REPRODUCTION

RESEARCH

Inhibition of MTOR signaling impairs rat embryo organogenesis by affecting folate availability

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Abstract

Mechanistic target of rapamycin (MTOR) is essential for embryo development by acting as a nutrient sensor to regulate cell growth, proliferation and metabolism. Folate is required for normal embryonic development and it was recently reported that MTOR functions as a folate sensor. In this work, we tested the hypothesis that MTOR functions as a folate sensor in the embryo and its inhibition result in embryonic developmental delay affecting neural tube closure and that these effects can be rescued by folate supplementation. Administration of rapamycin (0.5 mg/kg) to rats during early organogenesis inhibited embryonic ribosomal protein S6, a downstream target of MTOR Complex1, markedly reduced embryonic folate incorporation (−84%, $P < 0.01$) and induced embryo developmental impairments, as shown by an increased resorption rate, reduced embryo somite number and delayed neural tube closure. These alterations were prevented by folic acid administered to the dams. Differently, although an increased rate of embryonic rotation defects was observed in the rapamycin-treated dams, this alteration was not prevented by maternal folic acid supplementation. In conclusion, MTOR inhibition during organogenesis in the rat resulted in decreased folate levels in the embryo, increased embryo resorption rate and impaired embryo development. These data suggest that MTOR signaling influences embryo folate availability, possibly by regulating the transfer of folate across the maternal–embryonic interface.

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Introduction

Birth defects are a major cause of infant mortality. Periconceptional folate deficiency is a risk factor for fetal structural malformations such as neural tube defects (NTD) (Smithells *et al.* 1976, Czeizel & Dudas 1992), however, the molecular mechanisms linking folate availability to birth defects remain to be fully established. Furthermore, low maternal folate intake and red cell folate levels are linked to restricted fetal growth (Tamura & Picciano 2006, Fekete *et al.* 2012, van Uitert & Steegers-Theunissen 2013). We recently reported that mechanistic target of rapamycin (MTOR) signaling functions as a folate sensor in mammalian cells including placental trophoblast (Rosario *et al.* 2016, 2017*b*), providing a novel pathway by which folate modulates cell growth and function. However, if inhibition of MTOR signaling is involved in linking folate deficiencies to NTDs is unknown.

MTOR is a conserved serine/threonine protein kinase that functions as a nutrient sensor and regulates cell growth, proliferation and metabolism in accordance with nutrients availability. MTOR forms the catalytic subunit of two different protein complexes known as mTOR complex 1 (mTORC1) which is associated with the protein raptor (regulatory associated protein of MTOR) and mTOR complex 2 (mTORC2) which is associated with the protein rictor (rapamycin-insensitive companion of mTOR) (Laplante & Sabatini 2009). mTORC1 plays a central role in control cell growth and proliferation by regulating the balance between cellular anabolism and catabolism in response to environmental conditions (Hay & Sonenberg 2004). The eukaryotic translation initiation factor 4E binding protein 1 (EIF4EBP1) and the 70-kDa ribosomal protein S6 kinase polypeptide 1 (RPS6KB1) that phosphorylates the ribosomal protein S6 RPS6) are mTORC1 downstream targets (Saxton & Sabatini 2017) that regulate protein translation. mTORC2 plays key roles in cell survival, metabolism, proliferation and cytoskeleton organization and its main downstream targets are protein kinase B (AKT), protein kinase C (PKC), and

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serum- and glucocorticoid-regulated protein kinase 1 (SGK1) (Alessi *et al.* 2009). Rapamycin, which forms a ternary complex with the FK506-binding protein 1a (FKBP1A) and the FKBP12-Rapamycin-Binding domain of MTOR, is an allosteric inhibitor of mTORC1 (Yang *et al.* 2013), although it does not bind to mTORC2 (Jacinto *et al.* 2004).

MTOR is essential for embryonic development as a homozygous inactivation of the kinase activity of MTOR mutation results in embryonic lethality (Murakami *et al.* 2004). During the first stages of embryonic cleavage, MTOR is critical by balancing energy metabolism and nutrient availability and is involved in the highly specialized programs of tissue growth during organogenesis (Land *et al.* 2014).

Folate, also known as vitamin B9, is an essential B vitamin. The synthetic oxidized monoglutamate form of folate is folic acid which is used as a dietary supplement. Once absorbed, folic acid requires reduction to tetrahydrofolate (THF). THF is converted to 5-methyltetrahydrofolate (5-MTHF), which transfers a methyl group onto homocysteine to synthesize methionine (Lu 2000). Methionine is a substrate for S-adenosylmethionine synthesis, which is an important co-factor in methylation reactions (key mechanism of epigenetic regulation), including histone methylations, methylation of cytosine residues in DNA, as well as other methylation reactions (Lu 2000, Caudill 2010). Folates are also required for *de novo* purine and thymidylate synthesis (Fox & Stover 2008). Thus, folates are critically important in widespread cellular events, including sustained cell division occurring during embryo development. Indeed, it is well established that folic acid supplementation prevents neural tube defects. Both folic acid deficiency and impaired folic acid metabolism have long been recognized as important contributors to developmental anomalies and to increase the risk of intrauterine growth restriction (Rondo & Tomkins 2000, Scholl & Johnson 2000, Imbard *et al.* 2013, Christensen *et al.* 2015, Rosario *et al.* 2017*a*).

