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Cyclin G1 and Cyclin G2 Are Expressed in the Periimplantation Mouse Uterus in a Cell-Specific and Progesterone-Dependent Manner: Evidence for Aberrant Regulation with Hoxa-10 Deficiency

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Abstract

Because uterine cell-specific proliferation, differentiation, and apoptosis are differentially regulated during the periimplantation period, we speculated that negative cell cycle regulators are also operative in the uterus during this period. This prompted us to examine the roles of two negative growth-regulatory genes, cyclin G1 and cyclin G2, in the periimplantation mouse uterus. We show that cyclin G1 and cyclin G2 genes are differentially regulated in the uterus during this period (d 1–8 of pregnancy) in a spatiotemporal manner. The results suggest that cyclin G1 is primarily associated with epithelial cell differentiation before implantation and stromal cell proliferation and differentiation during decidualization, whereas cyclin G2 is associated with terminal differentiation and apoptosis of the luminal epithelial and stromal cells at the site of blastocyst after implantation. Pharmacological and genetic studies provide evidence that the expression of cyclin G1, not cyclin G2, is regulated by progesterone via its nuclear receptor. Furthermore, the expression of these genes is aberrantly up-regulated in homeo box A-10 mutant uteri, suggesting that cyclin G1 and cyclin G2 genes act as downstream targets of homeobox A-10 and negatively impact uterine cell proliferation. Collectively, our present and previous studies suggest that negative cell cycle regulators collaborate with growth-promoting regulators in regulating uterine cell-specific proliferation, differentiation, and apoptosis relevant to implantation and decidualization.

Blastocyst implantation is a complex process that requires two-way interactions between the implantation-competent blastocyst and the receptive uterus (1) and involves complex growth

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regulatory mechanisms leading to molecular, cellular, physiological, and functional changes in the uterus and embryo (2). The uterus is composed of heterogeneous cell types that undergo proliferation, differentiation, and apoptosis in a phase- and cell-specific manner during the progression of implantation. For example, on d 1 and 2 of pregnancy, preovulatory estrogen directs uterine epithelial cell proliferation, whereas on d 3, increasing progesterone (P_4) secretion from newly formed corpora lutea initiates stromal cell proliferation, which is further promoted by preimplantation estrogen secretion in the morning on d 4. In contrast, epithelial cells on this day cease to proliferate and become differentiated under the influence of both P_4 and estrogen. It is believed that differential uterine cell-specific functions on d 4 are essential to the preparation of the receptive uterus for implantation. After blastocyst attachment (2400 h on d 4), luminal epithelial cells at the site of blastocyst apposition progressively undergo apoptosis, whereas stromal cells surrounding the implanting blastocyst undergo intense proliferation. Late on d 5 (1800 h), stromal cells close to the implanting blastocyst cease to proliferate and initiate the process of differentiation forming the primary decidual zone (PDZ), which is fully established by d 6 morning. The cells within the PDZ subsequently undergo apoptosis and by d 8 most of these cells disappear. However, stromal cell proliferation and differentiation continue outside the PDZ, forming the secondary decidual zone (SDZ). This zone is primarily comprised of decidual cells that are mono- or multinucleated polyploid in nature (3). With time, this zone also undergoes apoptosis, enlarging the implantation chamber to accommodate the growing embryo.

Homeobox A (Hoxa)-10, a member of the Hox multigene family of transcription factors, is essential for female fertility in mice (4). Hoxa-10 is highly expressed in the proliferating and differentiated uterine stroma and P_4 is the primary inducer of Hoxa-10 in this tissue (4, 5). Whereas stromal cell proliferation is severely depressed in Hoxa-10 mutant mice in response to P_4 and estrogen, epithelial cell proliferation is normal in response to estrogen (reviewed in Ref. 2). Overall, female infertility in Hoxa-10-deficient mice correlates with the defects in uterine stromal cell responsiveness to proliferation and differentiation under the direction of P_4.

The cell cycle progression is primarily controlled by specific interaction of diverse pairs of cyclins and cyclin-dependent kinases (CDKs), triggering phase-specific cell cycle activation in an orderly fashion. CDKs are regulated by a number of proteins including cyclins that bind and activate the CDKs to form serine/threonine kinase holoenzyme complexes. These complexes are responsible for phosphorylation of numerous substrates, mediating progression through the cell cycle (6–9). There is evidence that cyclin/CDK complex is negatively regulated by the two cyclin-dependent kinase inhibitor families, p16 and p21. Each family also has a few submembers (10–12). Although, most cyclins are important for the progression of cell cycle, G-type cyclins are the exception. They participate primarily as inhibitory cyclins to negatively regulate cell proliferation.

The G-type cyclins consist of two members, cyclin G1 and cyclin G2. Cyclin G1 expression is apparently constitutive throughout the cell cycle, whereas that of cyclin G2 fluctuates during the cell cycle with a peak level of expression in the late S phase (13). Their cellular localization is different; cyclin G1 is primarily present in the nucleus, whereas cyclin G2 is

cytosolic. Cyclin G1 was identified as a homolog of c-*src* kinase and later characterized as a transcriptional target of p53 tumor suppressor gene (14, 15). Functions of G-type cyclins are controversial. Transactivation of cyclin G1 by p53 and its induction after γ-irradiation is implicated for G2/M arrest in the cell cycle for negative growth control or DNA damage repair (16–18). Cyclin G1's growth-inhibitory activity is also indicated by its abundant expression in highly differentiated tissues, such as the brain, skeletal muscle, and kidney. Furthermore, cyclin G1 is involved in many cell functions that are also regulated by p53. These include apoptosis and cell cycle checkpoint regulation (15–18). There is evidence that cyclin G1 also influences p53 stability (19). Conversely, cyclic G1 is considered a growth promoter because of its overexpression in certain cancer cell lines (20) and increased expression in leiomyoma (21). Reduced hepatic tumor incidence with the loss of cyclin G1 suggests that this cyclin is involved in cell growth (22). However, a recent study shows that cyclin G1's growth inhibitory activity is dependent on the magnitude of its expression (16), which may partly explain the reason for paradoxical observations. In contrast to cyclin G1, cyclin G2 is not a transcriptional target of p53 and its expression is independent of p53. In addition, cyclin G2 is an unconventional cyclin that is highly expressed in cells undergoing apoptosis resulting from cell cycle arrest at the G1/S phase or in response to growth-inhibitory stimuli (20, 22, 23). Although G-type cyclins are structurally similar to cyclin A in the cyclin box, they do not show any cyclin-dependent kinase activity (23).

Although both positive and negative regulatory cell cycle molecules coordinate cell proliferation, differentiation, and apoptosis, their mode of operation in uterine cell cycle regulation remains poorly understood (24, 25). Here we examined the status of expression of inhibitory G-type cyclins in the periimplantation mouse uterus and their regulation by steroid hormones. We further examined the expression of G-type cyclins in Hoxa-10(−/−) mice with defects in uterine cell proliferation. Our results show for the first time that G-type cyclins are expressed in a cell-specific and P_4 dependent manner in the periimplantation mouse uterus, but their expression become aberrant in the uterus lacking Hoxa-10. This suggests that G-type cyclins are involved in implantation and decidualization.

