The Cyclin-dependent Kinase Inhibitors p27Kip1 and p21Cip1 Cooperate to Restrict Proliferative Life Span in Differentiating Ovarian Cells*

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The timing of cellular exit from the cell cycle during differentiation is specific for each cell type or lineage. Granulosa cells in the ovary establish quiescence within several hours after the ovulation-inducing luteinizing hormone surge, whereas they undergo differentiation into corpora lutea. The expression of Cdk inhibitors p21Cip1/Waf1 and p27Kip1 is up-regulated during this process, suggesting that these cell cycle inhibitors are involved in restricting proliferative capacity of differentiating granulosa cells. Here we demonstrate that the lack of p27Kip1 and p21Cip1 synergistically renders granulosa cells extended a proliferative life span. Immunohistochemical analyses demonstrated that corpora lutea of p27Kip1, p21Cip1 double-null mice showed large numbers of cells with bromodeoxyuridine incorporation and high proliferative cell nuclear antigen expression, which were more remarkable than those in p27Kip1 single-deficient mice showing modest hyperproliferation. In contrast, differentiating granulosa cells in p21Cip1-deficient mice ceased proliferation similarly to those in wild-type mice. Interestingly, granulosa cells isolated from p27Kip1, p21Cip1 double-null mice exhibited markedly prolonged proliferative life span in culture, unlike cells with other genotypes. Cultured p27Kip1, p21Cip1 double-null granulosa cells maintained expression of steroidogenic enzymes and gonadotropin receptors through 8–10 passages and could undergo further differentiation in responses to CAMP accumulation. Thus, the cooperation of p27Kip1 and p21Cip1 is critical for withdrawal of granulosa cells from the cell cycle, in concert with luteal differentiation and possibly culture-induced senescence.

For tissue homeostasis, differentiation is usually coordinated with exit from the cell cycle. Proliferation stimulatory and inhibitory signals regulate the G1 phase of the cell cycle, governing the transition between proliferation and quiescence (1). Cyclin-dependent kinases (Cdks)1 form the central machinery of cell cycle progression (2–4). In G1 regulation, cyclin D- and cyclin E-dependent kinases play interacting roles. D-type cyclins, D1, D2 and D3, activate Cdk4 or Cdk6, whereas cyclin E activates Cdk2. Cdk-Drks phosphorylates some sites of retinoic acid protein followed by cyclin E-Cdk2-mediated phosphorylation of other specific sites (5). The sequential retinoic acid protein phosphorylation converts the E2F transcription factor from a repressor to activator form, leading to transactivation of a number of S-phase specific genes (6, 7). For withdrawal from the cell cycle, proper inactivation of the G1 Cdk is required, which largely depends on physical association with the Cdk inhibitor proteins. The Cdk inhibitor family consists of the Ink4-type Cdk4/Cdk6 inhibitors such as p16Ink4a, p15Ink4b, p18Ink4c, and p19Ink4d and the Kip/Cip-type Cdk2 inhibitors such as p21Cip1/Waf1/Cip200, p27Kip1, and p57Kip2 (8, 9).

