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The placental mTOR-pathway: correlation with early growth trajectories following intrauterine growth restriction?

F. B. Fahlbusch[1]*, A. Hartner[1], C. Menendez-Castro[1], S. C. Nögel[1], I. Marek[1], M. W. Beckmann[2], E. Schleussner[3], M. Ruebner[2], H. Huebner[2], H.-G. Dörr[1], R. L. Schild[4], J. Dötsch[5] and W. Rascher[1]

[1]*Department of Pediatrics and Adolescent Medicine, University of Erlangen-Nürnberg, Erlangen, Germany*
[2]*Department of Gynecology and Obstetrics, University of Erlangen-Nürnberg, Erlangen, Germany*
[3]*Department of Gynecology and Obstetrics, University of Jena, Jena, Germany*
[4]*Department of Obstetrics & Gynecology, Diakonische Dienste Hannover, Hannover, Germany*
[5]*Childrens' and Adolescents' Hospital, University of Cologne, Cologne, Germany*

Idiopathic intrauterine growth restriction (IUGR) is a result of impaired placental nutrient supply. Newborns with IUGR exhibiting postnatal catch-up growth are of higher risk for cardiovascular and metabolic co-morbidities in adult life. Mammalian target of rapamycin (mTOR) was recently shown to function as a placental nutrient sensor. Thus, we determined possible correlations of members of the placental mTOR signaling cascade with auxologic parameters of postnatal growth. The protein expression and activity of mTOR-pathway signaling components, Akt, AMP-activated protein kinase α, mTOR, p70S6kinase1 and insulin receptor substrate-1 were analysed via western blotting in IUGR *v.* matched appropriate-for-gestational age (AGA) placentas. Moreover, mTOR was immunohistochemically stained in placental sections. Data from western blot analyses were correlated with retrospective auxological follow-up data at 1 year of age. We found significant catch-up growth in the 1st year of life in the IUGR group. MTOR and its activated form are immunohistochemically detected in multiple placental compartments. We identified correlations of placental mTOR-pathway signaling components to auxological data at birth and at 1 year of life in IUGR. Analysis of the protein expression and phosphorylation level of mTOR-pathway components in IUGR and AGA placentas postpartum, however, did not reveal pathognomonic changes. Our findings suggest that the level of activated mTOR correlates with early catch-up growth following IUGR. However, the complexity of signals converging at the mTOR nexus and its cellular distribution pattern seem to limit its potential as biomarker in this setting.

Introduction

Intrauterine growth restriction (IUGR) is characterized by insufficient placental nutrient supply to the fetus.[1–4] It is associated with an increased risk of perinatal morbidity.[5] In addition, due to fetal programming, IUGR increases the likelihood of metabolic sequelae in adult life (i.e. type 2 diabetes and obesity), as well as cardiovascular disease.[6,7] Interestingly, early postnatal catch-up growth in the first 2 years augments the risk of childhood obesity at the age of 5 years.[8] These children are also more likely to suffer from the above negative long-term metabolic effects.[8] Therefore, catch-up growth itself was deemed an etiopathologic risk factor for the developing neonate post IUGR.[9] Subsequently, pediatric research shifted from focusing on the rate of postnatal catch-up growth to the identification of early placental biomarkers with predictive value for postnatal development itself.[10–12] Recently Roos *et al.*[3,4,13] identified the mammalian target of rapamycin (mTOR) pathway and its downstream effector p70S6kinase1 (p70S6k1) as an important regulatory link between nutrient and growth factor concentrations and the activity of system L-essential amino acid transporter in the human syncytiotrophoblast (SCT). In addition, mTOR was characterised as an important mediator of human trophoblast invasion through regulation of matrix-remodeling enzymes.[14] However, the correlation of its placental expression with postnatal auxologic data has not been assessed so far. It is known, that activation of mTOR by nutrients inhibits upstream PI3-kinase signaling of insulin-like growth factor 1 (IGF-I) and insulin signaling via a negative feedback loop from p70S6k1 to insulin receptor substrate-1 (IRS-1).[15] *P70S6k1*-deficient mice exposed to a high fat diet are protected against obesity, where high levels of circulating glucose and free fatty acids usually result in insulin receptor desensitization.[16] This is due to the non-functional negative feedback loop from p70S6k1 to IRS-1, blunting the phosphorylation of the S636/S639 site,[16] which is known to play a role for insulin resistance.[17] In contrast, the insulin resistant *ob/ob* (also known as Lep/Lep) obesity mouse model shows an elevated p70S6k1 activity and an increased phosphorylation of IRS-1 at S636/S639. Hence, p70S6k1

*Address for correspondence: F. B. Fahlbusch, MD, Department of Pediatrics and Adolescent Medicine, University of Erlangen-Nürnberg, Loschgestr. 15, Erlangen 91054, Germany.
(Email fabian.fahlbusch@uk-erlangen.de)

negatively regulates IRS-1 downstream signaling in conditions of nutrient excess[16] leading to insulin resistance.

