


Critical role of mTOR, PPAR γ and PPAR δ signaling in regulating early pregnancy decidual function, embryo viability and feto-placental growth

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STUDY QUESTION: What are the consequences of inhibiting mTOR, the mechanistic target of rapamycin (mTOR), and the peroxisome proliferator activated receptor gamma (PPAR γ) and PPAR δ pathways in the early post-implantation period on decidual function, embryo viability and feto-placental growth in the rat?

SUMMARY ANSWER: mTOR inhibition from Days 7 to 9 of pregnancy in rats caused decidual PPAR γ and PPAR δ upregulation on Day 9 of pregnancy and resulted in embryo resorption by Day 14 of pregnancy. PPAR γ and PPAR δ inhibition differentially affected decidual mTOR signaling and levels of target proteins relevant to lipid histotrophic nutrition and led to reduced feto-placental weights on Day 14 of pregnancy.

WHAT IS KNOWN ALREADY: Although mTOR, PPAR γ and PPAR δ are nutrient sensors important during implantation, the role of these signaling pathways in decidual function and how they interact in the early post-implantation period are unknown. Perilipin 2 (PLIN2) and fatty acid binding protein 4 (FABP4), two adipogenic proteins involved in lipid histotrophic nutrition, are targets of mTOR and PPAR signaling pathways in a variety of tissues.

STUDY DESIGN, SIZE, DURATION: Rapamycin (mTOR inhibitor, 0.75 mg/kg, sc), T0070907 (PPAR γ inhibitor, 0.001 mg/kg, sc), GSK0660 (PPAR δ inhibitor, 0.1 mg/kg, sc) or vehicle was injected daily to pregnant rats from Days 7 to 9 of pregnancy and the studies were performed on Day 9 of pregnancy ($n = 7$ per group) or Day 14 of pregnancy ($n = 7$ per group).

PARTICIPANTS/MATERIALS, SETTING, METHODS: On Day 9 of pregnancy, rat decidua were collected and prepared for western blot and immunohistochemical studies. On Day 14 of pregnancy, the resorption rate, number of viable fetuses, crown–rump length and placental and decidual weights were determined.

MAIN RESULTS AND THE ROLE OF CHANCE: Inhibition of mTOR in the early post-implantation period led to a reduction in FABP4 protein levels, an increase in PLIN2 levels and an upregulation of PPAR γ and PPAR δ in 9-day-pregnant rat decidua. Most embryos were viable on Day 9 of pregnancy but had resorbed by Day 14 of pregnancy. This denotes a key function of mTOR in the post-implantation period and suggests that activation of PPAR signaling was insufficient to compensate for impaired nutritional/survival signaling induced by mTOR inhibition. Inhibition of PPAR γ signaling resulted in decreased decidual PLIN2 and FABP4 protein expression as well as in inhibition of decidual mTOR signaling in Day 9 of pregnancy. This treatment also reduced feto-placental growth on Day 14 of pregnancy, revealing the relevance of PPAR γ signaling in sustaining post-implantation growth. Moreover, following inhibition of PPAR δ , PLIN2 levels were decreased and mTOR complex 1 and 2 signaling was altered in decidua on Day 9 of pregnancy. On Day 14 of pregnancy, PPAR δ inhibition caused reduced feto-placental weight, increased decidual weight and increased resorption rate, suggesting a key role of PPAR δ in sustaining post-implantation development.

LARGE SCALE DATA: Not applicable.

LIMITATIONS, REASONS FOR CAUTION: This is an *in vivo* animal study and the relevance of the results for humans remains to be established.

WIDER IMPLICATIONS OF THE FINDINGS: The early post-implantation period is a critical window of development and changes in the intrauterine environment may cause embryo resorption and lead to placental and fetal growth restriction. mTOR, PPAR γ and PPAR δ signaling are decidual nutrient sensors with extensive cross-talk that regulates adipogenic proteins involved in histotrophic nutrition and important for embryo viability and early placental and fetal development and growth.

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Key words: gestation / development / decidua / PPARs / histotrophic nutrition / lipids / embryo resorption / post-implantation period

Introduction

Emerging evidence suggests that the mechanistic target of rapamycin (mTOR) signaling functions as a placental nutrient sensor, regulating placental function and fetal growth in late pregnancy (Dimasuy et al., 2016). Placental mTOR is activated in women with obesity or gestational diabetes mellitus (GDM) who deliver large babies and in animal models associated with fetal overgrowth (Gaccioli et al., 2013; Jansson et al., 2013; Capobianco et al., 2016; Perez-Perez et al., 2013). In contrast, intrauterine growth restriction (IUGR) is characterized by inhibition of placental mTOR signaling in humans (Roos et al., 2007; Chen et al., 2015) and in animal models (Rosario et al., 2011; Kavitha et al., 2014).

mTOR is a serine/threonine kinase that is activated by increased nutrient levels and growth factor signaling, thereby regulating gene transcription and protein translation to control multiple cellular processes that promote cell metabolism, growth and survival (Laplante and Sabatini, 2013; Shimobayashi and Hall, 2014). mTOR forms two protein complexes, mTORC1 and mTORC2, in association with raptor or rictor, respectively (Shimobayashi and Hall, 2014). Ribosomal protein S6 (rpS6) and 4E-binding protein 1 (4E-BP1) are downstream effectors of mTORC1 activation that positively regulate protein synthesis (Shimobayashi and Hall, 2014). 4E-BP1 phosphorylation induces the release of the eukaryotic initiation factor 4E (eIF4E), activating protein synthesis and cell growth (Laplante and Sabatini, 2013). mTORC2 phosphorylates a number of targets, including serum and glucocorticoid-inducible kinase 1 (SGK-1), with important roles in implantation (Lou et al., 2017). Although mTOR signaling has been implicated in regulating implantation and trophoblast and embryo growth (Martin et al., 2003; Gardner and Harvey, 2015), the function of mTOR signaling in the early post-implantation period remains to be determined.

In the post-implantation period, the decidua plays a fundamental nutritional role, providing histotrophic nutrition to allow embryo survival until the formation of a mature placenta (Burton et al., 2010; Filant and Spencer, 2014). In murine embryos, the inverted yolk sac is crucial in histotrophic nutrition, as it is involved in the processes of endocytosis and pinocytosis of secretions from the uterine glands and decidual secretions (Holson et al., 2005; Zohn and Sarkar, 2010). Lipoprotein particles are internalized in the yolk sac through the multi-ligand endocytic receptor complex for further repackaging of lipids and transfer to the embryo (Herz and Farese, 1999; Zohn and Sarkar, 2010). The secretions from decidual cells and endometrial glands are rich in lipid droplets (Burton et al., 2007). Lipid droplets are characterized by the presence of the adipogenic protein perilipin 2 (PLIN2),

which is considered a marker for the lipid load of non-adipogenic cells (Heid et al., 1998). PLIN2 is transcriptionally regulated by peroxisome proliferator activated receptor γ (PPAR γ) and PPAR δ in trophoblasts and other cell types (Bildirici et al., 2003; Tobin et al., 2006). In addition, fatty acid binding protein 4 (FABP4), a protein involved in intracellular and transcellular lipid transfer and relevant for decidual function (Tian et al., 2011), is transcriptionally regulated by PPAR γ (Tan et al., 2002).

PPARs are transcription factors activated by ligands of lipid nature (e.g. mono- and polyunsaturated fatty acids) that function as master transcription factors to regulate gene expression in metabolic processes, critically important in maintaining lipid homeostasis and antioxidant/anti-inflammatory responses (Wahli and Michalik, 2012). Besides, they play relevant roles in embryo organogenesis and fetoplacental development and metabolism (Jawerbaum and Capobianco, 2011; Kadam et al., 2015; Lendvai et al., 2016). There are three PPAR isoforms, and among which, PPAR γ and PPAR δ play key roles in adipogenesis and are essential for embryo development (Barak et al., 2008; Barquissau et al., 2017; Wahli and Michalik, 2012). Indeed, there are specific placental developmental defects in PPAR γ and PPAR δ null mice, which lead to embryo death, suggesting that both isoforms are essential and the absence of compensatory effects by other PPAR isoforms that rescue the adverse phenotype (Wang et al., 2007; Barak et al., 2008). Our previous studies showed reduced embryonic PPAR δ and decidual PPAR γ in diabetic rats during early organogenesis, changes associated with impaired embryo morphogenesis (Higa et al., 2010, 2014). Moreover, changes in fatty acid metabolism involve extensive PPAR and mTOR cross-talk in a variety of cell types (Ishiguro et al., 2006; Blanchard et al., 2012; Angela et al., 2016) and these nutrient signaling pathways share targets such as FABP4 (Wang et al., 2017). In the current study, we hypothesized that there is a cross-talk between mTOR, PPAR γ and PPAR δ in the decidua that impacts post-implantation embryo survival and fetoplacental growth. To test this hypothesis, we determined the effects of pharmacological inhibition of mTOR, PPAR γ and PPAR δ signaling during the early post-implantation period in pregnant rats.

