The role of MTOR in mouse uterus during embryo implantation

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Abstract

Mammalian target of rapamycin (MTOR) is a protein kinase that plays a central role in cell growth and proliferation. It is a part of the signaling network transmitting growth factor signaling to translational control. Previous studies have shown that MTOR is involved in embryo implantation, but its expression in the uterus and its role in implantation are unclear. Here, we have investigated the expression and role of MTOR in mouse uterus during early pregnancy. RT-FQ PCR showed that the mRNA levels of Mtor in endometria of pregnant mice were higher than those of nonpregnant mice. The mRNA levels in the pregnant mice gradually increased from D3 of pregnancy, reached maximum on D5, and then declined afterward. In situ hybridization and immunohistochemical analysis showed that the mRNA and protein of MTOR were mainly located in stromal cells. The levels of the expressed MTOR protein correlate with those of mRNA. The number of implantation sites was greatly decreased by the intrauterine injection with rapamycin in the early morning of D4 of the pregnancy. These findings suggest that MTOR may play an important role in embryo implantation in mice.


Introduction

Embryo implantation is a complex physiological process and depends on a series of key events, including blastocyst migration, apposition and adhesion to the luminal epithelium, extensive degradation and remodeling of extracellular matrix, invasion of the maternal endometrium by the trophoblast cells of the developing blastocyst, and secretion of embryonic factors, which elicit a cellular reaction in the maternal endometrium (Carson et al. 2000, Achache & Revel 2006, Gentilini et al. 2007). Numerous signaling proteins such as ERKs, phosphatidylinositol 3-kinase (PI3K), RHO/ROCK, PTK2, or PI3K/AKT/mammalian target of rapamycin (MTOR) are involved in the process to coordinate and integrate these events so as to guarantee successful implantation (Pollheimer & Knoller 2005, Gentilini et al. 2007).

MTOR is a member of the PI3K-related kinase superfamily in which a lipid kinase homology domain functions as serine/threonine kinase (Rohde et al. 2001). As an important molecule of signal transduction, MTOR plays a crucial role in coordinating the cell cycle, protein synthesis, and energy metabolism and acts as a central coordinator in cell proliferation, growth, differentiation, and apoptosis (Fingar & Blenis 2004). In recent years, some studies indicate that the MTOR signaling pathway plays an important role during embryo implantation. Martin & Sutherland (2001) showed that exogenous amino acid signals can induce MTOR-dependent phosphorylation of RPS6KB1 to regulate the translation of proteins required for trophoblast differentiation. Gangloff and Murakami report that MTOR-deficient embryos die shortly following implantation in association with impaired cell proliferation in both the embryonic and extra-embryonic compartments. In vitro outgrowth experiments revealed that MTOR-deficient blastocysts were severely impaired in their ability to form trophoblasts (Gangloff et al. 2004, Murakami et al. 2004). These findings suggest that MTOR is essential for early mouse embryo growth and proliferation of embryonic stem cells. However, the expression and the role of MTOR in the uterus during early pregnancy are unclear. In the present study, we investigate the expression pattern of MTOR in the mouse uterus during early pregnancy.

Results

Mtor mRNA level in the uterus during early pregnancy

As a first step towards determining a possible role of MTOR in uterus, we examined the levels of Mtor mRNA in endometria by FQ-PCR and in situ hybridization.
The standard curve and amplification curve for FQ-PCR are shown in Fig. 1A and B respectively. The relative amounts of Mtor mRNA (Mtor/β-actin mRNA) in mouse endometria are shown in Fig. 1C. Expression of Mtor was detected in all endometria examined. The mRNA levels were elevated from D3 to D5 of pregnancy, but dramatically decreased on D6 and D7. The increase in mRNA levels on D4 and D5 of pregnancy was significant (P < 0.05) compared with other groups. These results indicate that a transient surge of Mtor mRNA expression occurs in a highly stage-specific manner between D3 and D5 of pregnancy (regarded as the ‘implantation window’ period), coincident with the events leading to embryo implantation. In situ hybridization of uterine sections confirmed the up-regulation of Mtor mRNA expression during this period (Fig. 2). No signal was detected when the antisense probe was replaced with the sense probe (Fig. 2 NTC). The weak signal corresponding to Mtor mRNA was detected in the luminal epithelium, glandular epithelium, and stromal cells of nonpregnant mice (Fig. 2D0). A significant signal was detected in the stromal cells on D3 and reached the maximum level on D5 of pregnancy (Fig. 2D3 and D5 respectively). By D6, the signal in the stromal cells became weak (Fig. 2D6). Only a weak signal of Mtor mRNA was observed in the pseudopregnant uterus. (Fig. 2P).

