Hormone-Induced Proliferation and Differentiation of Granulosa Cells: A Coordinated Balance of the Cell Cycle Regulators Cyclin D2 and $p27^{Kip1}$

Rebecca L. Robker and JoAnne S. Richards

Department of Cell Biology
Baylor College of Medicine
Houston, Texas 77030

The proliferation and terminal differentiation of granulosa cells are critical for normal follicular growth, ovulation, and luteinization. Therefore, the *in situ* localization and hormonal regulation of cell cycle activators (cyclin D1, D2, and D3) and cell cycle inhibitors ($p27^{Kip1}$ and $p21^{Cip1}$) were analyzed in ovaries of mice and rats at defined stages of follicular growth and differentiation. Cyclin D2 mRNA was specifically localized to granulosa cells of growing follicles, while cyclin D1 and cyclin D3 were restricted to theca cells. In hypophysectomized (H) rats, cyclin D2 mRNA and protein were increased in granulosa cells by treatment with estradiol or FSH and were increased maximally by treatment with both hormones. In serum-free cultures of rat granulosa cells, cyclin D2 mRNA was rapidly elevated in response to FSH, forskolin, and estradiol, indicating that estradiol as well as cAMP can act directly and independently to increase cyclin D2 expression. The levels of $p27^{Kip1}$ protein were not increased in response to estradiol or FSH. In contrast, when ovulatory doses of human CG (LH) were administered to hormonally primed H rats to stimulate luteinization, cyclin D2 mRNA and protein were rapidly decreased and undetectable within 4 h, specifically in granulosa cells of large follicles. Also in response to LH, the expression of the cell cycle inhibitor $p27^{Kip1}$ was induced between 12 and 24 h ($p21^{Cip1}$ was induced within 4 h) and remained elevated specifically in luteal tissue. A critical role for cyclin D2 in the hormone-dependent phase of follicular growth is illustrated by the ovarian follicles of cyclin $D2^{-/-}$ mice, which do not undergo rapid growth in response to hormones, but do express markers of FSH/LH action, cell cycle exit, and terminal differentiation. Collectively, these data indicate that FSH and estradiol regulate granulosa cell proliferation during the development of preovulatory follicles by increasing levels of cyclin D2 relative to $p27^{Kip1}$ and that LH terminates follicular growth by down-regulating cyclin D2 concurrent with up-regulation of $p27^{Kip1}$ and $p21^{Cip1}$. (Molecular Endocrinology 12: 924–940, 1998)

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INTRODUCTION

Cellular proliferation and differentiation are fundamental biological processes controlled by extracellular signals that impinge upon cell cycle regulatory machinery and modulate gene expression. In the ovary, hormones control the development of individual follicles by triggering sequential, dynamic changes in granulosa cell proliferation and gene expression (1–3). In nongrowing, primordial follicles, a single layer of granulosa cells arrested in G_0 surrounds the oocyte. In response to unknown signals primordial follicles leave the resting pool, and granulosa cells initiate a phase of growth in which proliferation is exceedingly slow. Studies measuring uptake of [^{3}H]thymidine have shown that granulosa cell proliferation at this time proceeds at a "glacial" rate, requiring an estimated 50 days for a multilayer preantral follicle to develop (1). The granulosa cells of these small growing follicles acquire FSH and estrogen responsiveness (4, 5) and when exposed to these hormones begin to proliferate rapidly (6). The resulting phase of exponential growth enables the development of large preovulatory follicles to occur within an additional 3 days (1). In the hypophysectomized rat (4, 7) as well as in mutant mice lacking either gonadotropins (8, 9), FSH (10), or estrogen receptor-α (11), follicular development is arrested at the preantral stage. These models illustrate that although the early/slow stages of granulosa cell proliferation and follicle growth occur in the absence of gonadotropins and estradiol, these hormones are required for normal growth, including the final rapid stages of development that form preovulatory follicles.

In response to the surge of LH that triggers ovulation, granulosa cells of preovulatory follicles cease dividing (1, 6) and initiate a program of terminal differentiation (12, 13). Whereas proliferation occurs over days, the cessation of granulosa cell division is rapid and within 7 h of exposure to LH, these cells are irreversibly committed to become luteal cells (14). These dramatic events indicate that hormones such as FSH, estradiol, and LH control both the rate of granulosa cell proliferation, as well as exit from the cell cycle during differentiation. With the recent explosion of information concerning cell cycle-regulatory molecules, the mechanisms involved in controlling successive stages of granulosa cell proliferation and terminal differentiation can begin to be elucidated.

Cellular proliferation is controlled by molecules positively or negatively affecting the kinase cascades that regulate transitions through the checkpoints of the cell cycle. Briefly summarized (for reviews, see Refs. 15–21), entry into the cell cycle is positively regulated by D-cyclins, D1, D2, or D3 (22, 23). The D-cyclins bind cyclin-dependent kinase (cdk) 4 or cdk6, which allows phosphorylation by cdk-activating kinase (CAK), thereby forming an active complex. The cyclin-cdk complex phosphorylates cellular substrates, including the retinoblastoma protein RB and related proteins, leading to activation of the E2F family of transcription factors. This results in the activation of numerous genes involved in DNA synthesis and ultimately enables cells to begin the transition from G_1 to S phase. Further progression depends on activation of cyclin E-cdk2 complexes and the sequential activation of additional cdks. In contrast to cyclins, cdk inhibitors (or CKIs) are negative regulators that arrest the cell cycle in G_1 by binding and inhibiting the activity of cyclin-cdk complexes. The Cip/Kip family of CKIs, $p21^{Cip1}$ (24–28), $p27^{Kip1}$ (29, 30), and $p57^{Kip2}$ (31, 32), bind cyclinD-cdk4/6 as well as cyclinE-cdk2 complexes and prevent their activation.

These regulatory molecules, D cyclins and Cip/Kips, although widely expressed in overlapping as well as tissue-specific patterns (28, 32–34), can exert dramatic effects on cell cycle progression when their relative amounts are altered by external signals. For instance, when overexpressed in fibroblasts, cyclin D2 stimulated a shortened G_1 phase and reduced cell generation time, and reduced cellular dependence on serum for growth (35). When proliferating macrophages were treated with cAMP, it caused p27 expression to increase and triggered cell cycle arrest (36). Expression of p27 was also increased when glial cell precursors were induced to differentiate into astrocytes (37). Of particular interest, the targeted deletion of cyclin D2 and p27 genes indicated that they are essential for normal ovarian function. In mice null for cyclin D2 (cyclin $D2^{-/-}$), granulosa cell proliferation is impaired, the ovarian follicles remain small, and ovulation fails to occur (38). In mice null for p27 ($p27^{-/-}$), follicular growth is not compromised but granulosa cells do not luteinize properly in response to LH leading to female infertility (39–41).

These advances in our understanding of hormonal control of ovarian function and of the roles of cell cycle regulatory molecules have proceeded independently. Therefore, we sought to determine when cyclin D2 and p27 are expressed in the ovary and whether they are regulated by hormones at specific stages of follicular growth and luteinization. Since cyclin D2 is a rate-limiting activator of cell cycle progression and essential for granulosa cell proliferation, we have investigated whether the hormones, FSH and estradiol, known to trigger rapid granulosa cell proliferation during the formation of preovulatory follicles, also regulate cyclin D2. Since p27 is known to be involved in exit from the cell cycle and, in particular, in the differentiation of granulosa cells to luteal cells, we analyzed its regulation by the surge of LH. Our results demonstrate that gonadotropins and steroids, acting via cAMP and estrogen receptor-mediated mechanisms, impinge upon the cell cycle regulatory machinery to control the function and fate of granulosa cells.

RESULTS

Expression of Cyclin D2, $p27^{Kip1}$, and Proliferating Cell Nuclear Antigen (PCNA) Is Cell Specific

The cellular localization of cyclin D2 and p27 expression in ovaries of normal mice was analyzed by *in situ* hybridization and related to cellular proliferation status by immunolocalization of PCNA, a cofactor of DNA polymerase δ and cyclin-cdk complexes (42). PCNA is expressed in proliferating cells (43) and has been shown in the rat ovary to be a sensitive marker of granulosa cell proliferation (44). In mice treated with PMSG to stimulate the growth of preovulatory follicles, cyclin D2 mRNA was localized specifically to the granulosa cells (Fig. 1). The abundance of PCNA in these cells indicates that they are proliferating. This pattern of expression was specific to cyclin D2. *In situ* hybridization using antisense probes for cyclin D1 and cyclin D3 showed their mRNA predominantly in the theca and interstitial cells. Therefore, the proliferating granulosa cells in the ovary specifically express cyclin D2.

When the expression of $p27^{Kip1}$ was analyzed in mouse ovaries, it was localized to the corpora lutea (Fig. 2) in ovaries from mice treated with PMSG followed by hCG to trigger ovulation and luteinization. PCNA was absent in the luteal cells that are no longer dividing and have terminally differentiated. Interestingly, mRNA for $p21^{Cip1}$, another member of the Cip/Kip family of cdk inhibitors, was also selectively localized to luteal cells (Fig. 2), indicating that these cdk inhibitors are specifically expressed in these terminally differentiated cells.

