Stimulation of ovarian follicle growth after AMPK inhibition

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Abstract

Previous studies showed that the protein kinase B (Akt)–mammalian target of rapamycin (mTOR) and Hippo signaling Yes-associated protein (YAP) pathways play important roles in promoting follicle growth. Additionally, other studies demonstrated that 5' adenosine monophosphate-activated protein kinase (AMPK) is an upstream regulatory element of mTOR and YAP. Here, we used AMPK inhibitor (Compound C) to *in vitro* cultured ovaries from 10-day-old mice followed by *in vivo* grafting into adult hosts or to *in situ* treated ovaries of 3-week-old mice by intrabursal injection followed by gonadotropin stimulation. We found that the phosphorylation of ovarian mTOR and downstream proteins (ribosomal protein S6 (S6) and eukaryotic translation initiation factor 4B (eIF4B)) was upregulated following Compound C administration, whereas tuberous sclerosis complex 2 (TSC2) phosphorylation was downregulated. Additionally, treatment with Compound C increased hypoxia-inducible factor 1- α (*Hif1a*), vascular endothelial growth factor A (*Vegfa*), VEGF receptor 2 (*Vegfr2*) and connective tissue growth factor (*Ctgf*) mRNA levels. Furthermore, treatment of 10-day-old mice with Compound C promoted the growth of preantral and antral follicles accompanied by enhanced angiogenesis. *In situ* intrabursal injection with Compound C, followed by controlled ovarian hyperstimulation, increased the number of ovulated oocytes in 3-week-old mice, and these oocytes could be successfully fertilized, leading to the delivery of healthy pups. Our results demonstrated that treatment with AMPK inhibitor resulted in the activation of the mTOR signaling pathway, increases in *Ctgf* expression in mouse ovaries, stimulation of follicle development and promotion of ovarian angiogenesis for ovary growth.

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Introduction

The ovarian follicle is an essential functional unit of the mammalian ovary. Previous studies on follicle development mainly focused on the early antral-to-preovulatory stages regulated by gonadotropin (Fauser & Van Heusden 1997, Richards 1980, Hsueh *et al.* 1984). However, few studies have focused on hormonal regulation of preantral follicle growth, including how to activate dormant primordial follicles and promote primary and secondary follicles to the early antral stage (Li *et al.* 2010, Kawamura *et al.* 2013, Cheng *et al.* 2015). The regulatory mechanisms associated with preantral follicles are directly related to the treatment and prognosis of patients exhibiting ovarian dysfunctions, including primary ovarian insufficiency, poor ovarian response to follicle-stimulating hormone (FSH) stimulation and low ovarian reserve, as well as cancer and those with cryopreserved ovarian tissues. Particularly for patients with primary ovarian insufficiency, the ability to activate primordial follicles is directly related to determining their ability to produce genetic descendants (Kawamura *et al.* 2013).

Although it remains unclear what specific hormonal factors are involved in the activation of primordial follicles, recent studies showed that the protein kinase B (Akt)–mammalian target of rapamycin (mTOR) signaling pathway plays an important role (Reddy *et al.* 2008, Jagarlamudi *et al.* 2009). mTOR is a serine/threonine kinase that integrates growth signals at the cellular level (Perl 2015), and treatment with an mTOR activator promotes follicle growth (Cheng *et al.* 2015). Moreover, the Hippo signaling pathway is essential for organism-size management, which is conserved in all metazoan animals (Pan 2007, Zhang *et al.* 2009, Halder & Johnson 2011, Hergovich 2012, Piccolo *et al.* 2014, Hansen *et al.* 2015). Disruption of Hippo signaling increases the expression of nuclear Yes-associated protein (YAP) and downstream connective tissue growth factor (CTGF) (de Winter *et al.* 2008), which results in CTGF stimulation of cell growth, survival and proliferation (Holbourn *et al.* 2008). These findings led to improved outcomes in mouse or human ovaries and the subsequent birth of healthy babies (Kawamura *et al.* 2013).

A recent study showed that the deletion of liver kinase B1 (LKB1) expression in mouse oocytes

overactivated the primordial follicle pool through an Akt-independent LKB1-5' adenosine monophosphate-activated protein kinase (AMPK) pathway by downregulating AMPK expression continuously (Jiang *et al.* 2016). AMPK coordinates glucose and lipid metabolism in response to changes in nutrients and intracellular energy levels in all eukaryotes. In one study, AMPK activators inhibited the proliferation of ovarian theca-interstitial cells through an AMPK-dependent mechanism (Will *et al.* 2012). Another study showed that AMPK activators decreased YAP-dependent CTGF expression under cellular energy stress conditions, whereas AMPK depletion blocked this inhibitory effect (Mo *et al.* 2015). By contrast, AMPK inhibition activates the mTOR signaling pathway in tumors (Shackelford & Shaw 2009). mTOR signaling activation promotes ovarian follicular development (Manning *et al.* 2002, Adhikari *et al.* 2009, 2010), and increased CTGF expression leads to ovarian follicle growth (Kawamura *et al.* 2013). Accordingly, we hypothesized that *in vitro* treatment with an AMPK inhibitor in a short term would activate primordial follicles and promote follicular development.