Granulosa cells in the adult ovary undergo dynamic regulation of cell cycle progression during folliculogenesis, ovulation, and luteinization (10–12). The importance of the cell cycle control for reproductive function is recapitulated by the observations that deficiency for cyclin D2, Cdk4, or p27Kip1 renders female mice infertile. Cdk2/Cnd2-null ovaries exhibit a block of folliculogenesis at the secondary follicle stage because of impaired proliferation of granulosa cells (13, 14). In contrast, ovarian function of Cdk4-null mice is intact, but maintenance of corpora lutea is perturbed by insufficient prolactin secretion from the pituitary (15, 16). Intriguingly, p27Kip1(Cdknb)-null ovaries show hyperproliferation of granulosa cells during luteinization, which is at least partly responsible for the sterility (17, 18). Thus, the G1-Cdk regulatory system plays critical roles in the homeostasis of granulosa/luteal cells. During luteal differentiation, the expression of both p21Cip1 and p27Kip1 is up-regulated (14, 18). Unlike p27Kip1-null mice, p21Cip1(Cdknb)-null mice show normal fertility (19, 20). To further investigate the significance of G1-Cdk down-regulation during luteal differentiation, we generated p21Cip1, p27Kip1 double-null mice by cross-breeding. Here we demonstrate that differentiating granulosa cells in p21Cip1, p27Kip1 double-null mice undergo more prolonged proliferation relative to those in p27Kip1 single null mice. Moreover, granulosa cells isolated from p21Cip1, p27Kip1 double-null mice are capable of dividing through a number of passages in culture, exhibiting markedly prolonged proliferative life spans. These data suggest that p21Cip1 and p27Kip1 age cytochrome P450; P450arom, aromatase cytochrome P450; BrdUrd, bromodeoxyuridine; PCNA, proliferating cell nuclear antigen; MEF, mouse embryonic fibroblast; 3β-H, 3β-hydroxysteroid dehydrogenase; DMEM, Dulbecco’s modified Eagle’s medium; RT, reverse transcription; TUNEL, TdT-mediated dUTP nick-end labeling.
Hyperplastic Ovaries of p21<sup>Cip1</sup>, p27<sup>Kip1</sup> Double-null Mice—To determine whether p21<sup>Cip1</sup> and p27<sup>Kip1</sup> cooperate for the cell cycle control in granulosa cells, we generated p21<sup>Cip1</sup>, p27<sup>Kip1</sup> double-null (p21<sup>Cip1</sup>-/- × p27<sup>Kip1</sup>-/-) mice. Mating of double-null females with wild-type males indicated that p21<sup>Cip1</sup>-/- × p27<sup>Kip1</sup>-/- females are sterile with no exception, although mating behaviors and vaginal plugs were observed. This phenotype is analogous to that of p27<sup>Kip1</sup> single-null female mice (17, 18). p21<sup>Cip1</sup> single-null mice exhibit normal fertility (19, 20), but their ovarian function has not been characterized previously. Examinations of prepubertal mice at 25 days of age showed that p27<sup>Kip1</sup> single-null mice displayed gigantism (Fig. 1A), as described previously (18, 22, 23). Their body weights were 44% higher than those of wild-type mice. No further increase in body weights was observed p21<sup>Cip1</sup>-/- × p27<sup>Kip1</sup>-/- mice, suggesting no cooperation of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> in body growth. Ovaries of p27<sup>Kip1</sup> single-null mice were enlarged, weighing 2.5-fold more than wild-type ovaries (Fig. 1, B and C). Ovaries of p21<sup>Cip1</sup>-/- × p27<sup>Kip1</sup>-/- mice were even heavier, showing a 3.3-fold increase over wild-type weights. In contrast, p21<sup>Cip1</sup> single-null mice did not show significant enlargement of bodies or ovaries. These observations indicate that double deficiency for the two Cdk inhibitors results in more remarkable effects on ovary tissue growth than p27<sup>Kip1</sup>-single deficiency.

Hyperproliferation of Luteinizing Granulosa Cells in p27<sup>Kip1</sup>, p27<sup>Kip1</sup> Double-null Mice—Ovulation and fertilization occur normally in p27<sup>Kip1</sup> single-null females. However, implantation is perturbed with sub-optimal steroid regulation (17). Examinations of oviducts after superovulation demonstrated that ovulation occurred in the p21<sup>Cip1</sup>-/- × p27<sup>Kip1</sup>-/- mice with...
slightly reduced numbers of ova compared with those in wild-type mice (30.0 ± 6.0 versus 38.8 ± 1.3, mean ± S.E., n = 4). After mating with normal fertile males, embryos in oviducts appeared intact with proper fertilization.

Within 4–6 h post-LH surge, luteinizing granulosa cells in wild-type mice ceased DNA replication, whereas p21Cip1-expressing cells continued to proliferate after passage 7. However, p27Kip1-null luteal cells continued to proliferate beyond passage 7. In contrast, p21Cip1-null ovaries showed minimum proliferation of luteal cells at 48 h post-hCG (Fig. 2, B and C). These data suggest that p27Kip1 plays a limiting role in terminal divisions of luteinizing granulosa cells, p21Cip1 cooperates in this regulation, which becomes obvious especially in the absence of p27Kip1.