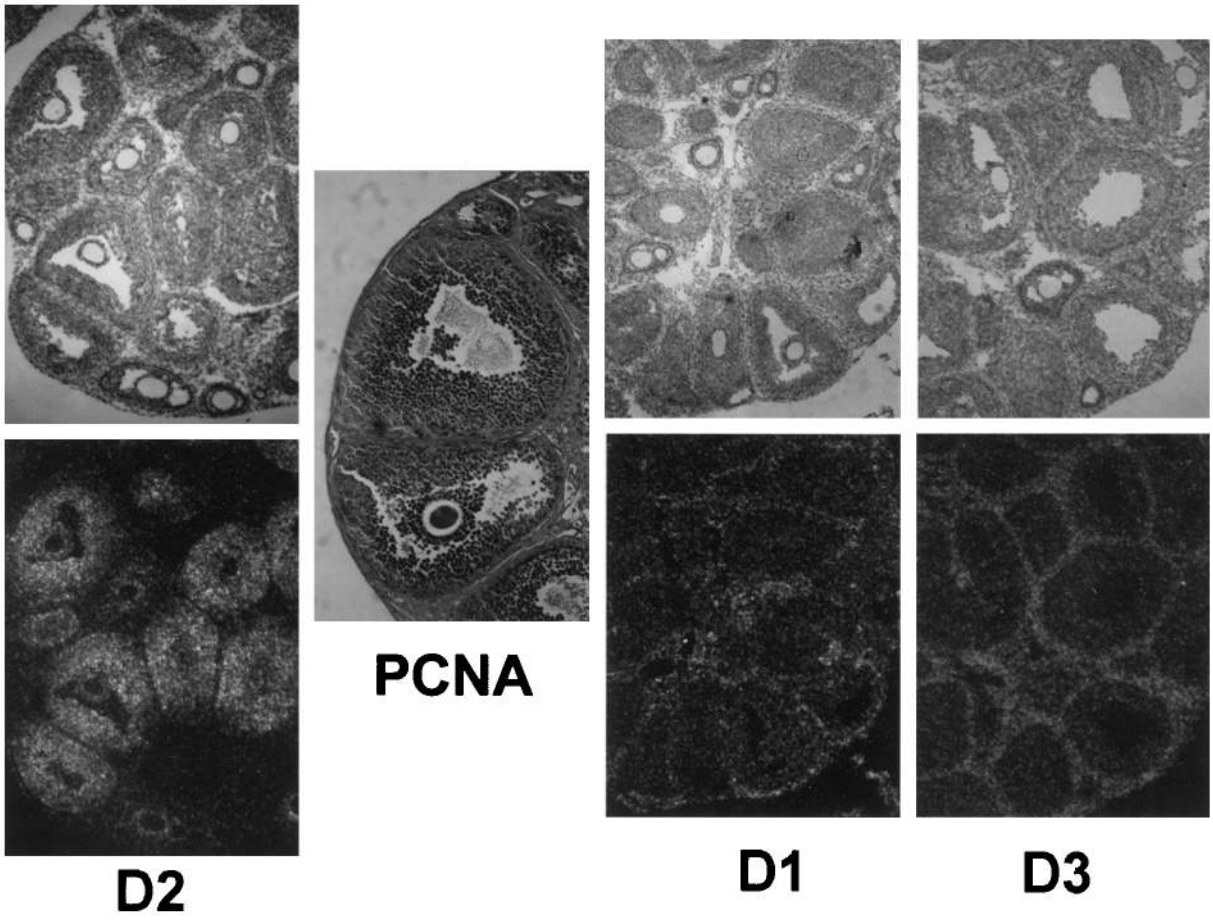

Fig. 1. Localization of D-type Cyclins and PCNA in the Preovulatory Mouse Ovary

Immature mice were treated with 5 IU PMSG to stimulate follicular growth, and ovaries were isolated 48 h later. Cyclin D2, cyclin D1, and cyclin D3 mRNA were analyzed by *in situ* hybridization using specific cDNA antisense probes. Hybridization signal is visualized in dark field illumination. Magnification is 100×. PCNA immunodetection of proliferating cells (intense black nuclear staining) was analyzed using anti-PCNA (Novacastra) and anti-IgG peroxidase-linked antibodies. Magnification is 200×.

Cyclin D2 Is Expressed during Early Follicular Growth

To determine whether cyclin D2 and p27 were expressed during the early stages of follicular development, *in situ* hybridization experiments were performed on ovaries from mouse pups on days 5 and 10 of age as well as ovaries from mutant mice in which follicular growth is arrested at early stages in development (Fig. 3). Ovaries from mice at day 5 (D5) and 10 (D10) of age contain growing follicles that consist of only one or two layers of granulosa cells. Although serum FSH increases between day 5 and 10, the follicles of these prepubertal mice do not mature and do not synthesize appreciable amounts of estradiol (45). Despite the small size of these follicles and the slow proliferative rate (1, 2, 46), cyclin D2 mRNA is expressed in the granulosa cells.

Recently, the transforming growth factor-β-related growth differentiation factor-9 (GDF-9), was shown to be essential for normal follicular development (47, 48). In mice null for gdf-9 (gdf-9$^{-/-}$), the ovaries appear abnormal but do contain follicles at an early/primary stage of development. Although the follicles contain only a single layer of granulosa cells, cyclin D2 mRNA is present (Fig. 3). FSH has long been known to be important for follicular growth (4). In mice null for the FSHβ subunit (FSH$\beta^{-/-}$), follicular growth progresses until follicles are comprised of several layers of granulosa cells, but arrests before antral formation indicating that only early stages of follicular growth are gonadotropin independent (10). Cyclin D2 is also expressed in the granulosa cells of the small follicles in the FSH$\beta^{-/-}$ mice (Fig. 3). *In situ* hybridization performed on ovarian sections from these same mice using a p27 antisense probe showed that p27 mRNA is expressed at low levels and not localized to any particular cell type (data not shown). Collectively, these results demonstrate that cyclin D2 is expressed very early during the course of follicular growth, *i.e.* in all follicles that have initiated growth from the primordial stage. Second, the expression of cyclin D2 mRNA in granulosa cells of small follicles is gonadotropin independent and can occur in the absence of FSH,

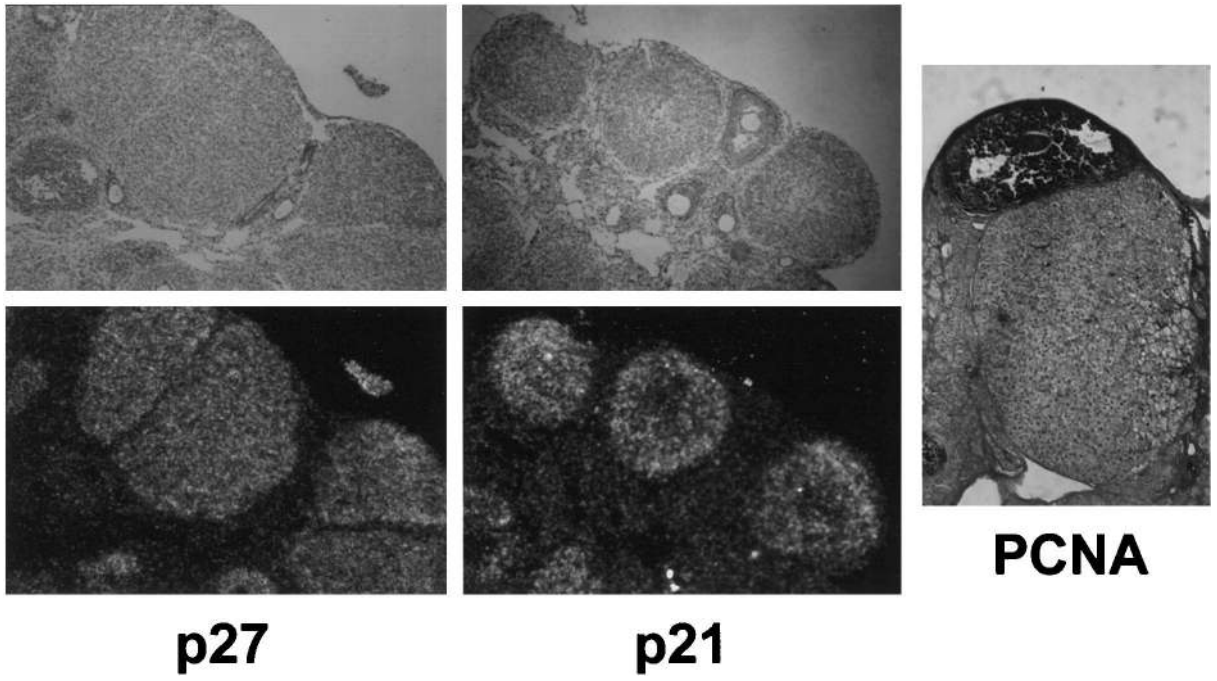

Fig. 2. Localization of $p27^{Kip1}$, $p21^{Cip1}$, and PCNA in the Luteinized Ovary

Immature mice were treated with 5 IU hCG 46 h after PMSG to trigger ovulation and luteinization, and ovaries were isolated 48 h later. Expression of p27 and p21 was analyzed by *in situ* hybridization using cDNA antisense probes. Magnification is 100×. PCNA was analyzed by immunodetection as in Fig. 1. Magnification is 200×.

estradiol, and GDF-9. Third, in these early stages of proliferation and follicular growth, granulosa cells lack appreciable levels of p27.

In the mice analyzed above, the follicles are in a gonadotropin-independent phase of growth, and granulosa cell proliferation is slow (1, 2). In contrast, the most rapid phase of granulosa cell proliferation occurs when small follicles become responsive to gonadotropins and begin to synthesize estradiol (1, 6). To determine whether these hormones regulate the expression of cyclin D2 or p27, we used the hypophysectomized rat model, which enables precise hormonal treatments, provides sufficient material for quantitative studies, and allows for comparison with the mouse. Most importantly, it is a physiological model in which the effects of estradiol, FSH, and LH on ovarian cell proliferation and function have been well characterized (4, 5, 7).