Materials and Methods

Animals

Albino Wistar rats were obtained from the certified animal facility of the School of Exact and Natural Sciences, University of Buenos Aires (UBA, Argentina). In our animal facility (CEFYBO-CONICET, UBA, Argentina), rats received water and food *ad libitum* (commercial rat chow, Asociación Cooperativa Argentina, Buenos Aires, Argentina), on a lighting cycle of

12 h light:12 h dark. The animal protocol was approved by the Institutional Committee for the Care and Use of Experimental Animals (CICUAL, Resolution CD No. 1497/2013), School of Medicine, University of Buenos Aires, and conducted according to the Guide for the Care and Use of Laboratory Animals, US National Institutes of Health (NIH Publication, 8th edition, 2011) <http://www.ncbi.nlm.nih.gov/books/NBK54050/?report=reader>. Females were mated and mating was confirmed by the presence of sperm in vaginal smears and this day was designated Day 1 of pregnancy. Pregnant females received three daily sc injections beginning on Day 7 of pregnancy: receiving either (i) rapamycin (mTOR inhibitor, 0.75 mg/kg, LC Laboratories, Massachusetts, USA); (ii) T0070907 (PPAR γ inhibitor, 0.001 mg/kg, Sigma Aldrich, Missouri, USA); (iii) GSK0660 (PPAR δ inhibitor, 0.1 mg/kg, Sigma Aldrich, Missouri, USA) or (iv) vehicle (control). The doses of the inhibitors used were chosen based on bibliography and preliminary experiments (Lee *et al.*, 2002; Harston *et al.*, 2011; Tsai *et al.*, 2014). The dosing interval was chosen aiming to address an early and pre-embryo post-implantation stage in which the decidua is crucial for embryo development and nutrition. Half of the animals were euthanized in a CO₂ chamber on Day 9 of pregnancy (3 h after the last injection) and the remainder on Day 14 of pregnancy. Using a stereomicroscope and microsurgical dissecting instruments, the balls of decidual tissue were explanted from each uterus on Day 9 of pregnancy ($n = 7$ dams in each experimental group). The decidua were prepared for immunohistochemical analyses of the levels of FABP4 and PLIN2, or homogenized in ice-cold buffer D (250 mM sucrose, 10 mM Hepes-Tris, pH 7.4) with protease inhibitor cocktail and phosphatase inhibitor cocktails 2 and 3 (Sigma Aldrich, Missouri, USA) and stored at -80°C for western blot analysis of PPAR γ , PPAR δ and functional readouts of the mTOR pathway. Decidua, fetuses and placentas from 14-day pregnant rats ($n = 7$ dams in each experimental group) were explanted from each uterus, examined morphologically under a stereomicroscope and weighed.

Immunohistochemistry

Immunostaining of FABP4 and PLIN2 was performed in the decidua of 9-day pregnant rats ($n = 7$ in each experimental group). Each decidua was paraffinized and serially sectioned (5 μm). Sections were deparaffinized, rehydrated through a graded series of ethanol and the endogenous peroxidase activity was blocked. The sections were processed using the corresponding primary antibodies: anti-FABP4 (rabbit polyclonal antibody (GTX54016), 1:100 dilution, GeneTex, CA, USA), and anti-PLIN-2 (mouse monoclonal antibody (LS-C348703 clone API25), 1:100 dilution, LifeSpan BioSciences, Washington, USA). All primary antibodies were checked for antibody specificity by Western blot and diluted in PBS-Tween with 1% bovine serum albumin (BSA). Negative controls were performed in the absence of primary antibody and by replacing the primary antibody by a pooled serum of the same species that contains a spectrum of the IgG subclasses (Vector Laboratories, Burlingame, CA, USA). Biotinylated antibodies (anti-rabbit IgG or anti-mouse IgG-rat absorbed), 1:200 PBS-Tween in 1.5% normal horse serum (Vector Laboratories, Burlingame, CA, USA), were applied, followed by an incubation with an avidin-biotin complex for 60 min (Vector Laboratories). Staining was visualized by adding 40% w/v 3,3'-diaminobenzidine tetrahydrochloride chromogen-buffer plus 0.02% (v/v) hydrogen peroxide in 0.05 M Tris (pH 7.6) for 10 min (Martinez *et al.*, 2012). Three entire sections per decidua were examined using light microscopy by two skilled blinded observers. Immunoreactivity intensity was quantified using the ImageProPlus software. Data are shown as relative to a value of 1, arbitrarily assigned to the control. We also performed a semiquantitative score, which led to similar results (data not shown). In addition, also in the yolk sac and the uterine glands a semiquantitative score was performed.

Western blot analysis

Proteins from homogenates of pooled decidua from the same litter ($n = 7$ rats in each experimental group) were separated by SDS-PAGE and transferred to PVDF membranes (35 V constant, overnight at 4°C), as previously described (Capobianco *et al.*, 2016). The membranes were stained with Ponceau S staining solution for total proteins (Sigma-Aldrich) to confirm equal loading and transfer. Blocking was carried out for 1 h at room temperature in 1% BSA in TBS-Tween and membranes were incubated with the primary antibody (diluted in 1% BSA in TBS-Tween) overnight at 4°C . Protein expression of total and phosphorylated rpS6, total and phosphorylated 4E-BP1 was determined using antibodies (monoclonal clone 5G10 and polyclonal 2211, 9452 and 9459, respectively) from Cell Signaling Technology (Massachusetts, USA). Total and phosphorylated SGK-1 was evaluated using polyclonal antibodies (AF3200) from RyD Systems (Minnesota, USA) and (SC16745) from Santa Cruz (Texas, USA), respectively. PLIN2 was determined using antibodies from LifeSpan BioSciences (LS-C348703 clone API25), while protein expression of PPAR γ and PPAR δ was determined using polyclonal antibodies (101 700 and 101 720, respectively) from Cayman Chemical Company, Michigan, USA. After washing, the membranes were incubated with the appropriate peroxidase conjugated secondary antibody and visualized using ECL detection solution (Thermo Scientific) and captured in a Chemiluminescence imaging system (GeneGnomeXRQ, Syngene). Densitometry analysis was performed with ImageJ software (Schneider *et al.*, 2012). The expression of the target protein in each individual lane was normalized for total protein staining to adjust for unequal loading. The mean of all the samples within a single gel was calculated and the expression of the target protein in each sample was calculated as a percentage of that mean (target/total protein).

Statistical analysis

Data are presented as the mean \pm SEM. Groups were compared by Student *t* test or Chi-square test as appropriate. A $P < 0.05$ was considered statistically significant.

Results

Effects of inhibition of mTOR signaling in the early post-implantation period

Aiming to address the impact of mTOR inhibition limited to an early post-implantation period, rapamycin (0.75 mg/kg, sc) was administered to pregnant rats on Days 7, 8 and 9 of pregnancy and the decidua were evaluated 3 h after the last injection on Day 9 of pregnancy. Rapamycin administration led to more than 50% reduction in the phosphorylation of downstream targets of mTORC1 (rpS6 and 4E-BP1) and mTORC2 (SGK-1) in the decidua (Supplementary Fig. S1). We determined the protein expression of FABP4 and found that the inhibition of mTOR signaling resulted in a 22% reduction of FABP4 levels in the decidua ($P < 0.05$), when compared to control rats (Fig. 1). A similar pattern of expression was observed in decidual cells and in the uterine glands, although only a weak staining, which did not change with the rapamycin treatment, was observed in the yolk sac (Fig. 1). We next evaluated the protein expression of PLIN2 and found that the inhibition of mTOR signaling resulted in a 25% increase in decidual PLIN2 levels ($P < 0.01$) when compared to controls (Fig. 1). This increase was also observed when PLIN2 protein

