Molecular Human Reproduction, Vol.15, No.12 pp. 765–770, 2009
Advanced Access publication on October 20, 2009 doi:10.1093/molehr/gap092

MHR Basic science of reproductive medicine

ORIGINAL RESEARCH

Disruption of *Tsc2* in oocytes leads to overactivation of the entire pool of primordial follicles

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ABSTRACT: To maintain the length of reproductive life in a woman, it is essential that most of her ovarian primordial follicles are maintained in a quiescent state to provide a continuous supply of oocytes. However, our understanding of the molecular mechanisms that control the quiescence and activation of primordial follicles is still in its infancy. In this study, we provide some genetic evidence to show that the tumor suppressor tuberous sclerosis complex 2 (Tsc2), which negatively regulates mammalian target of rapamycin complex 1 (mTORC1), functions in oocytes to maintain the dormancy of primordial follicles. In mutant mice lacking the *Tsc2* gene in oocytes, the pool of primordial follicles is activated prematurely due to elevated mTORC1 activity in oocytes. This results in depletion of follicles in early adulthood, causing premature ovarian failure (POF). Our results suggest that the Tsc1–Tsc2 complex mediated suppression of mTORC1 activity is indispensable for maintenance of the dormancy of primordial follicles, thus preserving the follicular pool, and that mTORC1 activity in oocytes promotes follicular activation. Our results also indicate that deregulation of Tsc/mTOR signaling in oocytes may cause pathological conditions of the ovary such as infertility and POF.

Key words: follicular activation / oocytes / Tsc/mTOR signaling / premature ovarian failure

Introduction

To maintain the lengthy female reproductive life, the majority of primordial follicles must be preserved in a quiescent state for later use (Hirshfield, 1991; McGee and Hsueh, 2000). In order to produce mature ova, primordial follicles are recruited from the reservoir of dormant follicles into the growing follicular pool, through a process termed follicular activation (McGee and Hsueh, 2000). The activation of primordial follicles is a highly regulated process, and its underlying mechanisms are not fully revealed (Liu *et al.*, 2006, 2007; Adhikari and Liu, 2009; Reddy *et al.*, 2009). Menopause, or ovarian senescence occurs when the pool of primordial follicles has become exhausted (Faddy *et al.*, 1992; Broekmans *et al.*, 2007; Hansen *et al.*, 2008).

Our earlier studies revealed that PTEN (phosphatase and tensin homolog deleted on chromosome 10), a negative regulator of phosphatidylinositol 3 kinase (PI3K), functions in oocytes as a suppressor of follicular activation (Reddy *et al.*, 2008). Inhibition with rapamycin, the specific inhibitor of the mammalian serine/threonine kinase mammalian target of rapamycin (mTOR), indicated a role for mTOR as well as P13K in follicle activation (Reddy *et al.*, 2008). Signaling events through mTOR, which regulate cell growth and proliferation in many types of cells, are closely related to the PI3K signaling pathway (Wullschleger *et al.*, 2006; Guertin and Sabatini, 2007). mTOR modulates important processes such as protein synthesis, ribosome biogenesis and autophagy (Sarbassov *et al.*, 2005; Wullschleger *et al.*, 2006; Guertin and Sabatini, 2007). In human cells, mTOR complex 1 (mTORC1) activity is negatively regulated by a heterodimeric complex consisting of two protein molecules: tuberous sclerosis complex 1 (TSC1, or hamartin) and tuberous sclerosis complex 2 (TSC2, or tuberin). TSC1 and TSC2 are products of two distinct tumor suppressor genes: *TSC1* and *TSC2*, respectively. These two genes are the genetic loci that are mutated in the autosomal dominant tumor syndrome tuberous sclerosis complex (TSC), which is characterized by numerous benign tumors (Kwiatkowski *et al.*, 2002; Crino *et al.*, 2006; Yang and Guan, 2007).

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The TSC1–TSC2 complex suppresses the activation of mTORC1 through a GTPase activating protein domain located in TSC2. The function of TSC1 is to stabilize TSC2 and protect it from ubiquitination and degradation (Chong-Kopera *et al.*, 2006).

