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Loss of Gremlin Delays Primordial Follicle Assembly but Does Not Affect Female Fertility in Mice[1]

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ABSTRACT

The transforming growth factor beta (TGFB) protein family is renowned for its diverse roles in developmental biology including reproduction. Gremlin is a member of the differential screening-selected gene aberrative in neuroblastoma (DAN)/cerberus family of bone morphogenetic protein (BMP) antagonists. Recent studies on gremlin focus on its involvement in embryonic skeletal, lung, and kidney development. To define the role of gremlin (*Grem1*) in female reproduction, we analyzed postnatal folliculogenesis using global and conditional knockout (cKO) mice for gremlin. *Grem1*$^{-/-}$ mice die within 48 h after birth, and ovaries collected from neonatal *Grem1*$^{-/-}$ mice demonstrated reduced oocyte numbers and delayed primordial follicle development. Transplanting *Grem1*$^{-/-}$ neonatal ovaries showed that folliculogenesis proceeded to large antral follicle stage, but *Grem1*$^{-/-}$ ovaries contained corpora lutea-like structures not found in control-transplanted ovaries. However, *Grem1* cKO mice had comparable fertility to control mice. These data suggest that gremlin plays a previously uncharacterized role in the regulation of oocyte numbers and the timing of primordial follicle development, but either it is not required for later folliculogenesis or its loss is possibly compensated by other BMP antagonists.

fertility, follicular development, folliculogenesis, ovary, primordial follicle, reproduction, transgenic, transgenic/knockout model

INTRODUCTION

Originally identified as a dorsalizing agent with bone morphogenetic protein (BMP) antagonistic activities during *Xenopus* embryonic development [1, 2], gremlin has emerged as a major regulator of growth, differentiation, and development. Gremlin is found as secreted or cell-associated forms and is a member of the differential screening-selected gene aberrative in neuroblastoma (DAN)/cerberus family of BMP antagonists. Gremlin is recognized mainly for its BMP antagonistic activities, and the majority of our understanding comes from its functions during developmental processes in the embryonic renal, lung, and bone systems. Much of these data were generated from mouse models either completely or cell-specifically devoid of gremlin as well as in mice with cell-specific overexpression [3–5].

BMPs have multiple described roles in embryonic and adult ovaries [6–8]. Knockout mice for the ligands and their downstream signaling proteins and receptors have established the BMP system as essential for primordial germ cell (PGC) specification, migration, and maintenance [9]. However, few mouse models are available to study the role of the BMP family in postnatal ovarian development because of embryonic lethality or genetic redundancy of the ligands or their signaling components. Exceptions to this include *Bmp15* or *Bmp6* knockout mice, which display subfertility on some genetic backgrounds [10–12], and *Bmpr1b (Alk6)* knockout mice, which are infertile with defects in cumulus expansion and uterine function [13]. More recently, mice with ovarian somatic cell deletion of BMP receptor-regulated SMADs [14] and type I receptors, *Bmpr1a*/*Alk3* and *Bmpr1b*/*Alk5* [15], develop infertility and metastatic granulosa cell tumors.

Gremlin (*Grem1*) is expressed in granulosa cells of the mouse ovary [16] along with *Grem2*, a closely related gene that shares 62% amino acid sequence homology in the mature region [17]. Gremlin regulation of BMP activity in granulosa cells has been reported from our laboratory [16] and others [18, 19]. Given the important developmental roles of gremlin in other systems [3–5], we hypothesized that gremlin plays an integral role in ovarian follicular development. We therefore generated knockout mice (*Grem1*$^{-/-}$) and ovary conditional knockout (*Grem1* cKO). Ovaries analyzed from neonatal *Grem1*$^{-/-}$ mice showed defects in germ cell numbers and primordial follicle development. However, transplanted *Grem1*$^{-/-}$ ovaries and *Grem1* cKO ovaries contained all stages of growing follicles, and *Grem1* cKO mice had similar fertility to controls. These data suggest that *Grem1* has important roles in ovary prior to the primordial follicle stage, but once primordial follicles are established, follicle development proceeds normally in the absence of gremlin.