In this work, we tested the hypothesis that MTOR functions as a folate sensor in the embryo and its inhibition result in embryonic developmental delay affecting neural tube closure and that these effects can be rescued by folate supplementation.

Materials and methods

Animals

Albino Wistar rats were purchased at the certified Animal Facilities of the Faculty of Exact and Natural Sciences, University of Buenos Aires, Argentina (UBA). 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, UBA, 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). Animals were fed with commercial rat chow (Asociación Cooperativa Argentina, Buenos Aires, Argentina) and water *ad libitum*, in a 12 h light:12 h darkness cycle.

Experimental design and tissue collection

Three-month-old Wistar female rats were mated with adult males. Mating was confirmed by the presence of sperm in vaginal smears and this day was designated day 0.5 of pregnancy. To study MTOR inhibition during early organogenesis without affecting the implantation process, pregnant rats received daily subcutaneous injections during three consecutive days beginning on day 8.5 of pregnancy (Fig. 1). Four experimental groups were constituted receiving either: (i) vehicle (control), (ii) rapamycin (MTOR inhibitor, 0.5 mg/kg, LC Laboratories, Massachusetts, USA), (iii) folic acid (15 mg/kg, Sigma), (iv) rapamycin (0.5 mg/kg) and folic acid (15 mg/kg). Each group comprised of 7 litters ($n = 7$). Doses of rapamycin and folic acid used were chosen based on previous reports (Anderl *et al.* 2011, Higa *et al.* 2012, Way *et al.* 2012, Roberti *et al.* 2018) and preliminary experiments.

Animals were euthanized in a CO_2 chamber 3 h after the last rapamycin injection on day 10.5 of pregnancy. The uterus was collected and transferred to Petri dishes with Krebs Ringer Bicarbonate (KRB) solution: 5 mM glucose, 145 mM Na^+,5.9 mM K^+, 2.2 mM Ca^{+2}, 1.2 mM Mg^{+2}, 127 mM Cl^-, 25 mM HCO_3^-, 1.2 mM SO_4^{2-} and 1.2 mM $PO4^{3-}$. The decidual tissue masses were collected from each uterus and gently opened to free the conceptuses using a stereomicroscope and microsurgical dissecting instruments. The embryos were dissected out of the yolk sacs and evaluated morphologically under a stereomicroscope. Viability was established by the presence of a beating heart. Embryos in resorption stages received no further analyses. Somite number was recorded and a score of the progression of the neural folds apposition

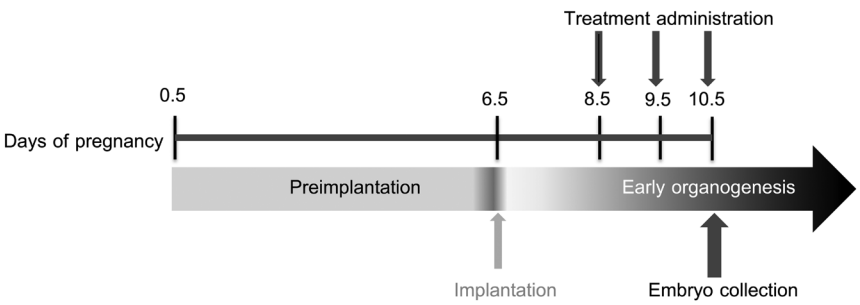

Figure 1 Experimental design: pregnant rats received daily subcutaneous injections during three consecutive days beginning on day 8.5 of pregnancy and embryos were collected on day 10.5 of pregnancy. Four experimental groups were constituted receiving either: (i) vehicle (control), (ii) rapamycin (MTOR inhibitor, 0.5 mg/kg), (iii) folic acid (15 mg/kg), (iv) rapamycin (0.5 mg/kg) and folic acid (15 mg/kg).

(1 = neural plate stage to 7 = neural folds completely closed) was performed for each embryo by two observers blinded to the source of the embryos (Higa *et al.* 2014).

Embryos at 10.5 days of gestation were categorized as having neural tube closure defects and as having rotation defects when the physiological process of embryo turning did not initiate on embryos that had more than 12 somites (Matsuda 1991, Higa *et al.* 2012). Viable embryos were preserved immediately at −80°C for further analyses described subsequently.