**Increased Apoptosis in Corpora Lutea of p21Cip1, p27Kip1 Double-null Mice—**Continued proliferation of p21Cip1, p27Kip1 double-null luteal cells beyond 96 h post-hCG suggested that the deficiency of the two Cdk inhibitors might immortalize or transform granulosa/luteal cells. However, we observed no spontaneous ovarian tumors in p21Cip1−/−p27Kip1−/− mice during 15-month-long observations. To investigate the fate of proliferating luteal cells, we examined apoptosis in ovaries after superovulation using the TUNEL assay (Fig. 4). Apoptosis was readily detectable in corpora lutea of p21Cip1−/−p27Kip1−/− ovaries at 48 h post-hCG and appeared more remarkable at 96 h. In contrast, wild-type ovaries did not display appreciable apoptosis in corpora lutea until 72 h. At 96 h, apoptosis was detected in wild-type corpora lutea, presumably because of the lack of luteotropins without mating. Therefore, highly proliferating luteinized granulosa cells in p21Cip1−/−p27Kip1−/− mice undergo robust apoptosis, possibly as a secondary response to the perturbed cell cycle control.

**Prolonged Proliferative Life Span of Cultured p21Cip1, p27Kip1 Double-null Granulosa Cells—**The continuous proliferation of luteal cells in superovulated p21Cip1−/−p27Kip1−/− ovaries prompted us to examine whether this phenotype is because of a change intrinsic to the granulosa/luteal cell or a change in the extracellular environment. We isolated granulosa cells from ovarian follicles of p21Cip1−/−p27Kip1−/− mice at 40 h after PMSG administration. Isolated cells were cultured under standard conditions, and proliferation was quantified by cell counts. Wild-type granulosa cells ceased proliferation after one passage (Fig. 5, A and B) and exhibited a flat enlarged phenotype after the first passage (Fig. 5C). The volume of each postmitotic cell was gradually increased through 10 days of continuous culture, and some cells became bi-nucleated, suggesting that these cells undergo senescence, as described previously (24). This is consistent the view that primary mouse cells are quite sensitive to culture stress-induced senescence (25, 26). p21Cip1 single-null granulosa cells displayed a proliferation kinetics similar to wild-type cells (Fig. 5, A and B) and underwent senescence (not shown). p27Kip1 single-null cells showed slow proliferation up to the second passage and then stopped dividing (Fig. 5B). In contrast, p21Cip1−/−p27Kip1−/− cells continued to proliferate beyond passage 7. However, p21Cip1−/−p27Kip1−/− granulosa cells were not completely immortal in vitro, as they slowed down and underwent senescence and/or cell death around passage 10 (data not shown).

**Differentialiation of Cultured p21Cip1, p27Kip1 Double-null Granulosa Cells—**To confirm that proliferating cells in...


p21Cip1 and p27Kip1 cultures are indeed granulosa/luteal cells, the expression of P450scc was examined by immuno-staining (Fig. 6A). Essentially all cells in p21Cip1 and p27Kip1 cultures examined at passage 5 expressed readily detectable levels of P450scc, indicating their steroidogenic activity. In contrast, human osteosarcoma U2OS cells, examined as a control, exhibited no significant staining for P450scc. Moreover, normal mouse embryonic fibroblasts (MEFs) displayed neither P450scc immunoreactivity (data not shown) nor P450scc mRNA in RT-PCR assays (Fig. 6B). These data indicate that p21Cip1 and p27Kip1 cultures expressed steroidogenic granulosa/luteal cells.

To determine the differentiation status of proliferating p21Cip1 and p27Kip1 granulosa cells, we examined the expression of genes characteristic to the granulosa/luteal cell type by semi-quantitative RT-PCR (Fig. 6C). The expression of cyclin D2 detected in cells at passage 4 was 40% reduced by treatment of cells with 0.5 mM 8-bromo-cAMP. The expression of LH receptor, which was relatively low in untreated cells, was up-regulated by 8-bromo-cAMP. Low levels of follicle-stimulating hormone receptor were also detected, which did not significantly respond to cAMP (data not shown). Prolactin receptor expression was undetectable in untreated proliferating cells, but both long and short forms of the transcripts were induced by 8-bromo-cAMP. P450scc expression was detectable in untreated cells and, remarkably, up-regulated by cAMP. In contrast, 3β-HSD expression was not significantly affected by 8-bromo-cAMP. Immunocytochemistry for P450scc confirmed that treatment with 8-bromo-cAMP increased expression of P450scc (Fig 6C). Cells became more round in morphology upon cAMP treatment, which is similar to the differentiation response in rat primary granulosa cells (24). Taken together, relatively high levels of P450scc and 3β-HSD suggest that p21Cip1, p27Kip1 double-null granulosa cells, which exhibit markedly prolonged proliferative life span in vitro, may be partially differentiated. The cells are still capable of undergoing further differentiation in response to cAMP accumulation, showing induction of prolactin receptor, accumulation of P450scc and P450arom, and down-regulation of cyclin D2.