MATERIALS AND METHODS

Experimental Mice

All mouse lines used in the present study were maintained on a mixed C57BL/6/129S7/SvEv genetic background and manipulated according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved protocols at Baylor College of Medicine. *Grem1*$^{flox/flox}$ mice (generated as previously described) [4] were rederived in the Transgenic Mouse

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Core facility at Baylor College of Medicine. *Ella-cre* (The Jackson Laboratory, http://jaxmice.jax.org/strain/003724.html) transgenic mice [20] were used to generate $Grem1^{+/-}$ mice that were then intercrossed to produce $Grem1^{-/-}$ homozygote mutants. To generate ovary conditional knockouts (cKO), $Grem1^{flox/flox}$ female mice were crossed to $Grem1^{+/-}$ $Amhr2^{cre/+}$male mice [14, 21–24] to generate $Grem1^{flox/-}Amhr2^{cre/+}$ experimental mice (denoted *Grem1* conditional knockout [cKO]). Control mice ($Grem1^{flox/-}$) were siblings of experimental mice but negative for cre. Mice were genotyped by PCR analysis of genomic tail DNA as reported [4, 24].

Tissue Collection

All mice were anesthetized by isoflurane inhalation (Abbott Laboratories, Abbott Park, IL) and euthanized by either decapitation (neonatal mice) or by cervical dislocation (adult mice). Ovaries for histology and immunohistology were collected in either Bouin's solution (Sigma, St. Louis, MO) or 10% neutral buffered formalin (Electron Microscopy Sciences, Hatfield, PA). Ovaries for gene expression studies were stored in RNA*later* (Ambion, Austin, TX) at −80°C for subsequent use. Neonatal ovaries for transplantation studies (described below) were collected on the day of birth (D0) and immediately placed in 1× PBS for transplantation under the kidney capsule.

Fertility Analysis

Four to six individually housed female mice of each genotype ($Grem1^{flox/-}Amhr2^{cre/+}$ or $Grem1^{flox/-}$) were bred at 6 wk of age continuously to wild-type males with known fertility. The number of litters and the number of pups were recorded over a 9-mo period. For the analysis of fertility over time, four control and four experimental female mice at 6 wk of age were crossed to adult wild-type males, the total number of pups generated that month was recorded, and the data were plotted over time.

Morphological, Histological, and Immunohistochemical Analysis

Tissue processing and embedding were performed by the Department of Pathology Core Facility (Baylor College of Medicine) using standard techniques. For counting, newborn ovaries (D0) were serially sectioned at 5 μm and all sections retained. Four mice per genotype were used for quantification. Sections were immunostained with a goat polyclonal antibody against NOBOX (1:500) using previously published protocols [25] to visualize the oocyte nucleus (Supplemental Fig. S1, available online at www.biolreprod.org). Images of ovarian sections were captured with a digital camera (AxioCam MRc5) at a low magnification (100×), and all NOBOX- labeled oocytes in every fifth section were counted manually. Every fifth section was counted to avoid double counting oocytes. From these values, the total sum of NOBOX labeled oocytes (as naked oocytes and primordial follicles) per ovary was calculated. Statistical differences in the total number of oocytes were assessed using Student's *t*-test. Statistical differences in the numbers of NOBOX-labeled cells between naked oocytes and primordial follicles were assessed by performing a chi-square test using Graph Pad Prism 5 (GraphPad Software, La Jolla, CA).

Immunohistochemistry

Immunohistochemistry was performed using the Vectastain ABC method (Vector Laboratories, Burlingame, CA) as previously described [24, 26] using the following antibodies: goat polyclonal anti-AMH (1:1500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), rat polyclonal anti-GCNA (kindly provided by G.C. Enders), and goat polyclonal NOBOX (1:500; kindly provided by A. Rajkovic). Immunoreactivity was visualized by diaminobenzidine (Vector Laboratories), and ovaries were counterstained with hematoxylin. Fluorescent immunohistochemistry was utilized to colocalize GCNA and rabbit polyclonal anti-YBX2 (MSY2; kindly provided by R.M. Schultz). Briefly, sections were subjected to antigen retrieval (0.01 M citric acid) and 0.1% Triton X (Sigma), followed by block incubation in normal horse serum (Vector). Rat anti-GCNA was incubated overnight (1:200 dilution) and detected using Alexa Fluor donkey anti-rat 488 (Invitrogen) diluted 1:500 in TBS for 1 h. Sections were reblocked with horse serum and incubated overnight with rabbit anti-YBX2 (MSY2) diluted 1:4000 and detected using Alexa Fluor donkey anti-rabbit 594 (Invitrogen) at 1:500 in TBS for 1 h. Nucleic acids were labeled with TOTO3 (Invitrogen) and washed and mounted with Prolong Gold mounting media (Invitrogen). Fluorescent images were captured using an LSM 510 Axiovert 100M confocal microscope (Carl Zeiss, Jena, Germany), and all images were compiled using Photoshop CS3 (Adobe Systems Inc., San Jose, CA).