Materials and Methods

Animals and tissue preparation

Adult CD-1 (Charles Rivers Laboratory, Raleigh, NC) and Hoxa-10(−/−) (129/SvJ/C57BL6) (4) mice were housed in our institutional animal care facility according to National Institutes of Health and institutional guidelines for laboratory animals. Females were mated with fertile or vasectomized males of the same strain to induce pregnancy or pseudopregnancy (d 1= vaginal plug), respectively. Pregnancy on d 1–4 was confirmed by recovering embryos from the reproductive tracts. On d 5 and 6 of pregnancy, implantation sites were identified by localized increased uterine vascular permeability at the site of blastocysts through iv injection of Chicago Blue B dye solution (1% in saline) (2). On d 7 and 8, implantation sites are distinct and their identification does not require any special manipulation. To stimulate experimentally induced decidualization, sesame oil (25 μl) was infused intraluminally in one uterine horn on d 4 of pseudopregnancy, whereas the contralateral horn without any infusion served as control. The decidual cell reaction was confirmed by recording uterine weight and

histological examination of uterine sections on d 6–8 of pseudopregnancy (26). Mice were killed between 0830 and 0900 h on each day of pregnancy or pseudopregnancy for the collection of uterine and brain tissues. Tissues were rapidly flash frozen and kept at −70 C for subsequent analyses.

Ovariectomy and ovarian steroid hormone treatments

Wild-type littermates and progesterone receptor knockout (PRKO) mice of the same genetic background (129SvEv/C57BL/6) were produced by crossing of heterozygous males with heterozygous females (27). Bert O'Malley (Baylor College of Medicine, Houston, TX) kindly provided PRKO mice for establishing a colony in our facility. Mice were genotyped by PCR analysis of tail DNAs. Adult mice (8–10 wk old) were ovariectomized and rested for 10–12 d before they received any injections. Ovariectomized mice were given a single sc injection of sesame oil (0.1 ml/mouse), estradiol-17β (E_2, 100 ng/mouse), or P_4 (2 mg/mouse) and killed at 0, 6, 12, and 24 h after the injection. The test agents were dissolved in sesame oil and injected sc (0.1 ml/mouse). Uterine tissues were collected as before and stored at −70 C for further studies.

Hybridization probes

Mouse-specific full-length cDNA clones for cyclins G1 and G2 were kindly provided by Dr. Mary C. Horne (University of Iowa, Iowa City, IA) (13). To prepare an appropriate length of cDNA clones for making cRNA probes, suitable fragments were generated from original clones by restriction digestion using *NotI* and *Kpn*I for cyclin G1 and *EcoR*V and *Kpn*I for cyclin G2 DNAs. These fragments were subcloned into pBlue-script II SK+ vector using the same restriction sites. The cDNA clone for the mouse-specific *p53* gene was generated by RT-PCR cloning into a pCRII TOPO vector. The authenticity of these clones was confirmed by nucleotide sequencing. The mouse ribosomal protein L7 (*rpL7*) cDNA clone has been described previously (24). For Northern blot hybridization, antisense ^{32}P-labeled cRNA probes were generated using appropriate RNA polymerases. For *in situ* hybridization, sense and antisense ^{35}S-labeled cRNA probes were generated using appropriate RNA polymerases. The probes had specific activities of 2×10^9 dpm/μg.

Northern blot hybridization

Total RNAs were extracted from whole uteri pooled from three to four mice by a modified guanidine thiocyanate procedure (24). Total RNA (6.0 μg) was denatured, separated by formaldehyde-agarose gel electrophoresis, and transferred to nylon membranes. RNA was cross-linked to the membranes by UV irradiation (Spectrolinker, XL-1500; Spectronics Corp., Westbury, NY), and the blots were prehybridized, hybridized, and washed as previously described by us (24). The stripping of hybridized probe for subsequent rehybridization was achieved as described (28). The hybrids were detected by autoradiography and the autoradiographic exposure times are indicated in the figure legends.

In situ hybridization

In situ hybridization was performed as previously described by us (28). In brief, frozen sections (11 μm) were mounted onto poly-L-lysine-coated slides and fixed in 4%

paraformaldehyde in PBS for 10 min at 4 C. Sections were prehybridized followed by hybridization with ^{35}S-labeled antisense or sense cRNA probes for 4 h at 45 C. After hybridization and washing, the sections were incubated with RNase-A (20 μg/ml) at 37 C for 20 min. RNase-A-resistant hybrids were detected by autoradiography using NTB-2 liquid emulsion (Eastman Kodak, Rochester, NY). The slides were poststained with hematoxylin and eosin. Sectioned hybridized with the sense probes served as negative controls.

Antibodies

The affinity-purified rabbit polyclonal antibodies for mouse cyclin G1 and cyclin G2 were used (17). These antibodies were previously used for the detection of cyclins G1 and G2 proteins by Western blot analysis in several mouse tissues (17). The affinity purified goat polyclonal antibody generated against mouse-specific synthetic peptide located at the carboxy terminus of actin (catalog no. sc-1615) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). For immunohistochemical staining, the affinity-purified rabbit polyclonal antibodies raised against peptides corresponding to rat cyclin G1 (catalog no. sc-320) and human cyclin G2 (catalog no. sc-7266) were purchased from Santa Cruz Biotechnology. These antibodies react with mouse-specific proteins. The corresponding blocking peptides were also purchased from Santa Cruz Biotechnology

Western blot analysis

The method followed the protocol as previously described by us (3). Proteins were extracted from the pooled uterine tissues ({sim]150–200 mg wet weight) on each day of pregnancy or the whole female brain irrespective of the day of pregnancy by homogenization in buffer containing 50 mM Tris (pH 7.4), 1 mM EDTA, 150 mM NaCl, and proteinase inhibitors (1 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1 μg/ml leupeptin). The homogenates were centrifuged at 2000 × *g* for 15 min at 4 C. The supernatants were separated and their protein concentrations were measured. The supernatants (50 μg protein) were boiled for 5 min in sodium dodecyl sulfate sample buffer [0.06 M Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 0.7 M β-mercaptoethanol] containing 0.01% bromophenol blue dye. After centrifugation, the samples were run on 10% SDS-PAGE gels under reducing condition and transferred onto nitrocellulose membranes. The membranes were blocked with 5% carnation milk in Tris-buffered saline [10 mM Tris-HCl (pH 8.0) and 150 mM NaCl] plus 0.05% Tween 20 overnight at 4 C and then incubated in 5% milk containing primary antibodies (1:1000 dilution for cyclin G1, 1:5000 dilution for cyclin G2, or 1:200 dilution for actin) overnight at 4 C. After incubation, membranes were washed three times (10 min each) with 5% milk, incubated with goat antirabbit IgG conjugated with horseradish peroxidase (HRP) (1: 5000) in 5% milk for 1 h at room temperature, and washed three times (5 min each) in Tris-buffered saline. The bands were detected using an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL).

Immunohistochemical staining

Immunostaining followed the protocol as previously described by us (3). In brief, formalin-fixed paraffin-embedded sections (6 μm) were deparaffinized, hydrated, and irradiated in the microwave oven for the antigen retrieval. Nonspecific reaction was blocked by incubation in

10% nonimmune serum. Sections were incubated with primary antibody at 4 C for 17 h, followed by incubation in secondary antibody for 10 min. Staining reaction was performed using a Histostain-SP kit (Zymed Laboratories, San Francisco, CA). Red deposits indicate the sites of positive immunostaining. Immunoneutralization was performed by incubating sections with preneutralized primary antibody with 250-fold molar excess of antigenic peptides.