To determine whether the Tsc/mTORC1 signaling in oocytes takes part in the regulation of follicular activation, in this study, we deleted the *Tsc2* gene from mouse oocytes in primordial and developing follicles. Deletion of *Tsc2* caused premature activation of all primordial follicles around the time of puberty, due to elevated mTORC1 activity in oocytes. This eventually led to follicular depletion in early adulthood, causing premature ovarian failure (POF). Thus, the Tsc1–Tsc2 complex mediated suppression of mTORC1 activity in oocytes is indispensable for sustaining the dormancy of primordial follicles, which is essential for preserving the female reproductive lifespan.

Materials and Methods

Mice

Tsc2$^{loxP/loxP}$ mice (Hernandez *et al.*, 2007) with a mixed 129S4/SvJae and C57BL/6J genomic background were crossed with transgenic mice carrying *growth differentiation factor 9* (*Gdf-9*) promoter-mediated Cre recombinase that had a C57BL/6J background (Lan *et al.*, 2004; Reddy *et al.*, 2008). After multiple rounds of crossing, we obtained homozygous mutant female mice lacking *Tsc2* in oocytes (referred to as Oo*Tsc2*$^{-/-}$ mice). Control mice that do not carry the Cre transgene are referred to as Oo*Tsc2*$^{+/+}$ mice. All comparisons were made between littermates. The mice were housed under controlled environmental conditions with free access to water and food. Illumination was on between 0600 and 1800 h. Experimental protocols were approved by the regional ethical committee of Umeå University, Sweden.

Reagents, antibodies and immunological detection methods

Rabbit polyclonal antibodies to phospho-rpS6 (S240/4), Tsc2/tuberin and phospho-S6K1 (T389), and also rabbit monoclonal antibody to S6K1 were obtained from Cell Signaling Technologies (Beverly, MA, USA). Mouse monoclonal antibody to rpS6 was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Mouse monoclonal antibody to β-actin was purchased from Sigma-Aldrich Sweden AB (Stockholm, Sweden). Western blots were carried out according to the instructions of the suppliers of the different antibodies and visualized using the ECL Plus Western Blotting Detection System (Amersham Biosciences, Uppsala, Sweden).

Histological analysis of ovarian tissues

Histological analysis of ovary was performed as previously described (Reddy *et al.*, 2008). Briefly, ovaries were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. Paraffin-embedded ovaries were serially sectioned at 8-μm thickness and stained with hematoxylin for morphological observation, and all sections from each ovarian block were examined. One or both ovaries from more than three mice of each genotype were used for each time point.

Isolation of oocytes from post-natal mouse ovaries

Mice were sacrificed by decapitation, and the ovaries were dissected free of fat and connective tissue using a dissection microscope. The ovaries were then minced with a pair of dissection scissors before being incubated in 0.05% collagenase dissolved in Dulbecco's modified Eagle's medium-F12 (DMEM/F12; Invitrogen) supplemented with 4 mg/ml bovine serum albumin (BSA), 100 units/ml penicillin and 100 μg/ml streptomycin, with frequent agitation and pipetting. After the tissues had mostly been digested by collagenase, usually within 45–60 min, EDTA was added to this mixture to a final concentration of 40 mM, and the mixture was incubated at 37°C with frequent pipetting for another 15–20 min until clusters of granulosa cells or other cells were completely dispersed. The mixture of cells and oocytes was then washed once and cultured in a 6 or 10-cm tissue culture dish with the above-mentioned serum-free DMEM/F12 medium for 12 h, to allow the granulosa cells and other ovarian cells to attach to the plastic. The unattached oocytes and red blood cells were then recovered by collection of the supernatant and centrifugation at 160 g for 5 min at room temperature. Red blood cells were subsequently removed using a hypotonic buffer containing 144 mM NH_4Cl and 17 mM Tris–HCl (pH 7.2). After several washes, oocytes were collected by centrifugation. They were then lysed in a buffer containing 50 mM Tris–HCl (pH 8.0), 120 mM NaCl, 20 mM NaF, 20 mM β-glycerophosphate, 1 mM EDTA, 6 mM EGTA (pH 8.0), 1% NP-40, 1 mM DTT, 5 mM benzamidine, 1 mM PMSF, 250 μM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 μg/ml pepstatin, followed by centrifugation at 14 576 g for 20 min at 4°C. The supernatants were collected and protein concentrations were measured using the bicinchoninic acid (BCA) protein assay, and equal amounts of proteins were used for western blot.