Western blot analysis

Proteins from four pooled embryos from the same rat ($n = 7$ 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 Amido Black staining solution for total proteins (Sigma-Aldrich) to confirm equal loading and transfer. Blocking was carried out for 1 h at room temperature in 5% BSA in Tris-buffered saline-Tween (TBS-T), and membranes were incubated with the primary antibody (diluted in 1% BSA in TBS-Tween) overnight at 4°C. The expression of the following proteins was determined using antibodies from Cell Signaling Technology: total and phosphorylated RPS6 (Ser-235/236), EIF4EBP1 (T-70) and AKT (Ser-473). After washing, the membranes were incubated with the appropriate peroxidase-conjugated secondary antibody, visualized using ECL detection solution (Thermo Scientific) and captured in a G:BOX gel imaging system (Syngene). Densitometry analysis was performed with ImageJ software. The expression of the target protein in each individual lane was normalized for total protein staining to adjust for unequal loading.

Folate measurement in embryos

One embryo from each rat ($n = 7$) was sonicated in distilled water, on ice, an aliquot was used for protein measurement and ascorbic acid (0.5%) was added followed by freeze-thaw. Embryonic folate content was measured by using a commercially available kit (ALPCO Diagnostic Products, Windham, NH, USA), according to the manufacturer's instructions.

Statistical analysis

Data are presented as the mean ± S.E. Groups were compared by two-way ANOVA. In all cases, differences were considered statistically significant at $P < 0.05$.

Results

Embryonic folate levels

We first determined if MTOR inhibition by maternal administration of rapamycin influenced folate levels in the embryo. Folate levels were markedly decreased in embryos from rapamycin-treated compared to embryos from control dams (−84%, $P < 0.01$, Fig. 2). Maternal treatments with folic acid increased folate content in embryos from both control group ($P < 0.05$, Fig. 1) and rapamycin-treated dams ($P < 0.001$, Fig. 2).

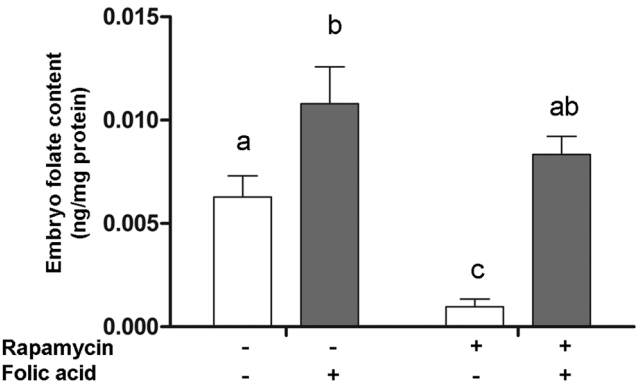

Figure 2 Effect of maternal administration of folic acid in control and rapamycin-treated dams from day 8.5 to 10.5 of gestation on embryo folate content. Values are means ± S.E.M. $n = 7$ litters per group. Two-way ANOVA in conjunction with Bonferroni's post-test was performed. Different letters denote significant differences between groups, $P < 0.05$.

mTORC1 pathway signaling

To determine if maternal administration of rapamycin (MTOR inhibitor, from days 8.5 to 10.5 of pregnancy) caused a robust inhibition of the mTORC1 pathway in the embryos, we measured the phosphorylation of two mTORC1 downstream targets, RPS6 and EIF4EBP1. Phosphorylation at Ser-235/236 of RPS6 was found to be decreased in embryos from rapamycin-treated dams compared to the control group (−81% $P < 0.01$, Fig. 3A). The decreased phosphorylation of RPS6 was similar in the rapamycin + folic acid-treated group (Fig. 3A). Total RPS6 expression in embryos was not different between the different groups (Fig. 3B). The ratio between P-RPS6 to total RPS6 showed a similar pattern than P-rpS6 (Fig. 3C). On the other hand, phosphorylation at T-70 of EIF4EBP1 or total EIF4EBP1 showed no differences between embryos from different treatments (Fig. 4).

We next measured AKT, a substrate downstream of mTORC2 pathway. Phosphorylation at Ser-473 of AKT was found increased in embryos from rapamycin-treated dams compared to embryos from control group (+65%, $P < 0.05$, Fig. 5A). No changes were observed in the rapamycin + folic acid-treated dams compared either with the control or the rapamycin-treated dams (Fig. 5A). Total AKT showed no differences between embryos from different treatment groups (Fig. 5B). The ratio between P-AKT to total AKT showed similar pattern than P-AKT (Fig. 5C).

