the effects of HIF-1 overexpression on placental development and preeclampsia is currently underway [46-47, Brown (data not shown)]. AMPK has been identified as an upstream regulator of HIF-1 activity under hypoxic conditions [26]. Therefore, using the Rcho-1 cell line to observe the effects of AMPK on the giant cell layer might give insight into the role of this gene in HIF-1 induction and preeclampsia.

Because of the dramatic morphological and functional changes observed in SM10 labyrinthine cells, using

lentiviral AMPK shRNA to look at placental AMPK knock down *in vivo* would be useful in determining if this gene reduction results in a pathological phenotype. This could be done by infecting embryos at the blastocyst stage with the shRNA-containing lentivirus and then implanting the transduced embryo into a surrogate mother. By infecting at the blastocyst stage, this insures that only the trophoctoderm, which will later form the placenta, is infected and not the inner cell mass (ICM), which forms the baby [48-49, Brown (data not shown)]. Infection of the ICM would likely lead to embryonic lethality as seen in the transgenic knockout mice. The placenta can then be observed for any changes in the structure of the layers and the babies can be measured to detect changes in fetal birth weight, which might indicate IUGR. These placental AMPK knockdown mice can then be injected with radiolabeled glucose and amino acids to determine if the same alterations of transport ability seen in SM10 cells is observed *in vivo*.

With the information documented in this study, it is possible to speculate that AMPK knockdown in labyrinthine progenitor cells will lead to decreased cell growth, fusion, and nutrient transport ability of labyrinthine

cells *in vivo*. These effects in the placenta could lead to an impaired ability to transport nutrients to the growing fetus. Since decreased nutrient transport is found in many placental disorders, namely IUGR, alteration of AMPK levels in the placenta could be the culprit. Further research as to the effects of AMPK knock down *in vivo* could help to identify a genetic reason behind these pregnancy-associated disorders and open a pathway to a possible cure in the future.

V. APPENDIX

Abbreviations

ACC- acetyl-CoA carboxylase
AICAR- 5-aminoimidazole-4-carboxamide ribonucleoside
AMP- adenosine monophosphate
AMPK- 5'-adenosine monophosphate-activated protein kinase
ATP- adenosine triphosphate
GLUT- glucose transporter
HIF-1- hypoxia-inducible factor-1
IUGR- intrauterine growth restriction
PBS- phosphate buffered saline
RT-PCR- reverse transcription polymerase chain reaction
shRNA- short hairpin ribonucleic acid
TGF- β - transforming growth factor beta

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