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High altitude regulates the expression of AMPK pathways in human placenta

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ABSTRACT

Introduction: High-altitude (>2500 m) residence augments the risk of intrauterine growth restriction (IUGR) and preeclampsia likely due, in part, to uteroplacental hypoperfusion. Previous genomic and transcriptomic studies in humans and functional studies in mice and humans suggest a role for AMP-activated protein kinase (AMPK) pathway in protecting against hypoxia-associated IUGR. AMPK is a metabolic sensor activated by hypoxia that is ubiquitously expressed in vascular beds and placenta.

Methods: We measured gene expression and protein levels of AMPK and its upstream regulators and downstream targets in human placentas from high (>2500 m) vs. moderate (~1700 m) and low (~100 m) altitude.

Results: We found that phosphorylated AMPK protein levels and its downstream target TSC2 were increased in placentas from high and moderate vs. low altitude, whereas the phosphorylated form of the downstream target translation repressor protein 4E-BP1 was increased in high compared to moderate as well as low altitude placentas. Mean birth weights progressively fell with increasing altitude but no infants, by study design, were clinically growth-restricted. Gene expression analysis showed moderate increases in *PRKAG2*, encoding the AMPK γ2 subunit, and mechanistic target of rapamycin, *MTOR*, expression.

Discussion: These results highlight a differential regulation of placental AMPK pathway activation in women residing at low, moderate or high altitude during pregnancy, suggesting AMPK may be serving as a metabolic regulator for integrating hypoxic stimuli with placental function.

1. Introduction

The chronic hypoxia of high-altitude (>2500 m) residence increases the risk of the pregnancy complications of intrauterine growth restriction (IUGR) and preeclampsia [1–3]. Contributing to these vascular disorders of pregnancy is a lesser pregnancy-dependent rise in uterine artery blood flow that precedes the altitude-associated reduction in fetal growth [4]. IUGR and preeclampsia at low altitudes are also often accompanied by uteroplacental hypoperfusion [5–7] and placental hypoxia [8].

Highland ancestry confers protection against the increased incidence of IUGR observed at high altitude. Among infants born to Andean high-

Abbreviations: 4E-BP1, translation repressor protein 4E-BP1; ACC1, acetyl-CoA carboxylase alpha; ADIPOR, adiponectin receptor; Akt, serine/threonine-protein kinase akt; AMPK, AMP-activated protein kinase; CAMKK2, calcium/calmodulin-dependent protein kinase kinase 2; INSR, insulin receptor; IUGR, intrauterine growth restriction; LEPR, leptin receptor; LKB1, liver kinase B1; mTOR, mechanistic target of rapamycin; PGC-1, peroxisome proliferator-activated receptor gamma coactivator 1; Raptor, regulatory associated protein of mTOR; S6K, ribosomal protein S6 kinase; TSC2, tuberin; p53, tumor protein p53.

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altitude residents, for instance, only 13% were small for gestational age, compared to 33% of infants born to women of European ancestry [9,10]. The fetal-growth protection afforded by Andean ancestry is accompanied by enhanced uteroplacental blood flow and oxygen delivery during pregnancy [9,11,12]. Implicating the involvement of genetic rather than developmental factors, no such protection was seen in women of European ancestry who were born and raised at high altitude [11], and we have previously reported several single nucleotide polymorphisms (SNPs) showing evidence of natural selection in Andeans that were positively associated with birth weight and uterine artery diameter at high altitude [13]. One such SNP, located approximately 50 kb upstream of PRKAA1, which encodes the catalytic subunit of AMP-activated protein kinase (AMPK), was also associated with pathways related to cellular metabolism and homeostasis that play key roles in the pathophysiology of IUGR [13].

AMPK, an ubiquitous protein kinase comprised of a catalytic subunit α with two isoforms (α1 and α2) and two regulatory subunits (β1-2, γ1-3), is a central regulator of metabolic homeostasis [14] that also affects vascular growth and function [15–18]. AMPK integrates diverse extracellular or intracellular stimuli (e.g., hypoxia, AMP/ATP levels) into a wide variety of cellular responses, such as decreased protein synthesis, and increased fatty acid oxidation and glucose uptake [19,20]. In human placental explants, AMPK activation has been shown to reduce fatty acid uptake and glycolysis and to induce mitochondrial toxicity at low altitude [21], suggesting that AMPK activation may also be a central regulator of placental function.

Although AMPK is positioned as a metabolic regulator for integrating hypoxic stimuli and placental function, the effect of chronic maternal hypoxia on AMPK activation in human placenta remains unclear. One previous report showed no differences in placental expression of total or phosphorylated AMPK (pAMPK) between women residing at high altitude (3100 m) vs. sea level [22]; however, the number of high-altitude placentas tested was small (n = 3) and AMPK protein levels were highly variable between subjects. Another study showed that high-altitude residence increased AMPKα mRNA expression in whole human placenta [23]. Studies in pregnant mice under hypoxic conditions revealed similar results as well. One report showed that hypoxia augmented AMPK phosphorylation in the placental labyrinthine zone [23], while a subsequent one showed the opposite effect in murine placental homogenates [24]. Such variation suggests that differences among animal models, types of tissues, and the duration or magnitude of hypoxic exposure have dissimilar effects on AMPK activation. We recently reported that *in vivo* treatment with the AMPK activator, AICAR, prevented half of the hypoxia-elicited decrease in murine fetal growth [24], strongly implicating a role of AMPK in the regulation of fetal growth under hypoxic conditions.

In the present study, we sought to determine whether placental AMPK signaling was augmented by the chronic hypoxia of high-altitude residence in the absence of clinically-defined IUGR. To accomplish this, we compared the levels of total and phosphorylated protein and mRNA transcription for AMPK, key upstream regulators and downstream targets, between placental villous homogenates obtained from healthy women delivering by Cesarean section or vaginal delivery at either low altitude (~100 m), moderate altitude (~1700 m) or high altitude (>2500 m). Localization of AMPK protein expression within the placenta was also assessed. Our results highlight a potentially novel role for AMPK for serving as a link between metabolic sensing and placental function in pregnancies at high altitude.

