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RESEARCH ARTICLE

Oocyte-dependent activation of MTOR in cumulus cells controls the development and survival of cumulus–oocyte complexes

Jing Guo[1,‡], Lanying Shi[1,‡], Xuhong Gong[1], Mengjie Jiang[1], Yaoxue Yin[1], Xiaoyun Zhang[1], Hong Yin[1], Hui Li[1], Chihiro Emori[2,*], Koji Sugiura[2], John J. Eppig[3] and You-Qiang Su[1,4,§]

ABSTRACT

Communication between oocytes and their companion somatic cells promotes the healthy development of ovarian follicles, which is crucial for producing oocytes that can be fertilized and are competent to support embryogenesis. However, how oocyte-derived signaling regulates these essential processes remains largely undefined. Here, we demonstrate that oocyte-derived paracrine factors, particularly GDF9 and GDF9–BMP15 heterodimer, promote the development and survival of cumulus-cell–oocyte complexes (COCs), partly by suppressing the expression of *Ddit4l*, a negative regulator of MTOR, and enabling the activation of MTOR signaling in cumulus cells. Cumulus cells expressed less *Ddit4l* mRNA and protein than mural granulosa cells, which is in striking contrast to the expression of phosphorylated RPS6 (a major downstream effector of MTOR). Knockdown of *Ddit4l* activated MTOR signaling in cumulus cells, whereas inhibition of MTOR in COCs compromised oocyte developmental competence and cumulus cell survival, with the latter likely to be attributable to specific changes in a subset of transcripts in the transcriptome of COCs. Therefore, oocyte suppression of *Ddit4l* expression allows for MTOR activation in cumulus cells, and this oocyte-dependent activation of MTOR signaling in cumulus cells controls the development and survival of COCs.

KEY WORDS: DDIT4L, MTOR, Oocyte, Cumulus cells, Apoptosis, Female infertility

INTRODUCTION

The ultimate goal of ovarian folliculogenesis is to produce a mature oocyte (egg) for reproduction, and to generate hormones and growth factors, particularly steroids, for endocrine functions (Eppig, 2001). To achieve these goals, the germinal and somatic compartments of the follicle must coordinate precisely in responding to the myriad endocrine, paracrine, autocrine and juxtacrine signals prevalent in the follicular microenvironment to ensure the proper development and normal functions of the follicle (Su et al., 2009). Bi-directional communication between the oocyte and its companion granulosa cells is a crucial factor in this coordination. Granulosa cells promote oocyte growth and global silencing of transcription (De La Fuente and Eppig, 2001; Hutt et al., 2006), maintain oocyte meiotic arrest (Zhang et al., 2010) and mediate the resumption of meiosis induced by the preovulatory luteinizing hormone surge (Park et al., 2004), whereas signals from oocytes promote granulosa cell proliferation and differentiation by coordinating with signals induced by follicle-stimulating hormone (FSH) (Diaz et al., 2007a; El-Hefnawy and Zeleznik, 2001; Gilchrist et al., 2001; Latham et al., 2004; Li et al., 1995; Vanderhyden et al., 1990, 1992), suppress granulosa cell apoptosis and premature luteinization (Eppig et al., 1997; Hussein et al., 2005; Orisaka et al., 2006), and enable expansion of the cumulus cell population and ovulation (Buccione et al., 1990; Joyce et al., 2001).

An 'oocyte–granulosa-cell regulatory loop' involving complementary metabolic and signaling pathways between oocytes and granulosa cells controls the development and functions of both compartments, and highlights a crucial role of oocytes in this dialog (Eppig, 2001; Su et al., 2004). Importantly, oocyte control of the metabolic cooperativity between the oocyte and its companion granulosa cells is a cornerstone of this regulatory loop (Su et al., 2009). For example, oocytes are unable to carry out some metabolic processes – including glycolysis and cholesterol biosynthesis – and to take up certain amino acids, such as L-alanine, owing to their deficient expression of genes encoding related enzymes and amino acid transporters, and therefore rely on cumulus cells to provide them with the corresponding metabolites that support their development (Biggers et al., 1967; Eppig et al., 2005; Su et al., 2008; Sugiura et al., 2005). Remarkably, oocytes enable this metabolic cooperativity by promoting the expression of these related key metabolic enzymes and amino acid transporters in cumulus cells (Eppig et al., 2005; Su et al., 2008; Sugiura et al., 2005, 2007). In addition to the role of compensating for its own metabolic deficiencies, the oocyte probably controls the growth rate and survival of follicles by promoting cumulus cell metabolism (Eppig et al., 2002; Su et al., 2009).

How cues from oocytes result in metabolic action in cumulus cells remains uncertain, but the mechanistic target of rapamycin (MTOR; also known as mammalian target of rapamycin, mTOR) pathway is likely to be involved. MTOR is an evolutionarily conserved kinase and an essential component of the classic energy-sensing pathway within cells, and plays essential roles in controlling cell growth and development by integrating and transmitting multiple extracellular cues, including nutrient supplies and signals affecting growth and stress (Albert and Hall, 2015; Aramburu et al., 2014; Huang and Fingar, 2014). Several studies have shown that this pathway might participate in the control of granulosa cell proliferation (Kayampilly and Menon, 2007; Yu et al., 2012), differentiation (Alam et al.,

[1]State Key Laboratory of Reproductive Medicine, Nanjing Medical University, Nanjing, Jiangsu 211166, People's Republic of China. [2]Department of Animal Resource Sciences, Graduate School of Agriculture and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan. [3]The Jackson Laboratory, Bar Harbor, ME 04609, USA. [4]Key Laboratory of Model Animal Research, Nanjing Medical University, Nanjing, Jiangsu 211166, People's Republic of China.
*Present address: The Jackson Laboratory, Bar Harbor, ME 04609, USA.
‡These authors contributed equally to this work

§Author for correspondence (youqiang.su@njmu.edu.cn)

J.G., 0000-0002-2595-7314; L.S., 0000-0002-2761-0787; X.G., 0000-0001-6650-0903; M.J., 0000-0002-2720-7517; Y.Y., 0000-0003-0098-3425; X.Z., 0000-0002-9496-8041; H.L., 0000-0001-5593-5004; Y.-Q.S., 0000-0003-3537-6246

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2004) and follicle growth (Yu et al., 2011). However, whether or not this pathway is regulated by oocyte-derived signals, and whether it is involved in the control of oocyte developmental competence and cumulus cell survival is unresolved and is therefore the focus of this study.

Many of the regulatory effects of oocytes on their companion granulosa cells are mediated by the oocyte-derived paracrine factors (ODPFs) – growth-differentiation factor 9 (GDF9) and/or bone morphogenetic protein 15 (BMP15), two members of the transforming growth factor superfamily (Pangas and Matzuk, 2004). Both GDF9 and BMP15 are predominantly expressed by oocytes, and are essential for female fertility in a variety of species studied (Pangas and Matzuk, 2004; Su et al., 2009). *Gdf9*-knockout (*Gdf9*$^{-/-}$) female mice are sterile owing to the arrest of primary follicle development (Dong et al., 1996), whereas *Bmp15*-null (*Bmp15*$^{-/-}$) females are subfertile owing to defective ovulation (Yan et al., 2001). *Gdf9*$^{+/-}$ *Bmp15*$^{-/-}$ (hereafter referred to as double mutants) females display a more severe subfertility than *Bmp15*$^{-/-}$ mice, which is largely caused by abnormal development of cumulus cells (Yan et al., 2001). Therefore, GDF9 and BMP15 are essential for cumulus cell development both before and after the luteinizing hormone surge (Su et al., 2004). GDF9 and BMP15 can form heterodimers that are more active than each of the homodimers (Mottershead et al., 2015; Peng et al., 2013; Wigglesworth et al., 2013).

Taking advantage of the defective development of the double-mutant cumulus cells, we have described previously a transcriptomic analysis of double-mutant cumulus cells using microarrays in order to gain a global perspective of the role of ODPFs in cumulus cell development (Su et al., 2008). Surprisingly, we have found that the major theme associated with GDF9- and BMP15-regulated transcripts in cumulus cells is metabolism, which includes cholesterol biosynthesis, glycolysis and inositol metabolism (Su et al., 2008). Moreover, levels of DNA-damage-inducible transcript 4-like (*Ddit4l*) mRNA is strongly upregulated in *Bmp15*$^{-/-}$ and double-mutant cumulus cells (Su et al., 2008). Because DDIT4L (also known as development and DNA-damage response 2, REDD2, or RTP801-like, RTP801L) is an upstream negative regulator of MTOR (Corradetti et al., 2005), we hypothesized that oocytes enable the activation of the MTOR pathway in cumulus cells, at least in part, by suppressing the expression of DDIT4L. Here, we tested this idea and investigated the potential biological functions relevant to oocyte-controlled activation of MTOR in cumulus cells.

RESULTS

Upregulation of *Ddit4l* in mutant cumulus cells

By mining our previously published dataset (Su et al., 2008), we found that the mRNA levels of *Ddit4l*, but not of its closely related paralog *Ddit4*, were significantly upregulated in *Bmp15*$^{-/-}$ and double-mutant cumulus cells (Fig. 1A; Fig. S1A). This upregulation was validated by quantitative real-time RT-PCR (qRT-PCR) analysis (Fig. 1A). Immunohistochemistry revealed that in wild-type large antral follicles, DDIT4L was predominantly expressed by mural granulosa cells adjacent to the follicular basal lamina, and there were very few cumulus cells that stained positively for DDIT4L (Fig. 1B,C; Fig. S1B). In contrast to the wild-type follicles, the difference in DDIT4L expression level between mural granulosa cells and cumulus cells was diminished in double-mutant antral follicles, and there was a large proportion (~60%) of cumulus cells that stained positively with the antibody against DDIT4L (Fig. 1B,C; Fig. S1B).