Cyclin D2 and $p27^{Kip1}$ Are Hormonally Regulated during Follicular Development

In the hypophysectomized (H) rat, follicular growth is initiated but not sustained due to lack of gonadotropins and steroids (4). Treatment of H rats with estradiol for 3 days (HE) promotes the proliferation of granulosa cells (6) and growth of large preantral follicles (4–6). Subsequent treatment of HE rats with FSH for 2 days (HEF) stimulates further proliferation and promotes the growth of large antral follicles that exhibit characteristics of normal preovulatory follicles (4–6). Finally, an ovulatory dose of hCG triggers ovulation and luteinization. The expression of cyclin D2 and p27 mRNA in the ovaries of these rats was analyzed by *in situ* hybridization (Fig. 4A). In H rats, cyclin D2 mRNA is expressed at low levels and is localized to the granulosa cells of the small growing follicles, a pattern similar to that observed in the follicles of $FSH\beta^{-/-}$ or prepubertal mice (Fig. 3). In ovaries of HEF rats, cyclin D2 mRNA expression was increased and specifically localized to the rapidly proliferating granulosa cells of large preovulatory follicles. In response to hCG, cyclin D2 mRNA was rapidly down-regulated within 4 h (HEF hCG4 h) in the large follicles that are committed to ovulate and luteinize and remained low in the terminally differentiated corpora lutea (HEF hCG48 h). In these same ovaries, cyclin D2 expression was not down-regulated in the small follicles; these lack LH receptor and therefore are not exposed to elevated levels of intracellular cAMP (3, 4, 49–51).

In situ hybridization of sections from these same animals using an antisense probe for p27 revealed a distinctly different pattern of expression (Fig. 4B). p27 mRNA was expressed at low levels throughout the ovary in H rats, but appeared to be preferentially localized to granulosa cells of the preovulatory follicles in HEF rats. Treatment with hCG resulted in decreased expression of p27 within 4 h but this effect was transient. Highest levels of p27 mRNA were observed 48 h

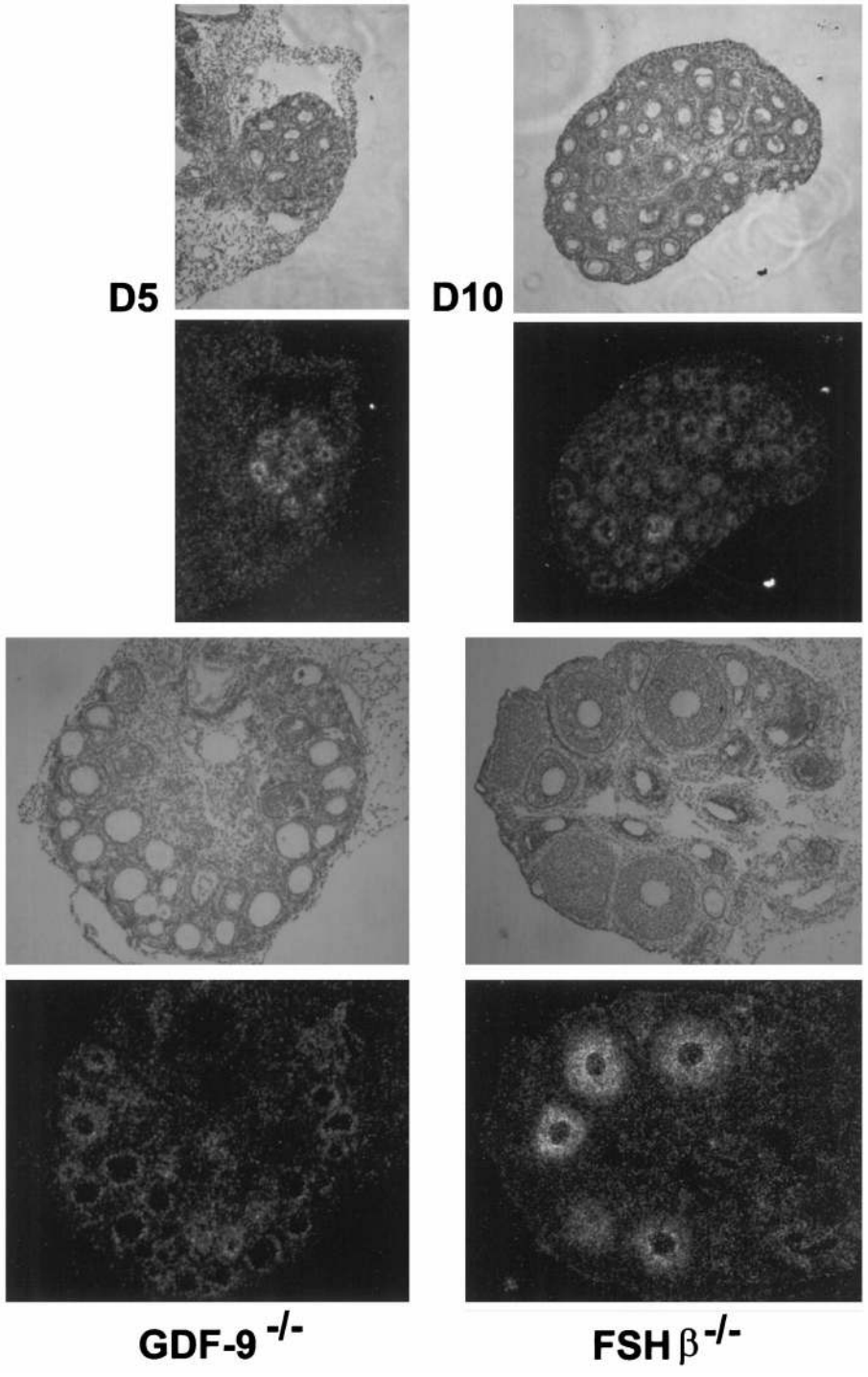

Fig. 3. Expression of Cyclin D2 in Small Follicles

In situ hybridization of cyclin D2 in ovaries from 5- (D5) and 10- (D10) day-old mouse pups and adult gdf-9$^{-/-}$ mice and FSH$\beta^{-/-}$ mice using a cDNA antisense probe. Magnification of D5 and D10 is 200×; gdf-9$^{-/-}$ and FSH$\beta^{-/-}$ are 100×.

after hCG specifically in the terminally differentiated luteal cells.

To provide a more detailed and quantitative analysis of the effects of estradiol, FSH, and LH on cyclin D2 and p27 expression, RNA and protein were obtained from granulosa cells and corpora lutea isolated from the ovaries of hormone-treated H rats. Northern analysis (Fig. 5A) using the cyclin D2 cDNA as probe showed that cyclin D2 transcripts of approximately 6 kb and 2.5 kb (34, 52) were present at low levels in granulosa cells of H rats. Cyclin D2 mRNA levels were greatly increased by estradiol treatment alone (HE)

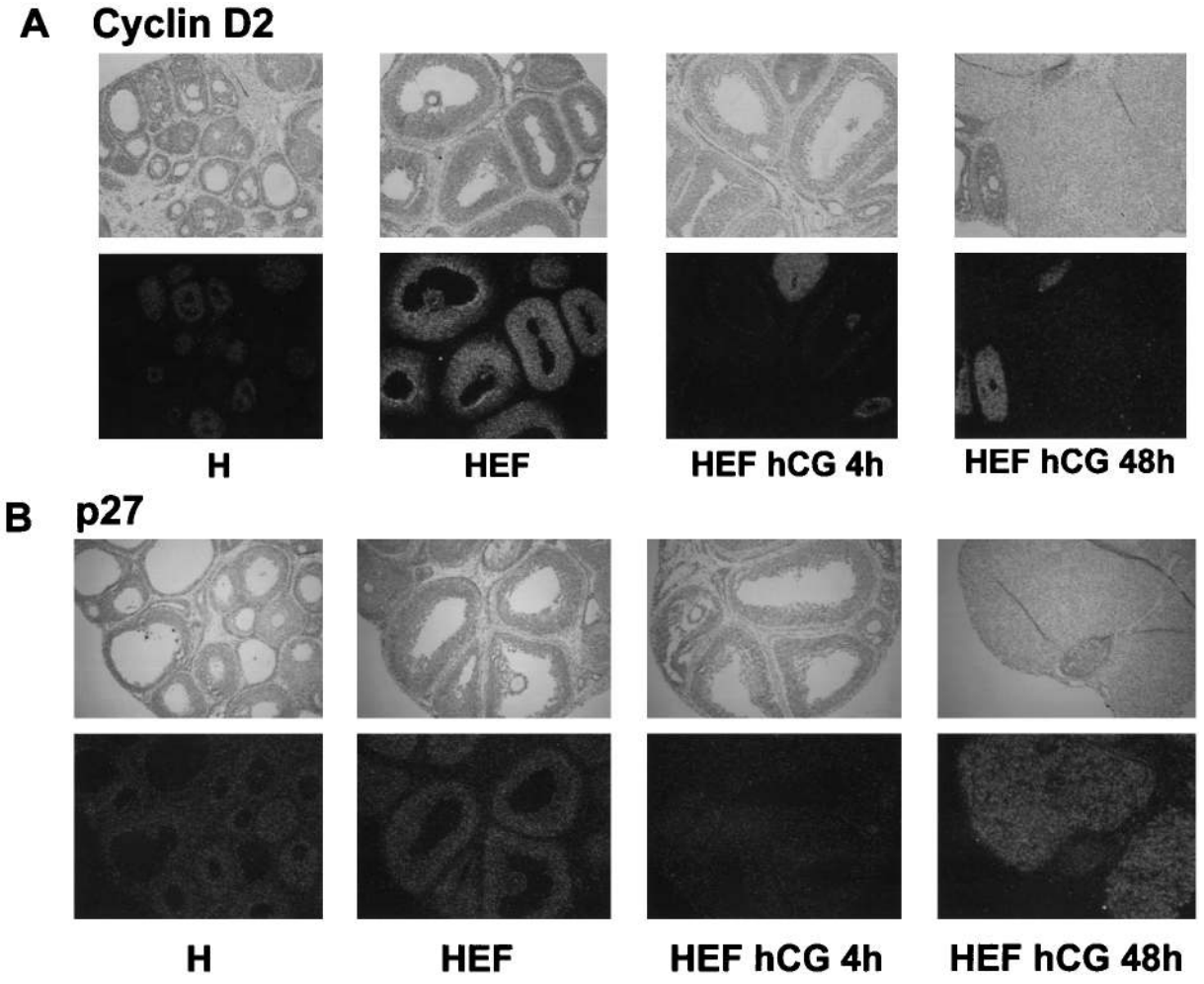

Fig. 4. Regulated Expression of Cyclin D2 and $p27^{Kip1}$ in the Rat Ovary

Hypophysectomized rats (H) were treated with estradiol (HE), estradiol followed by FSH (HEF), or HEF treatment followed by an ovulatory dose of hCG for 4 h (HEF hCG4 h) or 48 h (HEF hCG48 h), as described in *Materials and Methods*. Expression of cyclin D2 (A) or p27 (B) was analyzed by *in situ* hybridization of ovarian sections from the same animal using antisense probes. Similar results were observed in ovaries of other rats.