2. Materials and methods

2.1. Human subjects

Sixty-four pregnant women residing at low (~100 m, n = 18), moderate (~1700 m, n = 23) or high altitude (>2500 m, n = 23) and delivering by vaginal delivery or Cesarean section were included for analysis. Inclusion criteria were maternal age between 18 and 45 years, a pre-pregnant body mass index (BMI) less than 30 kg/m^2, no known risk factors for IUGR (i.e., diabetes [gestational, type 1 or type 2], chronic hypertension, gestational hypertension, preeclampsia, fetal congenital anomalies or smoking), no prior pregnancy complicated by IUGR or preeclampsia, and maternal residence at their respective altitude. Additionally, for the high-altitude group, women who spent more than two weeks (cumulatively) at lower altitudes during pregnancy were excluded. Recruitment, delivery and sample collection occurred at either the University of California San Diego Health (La Jolla, CA, 88 m), University of Colorado Hospital (Aurora, CO, 1640 m), or St. Anthony's Summit Medical Center (Frisco, CO, 2793 m) for low-, moderate- and high-altitude subjects, respectively. Demographic information, health and obstetric history, place of birth and childhood, labor and delivery information and newborn characteristics were obtained from medical records or self-reported questionnaire.

All subjects gave their written informed consent to study procedures approved by the University of California San Diego Institutional Review Board (Approval No. 090652), the University of Colorado Multiple Institutional Review Board (Approval No. 14–2178) or the Catholic Health Initiative Institute for Research and Innovation Institutional Review Board (Approval No. 1310). Our study was conducted in compliance with the principles outlined in the Declaration of Helsinki.

2.2. Tissue sampling

Placental biopsies were obtained and processed per established protocols [22]. Briefly, after removal of the basal plate, a 500 mg sample of exposed villous tissue was collected at a minimum of four randomly-selected sampling sites that were approximately midway between the placenta margin and cord insertion, and rinsed in ice-cold phosphate-buffered saline (PBS). At least four, 50 mg tissue sections were placed in 1 ml *RNAlater* for 48 h at 4 °C and then stored at −80 °C until processing. Since >45 min between placental sample collection and processing has been reported to affect protein phosphorylation [25], we completed processing of our as soon as the placenta was cleared for release by medical staff and within, on average, 30 ± 4 min after delivery. Additionally, we tested whether our processing times impacted the placental protein expression of total AMPK or pAMPK, and found no difference between processing times ranging from 10 to 60 min after delivery (Supplemental Figure S1). For immunohistochemistry, a full-thickness section of the placenta was washed in ice-cold PBS, placed in 4% paraformaldehyde, and processed for histology.

2.3. Protein extraction

Placental villous tissue (~20–30 mg/sample) was homogenized in ice-cold RIPA buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and 5 mM sodium vanadate) supplemented with 2x Halt Protease and Phosphatase Inhibitor cocktail (#1861281; Thermo Fisher Scientific, Waltham, MA) using a TT-30K Digital Handheld Homogenizer (Hercuvan Lab Systems, Cambridge, UK). Total protein concentration was determined by bicinchoninic acid analysis (Pierce BCA Protein Assay; Thermo Fisher Scientific) using a 1:10 dilution for each sample.

2.4. Western capillary electrophoresis

AMPK pathway proteins in placental villous tissue homogenates (1 mg/ml) were assessed by determining total AMPK and pAMPK protein expression, key upstream regulators and downstream targets using the Western capillary electrophoresis (WES) method (ProteinSimple, San Jose, CA). Primary antibodies and dilutions used are listed in Supplemental Table S1. We only reported the expression of the phosphorylated forms of liver kinase protein B1 (LKB1) and tuberin (TSC2) given that normalized values for total LKB1 and TSC2 were highly variable

between subjects and we were predominantly interested in the phosphorylated (activated) form of these proteins as an indicator of AMPK activity.

2.5. Immunohistochemistry

Full-thickness, paraffin-embedded placental specimens were sectioned (5 μm), adhered to slides, deparaffinized with xylenes and rehydrated. Slides were then treated with an antigen unmasking, citrate-based solution (Vector Laboratories, Burlingame, CA) for antigen retrieval and stained with either anti-AMPKα antibody or anti-phosphorylated-AMPKα antibody (Supplemental Table S1), and SignalStain Boost IHC Detection Reagent (HRP, goat anti-rabbit), then mounted and imaged with a confocal microscope (Olympus, Waltham, MA).

2.6. RNA extraction and gene transcription analysis

Gene expression analysis was performed on total RNA that had been extracted from human placental villous tissue homogenates using the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany). Quality control to assess RNA quantity and integrity was performed using Nanodrop spectrophotometry and DNA/RNA chips on the Agilent Bioanalyzer (Agilent 2100 Bioanalyzer and RNA 6000 Nano LabChip; Agilent Technologies, Inc., Santa Clara, CA). All samples included for analysis had an RNA Integrity Number (RIN) score of 7.0 or above. cDNA was synthesized from RNA using SuperScript III (Thermo Fisher Scientific). cDNA and RT^2 SYBR® Green qPCR Mastermix were prepared for real-time polymerase chain reaction (PCR) gene expression assays using a custom RT2 Profiler PCR Array (Qiagen) and Viaa7 real-time PCR system (Thermo Fisher Scientific). AMPK pathway genes included on the custom array are shown in Supplemental Table S2. Beta-2-microglobulin was used as the reference gene. Relative gene expression was determined through the ΔΔ cycle threshold (C_t) method [26]. C_t values, the number of PCR cycles required for fluorescent signal to exceed background levels, are inversely proportional to the amount of target nucleic acid present in the sample [26]. ΔC_t values were calculated by subtracting the C_t value for the reference gene, beta-2-microglobulin, from the C_t value for each target gene for a given sample.

2.7. Statistical analysis

After evaluation of data normality (Kolmogorov-Smirnov test), demographic characteristics were analyzed by one-way ANOVA or Chi-square test, as appropriate. Data obtained from Western capillary electrophoresis were extracted and quantified using Biotechne's Compass software. Placenta protein and gene expression, expressed as normalized protein abundance or ΔC_t values, respectively, were compared between low-, moderate- and high-altitude subjects using one-way ANOVA followed by Bonferroni's multiple comparisons. For gene expression, ΔC_t values were plotted and noted to be approximately normally distributed. Simple linear regression analyses were conducted to determine the relationship between expression of key AMPK pathway genes and birth weight. To determine whether altitudinal differences in placental protein expression levels were affected by fetal sex, mode of delivery or gestational age at delivery, estimated marginal means were compared between altitudes after adjusting for these covariates using univariate general linear models (GLM). A two-sided *P value* < 0.05 was taken as evidence of association or difference, and trends ("non-significant changes") were considered when a two-sided *P* value fell between 0.05 and 0.10. All statistical analyses were computed in GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA) or SPSS 26 (IBM, Armonk, NY).

