PDK1 signaling in oocytes controls reproductive aging and lifespan by manipulating the survival of primordial follicles

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Received March 17, 2009; Revised and Accepted May 5, 2009

The molecular mechanisms that control reproductive aging and menopausal age in females are poorly understood. Here, we provide genetic evidence that 3-phosphoinositide-dependent protein kinase-1 (PDK1) signaling in oocytes preserves reproductive lifespan by maintaining the survival of ovarian primordial follicles. In mice lacking the PDK1-encoding gene Pdk1 in oocytes, the majority of primordial follicles are depleted around the onset of sexual maturity, causing premature ovarian failure (POF) during early adulthood. We further showed that suppressed PDK1–Akt–p70 S6 kinase 1 (S6K1)–ribosomal protein S6 (rpS6) signaling in oocytes appears to be responsible for the loss of primordial follicles, and mice lacking the Rps6 gene in oocytes show POF similar to that in Pdk1-deficient mice. In combination with our earlier finding that phosphatase and tensin homolog deleted on chromosome 10 (PTEN) in oocytes suppresses follicular activation, we have now pinpointed the molecular network involving phosphatidylinositol 3 kinase (PI3K)/PTEN–PDK1 signaling in oocytes that controls the survival, loss and activation of primordial follicles, which together determine reproductive aging and the length of reproductive life in females. Underactivation or overactivation of this signaling pathway in oocytes is shown to cause pathological conditions in the ovary, including POF and infertility.

INTRODUCTION

In humans, the pool of ovarian primordial follicles is formed during fetal development. From a peak of 6–7 million at 20 weeks of gestation, the oocyte count falls dramatically so that by the time of onset of puberty, the number of oocytes that are enclosed in primordial follicles has decreased to around 400 000 in the ovaries, serving as the source of fertilizable ova (1–3). In order to ensure the proper length of reproductive life, certain primordial follicles must survive in the ovary for decades, in a resting state (Fig. 1A-1) (1,2,4). At the same time, progressive loss of primordial follicles, which is initiated by death of the oocyte (5), occurs in the ovary (Fig. 1A-2), dictating the process of reproductive aging (1,2,4). Only limited numbers of primordial follicles are continuously recruited into the growing follicle pool (Fig. 1A-3) (3,6). In humans, menopause occurs at about 50 years of age when the pool of primordial follicles has been virtually exhausted. The length of reproductive life and the timing of menopause in a woman...
Figure 1. Infertility and POF in OoPdk1^{-/-} mice. (A) Schematic illustrations of the courses of development of primordial follicles. In the mammalian ovary, the pool of dormant primordial follicles serves as the source of fertilizable ova for the entire duration of reproductive life. Three different courses of development for primordial follicles are illustrated: (1) they remain dormant (not growing but surviving); (2) they progressively die out directly from their dormant state, causing ovarian aging and (3) limited numbers of primordial follicles are continuously activated into the growing follicle pool. The reproductive lifespan and timing of menopause in a woman are decided by the duration of survival and the rate of loss of primordial follicles. (B) Illustration of the PI3K signaling pathway, with class IA P13K as an example. Upon ligand binding (e.g. of growth factors), the receptor protein tyrosine kinases (RPTK) are in most cases dimerized and autophosphorylated and present one or several phosphorylated tyrosine residues (pY) that are capable of binding to the SH2 domain of the p85 regulatory subunit of PI3K. This recruits PI3K from the cytoplasm to the inner membrane area of the cell and enables the p110 catalytic subunit of PI3K to phosphorylate the 3'-OH group of the inositol ring of inositol phospholipids, leading to the production of PIP3 from PIP2. The phosphatase PTEN converts PIP3 to PIP2, thereby negatively regulating PI3K activity. Kinases containing pleckstrin homology domain (PH domain), such as PDK1 and Akt, are recruited through binding of their PH domains to PIP3. (C and D) Oocyte-specific deletion of Pdk1 in mice. Western blots showing the absence of PDK1 protein expression in OoPdk1^{-/-} mouse oocytes. Oocytes were isolated from ovaries of 12–14-day-old OoPdk1^{+/+} and OoPdk1^{-/-} mice (C) or recovered from oviducts of OoPdk1^{+/+} and OoPdk1^{-/-} mice after PMSG-hCG primed ovulation (D), as described in Materials and Methods. For each experiment in (C), material from three to five mice was used per lane. For each lane, around 20 µg of protein was loaded. For experiments in (D), 100 ovulated oocytes were used in each lane. Levels of β-actin were used as internal controls. The experiments were repeated three times, and representative images are shown. (E) Comparison of average cumulative numbers of pups per OoPdk1^{+/+} (red line) and OoPdk1^{-/-} female (black line). (F–K) POF in OoPdk1^{-/-} females during early adulthood. Ovaries from 8- and 15-week-old OoPdk1^{+/+} and OoPdk1^{-/-} mice were embedded in paraffin, and sections of 8 µm thickness were prepared and stained with hematoxylin. Virtually, all follicular structures were depleted in OoPdk1^{-/-} ovaries (G and J). Arrows in the upper inset of (I) indicate primordial follicles. (L) Average weights of ovaries obtained from young adult (8–12 weeks old) OoPdk1^{+/+} and OoPdk1^{-/-} mice. (M and N) Levels of FSH and LH in sera of young adult (8–12 weeks old) OoPdk1^{+/+} and OoPdk1^{-/-} mice. For (E) and (L–N), the numbers of mice used (n) and P-values are shown in the figures.
are thus determined by the size and persistence of her primordial follicle pool (1–4).