and subsequent treatment with FSH (HEF) sustained the elevated expression of the 6-kb transcript and increased the 2.5-kb transcript. Consistent with the *in situ* hybridization results, cyclin D2 mRNA decreased rapidly within 2 h following treatment with hCG and further by 8 h. Cyclin D2 mRNA levels remained low in corpora lutea isolated 24 and 48 h after hCG. Western analysis of cellular extracts showed that cyclin D2 protein is regulated in a similar manner: major increases occurred with E alone, were sustained by subsequent treatment with FSH, and were down-regulated in response to hCG (Fig. 5A, *lower panel*).

When the same Northern blot was probed with p27 cDNA (Fig. 5B), a transcript of approximately 2.4 kb (53) was present in granulosa cells of H and HE rats and increased in response to FSH. Consistent with results from *in situ* hybridization, p27 mRNA levels were transiently decreased in HEF rats 2 and 8 h after treatment with hCG, but increased in corpora lutea isolated at 24 and 48 h. (The additional band at 4 kb hybridizing to the p27 cDNA appears to be nonspecific and is not hormonally regulated.) Western analysis of cellular extracts showed that changes in levels of p27 protein were quite similar to those of its mRNA: p27 protein was present in granulosa cells of small and preovulatory follicles of H rats, exhibited a transient decrease in response to ovulatory hCG, but then increased to highest levels in corpora lutea. However, unlike its mRNA, p27 protein was lower in granulosa cells of HE rats compared with H rats and did not exhibit a marked increase in granulosa cells of HEF rats, indicating that the half-life of p27 protein and mRNA in response to estradiol and FSH may differ.

To dissect the effects of FSH *vs.* estradiol, and to determine whether FSH can independently regulate the expression of cyclin D2 expression, H rats were treated for short periods with either FSH or estradiol, granulosa cells were isolated at selected timepoints,

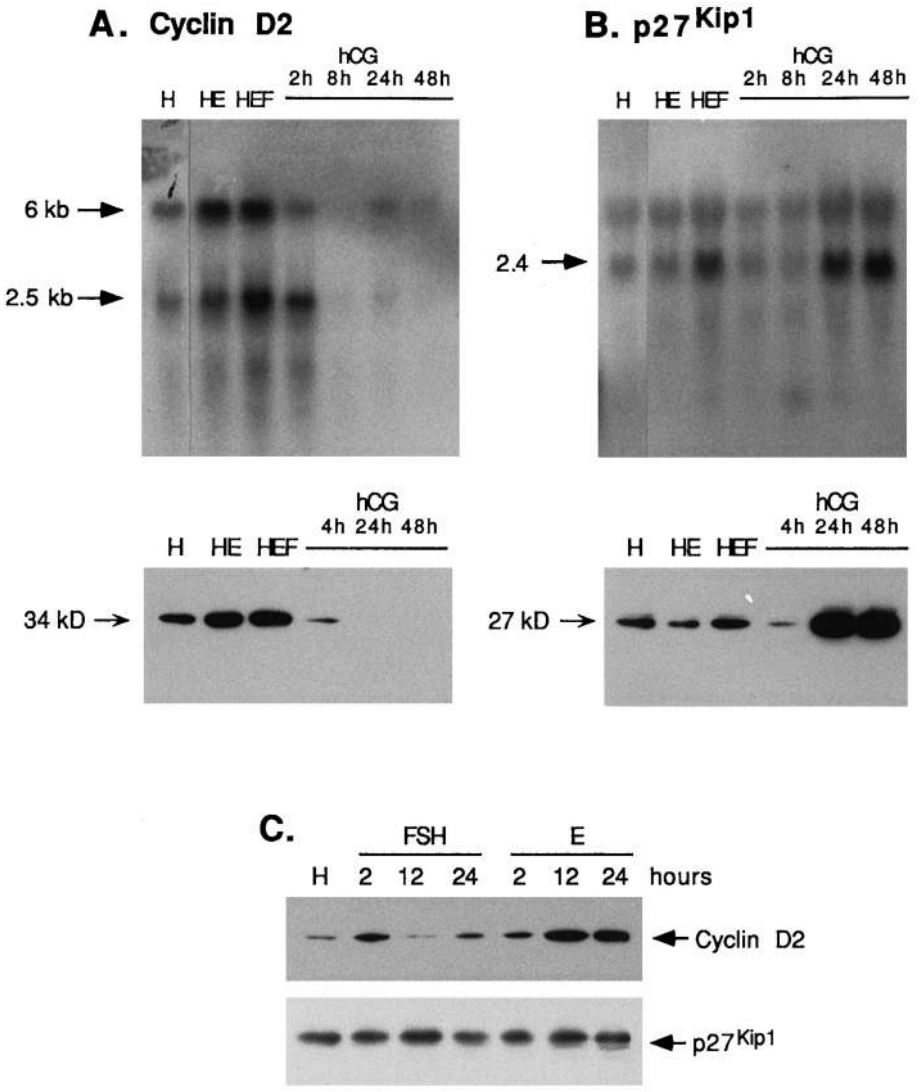

Fig. 5. Hormonal Regulation of Cyclin D2 and p27^{Kip1} mRNA and Protein

Expression of mRNA and protein for cyclin D2 (A) and p27 (B) using Northern (*upper panels*) and Western (*lower panels*) analysis, respectively. RNA and protein were isolated from granulosa cells and corpora lutea at the indicated times of hormone treatment. The Northern blot was initially probed with labeled cyclin D2 cDNA, allowed to decay, and subsequently reprobed with labeled p27 cDNA without stripping. Equal loading of total RNA was confirmed by acridine orange staining. For the Western blots, anti-cyclin D2 (Neomarkers) and anti-p27 (Santa Cruz) antibodies were used and the immunoreactive proteins detected by ECL. Levels of cyclin D2 and p27 protein in response to FSH or E by Western blotting (C). A single blot was sequentially probed with cyclin D2 and then p27. Data from densitometric analysis are included in the text.

and cellular protein was prepared. To stimulate a rapid response *in vivo* (*i.e*. at 2 and 12 h) rats were injected iv with 5.0 μg FSH, a dose previously shown to mimic the FSH surge by markedly elevating intracellular levels of cAMP (50). Additional rats were injected subcutaneously with FSH (1.0 μg) twice daily, a regimen that closely mimics basal levels of FSH and maintains granulosa cell function (5), and granulosa cells were isolated at 24 h. Estradiol treatments consisted of 1.5 mg estradiol injected ip (for 2 h) or subcutaneously (for 12 or 24 h). Western analysis of granulosa cell protein showed that each hormone alone regulates cyclin D2 protein levels and that each hormone causes a distinct temporal pattern of expression (Fig. 5C). Specifically, exposure of granulosa cells of H rats to elevated (surge) concentrations of FSH resulted in a marked 7.2-fold induction of cyclin D2 protein within 2 h compared to untreated H animals. Interestingly, the induction of cyclin D2 by elevated FSH was transient; protein levels returned to control levels (or below) after 12 h of FSH. In rats given the lower dose of FSH to mimic basal hormone secretion, cyclin D2 protein was elevated 2.6-fold by 24 h compared to untreated H controls. In contrast, the response to estradiol occurred more gradually, 2.8-fold increase by 2 h, but was then sustained at an elevated level, 9.7-fold at

24 h. When the same blot was reprobed using a p27 antibody, the levels of this protein were not acutely regulated by either FSH or estradiol. The fold induction in response to estradiol was never greater than 1.5-fold.

Cyclin D2 Is Regulated by FSH *in Vivo*

To further analyze the oscillating temporal expression pattern of cyclin D2 in response to FSH, H rats were injected iv with 5.0 μg FSH and granulosa cells were isolated at 2, 4, 8, and 12 h. Additional rats were injected subcutaneously with 1.0 μg FSH and granulosa cells were isolated at 24 and 48 h. Western analyses of granulosa cell extracts were analyzed using ECL (Fig. 6A, *upper*) and densitometric quantification (*lower*). As shown in Fig. 5C above and in additional experiments, the levels of cyclin D2 protein respond to acute as well as tonic treatment with FSH. High levels of FSH given iv stimulated a rapid increase (6.6 $\pm$ 1.7 fold; n = 3) in cyclin D2 protein at 2 h. This response was transient, and levels of cyclin D2 returned to near control levels at 4, 8, and 12 h (1.4 $\pm$ 0.5; n = 3) after FSH treatment. In response to the tonic dose of FSH given subcutaneously, cyclin D2 protein increased (2.8 $\pm$ 0.3; n = 3) at 24 h and 48 h. Analysis of p27 protein levels in these same extracts (Fig. 6B) showed that the increase in cyclin D2 in response to FSH is specific. When compared with untreated controls, the levels of p27 protein remained relatively constant at 2 h (1.2 $\pm$ 0.1; n = 3), 4 h, 8 h, 12 h (1.5 $\pm$ 0.1; n = 3), or 24 h (1.4 $\pm$ 0.5; n = 3) and were even decreased 50% at 48 h.