Embryo morphology

We also evaluated embryonic resorptions and found that they were increased by more than two-fold in the

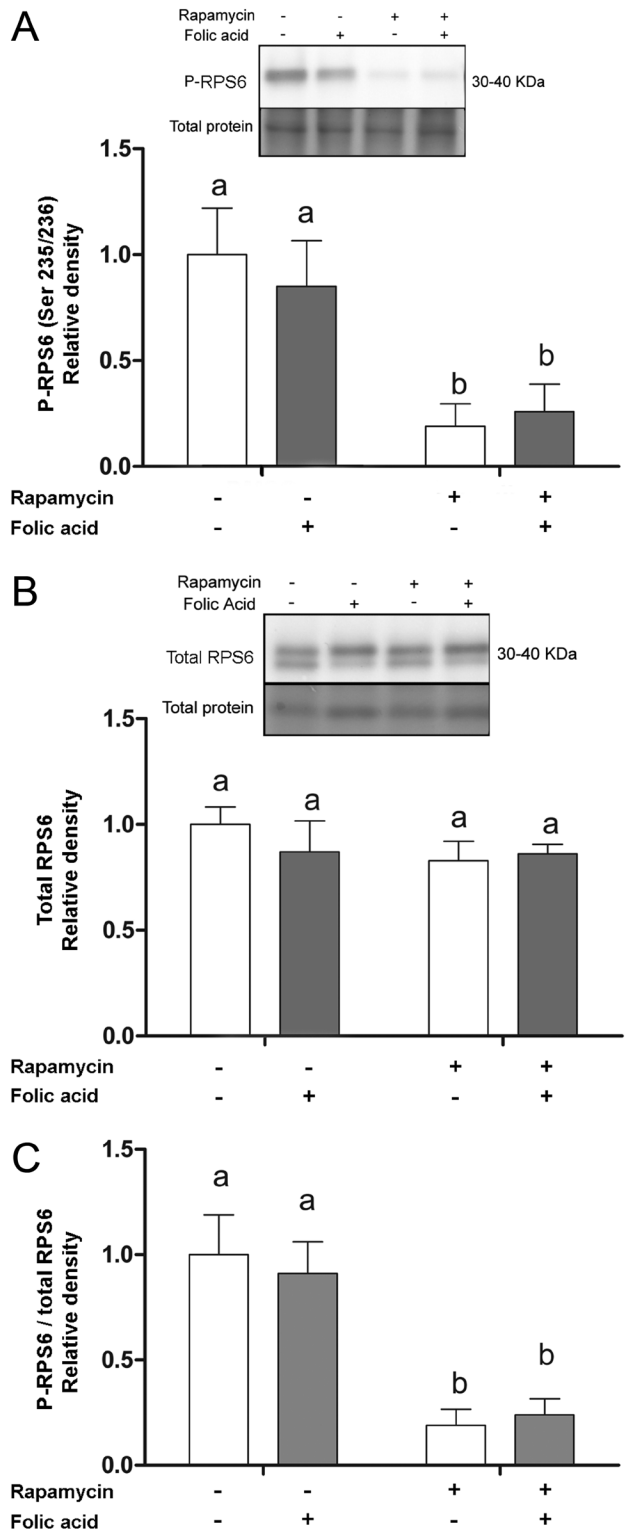

Figure 3 Effect of maternal administration of folic acid in control and rapamycin-treated dams from day 8.5 to 10.5 of gestation on: (A) embryonic protein levels of active RPS6 (phosphorylated at Serine 235/236), (B) embryonic protein levels of total RPS6. Values are means ± S.E.M. $n=7$ litters per group. Two-way ANOVA in conjunction with Bonferroni's post-test was performed. Different letters denote significant differences between groups, $P < 0.05$.

