Protein Related to DAN and Cerberus Is a Bone Morphogenetic Protein Antagonist That Participates in Ovarian Paracrine Regulation

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Bone morphogenetic proteins (BMPs) are important for body patterning and morphogenesis, whereas several BMP antagonists regulate the functions of BMPs during embryonic development and tissue differentiation. Protein related to DAN and cerberus (PRDC) is a secreted protein with a cystine knot structure identified by gene trapping in embryonic stem cells. Although PRDC shows sequence homology with proteins of the BMP antagonist family, its biological activity and physiological functions are unclear. We generated recombinant PRDC and its paralog, gremlin, and tested their ability to suppress actions initiated by diverse BMP proteins. Similar to the known BMP antagonist, gremlin, PRDC blocked ligand signaling induced by BMP2 and BMP4 but had minimal effects on reporter gene activation induced by GDF-9, activin, or transforming growth factor-β. Co-precipitation assays further demonstrated the direct protein-protein interactions between PRDC and BMP2 or BMP4. Reverse transcriptase-PCR analyses indicated that PRDC transcripts are widely expressed showing higher levels in ovary, brain, and spleen. In mouse ovary, PRDC transcripts were increased following gonadotropin treatment. In situ hybridization analyses further indicated that ovarian PRDC transcripts are localized in granulosa cells of selective follicles. In addition, co-treatment with PRDC antagonized the inhibitory effects of BMP4 on the follicle-stimulating hormone stimulation of progesterone production by cultured rat granulosa cells. Thus, PRDC is a potent BMP antagonist with a wide tissue expression pattern, and ovarian PRDC expressed in granulosa cells could be involved in follicular development by antagonizing the actions of theca cell-derived BMPs.

The TGF-β1 superfamily has more than 35 members in vertebrates, including TGF-βs, growth differentiation factors (GDFs), activins, inhibins, Müllerian inhibiting substance, Nodal, and Lefty proteins (1–5). Among these cystine knot proteins, bone morphogenetic proteins (BMPs) represent the largest subgroup. BMP proteins were first identified in the protein extracts of demineralized bone (6) and are involved in body patterning and morphogenesis (5, 7, 8). The developmental signaling pathway mediated by vertebrate BMPs and their fly orthologs, decapentaplegic, screw, and glass bottom boat (9), are highly conserved during animal evolution. These hormones are required for the regulation of dorsal-ventral patterning of the early embryo in both vertebrates and invertebrates (5, 7). Like other members of the TGF-β family, BMP proteins initiate cellular signaling by assembling type I and type II serine/threonine kinase receptor complexes that activate Smad transcription factors (1).

The BMP antagonists are also cystine knot-containing proteins and regulate BMP signaling through direct binding. By using a bioinformatic approach, we analyzed all human genes with unique cystine knot structures similar to known BMP antagonists (10). Based on the size of the cystine ring, BMP antagonists were classified into three subfamilies as follows: CAN (eight-membered ring), twisted gastrulation (nine-membered ring), and chordin and noggin (10-membered rings). The CAN family consists of seven members, including gremlin, protein related to DAN and cerberus (PRDC), and others. PRDC was discovered in mouse embryonic stem cells by gene trapping (11). Although PRDC, a close paralog of gremlin, was found to be expressed in commissural neurons of the spinal cord in the mouse embryo, its tissue expression pattern and potential role as a BMP antagonist are unclear. Here we produced recombinant human PRDC and examined its tissue expression pattern and biological functions as a BMP antagonist. Based on the high expression of PRDC in mouse ovary, its regulation by gonadotropins and ovarian expression pattern were tested. We also demonstrated that PRDC antagonized the inhibitory effects of BMP4 on FSH-induced progesterone production in granulosa cells.

MATERIALS AND METHODS

Animals—Adult Swiss-Webster mice were obtained from Charles River Breeding Laboratories (Wilmington, MA) and used for RT-PCR studies to elucidate tissue distribution of PRDC and gremlin. To investigate the regulation of PRDC in mouse ovary, 23-day-old female Swiss-Webster mice were injected with 4 IU of PMSG, followed by 10 IU of hCG 48 h later. For granulosa cell cultures, 25-day-old female Sprague-Dawley rats treated with diethylstilbestrol were used (12). Animals were housed in accordance with institutional and National Institutes of Health guidelines for the care and use of experimental animals.

