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Involvement of insulin in early development of mouse one-cell stage embryos

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Recent studies have suggested that growth factors and hormones play important roles in cell proliferation and differentiation during early embryonic development. In the present study, we examined the expression and localization of insulin in the mouse oocytes and one-cell stage embryos by quantitative ELISA, RT-PCR, Western blot and immunofluorescence. In the mouse oocytes and one-cell stage embryos, expression of insulin was uniformly distributed in the cytoplasm. We also examined the expression, activity and localization of mTOR (mammalian target of rapamycin) and p70S6K. The expression of mTOR and p70S6K was not significantly different at the cell cycle of mouse one-cell stage embryos. mTOR and S6K were distributed evenly in the cytoplasm at G1, G2 and M phase phase, but at S phase, the distribution of mTOR and S6K was around the pronucleus. At different phases, the activity of mTOR fluctuated. We also used the PI3K specific inhibitor-Wortmannin to investigate the cleavage rate of eggs. The result showed that the rate obviously decreased. When the mTOR specific inhibitor Rapamycin was used, the first mitotic division of the mouse one-cell stage embryo was delayed. These results suggested that insulin was expressed both in mouse oocytes and one-cell stage embryos, and may play functional roles in regulation of mouse early embryogenesis by activating the signal pathway of PI3K/PKB/mTOR/S6K.

mouse, embryo, insulin, mTOR, S6K

Insulin, which is synthesized and secreted by pancreatic beta cells, has been viewed classically as a vertebrate hormone. Studies using a wide variety of *in vivo* and *in vitro* systems have established its role in glucoregulation and related metabolic pathways[1]. Insulin plays its roles by binding receptors and interestingly, insulin receptors have been shown to display a developmentally-regulated expression pattern during embryogenesis[2], suggesting that the insulin family of growth factor may play an important role in metabolism, cell proliferation and differentiation at very early stages of mammalian development, and that early development may be regulated by factors from both maternal (e.g. insulin) and embryonic (e.g. IGF-Ⅱ) sources[3]. It has been shown that the PI3K–PKB–mTOR pathway is involved in the downstream signaling of insulin[4–6]. mTOR is a member of phosphatidylinositol kinase-related kinase family[7] and plays a key role in the cell growth. mTOR can be activated by insulin and the activated mTOR regulates protein biosynthesis through its downstream effectors, ribosome 40S subunit protein S6 kinase 1 (S6K1) and eIF initiate factor 4E binding protein 1 (4EBP1). Full activation of the mTOR pathway requires signals from both nutrients (e.g., amino acids and glucose) and growth factors (e.g. insulin) and can promote cell growth and inhibits apoptosis.

An early study reported no expression of insulin in mouse one-cell stage embryos[3]. However, in our re-

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cent study, we found that some of the insulin downstream effectors, such as mTOR and S6K, are expressed and play functional roles in mouse one-cell stage embryos. Since the PI3K/PKB/mTOR/S6K pathway requires insulin signaling, we decided to examine the expression of insulin and insulin receptor in mouse oocytes and one-cell embryos.

1 Materials and methods

1.1 Collection and Culture of Mouse Embryos

One-cell mouse embryos were collected and cultured according to the method described by Hogan and Constantini[8]. Female mice (KUMING strain, 4—5 weeks old) obtained from the Department of Laboratory Animals, China Medical University, were injected with 10 IU of pregnant mare serum gonadotropin (Shanghai BioSun Sci&Tech Co., Ltd., China) and then with 10 IU of HCG (Shanghai BioSun Sci&Tech Co., Ltd., China) 48 h later. A single female was placed with a single male of the same strain, and one-cell embryos were collected with M2 medium the next day from the oviducts of females possessing a vaginal plug. The embryos were then cultured in M16 medium (Sigma, USA) at 37℃ in a humidified atmosphere of 5% CO_2 in air.