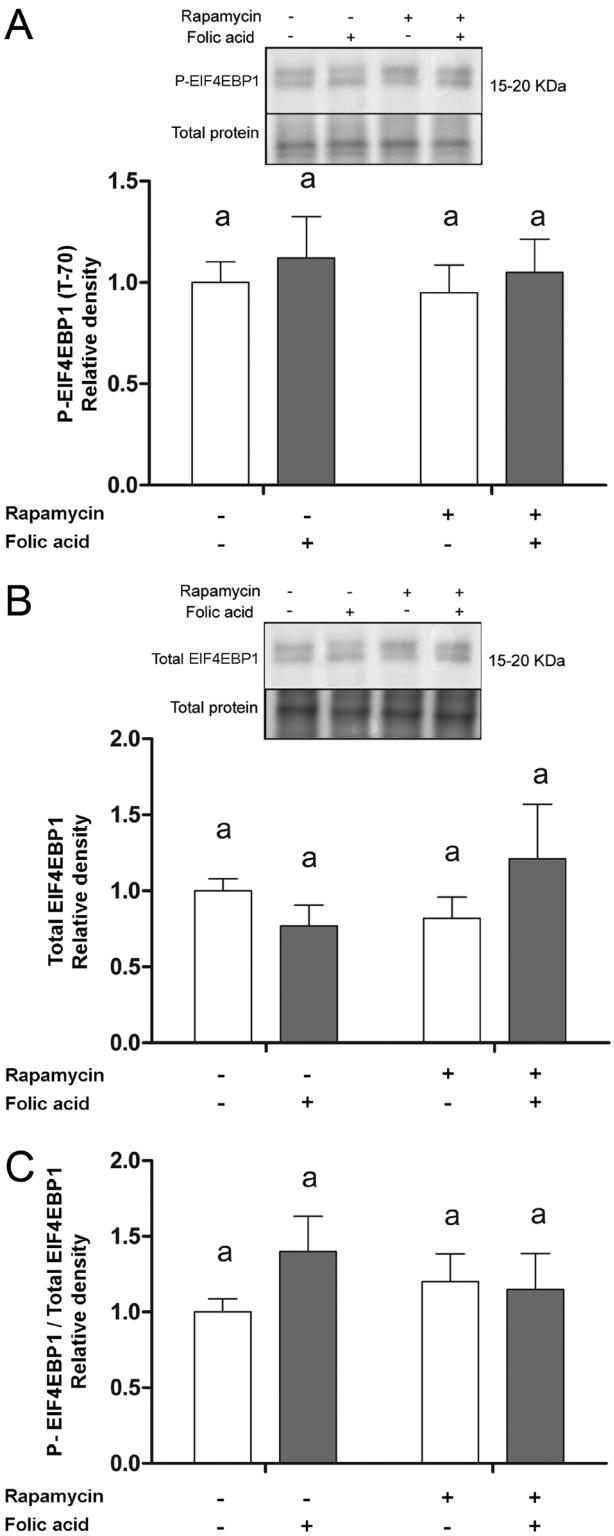

Figure 4 Effect of maternal administration of folic acid in control and rapamycin-treated dams from day 8.5 to 10.5 of gestation on: (A) embryonic protein levels of active EIF4EBP1 (phosphorylated at Threonine-70), (B) embryonic protein levels of total EIF4EBP1. Values are means ± S.E.M. $n=7$ litters per group. Two-way ANOVA in conjunction with Bonferroni's post-test was performed. Different letters denote significant differences between groups, $P < 0.05$.

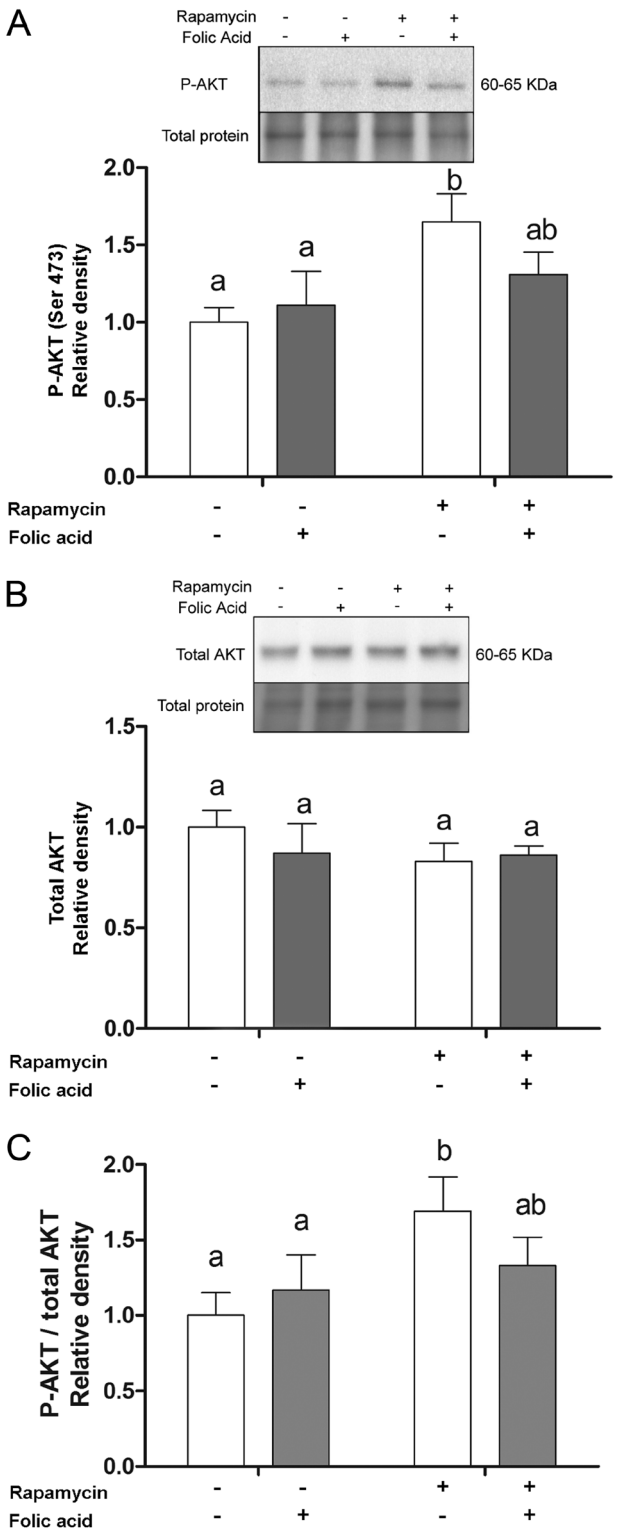

Figure 5 Effect of maternal administration of folic acid in control and rapamycin-treated dams from day 8.5 to 10.5 of gestation on: (A) embryonic protein levels of active AKT (phosphorylated at Serine-473), (B) embryonic protein levels of total AKT. Values are means ± S.E.M. $n=7$ litters per group. Two-way ANOVA in conjunction with Bonferroni's post-test was performed. Different letters denote significant differences between groups, $P < 0.05$.

embryos from the rapamycin-treated group compared with the embryos from control group (+212%, $P < 0.05$, Fig. 6A). Treatment with folic acid prevented the increased number of resorptions observed in the embryos from rapamycin-treated dams ($P < 0.05$, Fig. 6A).