As major anabolic hormones, IGF-I and insulin induce postnatal growth and development by signaling through IRS-1.[18] As postnatal catch-up growth following IUGR is accompanied by normal/reduced plasma concentrations of these hormones, an increased hormone action due to an increased peripheral sensitivity is suspected as pathomechanism[19] for catch-up growth. In sheep IUGR offspring, De Blasio *et al.*[19] found an increased insulin sensitivity of circulating free fatty acids, and insulin disposition indices for glucose and free fatty acids, suggesting that catch-up growth and early-onset visceral obesity after IUGR may have a common underlying cause. The onset of insulin resistance in these IUGR sheep is accompanied by, and can be accounted for, reduced expression of insulin signaling components (especially IRS-1) and glucose transporter genes in skeletal muscle.[20]

Taken together, post-IUGR catch-up growth and the development of insulin resistance seem to be mechanistically linked and might be triggered by increased IRS-1 sensitivity.[19,20] Because the mTOR pathway was identified as the placental nutrient sensor[4] and regulator of IRS-1 sensitivity,[16] we hypothesized that the underlying molecular changes for postnatal catch-up growth in IUGR could be visible at the level of the placenta at birth. We further hypothesized, that the IUGR placenta might show an increased sensitivity of IRS-1 due to a potentially low activity of the p70S6k1-to-IRS-1 feedback loop in growth-restricted neonates.[4] As a consequence, relative postnatal nutrient excess might trigger catch-up growth and consecutively induce insulin resistance and obesity in these children. Of further interest were the two signaling proteins Akt and AMP-activated protein kinase α (AMPKα), as positive and negative regulators of mTOR downstream activity, respectively. AKT signaling plays an essential role in the regulation of cell growth and proliferation via integration of growth factor signaling and via its function as an ATP and amino acid sensor by adjusting cellular protein synthesis to nutrient availability via control of the mTOR pathway.[21,22] Interestingly, homozygous disruption of the Akt1 gene in the mouse results in IUGR.[23] AMPK was shown to be upregulated in cotyledons of nutrient restricted ewes.[24] AMPK is activated by an increased AMP/ATP ratio under nutritional and environmental stress.[25] Moreover, AMPK activity is regulated by insulin via Akt signaling and by leptin.[26,27]

We hypothesized, that changes in the activity of placental mTOR pathway components following IUGR could be indicative of the intrauterine dysregulation and programming processes of the fetus. Such fetal programming might in turn influence postnatal growth. We chose to analyse the placental mTOR pathway due to its nexus function in metabolism and growth factor signaling. As placental metabolism and fetal growth are closely related, we further hypothesized that postnatal growth patterns in former IUGR newborns would be related to altered placental activity of mTOR signaling components.

Methods

Patients

Seven newborns with IUGR and seven healthy gestational age-matched newborns [(appropriate for gestational age (AGA)] were enrolled in this study. IUGR was defined by a pathologic flow impedance of the umbilical artery and/or uterine arteries as shown by Doppler sonography[28] in combination with the inclusion/exclusion criteria described before[29] (Supplementary Table 1). The IUGR cases studied are considered to be idiopathic,[30] with utero-placental insufficiency as the major cause. The clinical characteristics of IUGR and AGA groups are shown in Table 1. Auxological parameters were recorded at birth and 1 year of age. Standard deviation score (SDS) calculations for length/weight at birth were calculated based on Voigt *et al.*[31] SDS values for length and weight at 1 year of age were calculated according to references of Kromeyer-Hausschild *et al.*[32] Gestational age was determined from the date of last menstrual period of the mother and adjusted by fetal biometry before the 14th week of gestation.

Tissue preparation

Fresh samples of human placenta were obtained in a standardized sterile procedure immediately after placental delivery. A 2×2 cm segment, total depth ranging from basal plate to chorionic membrane, was excised at the central region of the placenta using a scalpel blade. Macroscopic calcifications were avoided. After a short rinse with normal saline, amniotic membranes and basal plate were removed. Samples were immediately snap frozen in liquid nitrogen and stored at −80°C until further processing.

Immunohistochemistry

Placental whole mount biopsies were excised at mid-proximity to the insertion of the umbilical cord, fixed with formalin and embedded in paraffin. Seven IUGR and AGA term placentas were analysed. Two-micrometre sections were cut and deparaffinized. Unmasking was performed with citrate buffer at pH 6.0. Subsequently endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 20 min at room temperature. Sections were then layered with the primary antibody and incubated overnight at 4°C. Immunostains were performed for total and phosphorylated mTOR. Primary antibodies were total mTOR (Abcam, Cambridge, UK; ab51089), dilution 1:100; phospho-mTOR (Ser2448; Cell Signaling, Frankfurt, Germany, #2976), dilution 1:100. After addition of biotin-conjugated goat anti-rabbit IgG as the secondary antibody (Vector Laboratories via Linaris, Dossenheim, Germany), staining was performed with a peroxidase detection method, as described previously.[33] Each slide was counterstained with hematoxylin. For negative control samples, we used equimolar concentrations of pre-immune rabbit IgG. Sections were analysed using a Leitz Aristoplan microscope (Leica Microsystems, Wetzlar, Germany).