**MTOR protein expression in the uterus during early pregnancy**

Figure 3 displays immunohistochemistry (IHC) staining for MTOR in endometria. The MTOR protein was located mainly in stromal cells of endometria. The MTOR levels were markedly increased from D3 to D5 (Fig. 3C and D; P < 0.05), but decreased on D6 (Fig. 3E). Only a slight increase in MTOR protein was seen in the pseudopregnancy group (Fig. 3F). The levels of MTOR protein analyzed by western blotting correlated with the mRNA levels detected by RT-PCR (Table 1 and Fig. 4).
Functional analysis of MTOR during embryo implantation

The results of functional tests showed that the number of implanted embryos in the right uterus horns by injecting with rapamycin was markedly fewer \((1.2 \pm 0.63)\) than those in the left side \((6.8 \pm 0.79); \text{Fig. 5}; \text{P}<0.05\). These data showed that the total number of implanted embryos was affected by an intrauterine injection with rapamycin in mice.

Discussion

Considerable attention has been devoted to the role of MTOR in embryo development, but little attention has been given to the role of MTOR in placental development. Evidence presented here shows that the expression of MTOR mRNA and protein in endometria was obviously increased when pregnant with a peak on D5, and reduced thereafter. The total number of implanted embryos was dramatically decreased by intrauterine administration of rapamycin on D4. These data indicate that MTOR may participate in the process of embryo implantation during early pregnancy. However, the mechanism by which MTOR fulfills its function during this period remains to be discovered.

Successful implantation requires a receptive endometrium, a normal and functional embryo at the blastocyst stage, and a synchronized dialogue between maternal and embryonic tissues (Simon et al., 2000, Lee & DeMayo 2004). The time from D1 to D5 of pregnancy is critical for the embryo implantation in mice and D4–D5 of pregnancy is regarded as the implantation window (Dey et al., 2004). During this period, blastocysts attach to, adhere, and invade the endometrium. At the same time, the endometria undergo great changes, such as vascular expansion and stromal cell proliferation, to become receptive for blastocyst growth, attachment, and the subsequent events of implantation (Tabibzadeh et al., 1999). In our study, the expression of MTOR mRNA and protein, which was mainly located in the stromal cells of uterus gradually increased during D3–D5 of pregnancy and reached the maximum level at the time of the implantation window. These results indicate that MTOR may participate in the proliferation of uterine stromal cells. During the process of implantation, a large number of molecular mediators were secreted by endometrium to support pregnancy, including cytokines, growth factors, lipids, and others (Achache & Revel, 2006, Guzeloglu-Kayisli et al., 2007). MTOR is a serine/threonine protein kinase that has been shown to regulate cell growth in response to nutrients and growth factors (Thomas & Hall, 1997). It has been reported that MTOR, as a nutrient sensor, can regulate the placental leucine transport that is critical for fetal growth (Regnaulta et al., 2007). In vitro studies have shown that MTOR can be activated by amino acid signaling and phosphorylates downstream proteins involved in the regulation of translation initiation, RPS6KB1 and EIF4EBP1 (Gingras et al., 2001a, 2001b, Volarevic & Thomas, 2001). These signaling pathways regulate trophoblast cell motility and stimulate trophoblast cell proliferation, which is essential for the embryo implantation (Martin et al., 2003, Wen et al., 2005). We hypothesized that the mechanism by which MTOR regulates uterine stromal cell growth, proliferation and differentiation is similar.

After implantation, the growth and proliferation of endometrial cells decrease, accompanied by partial cell decidualization and apoptosis in order to offer

Table 1 Results of mammalian target of rapamycin protein expression in different groups.

<table>
<thead>
<tr>
<th></th>
<th>D0</th>
<th>D3</th>
<th>D4</th>
<th>D5</th>
<th>D6</th>
<th>D7</th>
<th>Pseudopregnant</th>
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<tr>
<td>Relative expression</td>
<td>0.03±0.006</td>
<td>0.121±0.021*</td>
<td>0.151±0.020*</td>
<td>0.172±0.019*</td>
<td>0.08±0.017*</td>
<td>0.049±0.014*</td>
<td>0.058±0.012*</td>
</tr>
</tbody>
</table>

These results are shown as mean±s.o., \(n=10\). *\(P<0.05\) versus D0; †\(P<0.05\) versus D4 and D5.
a good microenvironment for maintaining embryo implantation. Previous work demonstrates that the expression of PTEN, which is a negative regulator of PI3K/AKT/MTOR signal pathway, is increased in the decidual membranes and glandular cells during early pregnancy and thus inhibits MTOR activity. The decrease in MTOR activity may affect the endometrial cells’ decidualization and further induce their apoptosis (Correia-da-Silva et al. 2004, Fingar & Blenis 2004).