Hormones and Reagents—DMEM/Ham’s F-12 (DMEM/F-12), McCoy’s 5A peptide N-glycosidase F; PRDC, protein related to DAN and cerberus; SBE, Smad-binding element; RT, reverse transcriptase; DMEM, Dulbecco’s modified Eagle’s medium; NT, N terminus; CT, C terminus.

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The abbreviations used are: TGF-β, transforming growth factor-β; BMP, bone morphogenetic protein; DAN, differential screening-selected gene aberrative in neuroblastoma; FBS, fetal bovine serum; FSH, follicle-stimulating hormone; GDF, growth differentiation factor; hCG, human chorionic gonadotropin; PMSG, pregnant mare serum gonadotropin; PNGase, peptide N-glycosidase F; PRDC, protein related to DAN and cerberus; SBE, Smad-binding element; RT, reverse transcriptase; DMEM, Dulbecco’s modified Eagle’s medium; NT, N terminus; CT, C terminus.

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5a medium, L-15 Leibovitz medium, α-minimum essential medium, and Dulbecco’s modified Eagle’s medium (DMEM) were obtained from Invitrogen. Recombinant human BMP2, BMP4, BMP6, BMP7, TGF-β1, activin-A, and recombinant mouse GDF-5 were purchased from R&D Systems, Inc. (Minneapolis, MN). Recombinant GDF-9 was generated in transfected human embryonic kidney 293T cells and characterized as described previously (13). Anti-FLAG M2 monoclonal antibodies and FLAG peptide were from Sigma. Biotinylated anti-mouse gelatin antibody and biotinylated anti-BMP-2/4 antibodies were purchased from R&D Systems, Inc. Anti-human PRDC antibodies were generated by using a synthetic peptide encoding the following sequence: Ac-CFY-IPRHVKKEEESFP-amide (BIOSOURCE, Camarillo, CA). This stretch of 16 residues was designed based on human PRDC and showed 93% homology to rat PRDC. The antibodies were enzyme-linked immunosorbent assay using the synthetic peptide as the antigen. This PRDC antibody does not cross-react with recombinant gremlin (data not shown).

**Generation of PRDC and Gremlin cDNAs—**PCR was performed using Advantage cDNA kit (Clontech Laboratories, Inc., Palo Alto, CA) and a human ovary Marathon-Ready cDNA library (Clontech Laboratories, Inc.). Fidelity of the PCR products was confirmed by sequencing on both strands of the final constructs before use in expression studies. All cDNA were subcloned into the expression vector pcDNA3.1/Zeo (Invitrogen). To allow efficient detection and purification of the recombinant protein, a FLAG epitope (DYKDDDDK) was added to the C terminus of PRDC (PRDC-CT). The C terminus of gremlin was fused to an FLAG epitope of tagged proteins using anti-FLAG M2 affinity column (Sigma) and verification by Coomassie Blue staining, the purified tagged proteins were used as standards to quantify nontagged recombinant PRDC and gremlin in conditioned media using immunoblotting.

**Expression of Recombinant Human PRDC and Gremlin Proteins in 293T Cells—**Human 293T cells were maintained in DMEM/F-12 supplemented with 10% FBS. Cells were transfected using 10 μg of plasmid by the calcium phosphate precipitation method (14). Clonal cell lines stably expressing wild type and FLAG-tagged PRDC and gremlin were selected under 500 μg/ml Zeocin (Invitrogen) and maintained in DMEM/F-12 containing 10% FBS and 100 μg/ml Zeocin. When the cells reached 60% confluence, the media was replaced in DMEM/F-12 without FBS or Zeocin. After 3 days of serum-free culture, the conditioned media were harvested, and cell debris was cleared. For some experiments, the supernatant was concentrated 20-fold using Amicon Ultra (Millipore Corp., Bedford, MA).