The molecular mechanisms controlling the balance between the survival and loss of primordial follicles are, however, poorly defined. In recent years, genetic factors have received increasing attention as determinants of ovarian aging and menopausal age (1). In a recent study, we showed that ablation of phosphatase and tensin homolog deleted on chromosome 10 (Pten) in oocytes results in excessive activation of primordial follicles (7). PTEN is a negative regulator of phosphatidylinositol 3 kinase (PI3K) (Fig. 1B) (8–10), and it appears to suppress the activation of primordial follicles and maintain them in a dormant state (7). However, whether or not the PI3K signaling in oocytes has direct effects on the survival and maintenance of the primordial follicle pool is not yet clear.

A considerable proportion of the signaling mediated by PI3Ks converges at 3-phosphoinositide-dependent protein kinase-1 (PDK1) (Fig. 1B). PDK1 activates Akt through co-binding to phosphatidylinositol 3,4,5-trisphosphate (PIP3) generated by PI3Ks (11,12). Apart from this, PDK1 also functions as a master kinase to activate other protein kinases of the AGC family (denoting protein kinases A, G and C), such as p70 S6 kinase 1 (S6K1) (Fig. 1B), p90 ribosomal S6 kinase (RSK) and protein kinase C (PKC) (11). The activation of substrates other than Akt does not, however, involve binding of PDK1 to the PI3K generated PIP3 (11). Previous reports have shown that conventional deletion of PDK1 in heart, pancreas, liver and T cells results in heart failure (14), diabetes (15), defective postprandial glucose disposal and liver failure (16,17) and impaired T cell differentiation (18), respectively.

In the present study, we found that deletion of Pdk1 in mouse oocytes resulted in accelerated ovarian aging and premature ovarian failure (POF), due to the severely compromised lifespan of primordial follicles, which was caused by suppressed PDK1–Akt–S6K1–ribosomal protein S6 (rPS6) signaling in oocytes. This finding was further corroborated by our results that mice lacking the Rps6 gene in oocytes exhibited POF similar to that seen in Pdk1-deficient mice. Thus, PDK1 signaling in oocytes appears to be indispensable for maintaining the survival of primordial follicles, which is in turn crucial for determining the duration of female fertility. As the demise of non-growing follicles is initiated by oocyte death (5), our results suggest that the suppression of PDK1 signaling in oocytes contributes to follicular loss, which eventually leads to the onset of ovarian senescence.

RESULTS
Infertility, accelerated ovarian aging and POF caused by deletion of Pdk1 in oocytes

To study how PDK1 signaling in oocytes controls the different courses of development of primordial follicles, we generated mutant mice in which the Pdk1 gene was deleted in oocytes of primordial and further developed follicles (referred to as OoPdk1−/− mice). This was achieved by crossing Pdk1<sup>loxP/loxP</sup>
mice (15,19) with transgenic mice expressing growth differentiation factor 9 (Gdf-9) promoter-mediated Cre recombinase (20). The Gdf-9 promoter becomes active in oocytes of primordial follicles (20), and primordial follicles in mice start to form on postnatal day (PD) 1–2 and finish on PD7–8 (21). By western blot analysis, we found that expression of PDK1 protein was almost completely absent in oocytes that were isolated from ovaries of 12–14-day-old OoPdk1−/− mice (Fig. 1C), and it was completely absent in ovulated OoPdk1−/− oocytes (Fig. 1D).