We also determined the number of somites for each embryo, an index of embryo development, and observed

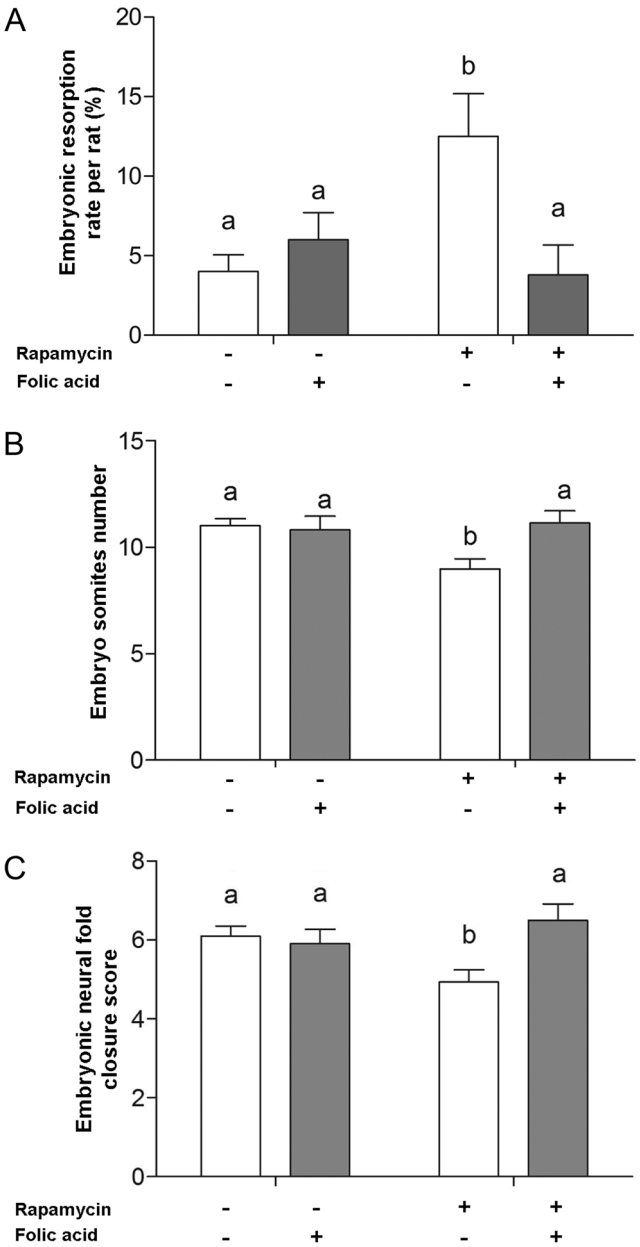

Figure 6 Effect of maternal administration of folic acid in control and rapamycin-treated dams from day 8.5 to 10.5 of gestation on: (A) resorption rate, (B) embryonic somite number, (C) Embryonic neural fold closure score. Values are means ± S.E.M. $n=7$ litters per group. Two-way ANOVA in conjunction with Bonferroni's post-test was performed. Different letters denote significant differences between groups, $P < 0.05$.

a decreased number of somites in embryos from the rapamycin-treated dams compared to embryos from control group (−18% $P < 0.01$, Fig. 6B). Supplementing with folic acid prevented the decreased somite number observed in the embryos from the rapamycin-treated dams ($P < 0.05$, Fig. 6B).

When we assessed the score of the progression of neural fold closure, we found that this score was lower in the embryos from the rapamycin-treated dams compared to embryos from control dams (−19%, $P < 0.05$, Fig. 6C). Treatment with folic acid prevented the decreased score of neural fold closure observed in the embryos from rapamycin-treated dams ($P < 0.01$, Fig. 6C).

There was no difference in the frequency of embryos with neural tube defects in the four groups (Fig. 7A). However, we observed an increased number of embryos with rotation defects in the rapamycin-treated dams ($P < 0.05$, Fig. 7B) compared with embryos from control group, that was not prevented with the folic acid supplementation (Fig. 7B).