**Characterization of Recombinant Human PRDC and Gremlin—**Conditioned media from 293T cells expressing PRDC or gremlin were electrophoresed using 12% SDS-PAGE before transfer of proteins to polyvinylidene difluoride membranes (Hybond-P, Amersham Biosciences). Immunoblots were performed using anti-PRDC antiserum (1:1000 dilution), anti-mouse gremlin antibody (0.15 μg/ml), or anti-FLAG M2 monoclonal antibody as primary antibodies. Signals were detected following chemiluminescent imaging using the ECL system (Amersham Biosciences).

To remove N-linked carbohydrate side chains, conditioned media were diluted in the deglycosylation buffer (50 mM sodium phosphate, pH 7.5, 1% Nonidet P-40, 0.5% SDS, and 1% β-mercaptoethanol) and incubated with 7.7 μIU of peptide N-glycosidase F (PNGase F) (New England Biolabs, Beverly, MA) at 37 °C for 1 h. Samples were mixed with loading buffer under reducing conditions (100 mM dithiothreitol and 5% β-mercaptoethanol) before immunoblotting.

** Luciferase Reporter Assay—**The reporter plasmids, SBE-lux and Luciferase Reporter Assay kit (Qiagen) and oligo(T)12-18 (Invitrogen). A mouse PRDC fragment (290 bp) was amplified using primers 5'-ctctctatcagtagaatt-3' and 5'-aagct-ttggcttcatc-3' by performing 35 cycles of PCR (94 °C, 30 s; 62 °C, 30 s; and 72 °C, 45 s). The PCR products were analyzed on a 1.5% agarose gels stained with ethidium bromide. For real time PCR, ovarian PRDC and β-actin cDNAs were amplified using the QuantiTete Probe PCR kit (Qiagen) and analyzed in the SmartCycler II System (Cepheid, Sunnyvale, CA). Webtool Primer3 www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi was used for designing PCR primers and probes.

**RT-PCR and Northern Blotting—**Total RNA from mouse tissue was isolated using RNeasy Mini kit (Qiagen, Valencia, CA). Samples were transcribed into cDNAs using Omniscript reverse transcriptase (Qiagen) and oligo(dT)12-18 (Invitrogen). A mouse PRDC fragment (290 bp) was amplified using primers 5'-ctctctatcagtagaatt-3' and 5'-aagct-ttggcttcatc-3' by performing 35 cycles of PCR (94 °C, 30 s; 62 °C, 30 s; and 72 °C, 45 s). The PCR products were analyzed on a 1.5% agarose gels stained with ethidium bromide. For real time PCR, ovarian PRDC and β-actin cDNAs were amplified using the QuantiTete Probe PCR kit (Qiagen) and analyzed in the SmartCycler II System (Cepheid, Sunnyvale, CA). Webtool Primer3 www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi was used for designing PCR primers and probes.

**In Situ Hybridization—**Mouse ovaries were fixed at 4 °C for 6 h in 4% paraformaldehyde in phosphate-buffered saline, followed by immersion in 0.5 M sucrose in phosphate-buffered saline overnight. Cryostat sections (14 μm thick) were mounted on Super Frost Plus (Fisher), fixed in 4% paraformaldehyde in phosphate-buffered saline and stored at 80 °C until analyzed. As described previously (18), hybridization was carried out at 52–55 °C overnight in a mixture containing the 35S-labeled mouse PRDC cDNA probe, 50% formamide, 0.3 M NaCl, 10 mM Tris-HCl, 5 mM EDTA, 1× Denhardt’s solution, 10% dextran sulfate, 1 μg/ml carrier transfer RNA, and 10 μM dithiothreitol. Posthybridization washing was performed under stringent conditions that included ribonuclease A (25 μg/ml) treatment at 37 °C for 30 min and a final stringency of 0.1× SSC. Slides were dipped into NTB-2 emulsion (Eastman Kodak Co.), exposed at 4 °C, and developed after 2 weeks. The slides were stained with hematoxylin and eosin and examined under the light microscope with bright- and dark-field illumination.

**Characterization of Granulosa Cells—**Granulosa cells were obtained from small antral follicles of diethylstilbestrol-treated rats (12). Ovaries were punctured in L-15 Leibovitz medium supplemented with 0.1% bovine serum albumin. Ovarian debris, oocytes, and small follicles were removed, and the remaining medium containing granulosa cells was collected after low speed centrifugation at 500 × g for 10 min.