Our results showed that the expression of MTOR is decreased dramatically on the D6–D7 of pregnancy compared with that of the implantation window. These findings suggest that the reduction in MTOR expression might reduce the growth and proliferation of endometrial cells.

In conclusion, we report a spatial–temporal expression pattern of MTOR in mouse endometria during early pregnancy. The high levels of MTOR in mouse endometria during the implantation window suggest that MTOR might be involved in the process of blastocyst implantation. However, the detailed mechanism by which MTOR fulfills its role in implantation needs further investigation. The present study has provided preliminary but important data for further study of the role of MTOR during embryo implantation.

Materials and Methods

Animals

Eight- to ten-week-old NIH mice (25–30 g) were caged in a controlled environment (14 h light:10 h darkness). All animal procedures were approved by the Ethical Committee, State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences. The estrus mice were mated with fertile males or vasectomized males of the same strain to induce pregnancy or pseudopregnancy (D1 = the day of vaginal plug). Pregnant mice were randomly divided into five groups (D3, D4, D5, D6, and D7) with 20 mice in each group. Twenty nonpregnant mice (D0) and 20 pseudopregnant mice (P) were chosen as controls. Mice in each group were killed at 0800–0900 h, after Trypan blue injection to the tail veins. Ten mouse uteri were collected and stored in liquid nitrogen for FQ-PCR and western blot. Ten uteri were fixed in 4% paraformaldehyde for IHC and in situ hybridization in each group. Another ten mice were injected with rapamycin through the intrauterine for functional tests at pregnant D4.

FQ-PCR of Mtor mRNA in mouse endometria

RNA extraction

Total RNA was extracted from the frozen mouse endometrial tissues using Trizol reagent (TAKARA Biotechnology Co. Ltd, Dalian, China) according to the manufacturer’s instructions and stored at –20 °C. Quantification and purity assessment were performed by optical density measurement at 260 and 280 nm. The integrity of the total RNA was examined by agarose gel electrophoresis.

FQ-PCR

The RT of cDNA was done with 5 μg total RNA in a 20 μl reaction. Briefly, 5 μl RNA (1 μg/μl), 4 μl 5× first strand buffer (250 mM Tris–HCl, pH 8.3, 375 mM KCl, and 15 mM MgCl2), 2 μl dNTPs (2.5 mmol/l), 1 μl Oligo (dT) (500 ng/μl), 1 μl RNAase inhibitor (40 U/μl), 1 μl M-MLV reverse transcriptase (200 U/μl), and an adequate volume of diethylpyrocarbonate-treated water were mixed to make up a total volume of 20 μl and then heated at 70 °C for 5 min, followed by incubation at 37 °C for 60 min and heated at 70 °C for another 10 min. cDNA

Figure 4 Western blot of MTOR protein expression in mice uterus. (A) The expression levels of MTOR during pregnancy were determined by western blotting using anti-MTOR antibody (from Santa Cruz Biotechnology Inc). Levels of β-actin were used as loading control. (B) A quantitative representation of the results is shown in (A). Data shown are from N – number of independent experiments. *P<0.05 versus D0; #P<0.05 versus D4 and D5.

Figure 5 Functional analysis of MTOR. The function of MTOR was blocked by intrauterine injection with rapamycin (from Invitrogen) between 0800 and 0900 h on D4 of pregnancy. The number of implanted embryos in the right uterus horns by injecting rapamycin was markedly decreased compared with those in the left side (P<0.05).
was stored at −80 °C for RT FQ-PCR. The standard preparation and standard curve were supplied by Shanghai GeneCore BioTechnologies Co., Ltd (Shanghai, China).

The specific primers and probes for the Mtor gene and β-actin are shown in Table 2 (Shanghai GeneCore Bio-Technologies Co., Ltd). Quantification of the transcripts was carried out in the ABI Prism 7000 HT. Reactions were prepared with a 96-well MicroAmp optical plate by the addition of 25 μl PCR master mixture consisting of 2.5 μl of 10× PCR buffer, 2 μl of 25 mM dNTPs, 2 μl of 25 mM MgCl2, 0.3 μl of 5U/μl Taq, 1.125 μl of 20 pmol/l primers, 0.625 μl of 20 pmol/l probe, 2 μl of cDNA, and 13.325 μl of ddH2O. The PCR conditions were set up as follows: initial denaturation at 94 °C for 5 min; 40 cycles of 15 s at 94 °C (denaturation); and 1 min at 57 °C (annealing and extension). Experiments were performed in duplicate for each sample. Target gene copy values were derived from standard curve. The gene expression rate was obtained by normalizing the amount of Mtor cDNA with that of β-actin.