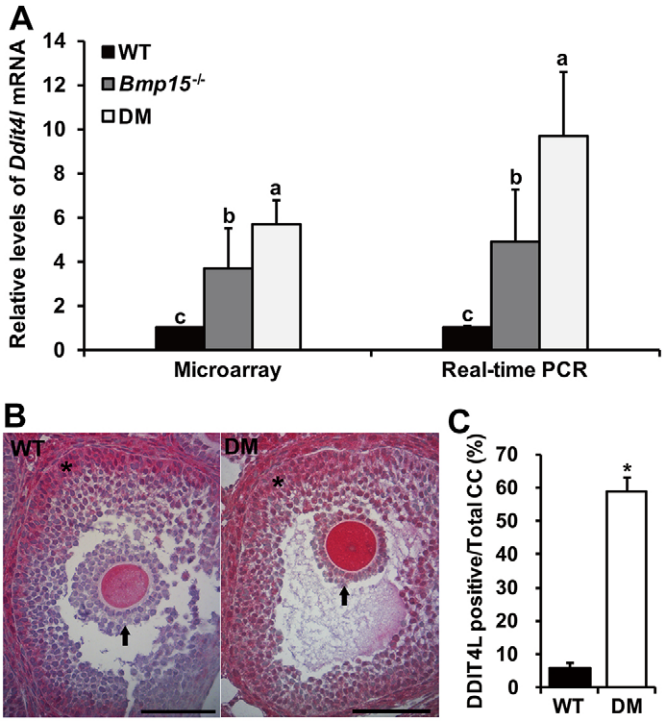

Fig. 1. Upregulation of *Ddit4l* expression in mutant cumulus cells. (A) Measurements of the steady-state levels of *Ddit4l* mRNA in wild-type (WT), double-mutant (DM) and *Bmp15*$^{-/-}$ cumulus cells by using microarray analysis (left bar graph) and quantitative real-time RT-PCR (qRT-PCR, right bar graph) analyses. Data are presented as mean±s.e.m. of fold changes relative to the wild-type group (n=3). Bars marked with different letters are statistically different, P<0.05 (one-way ANOVA followed by Tukey's HSD test). (B) Immunohistochemical detection of DDIT4L protein in the large antral follicles of wild-type and double-mutant ovaries. Arrows and asterisks indicate cumulus and mural granulosa cells, respectively. Scale bars: 100 µm. (C) Quantification of cumulus cells (CC) stained positively by a DDIT4L-specific antibody as shown in B. DDIT4L-positive and non-positive cumulus cells were counted, and the percentage of the positively staining cells relative to the total number of cumulus cells in the same follicle was calculated. A total of six large antral follicles per genotype was counted. *P<0.05 (t-test) compared with wild type.

Suppression of *Ddit4l* mRNA expression in cumulus cells by ODPFs

Because both *Bmp15* and *Gdf9* are exclusively expressed by oocytes, the upregulation of *Ddit4l* mRNA and protein in double-mutant cumulus cells implies that mouse oocytes suppress the expression of *Ddit4l*. We tested this possibility by using microsurgery to remove the oocyte from normal wild-type COCs and culturing the resultant oocytectomized (OOX) cumulus cells *in vitro*. As shown in Fig. 2A,B, *Ddit4l* mRNA was upregulated in oocytectomized cumulus cells after 20 h of culture, this upregulation was completely prevented by co-culture of oocytectomized cumulus cells with wild-type fully grown oocytes. However, neither the *Bmp15*$^{-/-}$ nor the double-mutant oocytes were able to prevent the increase of *Ddit4l* mRNA in oocytectomized cumulus cells as effectively as the wild-type oocytes; they only partially suppressed the upregulation caused by oocytectomization (Fig. 2B). Interestingly, *Ddit4* mRNA was unchanged in oocytectomized cumulus cells (Fig. S1A). Treating oocytectomized cumulus cells with recombinant mouse GDF9 (500 ng/ml) also effectively prevented the upregulation of *Ddit4l* mRNA. Recombinant mouse GDF9–BMP15 heterodimer elicited a stronger inhibitory effect on the expression of *Ddit4l* mRNA in oocytectomized cumulus cells; it

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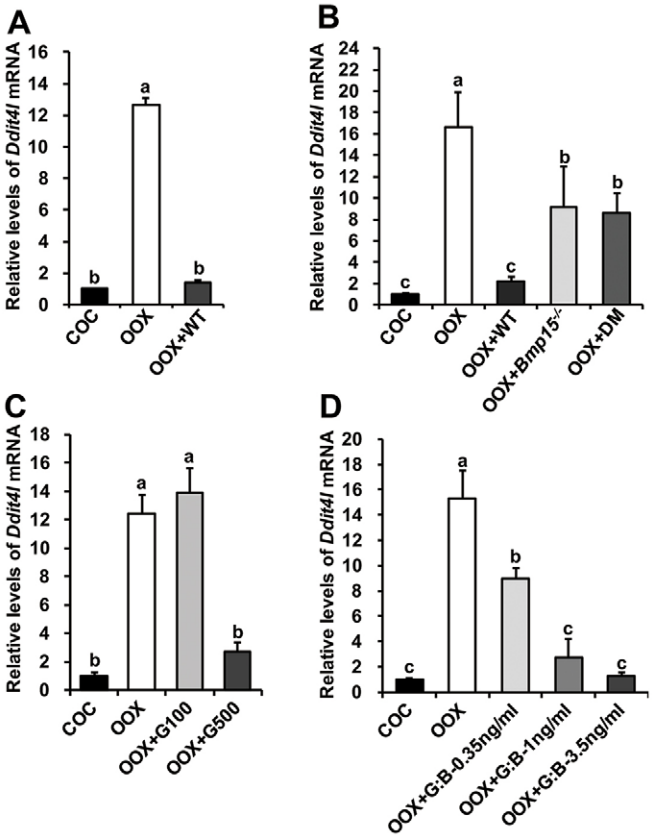

Fig. 2. Suppression of *Ddit4l* mRNA expression in cumulus cells by oocytes, GDF9 and GDF9–BMP15 heterodimer. (A) qRT-PCR analysis of *Ddit4l* mRNA expression in cumulus cells of normal wild-type mouse COCs, oocytectomized cumulus cells (OOX) and oocytectomized cumulus cells co-incubated with F1 mouse fully grown oocytes (OOX+WT) that were cultured for 20 h. (B) qRT-PCR analysis of *Ddit4l* mRNA expression in cumulus cells of normal wild-type mouse COCs, oocytectomized cumulus cells and oocytectomized cumulus cells co-incubated with wild-type, *Bmp15*$^{-/-}$ and double-mutant mouse fully grown oocytes (designated as OOX+WT, OOX +*Bmp15*$^{-/-}$ and OOX+DM, respectively) that had been cultured for 20 h. (C) qRT-PCR analysis of *Ddit4l* mRNA expression in cumulus cells of normal wild-type mouse COCs, oocytectomized cumulus cells and oocytectomized cumulus cells treated with 100 ng/ml or 500 ng/ml recombinant mouse GDF9 (designated as G100 and G500, respectively) and cultured for 20 h. (D) qRT-PCR analysis of *Ddit4l* mRNA expression in cumulus cells of normal wild-type mouse COCs, oocytectomized cumulus cells and oocytectomized cumulus cells treated with increasing doses (0.35, 1, 3.5 ng/ml) of recombinant mouse GDF9–BMP15 heterodimer (designated as G:B) and cultured for 20 h. Data are presented as the mean±s.e.m. of fold changes relative to those of the COC group (n=3). Bars marked with different letters are significantly different, P<0.05 (one-way ANOVA followed by Tukey's HSD test).

completely prevented the upregulation of *Ddit4l* mRNA even at the concentration of 1 ng/ml, which was 500 times as efficient as the GDF9 monomer (Fig. 2D).

The SMAD2-dependent pathway participates in oocyte-mediated suppression of *Ddit4l* mRNA expression in cumulus cells

The SMAD2-dependent pathway mediates regulatory signals from oocytes to companion granulosa cells (Diaz et al., 2007b; Mottershead et al., 2012; Su et al., 2010). We therefore tested whether this pathway also participates in oocyte-mediated suppression of *Ddit4l* mRNA expression in cumulus cells. As shown in Fig. 3A, when COCs were treated with 10 µM SB431542,

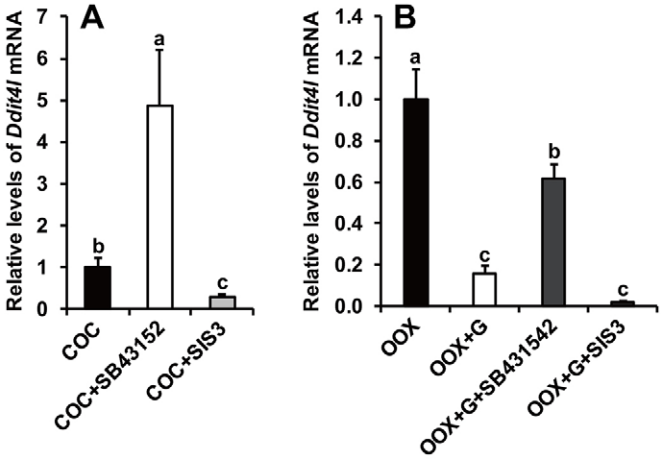

Fig. 3. Effects of SMAD2 and/or SMAD3 inhibitors on *Ddit4l* mRNA expression in cumulus cells. (A) qRT-PCR analysis of *Ddit4l* mRNA expression in cumulus cells of normal wild-type mouse COCs that had been treated with DMSO (designated as the 'COC' group), 10 µM SB431542 (COC+SB431542) or 20 µM SIS3 (COCs+SIS3) cultured for 20 h. (B) qRT-PCR analysis of *Ddit4l* mRNA expression in normal wild-type oocytectomized cumulus cells that had been treated with DMSO (the 'OOX' group), 500 ng/ml recombinant mouse GDF9 (OOX+G) or 500 ng/ml recombinant GDF9 in combination with 10 µM SB431542 (OOX+G+SB431542) or 20 µM SIS3 (OOX+G+SIS3) and cultured for 20 h. Data are presented as fold changes relative to control group (COC in A and OOX in B) and are shown as mean±s.e.m. (n=3). Bars marked with different letters are significantly different, P<0.05 (one-way ANOVA followed by Tukey's HSD test).