1.2 Isolation of mRNA, cDNA synthesis and PCR

Total RNAs were extracted from 300 mouse one-cell embryos using QuickPrep micro mRNA Purification Kit. An RNA PCR Kit (AMV) Ver3.0 (TaKaRa Biotechnology) was used to determine insulin mRNA levels. The RT reaction was carried out in one cycle at 50℃ 25 min; 99℃ 5 min; 5℃ 5 min. Aliquots of 5 μL of first-strand cDNA were mixed with 25 μL of the PCR mixture. The PCR reaction was carried out in three steps as follows: 94℃ for 2 min (one cycle); 94℃ for 30 s, 55℃ for 30s, and 72℃ for 1 min (35 cycles). PCR analysis was performed with primers specific for insulin I and II, which were designed to make the products across intron. Primers were synthesized by Sbsbio Company. Insulin type I primers: forward 5′-AGGACCCACAAGTGGAACA-3′; reverse 5′-GC TGGTAGAGGGAGCAAAT-3′ (size of the PCR products is 131 bp); insulin type II primers: forward 5′-AAGCCTATCTTCCAGGTTATT-3′, reverse 5′-TGGGTCCTCCACTTCACG-3′ (the size of PCR products is 211 bp). PCR products were visualized by electrophoresis on 12% polyacrylamide gels containing ethidium bromide.

1.3 Western blot

Protein extracts of mouse embryos were prepared by adding an appropriate number of embryos (see Results section for details) in a minimal volume of collection medium to 20 μL of protein extraction buffer (100 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 7.5, 0.5% Triton X-100, 0.5% NP-40) containing 1 mmol/L phenylmethylsulfonyl fluoride and 1 μg/ml leupeptin and pepstatin. The extracts were briefly vortexed, quickly frozen on dry ice, and stored at -20℃ until used. Laemmli sample buffer[9] was added to the protein extracts, and the mixture was boiled for 5 min and resolved on a SDS-PAGE gel. For immunoblotting, the fractionated proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 3% bovine serum albumin in Tris-buffered saline containing 0.05% Tween-20 and after blocking the antibodies against insulin (Santa Cruz Biotechnology, Inc.; Cat#: sc-9168) was added in a sealed plastic bag at 4℃ overnight. The primary antibodies against insulin, mTOR (Santa Cruz Biotechnology, Inc., USA), p70S6K (Santa Cruz Biotechnology, Inc., USA) were used at 1∶400 dilution. The membrane was then incubated with the horseradish peroxidase–conjugated rabbit antibody anti-mouse IgG at 1∶3000 (Beijing Zhongshan Biotechnology). The targeting proteins were detected by using an enhanced chemiluminescence detection system (Santa Cruz Biotechnology).

1. 4 mTOR kinase assays

The activity of mTOR kinase in mouse one-cell embryos was determined as described[10–12]. Briefly, 50 mouse one-cell stage embryos were lysed in a lysis buffer (20 mmol/L Tris-HCl, pH 7.5, containing 1% NP-40, 1 mmol/L EGTA, 1 mmol/L EDTA, 1 mmol/L sodium orthovanadate, and protease inhibitor cocktail [Boehouringer Mannheim]) at 4℃. The kinase activity was then assayed using 4EBP1 as a substrate in a reaction mixture containing 20 mmol/L Tris-HCl (pH 7.5), 75 mmol/L NaCl, 10 mmol/L $MgCl_2$, 1 mmol/L dithiothoureitol (DTT), 20 μmol/L ATP, 50 μmol/L Crosstide, and 1.75×10^5 Bq of [γ-^{32}P]ATP in a volume of 20 μL per assay. The reaction was allowed to proceed for 30 min at 30℃ and then terminated by the addition of an equal volume of 4×sample buffer. Phosphorylated proteins were separated by polyacrylamide gel electrophoresis and analyzed by autoradiography.

1.5 Indirect immunofluorescent staining

Mouse one-cell embryos were fixed in 4% formaldehyde (in PBS), followed by permeabilization in the wash buffer (0.1% Triton X-100, 1% BSA, in PBS) and blocked by the blocking buffer (10% BSA, in TTBS). Incubation with anti-mTOR, S6K and insulin antibodies was carried out in the wash buffer at room temperature, followed by incubation with a FITC–anti-mouse antibody in the wash buffer. Immunofluorescent images were obtained by Confocal microscopy.