Table 1. *Birth characteristics and auxological parameters of IUGR (intrauterine growth restriction) and AGA (appropriate-for-gestational age) neonates at birth and at 1 year of age*

	IUGR	AGA	*P*-value
At birth			
Birth length SDS	−2.1 ± 0.5	−0.04 ± 0.3	0.02*
Birth weight SDS	−2.25 ± 0.3	0.03 ± 0.1	<0.0006***
Gestational age (weeks)	34 ± 2	34 ± 1	0.90
Placental weight (grams)	306.0 ± 50.4	448.0 ± 57.9	0.22
Spontaneous delivery/elective caesarean section	1 *v.* 7	3 *v.* 7	
Gender distribution (female/male)	2 *v.* 7	2 *v.* 7	
At 1 year of age			
Longitudinal growth velocity normalised to age at follow-up (cm/year)	30.1 ± 1.5	25.2 ± 1.4	0.04*
Time interval of follow-up at 1 year of age (years)	1.2 ± 0.01	1.0 ± 0.02	0.60
Length SDS	−1.7 ± 1.1	−1.5 ± 0.5	0.53
Weight SDS	−1.9 ± 0.7	−1.1 ± 0.4	0.18
BMI SDS	−1.3 ± 0.24	−0.13 ± 0.47	0.05*
BMI	15.01 ± 0.25	16.1 ± 0.68	0.04*

BMI, body mass index; SDS, standard deviation score.
*$P < 0.05$, ***$P < 0.001$.

Protein extraction and western blot analysis

For western blotting, 0.1 g of placental tissue was manually grinded on dry ice with freshly prepared lysis buffer containing multiple protein inhibitors, as described by Sarbassov *et al.*[34] Subsequently, homogenates were thoroughly sonicated on ice, avoiding foaming. Homogenates were incubated with the buffer for 20 min before centrifuging at 12,000 rpm for 15 min at 4°C. The supernatant (cytosolic-enriched fraction) was collected. Protein concentrations were determined by a bicinchoninic acid-based Protein Assay Reagent Kit (Pierce, Rockford, IL, USA) with albumin as the standard, following the provider's instructions. Homogenates were snap-frozen in liquid nitrogen and stored at −80°C in aliquots to avoid repeated freeze-thaw cycles.

Subsequently, 40 µg/30 µl of total protein and pre-stained SDS molecular weight standard mixture (peqGOLD Protein-Marker V, PeqLab Biotechnologie GmbH, Erlangen, Germany) were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis. The cytosolic-enriched fraction of the trophoblast cell line BeWo (CLS, Eppelheim, Germany) was isolated as described for placental samples and served as a positive control for each run. Western blot analysis for each protein was performed three times. Conditions for separation gel concentrations, membrane blocking and anti-body-related data are given in Supplementary Methods 1. In general, semi-dry blotting technique at RT for 2 h was used, however, for mTOR/P-mTOR and IRS-1/P-IRS-1 tank-blotting at 4°C for 2 h was performed; 10X Towbin blotting buffer was used for the membrane transfer. Following blocking of the membrane, incubation with primary antibodies was carried out at 4°C overnight. Throughout this paper, phosphorylated signaling proteins (P-) are referred to as 'activated'. Following washes with 0.1% Tris-NaCl-Tween (TNT), membranes were incubated with the diluted secondary antibody for 45 min at RT. After washes with TNT, the bands were visualized on auto-radiographic light-max films (Kodak, Stuttgart, Germany) by chemiluminescence using the ECL plus reagent kit (Amersham Pharmacia Biotech, Freiburg, Germany) at a concentration of 1:40. Signal intensity was analysed by densitometry (Aida Image Analyzer v.4.15 software; Raytest GmbH, Straubenhardt, Germany). Primary antibodies directed *v.* total and activated protein forms were chosen based on their pathway connection, as described.[4,16,34] Peroxidase-labeled anti-rabbit antibody (#7074; Cell Signaling Technology®) was used as secondary antibody at 1:1500. Equal protein loading was verified by adding an antibody directed against β-actin (I-19, Santa Cruz Biotechnology Inc., Heidelberg, Germany). Coomassie Brilliant Blue R-250 (Fluka Cemie AG, Buchs, Switzerland) staining of the gel was used to assure the homogenous transfer of proteins to the membranes.[35]

Statistical analysis

Results were expressed as mean ± standard error of the mean (S.E.M.). Statistical analyses and data visualisation were performed using GraphPad Prism Version 4.0c (GraphPad Software, San Diego, CA, USA) and SPSS Statistics v20.0 (IBM Corporation, Armonk, NY, USA). Groups were compared using non-parametric Mann–Whitney *U* testing. Correlation analysis was performed using two-tailed non-parametric Spearman rho (*r*) analysis. A *P*-value of <0.05 was considered statistically significant.