Transplantation of Neonatal Ovaries

To assess the role of gremlin in growing follicles, neonatal (i.e., D0) control ($Grem1^{+/+}$ and $Grem1^{+/-}$) and mutant ($Grem1^{-/-}$) ovaries were surgically transplanted beneath the kidney capsule of bilaterally ovariectomized virgin adult female mice. Both ovaries were recovered from the neonatal donor mouse, dissected of surrounding bursal tissue, and placed in PBS. Immunocompetent recipient mice of the same genetic background (C57BL/6J; 129S7/SvEvBrd) at 6–8 wk of age were anesthetized with i.p. injections of 2.5% tribromoethanol (in t-amyl alcohol solution at 0.4–0.75 mg/g; Sigma) and ovariectomized via a dorso-horizontal incision. The recipient's left kidney was exteriorized, and, if possible, both neonatal ovaries (from the same donor) were inserted under the recipient kidney capsule using fine watchmakers' forceps, and the kidney was returned to its normal anatomical position. Incisions were closed with wound clips (BD, Franklin Lakes, NJ). Three weeks posttransplantation, recipient ovaries were collected for histological/immunohistochemical assessment. Transplantation was performed on three separate occasions. Eight recipient mice were used for each genotype. A total of 13 ovaries for $Grem1^{-/-}$ were transplanted; of these, eight $Grem1^{-/-}$ ovaries grafted successfully, and similar numbers were found for the controls.

RT-PCR and Quantitative PCR

RNA was isolated using the Qiagen (Valencia, CA) RNeasy Microkit. RNA concentration was quantified using a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, DE). Complementary DNA was reverse transcribed from 200 ng of total RNA in a 20-μl reaction using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA). Five-microliter templates were amplified using 10× ThermoPol reaction buffer and polymerase (New England Biolabs, Ipswich, MA). PCR conditions were as follows: incubation at 95°C for 2 min, 35 cycles of 94°C for 30 sec, annealing temperature for 30 sec, and 72°C for 30 sec, followed by 72°C for 7 min. Quantitative PCR was carried out as previously described [24, 26] using SYBR Green PCR Master Mix (Applied Biosystems) for *Grem1* transcript quantification (primers sequences availableon request) or Taqman Master Mix and Gene Expression Assays for *Grem1* (Mm00488615_s1) and *Grem2* (Mm0051909_m1; Applied Biosystems). All quantitative PCR data were analyzed by the ΔΔ cycle threshold method using the ABI 7500 System Software (version 1.2.3) and normalized to the endogenous reference (*Gapdh*). The mean of the control samples was used as the calibrator sample, and the mean ± SEM was calculated. Results were plotted using the relative expression of each target gene with each sample compared to the control.

Statistical Analyses

Statistical analysis was carried out using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Two-tailed unpaired Student *t*-tests were used for single comparisons. One-way analysis of variance followed by the Fisher least significant difference test was used for multiple comparisons. A minimum of at least three independent experiments were carried out at all times, and $P < 0.05$ was considered statistically significant.

RESULTS

Loss of Grem1 *Results in Fewer Germ Cells at Birth and Delays Primordial Follicle Assembly*

In mouse and human, the number of oocytes at birth is fixed [27, 28]. Because BMPs are essential for primordial germ cell specification and maintenance during embryogenesis [29–31], we analyzed whether loss of gremlin influenced oocyte numbers and primordial follicle formation. Histological analysis of $Grem1^{-/-}$ neonatal ovaries revealed germ cell nests and developing primordial follicles grossly similar to control mice (Fig. 1, A and B). However, the total number of oocytes was significantly decreased in $Grem1^{-/-}$ neonatal ovaries (Fig. 1C and Supplemental Fig. S1). In addition, when categorized into subpopulations (oocytes without somatic cell counterparts [naked oocytes] and oocytes with somatic cell counterparts [primordial follicles]), significantly fewer oocytes had transitioned into primordial follicles in the $Grem1^{-/-}$ ovaries (Fig. 1D; 7% versus 12%). Because there is a known interdependent relationship between meiotic markers during the onset of