Results

Generation of mutant mice with oocyte-specific deletion of *Tsc2*

We generated mutant mice in which the *Tsc2* gene was deleted in oocytes of primordial and developing follicles (Oo*Tsc2*$^{-/-}$ mice) by crossing *Tsc2*$^{loxP/loxP}$ mice (Hernandez *et al.*, 2007) with transgenic mice expressing *Gdf-9* promoter-mediated Cre recombinase (Lan *et al.*, 2004). Western blot result showed that the expression of the Tsc2 protein (tuberin) was completely absent in Oo*Tsc2*$^{-/-}$ oocytes (Fig. 1), indicating a successful deletion of the *Tsc2* gene from oocytes.

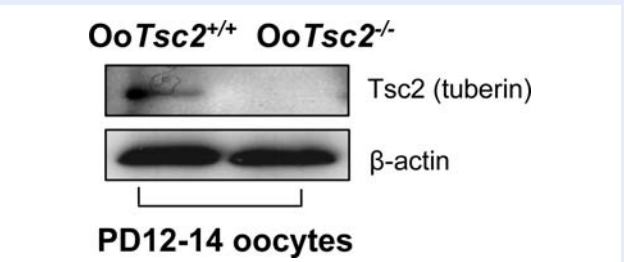

Figure 1 Oocyte-specific deletion of *Tsc2* in mice. Western blots showing the absence of Tsc2 (tuberin) protein expression in oocytes of Oo*Tsc2*$^{-/-}$ mice. Oocytes were isolated from ovaries of 12–14-day-old Oo*Tsc2*$^{+/+}$ and Oo*Tsc2*$^{-/-}$ mice as described in Materials and Methods. For each experiment, material from three to five mice was used per lane. For each lane, around 20 μg of protein was loaded. Level of β-actin was used as an internal control. The experiments were repeated three times and representative images are shown.

Shortened reproductive lifespan of Oo*Tsc2*$^{-/-}$ female mice

We found that the Oo*Tsc2*$^{-/-}$ females had a normal vaginal opening at the age of 5–6 weeks (which is the appropriate age). However, during the examined period from 7 to 27 weeks of age, Oo*Tsc2*$^{-/-}$ females were found to produce at most 2 litters of normal size, then they became infertile in young adulthood (i.e. after 12–13 weeks of age) (Fig. 2).

Enhanced mTORC1 activity in Oo*Tsc2*$^{-/-}$ oocytes

We found that loss of *Tsc2* led to enhanced mTORC1 activity in Oo*Tsc2*$^{-/-}$ oocytes, as indicated by the elevated phosphorylation of mTORC1 substrate S6K1 (p70 S6 kinase 1) (Fig. 3, p-S6K1, T389). Such elevated p-S6K1 had apparently led to an increase in activity, as the phosphorylation of the substrate of S6K1, rpS6 (ribosomal protein S6), was dramatically elevated in Oo*Tsc2*$^{-/-}$ oocytes (Fig. 3, p-rpS6, S240/4). These data showed that the mTORC1–S6K1–rpS6 signaling were elevated in Oo*Tsc2*$^{-/-}$ oocytes.