TUNEL assay

The terminal deoxynucleotide transferase mediated deoxyuridine 5-triphosphate-biotin nick end labeling (TUNEL) kit (DeadEnd Colorimetric TUNEL System, catalog no. G7130) was purchased from Promega (Madison, WI). In this assay, terminal deoxynucleotidyl transferase, which catalyzes polymerization of nucleotides to free 3′-OH DNA ends, is used to label DNA strand breaks. The experimental protocol was same as described by the manufacturer's instruction. Briefly, frozen tissue sections were fixed in 10% buffered formalin in PBS for 15 min and then permeabilized with proteinase K (20 μg/ml) in PBS at room temperature for 30 min. The reaction mix was then added to the sections and covered with plastic coverslips to ensure even distribution of the reagent. Slides were incubated at 37 C for 60 min inside a humidified chamber. The endogenous peroxidase activity was inactivated by incubation of sections with 0.3% hydrogen peroxide for 5 min at room temperature. After washing in PBS, sections were incubated in streptavidin-HRP solution (1:500 in PBS) for 30 min at room temperature. The color development was attained by exposing the sections to the diaminobenzidine solution. The sections were lightly counterstained with hematoxylin. Apoptotic cell nuclei are stained dark brown. For positive controls, the sections were treated with DNase I (5–10 U/ml) for 10 min at room temperature before incubation with the TUNEL reaction mixture.

Results

Cyclins G1 and G2 and p53 are differentially expressed in the periimplantation mouse uterus

Because G-type cyclins act as negative growth regulators in the cell cycle and because p53 regulates cyclin G1 expression as a transcriptional target, the primary objective of these experiments was to explore the overlapping and distinct expression profiles of cyclin Gs and p53 in the uterus during the periimplantation period (d 1–8 of pregnancy). We first analyzed the expression of mRNAs of these genes by Northern hybridization (Fig. 1A). As reported previously (13), single transcripts of approximately 3.4, 2.8, and 2.2 kb for cyclin G1, cyclin G2, and p53 mRNAs, respectively, were detected. Uterine expression of these three genes was detected on d 1–8 of pregnancy. However, the expression of p53 gene was low on d 1 and 2. Integrity and loading of RNA samples were confirmed by rehybridizing the same blot to a constitutive gene, rpL7-specific probe.

We next examined the spatiotemporal expression of these genes by *in situ* hybridization. As shown in Fig. 1B, the expression of cyclin G1 is very low to undetectable ond1and 2. However, on d 3 the expression is evident in epithelial cells. This pattern is maintained on d 4 but with higher intensity of signals. In contrast, on d 5 shortly after the initiation of

implantation, the expression is undetected at the site of implantation. However, with the progression of decidualization on d 6, the expression reappears at the site of implantation primarily in cells of the SDZ. A similar pattern of expression persists on d 7. In contrast, on d 8, the hybridization signals for cyclin G1 are attenuated at the SDZ with more localized expression at the mesometrial decidual bed, the presumptive site of placentation. The lack of cell-specific expression of cyclin G1 mRNA at the site of implantation on d 5, as opposed to its observed expression in the pooled uterine RNA samples on this day of pregnancy by Northern blot hybridization, suggests that cyclin G1 expression is present at the interimplantation area. Indeed, analysis of *in situ* hybridization results shows that cyclin G1 is expressed primarily in the luminal epithelium at the interimplantation region of the uterus on d 6 of pregnancy, suggesting an embryonic influence on implantation site-specific stromal expression (panel D6 IIS, Fig, 1B).

Cyclin G2 expression, in general, is very low to undetectable in the preimplantation uterus during d 1–4 of pregnancy, albeit at low levels in epithelial cells on d 4 (Fig. 1C). In contrast, uterine expression is predominant at the implantation site on d 5–8 (Fig. 1C). Notably, on d 5, the signals for cyclin G2 mRNA are distinctly localized in the luminal epithelium and few layers of underneath stromal cells at the site of the blastocyst toward the antimesometrial pole. On d 6, signals persist in the PDZ and in the remaining epithelium at the mesometrial pole of the implantation site, whereas the interimplantation region of the uterus had very little to undetectable level of expression (panel D6 IIS, Fig. 1C). The expression is even more intense at the PDZ on d 7 and 8. In contrast to cyclin Gs, p53 gene expression is primarily present in stromal cells on d 4 of pregnancy (Fig. 1D). However, after implantation on d 5 (Fig. 1D), the expression of p53 mRNA is predominantly localized in stromal cells in the subluminal region around the embryo both at the mesometrial and antimesometrial poles of the implantation chamber. On d 6 (Fig. 1D) and 7 (data not shown) of pregnancy, stromal expression at both poles still persists, albeit at higher intensity at the mesometrial pole. On d 8 (Fig. 1D), the intensity of uterine signals is somewhat reduced, but the developing embryo exhibits strong signals. Overall, these results suggest that cyclin G1, cyclin G2, and p53 mRNAs are differentially expressed in the periimplantation mouse uterus.

Cyclin G1 and cyclin G2 proteins are expressed in the periimplantation mouse uterus

Levels of cyclin G1 and cyclin G2 proteins in the whole uterus on d 3–8 of pregnancy were examined by Western blotting; brain tissue extracts were run as positive controls. As reported previously (17), the use of the same polyclonal antibodies detected a major band of approximately 34 kDa for cyclin G1 and approximately 40 kDa for cyclin G2 in all uterine tissue extracts (Fig. 2A). Overall, cyclin G1 is predominantly expressed in the preimplantation uterus on d 3 and 4, whereas that of cyclin G2 is detected at low levels. After implantation, both cyclins were detected.

To examine cell-specific localization of cyclin G1 and cyclin G2 proteins in the postimplantation uterus, immunohistochemical staining was performed on d 6 of pregnancy at the site of embryo implantation. Our results show that these two proteins are differentially localized in the uterine decidual bed (Fig. 2B). The nuclear staining for cyclin G1 is noted at

the antimesometrial pole of the decidual bed primarily in association with SDZ. Glandular epithelial cells were also positive for this staining. In contrast, both cytoplasmic and nuclear staining of cyclin G2 is present primarily in the PDZ. The luminal epithelium at the mesometrial pole of the decidual bed is also positive for cyclin G2. Overall, these results are consistent with our mRNA analysis.

Cyclins G1 and G2 are differentially expressed in the uterus during experimentally induced decidualization

To evaluate whether embryonic signals influence the expression of G-type cyclins during decidualization, experimentally induced decidualization was initiated in one uterine horn, whereas the noninfused contralateral horn served as a control. *In situ* hybridization was performed to assess the expression of cyclins G1 and G2 in both uterine horns on d 6–8 (Fig. 3). The results show that the decidual expression of cyclin G1 follows similar patterns as observed during normal pregnancy. However, the expression of cyclin G2 was weaker than what was noted during normal pregnancy, suggesting that embryonic signals influence its expression in the deciduum. The noninfused horns had little or undetectable expression of these genes.

Analysis of decidual cell apoptosis in the decidualizing uterus

During decidualization, a selective population of cells is eliminated from the decidual bed for the accommodation of the growing embryo. Because G-type cyclins and p53 are implicated in apoptosis, we examined the distribution of apoptotic cell nuclei in the decidual bed. TUNEL assays were performed on sections of the implantation sites retrieved on d 7 and 8 of pregnancy. It is to be recognized that antimesometrial decidualization primarily reaches to completion around this period. As shown in Fig. 4, numerous apoptotic cell nuclei (*arrows*) were evident in the decidual bed close to the implantation chamber. Apoptotic cells were also present in the luminal epithelium at the mesometrial pole.

Uterine expression of cyclin G1, but not cyclin G2, is regulated by P4

Our observations of uterine expression of cyclin G*s* in pregnancy suggested that ovarian steroid hormones regulate these genes. Thus, we analyzed steroid hormonal regulation of these genes in uteri of adult ovariectomized mice (Fig. 5). In wild-type mice, our results of *in situ* hybridization show that E_2 is unable to induce uterine expression of these genes (Fig. 5A). In contrast, an injection of P_4 induces the expression of cyclin G1 in the luminal and glandular epithelia by 12 h, reaching peak levels by 24 h (Fig. 5B). The combined treatment with P_4 and E_2 was also effective in inducing expression of cyclin G1 similar to that of P_4 alone (data not shown). However, the expression of cyclin G2 is not responsive to E_2 (Fig. 5A) or P_4 (Fig. 5B) or a combination of P_4 and E_2 (data not shown).