DISCUSSION

Cessation of proliferation is normally coordinated with progression of differentiation essentially in all cell types. Multiple Cdk inhibitors are involved in the down-regulation of Cdk activities during differentiation (8, 27). Here we show that p27Kip1 and p21Cip1 play cooperative roles in quiescence of differentiating granulosa/luteal cells. p27Kip1 single deficiency prolongs proliferation during luteal differentiation, and p27Kip1, p21Cip1 double deficiency further enhances the prolif-

Figure 2: Hyperproliferation in corpora lutea of mice deficient for p21Cip1 and p27Kip1. A, immunohistochemistry for cholesterol side chain cleavage (P450scc), PCNA, and BrdUrd (BrdU). Mice at 25 days of age were superovulated, and ovaries were harvested 96 h post-hCG administration. BrdUrd was injected 2 h before sampling. B, quantification of PCNA+ cells. C, quantification of BrdUrd+ cells in wild-type (WT, open columns), p21Cip1−/− (dotted columns), p27Kip1−/− (hatched columns), and p21Cip1−/− p27Kip1−/− (closed columns) mice. Ovaries were sampled at the indicated times after hCG administration. Data from three independent animals are expressed as mean ± S.E. *, p < 0.05.

Figure 3: Cells proliferating in corpora lutea of p21Cip1−/− p27Kip1−/− mice are luteinizing. Mice at 25 days of age were superovulated, and ovaries were harvested 96 h post-hCG administration. BrdUrd was injected 2 h before sampling. BrdUrd (BrdU) or PCNA (green) and P450scc (red) were visualized by double immunofluorescent staining.
p21Cip1 exhibit markedly decreased levels of cyclin D1-Cdk4 (28). Consistently, MEFs lacking p27Kip1 and p21Cip1 suggest that p27Kip1 is a major rate-limiting factor for the exit effects on proliferation of luteinizing cells. These observations contrast, the cooperative role for p21Cip1 in the timely establishment of differentiating granulosa cells from the cell cycle. In contrast, loss of p21Cip1 alone shows little capacity. In contrast, loss of p21Cip1 alone shows little effects on proliferation of luteinizing cells. These observations suggest that p27Kip1 is a major rate-limiting factor for the exit of differentiating granulosa cells from the cell cycle. In contrast, the cooperative role for p21Cip1 in the timely establishment of quiescence is dispensable by itself. In normal rodent ovaries, an increase in p21Cip1 expression is observed within 6 h after luteinization-inducing LH surge, preceding a marked increase in p27Kip1 expression after 12 h (14). The up-regulation in P450scc expression, an indicator of differentiation, occurs within 4–6 h after the LH receptor signal, regardless of the status of the p27Kip1 and p21Cip1 genes (data not shown). Although previous studies suggest a reciprocal correlation between differentiation and proliferation in various cell types, including granulosa cells (24), our studies indicate that luteal differentiation and cell cycle exit are regulated by distinct mechanisms and can be uncoupled. As another example of such uncoupling between cell cycle progression and differentiation, cyclin D2-null mice exhibit defective proliferation with intact luteiniztion (13, 14). Both p27Kip1 and p21Cip1 bind to cyclin D-Cdk4 (or Cdk6) and cyclin E (or A)-Cdk2 complexes (9). In proliferating cells, the association of the Kip/Cip proteins with cyclin D-Cdk is not inhibitory to the kinase activity, whereas their association with cyclin E (or A)-Cdk2 always inhibits the catalytic subunit. Indeed, p27Kip1 and p21Cip1 promote the assembly of D-type cyclins and Cdk4 (28). Consistently, MEFs lacking p27Kip1 and p21Cip1 exhibit markedly decreased levels of cyclin D-Cdk complexes and aberrantly high activity of cyclin E-Cdk2 (29). In MEFs lacking Cdk4, the ternary p27Kip1-cyclin E-Cdk2 complexes are increased, leading to a higher inhibitory threshold to the Cdk2 activation and delayed G1-S transition (30). Thus, cyclin D-Cdk complexes titrate p27Kip1 and p21Cip1, regulating the availability of the inhibitors to inactivate Cdk2. We observed that the ovaries of superovulated p27Kip1, p21Cip1 double-null mice have aberrantly high Cdk2 activity, suggesting that continuous proliferation of luteinized granulosa cells depends on elevated Cdk2 activity. In addition, cyclin D2-dependent kinase, which is required for proliferation of undifferentiated granulosa cells (13, 14), could also play a role in the prolonged proliferative life span. The large dose of 8-bromo-cAMP, which promotes further differentiation of cultured p27Kip1, p21Cip1 double-null granulosa cells, also inhibits cell cycle progression (data not shown), with concomitant suppression of cyclin D2 expression (Fig. 6B). Although the assembly of cyclin D and Cdk4 is diminished in MEFs lacking p27Kip1 and p21Cip1, the activities of cyclin D-dependent kinases are detectable, and the cells are sensitive to p16INK4a-induced G1 arrest (31, 32). These observations imply that in the absence of