a SMAD2–SMAD3 inhibitor (Inman et al., 2002), *Ddit4l* mRNA expression in cumulus cells was upregulated. However, the same effect did not occur when COCs were treated with 20 µM SIS3, which inhibits SMAD3 only (Jinnin et al., 2006), rather, there was a slight decrease in *Ddit4l* mRNA in cumulus cells. SB431542, but not SIS3, also effectively abolished the suppressive effect of GDF9 on *Ddit4l* mRNA expression in oocytectomized cumulus cells; SIS3 partially enhanced the suppressive effect of GDF9 on *Ddit4l* mRNA expression in oocytectomized cumulus cells (Fig. 3B).

Differential expression of *Ddit4l* mRNA and protein in mural and cumulus granulosa cells of normal wild-type mouse ovaries

In situ hybridization revealed that *Ddit4l* mRNA was robustly expressed by mural granulosa cells but was barely detectable in cumulus cells within large antral follicles of normal wild-type mouse ovaries (Fig. 4A,B). This differential pattern of *Ddit4l* mRNA expression was further confirmed by performing qRT-PCR analysis using cumulus and mural granulosa cells that had been isolated from large antral follicles (Fig. 4C). Similarly, immunohistochemical analysis revealed that DDIT4L protein was also differentially expressed within large antral follicles; the positive signal, as indicated by red staining, was enriched in granulosa cells lining the follicle wall, and a very low signal, if any, was found in the cumulus cells and peri-antral granulosa cells (Fig. 4D). However, unlike DDIT4L, DDIT4 was expressed uniformly in all types of granulosa cells within large antral follicles (Fig. S1C).

ODPFs control differential activation of the MTOR pathway in cumulus cells

Because DDIT4L is an upstream negative regulator of the MTOR signaling pathway (Corradetti et al., 2005), the observation that oocytes suppress the expression of *Ddit4l* mRNA in cumulus cells

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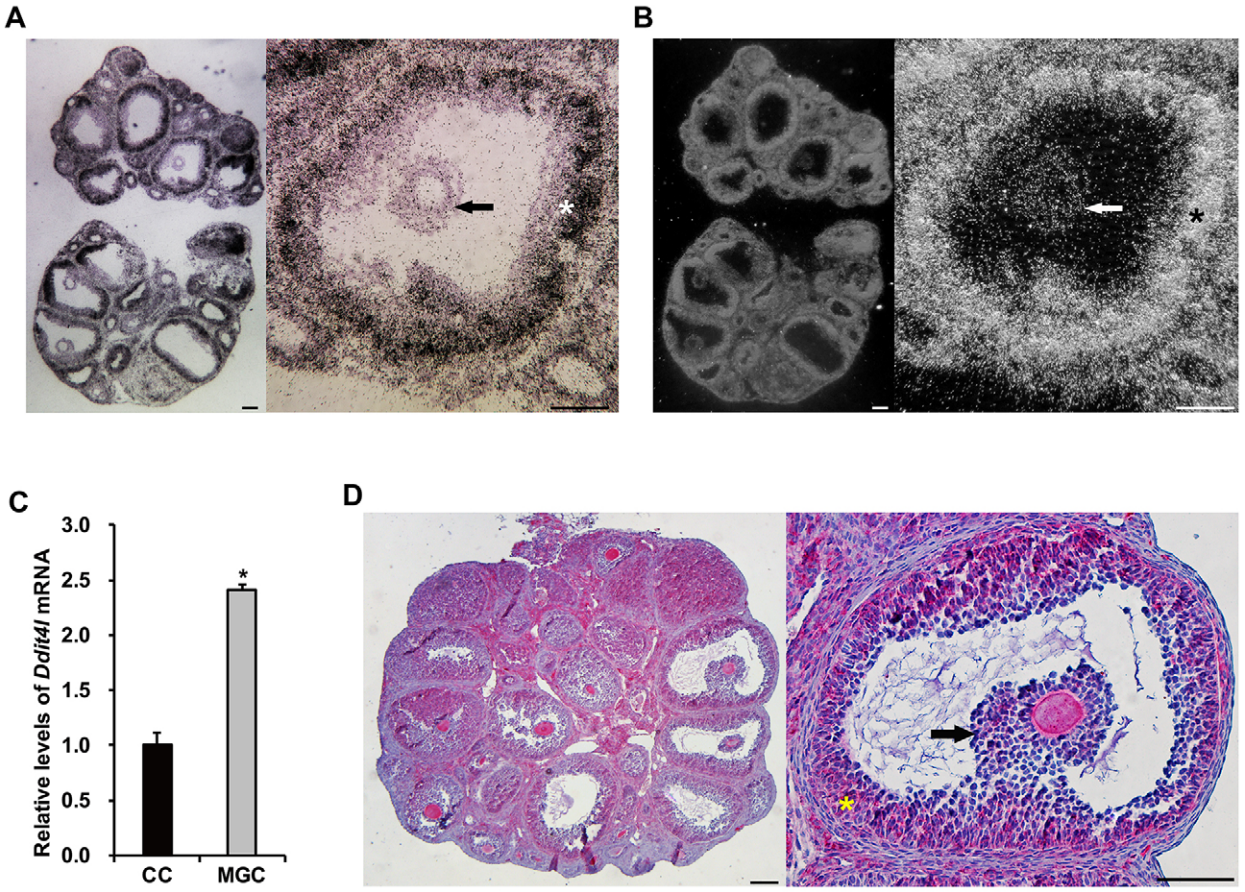

Fig. 4. Differential expression of *Ddit4l* in granulosa cells. Bright- (A) and dark- (B) field micrographs of ovarian sections after *in situ* hybridization analysis for *Ddit4l* mRNA. The magnified images of a large antral follicle are shown on the right-hand side of the whole ovarian images, with arrows and asterisks indicating cumulus and the mural granulosa cells, respectively. (C) qRT-PCR analysis of the levels of *Ddit4l* mRNA in isolated cumulus cells (CC) and mural granulosa cells (MGC). Data are presented as mean±s.e.m. (n=3) of fold changes relative to the CC group. *P<0.05 (t-test), compared with the CC group. (D) Micrographs of ovarian sections after immunohistochemical staining of DDIT4L protein. The magnified image of a large antral follicle is shown on the right-hand side of the whole ovarian image, with the arrow and asterisk pointing to cumulus and mural granulosa cells, respectively. Scale bars: 100 µm.

and that *Ddit4l* is highly expressed by mural granulosa cells indicates that oocytes might promote differential activation of the MTOR pathway in cumulus cells by suppressing *Ddit4l* expression. To test this possibility, we assessed whether the oocyte and its secreted paracrine factors, GDF9 and GDF9–BMP15 heterodimer, control the activation of MTOR in cumulus cells. As shown in Fig. 5A,B, when oocytes were removed from COCs by performing micro-surgery and cultured for 20 h, the levels of the active forms of MTOR (phosphorylated MTOR, p-MTOR) and its downstream effectors – RPS6KB1 (phosphorylated RPS6KB1, p-RPS6KB1) and EIF4EBP1 (phosphorylated EIF4EBP1, p-EIF4EBP1) (also known as S6K1 and 4E-BP1, respectively) – in the resultant oocytectomized cumulus cells were reduced. Co-culture of oocytectomized cumulus cells with normal wild-type fully grown oocytes effectively prevented this reduction in the levels of p-MTOR, p-RPS6KB1 and p-EIF4EBP1. Treating oocytectomized cumulus cells with 500 ng/ml of recombinant GDF9 or 3.5 ng/ml of GDF9–BMP15 heterodimer was also effective in preventing the reduction of p-MTOR, p-RPS6KB1 and p-EIF4EBP1. Is the MTOR pathway differentially activated in cumulus cells? Immunofluorescent analysis revealed that the active form of ribosomal protein S6, phosphorylated RPS6 (p-RPS6), another major downstream effector of MTOR activation, was strongly expressed in cumulus cells and peri-antral granulosa cells but barely detectable in mural granulosa cells (Fig. 5C).