We next wanted to examine whether P_4-dependent regulation of uterine gene expression is mediated through its nuclear receptor. Ovariectomized wild-type littermates and PRKO mice were given a single injection of P_4 (2 mg/mouse) and killed after 24 h to examine uterine gene expression. The results show that P_4 fails to induce the expression of cyclin G1 in PRKO uteri, whereas the same treatment effectively induces cyclin G1 in wild-type uteri (Fig. 5C). Consistent with our findings as described above, the expression of cyclin G2

mRNA is not induced by P_4 either in wild-type or PRKO uteri (Fig. 5C). Collectively, the results suggest that the expression of cyclin G1, but not cyclin G2, is regulated by P_4 in a PR-dependent manner in the mouse uterus.

We also analyzed their mRNA levels by Northern blotting in the uteri of ovariectomized wild-type and PRKO mice after injection of steroid hormones after 24 h. Overall, our Northern blot analysis is consistent with *in situ* hybridization results (Fig. 5D).

Uterine expression of G-type cyclins is aberrant in Hoxa-10(−/−) mice on d 4 of pseudopregnancy

Because G-type cyclins in general are growth inhibitory and because Hoxa-10(−/−) uterine stromal cells show attenuated proliferation, we speculated that expression of cyclin Gs in uteri of Hoxa-10(−/−) mice would be aberrant. Indeed, our results of Northern hybridization show that cyclin G1 and cyclin G2 expression in Hoxa-10 mutant uteri is markedly up-regulated, compared with that of wild-type littermates (Fig. 6A). Furthermore, *in situ* hybridization shows that these cyclins are aberrantly induced in uterine epithelial and stromal cells of Hoxa-10 mutant mice, compared with wild-type littermates on d 4 pseudopregnancy (Fig. 6B). These results suggest that Hoxa-10 is a key player in the regulation of cyclin Gs in the uterus.

Discussion

The highlights of this investigation are the expression of cyclin G1 in uterine epithelial cells undergoing differentiation for the preparation of the receptive uterus under the influence of P_4 and cyclin G2 with stromal cell differentiation and death in the decidual bed with the progression of implantation. In general, interplay between the positive and negative regulatory molecules associated with the cell cycle determines whether a cell would undergo proliferation, differentiation, or apoptosis. Our previous observations of expression of positive regulatory molecules and the present findings of expression of the negative cell cycle regulators of the G-type cyclins in the uterus before or during implantation and decidualization suggest that such interplay is also in place in the uterus. The absence of cell-specific expression of cyclin G1 on d 1 and 2 of pregnancy when the uterus is under the influence of ovarian estrogen but its expression in the uterine epithelium on d 3 and 4 with rising P_4 levels suggests that this cell cycle regulatory gene is modulated by P_4 and is important for cell differentiation but not proliferation. The absence of cyclin G1 mRNA expression in PRKO uteri after P_4 treatment is also consistent with P_4 regulation of this gene.

The significance of the expression of cyclin G1 on d 6–8 in the SDZ in which stromal cells are still proliferating is not clearly understood. It is possible that the presence of cyclin G1 in these cells restrict their unlimited growth because a majority of these cells become differentiated and ultimately die. These results are consistent with the role of this cyclin as previously reported (15–18). The lack of cell-specific expression of cyclin G2 mRNA in the uteri on d 1–4 suggests that this molecule is neither associated with proliferation or differentiation nor regulated by P_4 and/or estrogen. On the other hand, the expression of cyclin G2 in the luminal epithelium at the site of blastocyst attachment and in decidualizing

stromal cells that form the PDZ immediately surrounding the implantation chamber suggests that this cyclin heralds terminal differentiation and death signaling for these cells. It is to be recalled that epithelial cells undergo apoptosis for anchorage of the trophectoderm with the stroma, and stromal cells in the PDZ undergo apoptotic death to make room for the growing embryo. This observation is consistent with cyclin G2's role in cell growth inhibition and terminal differentiation (23, 24). It is interesting to note that whereas the expression of cyclin G1 is mostly limited to the SDZ, that of cyclin G2 is primarily restricted to the PDZ, suggesting that these two cyclins have different roles in the uterus.

Similar expression of cyclin G1 mRNAs in the experimentally induced deciduoma as the blastocyst-induced deciduum suggests that the induction of this gene does not require the presence of embryonic signals. On the other hand, much reduced expression of cyclin G2 mRNAs in the deciduoma, compared with the deciduum, suggests that an embryonic signal, at least partially, influences expression of this gene during decidualization. This may mean that cell death at the PDZ to accommodate the growing embryo during normal pregnancy is not an impeding issue for the experimentally induced deciduoma.

Although there is evidence that cyclin G1 expression is transcriptionally regulated by p53 (15, 18), our observations on the expression of p53 mRNAs and cyclin G1 in the periimplantation uterus reveal that these two genes are primarily regulated in uncoordinated manner (Fig. 2 *vs*. Fig. 4; compare d 4, 5, 6, and 8 of pregnancy). This suggests that regulation of cyclin G1 expression in the uterus does not involve p53; it is possible that some other mechanisms directed by P_4 are involved. In this regard, it should be noted that P_4 receptor B is specifically up-regulated in decidualizing stroma in mice lacking p21, an inhibitory protein for cdks (Li, M., and S. K. Das unpublished data). This suggests that an interplay between the two inhibitory-type proteins in the cell cycle plays a role in decidualization through P_4 receptor B.

Although the G-type cyclins are implicated in apoptosis (20, 22, 23, 29), our results of expression of cyclins G1 and G2, when compared with the occurrence of apoptosis at the implantation sites on d 7 and 8, indicate that the location of cyclin G2 expression, not cyclin G1, correlates with the distribution of TUNEL-positive cells. It is interesting to note that the progression of the apoptosis at the site of implantation is followed by the disappearance of cells enlarging the implantation chamber when compared between these two days of pregnancy. Our findings on apoptosis are consistent with previous observations (30). Overall, these results suggest that cyclin G2 influences apoptosis by acting as a proapoptotic inducer of the terminally differentiated cells closely located around the implanting embryo.

Estrogen induces proliferation of uterine epithelial cell, whereas P_4 as a differentiation factor counteracts estrogen-induced epithelial cell proliferation but stimulates epithelial cell differentiation in preparation for embryo implantation via steroid nuclear receptors (31). Our genetic and pharmacological studies provide evidence that progesterone, not estrogen, regulates cyclin G1 in the uterus, whereas the regulation of cyclin G2 appears not to be under the influence of ovarian steroid hormones. Previous studies have shown that progesterone-dependent inhibition of epithelial cell growth is associated with the antagonism of estrogen-induced cell cycle activities, such as nuclear translocation of cyclin

D1/cdk4 and activation of cyclin E/cyclin A/cdk2 kinase, which are associated with growth promotion (32, 33). Our results suggest that P_4 also involves cyclin G1 in inducing epithelial cell differentiation in the presence or absence of estrogen.