As the number of AMPK inhibitors is not so high, the best known one is Compound C. Its effect on inhibiting AMPK activation is confirmed when it was first mentioned as AMPK inhibitor in the literatures (Zhou *et al.* 2001, Kim *et al.* 2004, McCullough *et al.* 2005). It is a cell-permeable pyrazolopyrimidine, which acts as a potent, selective, reversible and adenosine triphosphate-competitive AMPK inhibitor that was used to counteract the effect of AMPK activators, including 5-aminoimidazole-4-carboxamide ribonucleotide or metformin (Tang *et al.* 2011). It is the main tool for studying the role of AMPK activation in a variety of biological processes (Novikova *et al.* 2015). Treatment with Compound C reduces the phosphorylation of tuberous sclerosis complex 2 (TSC2) in cardiac hypertrophy cells or tumor cells and increases the phosphorylation of mTOR/ribosomal protein S6 kinase beta-1 (S6K1)/eukaryotic initiation factor 4E-binding protein 1 *in vitro* (Yi *et al.* 2015, Zhang *et al.* 2015, Zhao *et al.* 2015, Zhou *et al.* 2015), whereas S6K1 is the upstream regulatory element of ribosomal protein S6 (S6) and eukaryotic translation initiation factor 4B (eIF4B) in tumorigenesis (Mamane *et al.* 2006). These findings confirmed that this drug might upregulate the expression of downstream proteins in the mTOR pathway; however, the differential effects of Compound C treatment in tumor and ovarian cells require verification.

Here, we aimed to investigate whether treatment with the AMPK inhibitor Compound C activates dormant follicles and promotes follicle development and vascularization in ovaries.

Materials and methods

Animals

CD-1 and C57BL/6 mice were purchased from Charles River Laboratories and housed in the animal facility of Stanford University under a 12-h light/darkness cycle with free access to water and food.

Ethical approval

Mice were treated in accordance with guidelines of the Stanford University Animal Research Committee, and the Stanford University Biosafety and Animal Research Committees approved all experimental protocols.

Experimental design

The details of the experimental design are illustrated in a flowchart in [Supplementary Fig. 1](#) (see section on [supplementary data](#) given at the end of this article). The three experiments are described as follows.

In vitro incubation of 10-day-old mouse ovaries (Experiment 1)

Ovaries were extracted and transferred individually to culture plate inserts (Millipore) and cultured in 400 µL Dulbecco's modified Eagle medium/F12 containing bovine serum albumin (Sigma-Aldrich), Albumax II (Invitrogen), insulin-transferrin-selenium (Invitrogen), L-ascorbic acid and penicillin-streptomycin, which was an explant culture model described previously (Li *et al.* 2010, Cheng *et al.* 2015). The ovaries were randomly divided into several groups ($n=5$ /group) according to different concentrations (0, 3, 10, 30 or 100 µM) or exposure times (0, 1, 4, 8, 24 or 96 h) for incubation with Compound C (TOCRIS, Bristol, UK). The control ovaries (0 µM or 0 h) were treated with vehicle only. Ovaries were cultured for 4 days, with changes of medium after 2 days to allow for weight determination. The Compound C powder was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) to create a 100-mM stock, followed by dilution with culture medium prior to use.

In vivo allograft of 10-day-old mouse ovaries (Experiment 2)

Paired ovaries from the same CD-1 10-day-old donor were incubated with vehicle or Compound C for different drug exposure concentrations and times (10 µM/1 h, $n=10$; 10 µM/8 h, $n=5$; or 30 µM/1 h, $n=4$), followed by grafting under separate sides of the kidney capsule in the same 9-week-old ovariectomized hosts. From 1 day after transplantation, hosts received daily intraperitoneal (i.p.) FSH injections (1 IU/animal) for 4 days as reported previously (Li *et al.* 2010, Cheng *et al.* 2015). The ovaries were collected 5 days after grafting for weight measurement.