Knockdown of *Ddit4l* in cumulus cells activates MTOR signaling

Given that DDIT4L is reported to be an upstream negative regulator of MTOR in other types of cells (Corradetti et al., 2005), the mutually exclusive patterns of expression of DDIT4L and p-RPS6 in mural and cumulus granulosa cells suggest that oocyte suppression of *Ddit4l* expression in cumulus cells might directly contribute to the activation of MTOR signaling. This possibility was tested here in cultured monolayer cumulus cells by knocking down *Ddit4l* expression and then assessing the activation of the MTOR pathway. Because the efficiency of gene knockdown in the multi-layered oocytectomized complexes cultured in the three-dimensional form is low (Sugiura et al., 2009), cumulus cells were cultured in monolayer. As shown in Fig. 6A, *Ddit4l* mRNA was upregulated about fourfold after being seeded in monolayer and cultured for 24 h. Transfection of these initially cultured monolayered cumulus cells with specific small interfering RNAs (siRNAs) against *Ddit4l*, but not with the control siRNAs, reduced the expression of *Ddit4l* mRNA by 97% (Fig. 6B). Importantly, levels of p-MTOR, p-RPS6KB1 and p-EIF4EBP1 were significantly elevated in cumulus cells with knocked down *Ddit4l*,

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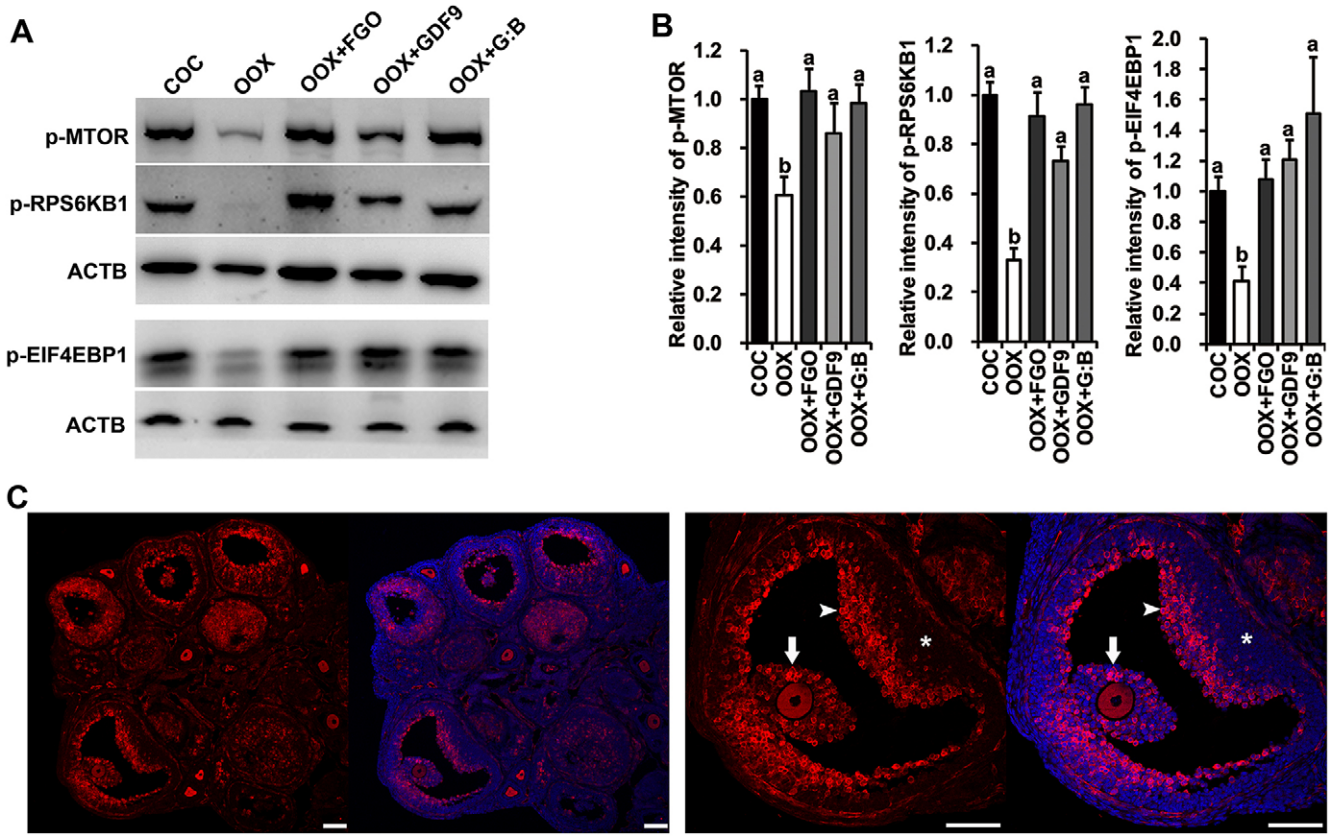

Fig. 5. ODPFs promote the differential activation of MTOR signaling in cumulus cells. (A,B) Western blot analysis of p-MTOR, p-RPS6KB1, p-EIF4EBP1 and ACTB in cumulus cells of COCs, oocytectomized cumulus cells (OOX) and oocytectomized cumulus cells co-cultured with normal wild-type fully grown oocytes (designated as OOX+FGO), or were treated with 500 ng/ml recombinant mouse GDF9 (designated as OOX+GDF9) or 3.5 ng/ml recombinant mouse GDF9–BMP15 heterodimer (designated as OOX+G:B) for 20 h. Representative western blot images are shown in A, and quantification of the signal intensity of each band from the western blot images is shown in B. Data are presented as the relative fold changes of signal intensity in each group compared to that in the COC after normalization to the corresponding ACTB expression values and are shown as mean±s.e.m. (n=3). Bars marked with different letters are significantly different, P<0.05 (one-way ANOVA followed by Tukey's HSD test). (C) Immunostaining of p-RPS6 on ovarian sections. The magnified images of a large antral follicle are shown on the right-hand side of the whole ovarian image, with arrows, arrowheads and asterisks pointing to cumulus, peri-antral and mural granulosa cells, respectively. p-RPS6 was stained in red, and DNA was stained in blue (4′,6-Diamidino-2-phenylindole, DAPI). Scale bars: 100 µm.

which were 2.4-, 2.3- and 1.8-fold the levels in control groups, respectively (Fig. 6C,D).

Inhibition of the MTOR pathway in COCs compromises oocyte developmental competence and cumulus cell survival

As a key intracellular energy-sensing pathway, MTOR signaling is crucial for sustaining the development and survival of a variety of cell types (Albert and Hall, 2015; Aramburu et al., 2014; Huang and Fingar, 2014). Because cumulus cells in COCs play an indispensable nurturing role in supporting oocyte development and maturation, we hypothesized that the oocyte-controlled activation of MTOR in cumulus cells promotes oocyte developmental competence and cumulus cell survival. We tested this possibility by inhibiting the MTOR pathway in intact COCs in culture and subsequently by assessing the post-fertilization developmental competence of oocytes and the levels of apoptosis in cumulus cells. As shown in Fig. 7A, Torin1, a specific inhibitor of MTOR (Liu et al., 2010), inhibited the phosphorylation of RPS6KB1 and EIF4EBP1 in isolated COCs at all the dosages tested. Both the rate of fertilization and the development of two-cell-stage embryos into blastocysts were significantly reduced in all the groups of COCs that had been initially treated with various doses of Torin1 for 24 h (Fig. 7B). Moreover, Torin1 dose dependently increased the occurrence of apoptotic cumulus cells, as revealed by whole-mount terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining of COCs (Fig. 7C,D).

Inhibiting the MTOR pathway in COCs results in specific changes in a subset of transcripts that are crucial for COC maturation and survival

In order to gain further insights into the roles that MTOR plays in cumulus cells, particularly those specific to cumulus cells, and to reveal the potential mechanisms responsible for Torin1-mediated reduction in oocyte developmental competence and cumulus survival, transcriptomic analysis of COCs that had been treated with 5 µM Torin1 or DMSO vehicle for 14 h in culture was performed using the Affymetrix® Mouse Gene 2.1 ST Arrays. A total of 1023 transcripts (indicated by the probe IDs) were changed significantly in COCs that had been treated with Torin1, of which 461 were upregulated and 549 were downregulated. Unsupervised hierarchical clustering revealed that these transcripts were changed to similar extents across all the samples tested (Fig. S2). Molecule Annotation System (MAS) software was used to identify the enriched pathways associated with the changed transcripts. Among the top 30 pathways identified, MAPK signaling and extracellular

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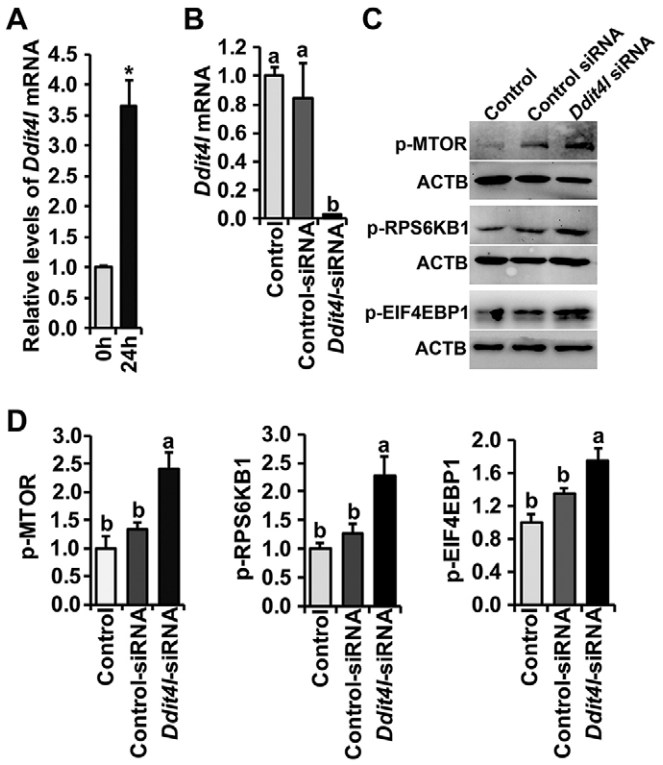