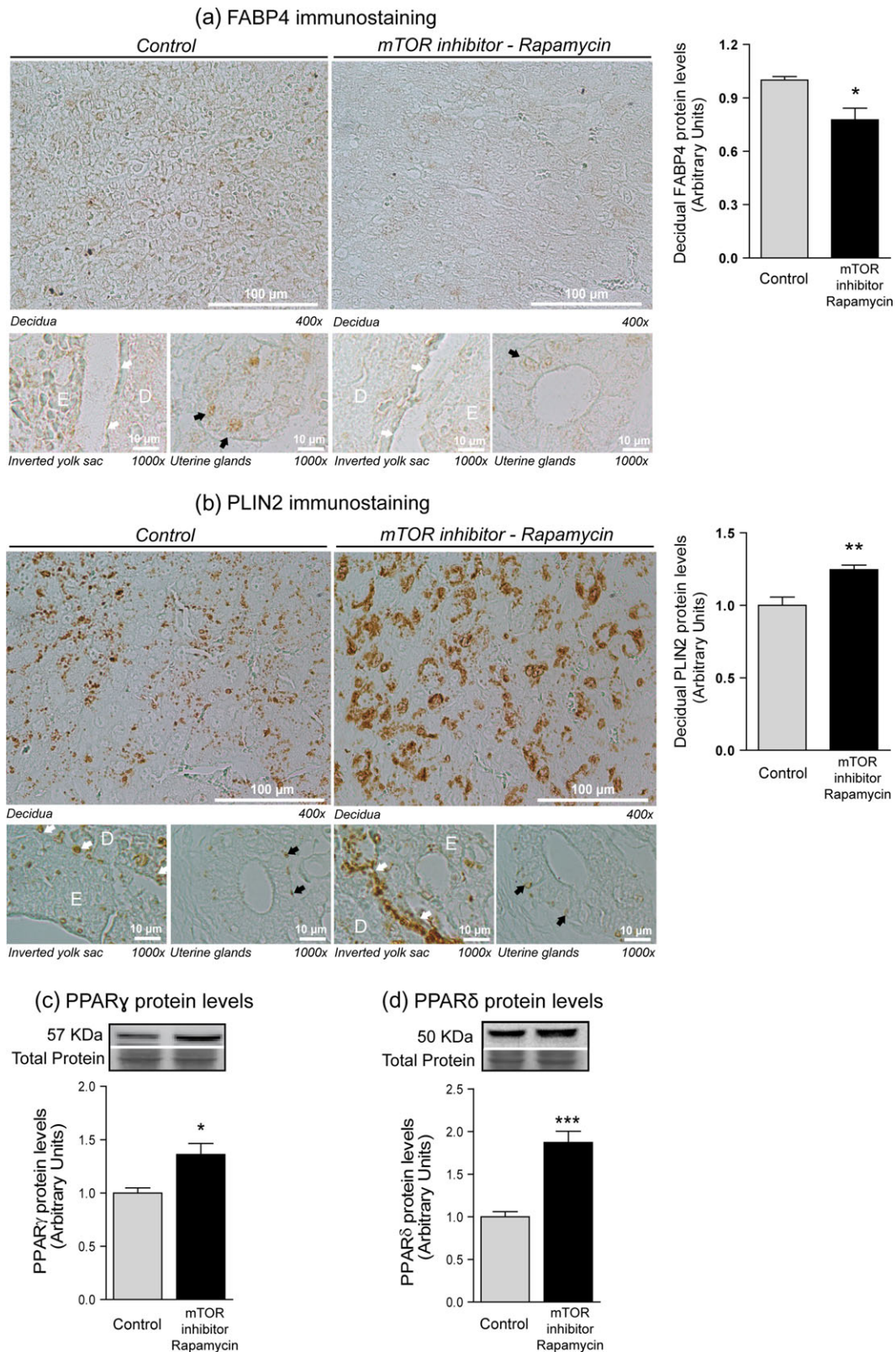


Figure 1 Effect of *in vivo* treatments with the mTOR inhibitor rapamycin during Days 7–9 of pregnancy on decidual protein levels of (a) FABP4, (b) PLIN2, (c) PPAR γ and (d) PPAR δ , evaluated on Day 9 of pregnancy. Values represent mean \pm SEM obtained from seven rats in each experimental group. Statistical analysis: Student *t* test. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ versus control. Representative photos of FABP4 and PLIN2 in the decidua, yolk sac and uterine glands are shown. D, decidua. E, embryo. White arrows point to the yolk sac. Black arrows point to immunostained uterine gland cells.

levels were determined by western blot analysis (Supplementary Fig. S2). Different from decidual cells, only a weak PLIN2 staining was observed in the uterine glands, although the yolk sac showed a strong PLIN2 staining, which increases when mTOR signaling was inhibited (Fig. 1). As PLIN2 is a classical PPAR target involved in the formation of lipid droplets (Heid *et al.*, 1998; Bildirici *et al.*, 2003), and mTOR and PPAR cross-talk has been described in different tissues/cell types (Blanchard *et al.*, 2012; Angela *et al.*, 2016), we next measured the protein expression of PPAR γ and PPAR δ . We observed that mTOR inhibition caused an increase in the protein levels of both PPAR γ (36%, $P < 0.05$) and PPAR δ (87%, $P < 0.001$) in the decidua (Fig. 1).

Effects of inhibition of PPAR γ signaling in the early post-implantation period

To next address the effect of PPAR γ inhibition limited to an early post-implantation stage, the PPAR γ inhibitor T0070907 (0.001 mg/kg, sc) was administered to rats on Days 7–9 of pregnancy and the decidua were evaluated on Day 9 of pregnancy. PPAR γ inhibition led to a decrease in the protein levels of FABP4 (40%) in the decidua when compared to controls ($P < 0.01$, Fig. 2). A reduced FABP4 expression was also observed in the uterine glands, although only a weak staining was observed in the yolk sac in both the control and the PPAR γ inhibited groups (Fig. 2). The inhibition of PPAR γ led to a decrease in the