1.6 Quantitative ELISA assay

The quantitative ELISA assay was performed using an immunoassay kit (Adlitteram Diagnostic Laboratories, Inc., Cat#: QRCT-33002EIA\UTL) according to the manufacturer's instructions. The optical density at 450 nm was read using a microtiter plate reader (R&D Systems Inc., USA). The average absorbance values (A450) for each set of reference standards, control, and samples were calculated and a standard curve was constructed by plotting the mean absorbance obtained for each reference standard against its concentration in nmol/L on linear graph paper, with absorbance on the vertical (y) axis and concentration on the horizontal (x) axis. The corresponding concentration of insulin was determined from the standard curve.

1.7 Treatment with rapamycin and wortmannin and observation of the mouse embryos

Rapamycin and Wortmannin were dissolved in DMSO. Zona pellucida-free (removed with Tyrode's buffer, pH 2.5) one-cell embryos at G1 phase were incubated with rapamycin at a final concentration of 3.3 μg/mL. Zona-free one-cell embryos at G1 phase were incubated with Wortmannin at a final concentration of 20 μmol/L. Control one-cell emrbyos were incubated with the same amount of vehicle. The percentages of egg cleavage were counted under a dissecting microscope 30—35 h after injection of HCG. Each experiment was repeated at least three times and the results were statistically analyzed by SPSS 12.0.

2 Results

2.1 Expression and localization of mTOR and S6K proteins in mouse one-cell embryos

The expression of mTOR and S6K proteins in mouse oocytes and one-cell embryos at G1, S, G2, or M phase were detected by Western blot analysis (Figure 1). Both mTOR and S6K were expressed in mouse oocytes and one-cell embryos at all of the 4 phases. We further examined the localization of mTOR and S6K1 in mouse oocytes and one-cell embryo by immunofluorescence (Figure 2). mTOR and S6K were detected with even intensity in the cytoplasm in mouse oocyte and one-cell stage embryo at G1 phase. Interestingly, the distribution of mTOR and S6K was more concentrated around the pronucleus following the entry into S phase. And at G2 and M phases, mTOR and S6K immunoreactivity appeared to be uniformly distributed in the cytoplasm again. The localization of mTOR and S6K might be responsible for the activation and effectiveness of mTOR and S6K.

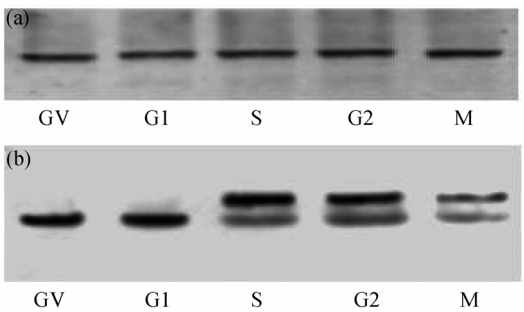

Figure 1 Western blot analysis of mTOR and p70S6K1 in mouse fertilized eggs. (a) and (b) show mTOR and p70S6K1 expression in mouse oocytes and different stages of mouse one-cell stage embryos, respectively. The collection and culture of mouse oocytes and embryos have been elucidated in Material and Methods. 200 eggs were used in each lane.

2.2 Activity of mTOR in mouse one-cell stage embryos

In the mouse one-cell stage embryos, the mTOR activity was assayed using 4E-BP1 as a substrate. The mTOR activity peak occurred at 18—21 h, corresponding to the late G1 and S phase, and a substantial decrease was detected between 23 and 31 h (Figure 3). This result suggested that mTOR might play a certain role during G1 and S phase at one-cell stage mouse embryos.

2.3 Specific inhibitors of mTOR and PI3K inhibit the first mitotic division of mouse one-cell embryos

We used Rapamycin and Wortmannin, the specific inhibitors of mTOR and PI3K respectively, to treat the mouse one-cell stage embryos at G1 phase, and then observed their effects on egg cleavage. Vehicle (DMSO, final concentration <0.5%) was added to the culture medium of the control groups. After 24 h incubation,