Granulosa cells (1 × 10^6) viable cells/ml were cultured using McCoy’s 5a media supplemented with androstenedione (10−7 M) in the presence or absence of different hormones. After 48 h of incubation, the supernatant was collected and stored at −80 °C until the determination of progesterone content by using antisera raised against progesterone-11-hemisuccinate/bovine serum albumin (19).

**RESULTS**

**Production and Characterization of Recombinant Human PRDC and Gremlin—**The mouse PRDC gene encodes a protein with a signal peptide for secretion and shares high homology with gremlin (11). We searched the human ortholog of PRDC in the GenBank™ using the BLAST program, and we performed RT-PCR using an ovarian cDNA library to obtain the full-length human PRDC cDNA. Human 293T cells were transfected with expression vectors containing human PRDC cDNA or PRDC tagged at its C terminus with a FLAG epitope (PRDC-CT).

Following selection of permanent clones, individual cell lines were cultured to obtain conditioned media. Immunoblotting was performed using PRDC antibodies or M2 antibodies.
against the FLAG epitope. Three prominent PRDC bands at 23, 19, and 18 kDa likely represented PRDC proteins with different degrees of N-linked glycosylation, whereas a lower 16-kDa band could be a proteolytic PRDC fragment (Fig. 1A). After treatment with PNGase F, only the 19-, 18-, and 16-kDa bands were evident. Based on sequence analyses (10), the PRDC gene encodes a peptide of 19 kDa after signal peptide removal. The mature peptide has two potential N-linked glycosylation sites (Asn<sup>26</sup> and Asn<sup>161</sup>) and one potential proteolytic processing site (Lys<sup>66</sup>). Thus, the 23-kDa band may represent glycosylated PRDC, whereas the 16-kDa band could be a proteolytic fragment. The PRDC antibodies also recognized two nonspecific high molecular weight bands in mock-transfected cells.

Because the present PRDC antibodies do not recognize the recombinant PRDC under nonreducing conditions, we also generated C-terminal tagged PRDC, and we analyzed its properties under both reducing and nonreducing conditions using PRDC (Fig. 1A) and M2 (Fig. 1B) antibodies. Compared with the native protein, the PRDC-CT migrated in a similar manner under reducing and deglycosylated conditions. Of interest, immunoblotting of tagged PRDC under nonreducing conditions yielded diffused high molecular mass bands of ~40 kDa, suggesting the presence of PRDC dimers.

We also characterized the recombinant human gremlin proteins generated by using a similar approach. Under reducing conditions, immunoblot analyses indicated that gremlin is also a glycosylated protein, with a higher molecular mass 24-kDa band migrating as a 20-kDa band following treatment with PNGase F (Fig. 2A). Under nonreducing conditions, a weak higher molecular mass band (40 kDa) corresponding to dimer forms was also detected. However, one cannot rule out the possibility that this band is nonspecific. By using either gremlin (Fig. 2A) or the M2 antibodies (Fig. 2B), the N-terminally tagged gremlin (gremlin-NT) also behaved as a glycosylated protein. Both PRDC-CT and gremlin-NT were purified with affinity chromatography and used to estimate the amount of their nontagged counterparts for functional studies as described below.

**Ability of PRDC and Gremlin to Suppress the BMP Induction of Luciferase Reporter Activities**—By taking advantage of luciferase reporter plasmids capable of responding to Smad activation (15, 16), we tested the inhibitory effect of PRDC and gremlin on luciferase reporter activities induced by various TGF-β superfamily ligands (Fig. 3). P19 cells transfected with the SBE promoter-luciferase construct responded to treatment with BMP2, BMP4, BMP6, and BMP7 but showed minimal stimulation by GDF-5. Of interest, co-treatment with either PRDC or gremlin suppressed BMP-induced luciferase reporter activities (Fig. 3A). At 25 nM, PRDC and gremlin completely suppressed luciferase reporter activity induced by BMP2 or BMP4. However, PRDC attenuated the actions of BMP6 and BMP7 by only 30% (p < 0.05), whereas gremlin did not significantly decrease the reporter activity induced by BMP6 or BMP7. Both PRDC and gremlin were ineffective in blocking GDF5 actions.