3. Results

3.1. Maternal and infant characteristics

As shown in Table 1, maternal age at delivery, height, pre-pregnant BMI, parity and ethnicity were similar between altitudes. Compared to low or moderate altitudes, Cesarean section rates were greater at high altitude. No altitudinal differences in gestational age, length, head circumference, ponderal index and sex were observed. However, high- compared to moderate- and low-altitude residence reduced birth weight by 8% and 6%, respectively. None of the infants included in this study were clinically diagnosed as IUGR or small for gestational age as defined by < the 10th percentile for gestational age and sex [27].

3.2. Placental AMPK protein expression is reduced, and phosphorylation increased by high altitude

Villous placental homogenates from high-altitude subjects showed lower total AMPK protein levels compared to moderate-altitude residents, and non-significantly increased protein levels in moderate- compared to low-altitude residents (Fig. 1A and B). Phosphorylated AMPK protein was higher at moderate than the other altitudes, and at high compared to low altitude (Fig. 1A and B). Consequently, the pAMPK:AMPK ratio was higher at both moderate and high altitudes compared to low altitude (Fig. 1B). AMPK and pAMPK staining were present in villous as well as chorionic plate tissue from low-, moderate- and high-altitude placentas, localizing in vascular cells and syncytiotrophoblasts in the villous tissue and the chorionic plate arteries (Fig. 1C and D).

3.3. Placental AMPK pathway protein expression varied by altitude

The protein expression of pLKB1, an upstream regulator of AMPK, was non-significantly higher in high vs. low altitudes (Fig. 2A). Moreover, the phosphorylated form of the downstream AMPK target pTSC2 was increased in high- or moderate-vs. low-altitude placentas (Fig. 2B).

Table 1
Maternal and infant characteristics.

Characteristic[a]	Altitude			*P* value[b]
Maternal	100 m (n = 18)	1700 m (n = 23)	>2500 m (n = 23)	
Age at delivery (y)	33.2 ± 1.3	31.2 ± 1.3	31.2 ± 1.2	0.4683
Height (cm)	166.2 ± 1.6	166.1 ± 1.4	164.6 ± 1.4	0.6648
Pre-gravid BMI[c] (kg/m^2)	23.5 ± 0.5	24.3 ± 0.6	24.1 ± 0.8	0.7388
Parity (no. of live births)	1.9 ± 0.3	1.9 ± 0.2	2.0 ± 0.2	0.8228
Ethnicity (% of Hispanic)	22.2	21.7	22.7	0.9968
Cesarean section (%)	33.3	47.8	73.9	**0.0290**
Infant				
Gestational age (weeks)	39.6 ± 0.3	39.6 ± 0.2	39.3 ± 0.2	0.4445
Birth weight (g)	3461 ± 99	3545 ± 73	3256 ± 68	**0.0277**
Adjusted birth weight (g)[d]	3479 ± 79	3518 ± 70	3267 ± 70	**0.0340**
Length (cm)	50.6 ± 1.1	50.3 ± 0.4	50.0 ± 0.5	0.1827
Head circumference (cm)	35.2 ± 0.9	35.3 ± 0.3	34.7 ± 0.2	0.3527
Ponderal Index	2.8 ± 0.3	2.8 ± 0.1	2.6 ± 0.1	0.5613
Sex (% female)	61.1	47.8	45.5	0.5774

[a] Values are means ± SEM, unless noted.
[b] *P* values were estimated by two-tailed Student's *t*-test or Fisher's test.
[c] BMI, body mass index.
[d] Birth weights were adjusted for gestational age at the time of delivery and fetal sex and compared between groups using analysis of covariance (ANCOVA).