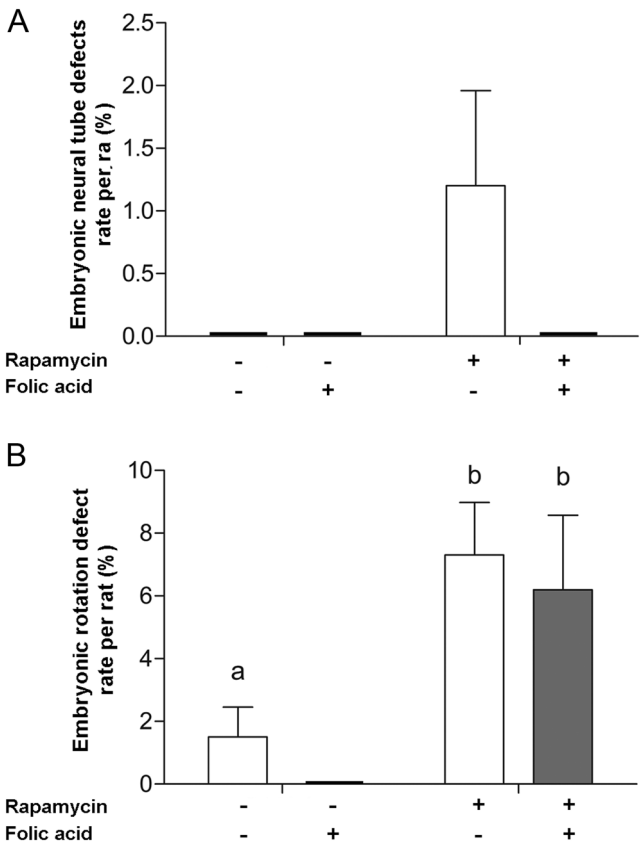

Figure 7 Effect of maternal administration of folic acid in control and rapamycin-treated dams from day 8.5 to 10.5 of gestation on: (A) embryonic neural tube defects rate, (B) embryonic rotation defects rate. Values are means ± S.E.M. $n = 7$ litter per group. Two-way ANOVA in conjunction with Bonferroni's post-test was performed. Different letters denote significant differences between groups, $P < 0.05$.

Discussion

We report that MTOR inhibition during organogenesis in the rat resulted in decreased folate levels in the embryo, increased embryo resorption rates and impaired embryo development. These effects of MTOR inhibition on the embryo are prevented by maternal folate administration suggesting that MTOR signaling influences embryo folate availability. These findings are consistent with the possibility that MTOR inhibition leading to impaired folate transfer to the embryo may underpin impaired early embryo development even when maternal folate levels are normal.

There are several pregnancy complications related to MTOR inhibition that should benefit from folic acid supplementation. For example, polymorphism in *Mtor* gene associated with recurrent spontaneous abortion, preeclampsia (which shows reduced MTOR pathways in the placenta), intrauterine growth restriction (which shows reduced MTOR signaling in both fetuses and placentas) and pregnancies in which MTOR inhibitors have been administered due to organ transplantation (Framarino-dei-Malatesta *et al.* 2013, Xiang *et al.* 2015, Gupta & Jansson 2019, Huang *et al.* 2020).

On the other hand, providing an excess of folates, the recovery of most embryo developmental patterns is likely not MTOR-dependent as this treatment allowed embryonic folate uptake despite the unrecovered MTOR signaling.

In this work, we used a dose of folic acid that had been previously found to be effective to prevent malformations in diabetic rats (Wentzel *et al.* 2005, Higa *et al.* 2012), which is higher than that recommended to be taken preconceptionally in woman, and thus provides an excess of folates. As a limitation, we did not use lower folic acid doses, and thus we were not able to determine the minimum dose required to prevent the reduced folate levels induced by MTOR inhibition in embryos from diabetic rats.

In vitro studies showed that the excess of extracellular folates induce its uptake by a mechanism that does not involve folate receptors binding proteins and are likely to be mediated by the process of passive diffusion (Antony *et al.* 1989). In HeLa cell line, a high dose of folates induces a posttranscriptional regulation of the reduced folate carrier that modified its stability inducing an increase in its protein and transcripts levels (Hou *et al.* 2014). However, little is known and further research is needed to elucidate the precise mechanisms that allow embryo access to folates when MTOR is inhibited but folates are simultaneously administered in excess.

Folates participate in nucleic acid and protein methylation reactions and in *de novo* purine and thymidylate synthesis and are essential for normal embryo development (Lu 2000, Fox & Stover 2008, Caudill 2010). Recently, we showed that MTOR functions as a folate sensor both in cultured primary

human trophoblast cells (Rosario *et al.* 2017*b*) and in pregnant mice *in vivo* (Rosario *et al.* 2017*a*).

At early post-implantation stages and before the establishment of the placenta, MTOR is a key regulator of nutrient transport in the maternal decidua (Roberti *et al.* 2018). Moreover, it was recently reported that activation of mTORC1 and mTORC2 increased the plasma membrane expression of the folate receptor-1 and the solute carrier family 19 (folate transporter), member 1 (SLC19A1, also known as reduced folate carrier) in primary human trophoblast cells (Rosario *et al.* 2016, 2017*b*). During early organogenesis, folate receptor 1, solute carrier family 46, member 1 (SLC46A1, also known as proton-coupled folate transporter), and SLC19A1 expression was found in the ectoplacental cone and the chorionic membrane (Cherukad *et al.* 2012). In the current study, maternal administration of rapamycin during the early organogenesis stage in pregnant rat, lead to a marked reduction in embryo folate content, suggesting that MTOR signaling regulates folate transport at the maternal–embryonic interface also at this early stage of pregnancy.