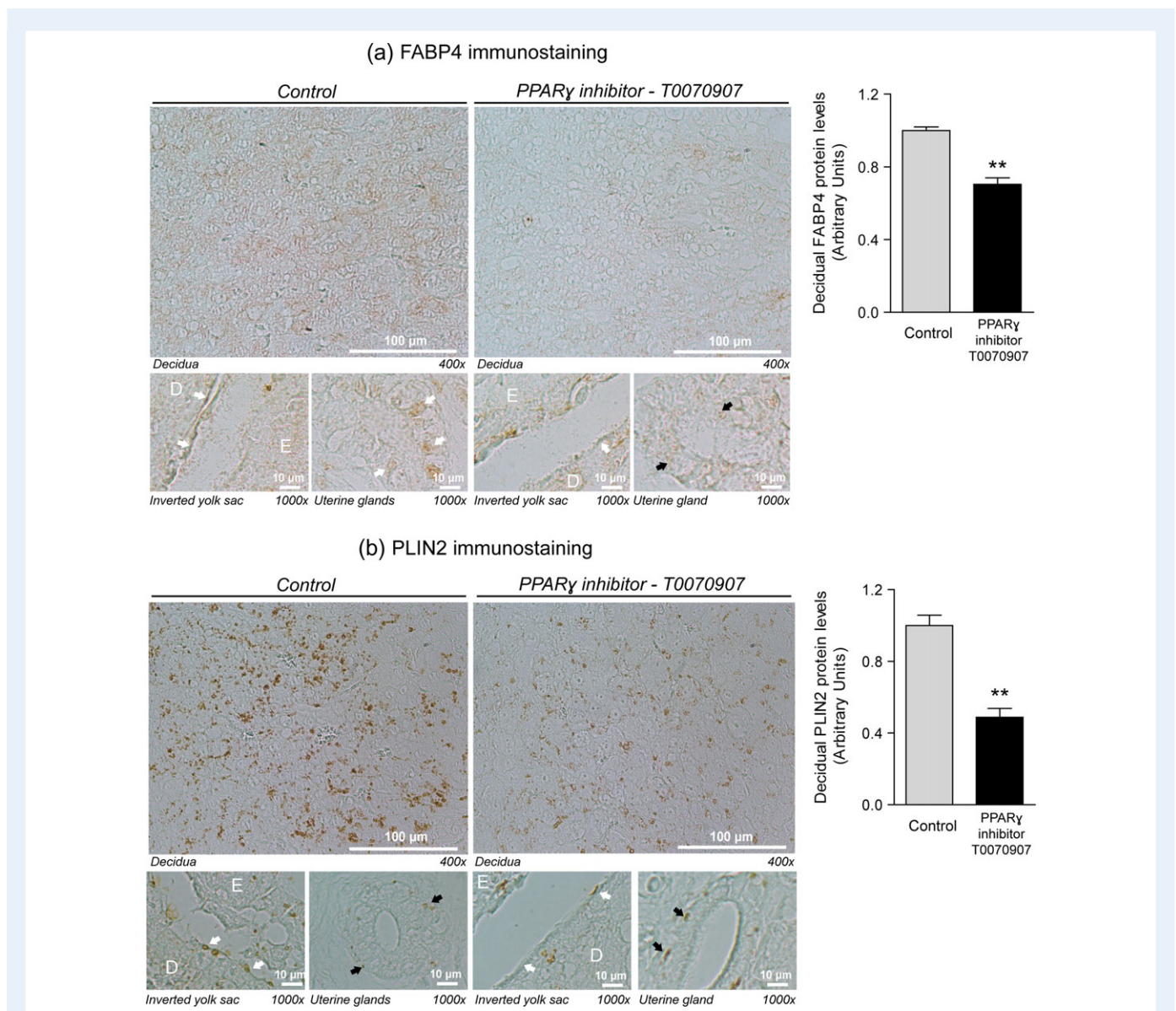


Figure 2 Effect of *in vivo* treatments with the PPAR γ inhibitor T0070907 during Days 7–9 of pregnancy on decidual protein levels of (a) FABP4 and (b) PLIN2, evaluated on Day 9 of pregnancy. Values represent mean \pm SEM obtained from seven rats in each experimental group. Statistical analysis: Student t test. ** $P < 0.01$ versus control. Representative photos of FABP4 and PLIN2 in the decidua, yolk sac and uterine glands are shown. D, decidua. E, embryo. White arrows point to the yolk sac. Black arrows point to immunostained uterine gland cells.

protein levels of PLIN2 (51%) in the decida when compared to controls ($P < 0.01$, Fig. 2). A decrease was also evidenced when decidual PLIN2 protein levels were determined by western blot analysis (Supplementary Fig. S2). In contrast to decidual cells, only a weak PLIN2 staining was observed in the uterine glands from the control and the PPAR γ inhibited group. Similarly to decidual cells, PLIN2 levels were reduced in the yolk sac when PPAR γ was inhibited (Fig. 2). In addition, PPAR γ inhibition decreased the phosphorylation of rpS6 ($P < 0.01$) by a 25% without affecting total rpS6 levels in the decida, whereas it did not change the levels of total and phosphorylated 4E-BP1 compared to controls (Fig. 3). Moreover, we observed a marked reduction of phosphorylated SGK-1 (50%, $P < 0.01$), despite unchanged total SGK-1 levels in the decida from T0070907-treated pregnant rats compared to controls (Fig. 3). Collectively, these data demonstrate that inhibition of decidual PPAR γ inhibits mTORC1 and mTORC2 signaling.

Effects of inhibition of PPAR δ signaling in the early post-implantation period

To address the effect of PPAR δ inhibition limited to an early post-implantation period, the PPAR δ inhibitor GSK0660 (0.1 mg/kg, sc) was administered to rats on days 7 to 9 of pregnancy and the decida were evaluated on Day 9 of pregnancy. PPAR δ inhibition did not alter decidual FABP4 protein levels compared to controls (Fig. 4). Similarly, PPAR δ inhibition did not change FABP4 levels in the uterine glands (Fig. 4). Only a weak FABP4 staining was detected in the yolk sac from rats treated or not with the PPAR δ inhibitor (Fig. 4). The inhibition of PPAR δ led to a decrease in the protein levels of PLIN2 (37%) in the decida when compared to controls ($P < 0.01$, Fig. 4). A similar decrease was evidenced when decidual PLIN2 levels were determined by western blot analysis (Supplementary Fig. S2). In the uterine glands, only a weak PLIN2 staining was observed in both the control and the PPAR δ inhibited groups (Fig. 4). Differently, the yolk sac showed a strong PLIN2 staining, which diminished when PPAR δ was inhibited, similarly to that observed in decidual cells (Fig. 4).

With respect to cross-talk with mTOR pathways, PPAR δ inhibition did not affect the total expression or phosphorylation of rpS6 in the decida, but markedly increased 4E-BP1 phosphorylation by a 73% ($P < 0.001$, Fig. 5) compared to controls, without affecting the total levels of this protein, suggesting the activation of this branch of the mTORC1 signaling pathway. In contrast, decidual levels of phosphorylated SGK-1 were found to be reduced by 53% ($P < 0.001$, Fig. 5) following PPAR δ inhibition, whereas no changes in the levels of total SGK-1 were observed, suggesting the inhibition of the mTORC2 signaling pathway.

Effects of mTOR and PPAR signaling inhibition on resorption rates and growth capacity

The number of viable embryos and the resorption rate on Day 9 of pregnancy were similar in the control rats and in the rats that received the mTOR inhibitor (rapamycin 0.75 mg/kg, sc) from Days 7 to 9 of pregnancy (Fig. 6). To further assess the effects of mTOR inhibition in the early post-implantation period on embryo viability and growth capacity, we evaluated the conceptus on Day 14 of pregnancy following administration of vehicle (control) or the mTOR inhibitor (rapamycin 0.75 mg/kg) from Days 7 to 9 of pregnancy. On Day 14 of pregnancy, a dramatic (92%) decrease in the number of viable fetuses

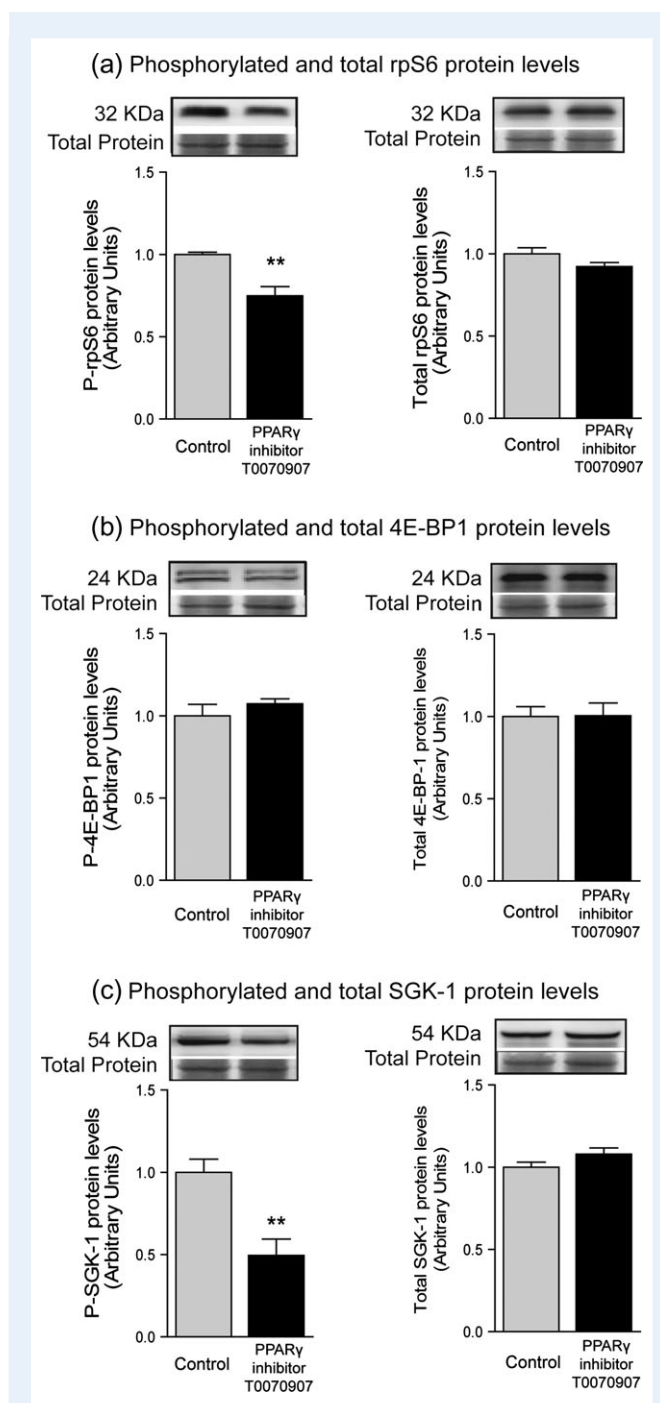


Figure 3 Effect of *in vivo* treatments with the PPAR γ inhibitor T0070907 during Days 7–9 of pregnancy on decidual protein levels of (a) phosphorylated and total rpS6, (b) phosphorylated and total 4E-BP1 and (c) phosphorylated and total SGK-1, evaluated on Day 9 of pregnancy. Values represent mean \pm SEM obtained from seven rats in each experimental group. Statistical analysis: Student *t* test. ** $P < 0.01$ versus control.