In situ intrabursal injection followed by controlled ovarian hyperstimulation (Experiment 3)

Immature 3-week-old C57BL/6 mice ($n=14$) were separated into two groups randomly for ovarian intrabursal injection as described previously (Flesken-Nikitin *et al.* 2003). They received a single ovarian intrabursal injection with 10 μ L saline on the left side as a blank control and 10 μ L vehicle (the control group) or 50 ng/10 μ L ($\approx 10 \mu$ M) Compound C (the treatment group) on the right side. After all of them received ovarian intrabursal injection and resuscitated from anesthesia, animals were then injected i.p. with 5 IU equine chorionic gonadotropin (eCG), followed by an injection of 5 IU human chorionic gonadotropin (hCG) after 48 h. The ovulated oocytes in the two oviducts of each mouse were collected and counted after 16 h, and ovaries were collected for weight determination and quantitative reverse transcription polymerase chain reaction (qRT-PCR). Meanwhile, the oocytes gained from the right sides were inseminated for *in vitro* fertilization and embryo transfer (IVF-ET) as described in previous studies (Kawamura *et al.* 2013, Cheng *et al.* 2015). In brief, oocytes were fertilized with sperm for 6 h, and then cultured for development into blastocysts. And for embryo transfer, some Compound C-pretreated 2-cell stage embryos were transferred into oviducts of pseudopregnant, 8-week-old mice followed by pregnancy and delivery.

Analyses after animal experimentation

Hematoxylin and eosin (H&E) staining and follicle counting

In vitro-cultured ovaries were treated with vehicle or 10 μ M Compound C and 8-h drug exposure time over a total 4-day culture prior to collection for H&E staining. Grafted ovaries were *in vitro* cultured with vehicle or 10 μ M Compound C for 1 h followed by *in vivo* allograft and 4 days of FSH injection prior to collection. The ovaries ($n=4$ /group) were collected and fixed in 10% buffered formalin for 1 day, embedded in paraffin, serially sectioned (4 μ m) and stained with H&E for evaluation of follicle dynamics as previously described (Flaws *et al.* 1997, Vitt *et al.* 2000, Li *et al.* 2010). Briefly, every third section from each ovary was used for counting, and only follicles with clearly stained oocyte nuclei were counted to prevent recounting of the same follicle.

CLARITY processing and immunofluorescence staining of whole ovaries

Whole paired ovaries were *in vitro* cultured for 1 h with vehicle or 10 μ M Compound C, followed by *in vivo* allograft and 4 days of FSH injection and collection for CLARITY processing as described previously (Chung *et al.* 2013). Briefly, ovaries were immersed in 5-mL conical tubes containing 3–4 mL of hydrogel solution with 4% (w/v) paraformaldehyde, 4% acrylamide, 0.05% bis-acrylamide, 0.25% VA-044, 0.05% Saponin and 1 $\times$ phosphate-buffered saline (PBS) for 3 days at 4°C (Chung *et al.* 2013). Tubes were then placed in a glass jar full of carbon dioxide to remove oxygen, followed by incubation in a 37°C water bath for 3 h to polymerize the hydrogel monomers.

Grafts were transferred into 10 mL clearing solution consisting of 200 mM boric acid, 4% (w/v) sodium dodecyl sulfate (SDS) dissolved in distilled H₂O and supplemented with NaOH to reach pH 8.5 (Chung *et al.* 2013). The clearing solution was replaced two to three times per week until the ovaries became transparent at 8–10 weeks.

Transparent ovaries were moved into 10-mL glass bottles containing PBST (PBS with 0.1% Triton-X) and shaken slowly for 1 day to remove residual SDS. Grafts were then incubated with primary antibodies (tyrosine hydroxylase (TH), 1:50, #ab76442; and platelet and endothelial cell adhesion molecule 1/cluster of differentiation-31 (CD31), 1:10, #ab28364; Abcam) for 2 days, washed in buffer for 1 day and incubated with secondary antibodies (Alexa FLUOR 488, 1:50, #A11039; and Alexa FLUOR 594, 1:50, #A11012; Thermo Scientific) for 2 days, followed by washing with buffer for 1 day. All procedures were conducted with shaking at 37°C. Before imaging, ovaries were immersed in FocusClear solution (CelExplore, Taiwan, China) for 3 h to correct for refractive index. Images were obtained using a Leica SP5 confocal microscope (20 $\times$ water-immersion objectives; numerical aperture: 1.0; working distance: 2.0 mm; Leica Microsystems). The total ovarian areas and vascular areas of sections were measured by Image-J software (National Institutes of Health). Ovarian vascular area percentage was defined as the area of vascular extent (signals of CD31 fluorescence) to total ovarian area (total CD31 and TH fluorescence signals).

Protein extraction and Western blot

In vitro-cultured ovaries were collected after a 1-h exposure to Compound C, followed by protein extraction using the Pierce BCA protein assay kit (Thermo Scientific) containing a protease-inhibitor cocktail and concentration determination. Protein samples were loaded onto a 4–12% NuPAGE Bis-Tris gel (Invitrogen), and then transferred to polyvinylidene fluoride membranes. The membranes were subsequently incubated with primary antibodies for TSC2, mTOR, S6 and eIF4B (Cell Signaling) and IRDye 800 rabbit secondary antibody (926-32211; LI-COR Biosciences, Lincoln, NE, USA) as previously described (Cheng *et al.* 2015). The primary antibodies are shown in Table 1. Images were collected using the LI-COR Odyssey infrared imager, and densitometry was performed using LI-COR Image Studio Lite, version 5.0 (LI-COR Biosciences).