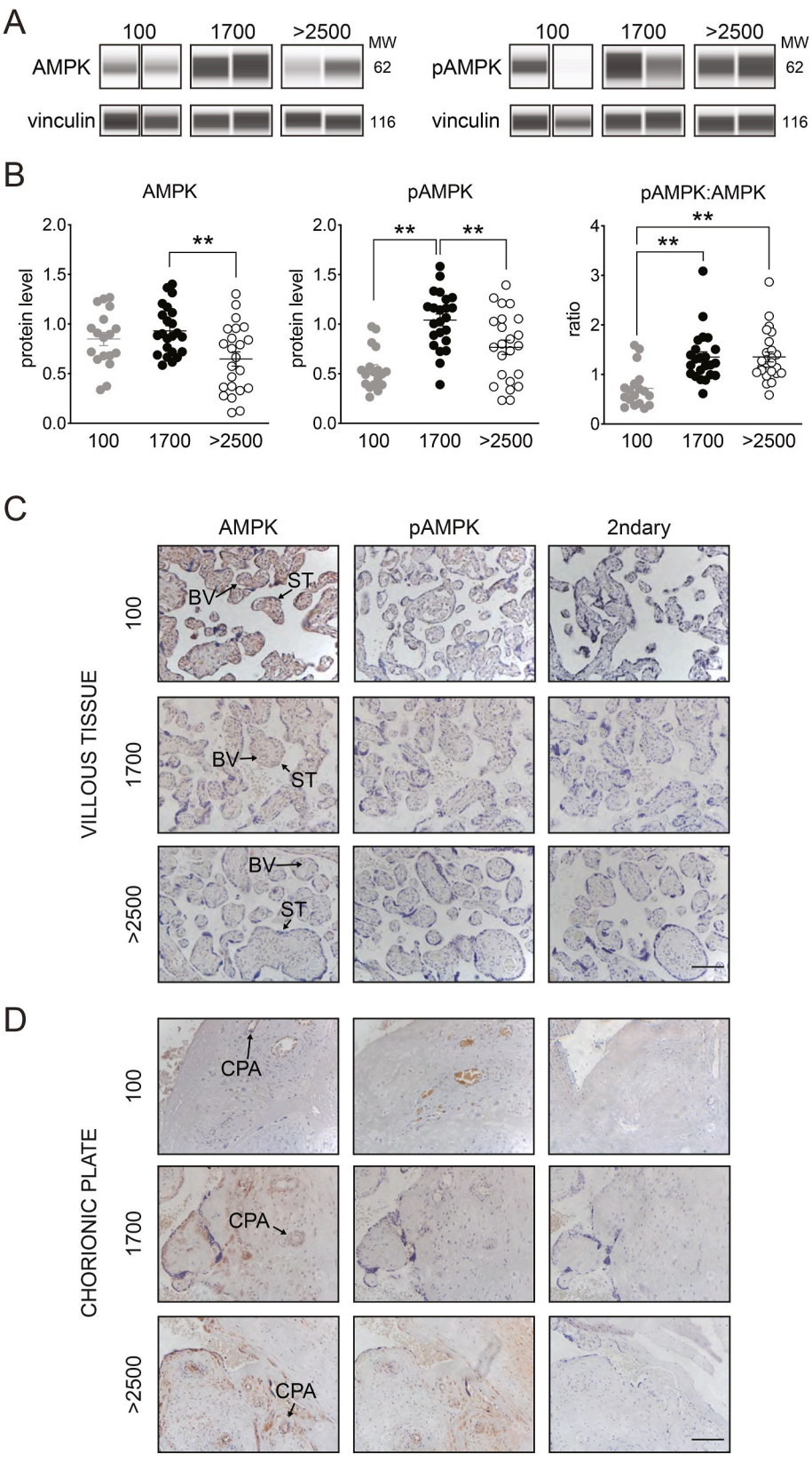

Fig. 1. Phosphorylated-to-total AMPK protein expression was increased at high altitude. A, Representative images of capillary electrophoresis bands for AMPK, pAMPK and vinculin in placentas collected from low- or high-altitude residents. MW, molecular weight in kDa. **B**, Protein abundance analysis of AMPK and pAMPK, and ratios pAMPK: AMPK from low- (100 m, gray symbols, n = 18) moderate- (1700 m, closed symbols, n = 23) and high-altitude (>2500 m, open symbols, n = 23) villous placental tissue. Levels were normalized to vinculin expression. Symbols are individual values per subject, lines are means ± SEM, $^{**}P < 0.01$. **C-D**, Representative microscope pictures of placental tissue from pregnant women residing at low (100 m), moderate (1700 m) or high altitude (>2500 m) showing staining of AMPK (left panels) or pAMPK (middle panels) in the villous tissue (**C**) or chorionic plate (**D**). Right panels show negative control staining using only secondary antibody (2ndary). BV, blood vessels; ST, syncytiotrophoblast; CPA, chorionic plate arteries. Scale bar = 100 μm.

Since AMPK activation inhibits mechanistic target of rapamycin (mTOR) signaling, in part by TSC2 phosphorylation and activation, we therefore examined two mTOR downstream targets, 4E-BP1 and S6K. Phosphorylated 4E-BP1 and the p4E-BP1:4E-BP1 ratio were elevated at high compared to low or moderate altitudes, whereas total 4E-BP1 protein levels were similar (Fig. 2C). In addition, although total S6K protein levels were higher at moderate than low altitude (Fig. 2D), phosphorylated S6K levels did not differ among altitudes. Similar results were obtained whether or not protein levels were corrected for infant sex or mode of delivery (Supplemental Tables S3 and S4). Since labor has been