Cyclin D2 mRNA Is Induced by FSH, Forskolin, and Estradiol

The oscillatory pattern of cyclin D2 expression observed in granulosa cells after FSH treatment *in vivo* is even more dramatic when primary granulosa cells are cultured in the presence of FSH. Granulosa cells were isolated from estradiol-primed rats, cultured overnight in serum-free media to remove hormonal influences and synchronize the cells in G_0, and then treated with FSH (100 ng/ml). Northern analysis (Fig. 7A) showed that FSH increased cyclin D2 mRNA expression within 1 and 2 h (9.0 $\pm$ 2.4, n = 3), but that expression was decreased to 1.6-fold by 6 and 10 h (not shown). After 24 h of FSH, cyclin D2 is again elevated to high levels (10.2-fold) compared with cells cultured 24 h in the absence of hormone (−). The increases in cyclin D2 mRNA are induced specifically by FSH since the addition of 10% FBS, which contains numerous growth factors, did not affect cyclin D2 expression at 2 or 6 h.

To determine whether estradiol, as well as stimulation of the cAMP/protein kinase A pathway, could directly regulate cyclin D2 mRNA, granulosa cells were isolated from untreated immature rats and cultured overnight in serum-free medium. The cells were then treated with forskolin (10 μM), a direct activator of adenylyl cyclase (54) known to mimic the FSH-mediated activation of protein kinase A or estradiol (10 nM). Northern analysis of total RNA (Fig. 7B) showed that forskolin (Fo) increased cyclin D2 mRNA expression within 1 and 2 h compared to untreated cells (−). Estradiol had only a minimal effect after 1 h, but increased cyclin D2 mRNA expression within 2 h. This increase was specific since the estrogen receptor an-

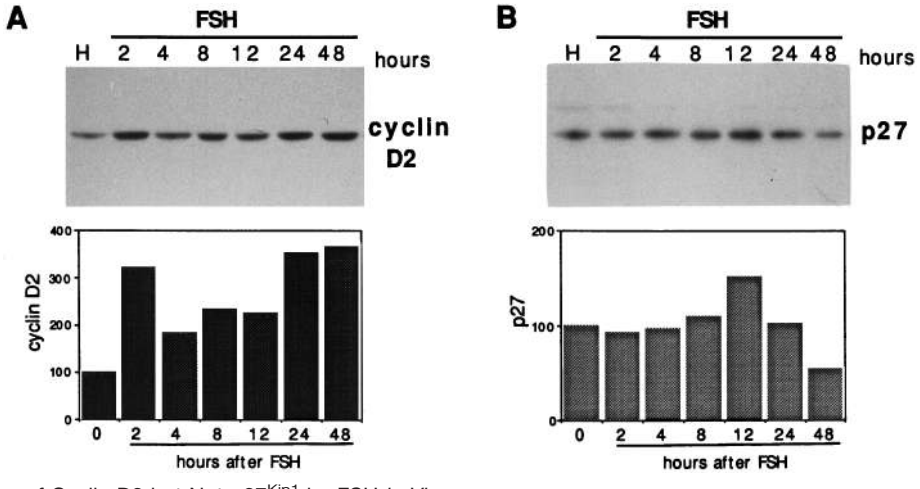

Fig. 6. Induction of Cyclin D2 but Not $p27^{Kip1}$ by FSH *in Vivo*

Western blot analysis of cyclin D2 (A) and p27 (B) protein in granulosa cell extracts from H rats treated with FSH for the indicated times. Experiments were analyzed using ECL detection (*autoradioagraph*) and densitometry (*graph*). Quantification depicts relative changes compared with untreated (H) rats. Similar results were obtained in three separate experiments as described in the text.

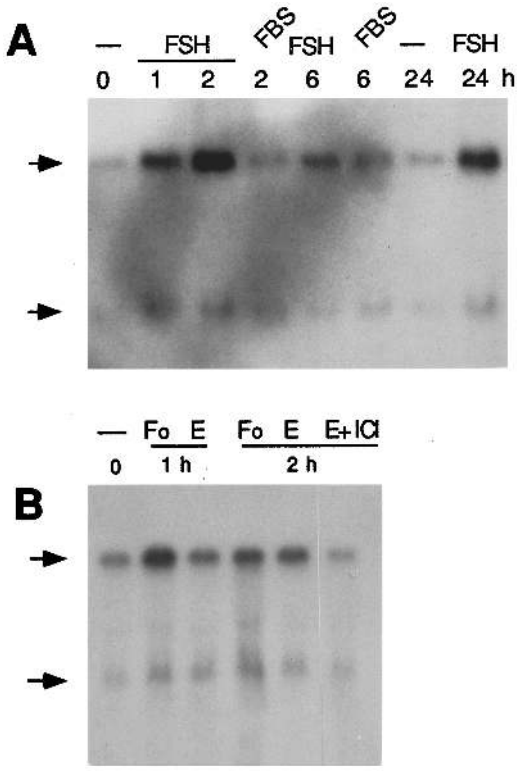

Fig. 7. Induction of Cyclin D2 by FSH, Forskolin, and Estradiol *in Vitro*

A, Granulosa cells isolated from immature E-primed rats were cultured and treated with FSH (100 ng/ml), FBS (10%), or untreated (−) for the indicated times and total RNA extracted. B, Granulosa cells isolated from immature, untreated rats were cultured and treated with forskolin (Fo, 10 μM), estradiol (E, 10 nM), estradiol plus 100 nM ICI (E+ICI) or untreated (−) for 1 or 2 h and total RNA extracted. Northern analysis was performed using labeled cDNA. Representative of three separate experiments with quantification provided in the text.

tagonist ICI 164,384 blocked the estradiol-mediated increase in cyclin D2 (E+ICI).

LH Regulates Expression of Cyclin D2, p27^{Kip1}, and p21^{Cip1}

Follicular growth is terminated by the LH surge. As a consequence of elevated cAMP, granulosa cells exit the cell cycle (Fig. 2 and Ref. 6), exhibit altered patterns of expression of cyclin D2 and p27 (Fig. 4), and undergo terminal differentiation (3, 12–14). To determine more precisely the time course of changes in cyclin D2 and p27 protein levels after the LH surge, granulosa cells and corpora lutea were isolated from hormone-primed H rats 2, 4, 8, 12, and 24 h after an ovulatory dose of hCG. Western analysis (Fig. 8) showed that the elevated levels of cyclin D2 protein present in preovulatory granulosa cells of HEF rats were decreased within 2 h, even lower at 4 h, and remained low in corpora lutea (24 and 48 h). In contrast, p27, although initially decreased by hCG after 2, 4, and 8 h, is elevated to high levels during the course of luteinization, between 12 and 24 h after hCG.

Because of the apparent delay in the increase of p27 during luteinization, we examined the expression of p21^{Cip1} in ovaries of these same rats using *in situ* hybridization. In contrast to p27, p21 is low in preovulatory follicles of HEF rats (Fig. 9) but is increased rapidly in response to hCG, within 2–4 h. Interestingly, at these early times, p21 mRNA was localized to the granulosa cells surrounding the antrum and oocyte. As luteinization proceeded, p21 was localized to granulosa cells throughout the follicle (hCG 12 h) and continued to be expressed in the luteal cells 24 h after hCG. Therefore, hCG induced rapid changes in the expression of p21, as well as cyclin D2 and p27, during

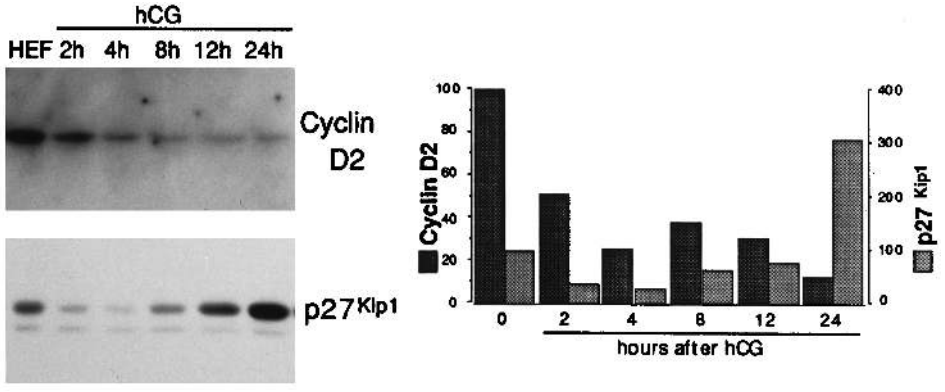

Fig. 8. Regulation of Both Cyclin D2 and p27^{Kip1} by LH

Western analysis of cyclin D2 (*upper panel*) and p27 (*lower panel*) protein levels in granulosa cell extracts prepared from H rats treated with estradiol and FSH (HEF) followed by an ovulatory dose of hCG for the indicated time. Immunopositive cyclin D2 and p27 were detected using specific primary antibodies followed by [^{125}I]protein A. Quantification depicts relative changes compared with non-hCG-treated HEF rats.