By using the CAGA promoter-luciferase reporter, the ability of PRDC and gremlin to modulate the actions of activin, TGF-β, and GDF-9 was tested (Fig. 3B). Treatment of cells transfected with the CAGA promoter with activin, TGF-β, or GDF-9 led to increases in reporter activities. In contrast to their regulation of BMP actions, PRDC or gremlin did not affect the reporter activity induced by activin, TGF-β, or GDF-9. Similar results were obtained when these experiments were performed by using the SBE promoter (data not shown). To evaluate the potency of PRDC and gremlin in antagonizing the BMP actions, we tested the dose dependence of these BMP antagonists by using the luciferase reporter assay (Fig. 4). Co-treatment with PRDC inhibited the stimulation of luciferase activity by BMP2 and BMP4 in a dose-dependent manner (IC<sub>50</sub> 3 and 2.5 nM, respectively). Likewise, co-treatment with gremlin inhibited the actions of BMP2 and BMP4 with a similar potency (IC<sub>50</sub> 3 and 2.8 nM, respectively). Similar to the recombinant nontagged PRDC, C-tagged PRDC also antagonized the actions of BMP2 and BMP4 in the present luciferase reporter activity assay (Fig. 4A). The tagged PRDC was then used for co-immunoprecipitation tests.

**Direct Interactions between PRDC and BMP2 or BMP4**—To elucidate the mechanisms underlying the antagonistic effects of PRDC on BMP2 or BMP4 actions, co-immunoprecipitation tests were performed using PRDC-CT and affinity beads containing covalently linked M2 antibodies. BMP2 or BMP4 proteins (1 μg each) were co-incubated with PRDC-CT or boiled PRDC-CT (1 μg each) before mixing with the M2-affinity beads. Proteins not bound by the M2 beads (pass-through fractions) and those eluted with a buffer containing the FLAG peptide (eluate) were analyzed in immunobLOTS using anti-BMP2/4 antibodies (Fig. 5). Although the BMP2 or BMP4 proteins preincubated with PRDC were retained in the eluate, BMP2 or BMP4 incubated with boiled PRDC was only found in the pass-through fraction. As expected, BMP2 or BMP4 proteins by themselves were also found in the pass-through fraction. These results demonstrated the direct binding between these two BMP proteins and tagged PRDC.
Tissue Expression Pattern of PRDC—Although an earlier study demonstrated the localization of PRDC mRNA in commissural neurons of the developing spinal cord of mouse embryos (11), the tissue expression pattern of PRDC in other stages and tissues is unclear. To elucidate the tissue expression pattern of PRDC in adult mice, we performed RT-PCR analyses using different amounts of the template cDNA. As shown in Fig. 6, PRDC was expressed most abundantly in the ovary, followed by brain, spleen, colon, kidney, and uterus. Even at a 100-fold dilution of the template cDNA, the ovary still showed a clear signal. These data suggest that PRDC is a BMP antagonist showing wide tissue expression in adult tissues.

Ovarian PRDC Expression Is Regulated by Gonadotropins—We further monitored PRDC mRNA levels in the ovary. As shown in Fig. 7A, quantitative real time PCR studies indicated that treatment of immature mice with PMSG to induce follicle maturation led to a small increase in PRDC expression. Subsequent treatment with hCG to induce ovulation was correlated with a decrease of PRDC message levels. Furthermore, Northern blot analyses (Fig. 7B) indicated similar regulation of a 4.0-kb PRDC transcript by gonadotropins. These results suggested that PRDC expressed in the ovary could participate in the regulation of follicle development.

Localization of PRDC Transcripts in Mouse Ovary—To investigate the ovarian cell types expressing PRDC, we performed in situ hybridization analyses (Fig. 8). The PRDC signal was localized to mural and cumulus granulosa cells in selective early antral follicles of immature mice without exogenous go-
nadotropin treatment. At 12 and 48 h after PMSG treatment, PRDC transcripts were found in mural and cumulus granulosa cells of preovulatory follicles. In contrast, neither oocytes nor theca-interstitial cells showed PRDC signals.

PRDC Antagonizes BMP2/4. BMP2 (A) or BMP4 (B) proteins were co-incubated with C-terminal FLAG-tagged PRDC (PRDC-CT) or boiled PRDC-CT before mixing with the affinity beads containing covalently linked M2 antibodies. Proteins not bound by the M2 beads (P, pass through) and those eluted from the beads with a buffer containing the FLAG peptide (E, eluate) were analyzed in immunoblots using biotinylated anti-BMP-2/4 antibodies.