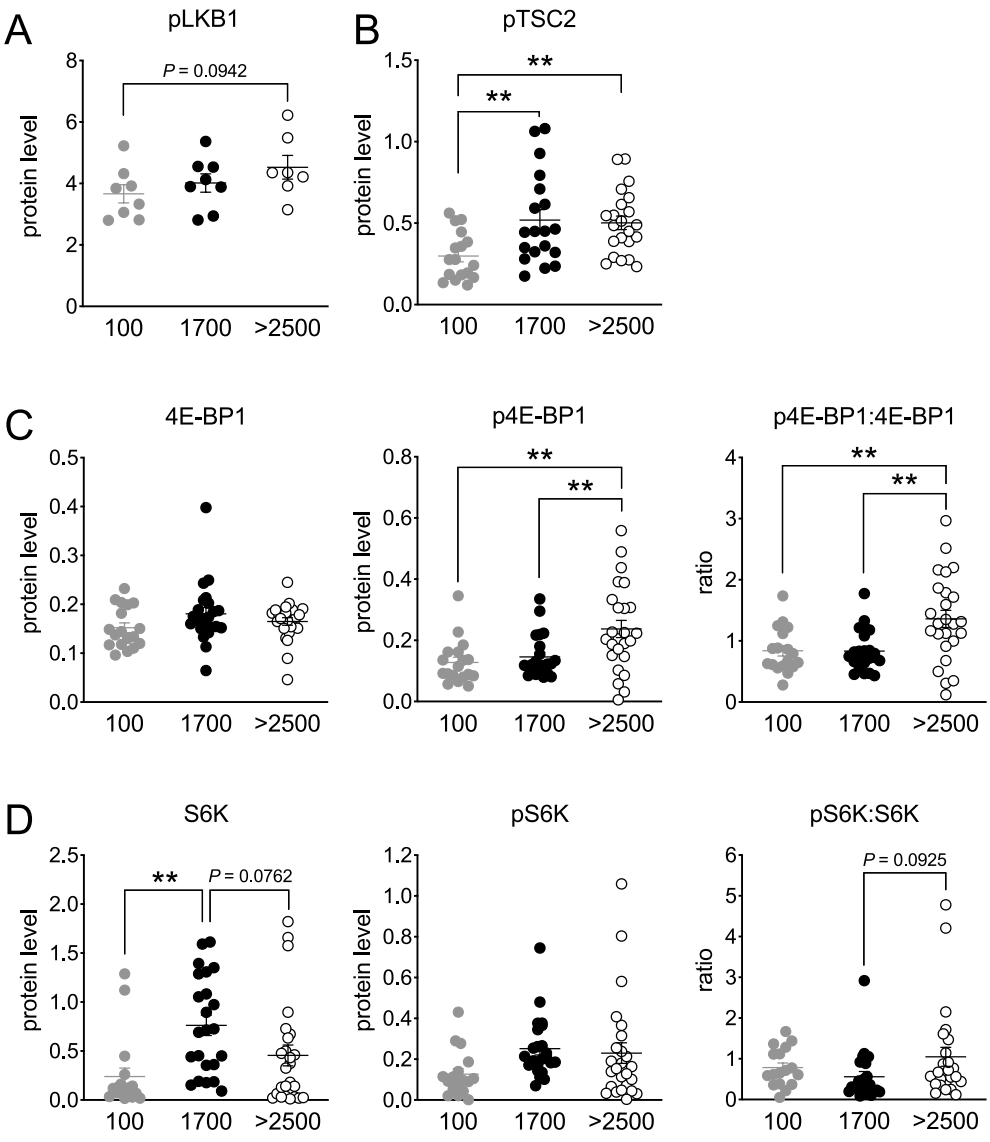

Fig. 2. Phosphorylated 4E-BP1 expression was increased at high altitude. Protein expression analysis of pLKB1 (**A**), pTSC2 (**B**), 4E-BP1 and p4E-BP1 (**C**), or S6K and pS6K (**D**) from low- (100 m, gray symbols, n = 8–18) moderate- (1700 m, closed symbols, n = 8–23) and high-altitude (>2500 m, open symbols, n = 7–23) villous placental tissue. pLKB1 and pTSC2 levels were normalized to β-actin; 4E-BP1, p4E-BP1, S6K and pS6K were normalized to vinculin expression. Righthand panels in C and D show the ratios of phosphorylated to total proteins. Symbols are individual values per subject, lines are means ± SEM, $^{**}P < 0.01$.

reported to inhibit placental mTOR signaling and phosphorylation of 4E-BP1 [28] and, as stated above, the rate of non-laboring Cesarean section was higher in the high-compared to moderate- and low-altitude women, we compared protein expression between vaginal and nonlaboring Cesarean section women. We did not find any differences in 4E-BP1, p4E-BP1 or their ratio at any altitude, but total AMPK levels were lower in Cesarean section compared to vaginal deliveries at moderate altitude, and the pAMPK:AMPK ratio increased at high altitude (Supplemental Figure S2). In addition, pTSC2 was increased and total S6K reduced in samples from Cesarean section compared to vaginal deliveries at moderate altitude, as was the ratio pS6K:S6K at moderate and high altitudes (Supplemental Figure S3).

3.4. Effect of altitude on the mRNA expression of AMPK-related pathways

We evaluated the altitudinal differences in mRNA transcript levels of AMPK-pathway genes in the placenta (Fig. 3A and Supplemental Table S2). Of the AMPK catalytic α subunits (α1 and α2) and non-catalytic isoforms (β1-2, γ1-2) examined, the *PRKAG2* gene regulating AMPKγ2 expression was insignificantly greater at high compared to moderate altitude (Fig. 3B). Neither the mRNA levels of membrane receptors regulating AMPK activation (e.g., adiponectin, leptin and insulin receptors) nor intracellular enzymes that regulate AMPK activity (e.g., CAMKK2, LKB1 [*SKT11*] or Akt), differed between moderate and high altitudes (Supplemental Figures S4 and S5).

With respect to downstream AMPK targets, high altitude non-significantly augmented mRNA expression of *MTOR* (Fig. 4A), but not other genes in the mTOR complex 1 (mTORC1) pathway (*RPTOR*, *EIF4EBP1*, *RPS6KB1* and *RPS6KB2*; Fig. 4B–E). Similarly, we assessed the expression of AMPK-regulated genes related to gluconeogenesis (PPARGC1A and PPARGC1B), fatty acid oxidation (ACACA) and cell cycle arrest (TP53), however, none of these target genes were changed by altitude (Supplemental Figure S6). We also found that mRNA levels of the mTORC1 pathway target 4E-BP1 (*EIF4EBP1*) were positively correlated with birth weight in moderate- but not high-altitude placentas (Fig. 4F), but we did not find correlation between birth weight and any of the other genes assessed (data not shown).

Interestingly, we observed an effect of fetal sex on some of the transcripts evaluated. Particularly, *AKT2*, *PRKAB1*, *RPS6KB1* and *RPS6KB2* were upregulated in placentas from male vs. female fetuses (Supplemental Table S5). Further, there was a non-significant increase in *AKT3*, *PRKAG1*, *PRKAG2* and *ACACA* mRNA in male vs. female placentas (Supplemental Table S5).