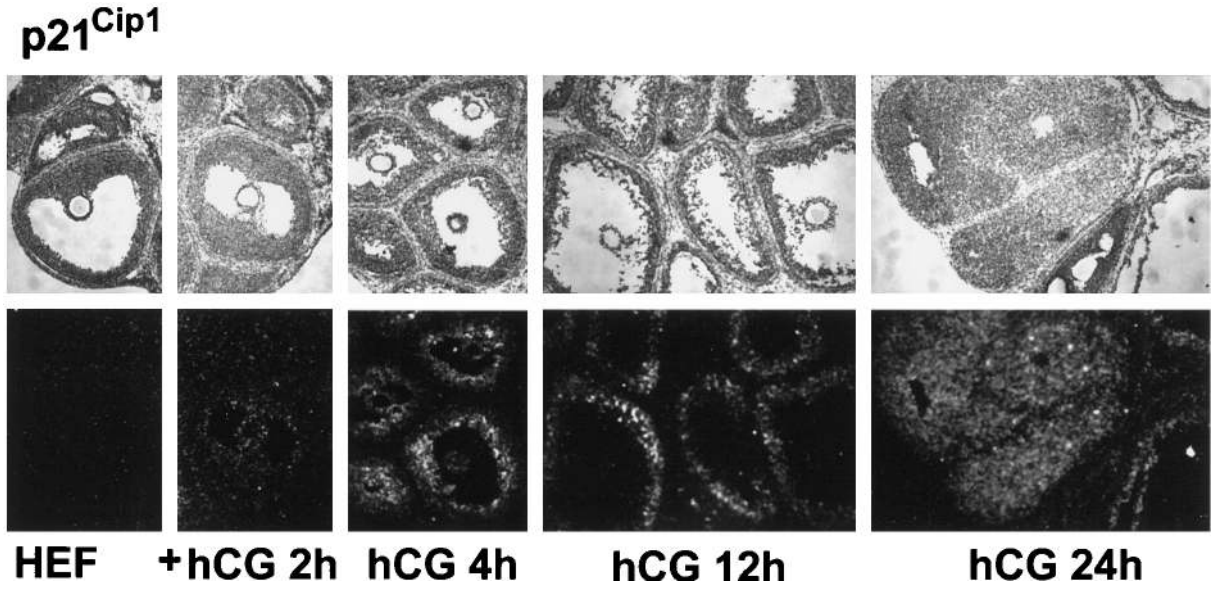

Fig. 9. LH Induces $p21^{Cip1}$ in Granulosa Cells

In situ hybridization of p21 in ovarian sections from hormone-treated H rats using an antisense probe. Rats were treated with estradiol and FSH (HEF) followed by an ovulatory dose of hCG for the indicated time.

the terminal differentiation of granulosa cells to luteal cells.

Granulosa Cell Differentiation Is Not Dependent On Cellular Proliferation

Based on our findings that cyclin D2 expression is increased by estradiol as well as FSH, we turned our attention to the cyclin $D2^{-/-}$ mice in which granulosa cell proliferation in response to PMSG (FSH) is impaired and follicles do not ovulate in response to the LH surge (38). Their phenotype shows that cyclin D2 is essential for normal gonadotropin-dependent follicular growth, but begs the question: is granulosa cell proliferation necessary for the differentiation that occurs in response to LH? Therefore, we analyzed the expression of PCNA, a marker of cellular proliferation, as well as a marker of granulosa cell differentiation, P450 aromatase (55), and markers of ovulation, prostaglandin synthase-2 (PGS-2) (56–58) and progesterone receptor (PR) (59, 60), in the ovaries of cyclin $D2^{-/-}$ mice (Fig. 10). In both normal and cyclin $D2^{-/-}$ immature mice, PCNA staining of granulosa cells was observed in the small follicles that are in the slow/gonadotropin-independent phase of growth (Fig. 10A). When normal mice were treated with PMSG, many more PCNA-immunopositive cells were present and associated with the rapidly dividing granulosa cells of large preovulatory follicles. However, when the cyclin $D2^{-/-}$ mice were treated with PMSG, proliferation did not occur, and few of the granulosa cells expressed PCNA.

Although PMSG did not stimulate proliferation of granulosa cells in cyclin $D2^{-/-}$ mice, antrum formation occurred as did the normal induction of markers of granulosa cell differentiation. RT-PCR of whole ovarian RNA (Fig. 10B) showed that aromatase was induced by PMSG in cyclin $D2^{-/-}$ mice, indicating that the FSH response system in granulosa cells of these mice is intact. Likewise, in cyclin $D2^{-/-}$ mice, hCG (LH) decreased aromatase mRNA and rapidly induced PGS-2 and PR mRNA, indicating that these responses of preovulatory granulosa cells to LH are not impaired. The lower levels of aromatase, PGS-2, and PR mRNA detected by RT-PCR in cyclin $D2^{-/-}$ mice most likely reflect the reduced number of granulosa cells in these ovaries. Lastly, hCG stimulated the formation of small corpora lutea that do not exhibit PCNA staining (Fig. 10C), indicating that the mechanisms mediating complete exit from the cell cycle are intact. However, despite the ability of cyclin $D2^{-/-}$ granulosa cells to differentiate in response to LH, the oocytes are not ovulated and remain trapped in the corpora lutea.

DISCUSSION

Evidence is rapidly accumulating which indicates that cell cycle progression and proliferation are controlled by altering the balance of positive and negative regulators that converge on cell cycle kinase cascades. Our results demonstrate that granulosa cell proliferation is associated with the regulated expression of both D-type cyclins and cdk inhibitors. We have shown that cyclin D2, a rate-limiting positive regulator of cdk activity and cell cycle progression, is the predominant D-type cyclin in granulosa cells, whereas cyclin D1 and cyclin D3 are localized to theca cells. In contrast, the cdk inhibitors, $p27^{Kip1}$ and $p21^{Cip1}$, are

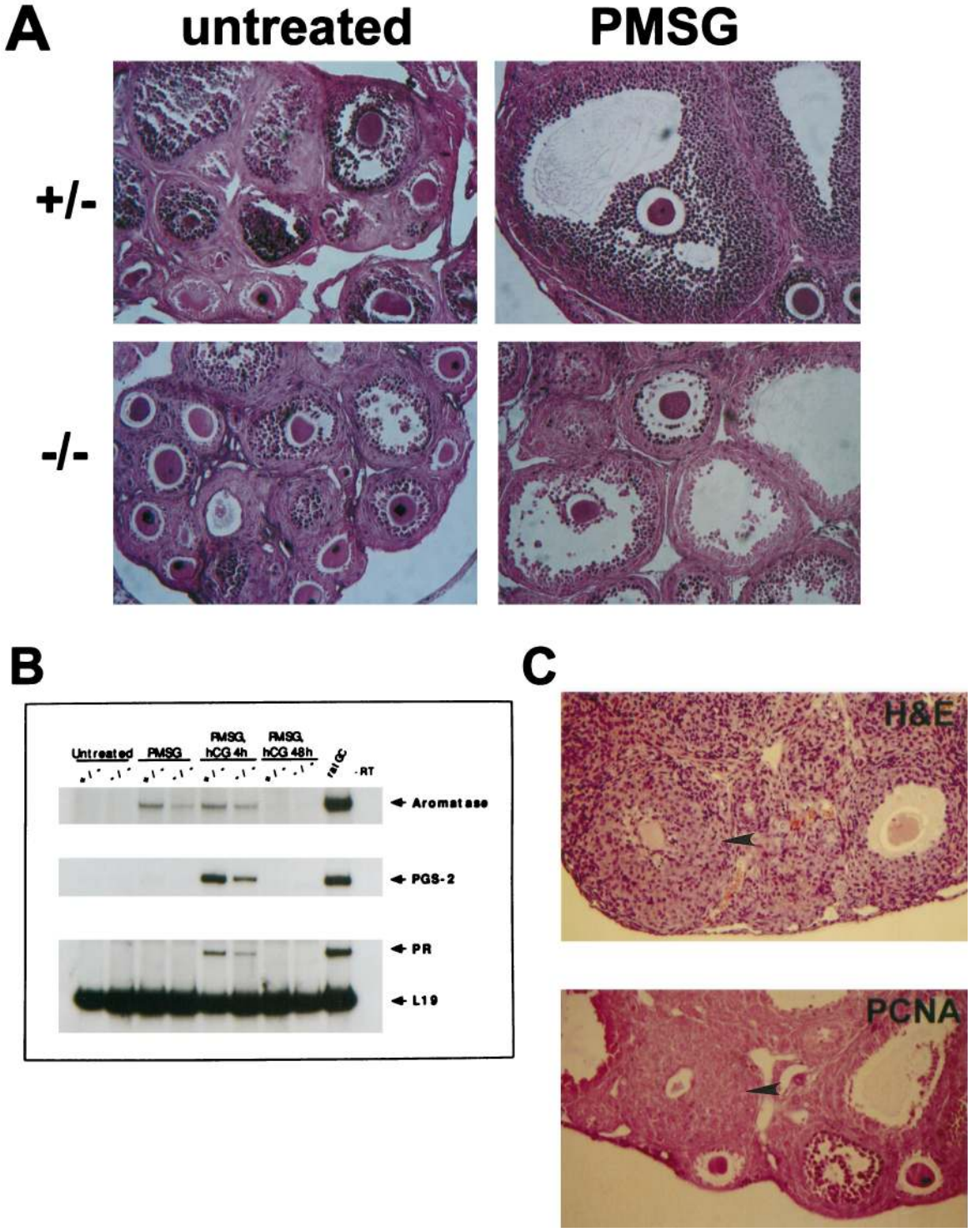

Fig. 10. Impaired Proliferation but Normal Differentiation in Follicles of Cyclin D2$^{-/-}$ Mice

Immunodetection of PCNA (A) in immature, untreated, and PMSG-treated mice heterozygous (±) or null (−/−) for cyclin D2. RT-PCR analysis (B) of whole ovarian RNA from hormone-treated mice heterozygous (±) or null (−/−) for cyclin D2. Rat granulosa cell RNA (rat GC) and omission of reverse transcriptase (−RT) were used as positive and negative controls, respectively. Hematoxylin and eosin staining and PCNA immunodetection (C) of dividing cells from cyclin D2$^{-/-}$ mice 48 h after hCG treatment. Remnants of nonovulated oocytes can be seen in the small corpora lutea (*arrowheads*).

selectively elevated in luteal cells. Most importantly, we have shown that the expression of each regulatory molecule is controlled by hormone-independent and hormone-dependent mechanisms that are associated with precise stages of follicular growth, granulosa cell proliferation, and luteal cell differentiation.