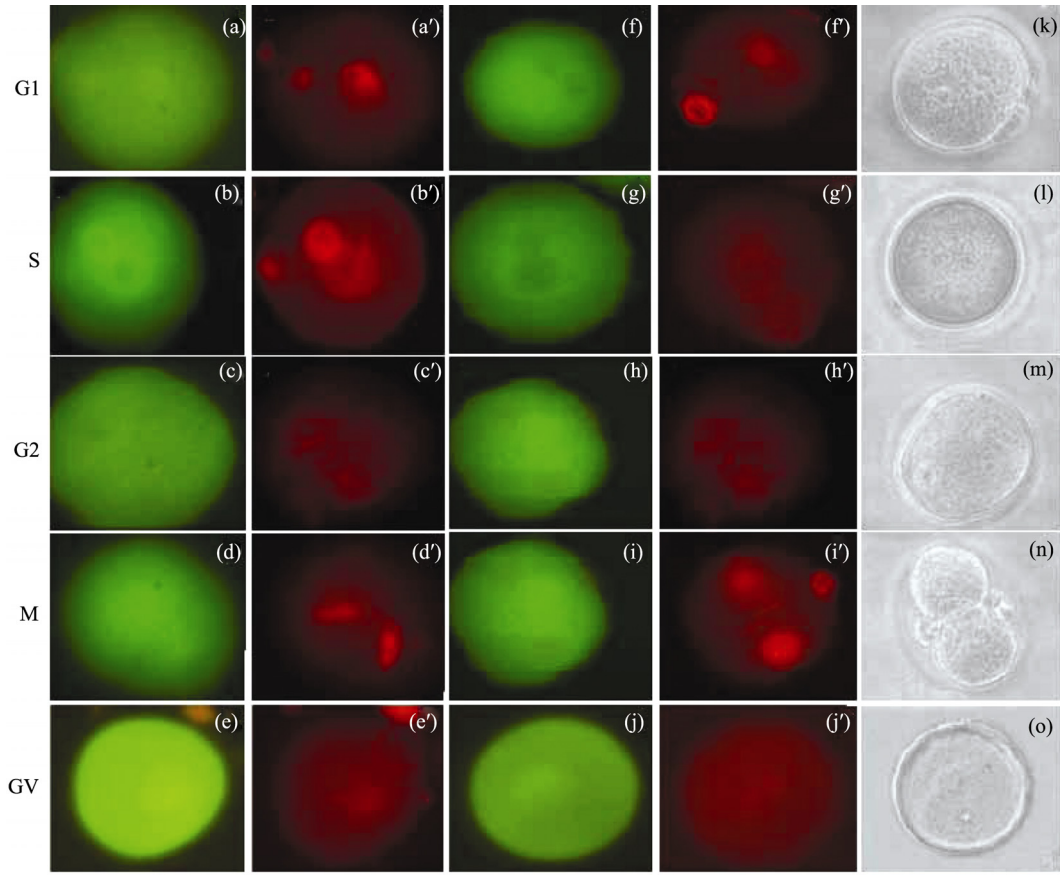

Figure 2 Immunofluorecence study of mTOR and p70S6K in mouse fertilized eggs. One-cell stage mouse embryos were collected and cultured according to the methods described in Material and Methods. The green fluorescence in (a)—(e) (G1, S, G2, M and oocytes, respectively), represents mTOR location, and the green fluorescence in (f)—(j) represents S6K location, while the red fluorescence in other images represents the nucleus stained by PI. Anti-mTOR, anti-S6K antibodies and FITC conjugated goat anti rabbit antibody were used in this analysis. Negative controls about mouse oocytes and one-cell stage embryos are shown in Figure 2K-O.

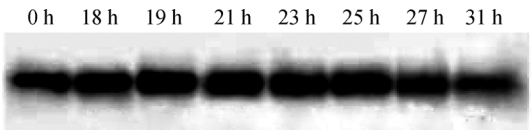

Figure 3 mTOR kinase activity in different phases of one-cell stage embryos. With 4EBP1 as the substrate and using autoradiography, the picture shows that in G1, S, and G2 phases, the activity remains at a high level through the whole cycle from 18 to 25 h until M phase of the first mitosis when the activity of mTOR decreased rapidly. 10 eggs were used in each lane. The [γ-^{32}P]-ATP was used in autoradiography.

we examined the percentage of the egg cleavage. In the presence of Rapamycin at a final concentration of 3.3 μg/mL, the egg cleavage was inhibited significantly (Figure 4).

Similarly, in the presence of Wortmannin at a final concentration of 20 μmol/L, a significant decrease in the rate of egg activation was observed (Figure 5). These results suggest that both Rapamycin and Wortmannin can interfere with G2/M transition in mouse one-cell embryos.