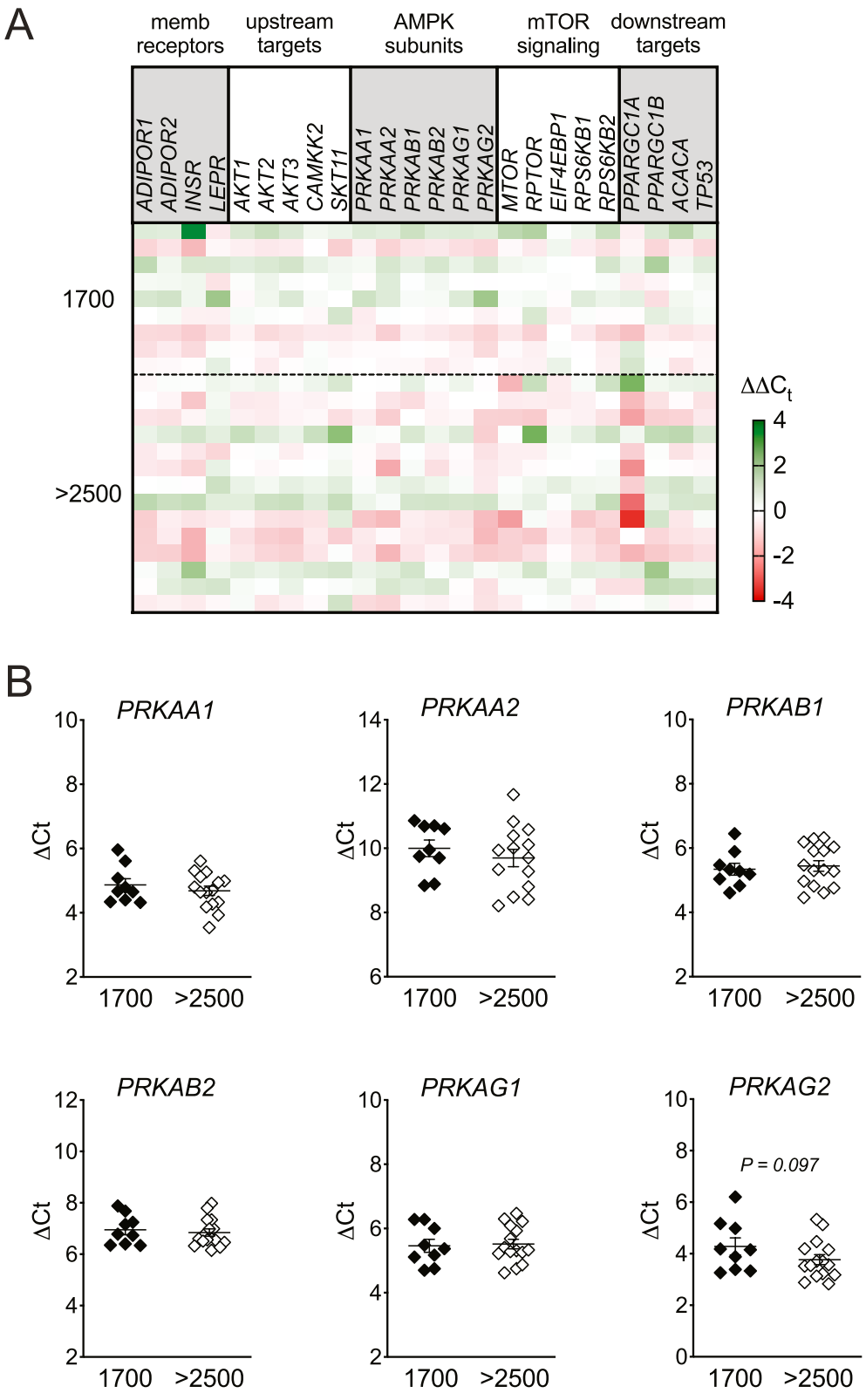

Fig. 3. High altitude modulated mRNA expression of placental AMPK pathway genes. A, mRNA heat-map representation showing differences in expression between moderate- (1700 m) and high-altitude (>2500 m) placentas. Green and red colors represent up- or down-regulation, respectively, expressed as $\Delta\Delta C_t$ values. Each subject is represented by a row and individual genes listed in the columns. **B**, ΔC_t values of mRNA encoding for the AMPK subunit genes (*PRKAA1*, *PRKAA2*, *PRKAB1*, *PRKAB2*, *PRKAG1* and *PRKAG2*) from moderate- (1700 m, closed symbols) or high-altitude (>2500 m, open symbols) villous placental tissue. Symbols are individual values per subject, lines are means ± SEM.

4. Discussion

High-altitude residence increases the risk of pregnancy complications such as preeclampsia and IUGR [1,4], both of which are associated with increased maternal and infant morbidity and mortality. Impaired placental function, such as failure of trophoblast cells to invade and remodel maternal spiral arteries and increased oxidative stress and inflammation, are often present in pregnancies characterized by preeclampsia or IUGR [29]. Therefore, studies designed to identify how pathways regulating placental metabolism are altered in hypoxia-related pregnancy complications are vital for the development of treatments and therapies to prevent or alleviate these disorders. In

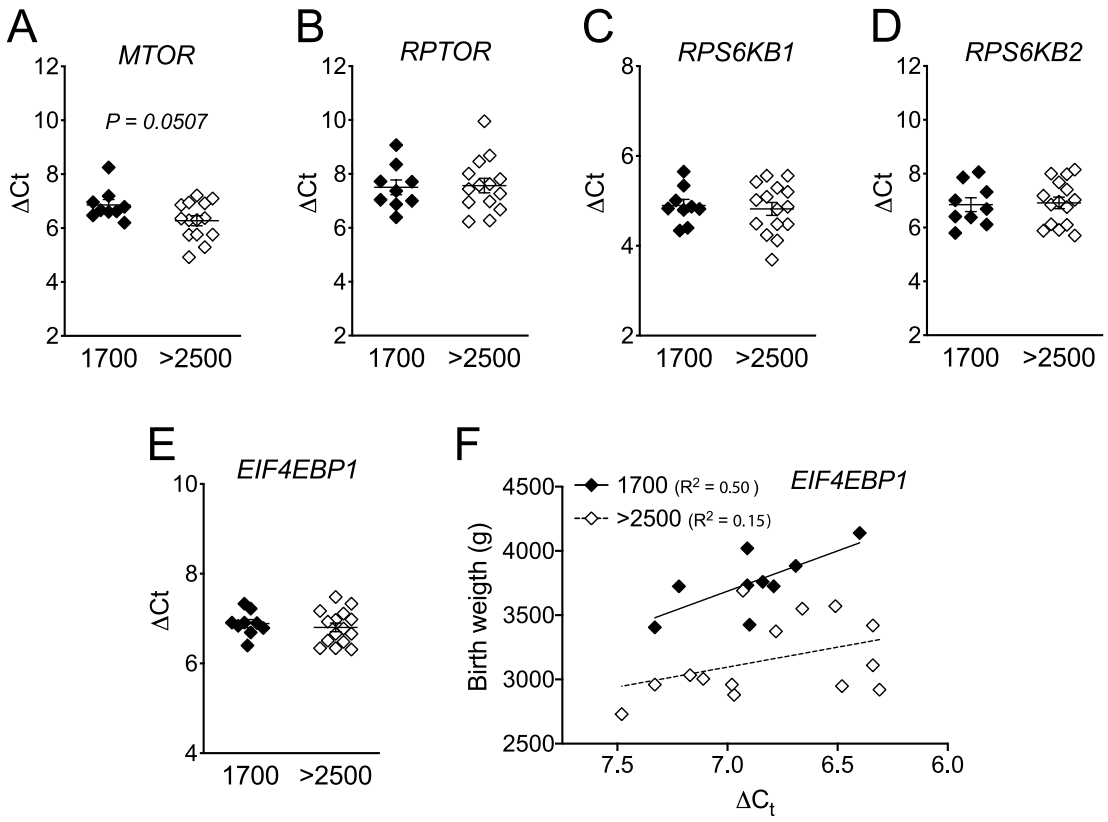

Fig. 4. mRNA expression of *mTOR* was non-significantly increased by high altitude, but the expression of other genes involved in mTOR complex 1 signaling were unaffected. ΔC_t values of mRNA encoding for the mechanistic target of rapamycin (*MTOR*, **A**), regulatory associated protein of mTOR, complex 1 (*RPTOR*, **B**), the ribosomal protein S6 kinase, 70 kDa, polypeptides 1 (*RPS6KB1*, **C**) and 2 (*RPS6KB2*, **D**) or the eukaryotic translation initiation factor 4E binding protein 1 (*EIF4EBP1*, **E**) from moderate- (1700 m, closed symbols) or high-altitude (>2500 m, open symbols) villous placental tissue. Symbols are individual values per subject, lines are means ± SEM. **F**, the placental *EIF4EBP1* mRNA expression levels were positively associated with birth weight at moderate (1700 m, closed symbols, $P < 0.05$) but not high altitude (>2500 m, open symbols, $P = 0.1657$). Symbols are individual values per subject, lines are those from least-squares linear regression analysis.