Accelerated activation of the entire pool of primordial follicles followed by premature follicular depletion in Oo*Tsc2*$^{-/-}$ mice

To learn how the elevated mTORC1–S6K1–rpS6 signaling in oocytes may lead to shortened reproductive lifespan in Oo*Tsc2*$^{-/-}$ mice, we compared the post-natal follicular development in Oo*Tsc2*$^{-/-}$ mice to that in Oo*Tsc2*$^{+/+}$ mice. We found that the pool of primordial follicles was formed normally in Oo*Tsc2*$^{-/-}$ mice, and no apparent morphological difference was observed in post-natal day (PD) 13 ovaries of Oo*Tsc2*$^{-/-}$ (Fig. 4B) and Oo*Tsc2*$^{+/+}$ (Fig. 4A) mice, where ovaries of both mutant and control mice had clusters of primordial follicles (Fig. 4A and B, inset, arrows). At PD23, the Oo*Tsc2*$^{-/-}$ ovaries (Fig. 4D) appeared somewhat larger than the Oo*Tsc2*$^{+/+}$ ovaries (Fig. 4C). By this age, all primordial follicles had been activated with enlarged oocytes in Oo*Tsc2*$^{-/-}$ ovaries (Fig. 4D, inset, red arrows). However, in Oo*Tsc2*$^{+/+}$ ovaries, cluster of primordial follicles were seen (Fig. 4C, inset, arrows). At PD35, Oo*Tsc2*$^{-/-}$ ovaries appeared larger, and contained many activated follicles with enlarged oocytes (Fig. 4F, inset, red arrows), whereas the control Oo*Tsc2*$^{+/+}$ ovaries were much smaller (Fig. 4E) and contained clusters of primordial follicles (Fig. 4E, inset, arrows). All types of activated follicles, including transient follicles (containing enlarged oocyte surrounded by flattened pregranulosa cells), type 3b, type 4, type 5 and type 6 follicles were seen in Oo*Tsc2*$^{-/-}$ ovaries. Thus, with *Tsc2* deleted in oocytes, the entire pool of primordial follicles was activated by PD23.

By 4 months of age, no healthy follicular structure could be identified in Oo*Tsc2*$^{-/-}$ ovaries (Fig. 4H), and only unhealthy dying oocytes were observed (Fig. 4H, inset, arrows). In contrast, the control Oo*Tsc2*$^{+/+}$ mice contained healthy follicles and corpus luteum (CL) (Fig. 4G). Thus, premature activation of the primordial follicle pool led to follicle depletion and POF in Oo*Tsc2*$^{-/-}$ mice in early adulthood.

Discussion

In this study, by using a mouse model with oocyte-specific deletion of the *Tsc2* gene, we showed that the tumor suppressor Tsc2 plays an essential physiological role in oocytes to preserve the female reproductive lifespan by suppressing activation of primordial follicles. We demonstrated that deletion of *Tsc2* in oocytes leads to premature activation of the entire pool of primordial follicles. We also showed that the driving force underlying the overactivation of primordial follicles in Oo*Tsc2*$^{-/-}$ mice is the elevated *intra-oocyte* mTORC1–S6K1–rpS6 signaling. We observed all types of activated follicles, including transient, types 3b, 4, 5 and 6 follicles in Oo*Tsc2*$^{-/-}$ ovaries. However, even though more primordial follicles were prematurely recruited

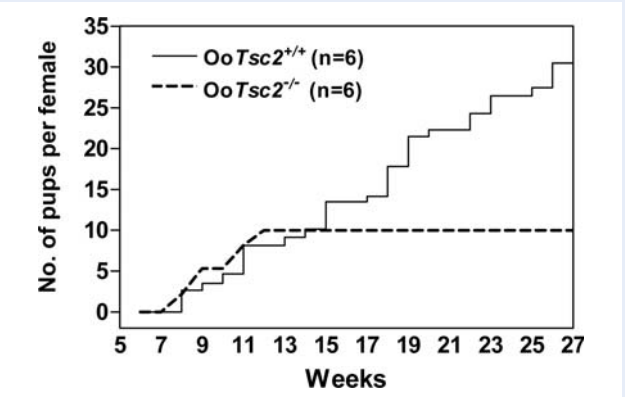

Figure 2 Subfertility in mice lacking *Tsc2* in oocytes. Comparison of the cumulative number of pups per Oo*Tsc2*$^{-/-}$ female ($n = 6$, dashed line) and per Oo*Tsc2*$^{+/+}$ female ($n = 6$, solid line). All Oo*Tsc2*$^{-/-}$ females became infertile in young adulthood after Week 12–13.