Real-time qRT-PCR

In vitro-cultured ovaries were collected after 0-, 1-, 4- or 8-h exposure to Compound C. Grafted ovaries were cultured with DMSO control or 10 μ M of Compound C for 1 h followed by *in vivo* allograft and 4 days of FSH injection prior to collection. *In situ*-treated ovaries from the right side of mice from each group were intrabursally injected with vehicle or Compound C, followed by controlled ovarian hyperstimulation. mRNAs were extracted using the Total RNA purification kit (#37500; Norgen, Thorold, ON, Canada) according to manufacturer protocol, and cDNA was synthesized using the iScript cDNA synthesis kit (#170-8891; Bio-Rad) prior to determining gene expression using the CFX96 Touch real-time PCR detection

Table 1 Western blot antibodies used in this study.

Primary antibody	Product number	Specificity	Isotype	Dilution
Phosphor-TSC2	#5584	Ser1387	Rabbit	1:1000
TSC2	#4308	Total	Rabbit	1:1000
Phosphor-mTOR	#5536	Ser2448	Rabbit	1:1000
mTOR	#2983	Total	Rabbit	1:1000
GAPDH	#5174	Total	Rabbit	1:1000
Phospho-rpS6	#4858	Ser235/236	Rabbit	1:1000
Phospho-eIF4B	#3591	Ser422	Rabbit	1:1000

system (Bio-Rad). The primer sequences for hypoxia-inducible factor 1- α (*Hif1a*), vascular endothelial growth factor A (*Vegfa*), VEGF receptor 1 and 2 (*Vegfr1* and *Vegfr2* respectively), *Ctgf* and beta-Actin (*Actb*) were generated using the Primer-Blast tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Primer sequences are shown in Table 2. The relative gene expression data were analyzed by the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001), and *Actb* expression was used to normalize mRNA levels.

Statistical analysis

Results are presented as the mean $\pm$ standard error of the mean (S.E.M.) of three or more independent assays. Statistical analyses were performed using Mann–Whitney *U* and Kruskal–Wallis tests and a $P < 0.05$ was considered statistically significant.

Results

AMPK inhibition activates the mTOR signaling pathway, increases *Ctgf* expression in the YAP signaling pathway and promotes ovarian growth in *in vitro*-cultured ovaries

To investigate the effects of AMPK inhibition on the mTOR and YAP signaling pathways in ovarian tissue, ovaries from 10-day-old mice were incubated for 1 h with 10 μ M of Compound C prior to immunoblot analysis or 0-, 1-, 4-, and 8-h treatment prior to mRNA quantitation. Ovarian phosphorylation of mTOR and downstream proteins (S6 and eIF4B) rose transiently, whereas phosphorylation-TSC2 levels decreased after Compound C treatment (Fig. 1A). Additionally, mRNA levels of *Hif1a* and *Vegfa* associated with the mTOR downstream signaling pathway and *Ctgf* in the YAP signaling pathway were elevated after Compound C treatment for 1–4 h (Fig. 1B).

Treatment with Compound C using an explant culture model led to both dose- and time-dependent increases in ovarian weights. As shown in Fig. 2A, ovarian weight increased as a gradient when the concentration of Compound C increased from 3 μ M to 30 μ M, but decreased significantly at concentrations of 100 μ M. Similarly, ovarian weight increased when the incubation time increased from 1 h to 8 h, reaching a plateau at drug exposure times > 8 h (Fig. 2B). Therefore, higher doses of or longer exposure times to Compound C is not conducive to ovarian activation. Histological analyses (Fig. 2C and D) showed that the percentage of primordial follicles decreased in ovaries treated with 10 μ M Compound C for 8 h, whereas the proportion of primary and secondary follicles increased, indicating the activation and growth of primordial follicles.

AMPK inhibition promotes ovarian growth and strengthens microvascular development in grafted ovaries

To determine whether *in vitro*-treated ovaries would continue growing after grafting into adult hosts, paired ovaries cultured with DMSO (vehicle control) or Compound C were transplanted under the kidney capsule of 9-week-old hosts. Graft weight significantly increased when pretreated with 10 μ M Compound C for 1 h or 8 h, but no differences were observed between controls and groups treated with 30 μ M Compound C for 1 h (Fig. 3A). Additionally, the percentage of primordial follicles decreased in ovaries pretreated with 10 μ M Compound C, whereas primary, antral and preovulatory follicles increased (Fig. 3B and C). These results indicated that the follicles of ovaries pretreated with Compound C continued to develop after *in vivo* grafting.

For further verification that AMPK inhibition promoted ovarian microvascular development, paired grafted ovaries pretreated with vehicle or Compound

Table 2 Target transcripts and primer sequences used for the quantification of mRNA levels in the ovaries.