per rat ($P < 0.001$) and an increased resorption rate ($P < 0.001$) were evident in the group that received the mTOR inhibitor compared to controls (Fig. 6). Indeed, most fetuses were resorbed, and the few fetuses that survived to Day 14 of pregnancy displayed morphological

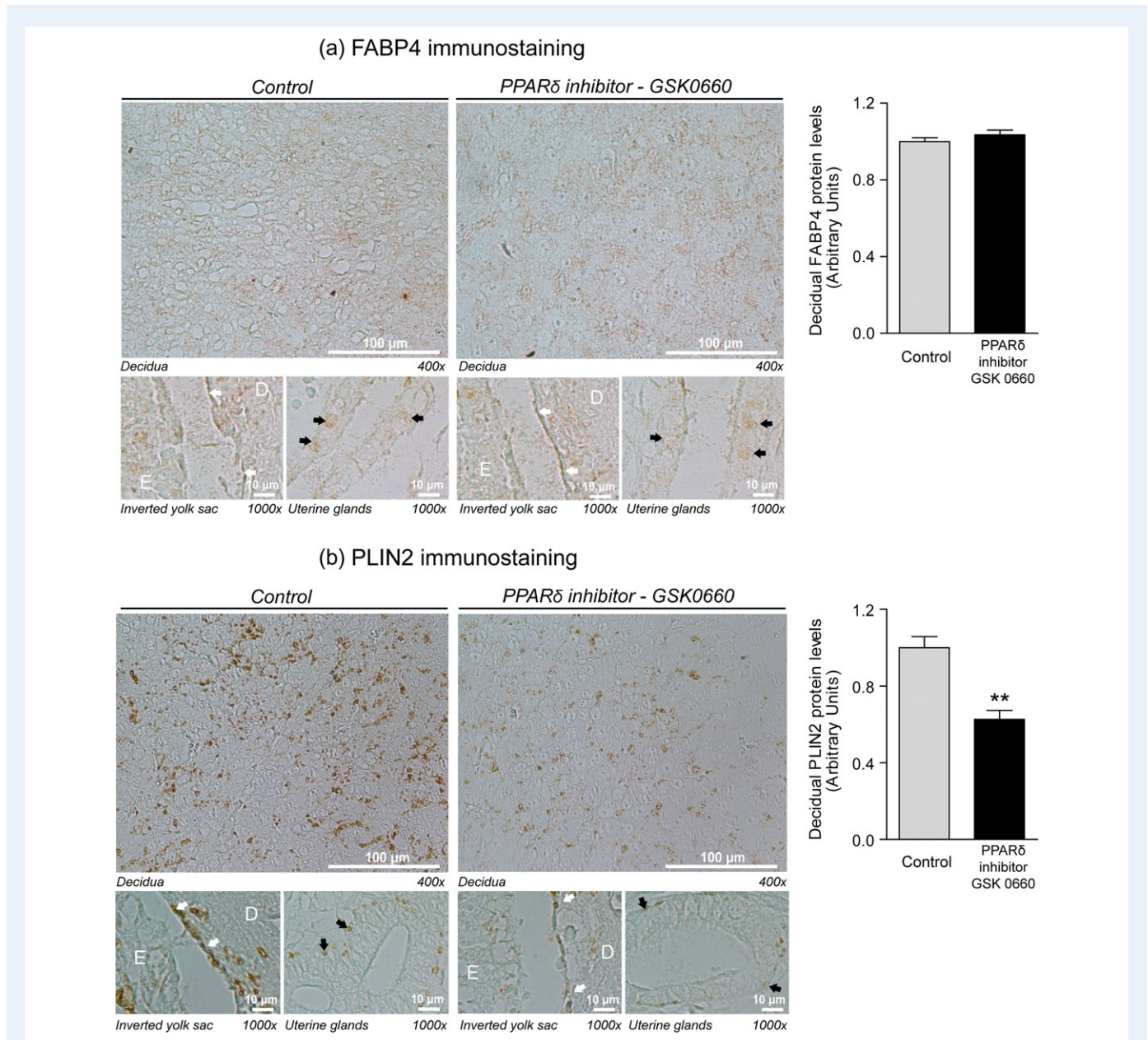


Figure 4 Effect of *in vivo* treatments with the PPAR δ inhibitor GSK0660 during Days 7–9 of pregnancy on decidual protein levels of (a) FABP4 and (b) PLIN2, evaluated on Day 9 of pregnancy. Values represent mean \pm SEM obtained from seven rats in each experimental group. Statistical analysis: Student *t* test. ***P* < 0.01 versus Control. Representative photos of FABP4 and PLIN2 in the decidua, yolk sac and uterine glands are shown. D, decidua. E, embryo. White arrows point to the yolk sac. Black arrows point to immunostained uterine gland cells.

alterations and a severe developmental delay likely leading to future resorptions (Fig. 7).

In the rats that received the PPAR γ inhibitor (T0070907 0.001 mg/kg, sc) from Days 7 to 9 of pregnancy, the number of viable embryos and the resorption rate on Day 9 of pregnancy were similar to those of control rats and remained similar on Day 14 of pregnancy (Fig. 8). The inhibition of PPAR γ did not affect gross fetal morphology on Day 14 of pregnancy (Fig. 7), but the reduced crown–rump length (*P* < 0.001) (Fig. 8) indicated a growth delay. In addition, placental weight was markedly reduced (51%, *P* < 0.001) following PPAR γ inhibition, although decidual weight was unchanged when compared to controls (Fig. 8).

In the rats that received the PPAR δ inhibitor (GSK0660 0.1 mg/kg, sc) from Days 7 to 9 of pregnancy, the number of viable embryos and the resorption rate on Day 9 of pregnancy were similar to those of control rats (Fig. 9). However, on Day 14 of pregnancy, the rats that received the PPAR δ inhibitor during the early post-implantation period showed a reduction in the number of viable fetuses (*P* < 0.05) and an increased resorption rate (*P* < 0.01) as compared to controls (Fig. 9). Although there were no gross malformations on the 14-day fetuses in the group that received the PPAR δ inhibitor (Fig. 7), they were growth restricted, as indicated by their reduced crown–rump length (*P* < 0.01) (Fig. 9). Furthermore, PPAR δ inhibition was associated with