Results

Auxological data

Auxological data at birth and in the 1st year of life are displayed in Table 1. The IUGR and AGA group were matched for their

gestational age (mean 34 weeks of gestation). The main mode of delivery was via caesarean section. Birth weight SDS ($P < 0.0006$) and birth length SDS ($P < 0.02$) were significantly lower in the IUGR group (Table 1). IUGR placental weight tended to be lower than in the AGA group, without reaching statistical significance (Table 1). The time of follow-up was not significantly different between groups. We found a significantly increased growth velocity in the IUGR group in the 1st year of life (Tables 1, $P < 0.04$), so that at the age of 1 year, body length was not different between IUGR and AGA children. While body weight did not show a significant difference between children of both groups at the age of 1 year ($P < 0.18$), body mass index (BMI, $P < 0.04$) and BMI SDS ($P < 0.05$) were significantly lower in the IUGR group.

Localisation of mTOR in the placenta

Total mTOR (Fig. 1a–1d) and activated mTOR (P-Ser2448; Fig. 1e–1h) proteins were expressed in multiple compartments within the villi and basal plates of IUGR and AGA placentas: we found positive staining in the SCT (Fig. 1a, 1b, 1e and 1f), in the villous cytotrophoblast (VCT, Fig. 1a, 1b, 1e and 1f) and in the extravillous trophoblasts (EVT, Fig. 1c, 1d, 1g and 1h) for both total and activated mTOR. We were not able to detect significant group differences in staining patterns between IUGR and AGA placentas.

Protein expression analysis of mTOR signaling pathway components

Protein expression in the cytosolic-enriched fraction from placental tissue was analysed via western blotting (Fig. 2a–2e). Values for fold changes of total and phosphorylated (activated) proteins of IUGR *v.* matched control placentas are given in Supplementary Table 2. Comparing total and activated levels of placental mTOR (Fig. 2a), p70S6k1 (Fig. 2b), IRS-1 (Fig. 2c), AMPKα (Fig. 2d) and Akt (Fig. 2e), we did not detect statistical differences between IUGR and AGA groups (data not shown). In addition, when comparing the activated-to-total ratios of the above proteins no significant differences were found between groups (Fig. 2). There was no correlation of mTOR pathway signaling components with gestational age (data not shown).

Auxological data and correlation with the level of mTOR-pathway component expression

We analysed possible correlations of mTOR signaling components with auxological parameters at birth and in the 1st year of life in the AGA (Fig. 3b) and IUGR group (Fig. 3a). As no group differences in the levels of these components were detected between IUGR and AGA placentas, the two groups were additionally analysed as one (Fig. 3c).

In the IUGR group, birth weight and length showed a significant negative correlation with activated-to-total ratio of mTOR (Spearman $r = -0.929$, $P < 0.003$, Spearman $r = -0.901$, $P < 0.006$, respectively; Fig. 3a), while birth weight and length correlated negatively with the level of total AMPKα in the total cohort (Fig. 3c; Spearman $r = -0.577$, $P < 0.031$; Spearman $r = -0.552$, $P < 0.041$, respectively).

Activated-to-total ratio of p70S6k1 and total p70S6k1 levels correlated with the increment in length in the 1st year of life in the IUGR group (Spearman $r = 0.943$, $P < 0.05$; Spearman $r = -0.829$, $P < 0.042$, respectively, Fig. 3a). Moreover, levels of activated mTOR significantly correlated positively with growth velocity in the 1st year of life (Fig. 3a; Spearman $r = 0.812$, $P < 0.05$). In the IUGR group, BMI SDS at 1 year of age correlated with activated-to-total ratio of p70S6k1 (Spearman $r = 1.0$, Fig. 3a). These correlations were IUGR-specific and not found in the AGA group (Fig. 3b).

Analysing the total cohort (Fig. 3c), the parameters weight and length and their respective SDS correlated with various mTOR-pathway components, however, correlations to Akt were most significant. Furthermore, total AMPKα correlated negatively with BMI SDS at 1 year of age (Spearman $r = -0.691$, $P < 0.019$, Fig. 3c). Taken together, analysis of placental mTOR signaling components and auxological parameters in the 1st year of life revealed correlations with placental mTOR and p70S6k1 in the IUGR group, while correlations with placental AMPKα and Akt were detected in the total cohort only. No correlations of auxological data with total and activated (Ser636/639) IRS-1, or the respective activated-to-total ratio, were observed.

Discussion

We analysed the placentas of gestational age-matched IUGR and AGA patients for changes in the protein expression and activity level of mTOR-pathway components (mTOR, p70S6k1, Akt, AMPKα and IRS-1) and evaluated correlation with postnatal development in the 1st year of life. We were especially interested in identifying placental mTOR pathway signaling components that correlate with catch-up growth in the 1st year of life, because of the etiopathologic relevance of catch-up growth for IUGR-associated sequelae. Our immunohistochemical analysis showed that mTOR and activated mTOR are located in multiple placental compartments. We did not observe a significant difference in staining patterns between IUGR and AGA. Accordingly, placental western blot analysis of mTOR pathway components expression and activity at birth did not reveal any IUGR-specific changes. Nevertheless, we were able to correlate the level of placental-activated mTOR to catch-up growth in the 1st year of life in the IUGR group only.