Hoxa-10 null uteri lack growth potential for cell proliferation and/or differentiation within the stromal compartment, and thus, Hoxa-10 mutant mice serve as a suitable model for studying cell cycle regulation in uterine biology during early pregnancy (2, 4, 5). We have previously shown Hoxa-10 null uteri after application of a deciduogenic stimulus (24). This suggests that Hoxa-10 is a regulator of cyclin D3, a growth promoter, during decidualization. In a similar notion, our present study showing up-regulated uterine expression of cyclin G1 and cyclin G2 on d 4 in Hoxa-10(−/−) mice suggests that these genes impose a negative regulation on uterine cell proliferation. Whether up-regulation of G-type cyclins in Hoxa-10 null uteri is a direct effect of Hoxa-10 deficiency or whether this up-regulation is the cause of reduced stromal cell proliferation in these null mice is not clearly understood. However, stromal cell expression of Hoxa-10 on d 4 of pregnancy and its continued expression in the stromal cell deciduum after implantation suggest that the expression of cyclin Gs is downstream of Hoxa-10. In conclusion, the present investigation adds new information regarding cell cycle regulation that involves negative regulators in the uterus with respect to cell proliferation, differentiation, and apoptosis, all of which occur during implantation and decidualization.

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Abbreviations

CDK	Cyclin-dependent kinase
E_2	estradiol-17β
Hoxa	homeobox A
HRP	horseradish peroxidase
P_4	progesterone
PDZ	primary decidual zone
PRKO	progesterone receptor knockout
SDZ	secondary decidual zone
TUNEL	terminal deoxynucleotide transferase mediated deoxyuridine 5-triphosphate-biotin nick end labeling

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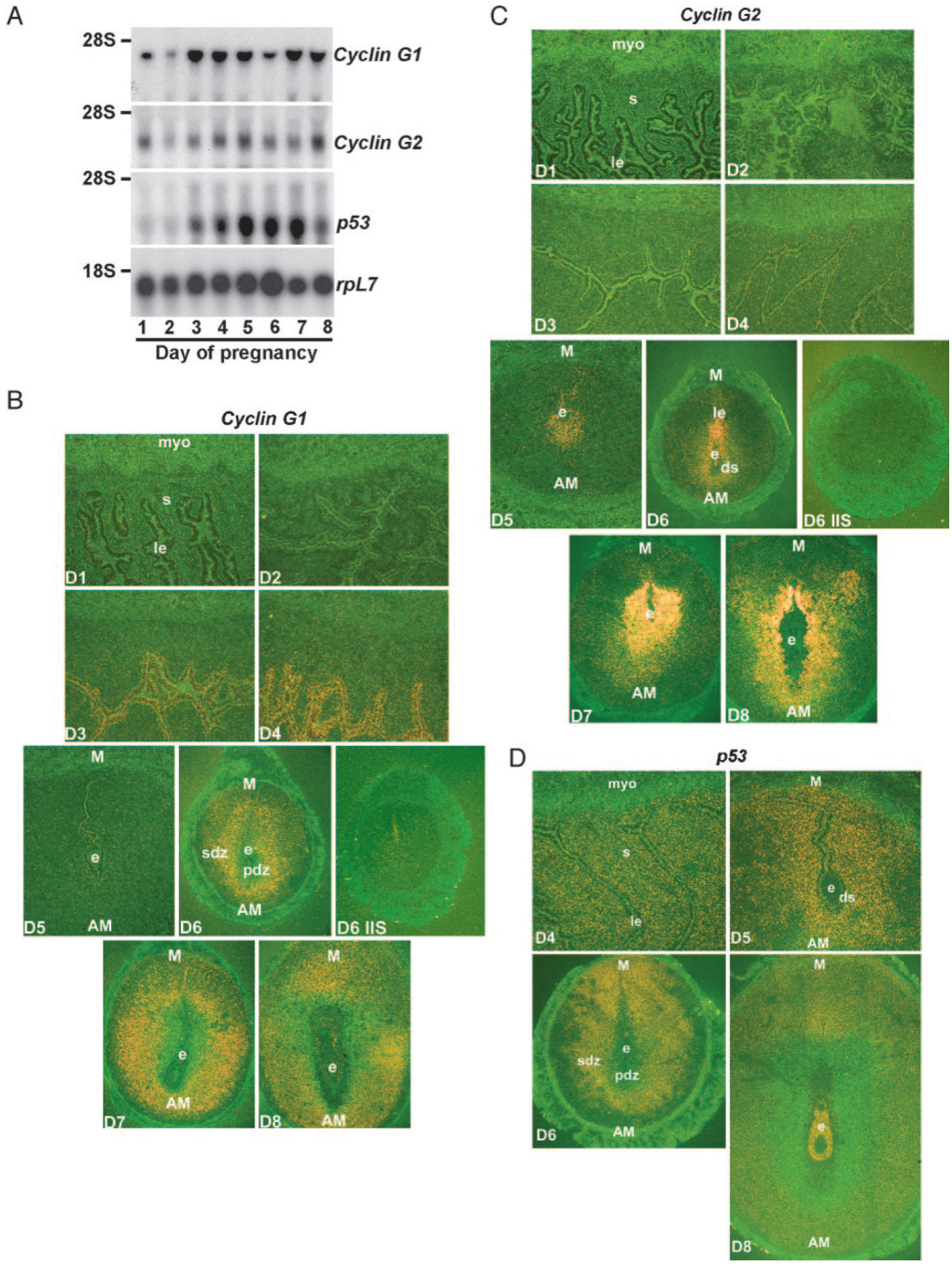

Fig. 1.

A, Northern blot hybridization of cyclin G1, cyclin G2, p53, and rpL7 mRNAs in the periimplantation mouse uterus (d 1–8). Total RNA (6 μg) was separated by formaldehyde-agarose gel electrophoresis, transferred and UV cross-linked to nylon membrane, and hybridized as described in *Materials and Methods*. Hybridization was performed using ^{32}P-labeled cRNA probes sequentially to cyclin G2, cyclin G1, p53, and rpL7. Autoradiographic exposures were 5 h for cyclin G1, 24 h for cyclin G2, 4 h for p53, and 1.5 h for rpL7. B, *In situ* hybridization of cyclin G1 expression in mouse uteri during d 1–8 (D1–D8) of

pregnancy. Frozen uterine sections (11 μm) were mounted onto poly-L-lysine-coated slides and fixed in 4% paraformaldehyde in PBS. Sections were hybridized with ^{35}S-labeled sense or antisense riboprobes for 4 h at 45 C. RNase A-resistant hybrids were detected after 5–7 d of autoradiography using Kodak NTB-2 liquid emulsion. Sections were poststained lightly with hematoxylin and eosin. Dark-field photomicrographs of representative longitudinal sections (D1–D4) and cross-sections for the implantation (D5–D8) and interimplantation sites (D6 IIS) are shown at ×100 (D1–D5) and ×40 (D6–D8). M, Mesometrial pole; AM, antimesometrial pole; e, embryo; pdz, primary decidual zone; sdz, secondary decidual zone; le, luminal epithelium; s, stroma; myo, myometrium. Sections hybridized with the sense probe did not show any positive signals (data not shown). C, *In situ* hybridization of cyclin G2 expression in mouse uteri during d 1–8 (D1–D8) of pregnancy. Dark-field photomicrographs of representative longitudinal sections (D1–D4) and cross sections for the implantation (D5–D8) and interimplantation sites (D6 IIS) are shown at ×100 (D1–D5) and ×40 (D6–D8). M, Mesometrial pole; AM, antimesometrial pole; e, embryo; ds, decidualized stroma; le, luminal epithelium; s, stroma; myo, myometrium. Sections hybridized with the sense probe did not show any positive signals (data not shown). D, *In situ* hybridization of p53 expression in the periimplantation mouse uteri on d 4 (D4), 5 (D5), 6 (D6), and 8 (D8) of pregnancy. Dark-field photomicrographs of representative longitudinal sections (D4) and cross-sections of the implantation sites (D5, D6, D8) are shown at ×100 (D4, D5) and ×40 (D6, D8). M, Mesometrial pole; AM, antimesometrial pole; e, embryo; ds, decidualized stroma; pdz, primary decidual zone; sdz, secondary decidual zone; le, luminal epithelium; s, stroma; myo, myometrium. Sections hybridized with the sense probe did not show any positive signals (data not shown). These experiments were repeated at least three times with similar results.