Fig. 6. Knockdown of *Ddit4l* in cumulus cells induces the activation of MTOR signaling. (A) qRT-PCR analysis of *Ddit4l* mRNA expression in cumulus cells before and after culture in monolayer for 24 h. Data are presented as the relative fold changes of *Ddit4l* mRNA in the cultured group (designated as 24 h) compared with the non-cultured group (designated as 0 h) and are shown as mean±s.e.m. (n=3). *P<0.05 (t-test), compared with the 0-h group. (B) qRT-PCR analysis of *Ddit4l* mRNA expression in monolayer-cultured cumulus cells after transfection with or without control siRNA or siRNA against *Ddit4l*. Data are presented as the relative fold changes of *Ddit4l* mRNA in the transfected group compared with that of the non-transfected group (designated as Control) and are shown as mean±s.e.m. (n=3). Bars marked with different letters are significantly different, P<0.05 (one-way ANOVA followed by Tukey's HSD test). (C,D) Western blot analysis of the expression of p-MTOR, p-RPS6KB1, p-EIF4EBP1 and ACTB in monolayer-cultured cumulus cells after transfection with or without control siRNA or *Ddit4l* siRNA. Representative western blot images are shown in C, and the quantitative analysis of the signal intensity of each band in the western blot images is shown in D. Data are presented as the relative fold changes of signal intensity in siRNA-transfected groups compared with those of the non-transfected groups (Control) after normalization with the corresponding ACTB expression values and are shown as mean±s.e.m. (n=3). Bars marked with different letters are significantly different, P<0.05 (one-way ANOVA followed by Tukey's HSD test).

matrix (ECM)-related processes were associated most significantly with upregulated transcripts (Fig. S3), whereas metabolic pathways, particularly cholesterol and sterol biosynthesis, were predominantly associated with downregulated transcripts (Fig. S4).

Because the MAPK pathway is essential for COC maturation and other ovulation-related processes after the luteinizing hormone surge (Fan et al., 2009; Su et al., 2002, 2003, 2006), and ECM formation is key for cumulus expansion (Richards, 2007), we analyzed the changed transcripts in more detail. To our surprise, many transcripts that have been reported previously to be up- or downregulated after the luteinizing hormone surge in COCs were also changed in the same direction in Torin1-treated COCs (Hernandez-Gonzalez et al., 2006). By comparing with another set of microarray data that we generated independently to examine the luteinizing-hormone-induced transcriptomic changes in cumulus cells *in vivo*, we found that 74.8% and 60.7% of the up- and downregulated transcripts, respectively, in Torin1-treated COCs were also changed in a similar manner in cumulus cells 6 h after injection of human chorionic gonadotropin (hCG) (Fig. 8A). In particular, transcripts essential for cumulus expansion – i.e. *Has2*, *Ptgs2*, *Tnfaip6* and *Ptx3*, and those encoding EGF-like growth factors, *Areg*, *Ereg* and *Btc* – were strongly upregulated by treatment with Torin1. These transcripts (Fig. 8D,E) and some of the other transcripts that were most dramatically upregulated (Fig. 8B) were validated with qRT-PCR analysis. For the downregulated transcripts, we validated those encoding the amino acid transporter SLC38A3, the glycolytic enzyme ALDOC and the essential enzymes for cholesterol biosynthesis, as well as those encoding important receptors (NPR2, EGFR, FSHR, AR and TGFBR3) on cumulus cells, by performing qRT-PCR (Fig. 8C, F).

DISCUSSION

Oogenesis and folliculogenesis are highly coordinated processes requiring complex cell-to-cell communication and a myriad of endocrine and paracrine factors that interact within the follicular microenvironment. Pituitary-derived FSH and factors produced by oocytes are of particular importance in the control of ovarian follicular development. FSH is classically known to be crucial for granulosa cell differentiation and follicle maturation, such that genetic deletion of *Fshr* or *Fshb* prevents the development of follicles significantly beyond the early antral stage (Dierich et al., 1998; Kumar et al., 1997). Whereas ODPFs have a more profound impact on follicular development starting from follicle formation up to the process of ovulation (Dong et al., 1996; McNatty et al., 2004; Soyal et al., 2000). Oocytes orchestrate the rate of follicle growth and appear to be a dominant factor in ovarian follicular development (Eppig et al., 2002). Interestingly, ODPFs and FSH exert opposing actions in directing patterns of gene expression in granulosa cells during the transition from preantral to antral follicles (Diaz et al., 2007b). By over-riding the effects of FSH in controlling the expression of *Lhcgr* mRNA and other genes that are characteristic of mural granulosa cells (Diaz et al., 2007b), oocytes promote the development and function of cumulus cells (Eppig, 2001). Accumulating evidence indicates that reciprocal signals and complementary metabolites generated by the oocyte and its companion granulosa cells promote the development and function of both compartments in a loop of interconnected regulatory processes (Eppig, 2001; Su et al., 2009). Here, we demonstrated that oocytes promote cumulus cell development and survival by suppressing *Ddit4l* expression, thereby enabling MTOR activation in cumulus cells, which in turn ensures the production of functionally normal eggs. These observations reveal an underlying mechanism governing the complex interplay between the oocyte and its companion granulosa cells in orchestrating the growth and function of ovarian follicles.

Differential expression of *Ddit4l* and activation of MTOR signaling in granulosa cells of large antral follicles

Mammalian DDIT4L is encoded by the mammalian ortholog of the *Drosophila* gene *charybde* that was originally identified to be a hypoxia-inducible negative regulator of the *Drosophila* TOR pathway (Reiling and Hafen, 2004). It is stimulated under certain stress conditions and functions as a negative regulator of MTOR by modulating the availability of free TSC2, the immediate upstream inhibitor for MTOR (Miyazaki and Esser, 2009). Despite the well-studied roles of DDIT4L in the pathogenesis of certain diseases (Cuaz-Perolin et al., 2004; Imen et al., 2009), virtually nothing was known about its ovarian expression and function. Nevertheless, we

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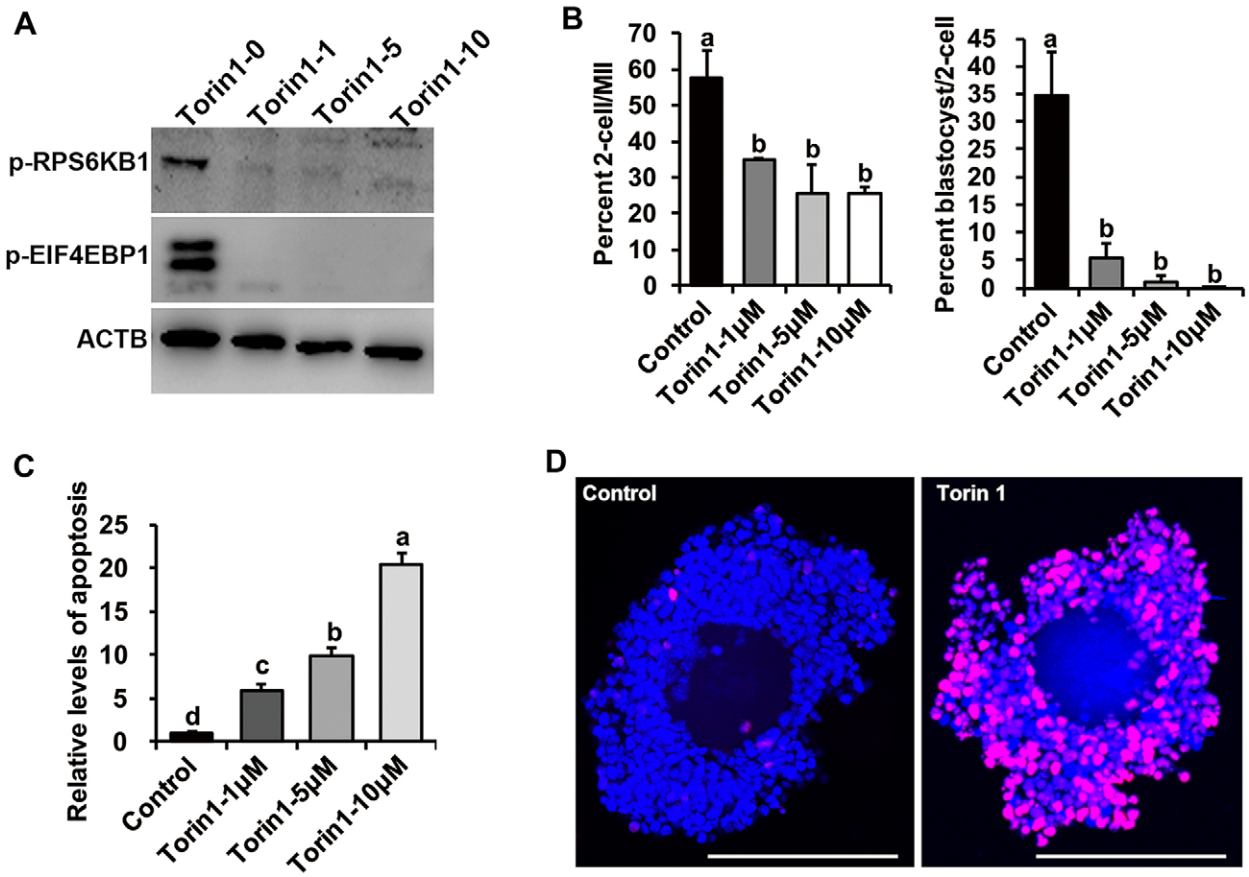