**Fig. 4. Enhanced apoptosis in ovaries lacking p21Cip1 and p27Kip1.** A, Wild-type (WT) and p21Cip1−/− p27Kip1−/− mice at 25 days of age were superovulated, and ovaries were harvested at the indicated times after hCG administration. Cells undergoing apoptosis were visualized by the fluorescein-based TUNEL assay. B, quantification of TUNEL-positive cell counts in corpora lutea at 48 and 96 h after hCG administration from wild-type (open columns) and p21Cip1−/− p27Kip1−/− (closed columns) mice. Numbers of TUNEL-positive cells per field (40× magnification) were counted. Fields were randomly chosen within each corpus luteum, and three corpora lutea per mouse were analyzed. Data are expressed as mean ± S.E. from three individual mice. *, p < 0.05.

**Fig. 5. Markedly prolonged proliferative life span of p21Cip1−/− p27Kip1−/− granulosa cells in culture.** A, growth curves of granulosa cells at the first passage. Cells were isolated from preovulatory follicles in PMSG-treated mice with the indicated genotypes. Culture was initiated at the density of 2×105 cells/2.2-cm well (3.8 cm2) as described under “Experimental Procedures.” B, cells were passed every 5 days, and cumulative cell numbers are shown. Data are shown as the averages of three independent preparations. C, the morphology of cultured granulosa cells after one passage. The asterisks indicate a senescence-like morphology, and the arrows show luteinized cells. WT, wild type.
Granulosa Cells from p27- and p21-null Mice

Fig. 6. Granulosa cells lacking p21Cip1 and p27Kip1 retain differentiation responses to cAMP after prolonged culture. A. P450scc immunostaining and 4,6-diamidino-2-phenylindole (DAPI) nuclear staining with cultured p21Cip1−/− p27Kip1−/− granulosa cells (GC) at passage 5 and human osteosarcoma U2OS cells (U2OS). B, the expression of granulosa/fetal cell-specific genes in p21Cip1−/− p27Kip1−/− cells at passage 4 was examined by RT-PCR after 48 h of incubation with 0.5 mM 8-bromo-cAMP. Data from two different cell preparations are shown as duplicates. MEFs and whole ovaries from 6-week-old wild-type mice were used as negative and positive controls. LHR, luteinizing hormone receptor; PRLR, prolactin receptor; P450arom, aromatase; L19, ribosomal RNA L19. C, the morphology and the expression of P450scc in p21Cip1−/− p27Kip1−/− cells at passage 4 treated with or without 0.5 mM 8-bromo-cAMP for 48 h.

p27Kip1 and p21Cip1, cyclin D-Cdk complexes are still required for proliferation.