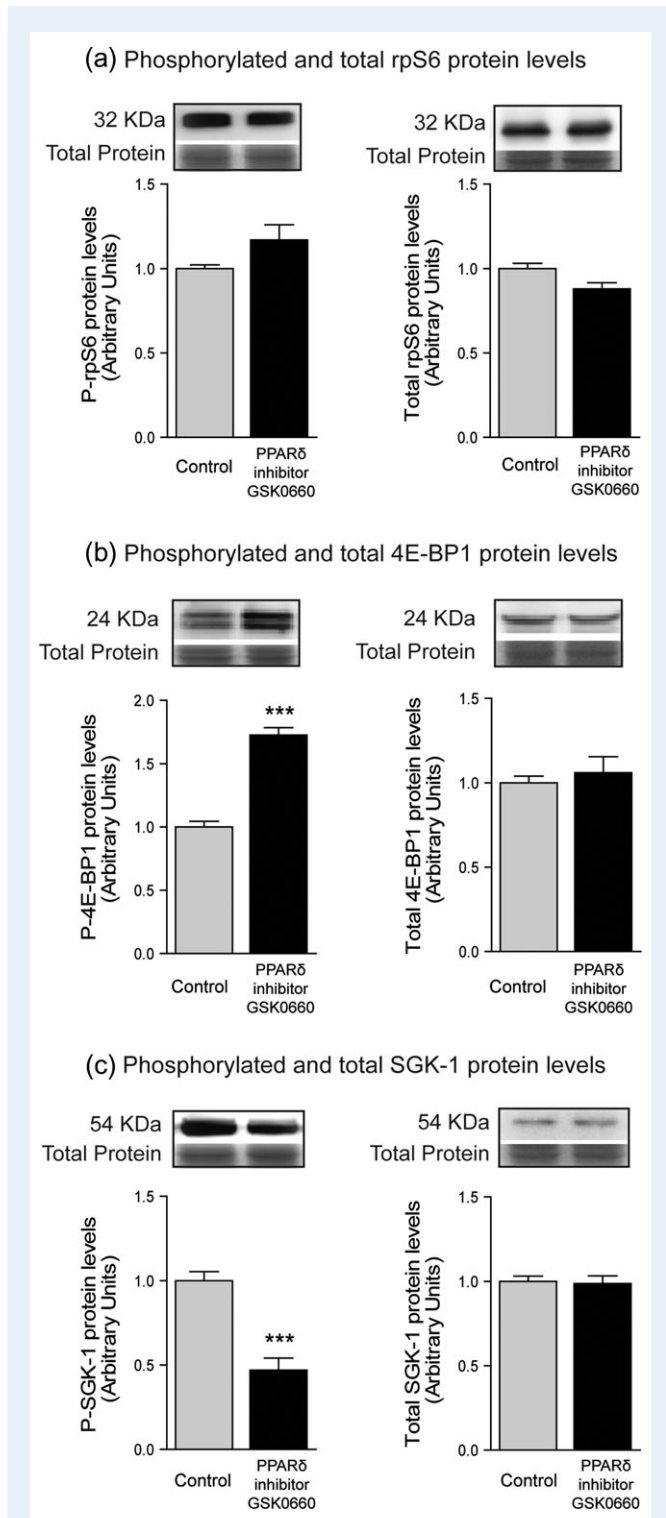


Figure 5 Effect of *in vivo* treatments with the PPAR δ inhibitor GSK0660 during Days 7 to 9 of pregnancy on decidual protein levels of (a) phosphorylated and total rpS6, (b) phosphorylated and total 4E-BP1 and (c) phosphorylated and total SGK-1, evaluated on Day 9 of pregnancy. Values represent mean \pm SEM obtained from seven rats in each experimental group. Statistical analysis: Student t test. *** $P < 0.001$ versus control.

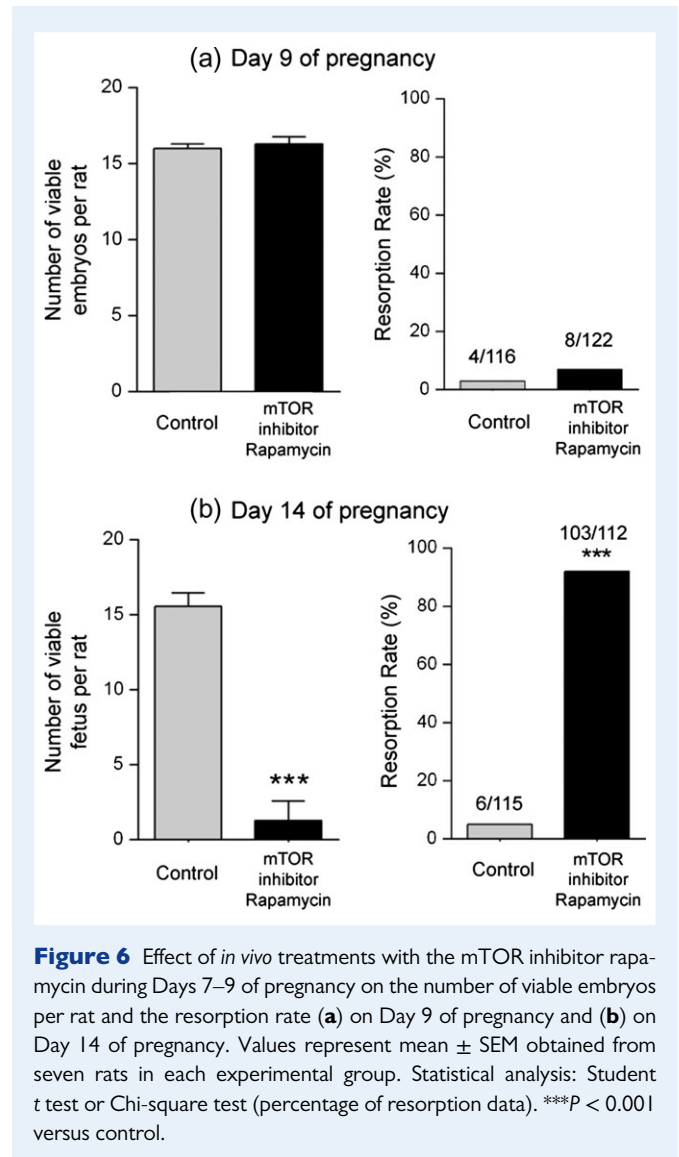


Figure 6 Effect of *in vivo* treatments with the mTOR inhibitor rapamycin during Days 7–9 of pregnancy on the number of viable embryos per rat and the resorption rate (a) on Day 9 of pregnancy and (b) on Day 14 of pregnancy. Values represent mean \pm SEM obtained from seven rats in each experimental group. Statistical analysis: Student t test or Chi-square test (percentage of resorption data). *** $P < 0.001$ versus control.

reduced placental weight (35%, $P < 0.01$), and increased decidual weight on Day 14 of pregnancy (35%, $P < 0.001$) (Fig. 9).

Discussion

Following implantation and before the mature placenta is established, the embryo relies on histotrophic nutrition through secretions from decidual cells and endometrial glands in both humans and rodents (Burton et al., 2010; Filant and Spencer, 2014). In rodents, the inverted yolk sac favors the absorption of nutrients (Zohn and Sarkar, 2010). Although the role of placental mTOR and PPARs as nutrient sensors in late pregnancy is established, the function of these signaling pathways in regulating histotrophic nutrition in the early post-implantation period is largely unknown. We report for the first time that when mTOR, PPAR γ or PPAR δ pathways are inhibited in the early post-implantation period, a complex cross-talk between these signaling pathways is observed in the decidua, leading to reduced levels of adipogenic proteins relevant for histotrophic nutrition. Specifically, although inhibition

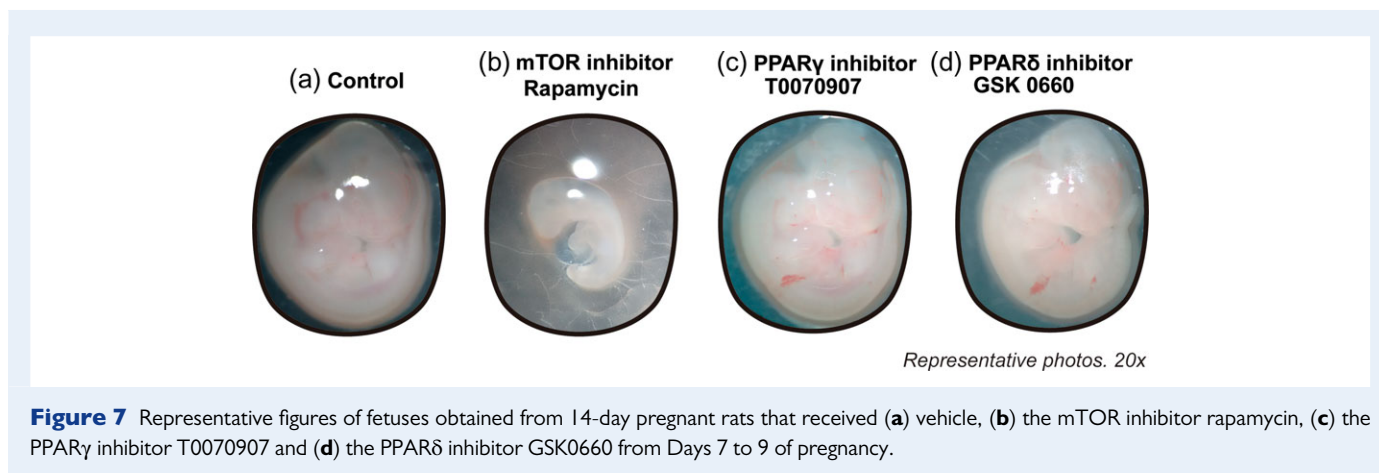


Figure 7 Representative figures of fetuses obtained from 14-day pregnant rats that received (a) vehicle, (b) the mTOR inhibitor rapamycin, (c) the PPAR γ inhibitor T0070907 and (d) the PPAR δ inhibitor GSK0660 from Days 7 to 9 of pregnancy.

of these nutrient sensors does not affect early post-implantation embryo viability, it leads to embryo loss, growth delay or placental/decidual growth impairments in mid-gestation.

There is a growing interest in understanding the first trimester of human pregnancy, not only to improve the understanding of implantation failure and miscarriage but also in the search of possible early causes of pregnancy complications that typically are diagnosed later in pregnancy, including gestational diabetes and IUGR (Albu *et al.*, 2014; Kennelly and McAuliffe, 2016). The first trimester of pregnancy is characterized by histotrophic nutrition, which provides essential nutrients to the growing embryo and allows a proper placental development (Burton *et al.*, 2010; Filant and Spencer, 2014). To address the impact of nutrient sensors in the early post-implantation period, we administered mTOR, PPAR γ and PPAR δ inhibitors on Days 7–9 of rat pregnancy, a developmental stage corresponding to the very early post-implantation embryonic and placental development that occurs in the second week of human pregnancy. Our data reveal the importance of this early post-implantation period in determining fetal growth and identify distinct effects of the inhibition of mTOR, PPAR γ or PPAR δ signaling on decidual proteins relevant for histotrophic nutrition.