PRDC Antagonizes BMP4-induced Suppression of Progesterone Production by Cultured Granulosa Cells—Earlier studies have shown that co-treatment with BMP4 suppressed progesterone production induced by FSH in cultured rat granulosa cells (20). Based on these findings, we investigated the potential antagonizing effect of PRDC on BMP4 actions. Granulosa cells were treated with FSH to stimulate progesterone production (Fig. 9). Some cells were also treated with BMP4 with or without PRDCs that were preincubated together for 30 min at 37 °C. Forty eight hours after cell culture, progesterone levels in the conditioned medium were determined. Co-treatment with BMP4 suppressed the FSH stimulation of progesterone production by 60%. However, preincubation of BMP4 with
PRDC Antagonizes BMP Actions in the Ovary

62% amino acid sequence homology in their mature region. In contrast to the presence of orthologs for PRDC and gremlin in human, mouse, and fugu, only one ortholog for both PRDC and gremlin was found in Caenorhabditis elegans (29) and Ciona intestinalis (10). These results indicate that during early vertebrate evolution, gene duplication allowed the divergence of PRDC and gremlin functions. Mouse gremlin is glycosylated, and both glycosylated and nonglycosylated forms of gremlin are potent BMP antagonists (28). The present recombinant human gremlin proteins are also glycosylated, consistent with the presence of a putative glycosylated site in the deduced human protein sequence. Similar to gremlin, multiple forms of the human recombinant PRDC protein were found. Because the predicted human PRDC protein has a putative N-glycosylation site (Asn40) and a potential proteolytic processing site (Lys65), the observed forms could represent glycosylated, nonglycosylated, and proteolytic processed PRDC. Immunoblotting analyses further suggested that both PRDC and gremlin may form dimers. Based on the crystallographic analyses of the hCG-β structure, both gremlin and the closely related PRDC have an additional free cysteine residue near the cystine knot likely to be involved in dimer formation (10).

During evolution, BMP family genes expanded and comprised a large family of ligands including multiple paralogs in vertebrates. Based on phylogenetic analyses, BMP2 and BMP4 are highly related, with BMP2 containing an additional heparin-binding domain at the N terminus (30, 31). In contrast, BMP5, BMP6, BMP7, and BMP8 comprise the second subgroup, and GDF6/BMP13, GDF7/BMP12, and GDF5 are classified into the third subgroup. To evaluate the ability of PRDC to antagonize BMP signaling, we took advantage of the luciferase reporter activity assay (15, 16). The luciferase reporters carry the tandem repeats of short consensus sequences that are identified as Smad-binding elements in the promoters of target genes up-regulated by TGF-β superfamilies. In P19 cells, treatment with BMP2, BMP4, BMP6, BMP7, and GDF5 stimulated the SBE promoter. SBE is composed of the CAACAG sequence found in the promoter of the JunB gene, a known target of TGF-β, activin, and BMP2. Of interest, PRDC shows broad and strong inhibitory effects on the activation of the SBE promoter induced by BMP2, BMP4, BMP6, and BMP7. Although gremlin also antagonized the SBE promoter activity induced by BMP2 and BMP4, it showed lower potency in blocking BMP6 or BMP7 actions. These data suggest that PRDC antagonized the actions of both first and second subgroups of BMP protein, whereas gremlin was mainly acting on the first subgroup. In contrast, both PRDC and gremlin showed no antagonism of the actions of activin, TGF-β, or GDF-9 based on studies using both SBE and CAGA promoters. These results indicate that both PRDC and gremlin antagonize BMP but not TGF-β/activin signaling and that PRDC has a wider specificity than gremlin.

Co-precipitation analyses further demonstrated the direct binding between PRDC and BMP2 or BMP4, and that antagonism of BMP signaling by PRDC is likely due to direct binding of PRDC to BMP proteins. Recently, the crystal structure of Noggin complexed to BMP7 was reported suggesting that Noggin inhibits BMP signaling by blocking the molecular interfaces of the BMP-binding epitopes for both type I and type II receptors (32). This model suggests that other BMP antagonists might also prevent BMP ligand-receptor assembly by occupying the binding interfaces of BMPs to receptors.