Analysing auxological data, we found significant catch-up growth in terms of weight and length in our IUGR cohort in the 1st year of life. Interestingly, the catch-up in length was more pronounced, leading to a reduced BMI in IUGR infants at 1 year of age. Postnatal catch-up growth usually occurs in the first 2 years of life, with a marked gain in length and weight in the first months of life.[36–38] While there is some evidence for

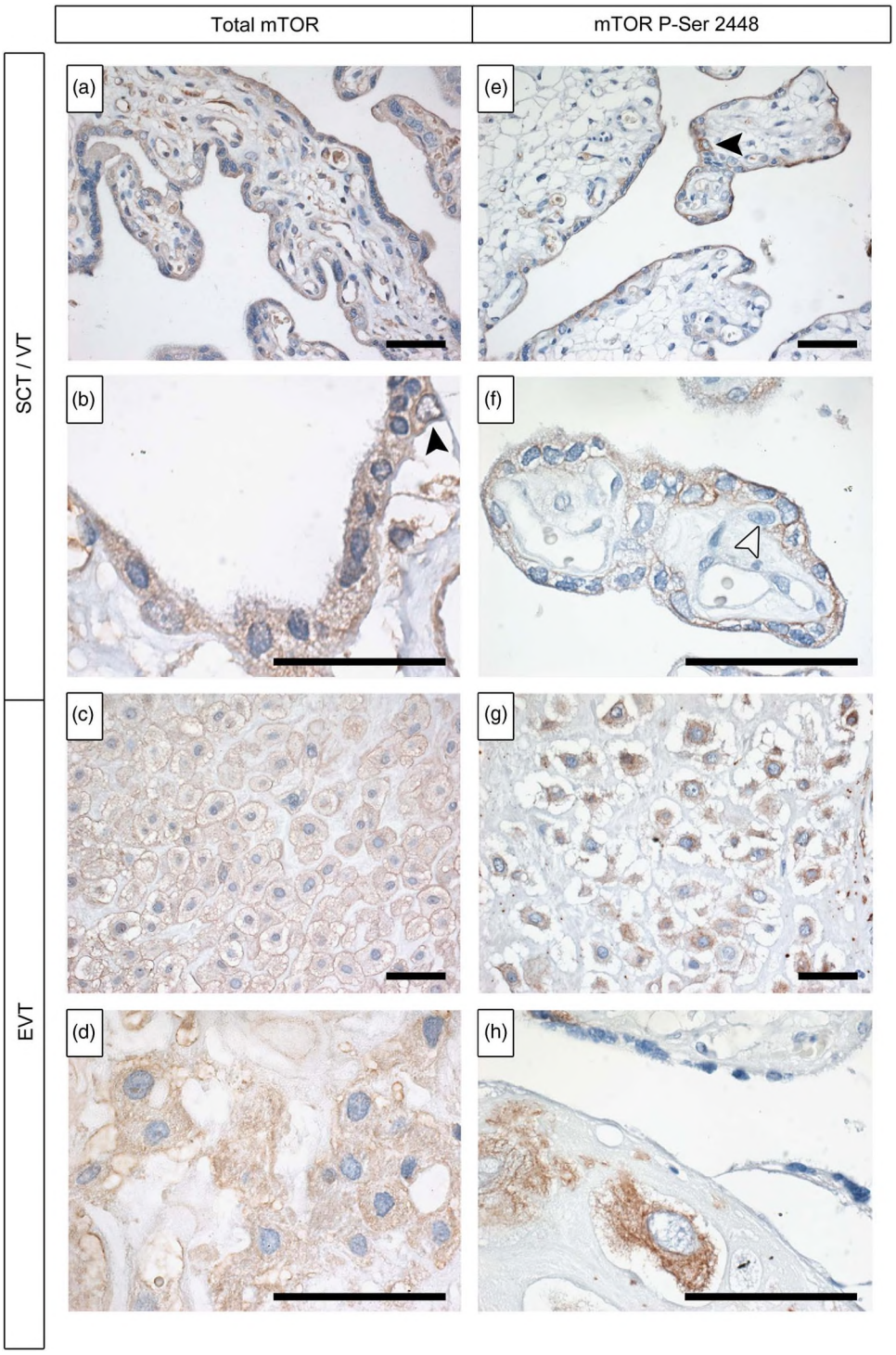

Fig. 1. Immunohistochemical staining of total *v.* activated phospho (P)-Ser2448 mammalian target of rapamycin (mTOR). The placental sections show placental villi stained for both total (a–d) and activated (e–h) mTOR. While total mTOR was equally distributed within the syncytiotrophoblast (SCT, a-b), activated mTOR showed an apico-basal distribution pattern in the SCT (e–f). Villous cytotrophoblasts (VT) are marked with arrows (black arrow = positive staining, white arrow = negative staining). For both total and activated mTOR, we found positively and negatively stained VT, without a group specific pattern. Basal plate extravillous trophoblasts (c–d and g–h) stained positive for both total (c–d) and activated mTOR (g–h). The bar represents 100 µm.