During the earliest stages of follicle growth, granulosa cell proliferation occurs by mechanisms that are independent of FSH and estradiol as well as of cyclin D2. Specifically, we have shown that cyclin D2 mRNA is present in granulosa cells of small follicles in which follicular growth is restricted by the absence of hormones or growth factors; FSH$\beta^{-/-}$ mice, gdf-9$^{-/-}$ mice, prepubertal mice, and hypophysectomized rats. Conversely, some, albeit limited, proliferation of granulosa cells occurs in cyclin D2$^{-/-}$ mice. These observations indicate that in small follicles, cyclin D2 is one, but not the exclusive, factor regulating granulosa cell entry into the cell cycle and that its expression occurs independently of gonadotropins and steroids. The slow rate of growth is not related to elevated amounts of p27 or p21; expression of both was low in these small follicles. Therefore, this protracted phase of granulosa cell proliferation appears to involve a unique set of cell cycle regulators (or inhibitors)—a specific hormone/growth factor (GDF-9 or one yet to be identified) or the controlled lack of responsiveness to stimulatory molecules such as FSH and estradiol.

Once the follicles begin to mature, respond to the gonadotropins, and produce estradiol, the rate of granulosa cell proliferation is exponentially increased (1). Based on data from the H rat, this period of rapid proliferation is due, at least in part, to the combined effects of estradiol and FSH/cAMP (6). Our data indicate that FSH/cAMP and estradiol increase cyclin D2 expression by specific receptor-mediated mechanisms in a fashion that mimics the effects of these hormones on the labeling index/proliferation of granulosa cells in H rats (6). Previous studies have estimated that the doubling time of granulosa cells during this time is reduced to 24 h (1). Consistent with this report, the results presented herein show that *in vivo* cyclin D2 expression not only increased rapidly in response to a surge concentration of FSH, but also increased between 12 and 24 h in response to tonic, basal levels of FSH. Likewise, when cultured granulosa cells were exposed to FSH *in vitro*, cyclin D2 expression showed a transient increase at 2 h followed by a secondary increase at 24 h. These observations combined with the markedly impaired proliferation in FSH-stimulated cyclin D2$^{-/-}$ mice indicate that cyclin D2 is an essential downstream mediator of hormone-stimulated proliferation at this period of exponential cell growth. The ability of estradiol and FSH to increase cyclin D2 is specific. Neither hormone increases the other D-cyclins or p27. Therefore, our data suggest that FSH and estradiol stimulate granulosa cell proliferation by increasing the levels of cyclin D2 relative to those of p27, thereby triggering a burst of proliferation that results in the development of large preovulatory follicles.

The individual effects of estradiol and FSH/cAMP indicate that each hormone may regulate distinct points in the cell cycle and may utilize parallel mechanisms. For example, the temporal increase in expression of cyclin D2 expression within 24 h in response to FSH, as well as estradiol, is consistent with the doubling time of granulosa cells during the period of exponential growth (1), indicating that cyclin D2 is directly or indirectly involved in timing cell cycle transit. An oscillatory pattern of cyclin D2 expression has been observed in other cell types in response to stimulatory factors (18, 52). However, the apparent oscillatory pattern observed in response to FSH may also involve an inherent periodicity in the activation of the A-kinase pathway (61) in granulosa cells, thereby integrating many facets of granulosa cell function. Additionally, FSH (in the presence of insulin) has been shown to induce c-*myc* and c-*fos* (62), and cAMP has been shown to regulate the levels of cyclin B (63), indicating that FSH may act at multiple steps during the cell cycle. Estradiol, in contrast to FSH, not only stimulates a greater increase in cyclin D2 mRNA and protein, but may also decrease p27 protein levels. The apparent down-regulation of the cell cycle inhibitor p27 concurrent with enhanced induction of cyclin D2 may be responsible for the greater mitogenic activity of estradiol compared with FSH in the H rat (5, 6). Furthermore, in other cell types, estradiol has been observed to induce cyclin E (64), whose activation of cdk2 is essential for cells to enter S phase. Therefore estradiol may also induce cyclin E in granulosa cells providing another explanation for its greater effect on granulosa cell proliferation compared with FSH.

In addition to FSH and estradiol, other growth factors are able to stimulate granulosa cell proliferation implicating a role in follicular growth. For example, activin has been observed to stimulate DNA synthesis (65) and maintain proliferation (66) in cultured granulosa cells, and insulin-like growth factor I (IGF-I) expression *in situ* has been correlated with granulosa cell DNA synthesis (67). However, targeted deletion of the activin βB subunit in mice (68, 69) did not result in an ovarian phenotype, indicating that activins alone are not the essential mediators of granulosa cell proliferation *in vivo*. In mice null for IGF-I, although the females are unable to ovulate, the ovarian follicles develop to the large antral stage, indicating IGF-I may be involved primarily in granulosa cell differentiation and responsiveness to ovulatory hormones as opposed to granulosa cell proliferation, *per se* (70).

The importance of gonadotropin stimulation for granulosa cell proliferation during the formation of preovulatory follicles is highlighted by observations in mice deficient in the inhibin subunit α. In these mice, the appearance of ovarian tumors is coincident with puberty (71), when the first group of growing, gonadotropin-responsive follicles reaches the phase of ex-

ponential proliferation. However, if the inhibin α null mice are cross-bred with hypogonadal mice that lack FSH/LH, no ovarian tumors form (72), suggesting that when gonadotropin/steroid action is unopposed, such as in the absence of inhibin, granulosa cell proliferation is uncontrolled. Consistent with this idea, chronic overexpression of LHβ results in elevated levels of estradiol and occasional ovarian tumors (73). Therefore, granulosa cell proliferation is tightly regulated, and it appears that appropriate ratios of estradiol, FSH/LH, and transforming growth factor-β family members (activin, inhibin, GDF-9), as well as growth factors such as IGF-I, determine the progression of proliferative and antiproliferative events within these cells. Interestingly, the human granulosa cell tumors that have been studied overexpress cyclin D2 but not cyclin D1 or D3 (38), supporting the evidence that expression of cyclin D2 causes rapid granulosa cell proliferation and thereby, may contribute to enhanced oncogenic potential.

Most dramatic is the ability of the LH surge to rapidly and completely halt cell cycle progression as well as initiate the process of luteinization, potentially utilizing distinct mechanisms for each. Our results indicate that the LH surge (high cAMP) induces exit from the cell cycle not only by elevating the levels of cell cycle inhibitors, such as p27 and p21, but by completely down-regulating the activator, cyclin D2. Cyclin D2 mRNA and protein are undetectable within 2–4 h after the LH surge. The temporal differences in the expression of cyclin D2, p27, and p21 may be indicative of distinct roles for each of these regulatory molecules in the control of cell cycle progression and cellular differentiation. Specifically, granulosa cell exit from the cell cycle occurs rapidly after the LH surge and is likely due to the drastic down-regulation of cyclin D2, since its absence would prevent or markedly reduce progression through G_1 phase. The early induction of p21 in response to hCG may indicate it plays a role in exit from the cell cycle as well. Since p27 is not elevated until 12–24 h after hCG, it may not affect the immediate exit of granulosa cells from the cell cycle but rather contributes to cell differentiation and maintenance of cell cycle arrest. These observations are consistent with those presented by the phenotype of p27$^{-/-}$ mice (39–41) as well as evidence that cAMP increases p27 in glial cells (36). Furthermore, p27 and p21 inhibit many cdks and may be most important for blocking the activity of cyclin E (and cyclin A)-cdk2 complexes (15, 18); therefore, the LH-mediated induction of high levels of these CKIs would inhibit cell cycle progression at multiple points. Collectively, these (and additional) mechanisms permit the LH surge (high cAMP) to rapidly reprogram granulosa cells to enter a nonproliferative luteal state. In contrast, FSH and low levels of cAMP promote granulosa cell proliferation by increasing expression of cyclin D2. Such different effects in response to changes in cAMP levels underscore the diverse roles that the A-kinase pathway plays in regulating ovarian cell function.

Lastly, our results offer insight into the relationship between cell number and follicular function. Specifically, we have shown that although follicles in the cyclin D2$^{-/-}$ mice have a reduced number of granulosa cells and fail to ovulate, the hormonal activation of the PGS-2 and PR genes that are essential for ovulation (74–76) occurs normally in these follicles. These results implicate cell number as a critical factor in ovulation, presumably due to the production by granulosa cells of a critical (as yet unknown) factor controlling an event in the ovulatory process. In contrast, cell number is not a factor for differentiation and the formation of the corpus luteum. In response to the LH surge, granulosa cells of cyclin D2$^{-/-}$ mice exit the cell cycle (loss of PCNA staining) and undergo terminal differentiation including hypertrophy and expression of the luteal-specific gene, P450 side-chain cleavage (14, 38).