During a testing period from 5 to 30 weeks of age, the OoPdk1−/− females were found to be infertile (Fig. 1E). To determine how the deletion of Pdk1 from oocytes impedes fertility, we studied the ovarian morphology of young adult mutant mice. As shown in Figure 1G, in ovaries of 8-week-old OoPdk1−/− mice, basically all follicular structures were diminished, with some degenerating oocytes in growing follicles being seen (Fig. 1H, arrows). Ovaries from older OoPdk1−/− mice showed a similar morphology; they were devoid of oocytes and follicles (Fig. 1J and K). In comparison, ovaries from adult OoPdk1+/− ovaries (Fig. 1F and I) contained follicles and corpora lutea (CL), and most importantly, primordial follicles that would retain their fertility for the rest of the reproductive life (Fig. 1I, upper inset, arrows).

In young adult OoPdk1−/− mice, the ovaries were lighter (Fig. 1L), which was accompanied by significantly elevated serum levels of follicle-stimulating hormone (FSH) (Fig. 1M) and luteinizing hormone (LH) (Fig. 1N). These results show that deletion of Pdk1 from oocytes causes POF in OoPdk1−/− mice.

PDK1 in oocytes is required to maintain the survival of primordial follicles

The follicular resource available in the ovary is the direct determinant of reproductive lifespan (1,2). To investigate how the absence of Pdk1 in oocytes leads to POF, we studied postnatal follicular development in OoPdk1−/− mice. We found that at PD8 (Fig. 2A, B and G) and PD23 (Fig. 2H), the numbers of primordial follicles and of all follicles were similar in OoPdk1−/− and OoPdk1+/+ ovaries, indicating that comparable numbers of follicles are formed in the wild-type and mutant ovaries and that deletion of Pdk1 in oocytes does not have an immediate effect on follicular survival up to PD23. This may be caused by some temporary compensatory mechanisms that take over the functions of PDK1 signaling in oocytes. Alternatively, as the deletion of Pdk1 in oocytes only occurs after the primordial follicles are formed, it may take some time for PDK1-mediated signals to become downregulated or inactivated in oocytes.

However, at PD35, the time of onset of sexual maturity, the OoPdk1−/− ovaries were smaller (Fig. 2D), due to markedly reduced numbers of follicles (Fig. 2I). Specifically, compared with OoPdk1+/+ ovaries where clusters of primordial follicles were seen (Fig. 2E, arrows), the numbers of healthy-looking primordial follicles in OoPdk1−/− ovaries were substantially reduced (Fig. 2F, arrow), corresponding to 27.7% of those in OoPdk1+/+ ovaries (Fig. 2I). In OoPdk1−/− ovaries, the total numbers of follicles corresponded to 33.7% of those in OoPdk1+/+ ovaries at PD35 (Fig. 2I), with also reduced numbers of activated follicles (including type 3b, type 4, type 5 and type 6 follicles) (61.6%) (Fig. 2I). Thus, primordial follicles were more susceptible to the loss of Pdk1 in oocytes and were the main population of follicles to disappear from OoPdk1−/− ovaries.

At 8 weeks of age, primordial follicles that normally survive as the source of ova for the entire reproductive lifetime had been completely abolished in OoPdk1−/− ovaries (Fig. 1G). Consequently, healthy-looking growing follicles were not observed in the mutant ovaries at this age (Fig. 1G). These results show that the accelerated depletion of primordial follicles is the direct cause of POF in OoPdk1−/− mice.

In this mutant mouse model, the Gdf-9 promoter used to mediate Cre recombinase expression becomes active in oocytes of primordial follicles (20). As a synchronous wave of follicular activation takes place once the primordial follicles are formed (3,22), it is most likely that the first wave of postnatal follicular activation has occurred in OoPdk1−/− ovaries before the complete clearance of Pdk1 mRNA and PDK1 protein in oocytes.

At PD23, female OoPdk1−/− mice can respond normally to gonadotropin treatment and ovulate. At the age of 5–6 weeks, some type 5–6 follicles from the first wave of postnatal follicular activation were still present in OoPdk1−/− ovaries (Fig. 2D, arrows), indicating that PDK1 in oocytes mainly maintains the survival of primordial follicles in a stage-specific manner, but may not greatly affect the follicles that have entered the growing phase. The OoPdk1−/− females had a normal vaginal opening at the age of 5–6 weeks (which is the appropriate age) and they showed normal estrous cycles. These mice were found to ovulate normally when mated with wild-type male mice, based on the fact that similar numbers of oocytes/embryos were recovered from their oviducts (Supplementary Material, Fig. S1A). This indicates that before the follicles become depleted in OoPdk1−/− ovaries, ovulation per se is not affected even if Pdk1 is deleted from oocytes. However, zygotes from the OoPdk1−/− females mated with wild-type males showed arrest of development at the two-cell stage, making the OoPdk1−/− females completely infertile (Supplementary Material, Fig. S1B).