Transcripts	Forward (5'–3')	Reverse (5'–3')
<i>Actb</i>	GATGTGGATCAGCAAGCAGGA	AAACGCAGCTCAGTAACAGTC
<i>Hif1a</i>	TGCTGGCTCCCTATATCCCA	GTGCTCATACTTGGAGGGCT
<i>Vegfa</i>	GGAGTCTGTGCTCTGGGATT	TGGCAGGATTAAGAGGGGA
<i>Vegfr1</i>	CGTGCAAGGAACCTCAGACA	CGGAAGAAGACCGCTTCAGT
<i>Vegfr2</i>	GATGCATTGTGCTGGCTCTG	CTGTGAAGAGCACGCAAACC
<i>Ctgf</i>	GTCAAGCTGCCTGGGAAATG	TGGGCCAAATGTGTCTTCCA

Figure 1 AMPK inhibition increases the expression of targets involved in the mTOR and YAP signaling pathways in *in vitro*-cultured mouse ovaries. (A) Ovaries of 10-day-old CD-1 mice were treated with DMSO (control) or Compound C (10 μ M) for 1 h. Protein levels (TSC2, mTOR, S6 and eIF4B) were analyzed by Western blot. Western blot quantification was undertaken using ImageJ software ($n=3$; mean $\pm$ S.E.M.). * $P<0.05$. (B) The relative mRNA expression (*Hif1a*, *Vegfa* and *Ctgf*) in *in vitro*-cultured 10-day-old mouse ovaries treated with DMSO (control) or Compound C (10 μ M) for different times (0–8 h). Relative gene expression in fresh ovaries collected from 10-day-old mice (no treatment) was used as the baseline in the graph (1.0), and *Actb* expression was used to normalize mRNA levels ($n=3$; mean $\pm$ S.E.M.; $P<0.001$).

C were collected for CLARITY processing prior to immunohistochemistry staining and confocal scanning. We observed a larger number of preovulatory, but fewer primordial and primary follicles in the Compound C-pretreated group (Fig. 4A). Additionally, CLARITY processing revealed larger distributions of bright signals in endothelial cells in the surrounded area of each follicle, implying that AMPK inhibition intensified vascular development in grafted ovaries (Fig. 4A). The percentage of the vascular area also increased in the Compound C-pretreated group as compared with that observed in the control group (Fig. 4B). Furthermore, the expression of *Hif1a*, *Vegfa*, *Vegfr2* and *Ctgf* mRNA in Compound C-pretreated ovaries was higher than that measured in

the vehicle group; however, we observed no difference in *Vegfr1* mRNA levels (Fig. 4C). These results suggested that Compound C treatment stimulated *in vivo* follicle and blood vessel growth in grafted ovaries.

AMPK inhibition increases ovulated oocytes after *in situ* injection

To further investigate the *in vivo* effect of Compound C treatment, *in situ* ovarian intrabursal administration was performed. Although we observed no significant changes in ovarian weights after ovulation (Fig. 5A), the number of ovulated oocytes increased in Compound C-treated ovaries (Fig. 5B). Moreover, the expression of *Htf1a*, *Vegfa*, *Vegfr2* and *Ctgf* mRNA in Compound C *in situ*-treated ovaries was higher than that observed in the vehicle group; however, we observed no difference in *Vegfr1* mRNA levels (Fig. 5C). In addition, the Compound C-pretreated oocytes could develop into blastocysts (Fig. 5D), had comparable rate of early embryonic development as compared with controls (Fig. 5E) and healthy pups were delivered (Fig. 5F).

Discussion

This study showed that AMPK inhibition followed by Compound C treatment-stimulated follicle development and promoted ovarian angiogenesis but only in a narrow range of drug dose. The effect of Compound C on ovarian growth is very sensitive to dose concentration and exposure time according to our results. A study showed that Compound C may be a potent AMPK-independent anti-glioma agent (Liu *et al.* 2014), whereas another paper pointed out that it may be an attractive candidate for anticancer drug development because it induces apoptosis in cancer cells (Dai *et al.* 2013). Although the mechanisms may be different between normal ovarian cells and cancer cells, those who tried to use it for clinical applications should be very careful.