The inhibition of mTOR limited to this short post-implantation period had a pronounced impact on mid-gestation outcomes. Indeed, the resorption rate on Day 14 of pregnancy was extremely high and occurred despite the observed upregulation of PPAR γ and PPAR δ in the decidua, which may be compensatory. The cross-talk between mTOR and PPARs seems to be dependent on the tissue and cell type, as it is well described that in adipose tissue, mTOR activation stimulates PPAR γ -induced lipid uptake and fat accretion (Kim and Chen, 2004; Blanchard *et al.*, 2012). The increased abundance of PLIN2 protein in the decidua is likely to be the result of PPAR γ activation, given the well established role of PPAR γ as a transcriptional regulator of PLIN2 in trophoblasts and other cell types (Bildirici *et al.*, 2003). In this study, PLIN2 levels were low in the uterine glands but high in the yolk sac, which show a further increase in their levels when mTOR is inhibited, suggesting a common regulation in the decidual cells and the yolk sac.

mTOR inhibition caused a decrease in FABP4 protein expression, in both decidua and uterine glands, similarly to that observed in other cell types (Wang *et al.*, 2017), and despite PPAR γ activation, which has been reported to positively regulate FABP4 in different tissues (Tan *et al.*, 2002).

Although it is well established that IUGR is associated with inhibition of placental mTORC1 and mTORC2 signaling (Roos *et al.*, 2007; Rosario *et al.*, 2011; Kavitha *et al.*, 2014; Chen *et al.*, 2015), the severity of the phenotype observed in this work demonstrates that the early post-implantation period is highly susceptible to mTOR inhibition, implicating mTOR as a nutrient sensing and growth promoting signaling pathway critical for normal development and survival at this developmental stage. mTOR is important for the growth and survival of the trophoblast and embryo prior to implantation as evidenced by the lack of viability of mTOR null mice (Murakami *et al.*, 2004), and impaired trophoblast outgrowth following mTOR inhibition in pre-implantation embryos in culture (Martin *et al.*, 2003). These reports, together with the findings in the present study, are consistent with a critical role of mTOR in embryo viability from conception to the early post-implantation stage. In addition to the well-known role of mTOR modulating placental nutrient transport and mitochondrial respiration and thereby regulating fetal growth (Kavitha *et al.*, 2014), a recent study in which rapamycin was given to rats during late pregnancy has shown that mTOR signaling is involved in the programming of heart defects in the offspring (Hennig *et al.*, 2017). Moreover, placental mTOR signaling activity in IUGR correlates with growth during the first year of life (Fahlbusch *et al.*, 2015). Therefore, mTOR signaling appears to play an important role in determining growth and survival from early pregnancy to the postnatal period.

In the present study, a less severe phenotype was observed when PPAR γ was inhibited in the early post-implantation period in the rat. Our results are consistent with a role for PPAR γ as a nutrient sensor, capable of regulating the levels of FABP4 in the decidua and uterine glands and the levels of PLIN2 in the decidua and yolk sac, adipogenic proteins involved in cellular and tissue lipid metabolism (Heid *et al.*, 1998; Li *et al.*, 2018; Scifres *et al.*, 2011). As FABP4 is involved in decidualization and PLIN2 modulates lipid accumulation and transfer in different tissues, including the placenta (Bildirici *et al.*, 2003; Scifres *et al.*, 2011; Frank *et al.*, 2015; Tian *et al.*, 2011; Li *et al.*, 2018), the downregulation of these adipogenic proteins in the decidua, uterine glands and yolk sac may be directly linked to the fetoplacental growth restriction observed following PPAR γ inhibition. The proposed histotrophic and growth promoting role of these adipogenic proteins in the decidua is supported by the reported metabolic and histotrophic role of PLIN2 in tumor cell growth and the ability of FABP4 to regulate proliferation,

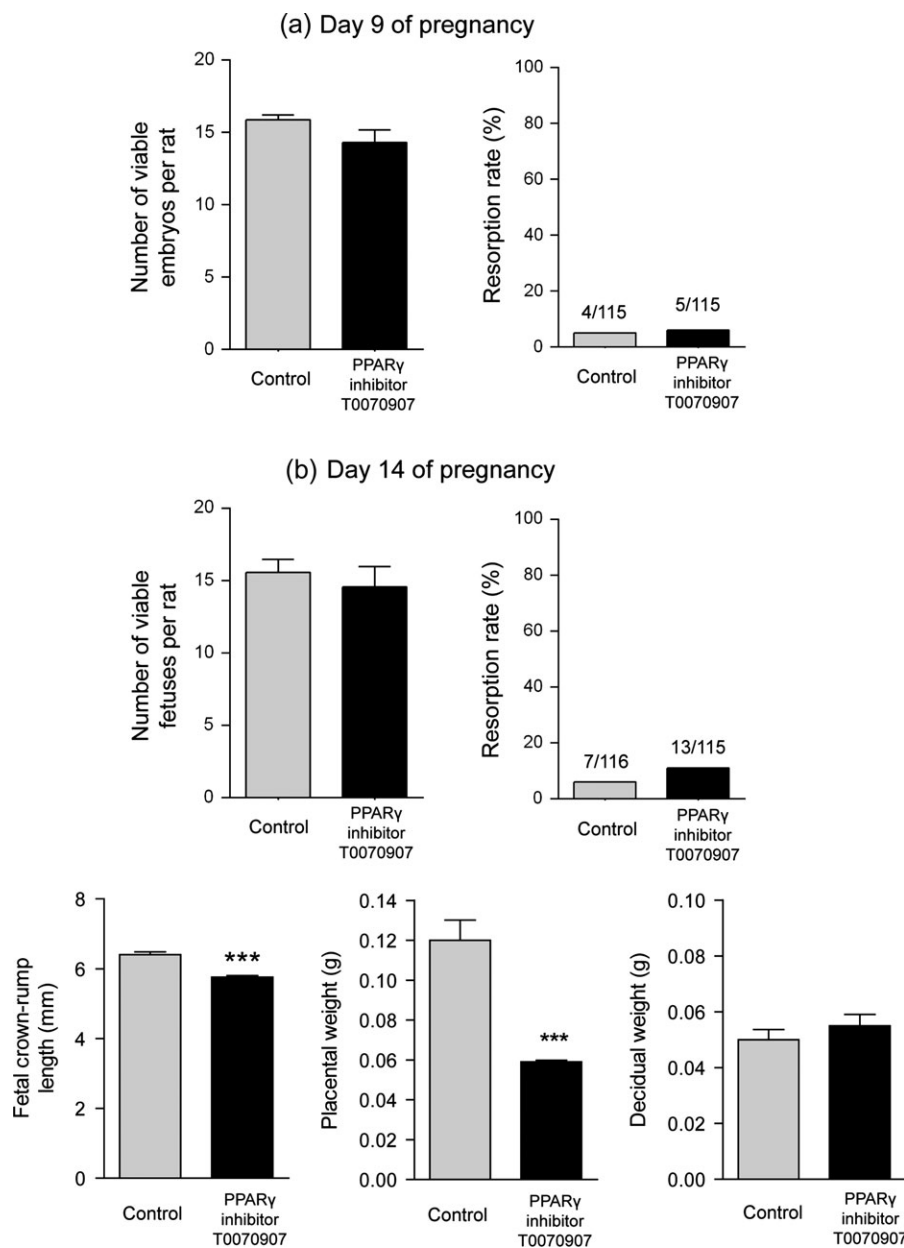


Figure 8 Effect of *in vivo* treatments with the PPAR γ inhibitor T0070907 during Days 7 to 9 of pregnancy on (a) the number of viable embryos per rat and the resorption rate on Day 9 of pregnancy and (b) the number of viable fetuses per rat, the resorption rate, the fetal crown-rump length and the placental and decidual weight on Day 14 of pregnancy. Values represent mean \pm SEM obtained from seven rats in each experimental group. Statistical analysis: Student t test or Chi-square test (percentage of resorption data). *** $P < 0.001$ versus control.

migration and invasion of human uterine endometrial epithelial cells (Zhu et al., 2015; Koizume and Miyagi, 2016).