Deletion of Pdk1 in oocytes reverses the excessive follicular activation and survival caused by loss of Pten

The rapid depletion of primordial follicles in OoPdk1−/− ovaries makes it difficult to study the function of oocyte PDK1 in mediating follicular activation. To circumvent this problem, we crossed OoPdk1−/− mice with mice lacking Pten in oocytes, which exhibit premature activation of the primordial follicle pool (referred to as OoPten−/− mice in this study) (7), and studied follicular development in progeny mice with concurrent loss of Pdk1 and Pten in oocytes (referred to as OoPten−/−;Pdk1−/− mice).

In OoPten−/− ovaries, all primordial follicles were activated by PD23, with noticeably enlarged oocytes (Fig. 3A, arrows), and the percentage of primordial follicles was 0% (Fig. 3D) (7). In OoPten−/−;Pdk1−/− ovaries at PD23, however, clusters of primordial follicles were observed (Fig. 3B, arrows), as with OoPten+/+ ovaries (Fig. 3C,
Deletion of Pdk1 impairs PDK1→Akt→S6K1→rpS6 signaling in OoPdk1−/− oocytes

PDK1 plays an essential role in phosphorylating and activating Akt and S6K1 (11). To study the signaling pathways underlying the accelerated loss of follicles in OoPdk1−/− oocytes, we first studied Akt signaling in mutant and control oocytes. We stimulated starved oocytes with Kit ligand (KL), which we first studied Akt signaling in mutant and control oocytes.

Figure 2. Deletion of Pdk1 from oocytes causes accelerated depletion of primordial follicles in OoPdk1−/− mice. (A–F) Morphological analysis of ovaries from OoPdk1−/− and OoPdk1+/+ littermates at PD8 and PD35. Ovaries from 5- and 35-day-old OoPdk1+/+ and OoPdk1−/− mice were embedded in paraffin, and serial sections of 8 μm thickness were prepared and stained with hematoxylin. At PD8, similar ovarian morphologies were seen in OoPdk1−/− and OoPdk1+/+ littermates (A and B). At PD35, however, the OoPdk1−/− ovaries were smaller (D), and a dramatic loss of primordial follicles took place in OoPdk1−/− ovaries (F, arrow) compared with OoPdk1+/+ ovaries (E, arrows). The proportion of primordial follicles in OoPten−/−; Pdk1−/− ovaries was elevated to 71.3%, which was similar to the proportions in OoPten+/+ ovaries (69.2%) and OoPdk1−/− ovaries (76.2%) (Fig. 3D). In addition, the temporarily enhanced postnatal follicular survival seen in OoPten−/− ovaries, as judged by the elevated total number of follicles at PD23 (Fig. 3E) (7), was found to be efficiently reversed in OoPten−/−;Pdk1−/− ovaries, to levels similar to those seen in OoPten+/+ and OoPdk1−/− ovaries (Fig. 3E).

The above results indicate that the excessive follicular activation and elevated postnatal follicular survival observed in OoPten−/− ovaries (7) were mostly, if not all, mediated by PDK1, suggesting that the activation and survival of primordial follicles are dependent on PDK1 signaling in oocytes. Through its receptor Kit (23). We found that KL was able to trigger phosphorylation of Akt (p-Akt, T308) in OoPdk1+/+ oocytes, but not in OoPdk1−/− oocytes (Fig. 4A). This shows that the phosphorylation of Akt at threonine 308 by PDK1, which is essential for activation of Akt (11,24), is disabled in OoPdk1−/− oocytes. As an indicator of Akt activity, elevated phosphorylation of Foxo3a at serine 253 (p-Foxo3a, S253) upon stimulation by KL was seen in OoPdk1+/+ oocytes, but not in OoPdk1−/− oocytes (Fig. 4A), implying that Foxo3a can not be efficiently phosphorylated and suppressed by Akt (25) in the mutant oocytes, which can be a reason for the accelerated follicular loss, as previously proposed (26). Elevated phosphorylation of another Akt substrate, tuberin/Tsc2, was triggered by treatment with KL in OoPdk1+/+ oocytes, but again not in OoPdk1−/− oocytes (Fig. 4A, p-Tsc2, T1462), indicating that Tsc2 may not be efficiently suppressed by Akt in OoPdk1−/− oocytes. Thus, the loss of Pdk1 in oocytes leads to suppressed Akt signaling.