Homozygous MTOR knockout embryos die shortly after implantation due to impaired cell proliferation in both embryonic and extraembryonic compartments (Murakami *et al.* 2004), demonstrating the critical role that mMTOR signaling plays in early embryonic development. Other studies have highlighted MTOR role in neural tube development (Rennebeck *et al.* 1998, Hentges *et al.* 2001) and in survival and proliferation of cardiomyocytes in the developing heart (Zhu *et al.* 2013). To the best of our knowledge, this is the first report that examines the effects of maternal rapamycin administration on embryos at an early organogenesis stage to evaluate the regulation of embryonic folic acid availability. However, there are several previous reports in the literature exploring the effects of maternal administration of rapamycin in pregnancy. Studies performed during late gestation showed that a single dose of rapamycin (1 mg/kg) given to pregnant dams at day 16 of pregnancy resulted in inhibition of S6 pathway of mTORC1 in neonatal brain (Anderl *et al.* 2011). Administration of rapamycin from day 15.5 of gestation until delivery induced IUGR and altered postnatal cardiac growth, morphology and function (Hennig *et al.* 2017). Maternal rapamycin administration from day 12 of pregnancy inhibited ribosomal protein S6 signaling in the rat embryo at day 14 of pregnancy (Ozmen *et al.* 2019). However, there are only few studies of rapamycin administration in dams during early gestation. We recently found that maternal rapamycin administration during the immediate post-implantation stage (days 7–9 of gestation) lead to increased embryo resorptions at day 14 of pregnancy (Roberti *et al.* 2018). In the current work, rapamycin administration to pregnant dams from 8.5 to 10.5 days of gestation did not alter phosphorylation of EIF4EBP1 in the embryo, but resulted in a marked inhibition of the mTORC1-downstream target RPS6. This inhibition was also observed in the embryos from dams simultaneously treated with rapamycin and folate. Rapamycin is known to specifically inhibit mTORC1 without decreasing the activity of mTORC2 (Jacinto *et al.* 2004). In this work, we found increased AKT phosphorylation at Ser-473, suggesting an activation of mTORC2, in the embryos from rapamycin-treated dams, an alteration that was not observed in the rapamycin and folate-treated group compared to controls. This likely reflects cross-talk between mTORC1 and 2 in the embryo. For example, it is well established that inhibition of mTORC1 may activate insulin/IGF1 signaling by a feedback loop from S6K to IRS1 (Saxton & Sabatini 2017), which in turn can lead to an activation of mTORC2.

In this work, we found that rapamycin administration to pregnant dams during 3 consecutive days of early organogenesis increased resorption rate and reduced somite number and the neural fold closure score, demonstrating that mTORC1 inhibition impairs normal embryo development. Interestingly, with exception of rotation defects, folate supplementation prevented the adverse effect on mTORC1 inhibition. However, because folate supplementation failed to normalize embryo RPS6, folate recovery of the adverse effect of rapamycin on embryo development is likely independent of embryonic mTORC1 activation.

Despite the known fact that folic acid administration prevents neural tube defects (Copp & Greene 2013, Sarmah *et al.* 2016), the mechanisms involved are not fully established. We were not able to observe any changes in increased neural tube defects in the embryos from rapamycin-treated group that had reduced levels of folate. Other studies have also found a lack of neural tube defects induction in folate-deficient embryos without genetic predisposition (Heid *et al.* 1992, Burren *et al.* 2008). Interestingly, an increase rate of embryos with a rotation defect (fail to perform the physiological process of rotation around the body axis) was found in the rapamycin-treated group. This defect was previously described in MTOR mutant mice (Hentges *et al.* 1999). However, folate treatment was not able to prevent the rotation defects in the rapamycin-treated group, suggesting that this process is dependent on mTORC1 signaling pathway and not related to the folate metabolism.

In conclusion, our results identified a link between mTORC1 and folates relevant for proper embryo development during organogenesis. Indeed, maternal administration of rapamycin during early organogenesis leads to reduced mTORC1 activity, developmental impairments and reduced folate content in the embryo. Of relevance, we found that maternal folate supplementation limited to early organogenesis is an effective treatment for embryo developmental impairments in a context in which MTOR activity is reduced.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

The authors' contributions to the manuscript were as follows: A J, T J: designed research; R H, F J R: conducted the research; R H: performed statistical analysis; R H, A J, T J: wrote manuscript; T L P, F J R: interpretation of data and critical reading of the manuscript; R H, A J, T J: had primary responsibility for the final content of the manuscript; all authors: read and approved the final manuscript. T J and A J contributed equally to this work.

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