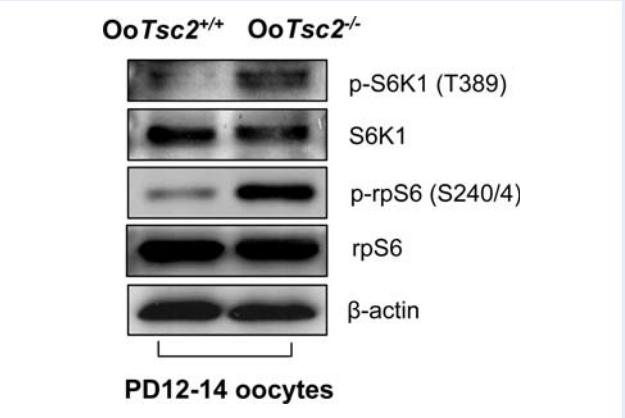

Figure 3 Studies of mTORC1 signaling in Oo*Tsc2*$^{-/-}$ and Oo*Tsc2*$^{+/+}$ oocytes. Comparison of Tsc2/mTORC1 signaling in Oo*Tsc2*$^{-/-}$ and Oo*Tsc2*$^{+/+}$ oocytes. Oocytes were isolated from ovaries of mice at PD12–14 and western blot was performed as described in Materials and Methods. mTORC1 signaling in Oo*Tsc2*$^{-/-}$ oocytes was enhanced, as indicated by elevated levels of phosphorylated S6K1 (p-S6K1, T389) and phosphorylated rpS6 (p-rpS6, S240/4). Levels of total S6K1, rpS6 and β-actin were used as internal controls. The experiments were repeated three times. For each experiment, material from three to five mice was used per lane. Representative images are shown.