Activation of S6K1 requires phosphorylation of its threonine 229 by PDK1 (11). S6K1 can then phosphorylate and activate one of the 40S ribosomal proteins, rpS6, which is needed for protein translation and ribosome biogenesis during cell growth (27,28). In an earlier study (7), we reported that overactivation of PI3K signaling in OoPten−/− oocytes leads to...
elevated activation of rpS6, indicating that rpS6 may act downstream of PI3K–Akt–S6K1 signaling to mediate the protein translation needed for oocyte growth during follicular activation. In this study, we investigated whether the S6K1–rpS6 signaling is deregulated in OoPdk1−/− oocytes. As shown in Figure 4B, compared with OoPdk1+/+ oocytes where phosphorylation of S6K1 (p-S6K1, T229) was triggered by KL treatment, the phosphorylation of S6K1 at T229 was completely abolished in OoPdk1−/− oocytes, showing that S6K1 cannot be activated in the mutant oocytes. Moreover, in OoPdk1−/− oocytes, phosphorylation of rpS6 was substantially downregulated (Fig. 4B, p-rpS6, S240/4), indicating that there were low levels of rpS6 activation and of rpS6-mediated protein translation and ribosome biogenesis in the mutant oocytes.
To determine the upstream and downstream relationships between Akt and S6K1 signaling in oocytes, we treated cultured wild-type oocytes with the PI3K-specific inhibitor LY294002. As shown in Figure 4C, LY294002 (LY) largely suppressed the phosphorylation of Akt (p-Akt, S473), S6K1 (p-S6K1, T389) and rpS6 (p-rpS6, S240/4) in oocytes. As a control, treatment of cultured oocytes with the mammalian target of rapamycin complex 1 (mTORC1) inhibitor rapamycin (Rap) only suppressed the phosphorylation of S6K1 and rpS6, but not that of Akt (Fig. 4C). In addition, the phosphorylation of S6K1 at T229 (p-S6K1, T229) that was triggered by KL treatment was completely blocked by pretreatment of the oocytes with LY294002 (Fig. 4D). These results indicate that activation of S6K1–rpS6 in primary mouse oocytes is downstream of PI3K–Akt signaling.

Therefore, we conclude that the absence of Pdk1 in oocytes impairs the PDK1–Akt–S6K1–rpS6 signaling, which appears to be responsible for the premature loss of primordial follicles in OoPdk1−/− ovaries. To determine whether the premature loss of primordial follicles in OoPdk1−/− ovaries occurred through accelerated
apoptosis in the mutant oocytes, we measured the levels of some apoptotic molecules in OoPdk1\(^{-/-}\) and OoPdk1\(^{+/+}\) oocytes that were isolated from PD23–25 mice, which is a time point that is prior to the disappearance of primordial follicles. However, the levels of cleaved poly (ADP-ribose) polymerase [PARP] and cleaved caspase-7 were found to be unaltered in OoPdk1\(^{-/-}\) oocytes as compared to OoPdk1\(^{+/+}\) oocytes (Supplementary Material, Fig. S2).

DISCUSSION

In this study, by using a mouse model with oocyte-specific deletion of Pdk1, we found that PDK1 signaling in oocytes plays an essential role in preserving the normal reproductive lifespan in females by maintaining the survival of primordial follicles during their long dormancy. Suppressed PDK1–Akt–S6K1–rpS6 signaling in oocytes was shown to be responsible for the clearance of non-growing follicles, which causes reproductive aging and eventually ovarian senescence. We therefore propose that maintenance of the primordial follicle pool, which is a prerequisite for preservation of the normal length of reproductive life in females, is dependent on PDK1 signaling in oocytes (Fig. 6A).

Recently, PTEN, the upstream negative regulator of P3K–PDK1 signaling, was identified as a suppressor of follicular activation (7). PTEN in oocytes prevents primordial follicles from being activated prematurely, and it also promotes their death (7). It is likely that PTEN activity adjusts the PDK1 signaling in oocytes to an optimal level so that the pool of primordial follicles can be maintained in a dormant and surviving condition (Fig. 6A). On the other hand, loss of primordial follicles can be caused by suppressed PDK1 signaling in oocytes (Fig. 6C); and activation of primordial follicles can be a result of elevated PDK1 signaling in oocytes (Fig. 6B). Based on the evidence accumulated, we believe that the PTEN/P3K–PDK1 signaling network in oocytes controls the survival during dormancy, the activation, and the loss of primordial follicles, which together govern the length of reproductive life in females.

Signal transduction studies with oocytes lacking Pdk1, Pten or both Pdk1 and Pten revealed that the phosphorylation and activation of rpS6 in oocytes via PDK1–Akt–S6K1 signaling are key downstream events in determining the fates of primordial follicles. Underactivation of rpS6 in oocytes by deletion of Pdk1, or deletion of Rps6, leads to accelerated loss of primordial follicles. In contrast, overactivation of rpS6 in oocytes as a result of loss of Pten is accompanied by enhanced follicular activation and survival (7). Furthermore, simultaneous ablation of Pdk1 and Pten in oocytes reverses the excessive follicular activation and survival seen in OoPten\(^{-/-}\);Pdk1\(^{-/-}\) oocytes, which is likely achieved by downregulation of rpS6 phosphorylation in OoPten\(^{-/-}\);Pdk1\(^{-/-}\) oocytes. We therefore propose that the up- and downregulation of rpS6-mediated protein translation and ribosome biogenesis in oocytes may be critical for controlling the courses of development of primordial follicles regarding their survival during dormancy, their activation, or their loss.