this study, we evaluated the expression of AMPK, its upstream regulators and downstream targets in human placentas from low-, moderate- and high-altitude residents. Our findings showed that high altitude reduced total AMPKα protein but increased relative AMPK activation, as well as the activation of its downstream targets TSC2 and 4E-BP1 in appropriate for gestational age pregnancies, suggesting that AMPK and its targets upstream and downstream of mTORC1 signaling may be important adaptive mechanisms to prevent or limit the reduction in fetal growth at high altitude.

AMPK is a metabolic sensor that responds to external and cellular inputs and thereby serves to control multiple cellular functions through the regulation of several signaling pathways (Fig. 5) [19]. AMPK activity is positively and directly regulated through the binding of AMP or ADP binding to its non-catalytic γ subunit. In contrast, ATP binding to the γ subunit returns AMPK to its basal inactive state [14]. Extracellular metabolic signals such as adipokines and insulin activate AMPK, and several intracellular signals can also regulate AMPK activity. For instance, CAMKK2 and LKB1 enzymes activate AMPK, whereas Akt has an inhibitory effect [19]. Once active, the AMPK catalytic α subunit activates specific targets, such as PGC-1, ACC1, p53 and TSC2, thus integrating cellular metabolic state with cellular function. In the placenta, an especially metabolically-active organ, AMPK also seems to have a key role. In particular, *in vitro* pharmacologic activation of AMPK evokes mitochondrial toxicity and reduces metabolism by decreasing fatty acid uptake and glycolysis in placental explants from healthy women [21]. In addition, AMPK knockdown decreases cell growth, differentiation and glucose transport in trophoblast cells [30].

Hypoxia shifts cellular metabolism toward an anaerobic state, increasing the AMP:ATP ratio, which would be expected to activate AMPK [31]. In the present study, we identified key genes and proteins in the AMPK-signaling pathway that were differentially regulated in placentas from high to low altitudes, across a ~2500 m altitude range. Specifically, we found that high-altitude residence decreased placental AMPKα protein expression while increasing its phosphorylation. These observations disagree with a previous report showing similar AMPKα protein levels in human placenta from sea level and high-altitude (~3100 m) residents [22]; such variability could be due to the small sample size in the previous study (n = 3 high-altitude placentas) and high inter-subject variability. We also found substantial variability in protein expression within altitudes, but our larger sample size (n = 23 high-altitude placentas) provided stronger statistical power to identify differences.

Activated AMPK can inhibit mTOR by at least two mechanisms: a) reducing the activity of the mTOR activator Raptor, or b) phosphorylating and activating the mTOR inhibitor TSC2 [32]. mTOR-dependent phosphorylation of 4E-BP1 prevents the latter from repressing translation initiation, increasing protein synthesis. Hence, increased AMPK activity may promote reduced protein synthesis though inhibition of mTOR. Our results showed that phosphorylated forms of AMPK and TSC2 were increased in high-altitude placentas, so was 4E-BP1. Although we did not directly assess Raptor phosphorylation, we consider the possibility that the increased 4E-BP1 phosphorylation that we observed in high-altitude placentas may occur through a mechanism other than mTOR-dependent phosphorylation. Glycogen synthase kinase 3 beta, p38 mitogen-activated protein kinase and leucine-rich repeat kinase 2 have been shown to phosphorylate 4E-BP1 at