Fig. 7. Inhibition of MTOR signaling in COCs compromises oocyte developmental competence and cumulus cell survival. (A) Western blot analysis of p-RPS6KB1, p-EIF4EBP1 and ACTB in COCs that had been treated for 14 h with various doses (i.e. 0, 1, 5 and 10 μM) of Torin1. One set of representative images is shown, and similar results were obtained in three independent experiments. (B) The effect of initially treating COCs for 24 h with various doses (i.e. 0, 1, 5 and 10 μM) of Torin1 on subsequent oocyte fertilization (left panel; percentage of two-cell-stage embryo calculated from the total number of MII- oocytes) and preimplantation development (right panel; percentage of blastocysts calculated from the total number of two-cell-stage embryos). Data are shown as mean±s.e.m. (n=3). Bars marked with different letters are significantly different, P<0.05 (one-way ANOVA followed by Tukey's HSD test). (C,D) Analysis of apoptosis by TUNEL staining in COCs after being treated for 14 h with various doses (i.e. 0, 1, 5 and 10 μM) of Torin1. The quantification of TUNEL-staining-positive cells in COCs is shown in C. Data are presented as mean±s.e.m. (n=3) of the fold changes in the number of apoptotic cells in the treated groups relative to those in the Control group, which was treated only with DMSO. Bars marked with different letters are significantly different, P<0.05 (one-way ANOVA followed by Tukey's HSD test). Representative confocal microscope images of TUNEL-stained COCs are shown in D. Apoptotic cells are marked in magenta, and DNA is stained in blue (4′,6-Diamidino-2-phenylindole, DAPI). Scale bars: 100 μm.

demonstrated here that *Ddit4l*, but not its closely related paralog *Ddit4* (Corradetti et al., 2005), is differentially expressed in mouse ovarian large antral follicles before the luteinizing hormone surge, with its mRNA and protein expressed strongly in mural granulosa cells lining the follicle wall but not in the peri-antral granulosa cells or cumulus cells that are juxtaposed to the oocyte and follicular antrum. Notably, some DDIT4L protein was also detected in oocytes despite the presence of relatively lower levels of *Ddit4l* mRNA. This suggests that the expression of DDIT4L protein in oocytes might be subjected to different control mechanisms – it might be either actively translated or be stabilized immediately after translation. In striking contrast to the pattern of *Ddit4l* expression, p-RPS6 was predominantly found in cumulus cells and peri-antral granulosa cells but not in the mural granulosa cells. Given that DDIT4L is reported to be a negative regulator of the MTOR pathway in other cell types, and that *Ddit4l* expression and MTOR activation were found here to be mutually exclusive in granulosa cells, it is conceivable that DDIT4L expression contributes to the regulation of MTOR signaling in follicles. This possibility was supported by our observation that *Ddit4l* knockdown in cumulus cells significantly enhanced the activation of MTOR pathway. Therefore, before the luteinizing hormone surge, lower levels of *Ddit4l* in cumulus cells and peri-antral granulosa cells result in high levels of p-RPS6, whereas higher levels of *Ddit4l* in mural granulosa cells attenuate the levels of p-RPS6.

Contribution of oocytes to the control of the differential expression of *Ddit4l* and activation of MTOR signaling in granulosa cells

DDIT4L is upregulated under hypoxia (Shoshani et al., 2002). Although there is some controversy whether the microenvironment within antral follicles is hypoxic, it is generally accepted that the absence of a vasculature within the follicular basal lamina results in a poorly oxygenated environment for oocytes and granulosa cells before the luteinizing hormone surge (Neeman et al., 1997; Thompson et al., 2015). Therefore, low oxygen could be an initiating factor that promotes *Ddit4l* expression in mural granulosa cells, although other factors, particularly FSH, might be also involved. Interestingly, oxygen is also predicted to be lower in the vicinity of COCs (Clark and Stokes, 2011); and cumulus and peri-antral granulosa cells express higher levels of vascular endothelial growth factor (VEGF), a known effector of hypoxic stress (Shweiki

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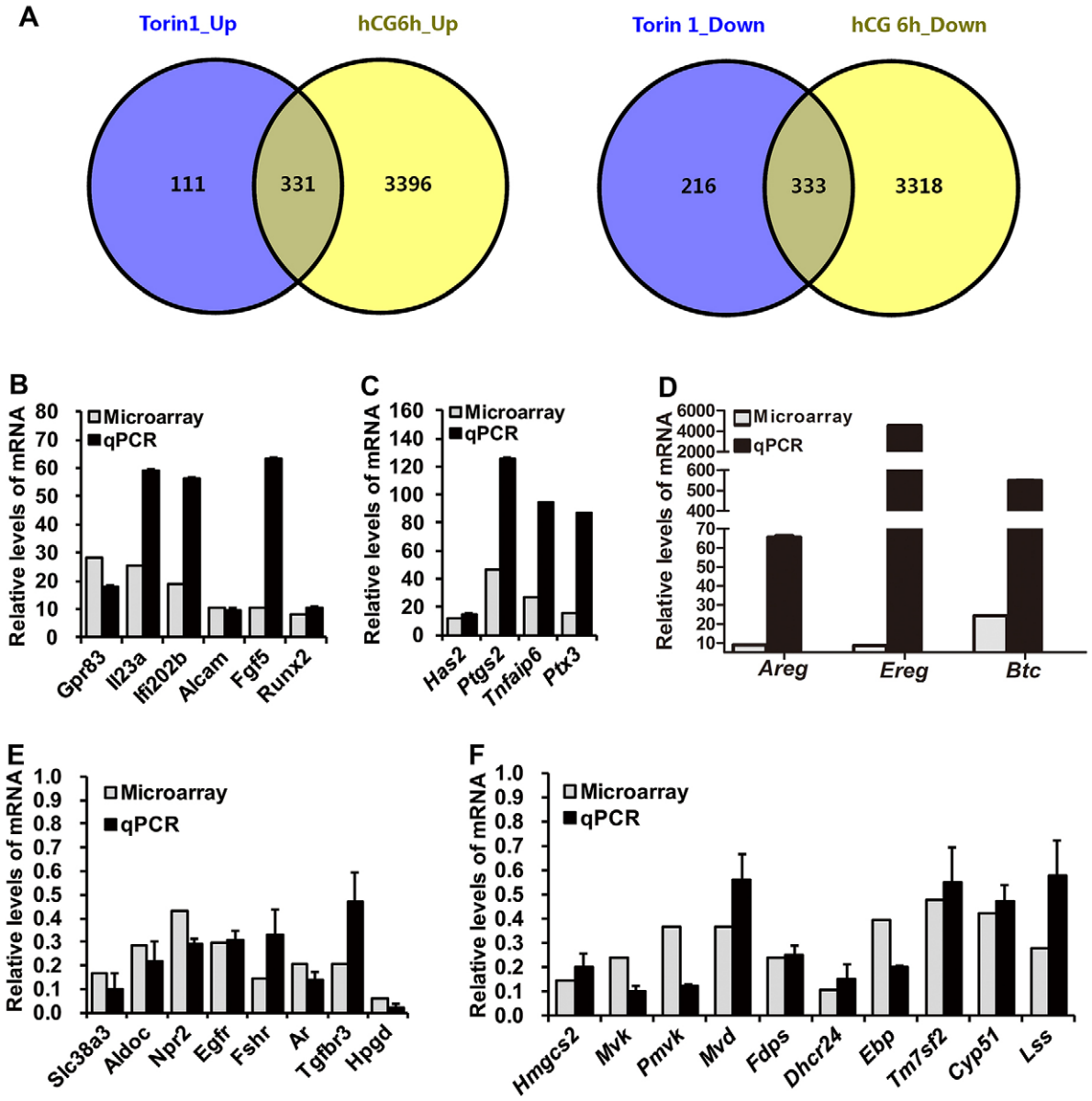

Fig. 8. Comparison of transcripts changed by MTOR inhibition *in vitro* and hCG administration *in vivo*, and qRT-PCR validation of a subset of transcripts identified by microarray analysis to be changed after MTOR inhibition. (A) Venn diagrams showing the transcripts that were commonly up- (left panel) and downregulated (right panel) through MTOR inhibition *in vitro* and hCG administration *in vivo*. (B) qRT-PCR (qPCR) validation of transcripts that were most dramatically upregulated by Torin1 treatment in COCs. (C) qRT-PCR validation of transcripts that were either most dramatically downregulated or that encode important proteins essential for cumulus cell development and function. (D) qRT-PCR validation of transcripts encoding EGF-like growth factors. (E) qRT-PCR validation of transcripts essential for cumulus expansion. (F) qRT-PCR validation of transcripts that encode enzymes for cholesterol biosynthesis. Gray bars indicate expression detected by using a microarray; black bars indicate levels detected by using qRT-PCR. Four sets of COC samples were used for qRT-PCR analysis, and data are presented as the mean±s.e.m. of fold changes relative to levels in the DMSO-treated (Control) group. The changes in the expression of all the transcripts examined were statistically significant (P<0.05; one-way ANOVA followed by Tukey's HSD test).

et al., 1993; Wigglesworth et al., 2015). Cumulus and peri-antral granulosa cells should, in theory, express more *Ddit4l*; however, they were observed here unexpectedly to express much lower levels of *Ddit4l* and to possess higher levels of p-RPS6. These data suggest that there must be additional key factors restricting *Ddit4l* expression in cumulus cells to facilitate the activation of the MTOR pathway. Indeed, our data indicate that ODPFs, specifically GDF9 and GDF9–BMP15 heterodimer, suppress the expression of *Ddit4l* and enable MTOR activation in cumulus cells. Therefore, oocytes control the differential expression of *Ddit4l* and activation of the MTOR pathway in granulosa cells before the luteinizing hormone surge.