One remaining question is that the upstream regulation of PTEN/P3K–PDK1–Akt–S6K1–rpS6 signaling in oocytes, such as the extra-oocyte signals from the surrounding somatic cells that activate or suppress the signaling cascades within the oocyte, is not completely understood.

rpS6 is the key downstream effector of PTEN/P13K–PDK1–Akt–S6K1 signaling in oocytes

rpS6 is an important substrate of S6K1. When PI3K signaling was overactivated in OoPten\(^{-/-}\) oocytes, elevated phosphorylation (indicating activation) of both rpS6 (Fig. 4E, p-rpS6, S240/244) and S6K1 (Fig. 4E, p-S6K1, T229) could be seen. In the present study, we also found that the elevated phosphorylation of rpS6 in OoPten\(^{-/-}\) oocytes was prevented by the concurrent loss of Pdk1 in OoPten\(^{-/-}\);Pdk1\(^{-/-}\) oocytes (Fig. 4E, p-rpS6), which was most likely caused by downregulated activation of S6K1 (Fig. 4E, p-S6K1, T229) and of Akt (Fig. 4E, p-Akt, T308) in OoPten\(^{-/-}\);Pdk1\(^{-/-}\) oocytes, when compared with that in OoPten\(^{-/-}\) oocytes. Thus, downregulation of phosphorylation of rpS6 in OoPten\(^{-/-}\);Pdk1\(^{-/-}\) oocytes may be the reason behind the prevention of excessive follicular activation and survival seen in OoPten\(^{-/-}\) oocytes. We propose that the phosphorylation and activation of rpS6, as downstream events of PTEN/P13K–PDK1–Akt–S6K1 signaling in oocytes, are important for maintaining the survival and activation of primordial follicles.

Oocyte-specific deletion of Rps6 causes POF in OoRps6\(^{-/-}\) mice

Of the 33 40S ribosomal proteins, rpS6 is the only ribosomal protein that is known to be a substrate of S6K1 (28). To determine whether rpS6 in oocytes is indispensable for maintenance of the normal reproductive lifespan, we conditionally deleted the Rps6 gene in mouse oocytes by crossing Rps6\(^{loxP/loxP}\) mice (28) with the Gdf-9-Cre mice (20). The mutant animals are referred to as OoRps6\(^{-/-}\) mice.

Our results showed that Rps6 protein expression was almost absent in OoRps6\(^{-/-}\) oocytes (Fig. 5A). At PD8, the morphology of OoRps6\(^{-/-}\) ovaries was similar to that of OoRps6\(^{+/+}\) ovaries (Fig. 5B and C). At PD23, however, the mutant ovaries were much smaller (Fig. 5E) and lighter (Fig. 5L), apparently due to dramatically reduced numbers of follicles (Fig. 5E). By PD35, even fewer healthy primordial follicles or other types of follicles were found in OoRps6\(^{-/-}\) ovaries (Fig. 5G and H). At 8 weeks of age, the follicular structure completely disappeared in ovaries of OoRps6\(^{-/-}\) mice (Fig. 5J and K). In accordance with this, the OoRps6\(^{-/-}\) mice were found to be infertile due to lack of preovulatory follicles (Fig. 5E and G) and ovulation. It is thus clear that the loss of Rps6 in oocytes causes POF in OoRps6\(^{-/-}\) mice, which was corroborated by the elevated levels of FSH and LH in sera (Fig. 5M and N).

Collectively, these results strongly suggest that rpS6 in oocytes plays an important role in maintaining the survival of primordial and developing follicles. The PDK1–Akt–S6K1–rpS6 network in oocytes is therefore crucial for preservation of the normal length of reproductive life in females.
Also, it is not yet clear whether deletion of Pdk1 in oocytes triggers the loss of primordial follicles via apoptosis of the oocytes. From a practical point of view, it has been difficult to detect apoptosis in mouse primordial follicles using normal apoptotic markers, such as by TUNEL assay or by detection of cleaved PARP (our own data; Dr Jodi Anne Flaws, personal communications, University of Illinois, USA). This is likely due to the rapid and transient nature of the apoptotic process. On the other hand, although apoptosis has been suggested as the mechanism underlying oocyte death during primordial and primary follicle atresia in the postnatal ovary (29,30), other studies, however, show no apoptosis in primordial follicles in postnatal life (31). Some previous studies have suggested that there are more differences than similarities between physiological oocyte cell death and apoptosis, suggesting that oocyte death should be assigned to a different class of cell death other than apoptosis (32,33). It has been shown recently that the clearance of primordial follicles in postnatal mouse ovaries does not occur via apoptosis (34). Whether suppressed PDK1 signaling in oocytes triggers fol-