Studies using mutant mice indicated that both BMP2 and BMP4 null mice showed embryonic lethality, with the former exhibiting defects in amnion/chorion and cardiac development (33) and the latter showing a lack of mesoderm differentiation.

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**DISCUSSION**

We generated recombinant PRDC and showed it to be a glycoprotein capable of forming dimers that could function as a BMP antagonist. Compared with gremlin, a known BMP antagonist, PRDC showed broader inhibitory effects by blocking the Smad signaling induced by BMP2, BMP4, BMP6, or BMP7. Co-precipitation tests indicated that the observed antagonistic effect of PRDC is likely due to the direct binding of PRDC to BMP proteins. Of interest, the PRDC transcript was abundantly expressed in the ovary and regulated by gonadotropins. In situ hybridization demonstrated that mouse PRDC transcripts were expressed in mural and cumulus granulosa cells. In cultured granulosa cells, PRDC treatment antagonized the inhibitory effects of BMP4 on the FSH stimulation of progesterone production (p < 0.01; Fig. 9B).

PRDC prevented the suppressive effect of BMP4 on FSH actions (p < 0.01; Fig. 9A). Similar to PRDC, preincubation of BMP4 with gremlin also antagonized the inhibitory effect of BMP4 on the FSH stimulation of progesterone production (p < 0.01; Fig. 9B).

**FIG. 7. Gonadotropin regulation of PRDC expression in the ovary.** Immature mice were treated with PMSG (4 IU) to induce follicle development followed 48 h later by hCG (10 IU) to induce ovulation. Ovarian RNA was extracted at different times after hormonal treatment before real time RT-PCR (A) or Northern blot (B) analyses. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) expression served as an internal control.

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Both **PRDC** and **gremlin** are single exon genes and share
and deficiency of primordial germ cells (35). These results indicate that BMP2 and BMP4 are essential for early embryonic development. It is likely that mis-expression of PRDC during embryonic development could lead to embryonic abnormalities. Although gremlin null mice showed abnormalities in limb skeletal development (36), the role of PRDC in embryonic development is unclear. In addition to earlier studies showing PRDC expression in embryos (11), our data demonstrated the expression of PRDC in diverse tissues of adult mice, suggesting potential roles of this protein in regulating BMP signaling during adult life in ovary, brain, and other adult tissues. Of interest, gremlin was also shown to be expressed in diverse adult organs in addition to embryonic tissues (27). PRDC expression in mouse ovary was regulated by gonadotropins, and in situ hybridization demonstrated the localization of PRDC in mural and cumulus granulosa cells of antral follicles. After PMSG treatment, the signals were seen not only in antral but also in preovulatory follicles.

BMP4 is produced by theca-interstitial cells, and its expression is augmented during follicle development (37). BMP4 expression reaches a maximum in the theca cells of preovulatory follicles, and treatment of granulosa cells with BMP4 suppresses FSH-stimulated progesterone production by rat granulosa cells (20). It was suggested that BMP4 could suppress premature progesterone elevation associated with follicle atresia and function as a luteinizing inhibitor (20). Our data suggested that granulosa cell-derived PRDC could antagonize the actions of theca-derived BMP4 to modulate ovarian follicle development and luteinization via local paracrine mechanisms. The precise fate of individual developing follicles could be modulated by pituitary gonadotropins as well as ovarian BMP proteins and their antagonists.

BMP signaling pathways are finely regulated by different extracellular and intracellular molecules including BMP antagonists, BMP and activin membrane-bound inhibitors (38), inhibitory Smad proteins (31, 39, 40), Smad ubiquitination regulatory factors (41, 42), and other Smad-interacting nuclear cofactors (40). Here we demonstrated that PRDC is more potent than the known BMP antagonist, gremlin, and exhibits a wide tissue expression pattern in adults. In addition, the ovarian PRDC expressed in granulosa cells may participate in follicular development by antagonizing the actions of BMP proteins. These studies provide the basis for future investigation of the interactions between BMP proteins and BMP antagonists in diverse embryonic and adult tissues.

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