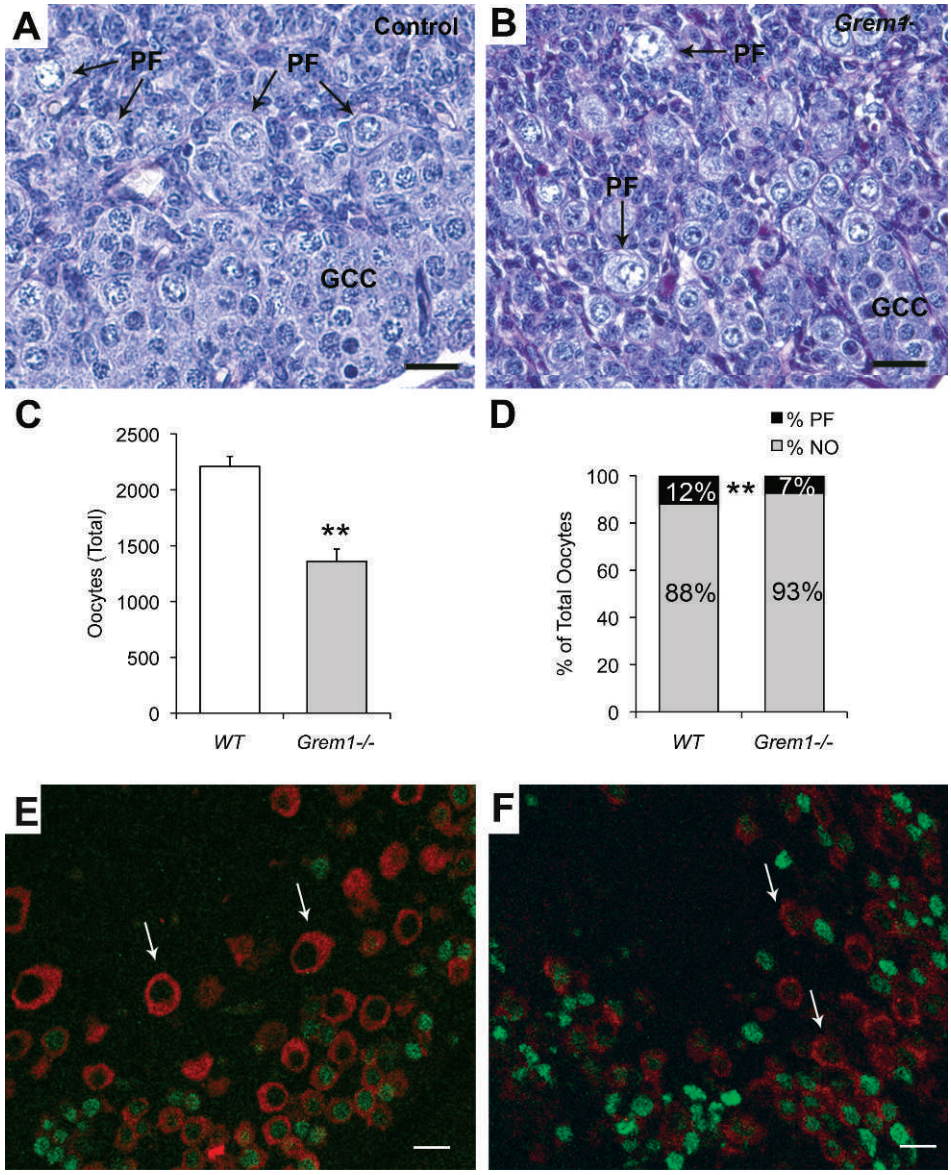

FIG. 1. *Grem1*$^{-/-}$ ovaries exhibit a delay in the formation of primordial follicles. **A**, **B**) Germ cell cysts (GCC) and primordial follicle (PF) formation are evident in control (**A**) and *Grem1*$^{-/-}$ (**B**) neonatal ovaries (Postnatal Day 0). **C**) Analysis of total oocyte numbers reveals that germ cells are decreased in *Grem1*$^{-/-}$ ovaries. Error bars are the mean ± SEM of n = 4 ovaries per genotype. **D**) The percentage of oocytes found as primordial follicles (PF) versus those found as naked oocytes (NO) is significantly reduced in *Grem1*$^{-/-}$ ovaries ($P < 0.01$ by chi-square test). **E**) Stage-specific molecular markers show that pre-meiotic-arrested oocytes positive for GCNA (green) are more prominent in *Grem1*$^{-/-}$ *ovaries* (**F**) compared to control ovaries (**E**), which have more oocytes in the diplotene stage of Meiosis I (arrows in **E**, **F**; red, positive for YBX2). **Indicates statistical significance at $P < 0.01$. Bars = 20 μm.

follicular assembly [32], we further characterized neonatal ovaries utilizing GCNA (a prediplotene marker) and YBX2 (MSY2; a diplotene marker). Oocytes in primordial follicles of control ovaries are GCNA negative and YBX2 positive [32] (Fig. 1E). *Grem1*$^{-/-}$ ovaries show abundance of GCNA staining (green in Fig. 1F) and less for YBX2 (red; Fig. 1F compared to Fig. 1E). Collectively, these results indicate that loss of gremlin results in developmentally delayed oocytes in the neonate; fewer oocytes are arrested in the diplotene stage of meiosis and found as primordial follicles.

Transplanted Grem1$^{-/-}$ *Ovaries Display Follicles of All Growing Stages but Exhibit Luteal Tissue Not Observed in Control Ovaries*