**Immunohistochemistry**

Mouse uteri were excised and fixed immediately in 4% paraformaldehyde and embedded in paraffin. Sections (6 μm) were cut, deparaffinized, and rehydrated. Endogenous peroxidase activity was inhibited with methyl alcohol containing 0.5% H2O2. The sections were digested with 0.1 mg/ml proteinase K at room temperature for 10–15 min, and washed thrice with PBS. Following the prehybridization in hybridization buffer for 3 h at 42 °C, the sections were hybridized at 42 °C overnight in hybridization buffer with 1–5 mg/ml DIG-labeled antisense or sense oligonucleotide probe. After hybridization, the sections were washed twice with 2×SSC, 0.5×SSC, and 0.2×SSC for 15 min at 37 °C. After blocking with 0.5% (w/v) block mix, the sections were incubated in rabbit anti-DIG antibody (1:100) for 1 h at 37 °C and goat anti-rabbit IgG conjugated to alkaline phosphatase (1:100) for 1 h at 37 °C. Sections were washed thrice in PBS for 5 min. The signal was visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

**Western blotting**

Proteins were extracted from the uteri of each group using TriPure reagent and protein concentration was determined by using a bicinchoninic acid protein assay kit according to the manufacturer’s protocol (Sigma Chemical Co). Samples were boiled in SDS-sample buffer for 5 min, and then loaded to a 4% SDS-PAGE gel. Following electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories). Membranes were incubated for 1 h at room temperature in blocking Tris-buffered saline solution containing 0.1% Tween-20 (TBST) and 5% nonfat milk. Immunoblot analyses were performed by incubating the membranes with monoclonal mouse anti-MTOR antibody (Santa Cruz Biotechnology Inc.) at 1:1000 dilution in 5% milk-TBST overnight at 4 °C. Membranes were washed in TBST for 45 min followed by incubation with a secondary antibody (goat anti-mouse IgG) conjugated with HRP (Zhongshan Biosciences Inc). Positive bands were detected by DAB after a final washing in TBST for 1 h. Densitometry was performed using Quantity One version 4.4.0 analysis software.

**MTOR function study during embryo implantation**

To determine whether MTOR has a role in embryonic implantation, ten mice were given an intrauterine injection of rapamycin (an inhibitor of MTOR) between 0800 and 0900 h on D4 of pregnancy before implantation. Rapamycin was dissolved in DMSO for stock solution. Before injection, the stock solution was diluted with PBS to make a working solution. After being anesthetized and disinfeeted, the right uterus horns of pregnant D4 mice were injected with the rapamycin working solution according to their body weight (bw; 10 mg/kg bw). As a control, the left uterus horns were injected with the same volume of 0.1% DMSO (diluted with PBS). Mice were killed between 0800 and 0900 h on D9 of pregnancy and then the number of embryos in the implantation sites was recorded.

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Table 2 Primer and probe sequences for real-time quantitative RT-PCR analyses.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Primer-F-STND</td>
<td>TGCTACGGACGGCAGCTAGAT</td>
</tr>
<tr>
<td>Primer-R-STND</td>
<td>CCGTAGAGGAGAGGGTTGGAAAC</td>
</tr>
<tr>
<td>Mtor-TAMRA-FP</td>
<td>TGCTAAGGTAAGCTTGTGCTGGAA</td>
</tr>
<tr>
<td>Mtor-TAMRA-RP</td>
<td>CGTCATATGGGTTTAATAACTCCTTGGA</td>
</tr>
<tr>
<td>Mtor-TAMRA-FAM</td>
<td>FTTGTTGCCGTCCCTGCTTCATTTCTTCTTP</td>
</tr>
<tr>
<td>β-Actin-TAMRA-FP</td>
<td>CCGAGGGCTCTTTCCTCACCC</td>
</tr>
<tr>
<td>β-Actin-TAMRA-RP</td>
<td>TAGAGGCTTACGAGTGTAACGT</td>
</tr>
<tr>
<td>β-Actin-TAMRA-FAM</td>
<td>FTCCTTTCTGGTATGGATTCCTGGCP</td>
</tr>
</tbody>
</table>

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The role of MTOR in mouse uterus  355


**Statistical analysis**

Statistical analysis was performed using Statistical Package for Social Science (SPSS for Windows package release 10.0; SPSS Inc., Chicago, IL, USA). Statistical significance was determined by one-way ANOVA. Post-hoc comparisons between treatment group means were made using Fisher’s protected least significant difference test. Differences were considered significant if $P<0.05$. Values shown in all the figures were given as the mean ± S.D.

**Declaration of interest**

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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**References**


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