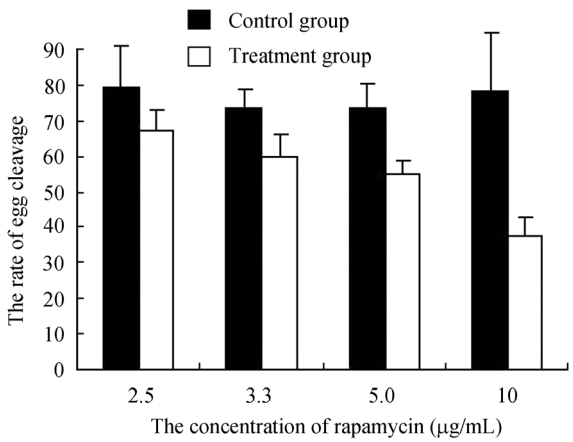

Figure 4 Rapamycin inhibits the egg cleavage in mouse one-cell stage embryos. The cleavage rate of mouse eggs was examined with the treatment of Rapamycin at various concentrations (2.5, 3.3, 5.0 and 10 μg/mL). The experiment was repeated three times.

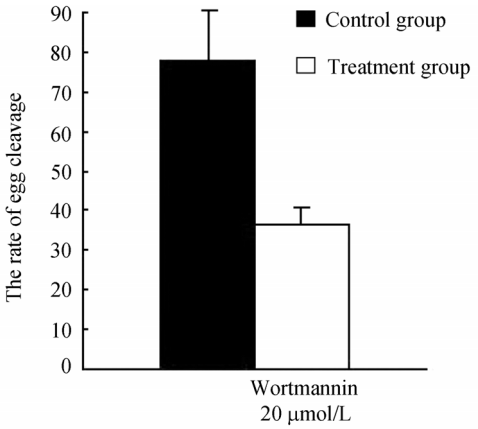

Figure 5 Wortmannin inhibits the egg cleavage in mouse one-cell stage embryos. The cleavage rate of mouse eggs was examined with the treatment of Wortmannin at the final concentration 20 μmol/L. At least three independent experiments were performed.

2.4 Expression and localization of insulin in mouse oocytes and one-cell embryos

RT-PCR was performed to detect insulin mRNA in GV oocytes and one-cell embryos (Figure 6). The expected

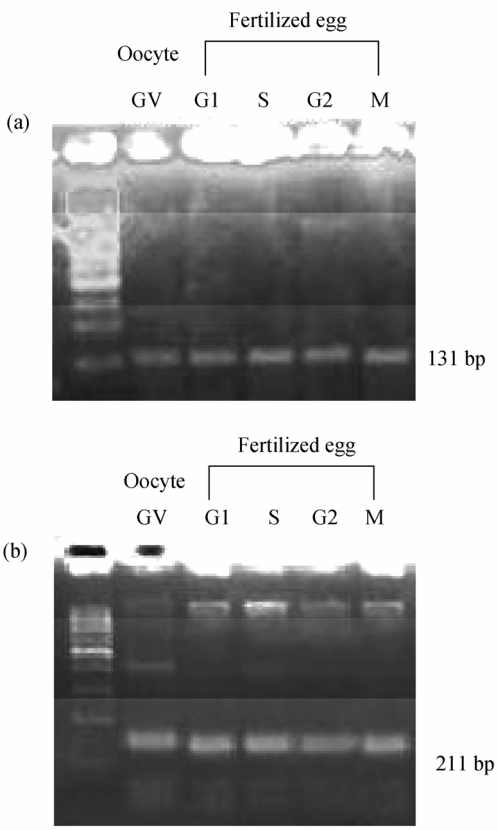

Figure 6 RT-PCR analysis of insulin in mouse oocytes and one-cell stage embryos. (a) The mRNA level of insulin. (b) The mRNA level of insulin Ⅱ. The collection and culture of mouse embryos have been described in Material and methods. 300 eggs were used in each lane.