Because of the absence of kidney formation, *Grem1*$^{-/-}$ mice die within 48 h of birth [5]. Therefore, to investigate whether folliculogenesis proceeds in the absence of gremlin, we transplanted neonatal (i.e., D0) *Grem1*$^{-/-}$ ovaries under the kidney capsule of ovariectomized wild-type recipient mice for 3 wk. Approximately 60% of control and 60% of *Grem1*$^{-/-}$ transplanted ovaries survived and exhibited healthy follicles (Fig. 2, A and B). Three weeks posttransplantation, all surviving transplanted ovaries had grown in size and measured between 1.5 and 2.5 mm across their longest axis for both genotypes. Histological assessment of the transplants (n = 8 ovaries for each genotype) revealed that follicles at all stages of development developed in both genotypes (Fig. 2, C and D). Interestingly, in three of the eight *Grem1*$^{-/-}$ ovarian transplantations, large structures that histologically resemble corpora lutea were observed (Fig. 2D), suggesting that postantral stage follicle development may be altered in the absence of *Grem1*.

In the postnatal ovary, anti-Müllerian hormone (AMH) is detected in nonatretic preantral and small antral follicles [33, 34] and decreases when follicles become responsive to follicle-stimulating hormone (FSH) [35]. Immunohistochemistry of control-transplanted ovaries confirmed positive AMH staining patterns in secondary follicles, with loss of the protein in the more advanced larger antral follicles (Fig. 2E). Similar patterns were also observed in preantral and antral follicles in transplanted *Grem1*$^{-/-}$ ovaries (Fig. 2F). Rarely, *Grem1*$^{-/-}$ mice survive to adulthood, and in the 24 mo of breeding for these studies, three mice (one female and two males) were found at weaning that exhibited the skeletal deformities