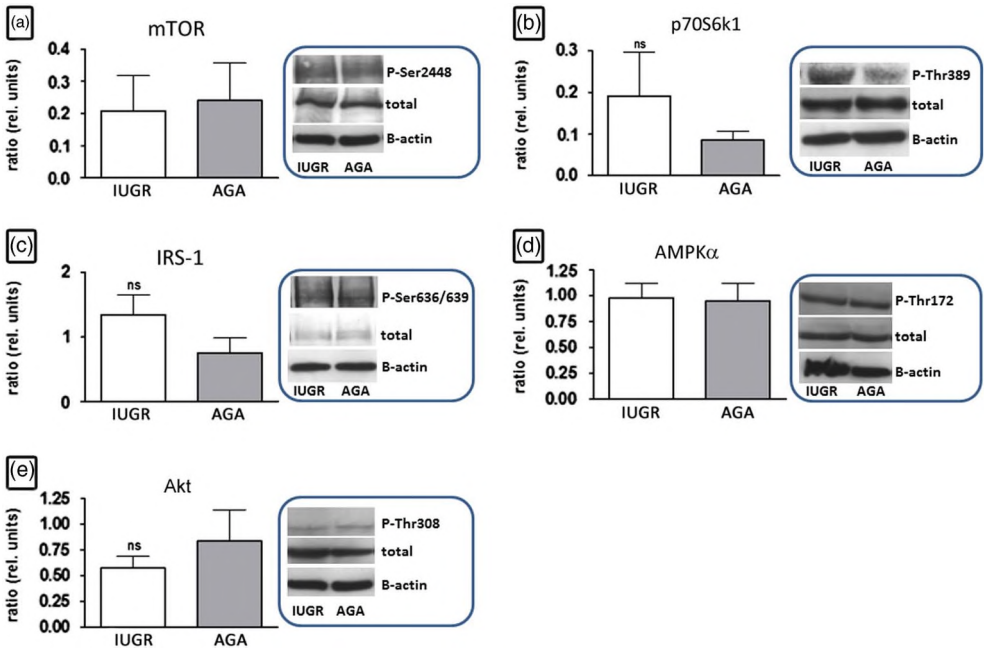

Fig. 2. Western blot analysis of protein expression of mammalian target of rapamycin (mTOR) pathway components in cytosol-enriched fractions of placental homogenates. On the left: densitometric evaluation of activated (phosphorylated)-to-total ratio of intrauterine growth restriction (IUGR; white bars) and appropriate-for-gestational age (AGA; grey bars) placentas. On the right: representative western blot bands, including the β-actin (beta actin) band as housekeeping protein. $n = 7$ per group, *P*-value was ns for all proteins. (a) mTOR, mammalian target of rapamycin; (b) p70S6k1, p70S6kinase1; (c) IRS-1, insulin receptor substrate 1; (d) AMPKα, adenosine monophosphate-activated protein kinase; (e) Akt, protein kinase B.

beneficial effects of catch-up growth,[39,40] it is believed that catch-up growth has mostly adverse long-term consequences for individuals born with IUGR (reviewed by[41]). These children have increased subcutaneous and visceral obesity[8] leading to type 2 diabetes and an increased cardiovascular risk in adulthood.[42,43] We determined, that the level of phosphorylated mTOR in IUGR placentas correlates positively with the growth velocity in the 1st year of life. This finding was IUGR-specific. At birth, mTOR activation (phosphorylated-to-total ratio) correlated negatively with birth weight/length. Hence, higher levels of placental mTOR phosphorylation correlated with a reduced birth weight and length, yet with an increase of catch-up growth in our IUGR cohort. Accordingly, activation of the mTOR downstream signaling intermediate p70S6k1 correlated positively with BMI and the increment in length at 1 year of age. While the presented study only covers the development in the 1st year of life, further long-term follow-up of these children is needed, with regard to catch-up growth-related IUGR sequelae.

When looking at correlations of mTOR pathway signaling components with auxological parameters in our total cohort (IUGR and AGA placentas), we found little correlation to the placental levels of activated mTOR and p70S6k1. In contrast, birth weight/length and BMI at 1 year of age correlated negatively with placental levels of total AMPKα. As the growth of our AGA cohort was normal, we were interested in the increment in weight and length in the 1st year of life in our total cohort. Surprisingly, we found a negative correlation with the level of Akt phosphorylation. This was somehow unexpected, as maternal low protein diet in the pregnant rat inhibits placental Akt phosphorylation (Thr308)[23] and (contrary to our findings) in human IUGR a lower activated-to-total ratio of Akt is found.[44] Akt activation is driven by growth factors, hormones and cytokines, such as IGF-I and insulin, via PI3K activation of PDK1.[45] Our finding that activated placental Akt is inversely correlated with the rate of weight and length gain in the total cohort might be indicative of an increased placental sensitivity for growth factors, such as IGF-I, in smaller neonates, with subsequent activation of Akt and vice versa in larger neonates. However, IRS-1 sensitivity, measured via phosphorylation of IRS-1 did not differ between the IUGR and AGA group in our study. Moreover, we did not observe correlations of auxological parameters with Akt in our IUGR group. Hence, at this time, the patho-physiological relevance of the negative correlation of Akt phosphorylation with auxological data at the age of 1 year remains unclear.