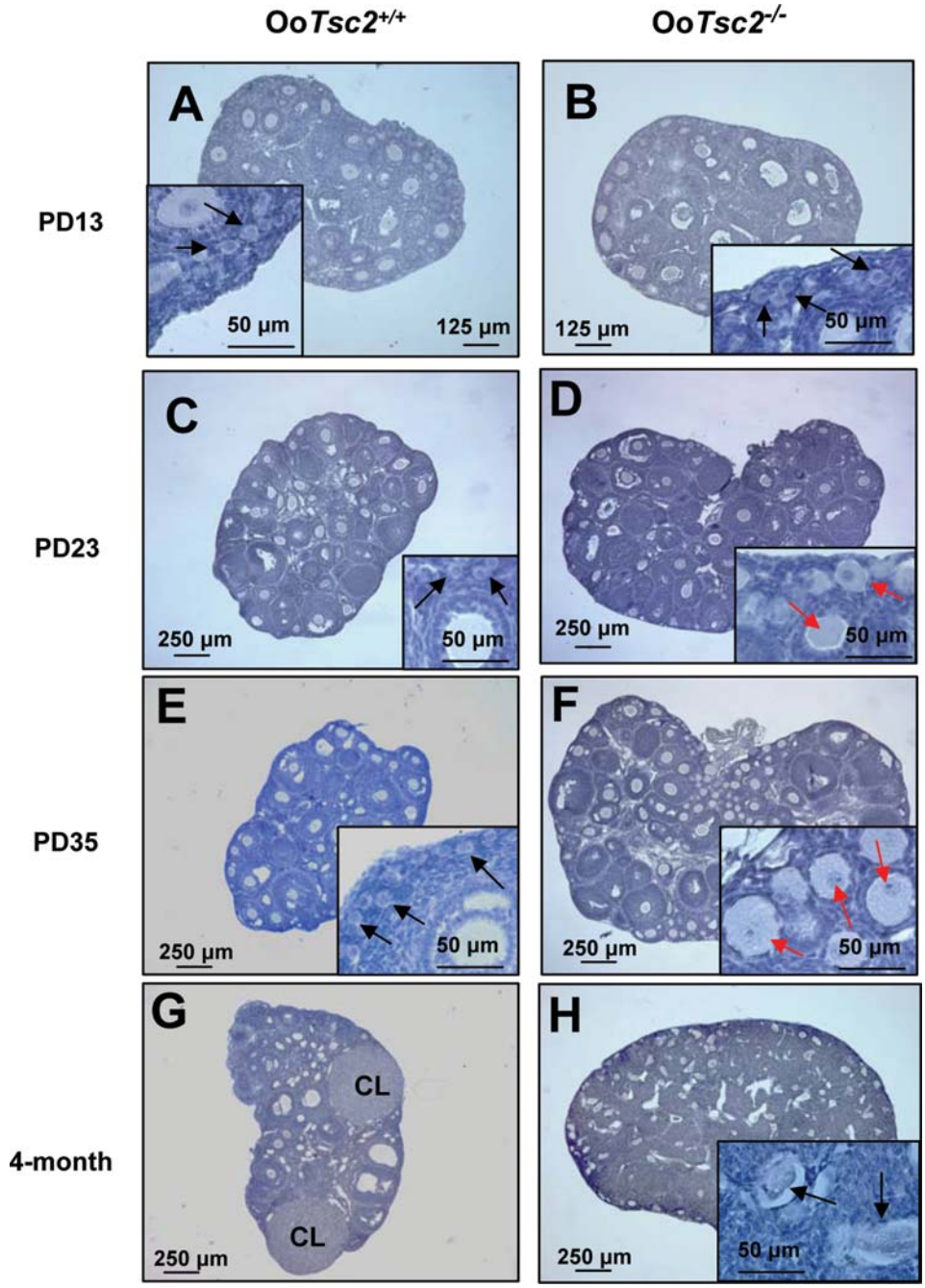

Figure 4 Activation of the entire pool of primordial follicles leading to follicle depletion and POF at young adulthood in Oo*Tsc2*$^{-/-}$ mice. (**A**–**F**) Morphological analysis of ovaries from Oo*Tsc2*$^{-/-}$ and Oo*Tsc2*$^{+/+}$ littermates at PD13, PD23 and PD35. Ovaries from13-, 23- and 35-day-old Oo*Tsc2*$^{+/+}$ and Oo*Tsc2*$^{-/-}$ mice were embedded in paraffin, and serial sections of 8-μm thickness were prepared and stained with hematoxylin. At PD13, similar ovarian morphologies were seen in sections from Oo*Tsc2*$^{+/+}$ (A) and Oo*Tsc2*$^{-/-}$ (B) ovaries. Cluster of primordial follicles were seen in both Oo*Tsc2*$^{+/+}$ (A, inset, arrows) and Oo*Tsc2*$^{-/-}$ (B, inset, arrows) ovaries at PD13. At PD23, Oo*Tsc2*$^{-/-}$ ovaries were larger (D) than Oo*Tsc2*$^{+/+}$ ovaries (C). No primordial follicles could be seen by PD23 in Oo*Tsc2*$^{-/-}$ ovaries, and almost all the primordial follicles were activated with apparently enlarged oocytes surrounded by flattened granulosa cells (D, inset, red arrows). In Oo*Tsc2*$^{+/+}$ ovaries primordial follicles could be readily observed at PD23 (C, inset, arrows). At PD35, Oo*Tsc2*$^{-/-}$ ovaries were much larger (F), with all the follicles activated (F, inset, red arrows) than Oo*Tsc2*$^{+/+}$ ovaries (E), which still had cluster of primordial follicles (E, inset, arrows). (**G**–**H**) Morphological analysis of ovaries from Oo*Tsc2*$^{-/-}$ and Oo*Tsc2*$^{+/+}$ littermates at 4 months of age. At 4 months of age, almost all follicles had degenerated and healthy follicular structures had completely disappeared in Oo*Tsc2*$^{-/-}$ ovaries (H). Two degenerating oocytes were shown (H, inset, arrows). As a control, Oo*Tsc2*$^{+/+}$ mice had normal ovarian morphology (G). CL was seen showing that Oo*Tsc2*$^{+/+}$ mice had been ovulating (G). The experiments were repeated at least four times, and for each time and each age, ovaries from one mouse of each genotype were used. CL, corpus luteum.