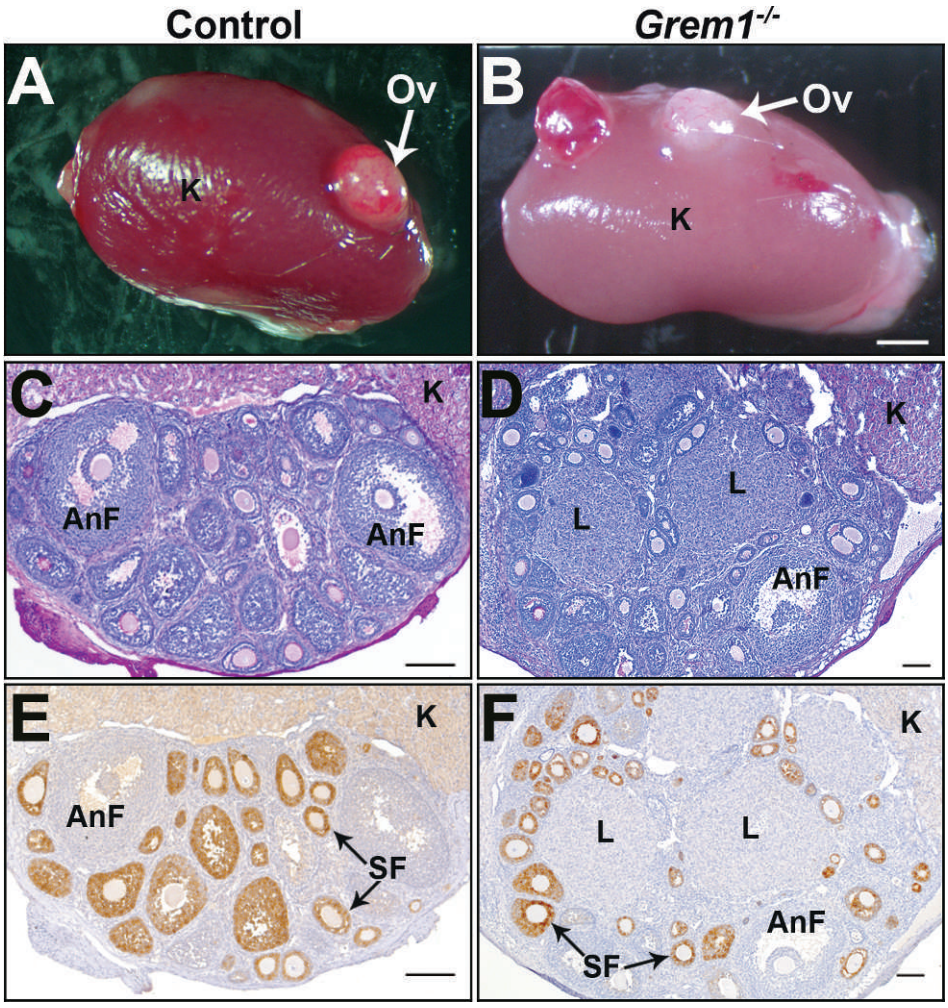

FIG. 2. Transplanted *Grem1*$^{-/-}$ ovaries display mature follicles and contain luteinized structures not found in control ovaries. **A**, **B**) Gross morphology of neonatal control (**A**) and *Grem1*$^{-/-}$ (**B**) ovaries (Ov) 3 wk posttransplantation under the kidney (K) capsule of ovariectomized adult recipient mice. **C**, **D**) Histological assessment of control (**C**) and *Grem1*$^{-/-}$ (**D**) transplanted ovaries reveal follicles at all stages of development. Mature antral follicles (AnF) are present in both genotypes. Interestingly, only *Grem1*$^{-/-}$ (**D**) ovaries display luteinized structures (L), suggesting that they are more advanced than control (**C**) ovaries. **E**, **F**) Immunoreactivity for anti-Müllerian hormone (AMH) confirms normal positive staining patterns in secondary follicles (SF) and loss of the protein in the more advanced antral follicles (AnF) and luteinized structures in both wild-type (**E**) and *Grem1*$^{-/-}$ (**F**). Bars in **A** and **B** = 1 mm; bars in **C**–**H** = 100 μm.