Most Cdk inhibitors are ubiquitously expressed, and cooperation between multiple Cdk inhibitors seems to be critical for development and function of various tissues. We have recently demonstrated that increased p21Cip1 expression plays a role in maintaining quiescence of hepatocytes in adult p27Kip1-deficient mice (33). Cooperation between p27Kip1 and p57Kip2 in development of the lens and placenta has been demonstrated in mice deficient for these two Cdk inhibitors (34). p21Cip1 and p57Kip2 cooperate in differentiation of skeletal muscle and alveoli in the lung (35). The two Ink4-type inhibitors, p18Ink4a and p19Arf, cooperate for spermatogenesis (36). Thus, the negative regulation of cell cycle progression, particularly during development, could depend on various combinations of Cdk inhibitors in cell type-specific manners.

The present study is unique in demonstrating that the absence of multiple Cdk inhibitors prolongs the proliferative life span of primary rodent cells ex vivo. p27Kip1, p21Cip1 double-null granulosa cells could continue proliferation up to 10 passages, whereas wild-type and p21Cip1 or p27Kip1 single-null granulosa cells cease proliferation almost immediately when placed in culture. Consistently, a previous study showed that primary rat granulosa cells can divide no more than once in culture (24). It has been thought that the culture environment rapidly leads primary granulosa cells to spontaneous differentiation. Deficiency for p27Kip1 and p21Cip1 overcomes the cell cycle inhibitory effect of culture, but these cells are neither immortal nor transformed. p27Kip1, p21Cip1 double-null granulosa cells continue to express granulosa cell-specific genes such as P450scc, 3β-HSD, follicle-stimulating hormone receptor, and LH receptor, through 8–10 passages. Relatively high expression of P450scc and low expression of follicle-stimulating hormone receptor suggest that these proliferating cells are differentiated (luteinized) to some extent, presumably in response to the culture environment. However, treatment with 8-bromo-cAMP induces prolactin receptors and P450arom and further increases P450scc and LH receptors. Progesterone secretion into the culture medium is also increased by cAMP treatment (data not shown). These data indicate that p27Kip1, p21Cip1-deficient granulosa cells are capable of further differentiation even after several passages in culture. The large dose of 8-bromo-cAMP also inhibits proliferation of p27Kip1, p21Cip1-deficient granulosa cells with a reduction in cyclin D2 expression. Thus, the absence of the two Cdk inhibitors does not create complete resistance to differentiation-associated cell cycle arrest, despite its proliferation-enhancing effect.

Continuous passages of primary fibroblasts or epithelial cells in culture induce replicative senescence with the characteristic flat-enlarged morphology (37). Senescence involves the Arf-p53-p21Cip1 and p16Ink4a-retinoblastoma protein tumor-repressive pathways (26, 38, 39). MEFs lacking p53 or Arf display an immortal phenotype ex vivo devoid of senescence (26, 40). In contrast, MEFs deficient for p16Ink4a or p21Cip1 show normal senescence in culture (41–43). MEFs lacking p53 or Arf also exhibit a normal senescence response. Wild-type granulosa cells develop a senescence phenotype within several days in culture, sometimes displaying a bi-nucleated morphology similar to senescent fibroblasts (Fig. 5C) (24). The markedly prolonged proliferative life span of p27Kip1, p21Cip1 double-null granulosa cells implies that these two inhibitors may cooperate not only in differentiation-associated withdrawal from the cell cycle but also in inducing senescence to maintain irreversible quiescence. However, it should be noted that p27Kip1-null granulosa cells are not completely devoid of senescence. Continuous culture beyond passage 10 results in dominance of the senescent morphology, and most cells undergo apoptosis thereafter. This crisis-like cell death may be associated with enhanced apoptosis of corpora lutea in p27Kip1, p21Cip1 double-null mice (Fig. 4), although the mechanism of apoptosis is unclear.

In summary, the absence of the two Cdk inhibitors p27Kip1 and p21Cip1 creates a unique cellular condition that extends the proliferative life span of granulosa cells without losing the

3 H. Kiyokawa, unpublished observations.
potential of differentiation. This experimental model provides a useful tool for the study of cell cycle control during differentiation and senescence.

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