Although diverse studies have shown that PPAR γ activation negatively regulates mTOR signaling in different tissues (San et al., 2015; Osman and Segar, 2016; Capobianco et al., 2017), by studying the decidua, we found that the activities of both mTORC1 and mTORC2 signaling pathways were reduced when the rats were treated with the PPAR γ inhibitor. Despite this reduction, there was no increased resorption rate on Day 14 of pregnancy. The different outcomes in pregnancies in which mTOR was inhibited using rapamycin (causing a

high resorption rate) and in pregnancies in which PPAR γ was inhibited (associated with mTOR inhibition but no effect on resorption rates) may be related to a less pronounced inhibition of mTOR in rats following PPAR γ inhibition as compared to rats treated with the mTOR inhibitor.

In rats treated with the PPAR γ inhibitor during the early post-implantation period, both the reduction in levels of adipogenic proteins, which is expected to limit lipid transfer to the developing embryo (Bildirci et al., 2003; Scifres et al., 2011), and the reduced mTOR signaling, expected to inhibit transfer of amino acids and cell growth

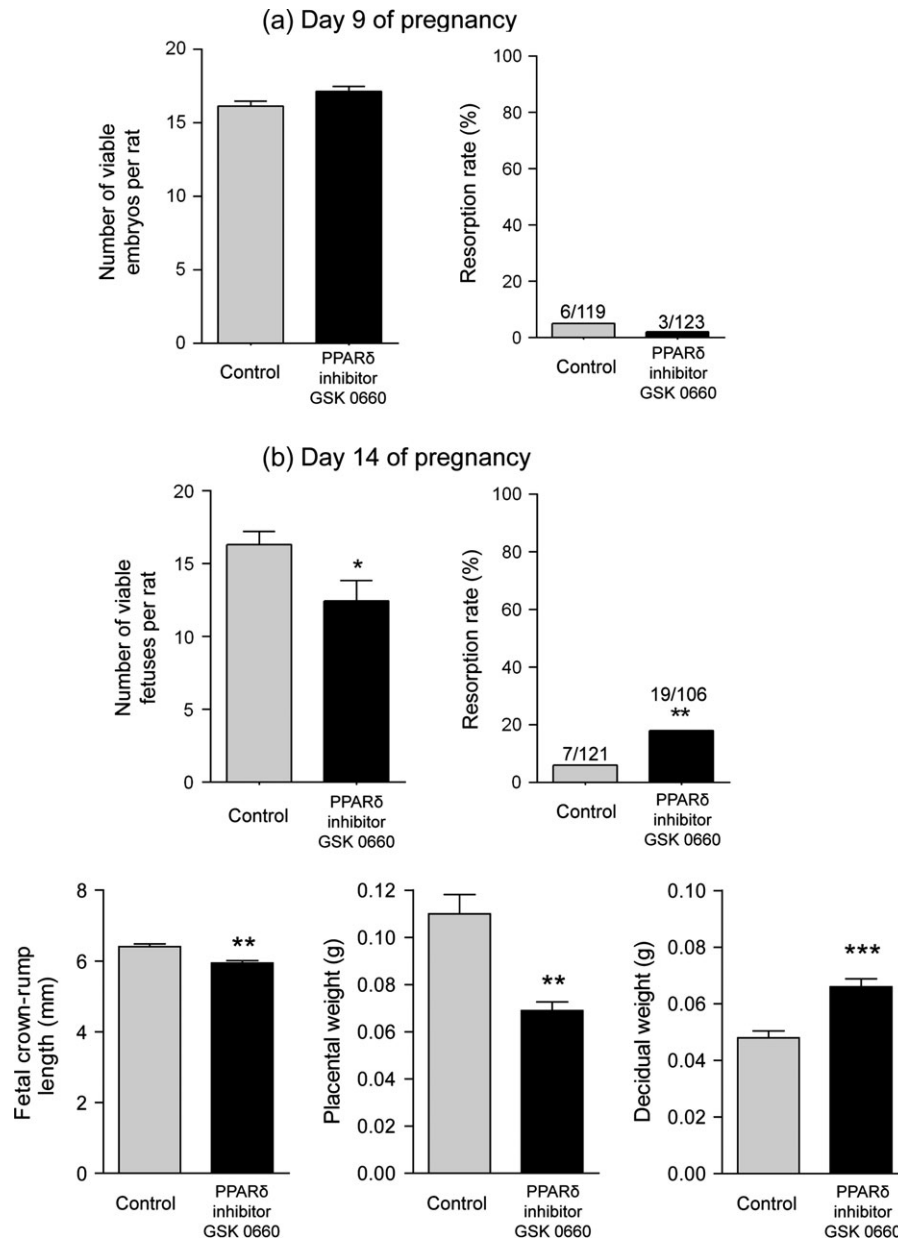


Figure 9 Effect of *in vivo* treatments with the PPAR δ inhibitor GSK0660 during Days 7–9 of pregnancy on (a) the number of viable embryos per rat and the resorption rate on Day 9 of pregnancy and (b) the number of viable fetuses per rat, the resorption rate, the fetal crown–rump length and the placental and decidual weight on Day 14 of pregnancy. Values represent mean \pm SEM obtained from seven rats in each experimental group. Statistical analysis: Student *t* test or Chi-square test (percentage of resorption data). **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 versus control.

(Shimobayashi and Hall, 2014; Dimasuy *et al.*, 2016), may be involved in the reduced fetal and placental weight evidenced in the post-placentation period. The mechanisms linking the inhibition of critical nutrient sensing signaling pathways on Days 7–9 of gestation to the outcomes on Day 14 of pregnancy remain to be established but may involve epigenetic mechanisms or effects on stem/progenitor cell populations. Indeed, PPAR and mTOR signaling regulates epigenetic and stem cell differentiation in different tissues/cell types, leading to developmental and metabolic changes (Meyer *et al.*, 2016; Vadla and Haldar, 2018).

PPAR δ inhibition in pregnant rats in the early post-implantation period caused no changes in FABP4 levels in the decidua and uterine glands, but resulted in a reduction in PLIN2 protein levels in the decidua and yolk sac, which may contribute to an impaired lipid histotrophic nutrition. To the best of our knowledge, PLIN2 has not been previously studied in the context of decidual function, but our data are consistent with the possibility that PLIN2 is regulated by mTOR and PPAR nutrient sensing pathways and is involved in histotrophic nutrition in early pregnancy. Indeed, the strong PLIN2 immunostaining evident in the inverted yolk sac suggests its contribution to the various

mechanisms described in the yolk sac that allow a proper transfer of lipids to the developing murine embryos (Herz and Farese, 1999; Zohn and Sarkar, 2010).

PPAR δ inhibition on Days 7–9 of pregnancy resulted in increased decidual levels of phosphorylated 4E-BP1, a branch of the mTORC1 pathway that promotes initiation of protein translation, thus leading to increased protein synthesis. It is possible that these changes contribute to the decidual overgrowth observed in this experimental group. On the other hand, decidual mTORC2 signaling was inhibited following PPAR δ inhibition, which, together with the reduction of decidual PLIN2 levels, may have detrimental effects and lead to the reduced fetoplacental growth evidenced on Day 14 of pregnancy. Moreover, embryo resorptions were also observed in this experimental group, highlighting an important role of PPAR δ in the decidua in the early post-implantation period. These observations extend to the early post-implantation period the previously established critical role of PPAR δ in implantation (Wang et al., 2007).

Our results point to an impaired nutritional effect in the decidua, which leads to the adverse results observed in embryo and placental development. Nevertheless, as a limitation of this work, it is unknown whether some of these effects are secondary to systemic (endocrine) effects in the mother. Moreover, as PPARs and mTOR pathways are involved in angiogenic pathways in the placenta and other tissues/cell types (Wang et al., 2007; Jiang and Liu, 2008), a possible regulation of angiogenic pathways that may be involved in the observed effects deserves further research. Finally, further studies addressing a rescue of the phenotype through mTOR and PPARs activation will be needed to improve the understanding of mTOR and PPAR signaling in embryo organogenesis.

In conclusion, our findings are consistent with an important role of decidual mTOR and PPAR nutrient sensing pathways in a very early post-implantation period. Possibly by regulating histotrophic nutrition, these nutrient sensors determine embryo viability and the growth of the placenta and the fetus.

Supplementary data

Supplementary data are available at *Molecular Human Reproduction* online.

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Authors' role

S.R., R.H. and V.W. performed the experiments and contributed to the analysis and interpretation of the data. T.P. and T.J. contributed to the analysis and interpretation of the data and critically revised the article. A. J. designed the study, contributed to the analysis and interpretation of the data and drafted the article. All the authors contributed to the construction of this article and approved the final version to be published.

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Conflicts of interest

None.

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