Although its effect of AMPK inhibition has been confirmed in early studies (Zhou *et al.* 2001, Kim *et al.* 2004, McCullough *et al.* 2005), Compound C was also found to be an inhibitor of bone morphogenetic protein type 1 (BMP1) receptors ACTR-I (ALK2), BMPR-IA (ALK3) and BMPR-IB (ALK 6), which would affect embryogenesis and iron metabolism (Yu *et al.* 2007). BMP1 is highly conserved among different species (Maéno *et al.* 1993, Fukagawa *et al.* 1994, Bond & Beynon 1995, Takahara *et al.* 1995, Lin *et al.* 1997, Reynolds *et al.* 2000). One study found out that BMP1 levels were stable in sheep granulosa cells, whereas its activity in follicular fluid decreased with follicular growth (Canty-Laird *et al.* 2010). Another study showed that BMP1 recombinant protein increased the expression of the key cell cycle regulators and decreased cell apoptosis pathway genes in *in vitro*-cultured buffalo

Figure 2 AMPK inhibition improves ovarian growth *in vitro*. (A) Ovaries from 10-day-old mice were treated with different doses of Compound C (0–100 μM) and incubated for 4 days, with medium changes after 2 days. Ovarian-explant weights were determined at the end of the culture ($n=5$; mean $\pm$ S.E.M.). **** $P < 0.0001$. (B) Ovaries from 10-day-old mice were treated with 10 μM Compound C for different times (0–96 h), washed twice and then incubated for 4 days with medium changes after 2 days. Ovarian-explant weights were determined at the end of culture ($n=5$; mean $\pm$ S.E.M.). ** $P < 0.01$. (C) Representative histologic sections of explants. Ovarian explants in the presence or absence of treatment with 10 μM Compound C for 8 h and incubation for 4 days. (D) Distribution of follicles at different developmental stages in ovaries in the presence or absence of treatment with 10 μM Compound C for 8 h ($n=4$; mean $\pm$ S.E.M.). *** $P < 0.001$; **** $P < 0.0001$.

granulosa cells (Lei *et al.* 2016). Anyway, there is no study about if Compound C will affect folliculogenesis through BMPs signaling till now. The preliminary data in our lab showed that the expression of *Alk3* was very low in mouse ovaries (Cq value ≥ 35), whereas *Alk2* and *Alk6* mRNA levels increased short term after Compound C treatment but had no significant change in graft ovaries and *in situ* treatment ovaries (Supplementary Fig. 2). Therefore, it may be another story with some unclear mechanisms, which we will try to figure out in the future.

The results of this study showed that AMPK inhibition activated the mTOR signaling pathway and increased YAP-dependent *Ctgf* expression in mouse ovaries. Additionally, our *in vitro* results indicated that AMPK inhibition decreased phosphorylation of TSC2 at Ser1387 and increased the expression of mTOR and downstream proteins. These findings were similar to results reported after selective disruption of LKB1 in mouse oocytes (Jiang *et al.* 2016) and conditional knockout of *Tsc1* in granulosa cells (Huang *et al.* 2013). Furthermore, we observed that mTOR activation stimulated dormant ovarian follicle growth through increased mTOR phosphorylation at Ser2448 similar to results reported after treatment with an mTOR activator (Cheng *et al.* 2015). We also observed an increased

expression of downstream eIF4B and S6 proteins after treatment with the AMPK inhibitor, similar to the results observed following treatment with an mTOR activator (Cheng *et al.* 2015). Activated mTOR signaling observed in primordial follicle granulosa cells also stimulates the secretion of stem-cell-factor ligand, activates the phosphoinositide-3-kinase (PI3K)-signaling pathway and stimulates dormant oocyte growth (Hsueh 2014, Zhang & Liu 2015). Our results showed that AMPK inhibition resulted in increased primary and antral follicle growth and ovarian weight in *in vitro*-cultured and *in vivo*-grafted 10-day-old mouse ovaries, as well as elevated numbers of ovulated oocytes in 3-week-old mice. Therefore, treatment with an AMPK inhibitor promoted ovarian follicle development via an AMPK-TSC2-mTOR-eIF4B/S6 signaling pathway.

In addition, it had been previously demonstrated that FSH inhibited AMPK phosphorylation and promoted granulosa cell (Kayampilly & Menon 2009, 2012) or Sertoli cell (Riera *et al.* 2012) proliferation, which means FSH may also promote ovarian growth via AMPK inhibition in a way similar to Compound C, and this may help explain our results in *in vivo* allograft and *in situ* intrabursal injection models.

Hif1a is a downstream protein involved in the mTOR signaling pathway (Shackelford & Shaw 2009, Perl 2015)

Figure 3 AMPK inhibition contributes to ovarian development *in vivo*. (A) Paired ovaries were treated with DMSO (control) or Compound C at different drug exposure concentrations and times (X-axis) prior to grafting into adult hosts treated daily with FSH for 4 days. After graft removal, graft weights were determined. Numbers

that plays a critical role along with its target gene (*Vegfa*) in folliculogenesis and corpus luteum formation in mammalian ovaries (Yamamoto *et al.* 1997, Nishimura & Okuda 2010, Rico *et al.* 2014, Wu *et al.* 2015, Yalu *et al.* 2015). Additionally, *Hif1a* activity is required for gonadotropin-regulated *Vegfa* expression and follicle growth (Rico *et al.* 2014). *Vegfa* expression constitutes a marker indicating ovarian function status where strong *Vegfa* expression in the presence of drug treatment or transcription factors suggests their effectiveness at stimulating follicle development (Joo *et al.* 2010, Park *et al.* 2011, 2013, Scotti *et al.* 2011, Choi *et al.* 2012). Here, treatment with an AMPK inhibitor increased mRNA levels of *Hif1a*, *Vegfa* and *Vegfr2*, enhanced CD31-signal intensity in the surrounded area of follicles and increased the vascular area percentage in treated ovaries. These results indicated that AMPK inhibition promoted blood vessel and follicle growth in mammalian ovaries.