131 bp (for type I insulin) and 231 bp (for type II insulin) PCR products were obtained, and sequence analysis of the PCR fragment revealed that these PCR products were indeed derived from the insulin mRNAs. There were the insulin I and II mRNA in the mouse oocytes and one-cell stage embryos (Figure 6(a) and (b)). We further detected the expression of insulin protein by Western blot in mouse oocytes and one-cell stage embryos. The quantity of insulin protein remained stable during meiotic maturation, and this pattern of expression was unchanged after fertilization (Figure 7). In addition, we also used the ELISA to assay the expression of insulin. The experiment was repeated 5 times and the concentration of insulin in mouse oocytes and one-cell stage embryos was respectively detected at 308±17 and 203±35 nmol/L.

At the same time, we used immunofluorescence to investigate the distribution of insulin in mouse oocytes and one-cell stage embryos. Whatever before or after fertilization, insulin was distributed evenly in the cytoplasm (Figure 8). The results suggested that insulin was expressed in mouse oocytes and one-cell stage embryos.

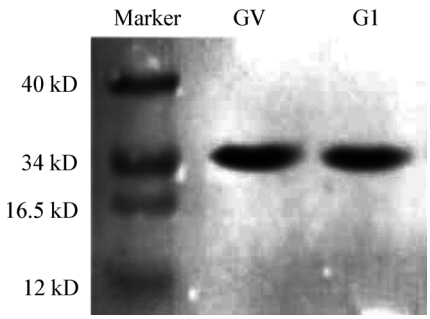

Figure 7 Western blot analysis of insulin in mouse oocytes and one-cell stage embryos. The first lane is the marker. The second and third lanes are the expression of insulin in the mouse oocytes and one-cell stage embryos with ECL, respectively. The collection and culture of mouse embryos has been described in Material and Methods. 200 eggs were used in each lane.

3 Discussion

Our research indicated that insulin is expressed at both the protein and mRNA level in mouse oocytes and one-cell stage embryos. Moreover we also used BLAST to detect the specificity of insulin primers, which could examine accurately the mRNA level of insulin but not the mRNA level of analogs of insulin such as IGF. For the expression of insulin in mouse oocytes, we considered that insulin possibly came from the maternal source. It has been known that insulin signaling plays complex

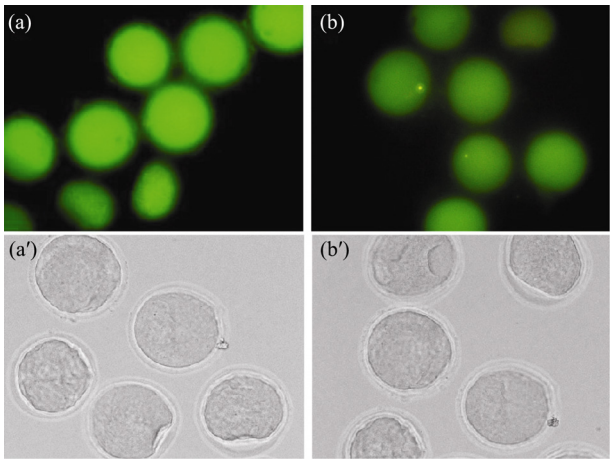

Figure 8 The immunofluorescence analysis of insulin in mouse oocytes and one-cell stage embryos. The collection and culture of mouse embryos have been elucidated as above. The green fluorescence indicates the location of insulin in the mouse oocytes (a) and one-cell stage embryos (b). Negative controls about mouse oocytes and one-cell stage embryos are shown in Figure 8(a′) and (b′).