Figure 5. Deletion of Rps6 in mouse oocytes leads to POF. (A) Effective removal of rpS6 protein expression from mouse oocytes. Oocytes were isolated from ovaries of 14–16-day-old OoRps6+/+ and OoRps6−/− mice as described in Materials and Methods, and the extracted proteins were used for western blot. The experiments were repeated three times. For each experiment, material from five to ten mice was used per lane. In each lane, about 20 μg of protein sample was loaded. Levels of β-actin were used as internal controls. Representative images are shown. (B–K) Morphological analysis of ovaries from OoRps6+/+ and OoRps6−/− littermates. Ovaries were embedded in paraffin, and sections of 8 μm thickness were prepared and stained with hematoxylin. At PD8, similar ovarian morphologies were seen in OoRps6+/+ and OoRps6−/− mice (B and C). At PD23, however, the OoRps6−/− ovaries appeared to be much smaller (E), due to dramatic loss of primordial and activated follicles. By PD35, the follicular structures in OoRps6−/− ovaries were almost depleted, and few primordial or other types of follicles could be identified (G and H). Ovaries from 8-week-old OoRps6−/− mice showed a complete loss of follicular structures (I and K). The experiments were repeated more than three times, and for each time and each age, ovaries from one mouse of each genotype were used. (L) Average weights of ovaries that were obtained from 23- to 25-day-old OoRps6+/+ and OoRps6−/− mice. The numbers of mice used (n) and P-values are given. (M and N) Elevated levels of FSH and LH in 8–12-week-old OoRps6−/− mice. This confirms the POF in OoRps6−/− mice, as shown in (J) and (K). Measurements of FSH and LH levels in mouse serum were performed as described in Materials and Methods. The numbers of mice used (n) and P-values are shown.
Oocyte loss through apoptosis will be revealed by in vivo studies; for example, by crossing mice lacking the pro-apoptotic molecule Bax to OoPdk1/−/− mice, to see whether the accelerated loss of primordial follicles in OoPdk1/−/− mice can be rescued by the simultaneous loss of Bax.

Ablation of either Pten or Pdk1 in mouse oocytes results in POF, but from opposite directions. Pten deficiency in oocytes leads to POF due to excessive follicular activation that is followed by follicular atresia (7), whereas deletion of Pdk1 causes POF as a result of accelerated clearance of primordial follicles directly from their dormant state. The two types of POF, resulting from loss of Pten or Pdk1, may represent distinct etiologies of POF in humans. In women, the normal length of reproductive life and menopausal age are determined by the reserve of primordial follicles in the ovaries. It is possible that PTEN/P13K–PDK1 signaling in oocytes also controls the courses of development of primordial follicles in humans, thereby maintaining the duration of fertility and contributing to the timing of menopause in women. Underactivation or overactivation of the PDK1–Akt–S6K1–rpS6 signaling in oocytes may also cause defects in primordial follicle survival and development in humans, resulting in pathological conditions in the ovary, including POF and infertility. In this sense, our work may have broad physiological and clinical implications. We believe that comprehension of the signaling networks in oocytes will open up new avenues for a better understanding of ovarian physiology and pathology.

MATERIALS AND METHODS

Mice

Pdk1loxP/loxP mice (15,19), PtenloxP/loxP mice (7) and Rps6loxP/loxP mice (28) with C57BL/6J genomic background were crossed with transgenic mice carrying Gdf-9 promoter-mediated Cre recombinase that also had a C57BL/6J background (7,20). After multiple rounds of crossing, we obtained homozygous mutant female mice lacking Pdk1 in oocytes (OoPdk1−/− mice), mice lacking Pten in oocytes (OoPten−/− mice), mice lacking Pten in oocytes (OoPten−/− mice), mice lacking both Pdk1 and Pten concurrently in oocytes (OoPten−/−;Pdk1−/− mice) and mice lacking Rps6 in oocytes (OoRps6−/− mice). Control mice that do not carry the Cre transgene are referred to as OoPdk1+/+, OoPten+/+ or OoRps6+/+ mice. The mice were housed under controlled environmental conditions with free access to water and food. Illumination was on between 06:00 and 18:00 h. Experimental protocols were approved by the regional ethical committee of Umeå University, Sweden.