Potential mechanisms for oocyte control of the differential expression of *Ddit4l* and activation of MTOR signaling in granulosa cells

Cumulus cells surround the oocytes intimately, and along with peri-antral granulosa cells, also directly contact the follicular fluid, they thus have early access to GDF9 and GDF9–BMP15 heterodimer diffusing from the oocyte, as well as to other ODPFs moving into the follicular fluid. In contrast, mural granulosa cells are sequestered from follicular fluid by the adjacent peri-antral granulosa layers and therefore have limited exposure to ODPFs. This spatial difference between mural granulosa cells and cumulus cells in their relative

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proximity to the oocyte and follicular fluid might be the key for oocytes to drive the differential expression of *Ddit4l* and activation of MTOR pathway within antral follicles. A similar mechanism has been proposed for oocyte control of the differential expression of many other functionally important genes within antral follicles (Eppig et al., 1997; Eppig et al., 2005; Su et al., 2008; Sugiura et al., 2005) and to direct the global patterns of gene expression in granulosa cells (Diaz et al., 2007b; Emori et al., 2013; Wigglesworth et al., 2015). Although the actual concentration of GDF9 in follicular fluid is unlikely to be as high as the dose (500 ng/ml) that we have observed here to be effective in regulating *Ddit4l* expression and MTOR activation, genetic studies clearly show that both GDF9 and BMP15 are present in follicular fluid and function in synergy (Su et al., 2004). Interestingly, GDF9 and BMP15 have recently been found to form a heterodimer that strongly stimulates cumulus cell functions *in vitro* (Mottershead et al., 2015; Peng et al., 2013; Wigglesworth et al., 2013). We also observed here that mouse recombinant GDF9–BMP15 heterodimer suppressed *Ddit4l* expression in oocytectomized cumulus cells even at 1 ng/ml, which was 500 times as potent as GDF9 homodimer. Given that the same heterodimer might also form *in vivo*, GDF9–BMP15 heterodimer that is present in follicular fluid, even at a very low concentration, could preferentially reach peri-antral and cumulus cells. In addition, as proposed in a recent study (Watson et al., 2012), the differential expression of certain types of heparan sulfate proteoglycan co-receptors for GDF9 and/or GDF9–BMP15 heterodimer in mural, peri-antral and cumulus granulosa cells could facilitate the cell-type specific regulatory functions of GDF9 and GDF9–BMP15 heterodimer. The same scenario might also account for the differential expression of *Ddit4l* and activation of MTOR in these cells.

Oocyte-dependent activation of MTOR signaling in cumulus cells controls the development and survival of COCs

We observed that even though oocytes could still mature to the metaphase stage of meiosis II (MII) after MTOR activity in COCs was inhibited with Torin1 for 24 h, their post-fertilization developmental competence was dramatically compromised. These data suggest that the oocyte-dependent activation of MTOR in cumulus cells is essential for maintaining oocyte developmental competence. Although completely disrupting the activation of MTOR complex 1 (MTORC1) in mouse oocytes through conditional knockout of *Rptor* (also known as *Raptor*), a specific and essential component of MTORC1, does not affect folliculogenesis and female fertility (Gorre et al., 2014), we still can not rule out the possibility that some of the effects of Torin1 on oocyte developmental competence in COCs observed here could be attributable to the direct inhibition of MTOR signaling in oocytes. The role of MTOR in oocytes awaits further investigation. Interestingly, exogenous ODPFs augment the developmental competence of oocytes in cultured COCs (Mottershead et al., 2015; Yeo et al., 2008), and the developmental competence of double-mutant mouse oocytes and human oocytes expressing low levels of GDF9 and/or BMP15 is low (Gode et al., 2011; Su et al., 2004; Wu et al., 2007). Therefore, oocytes influence their own development through an integrated 'oocyte–granulosa cell regulatory loop', and activation of MTOR in cumulus cells is an essential step in getting this loop fully connected and functioning.

We also observed that Torin1 dose dependently increased cumulus cell apoptosis in COCs. Hence, MTOR activation in cumulus cells is essential for COC survival. This anti-apoptosis role of the oocyte-dependent activation of MTOR in cumulus cells is consistent with a previous finding made by Hussein et al. (2005), which showed that oocytes suppress apoptosis in cumulus cells through specific ODPFs. Therefore, oocyte suppression of *Ddit4l* expression, thereby enabling MTOR activation in cumulus cells, is essential for maintaining the healthy vigor of COCs. Given the well-conserved role of MTOR in stimulating cell growth and proliferation, and the fact that cumulus cells are more proliferative than mural granulosa cells (Hirshfield, 1986), oocyte-dependent activation of MTOR in cumulus cells might also contribute to the promotion of cumulus cell proliferation. Indeed, oocytes are known to be able to promote cumulus cell proliferation (Vanderhyden et al., 1992).

Lastly, we revealed that the oocyte-dependent activation of MTOR in cumulus cells is crucial for maintaining the integrity of the transcriptome of COCs. Torin1 treatment caused significant alteration of the global profile of COC gene expression, and most strikingly, the expression of genes that was changed by Torin1 treatment in COCs was also found to be changed in a similar way in cumulus cells isolated from mice that had been primed with hCG for 6 h. In particular, the most dramatically upregulated genes included those that are essential for cumulus expansion – i.e. *Has2*, *Ptgs2*, *Ptx3* and *Tnfaip6* – and those encoding EGF-like growth factors – i.e. *Areg*, *Ereg*, and *Btc*. The expression of these genes is required for re-initiation of oocyte meiotic maturation and cumulus expansion, two normally integrated processes that are commonly referred to as COC maturation (Park et al., 2004; Shimada et al., 2006). Therefore, it is plausible that Torin1 treatment could induce phenotypic changes similar to those of COC maturation. However, because the culture system applied in this experiment (i.e. serum-free medium containing 5 µM milrinone that maintains oocyte meiotic arrest and does not support cumulus expansion) was not optimal for assessing COC maturation, we did not observe evident changes in these parameters. Further studies are needed to resolve this issue. Because COC maturation only takes place in follicles after the luteinizing hormone surge, upregulation of the genes essential for COC maturation through MTOR inhibition suggests that the oocyte-dependent activation of MTOR in cumulus cells prevents the precocious onset of COC maturation before the luteinizing hormone surge. Therefore, fine-tuned MTOR signaling in cumulus cells is crucial for synchronizing COC maturation and ovulation. Downregulation of a specific set of metabolism-associated transcripts, especially those encoding the essential enzymes for cholesterol biosynthesis, might account for the greatly reduced oocyte developmental competence and cumulus survival. This is because oocyte and preimplantation embryos up to the blastocyst stage are deficient in producing cholesterol and/or other sterols, and require the surrounding cumulus cells to synthesize and provide cholesterol to them in order to support preimplantation development (Comiskey and Warner, 2007; Pratt 1982; Su et al., 2008). Apart from these transcripts that are involved in metabolism, the downregulation of some other transcripts, such as those encoding important receptors – e.g. *Fshr*, *Ar*, *Npr2* and *Tgbr3* – might also contribute to the defects observed in Torin1-treated COCs. Of note, conditional deletion of *Ar* in granulosa cells results in decreased follicular health and fewer competent oocytes (Walters et al., 2012).

Conclusion

We propose that *Ddit4l*, a heretofore functionally unexplored gene in mammalian ovaries, is differentially expressed in mural granulosa cells and that the MTOR pathway is selectively activated in cumulus and peri-antral granulosa cells, within the large antral follicles before the luteinizing hormone surge. ODPFs, specifically GDF9

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and/or GDF9–BMP15 heterodimer, regulate the differential expression of *Ddit4l* and activation of MTOR in granulosa cells. The oocyte-dependent activation of MTOR signaling in cumulus cells encompasses a myriad of aspects of the metabolic and nutritional processes – key elements of the oocyte-cumulus cell regulatory loop – that promote the health and function of the cumulus cells and, in turn, the oocytes themselves.

MATERIALS AND METHODS

Chemicals and reagents

Unless otherwise specified, all chemicals and reagents were purchased from Sigma-Aldrich.

Mice

Female 22- to 24-day-old B6SJLF1 mice, as well as *Bmp15*$^{-/-}$, double-mutant and the wild-type littermate control mice on a B6129 mixed genetic background, were used in experiments. Mice were raised under the standard conditions at the investigators' colonies at the Jackson Laboratory and/or the Research Animal Center at Nanjing Medical University. All mouse procedures and protocols were approved by the Animal Care and Use Committee of each institution, and conducted in accordance with the institutional guides for the care and use of laboratory animals.

Cell isolation and culture

Female mice were primed with 5 IU equine chorionic gonadotropin (eCG; EMD Biosciences) for 46 h to stimulate follicular development, and COCs, cumulus cells and oocytes were then isolated as described previously (Su et al., 2008). Oocytectomized cumulus cells were prepared through microsurgical removal of oocytes from COCs, as described previously (Buccione et al., 1990). COCs, oocytectomized cumulus cells and oocytectomized cumulus cells plus oocytes (two oocytes/µl of medium) were cultured in a drop of medium covered with washed mineral oil at a density of one COC or oocytectomized cumulus cells/µl of medium. The culture medium used in all experiments was minimum essential medium α (MEMα) that had been prepared exactly as described previously (Su et al., 2008). Cells were cultured for various times according to each experimental design and then collected for mRNA or protein expression analyses.