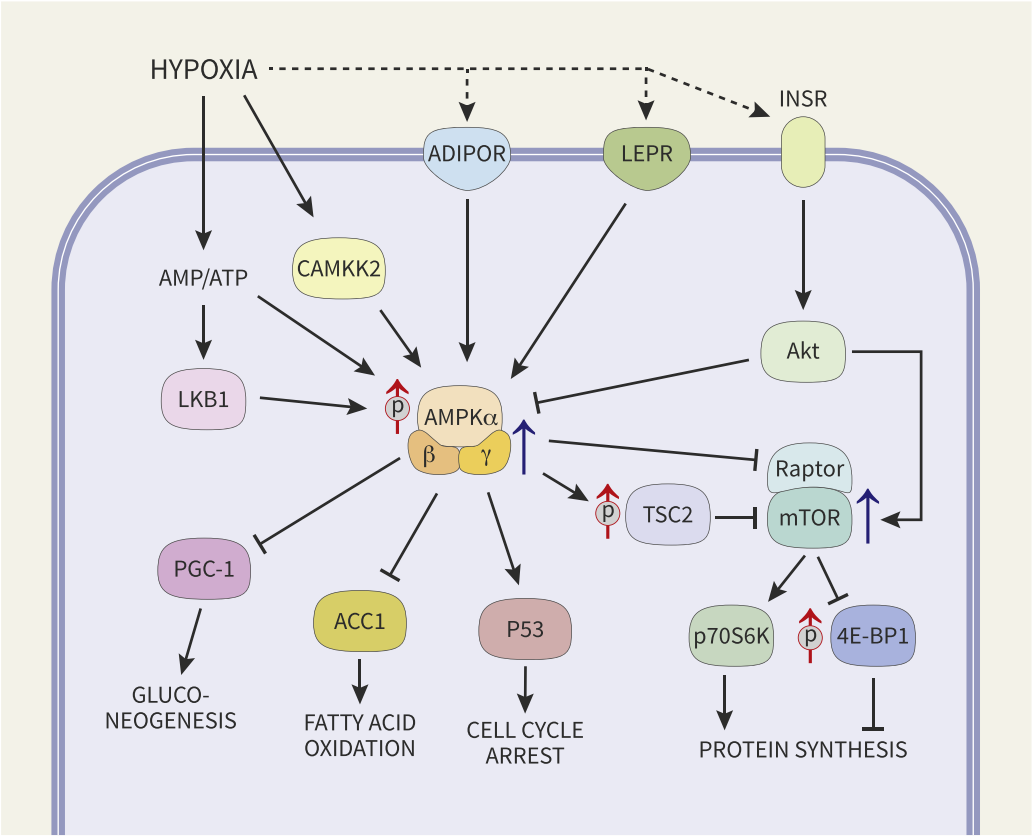

Fig. 5. Schematic representation of the effect of chronic hypoxia of high-altitude residence on the expression and activation of AMPK pathways in the human placenta. The AMPK enzymatic complex is composed of catalytic α-, and non-catalytic β- and γ-subunits. External stimuli, such as hypoxia, modulate the activation of AMPK by several membrane receptors, such as adiponectin (ADIPOR), leptin (LEPR) and insulin receptors (INSR). Intracellular enzymes also regulate AMPK activity, calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK2), serine/threonine kinase 11 (also known as liver kinase B1, LKB1) and serine/threonine-protein kinase akt (Akt) which is activated by INSR. Hypoxia increases the intracellular AMP/ATP ratio, which in turn can activates LKB1 and AMPK. Hypoxia can also indirectly activate AMPK through activation of CAMKK2. Hypoxia can also regulate ADIPOR, LEPR and INSR in other tissues (dotted lines) but whether this is also true in the human placenta has not been established. AMPK controls a wide variety of cellular processes such as gluconeogenesis, fatty acid oxidation, cell cycle and protein synthesis through downstream effectors including the peroxisome proliferator-activated receptor gamma, coactivator 1 (PGC-1), the acetyl-CoA carboxylase alpha (ACC1), the tumor protein p53 (p53) and the mechanistic target of rapamycin (mTOR) complex 1. mTOR signaling is inhibited by AMPK through inhibition of the regulatory associated protein of mTOR (Raptor) and by activating tuberin (TSC2), which, in turn, inhibits mTOR. Activation of mTOR regulates protein synthesis by activating the ribosomal protein S6 kinase (70 kDa, p70S6K) and inhibiting eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1). In placentas from high-altitude residents, we observed a non-significant increase in transcript levels of AMPK γ subunit 2 and mTOR (*blue arrows*), a significant increase in pAMPK, pTSC2 and p4E-BP1, and reduced total S6K protein levels, suggesting that high altitude increases activation of AMPKα, phosphorylation of TSC2 and phosphorylation of the mTORC1-target 4E-BP1, thereby serving to help maintain protein synthesis and fetal growth in these high-altitude residents with appropriate for gestational age infants.

Thr36/Thr46 in a wide variety of tissues [33–35]; further studies aimed to assess the contribution of these pathways for human placental 4E-BP1 phosphorylation are needed. We speculate that, in these high-altitude pregnancies with appropriate for gestational age fetuses, the increase in 4E-BP1 phosphorylation helped to maintain protein synthesis and, consequentially, acted as a compensatory mechanism to minimize the high altitude-dependent reduction in fetal growth.

Supporting our hypothesis that the hypoxia of high-altitude residence activated AMPK, we found augmented AMPK protein phosphorylation. Our high-altitude sample was comprised of women with no overt pathology or known (apart from residence at high altitude) risk of IUGR. While birth weights were lower at high compared to moderate or low altitude, none (by design) were below the 10th percentile for gestational age and sex. Therefore, we thought it likely that in these appropriate for gestational age high-altitude pregnancies, uteroplacental oxygen supply was not sufficiently reduced to inhibit mTOR signaling. However, since birth weight was diminished in high-altitude infants our data imply that chronic hypoxia is still affecting these pregnancies. In keeping with such a possibility, our previous studies at the same altitude (>2500 m) have shown that residence at high altitude reduced the vasodilatory response to acetylcholine in isolated maternal myometrial arteries [36], suggesting increased uterine vascular resistance and reduced uteroplacental blood flow. Furthermore, high altitude increases the sensitivity of AMPK to vasodilate myometrial arteries, suggesting a maternal vascular adaptation to maintain uteroplacental blood flow in these appropriate for gestational age high-altitude pregnancies [37]. Another possibility is that the increase in p4E-BP1, suggesting an augmented protein synthesis in the high-altitude placentas, might not be sufficient to fully reverse the effect of chronic hypoxia on fetal growth. Supporting this latter possibility, our transcript expression analysis revealed that *EIF4EBP1*, which encodes 4E-BP1, was positively correlated with birth weight at moderate altitude but not at high altitude. Our study aimed to identify factors related to AMPK pathways that are protecting fetal growth at high altitude in relatively healthy pregnancies, however, further studies are needed to identify whether placental protein synthesis is related to the maintenance of normal

uterine artery blood flow during pregnancy at high altitude, and whether placental protein synthesis differs in IUGR vs. appropriate for gestational age pregnancies at high (or lower) altitudes. Also needed are further studies in the Andean high-altitude residents in whom we identified a relationship between gene variants that had been acted upon by natural selection near the catalytic AMPK α subunit, which were also associated with preservation of uterine artery blood flow and birth weight at high altitude [13].

Among the strengths of our study are the comparison between three altitudes including a sea-level control, expanded sample sizes compared to prior studies, and similar maternal ethnic composition of study cohorts at each altitude. Furthermore, including measurements of both gene-and protein-expression is an advantage given that, although gene transcription generally shows a positive correlation with protein expression, mRNA transcription is an imperfect predictor of protein levels when considered in isolation. Future experimental studies will build upon our observations by determining the mechanism(s) underlying these differences in expression by performing *in vitro* studies using primary trophoblast cultures from placental explants or other isolated cell preparations. Also, although we strived to match the delivery mode from all altitudes there were more specimens from Cesarean section deliveries at high altitude, likely due to the fact that our past [36,37] and current studies at high altitude prioritized collections from Cesarean section deliveries to obtain myometrial biopsies as required for myometrial artery vasoreactivity experiments.

In summary, we evaluated the transcript and protein expression levels in several key pathways related to AMPK signaling in placentas from women residing at low, moderate or high altitude. We found that residence at high altitude augmented activation of AMPK protein and increased the activation of TSC2 and the mTORC1 target 4E-BP1 as well in placentas from pregnancies with no overt pathology. Future work designed to identify the mechanisms by which the chronic hypoxia of residence at high altitude affects placental function and fetal growth may reveal new therapeutic candidates for preventing or improving fetal growth under hypoxic conditions.

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Declaration of competing interest

The authors declare that they have no conflicts of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2021.01.010.

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