roles in ovarian function including the regulation of ovarian steroidogenesis, follicular development and granulosa cell proliferation[13–15]. According to the report of Frias MA, insulin can regulate the activity of two mTOR related multiprotein complexes[16]. On the other hand, insulin and nutrients can also activate S6K1 to regulate protein synthesis[17]. Insulin stimulates protein synthesis by promoting phosphorylation of the eIF4E-binding protein, 4EBP1. This effect is rapamycin-sensitive and mediated by mTOR complex 1[18]. Several research groups have found that PI3K–PKB-mediated activation of mTOR also leads to attenuation of insulin signal (Figure 9) Their studies have demonstrated insulin may be involved in the cell cycle regulation in various cell lines by activating the PI3K–PKB–mTOR pathway[4–6]. Usually, insulin plays roles via its receptors on cell membrane which activates the insulin receptor substrate (IRS). And then IRS activated phosphoatidylinositol 3 kinase (PI3K). But our experiment showed that there was not the expression of insulin receptor and IRS in mouse one-cell stage embryos（data not shown）which is coincident with the report of Susan Heyner[3]. Furthermore, Rosenblum also reported that there was no evidence for insulin binding during the early development of mouse embryos[19]. A few last reports in eukaryotic models have shown that insulin is able to activate PI3K/PKB/mTOR signal pathway by the association with insulin receptor[20–23]. So it is very important to study the function of PI3K/mTOR pathway. For further research, we used Rapamycin and Wortmannin, the specific inhibitors of mTOR and PI3K respectively, to determine the relationship between insulin and mTOR and whether insulin functions via PI3K/PKB in mouse one-cell stage embryos. Our results also indicated that both Wortmannin and rapamycin were able to inhibit the egg cleavage. So we supposed that insulin might play functional roles in the development of mouse one-cell stage embryos by the signal pathway of PI3K/PKB/mTOR.

Then we examined the expression, localization and activity of mTOR. The results showed that the distribution of mTOR and S6K was around the pronucleus at S phase. The activity of mTOR fluctuated at four phases (G1/S/G2/M)of mouse one-cell stage embryos. Our data showed that mTOR and S6K were expressed and activated in mouse one-cell stage embryos, which indicated that mTOR and S6K play an important role in the development of mouse one-cell stage embryos.

In the PI3K/PKB/mTOR pathway, PKB is another important component. Previous experiments of our group have demonstrated that PI3K and PKB were expressed in mouse one-cell stage embryos and promoted

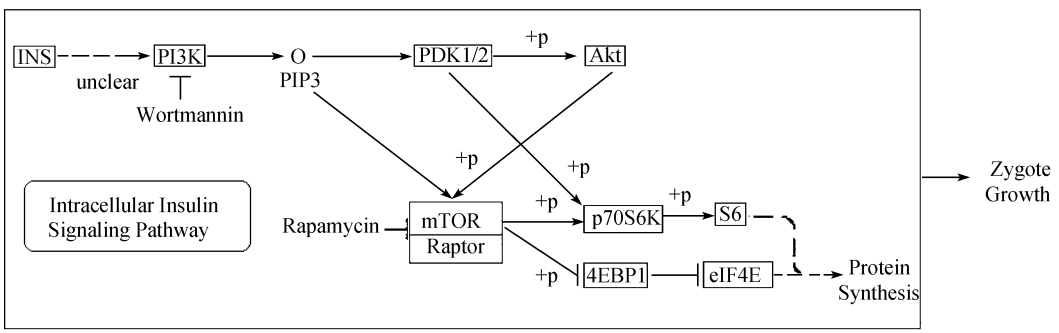

Figure 9 The diagram of the possible relationship between insulin and mTOR in mouse one-cell stage embryos.

the early development of mouse embryos[24]. According to our results, we suggested that insulin has an effect on the development of mouse one-cell stage embryos through the PI3K/PKB/mTOR signal pathway. In future research, the study should be focused on whether there is a direct or indirect relationship between the insulin, PI3K and PKB in mouse one-cell stage embryos. It will help us better understand the mechanism of the regulation of cell cycle progression in early embryo development.

1 Stockhorst U, de Fries D, Steingrueber H J, et al. Insulin and the CNS: effects on food intake, memory, and endocrine parameters and the role of intranasal insulin administration in humans. Physiol Behav, 2004, 83: 47－54

2 Petruzzelli L, Herrera R, Garcia-Arenas R, et al. Acquisition of insulin-dependent protein tyrosine kinase activity during Drosophila embryogenesis. J Biol Chem, 1985, 260(30): 16072－16075

3 Heyner S, Smith R M , Schultz G A.. Temporally regulated expression of insulin and insulin-like growth factors and their receptors in early mammalian development. Bioessays, 2004, 11: 171－176[DOI]

4 Haruta T, Uno T, Kawahara J, et al. A rapamycin-sensitive pathway down-regulates insulin signal via phosphorylation and proteasomal degradation of insulin receptor substrate-1. Mol Endocrinol, 2000, 14: 783－794[DOI]