into the growing follicular pool in Oo*Tsc2*$^{-/-}$ mice, the litter sizes of Oo*Tsc2*$^{-/-}$ mice do not seem to be apparently different from those of Oo*Tsc2*$^{+/+}$ mice.

In our parallel study with a mouse model carrying oocyte-specific deletion of *Tsc1* (referred to as Oo*Tsc1*$^{-/-}$ mice), we found a similar phenotype in that the entire pool of primordial follicles was prematurely activated in young adulthood (Adhikari *et al.*, 2009). These studies indicate that the tumor suppressors Tsc1 and Tsc2 do play physiological roles in oocytes of primordial follicles to suppress their activation. On the basis of the current study and our parallel study with the *Tsc1* in mice (Adhikari *et al.*, 2009), we confirm that the Tsc1–Tsc2 complex mediated suppression of mTORC1 activity in oocytes is indispensable for maintenance of the dormancy of primordial follicles, thus preserving the follicular pool.

Nevertheless, one major unsolved question is that how the expressions and functions of Tsc1 and Tsc2 in oocytes are regulated, in order to orchestrate follicular activation throughout the female reproductive life.

It was hypothesized more than a decade ago that dormant primordial follicles may be under constant inhibitory influences of local origin to remain quiescent (Wandji *et al.*, 1996). Our earlier reports have shown that PTEN in oocytes functions as a suppressor of follicular activation (Reddy *et al.*, 2008). It is clear now that Tsc2 and Tsc1 (Adhikari *et al.*, 2009) in oocytes are also parts of the inhibitory mechanisms. Other recognized inhibitory molecules include the cyclin-dependent kinase (Cdk) inhibitor p27^{kip1} (p27, or Cdkn1b) (Rajareddy *et al.*, 2007), which functions in both oocytes and pregranulosa cells to suppress follicular activation; and Foxo3a, which is a downstream transcription factor of the PTEN/PI3K pathway that functions in oocytes to suppress follicular activation (Castrillon *et al.*, 2003; Reddy *et al.*, 2008).

In women, reproductive lifespan and menopausal age are determined by their ovarian reserve, i.e. the size and persistence of the primordial follicle pool. Our results from the current study and several recent reports using genetically modified mouse models imply that deregulation of signaling events in oocytes, such as the Tsc1–Tsc2/mTORC1 signaling (Adhikari *et al.*, 2009), the PTEN/PI3K signaling (Reddy *et al.*, 2008, 2009) and the p27-Cdk system (Rajareddy *et al.*, 2007), may contribute to defects in primordial follicle development in humans, which may result in pathological conditions of the ovary, such as POF and infertility. The possible involvement of the above-mentioned molecules in human POF, however, needs to be investigated. In this sense, our work may have both physiological and clinical implications.

Acknowledgements

We thank Dr Austin Cooney (Baylor College of Medicine, TX, USA) for kindly providing the *Gdf-9*-Cre mice.

Funding

This work was supported by grants from the Swedish Research Council (K.L.), the Swedish Cancer Foundation (K.L.), the Young Researcher Award of Umeå University, Sweden (K.L.), the Lions Cancer Research Foundation in Norrland, Sweden (K.L.), and the Novo Nordisk Foundation, Denmark (K.L.), and the Cutting-Edge Research Grant from the County Council of Västerbotten, Sweden (E.L.).

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Submitted on October 9, 2009; resubmitted on October 16, 2009; accepted on October 17, 2009