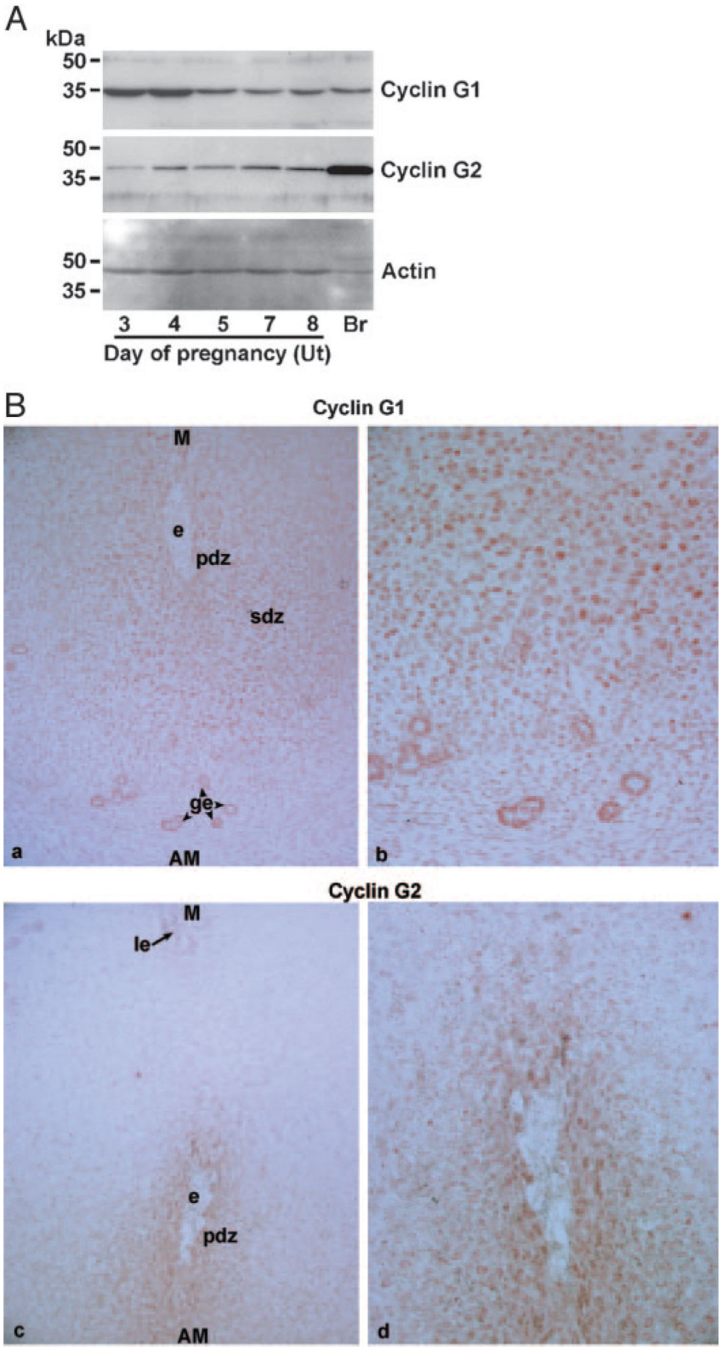

Fig. 2.

A, Western blot analysis of cyclin G1, cyclin G2, and actin proteins in whole uterine (Ut) extracts on d 3–8 of pregnancy and the brain (Br). Immunoblotting with the primary antibodies was performed as described in *Materials and Methods*. A constitutive protein actin was detected to analyze the levels of equal loading. B, Immunostaining of cyclins G1 and G2 in the mouse uterus on d 6 of pregnancy. The photomicrographs of representative uterine sections are shown for cyclin G1 (a and b) and cyclin G2 (c and d) with magnifications at ×100 (a and c) and ×200 (b and). *Red staining* indicates the localization of

immunoreactive proteins. No immunostaining was noted when similar sections were incubated with the primary antibody preneutralized with excess antigen (data not shown). M, Mesometrial pole; AM, anti-mesometrial pole; e, embryo; pdz, primary decidual zone; sdz, secondary decidual zone; le, luminal epithelium; ge, glandular epithelium. These experiments were repeated at least three times with similar results.

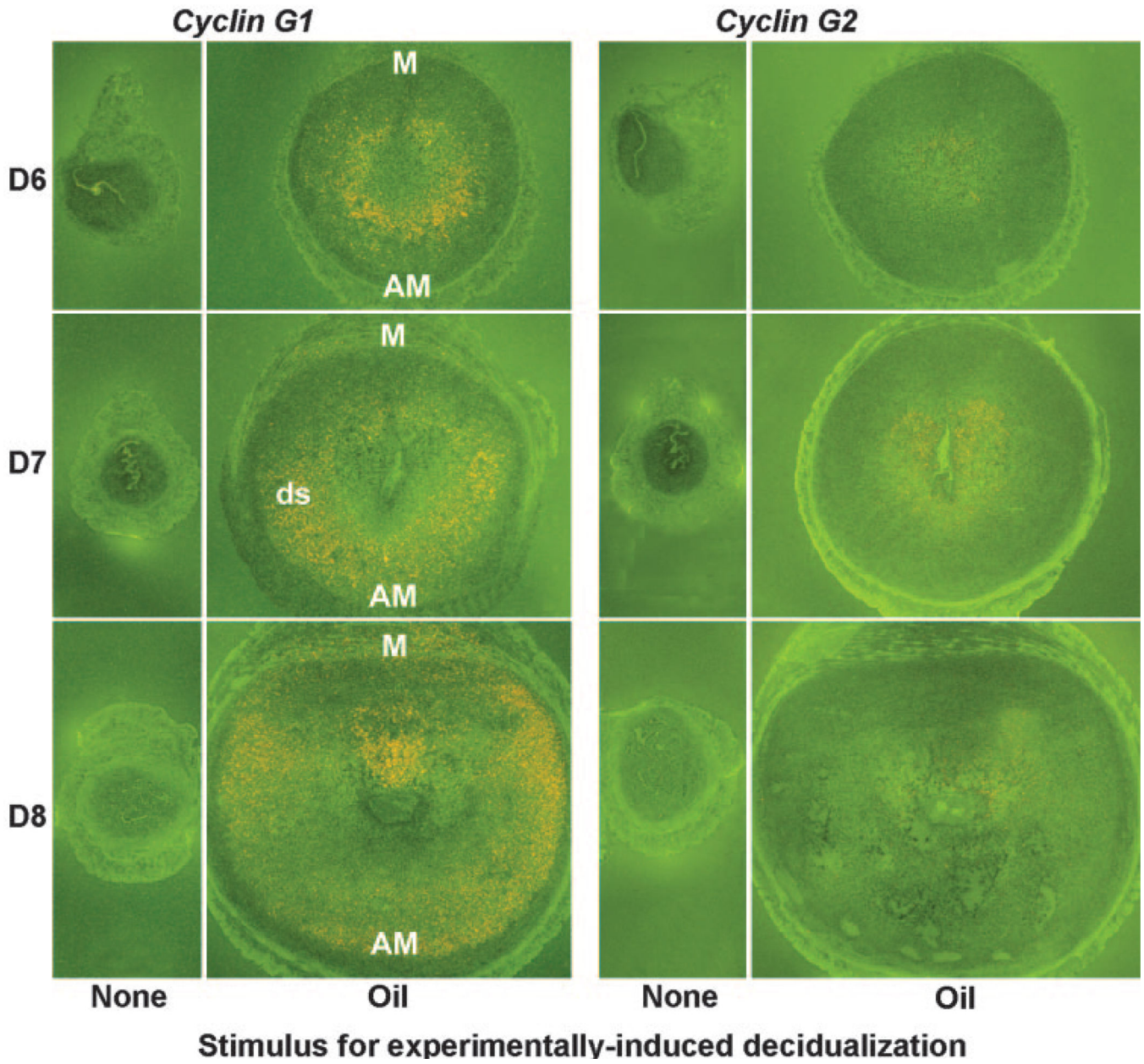

Fig. 3.