In summary, the control of granulosa cell proliferation is complex, essential for normal follicular growth, and critical for ovulation. It is dependent on the cell-specific expression of regulators of G_1 progression: the cdk activator cyclin D2 and the cdk inhibitor p27^{Kip1}. After the initial gonadotropin-independent phase of growth, the regulation of these molecules by FSH, LH, and estradiol controls the exponential phase of granulosa cell proliferation as well as the exit of granulosa cells from the cell cycle and their terminal differentiation to luteal cells. Critical to the entry into and exit from the period of rapid proliferation are the differential responses of the granulosa cells to estradiol, as well as to the low *vs.* high levels of cAMP stimulated by FSH and LH, respectively. Thus, the coordinated interactions of estradiol and cAMP are able to tightly control the highly proliferative and nonproliferative events in granulosa cells.

MATERIALS AND METHODS

Hormone Treatments

Mice and rats were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Female mice (C57/129) at approximately 21 days of age were injected ip with 5 IU PMSG (Gestyl, Professional Compounding Center of America, Houston, TX) to stimulate follicular growth. They were injected ip 46 h later with 5 IU hCG (Pregnyl, Organon Special Chemicals, West Orange, NJ) to trigger ovulation and luteinization. FSH$\beta^{-/-}$ mice and tissue from gdf-9$^{-/-}$ mice were generously provided by Dr. Martin Matzuk, Department of Pathology, Baylor College of Medicine (Houston, TX). Female Holtzman Sprague Dawley rats were hypophysectomized (H) on day 26 of age and were purchased from Harlan (Indianapolis, IN). Untreated (H) rats were given sc injections of 1.5 mg/0.2 ml 17β-estradiol (Sigma Chemical Co, St. Louis, MO) once daily for 3 days beginning on day 28 of age (HE); estradiol treatment followed by sc injections of 1.0 μg/0.1 ml ovine FSH (NIH oFSH-16, National Hormone and Pituitary Agency, Rockville, MD) twice daily for two days (HEF); or estradiol and FSH treatments followed by a single injection of 10 IU hCG (Organon Special Chemicals) for the indicated time (HEF hCG). hCG was administered iv

for acute treatments (2, 4, 8, and 12 h) and ip for longer timepoints (24 and 48 h).

In Situ Hybridization

In situ hybridization was done as described by Wilkensen (77). Briefly, [^{35}S]UTP-labeled antisense and sense probes of mouse cyclin D1, cyclin D2, and cyclin D3 cDNAs (provided by Dr. Piotr Sicinski and Dr. Robert Weinberg, Whitehead Institute, MIT, Boston, MA) and mouse p27^{Kip1} and p21^{Cip1} cDNAs (provided by Dr. Stephen Elledge, Department of Biochemistry, Baylor College of Medicine) were made using the Riboprobe *In Vitro* Transcription Systems kit (Promega, Madison, WI). Ovaries were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 6 μm onto silane-coated slides (Histology Control Systems, Glen Head, NY). After rehydration, sections were pretreated with 20 μg/ml proteinase K and 0.1 M triethanolamine/acetic anhydride before coating with the labeled probe and then incubated at 55 C overnight. Slides were washed at high stringency including two washes of 50% formamide, 2× saline sodium citrate, 100 mM β-mercaptoethanol for 30 min each at 65 C, then treated with 20 μg/ml RNase A and dehydrated. After drying, slides were dipped in photographic NTB-2 emulsion (Kodak, Rochester, NY) and exposed at 4 C for 2–5 days. The slides were developed with D-19 developer and fixer (Kodak) and counter-stained with hematoxylin for 30 sec. Light field photography shows the tissue histology while dark field illumination allows visualization of the mRNA probe.

Northern Analysis

Granulosa cells were isolated from ovaries by needle puncturing (78). Northern analysis was done according to the method of Fitzpatrick and Richards (79). Briefly, total cellular RNA was obtained by homogenizing granulosa cells in buffer (140 mM NaCl, 5 mM KCl, 3 mM MgCl2, 25 mM Tris-HCl) containing 1% NP-40 followed by phenol extraction and ethanol precipitation. The RNA (20 μg) was separated on formaldehyde-agarose gels and transferred to 0.2-μm Biotrans nylon membranes (ICN Biomedicals Inc, Aurora, OH). Probes consisted of isolated cDNA inserts labeled by random hexanucleotide priming (79) using [^{32}P]dCTP (ICN Radiochemicals, Los Angeles, CA) and were incubated with the membrane at 42 C overnight, according to the specifications of ICN. Membranes were washed for high stringency with 0.5× saline sodium citrate, 0.1% SDS at 55 C for 1 h and exposed to X-OMAT film (Kodak).

Western Analysis

Soluble cellular protein was obtained by suspending isolated granulosa cells in whole cell extract buffer: 10 mM $NaHPO_4$, pH 7.4, 1 mM Na_2EDTA, 1 mM dithiothreitol, 400 mM KCl, 10% glycerol, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 μM pepstatin, 1 mM phenylmethylsulfonyl fluoride, 5 μM NaF, 0.5 μg/ml ocadeic acid; followed by two freeze/thaw cycles and centrifugation at 4 C. Fifty micrograms of soluble protein were run on 12% acrylamide/SDS gels and transferred to Immobilon-P nylon membranes (Millipore Co, Bedford, MA). Membranes were blocked with 5% nonfat milk followed by incubation with the recommended dilution of primary antibody in 5% milk for 1 h at room temperature. Antibodies used were mouse monoclonal anticyclin D2 (DCS-3.1, Neomarkers Inc, Fremont, CA) at 2 μg/ml and rabbit polyclonal anti-p27 (C-19, Santa Cruz Biotechnology Inc, Santa Cruz, CA) at 1 μg/ml. Blots were then washed extensively in 10 mM Tris-buffered saline containing 0.5% Tween-20 (TBS-T) and incubated with 1:10,000 dilution of antimouse IgG (or antirabbit IgG) peroxidase-linked antibody (Amersham Life Sciences, Inc., Arlington Heights, IL) in 5% milk for 1 h at room temperature. After washing in TBS-T, ECL was performed using Supersignal chemiluminescent detection reagents (Pierce, Rockford, IL). Western blots were quantitated by densitometry (Molecular Dynamics, Sunnyvale, CA) of autoradiographic films. For quantification of Western blots shown in Fig. 8, proteins were transferred to Protran nitrocellulose membrane (Schleicher & Schuell, Keene, NH), blocked with 5% milk, and incubated with primary antibody as above but detected with ^{125}I-labeled protein A (ICN) and exposed to film. Bands detected by autoradiography were cut from the membrane, dissolved in scintillation fluid, and counted in a Beckman LS-6500 multipurpose scintillation counter set for γ-detection.

Cell Culture

For *in vitro* experiments, granulosa cells were isolated from either immature (day 22 of age) rats (Holtzman, Madison WI) or immature rats primed with 1.5 mg/0.2 ml 17β-estradiol (Sigma) once daily for 3 days, as indicated. The cells were treated with 20 μg/ml trypsin and 160 μg/ml DNaseI as described previously (78). The cells were then plated on bovine serum-coated dishes in serum-free DMEM:F12 medium and cultured overnight at 37 C, 95% air/5% CO_2 (79). Hormone treatments included 100 ng/ml FSH (NIH oFSH-16, National Hormone and Pituitary Agency, Rockville, MD), 10% FBS (Hyclone Labs., Logan, UT), 10 μM forskolin (Calbiochem, La Jolla, CA), 10 nM estradiol (Sigma), and 100 nM ICI 164,384 (Zeneca Pharmaceuticals, Macclesfield, UK).

Immunocytochemistry

Proliferating Cell Nuclear Antigen (PCNA) was detected by immunolocalization as described previously (44) with modifications. Briefly, rehydrated paraffin sections were washed in PBS, blocked with 1% equine serum for 10 min at 37 C followed by overnight incubation at room temperature with 1:150 dilution monoclonal anti-PCNA antibody (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK). Slides were washed with PBS and then incubated with 15 μl/ml mouse IgG peroxidase-linked antibody (Amersham Life Sciences) at 37 C for 30 min. After washing with PBS, slides were incubated in 50 ml $NaPO_4$ buffer containing 25 mg 3,3′-diaminobenzidine substrate (Amersham) for 10 min, rinsed with H_2O, and counter-stained with eosin.

RT-PCR

RT-PCR was performed as previously described (80). Total RNA (100 ng for aromatase; 350 ng for PGS-2 and PR) was reverse transcribed using 500 ng poly dT (Pharmacia Biotech Inc, Piscataway, NJ) and 0.25 U avian myeloblastosis virus (AMV) reverse transcriptase (Promega) at 42 C for 75 min. After the addition of 500 ng of each specific primer, 2 μCi [^{32}P]dCTP (ICN), 2.5 U *Taq* Polymerase (Promega) and Thermocycle Buffer (Promega), the 100 μl reaction completed 20 cycles of PCR at 94 C for 1 min, 55 C (aromatase) or 65 C (PGS-2, PR) for 2 min, and 72 C for 3 min. Primers for the ribosomal protein L19 were included in each experiment as an internal control (60). Products were separated on a 5% polyacrylamide gel, which was then dried and exposed to film. PCR primers are: aromatase for 5′-TGCACAGGCTCGAGTATTTCC-3′; aromatase rev 5′-ATTTCCACAATGGGGCTGTCC-3′ (80); PGS-2 for 5′-TGTACAAGCAGTGGCAAAGG-3′; PGS-2 rev 5′-GCTGTGGATCTTGCACATTG-3′; PR for 5′-CCCACAGGAGTTTGTCAAGCT-3′; PR rev 5′-TAACTTCAGACATATTCCGG-3′ (60); and L19 for 5′-CTGAAGGTCAAAGGGAATGTG-3′; L19 rev 5′-GGACAGAGTCTTGATGATCTC-3′ (60), which result in products of 271 bp, 430 bp, 328 bp, and 194 bp, respectively.

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Address requests for reprints to: JoAnne S. Richards, Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030. E-mail: joanner@bcm.tmc.edu.

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