Treatments

To test the effects of GDF9 and GDF9–BMP15 heterodimer on the expression of *Ddit4l* mRNA and phosphorylation of MTOR, RPS6KB1 and EIF4EBP1 protein in cumulus cells, oocytectomized cumulus cells were treated with 100- or 500-ng/ml recombinant mouse GDF9 (catalog no. 739-G9-010, R&D Systems) and/or various doses (0, 0.35, 1, 3.5 ng/ml) of recombinant mouse GDF9–BMP15 heterodimer (a gift from Prof. Martin M. Matzuk, Baylor College of Medicine, TX) and cultured for 20 h. To test the effect of inhibitors for SMAD 2 and/or SMAD3 on the expression of *Ddit4l* mRNA in cumulus cells of COCs, COCs were cultured in medium supplemented with or without 10 µM SB431542 or 20 µM SIS3 (EMD Biosciences) for 20 h. To test the effect of the same SMAD inhibitors on GDF9-controlled expression of *Ddit4l* mRNA, oocytectomized COCs cells were first treated with 10 µM SB431542 or 20 µM SIS3 for 1 h, and then treated together with 500 ng/ml GDF9 for another 19 h. The doses of SB431542 and SIS3 were chosen based on our previous studies (Diaz et al., 2007b; Su et al., 2010). SB431542 and SIS3 were dissolved in DMSO at stock concentrations of 10 and 20 mM, respectively. To study the effect of MTOR inhibition on COCs, COCs were treated with various concentrations (0, 1, 5, 10 µM) of Torin1 and cultured for either 14 or 24 h depending on the specific experimental designs. Torin1 was dissolved in DMSO at a stock concentration of 10 mM. Groups treated with only 10 µM DMSO served as controls.

Knockdown of *Ddit4l* with siRNAs

Cumulus cells were stripped from intact COCs by repeated mouth-controlled pipetting, and seeded into the wells of a 96-well plate at a density of 50 COCs/150 µl/well. Cumulus cells were firstly cultured in 10%-FBS-containing medium for 24 h to form monolayers. Then the medium was changed into Opti-MEM containing no antibiotics, and the cells were transfected with *Ddit4l*-specific siRNAs (a mixture of three siRNAs targeting different regions of *Ddit4l* mRNA) or control siRNA at final concentrations of 40 nM for 24 h. Then, Opti-MEM was replaced by MEMα medium containing 10% FBS and antibiotics, and cells were further cultured for another 24 h. At the end of culture, cells were collected for both western blot and real-time RT-PCR analyses. *Ddit4l* siRNA and control siRNA were purchased from GenePharma company (Beijing, China). Their sequences were: *Ddit4l*-mus-392: 5′-CCUCAACGAGGUGGUAUUUTT-3′ and 5′-AAAUACCACCUCGUUGAGGTT-3′; *Ddit4l*-mus-460: 5′-CCAGAUCAAAGCAAACCAATT-3′ and 5′-UUGGUUUGCUUUGAUCUGGTT-3′; *Ddit4l*-mus-626: 5′-GGAUAGGAUCGUGUGUGAUTT-3′ and 5′-AUCACACACGAUCCUAUCCTT-3′. Control siRNA: 5′-UUCUCCGAACGUGUCACGUTT-3′ and 5′-ACGUGACACGUUCGGAGAATT-3′.

Oocyte *in vitro* maturation, fertilization and embryo culture

After being treated with various doses (0, 1, 5, 10 µM) of Torin1 in medium containing 5 µM milrinone for 24 h, COCs were washed with medium containing 10% FBS but without milrinone three times and were then cultured in the same medium and treated with 10 ng/ml of EGF for 14 h to induce maturation. After maturation, cumulus cells were removed by treating COCs with 100 ng/ml of hyaluronidase, and oocytes with extruded first polar bodies were counted and inseminated with normal sperm, as described previously (Su et al., 2004). The formation of two-cell-stage embryos was scored 24 h after *in vitro* fertilization. Thereafter, two-cell-stage embryos were cultured in KSOM medium (EMD Millipore Corporation, Billerica, MA), and the number of blastocysts was counted 3–5 days later.

Microarray analysis

To examine the effect of MTOR inhibition on transcriptomic changes in cumulus cells, COCs were cultured in medium containing milrinone (5 µM) and treated with 5 µM Torin1 or an equal concentration of DMSO for 14 h, and then were collected in RLT lysis buffer for RNA extraction. To compare global changes of gene expression in cumulus cells after hCG administration *in vivo*, cumulus cells were isolated from large antral follicles of mice at the time of hCG injection (designated as hCG 0 h) and at 6 h after hCG injection (designated as hCG 6 h). Three sets of samples were collected for each experiment, with each sample containing 100 COCs or cumulus cells from 100 COCs. Total RNA extraction, quantification and quality evaluation, sample amplification, labeling and hybridization, as well as data acquisition and analysis were performed as described previously (Su et al., 2008). Pairwise comparison was used to resolve the statistical differences of gene expression between groups with different treatments. Differentially expressed genes are defined at a false discovery rate (FDR) q-value threshold of 0.05. Molecule Annotation System (MAS) software (http://bioinfo.capitalbio.com/mas3/) was used to identify the enriched pathways and gene ontology (GO) terms associated with the changed transcripts.

qRT-PCR analysis

Total RNA extraction, *in vitro* transcription and real-time PCR analyses were performed as described previously (Su et al., 2008). The primer sequences for the tested genes are listed in Table S1. The relative fold change in mRNA levels was calculated using the method of $2^{-\Delta\Delta Ct}$, as described previously (Su et al., 2007), and is presented as relative changes to a specific group (control) in which the expression level of the mRNA of interest was set at 1. *Rpl19* served as an internal control.

Western blot analysis

Protein sample preparation, SDS-PAGE gel electrophoresis and transfer, and the subsequent immunodetection were carried out following the exact procedures as described previously (Su et al., 2001). The primary antibodies used were against p-MTOR (phosphorylated at Ser2448; clone D9C2; XP® rabbit monoclonal; catalog no. 5536; 1:1000), p-RPS6 (phosphorylated at Ser235 and Ser236; catalog no. 2211; 1:1000), p-4E-BP1 (phosphorylated at Thr37 and Thr46; clone 236B4; rabbit monoclonal; catalog no. 2855; 1:1000) and phosphorylated p70 S6 Kinase (phosphorylated at Thr389;

catalog no. 9205; 1:1000). All these antibodies were purchased from Cell Signaling Technology. The expression of β-actin (ACTB) detected by anti-β-actin (ACTB) antibody (clone AC-15; mouse monoclonal; catalog no. A1978; 1:5000) was used as internal control of each sample.

In situ hybridization, immunohistochemistry and immunofluorescence

Female mice were primed with eCG for 46 h to stimulate antral follicle development. Ovaries were then isolated and fixed in 4% paraformaldehyde solution for 4 h. The fixed ovaries were then embedded in paraffin and sectioned at 5-µm thickness for *in situ* hybridization, immunohistochemistry and immunofluorescence analyses. *In situ* hybridization was performed as described previously (Eppig et al., 2002) using the ^{33}P-labeled anti-sense riboprobe spanning nucleotides 571–1480 of the ovarian *Ddit4l* mRNA sequence. Immunohistochemistry and immunofluorescence detection of DDIT4L and/or p-RPS6 proteins was performed following the exact procedure as described previously by Diaz et al. (2007b). The VECTASTAIN ABC-AP KIT (Rabbit IgG, catalog no. AK-5001) and VECTOR Red Alkaline Phosphatase (AP) Substrate Kit (catalog no. AK-5100) purchased from Vector Laboratories were used for immunohistochemistry staining. Primary antibodies used were: anti-DDIT4L polyclonal antibody (catalog number 12094, Proteintech Group; 1:50 dilution); anti-p-RPS6 (phosphorylated at Ser235 and Ser236) antibody (catalog no. 2211, Cell Signaling Technology; 1:200 dilution for immunohistochemistry, 1:100 dilution for immunofluorescence). For immunofluorescence detection of p-RPS6, the Alexa-Fluor-594 donkey anti-rabbit IgG was used as the secondary antibody.

In situ detection of cell apoptosis

Apoptosis was detected using the TMR red *in situ* Cell Death Detection Kit (catalog no. 12156792910, Roche Diagnostics GmbH) according to the manufacturer's instructions. Images were taken under the LSM 710 confocal laser scanning microscope (Zeiss). *Z*-stack images were recorded at 0.2-µm intervals over the entire depth of the nuclei using the same image acquisition settings for controls and inhibitor-treated groups, and the maximum projection images were computed. The fluorescence intensity of the projected images was calculated using the ImageJ software (National Institutes of Health) and was presented as levels relative to those of the control group.

Statistical analysis

All experiments were repeated at least three times, and data are presented as mean±s.e.m. In experiments with more than two treatments, differences between groups were calculated by one-way ANOVA followed by Tukey's honest significant difference test (HSD) test using JMP software (SAS Institute, Cary, NC), whereas for experiments with only two treatments, differences were analyzed with a *t*-test using Microsoft Excel. $P<0.05$ was defined as a significant difference.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Y.-Q.S. designed the study. J.G., L.S., X.G., M.J., Y.Y., X.Z., H.Y., H.L., C.E., K.S. and Y.-Q.S. performed the experiments. Y.-Q.S. analyzed and interpreted the data. Y.-Q.S. and J.J.E. wrote the manuscript.

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Data availability

Full microarray datasets are available in the Gene Expression Omnibus database under accession numbers GSE79862 and GSE80255.

Supplementary information

Supplementary information available online at
http://jcs.biologists.org/lookup/doi/10.1242/jcs.182642.supplemental

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