In situ hybridization of cyclins G1 and G2 expression in experimentally induced deciduoma on d 6–8 of pseudopregnancy. To induce artificial decidualization, sesame oil was infused intraluminally in one uterine horn on d 4 of pseudopregnancy, whereas the contralateral horn without any infusion served as a control. Dark-field photomicrographs of representative cross-sections of oil-infused and noninfused horns on d 6 (D6), 7 (D7), and 8 (D8) of pseudopregnancy are shown at ×40. M, Mesometrial pole; AM, antimesometrial pole; ds, decidualized stroma. Sections hybridized with the sense probe did not show any positive signals (data not shown). These experiments were repeated at least three times with similar results.

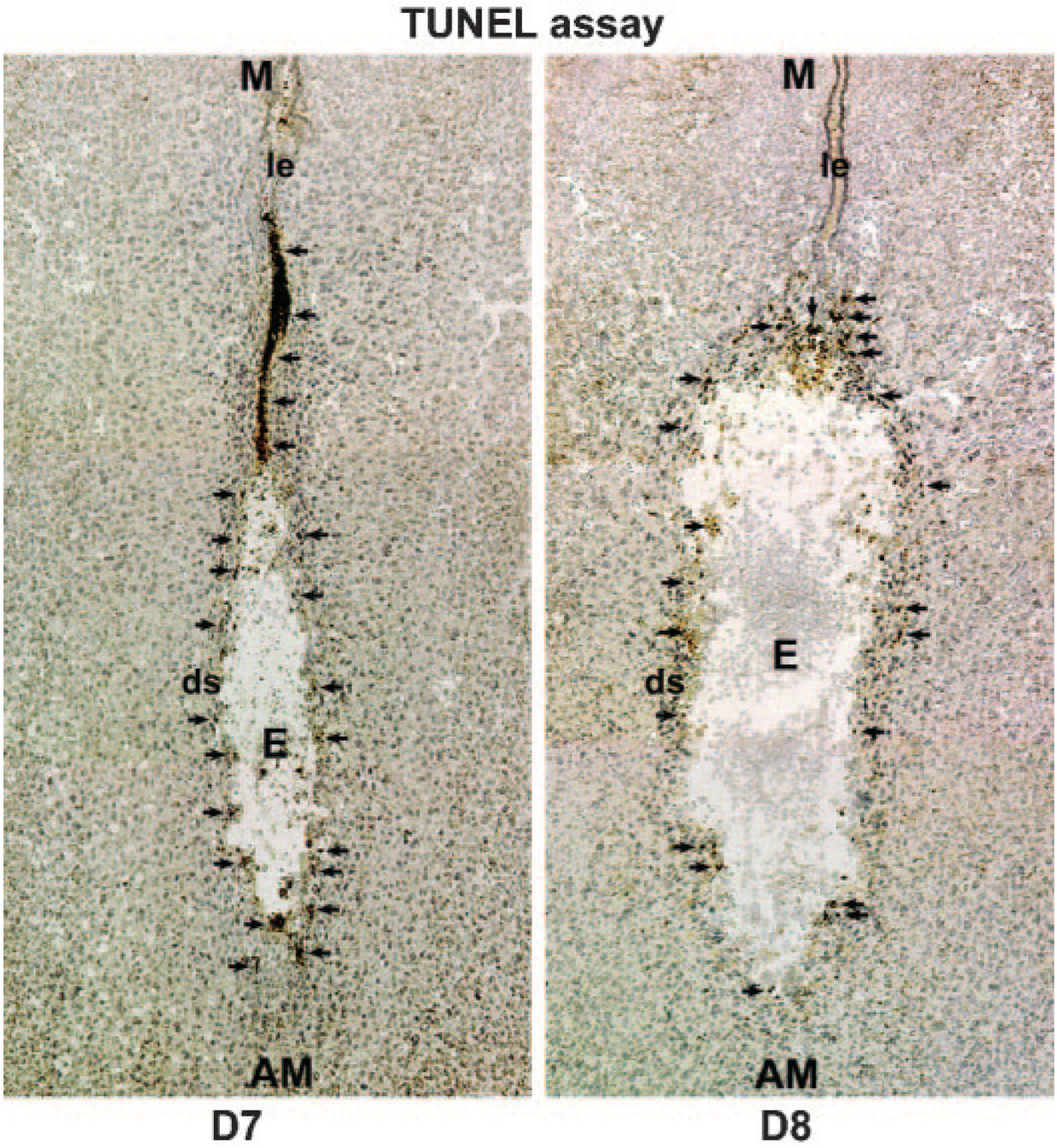

Fig. 4.

TUNEL staining in uterine implantation sites of d 7 and 8 of pregnancy. Uterine sections were incubated with the TUNEL reaction mixture according to the manufacturer's instruction. After a brief rinse in PBS, sections were incubated in streptavidin-HRP solution, followed by incubation in diaminobenzidine solution for color development. Sections were viewed after light hematoxylin counterstaining. Apoptotic cells are detected by *dark brown staining* (*arrows*). For controls, sections were treated with DNase I before incubation with the TUNEL reaction mix (data not shown). Photomicrographs of uterine implantation sites

on d 7 (D7) and 8 (D8) of pregnancy are shown at ×100. M, Mesometrial pole; AM, antimesometrial pole; e, embryo; ds, decidualized stroma; le, luminal epithelium. These experiments were repeated at least three times with similar results.