Our western blot analysis did not reveal significant group differences between IUGR and AGA placentas. This result is in disagreement with previous studies analysing the placental mTOR-pathway in IUGR.

In human IUGR placentas at term (38 weeks of gestation, $n = 9$), Roos *et al.*[46] found a significantly reduced protein expression of phosphorylated p70S6k1 (Thr-389), a key downstream target of mTOR and a measure of the activity of the mTOR signaling pathway,[47] while expression of total mTOR

(a)

IUGR

At birth

protein	parameter	coefficient of correlation	p-value
P/T-mTOR	Birth length	-0.901**	.006
P/T-mTOR	Birth weight	-0.929**	.003

At 1 year of age

Length

protein	parameter	coefficient of correlation	p-value
P/T-p70S6k1	Δ length	0.943**	.005
total p70S6k1	Δ length	-0.829*	.042
P-mTOR	Growth velocity (cm/year)	0.812*	.050

BMI

protein	parameter	coefficient of correlation	p-value
total p70S6k1	BMI	-0.886*	.019
P/T-p70S6k1	BMI SDS	1.000**	
total p70S6k1	BMI SDS	-0.943**	.005

(b)

AGA

At birth

protein	parameter	coefficient of correlation	p-value
P-IRS-1	Birth weight SDS	.857*	.014

At 1 year of age

Length

protein	parameter	coefficient of correlation	p-value
total mTOR	Δ length SDS	.900*	.037
P-Akt	Δ length SDS	-.900*	.037
P/T-Akt	Δ length SDS	-.900*	.037
P-AMPK alpha	Growth velocity (cm/year)	-.900*	.037

Weight

protein	parameter	coefficient of correlation	p-value
total mTOR	Δ weight	.900*	.037
P-mTOR	Δ weight	.900*	.037
P-Akt	Δ weight	-.900*	.037
P/T-Akt	Δ weight	-.900*	.037
P-IRS-1	Δ weight	.900*	.037
total mTOR	Δ weight SDS	.900*	.037
P-mTOR	Δ weight SDS	.900*	.037
P-Akt	Δ weight SDS	-.900*	.037
P/T-Akt	Δ weight SDS	-.900*	.037
P-IRS-1	Δ weight SDS	.900*	.037

BMI

protein	parameter	coefficient of correlation	p-value
P-mTOR	BMI	.900*	.037
P/T-AMPK alpha	BMI	1.000**	
P-IRS-1	BMI	.900*	.037
P/T-AMPK alpha	BMI SDS	.900*	.037

(c)

Total cohort

At birth

protein	parameter	coefficient of correlation	p-value
total AMPKα	Birth length	-0.577*	.031
total AMPKα	Birth weight	-0.552*	.041

At 1 year of age

Length

protein	parameter	coefficient of correlation	p-value
P/T-Akt	Δ length SDS	-0.664*	.026
P-Akt	Δ length SDS	-0.636*	.035
total Akt	Δ length SDS	0.618*	.043
total mTOR	Length SDS	0.682*	.021
P-Akt	Length	-0.606*	.048

Weight

protein	parameter	coefficient of correlation	p-value
P/T-Akt	Δ weight SDS	-0.745**	.008
total p70S6k1	Δ weight SDS	-0.718*	.013
P-Akt	Δ weight SDS	-0.718*	.013
total mTOR	Δ weight SDS	0.673*	.023
P-mTOR	Δ weight SDS	0.618*	.043
total p70S6k1	Δ weight	-0.627*	.039
P-Akt	Weight	-0.606*	.048

BMI

protein	parameter	coefficient of correlation	p-value
total AMPKα	BMI	-0.736**	.010
total AMPKα	BMI SDS	-0.691*	.019

(d)

At birth	IUGR			AGA			Total cohort		
	length	weight	BMI	length	weight	BMI	length	weight	BMI
Total mTOR									
P-mTOR									
P/T-mTOR									
Total p70S6k1									
P-p70S6k1									
P/T-p70S6k1									
Total IRS-1									
P-IRS-1									
P/T-IRS-1									
Total AMPK									
P-AMPK									
P/T-AMPK									
Total Akt									
P-Akt									
P/T-Akt									

(e)

At 1 year of age	IUGR			AGA			Total cohort		
	length	weight	BMI	length	weight	BMI	length	weight	BMI
Total mTOR									
P-mTOR									
P/T-mTOR									
Total p70S6k1									
P-p70S6k1									
P/T-p70S6k1									
Total IRS-1									
P-IRS-1									
P/T-IRS-1									
Total AMPK									
P-AMPK									
P/T-AMPK									
Total Akt									
P-Akt									
P/T-Akt									