We used a recently developed CLARITY method (Chung *et al.* 2013, Tomer *et al.* 2014) to evaluate the ovarian vascular function rather than using traditional sliced tissue, followed by immunohistochemistry staining. The CLARITY approach causes intact tissues to become transparent, thereby enabling immunostaining of markers to elucidate detailed structures from whole organs (Chung *et al.* 2013). Our results revealed that the control groups consisted primarily of primordial, primary and early secondary follicles, whereas late secondary and antral follicles were observed in the treated groups. This result agreed with those derived from H&E staining. Additionally, we observed the formation of a bright, network-like structure based on CD31 signal intensity in the treated ovaries, indicating active ovarian angiogenesis and promotion of follicular development in Compound C-treated ovarian grafts. This finding also confirmed qRT-PCR results showing increases in *Vegfa* and *Vegfr2* mRNA levels in the treated group.

We also observed that *Vegfr2* mRNA levels increased in treated ovaries in both the grafted and *in situ* injection groups, whereas no significant change in *Vegfr1* mRNA levels was observed in the control groups. This may be a consequence of their different functions. *Vegfr1* is primarily expressed in the blood cells and regulates the recruitment of hematopoietic stem cells and migration of monocytes and macrophages (Shibuya 2006). However, *Vegfr2* is the principal mediator of *Vegfa* in endothelial cells, the binding of which activates intracellular signaling cascades that result in cell proliferation, migration, survival and increased permeability

in parentheses indicate the number of grafts used (mean $\pm$ S.E.M.).

* $P < 0.05$. (B) Representative histologic sections of grafts. Paired ovaries were treated with DMSO (control) or 10 μ M Compound C for 1 h before grafting into adult hosts treated daily with FSH for 4 days. (C) Follicle dynamics of paired grafts exposed to DMSO or 10 μ M Compound C for 1 h ($n = 4$; mean $\pm$ S.E.M.). ** $P < 0.01$; *** $P < 0.001$.

Figure 4 AMPK inhibition increases mRNA expression and strengthens vascular development *in vivo*. (A) Grafts with immunohistochemistry staining for TH and CD31 antibodies after CLARITY processing (left panels) and staining with CD31 antibody in endothelial cells only (right panels). Paired ovaries from 10-day-old mice were cultured with vehicle or 10 μ M Compound C for 1 h before grafting into adult hosts treated daily with FSH for 4 days. The hollow arrow indicates the primordial and primary follicles, whereas the solid arrow indicates preovulatory follicles. (B) Vascular area percentage of grafted ovaries was calculated using ImageJ software ($n = 10$; mean $\pm$ S.E.M.). **** $P < 0.0001$. (C) Different mRNA levels (*Hif1a*, *Vegfa*, *Vegfr1*, *Vegfr2* and *Ctgf*) measured in 10-day-old grafted ovaries. Paired ovaries were collected after treatment with 10 μ M Compound C or vehicle. The relative mRNA expression from the control group was used as the baseline in the graph (1.0), and *Actb* expression was used to normalize mRNA levels ($n = 3$; mean $\pm$ S.E.M.; $P < 0.05$).

(Xu 2005, Holmes *et al.* 2007). Therefore, increased *Vegfr2* mRNA levels in treated ovaries may be a consequence of increased *Vegfa* expression in ovarian cells. This finding offered indirect proof that AMPK inhibition stimulated ovarian angiogenesis and follicular development.

CTGF is a matricellular protein that plays important roles in cell adhesion, migration, proliferation, angiogenesis and other processes (Hall-Glenn & Lyons 2011, Jun & Lau 2011, Kubota & Takigawa 2011). It has been confirmed that the increase of CTGF in Hippo signaling indicates cell growth, survival and proliferation in ovaries in turn (Kawamura *et al.* 2013), and our preliminary data have exhibited that YAP expression increased in Compound C-treated nuclei of oocytes (Supplementary Fig. 3), which means that YAP proteins had gone into nuclei and will interact with gene promoters to increase the expression of downstream CCN growth factors, including CTGF (Pan 2007). Thus, we chose this most representative gene, CTGF, to test in our experiments. Previous studies have

demonstrated that AMPK activation decreased YAP-dependent *Ctgf* expression (DeRan *et al.* 2014, Mo *et al.* 2015). However, our results showed that *Ctgf* expression increased in ovaries treated with the AMPK inhibitor Compound C in *in vitro* culture, *in vivo* grafting and *in situ* intrabursal injection experiments. These findings demonstrated that AMPK inhibition increased *Ctgf* expression in mammalian ovaries and offered insight into another mechanism associated with follicle growth. In consequence, our model indicates the mechanism of AMPK inhibition in promoting follicle proliferation as shown in Fig. 6.