5 Greene M W, H. Sakaue, Wang L, et al. Modulation of insulin-stimulated degradation of human insulin receptor substrate- 1 by Serine 312 phosphorylation. J Biol Chem, 2003, 278: 8199－8211[DOI]

6 Carlson C J, White M F, Rondinone C M. Mammalian target of rapamycin regulates IRS-1 serine 307 phosphorylation. Biochem Biophys Res Commun, 2000, 316: 533－539[DOI]

7 Murakami M, Ichisaka T, Maeda M, et al. mTOR is essential for growth and proliferation in early mouse embryos and embryonic stem cells. Mol Cell Biol, 2004, 24: 6710－6718[DOI]

8 Hogan B, Constantini L E. Manipulating the mouse embryos: A laboratory manual. NewYork: Cold Spring Harbor Laboratory Press, 1986. 249－250

9 Laemmli V K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 1970, 227: 680－685[DOI]

10 Gallicano G L, Mcgaughey W M, Capco D G. Activation of protein kinase C after fertilization is required for remodeling the mouse egg into the zygote. Mol Reprod Dev, 1997, 46: 587－601[DOI]

11 Yu B Z, Zheng J, Yu A M, et al. Effects of protein kinase C on M-phase promotin factor in early development of fertilized mouse eggs. Cell Biochem Funct, 2004, 22: 291－298[DOI]

12 Yu B, Wang Y, Liu Y, et al. Protein kinase A regulates cell cycle progression of mouse one-cell stage embryos by means of MPF. Dev Dynam, 2005, 232(1): 98－105[DOI]

13 Adashi E Y, Resnick C E, Payne D W, et al. The mouse intraovarian insulin-like growth factor I system: Departures from the rat paradigm. Endocrinology, 1997, 138: 3881－3890[DOI]

14 Poretsky L, Cataldo N A, Rosenwaks Z, et al. The insulin-related ovarianregulatory system in health and disease. Endocr Rev, 1999, 20: 535－582[DOI]

15 Willis D, Mason H, Gilling-Smith C, et al. Modulation by insulin of follicle stimulating hormone and luteinizing hormone actions in human granulosa cells of normal and polycystic ovaries. J Clin Endocrinol Metab, 1996, 81: 302－309

16 Frias M A, Thoreen C C, Jaffe J D, et al. mSin1 is necessary for PKB/PKB phosphorylation, and its isoforms define thouree distinct mTORC2s. Curr Biol, 2006, 16(18): 1865－1870[DOI]

17 Matsushima R, Harada N, Webster N J, et al. Effect of TRB3 on insulin and nutrient-stimulated hepatic p70 S6 kinase activity. J Biol Chem, 2006, 281(40): 29719-29729 [DOI]

18 Wang L, Rhodes C J, Lawrence J C Jr. Activation of mammalian target of rapamycin (mTOR) by insulin is associated with stimulation of 4EBP1 binding to dimeric mTOR complex 1. J Biol Chem, 2006, 281: 24293－24303[DOI]

19 Rosenblum I Y, Mattson B A, Heyner S. Stage-specific insulin binding in mouse preimplantation embryos. Dev Biol, 1986, 116: 261－263[DOI]

20 Han E K, Leverson J D, McGonigal T, et al. Akt inhibitor A-443654 induces rapid Akt Ser-473 phosphorylation independent of mTORC1 inhibitor. Oncogene, 2007, 26(38): 5655-5661[DOI]

21 Alpert E, Gruzman A, Tennenbaum T, et al. Selective cyclooxygenase-2 inhibitors stimulate glucose transport in L6 myotubes in a protein kinase Cdelta-dependent manner. Biochem Pharmacol, 2007, 73: 368－377[DOI]

22 Dann S G, Thomas G. The amino acid sensitive TOR pathway from yeast to mammals. FEBS Lett, 2006, 580: 2821－2829[DOI]

23 Yeshao W, Gu J, Peng X, et al. Elevated glucose activates protein synthesis in cultured cardiac myocytes. Metabolism, 2005, 54: 1453－1460[DOI]

24 Chen F, Yu A M, Feng C, et al. Activity and expression changes of protein kinase B in 1-cell stage fertilized eggs of mouse. Chin J Biochem Mol Biol, 2003, 19(4): 542－545