Fig. 3. Correlation of placental mTOR pathway signaling components with auxological parameters at birth and at 1 year of age. Displayed are data of the intrauterine growth restriction group (IUGR, a), control group (AGA, b) and total cohort (c). (d and e) give an overview (heatmap) of observed correlations (green = positive correlation; red = negative correlation) sorted by their auxologic category (weight, length and BMI) at birth (d) and at 1 year of age (e). Analysis was performed using the non-parametric Spearman rho (*r*) correlation. SDS = standard deviation score; IUGR = intrauterine growth restriction; AGA, appropriate-for-gestational age; BMI, body mass index; P, phosphorylated (activated); P/T, activated-to-total ratio; $^{*}P < 0.05$, $^{**}P < 0.01$.

was increased.[46] Phosphorylated mTOR (Ser2448) and total p70S6k1 levels, however, were not examined in this study. Street *et al.*[44] found a significant reduction of total and activated Akt levels in human IUGR placentas at term and determined a low activated-to-total ratio of Akt expression levels in IUGR *v.* AGA placentas.

Comparing a smaller number of human preterm IUGR placentas with term AGA placentas, Laviola *et al.*[48] observed a reduced content of total Akt and an unchanged content of activated Akt. Akt-1-deficient mice[23] develop fetal growth restriction as a result of placental insufficiency and in the rat, maternal low protein diet during pregnancy inhibits placental-activated Akt (P-Thr308) without changing total Akt and inhibits ribosomal protein S6 via P-Ser 235/236, without changing total ribosomal protein S6 content.[49]

Taken together, there is evidence from the literature that IUGR affects the activity of placental mTOR pathway components. The design of these studies (definition of IUGR, term *v.* preterm, animal model *v.* human) and the results obtained, however, vary greatly. The fact, that we did not find significant differences in our placental mTOR signaling components of IUGR, might be due to the complexity of the mTOR pathway. As an important homeostatic nexus protein, mTOR integrates converging extracellular signals from growth factors (e.g. insulin, IGF-I, leptin), nutrients and stressors (e.g. hypoxia) and matches them to the intracellular energy level.[4,50] Subsequently, the finding, that placental weight was significantly reduced in other studies[46] while unaffected in our cohort might point to a more pronounced IUGR in these studies that might in part have accounted for the observed differencein the activity of placental mTOR signaling components. Interestingly, Kavitha *et al.*[51] – studying the effects of intrauterine malnutrition in the baboon – did not find a significant difference in placental weight, albeit a significant IUGR and an inhibition of placental p70S6k1 and insulin/IGF-I downstream signaling. Hence, further influential factors for the variance of our findings have to be taken into account: as stress and endocrine signals are important regulators of mTOR, the mode of delivery might be of significant relevance when analysing mTOR pathway components. In a recent study, Lager *et al.*[52] showed that placental mTOR signaling is significantly inhibited in response to labor.

Hence, the summation of various upstream signals, each only modest in magnitude, could account for the difference in study results.

Moreover, sample handling *in vitro* is a critical factor in this context. The mTOR nexus shows a rapid responsiveness with changes in phosphorylation occurring within 15 min and vanishing as early as after 40 min.[53] This poses a big challenge for rapid sampling, snap freezing and tissue processing. Furthermore, placental *in vivo* results may vary from *in vitro* findings in isolated trophoblasts. Using *in vitro* studies, Roos *et al.*[13] found mTOR expression mainly in SCT. Our immunohistochemical *in vivo* data, however, show mTOR expression in SCT, VCT and EVT, as well as in DSC. This finding could argue for an examination of the mTOR system in functional placental compartments, instead of in isolated placental samples of whole placenta. Possibly, certain differences in mTOR activation might have been masked by our whole organ analysis. Finally, we cannot exclude the possibility that further technical differences, especially such as the choice of western blot antibodies, may contribute to the contrasting results found in the literature and may also lead to the differences in the results we obtained compared with other studies.

Taken together, mTOR is an important regulator of placental homeostasis, integrating many converging signals. We were able to determine an IUGR-specific correlation of activated placental mTOR with catch-up growth in the 1st year of life. However, western blot analysis of placental mTOR pathway signal components showed limitations regarding the identification of group differences, possibly owing to the complexity of this signaling network, as discussed. Thus, the role of the placental mTOR pathway as potential biomarker for assessing the risk of catch-up growth post IUGR needs to be re-evaluated in a bigger cohort, to better account for possible intrinsic and extrinsic confounders.

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Conflicts of Interest

The authors report no conflict of interest.

Ethical Standards

The authors assert that all procedures contributing to this work comply with the Helsinki Declaration of 1975, as revised in 2008, and the study was reviewed and approved by the Ethical Committee of the Medical Faculty of the University of Erlangen-Nürnberg (#2625 02/28/02). Written consent was given by the parents of every patient.

Supplementary material

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