Previous methods for primordial follicle activation included *in vitro* culture involving combined treatment with a phosphatase and tensin homolog inhibitor and a PI3K-activating peptide (Jagarlamudi *et al.* 2009, Li *et al.* 2010), treatment with an mTOR activator (Cheng *et al.* 2015) or dissection of the ovary into small pieces to disrupt Hippo signaling (Kawamura *et al.* 2013). Our results demonstrated that AMPK inhibition could also promote follicle development and angiogenesis

Figure 5 AMPK inhibition increases ovulated oocytes and mRNA expression in 3-week-old mice after *in situ* injection of Compound C followed by gonadotropin stimulation and leads to healthy pups. (A) Fourteen 3-week-old mice were randomly separated into two groups for ovarian intrabursal injection of 10 μ L saline on the left side and vehicle (control) or 50 ng/10 μ L Compound C (treatment) on the right side, followed by immediate injection with 5 IU eCG and then 5 IU hCG after 48 h. Ovarian weight was determined after 16 h ($n=7$; mean $\pm$ S.E.M.). (B) The number of ovulated oocytes from *in situ* injection-treated ovaries ($n=7$; mean $\pm$ S.E.M.). $*P<0.05$. (C) mRNA expression profiles (*Hif1a*, *Vegfa*, *Vegfr1*, *Vegfr2* and *Ctgf*) in ovaries treated with DMSO (control) or 50 ng/10 μ L Compound C. mRNA expression from the vehicle group was used as the baseline in the graph (1.0), and *Actb* expression was used to normalize mRNA levels ($n=3$; mean $\pm$ S.E.M.; $P<0.001$). (D) Early embryonic development of oocytes after Compound C treatment. (E) Percentage of oocytes developed into each embryonic stage ($n=3$; mean $\pm$ S.E.M.). (F) Healthy pups were delivered followed by some Compound C-pretreated 2-cell stage embryos transferred into pseudopregnant hosts.

Figure 6 A model for follicle growth regulated by the mTOR and YAP signaling pathways and following treatment with an AMPK inhibitor. AMPK inhibition inactivates TSC2, but promotes phosphorylation and activation of mTOR downstream proteins S6 and eIF4B, increased *Hif1a* and *Vegfa* mRNA levels, and follicle growth. In contrast, as a secondary pathway, AMPK inhibition also results in YAP localization to the nucleus where it promotes *Ctgf* expression, which subsequently stimulates follicle growth.

in mammalian ovaries via *in vitro*, *in vivo* or *in situ* treatment through the AMPK–mTOR (mTOR activation) or AMPK–CTGF pathway. Moreover, the oocytes gained from ovaries *in vitro* pretreated with the mTOR activator (Cheng *et al.* 2015) or *in situ* pretreated with AMPK inhibitor in our experiments were subsequently fertilized and resulted in healthy mouse offspring. These findings implied that AMPK inhibitors might potentially constitute a therapeutic method to promote ovary activation. Furthermore, current ovarian activation protocols all require removal of the ovaries for *in vitro* treatment procedures due to drug toxicity or the need for ovarian dissection. This requires patients with ovarian insufficiency to undergo an invasive procedure to gain usable oocytes. However, our results showed that *in situ* injection of Compound C resulted in ovarian activation in live mice, leading to healthy pups. This could be a clue that we may activate human oocytes *in situ* without invasive procedures someday in the future.

This study has some limitations. First, though we gained healthy pups from *in situ* pretreated ovaries, we did not perform additional experiments on oocytes obtained from grafted ovaries; therefore, we cannot completely confirm the function of oocytes treated with Compound C. Additionally, the AMPK inhibitor was not tested on human ovarian tissue, precluding our recommending direct application of these results to human clinical practice.

In conclusion, we showed that treatment with AMPK inhibitor resulted in the activation of the mTOR signaling pathway, increases in *Ctgf* expression in mouse ovaries,

stimulation of follicle development, promotion of ovarian angiogenesis for ovary growth, leading to the delivery of healthy pups in *in situ* treatment model. This study provided some very early evidence supporting the use of AMPK inhibitors as potential therapeutic agents for patients exhibiting ovarian insufficiency to possibly improve fertility. Further studies are needed in Compound C-pretreated grafted mouse ovaries and human subjects to verify the health and efficacy of ovulated oocytes, as well as their ability to be fertilized, leading to viable offspring.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-16-0577>.

Declaration of interest

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

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