Reagents, antibodies and immunological detection methods

Mouse monoclonal antibodies to PDK1 (PKB kinase) and rpS6 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Rabbit polyclonal antibodies to Akt, phospho-Akt (S473), S6K1, phospho-S6K1 (T389), phospho-rpS6 (S240/244), tuberin/Tsc2, cleaved PARP,
cleaved caspase-7, and also rabbit monoclonal antibody to phospho-tuberin/Tsc2 (T1462), were obtained from Cell Signaling Technologies (Beverly, MA, USA). Mouse monoclonal antibody to phospho-Akt (T308) was purchased from R&D Systems (Minneapolis, MN, USA). Pregnant mare serum gonadotropin (PMSG), human chorionic gonadotropin (hCG) and mouse monoclonal antibody to β-actin were purchased from Sigma-Aldrich Sweden AB (Stockholm, Sweden). The PI3K-specific inhibitor LY294002, the mTORC1-specific inhibitor rapamycin and recombinant mouse KL were obtained from EMD Biosciences (San Diego, CA, USA). 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).

Quantification of ovarian follicles and histological analysis

Quantification of ovarian follicles was performed as previously described (7). Briefly, ovaries were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. To count the numbers of follicles, paraffin-embedded ovaries were serially sectioned at 8 μm thickness and stained with hematoxylin for morphological observation. Ovarian follicles at different stages of development, including primordial follicles and activated follicles (including transient follicles containing enlarged oocytes surrounded by flattened pregranulosa cells, type 3b, type 4, type 5 and type 6 follicles), were counted in all sections of an ovary, based on the well-accepted standards established by Pedersen and Peters (35). Follicles that contained oocytes with clearly visible nuclei were scored in each section, as previously reported (36). Judged from careful morphological analysis, the incidence of counting the same follicle twice or of missing a follicle was low.

Isolation of oocytes from postnatal mouse ovaries and stimulation of starved oocytes with KL

Isolation and lysis of oocytes were performed as previously described (7). For stimulation with KL, equal amounts of oocytes were aliquoted into wells of a 24-well plate. Typically, each well contained oocytes obtained from three to five mice that were 12–14-days old. The oocytes were first starved by culturing them in serum-free DMEM/F12 medium for 4 h, followed by treatment with 100 ng/ml KL for 2 min. After stimulation with KL, the 24-well plate was chilled on ice and oocytes were lysed for western blot analysis.

Measurement of serum hormone levels

Adult female OoPdk1−/− or OoRps6−/− mice from weeks 8–12 were killed randomly due to lack of regular estrus cycles; control OoPdk1+/+ or OoRps6+/+ female mice of similar ages were killed at the estrus stage based on vaginal smears, in order to measure gonadotropin levels during the follicular growth phase but not the ovulation phase. Serum hormone levels were determined by immunoassay as described previously for FSH (37) and LH (38).

Gonadotropin-induced ovulation

To induce synchronized follicular growth and ovulation in order to obtain ovulated oocytes for western blot, immature 23-day-old female mice were injected intraperitoneally with 5 IU of PMSG to stimulate follicular development and with 5 IU hCG 48 h later to induce ovulation. Ovulation normally takes place 10–12 h after hCG treatment (39). Cumulus-oocyte complexes were recovered from oviducts and treated with hyaluronidase (0.1%) before oocytes were collected.

Natural ovulation and embryo culture

OoPdk1−/− and OoPdk1+/+ female mice of 5–6 weeks old were housed with wild-type males, and vaginal plugs were checked every morning. Embryonic day 0.5 (E0.5) refers to the day that a vaginal plug was found. The mated female mice were sacrificed at E1.5 and zygotes/oocytes were recovered from their oviducts; these were cultured further for 72 h in KSOM medium supplemented with amino acids (Chemicon) in an incubator at 37°C with an atmosphere of 5% CO2. The numbers and stages of development of the zygotes/oocytes were recorded.

Statistical analysis

All experiments were repeated at least three times. For comparisons of follicle numbers, ovarian weights and hormone levels, differences between the two groups were calculated with Student’s t-test, and a difference was considered to be significant if P < 0.05.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

We thank Dr Austin Cooney (Baylor College of Medicine, TX, USA) and Dr Zijian Lan, University of Louisville Health Sciences Center, Louisville, Kentucky, USA) for kindly providing the Gdf-9-Cre mice.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by grants to K.L. from the Swedish Research Council, the Swedish Cancer Foundation, the Young Researcher Award of Umeå University, Sweden, the Lions Cancer Research Foundation in Norrland, Sweden, and the Novo Nordisk Foundation, Denmark.
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