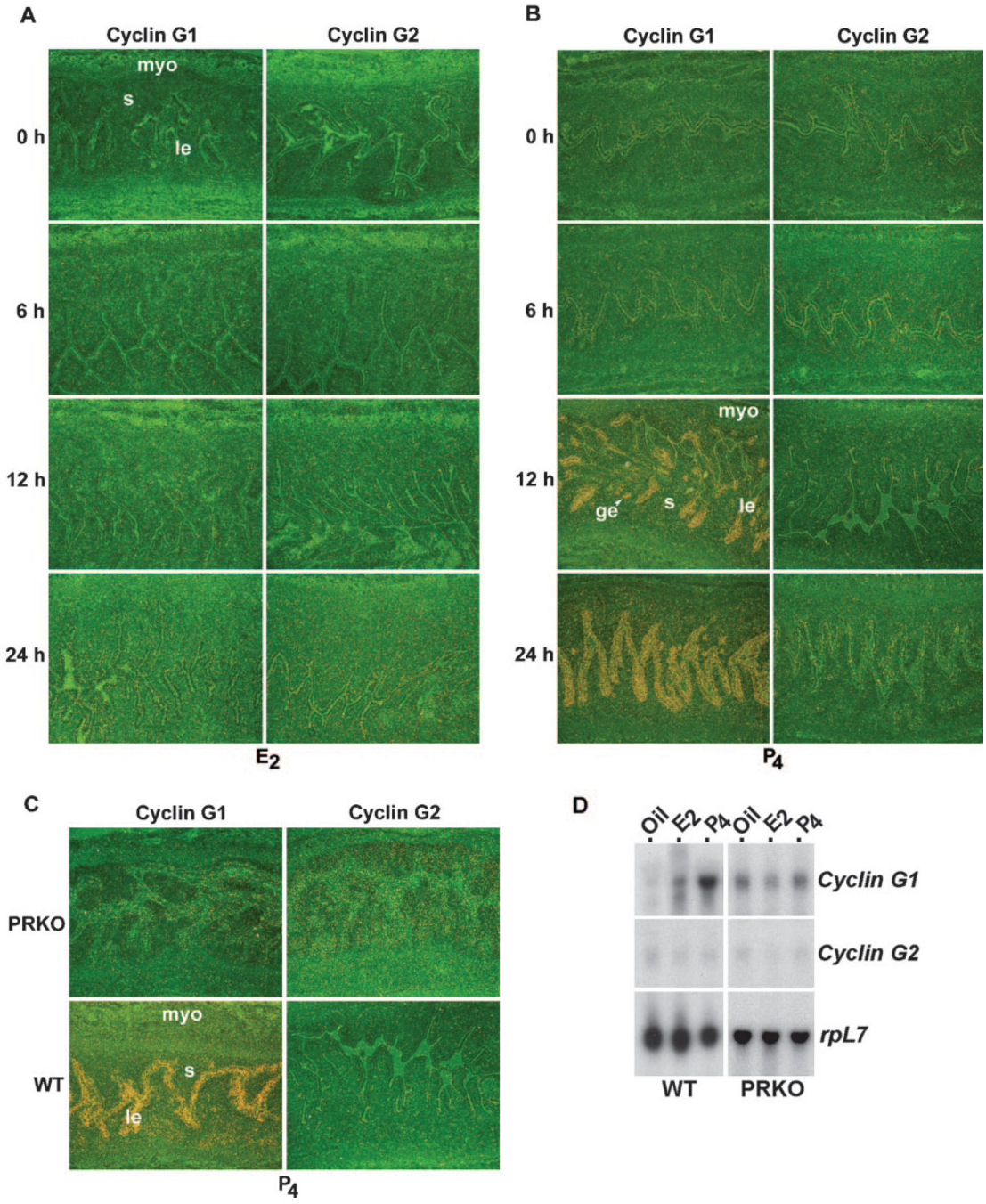

Fig. 5.

In situ hybridization of cyclin G1 and cyclin G2 expression in uteri of ovariectomized wild-type and PRKO mice after steroid hormone treatments. Adult ovariectomized wild-type mice were given a single injection of E_2 (A) (100 ng/mouse) or P_4 (B) (2 mg/mouse) and uteri collected at indicated times. C, Adult ovariectomized wild-type (WT) or PRKO mice were given a single injection of P_4 (2 mg/mouse) and examined after 24 h. Dark-field photomicrographs of uterine sections are shown at 100×. le, Luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium. D, Northern blot hybridization of cyclin

G1 and cyclin G2 expression in uteri of ovariectomized wild-type and PRKO mice undergoing similar treatments as in Fig. 5C. Total RNA (6 μg) was separated by formaldehyde-agarose gel electrophoresis, transferred and UV cross-linked to nylon membrane, and hybridized as described in *Materials and Methods*. Hybridization was performed using ^{32}P-labeled cRNA probes sequentially to cyclin G2, cyclin G1, and rpL7. Autoradiographic exposures were 5 h for cyclin G1, 24 h for cyclin G2, and 1.5 h for rpL7. These experiments were repeated at least three times with similar results.

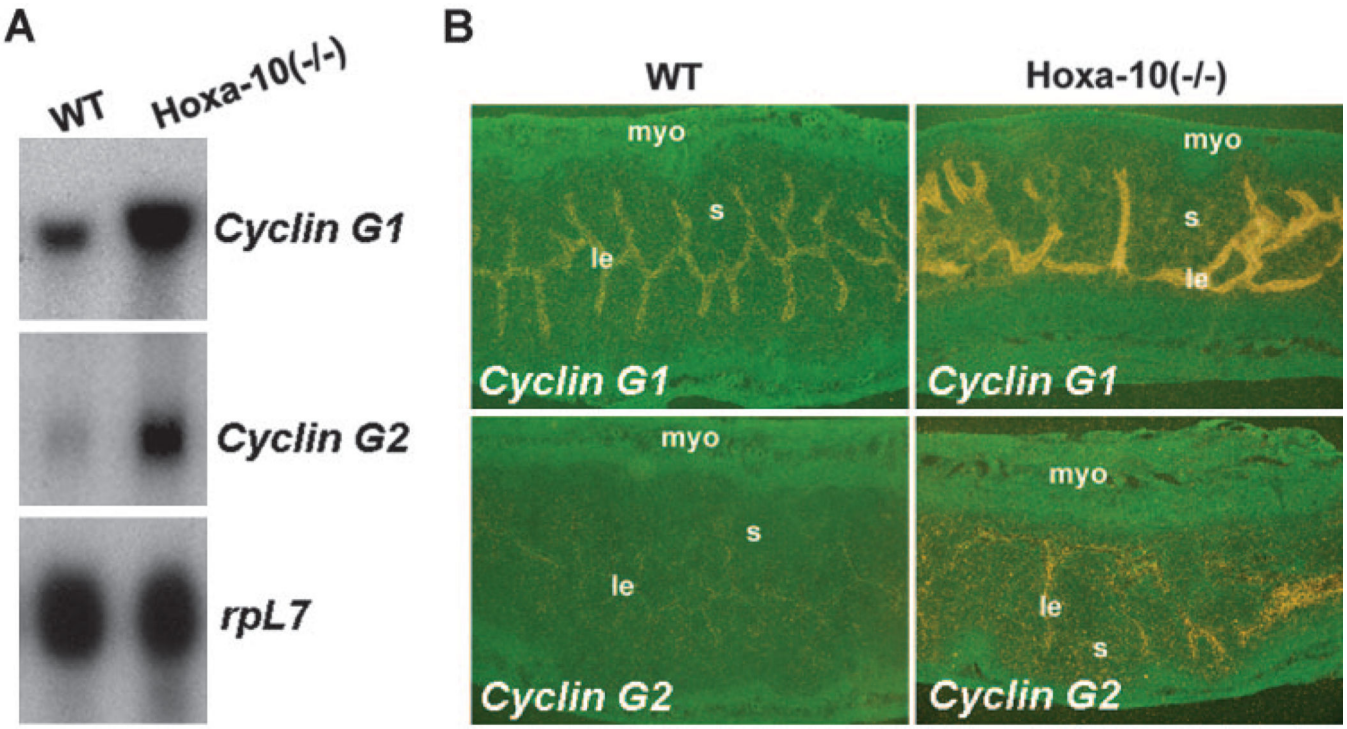

Fig. 6.

A, Northern blot analysis of cyclin G1 and cyclin G2 expression in uteri on d 4 pseudopregnancy of wild-type (WT) and Hoxa-10(−/−) mice. Whole uterine total RNA samples (6μg) isolated from wild-type and Hoxa-10(−/−) mice were analyzed by Northern blotting. rPL7 is a housekeeping gene. B, *In situ* hybridization of expression for cyclin G1 and cyclin G2 in uteri of wild-type (WT) and Hoxa-10(−/−) mice on d 4 pseudopregnancy. Representative dark-field photomicrographs of longitudinal uterine sections are shown at ×40. le, Luminal epithelium; s, stroma; myo, myometrium. Sections hybridized with sense probes did not show any positive signals (data not shown). These experiments were repeated at least three times with similar results.