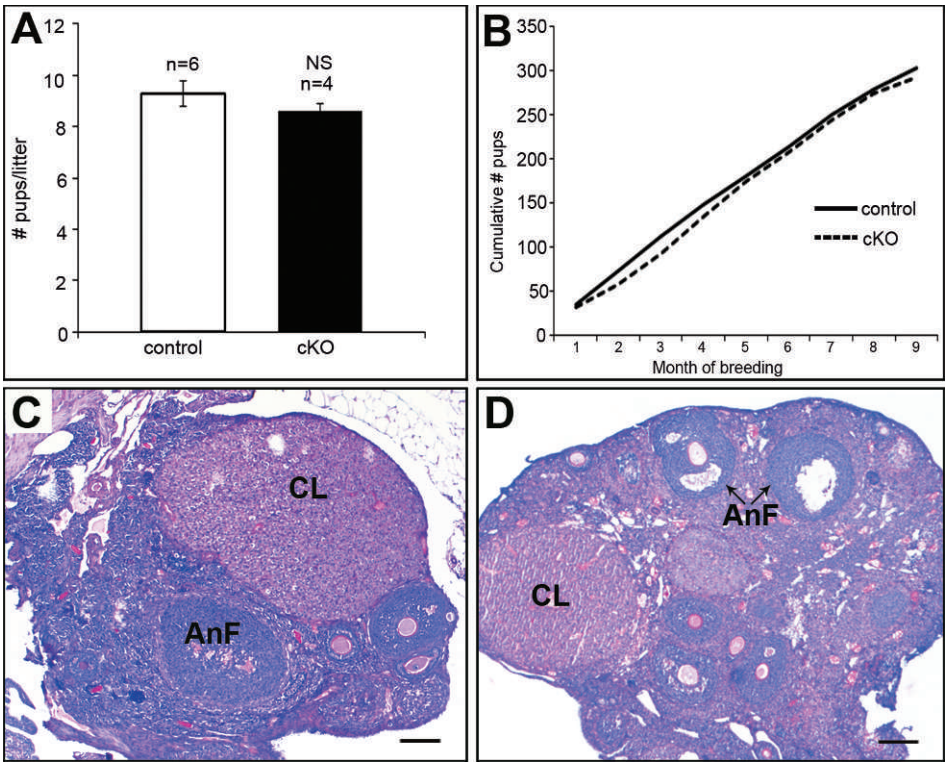

FIG. 3. Conditional deletion of gremlin in granulosa cells does not affect overall fertility in adult female mice. **A**) Adult female mice at 6 wk of age were bred to wild-type males for a 9-mo period (control, n = 6; cKO, n = 4). Gremlin conditional knockout mice (cKO) had comparable litter sizes to control mice. The error bars are mean ± SEM. NS, not significant. **B**) The cumulative number of pups generated from four breeding females of each genotype is similar. **C**, **D**) Histological analysis from adult cycling control (**C**) and *Grem1* cKO (**D**) mice display normal folliculogenesis with secondary follicles, antral follicles (AnF), and postovulatory corpora lutea (CL). Bars = 200 μm.

characteristic of *Grem1*$^{-/-}$ mice [5]. Genotyping confirmed that all were *Grem1*$^{-/-}$ and that all had developed one kidney, consequently allowing for longevity after the neonatal period. Gonads from the one 3-wk-old female (Supplemental Fig. S2) and two males (data not shown) were histologically normal, further confirming that prepubertal folliculogenesis to 3 wk of age did not appear to be compromised in the absence of gremlin. No luteinized follicles were visible in these ovaries (such as in the transplants), but none would be expected, as these surviving mice were collected prior to puberty (i.e., at 3 wk of age).

Conditional Deletion of Gremlin in Granulosa Cells Does Not Affect the Overall Fertility in Adult Mice

Previous studies from our laboratory [16] and others [19] have proposed a role for gremlin as an important inhibitor of BMPs during ovulation. To assess the in vivo interactions of gremlin in the later stages of folliculogenesis and ovulation, we generated a conditional knockout (*Grem1* cKO) using *Amhr2*$^{cre/+}$ mice. Confirmation of deletion was tested by quantitative PCR by measuring *Grem1* transcript levels in preovulatory granulosa cells isolated from 3-wk-old female mice. Relative levels of *Grem1* transcript were approximately 75% lower than controls (Supplemental Fig. 3). *Grem1* cKO mice had comparable litter sizes (8–10 pups; Fig. 3A) and a similar reproductive profile (Fig. 3B) as control mice, revealing that conditional loss of gremlin did not affect the overall fertility. Histologic analysis of ovaries from adult *Grem1* cKO mice showed normal folliculogenesis with secondary follicles, antral follicles, and postovulatory corpora lutea structures (Fig. 3, C and D).

Grem1 *and* Grem2 *Are Coexpressed in the Postnatal Mouse Ovary*

Loss of gremlin in our mouse models could potentially be compensated by expression of other BMP antagonists, including gremlin 2 (*Grem2*), which has been shown to be expressed in rodent granulosa cells [17]. We analyzed the expression profiles of *Grem1* and *Grem2* in the postnatal ovary. Both *Grem1* and *Grem2* are detected in postnatal ovaries from Day 0 to Day 21 (Fig. 4). The mean value of *Grem1* expression did not change in ovaries from Day 0 to Day 21. *Grem2* showed statistically significant changes in expression during this time frame ($P < 0.05$; Fig. 4B).

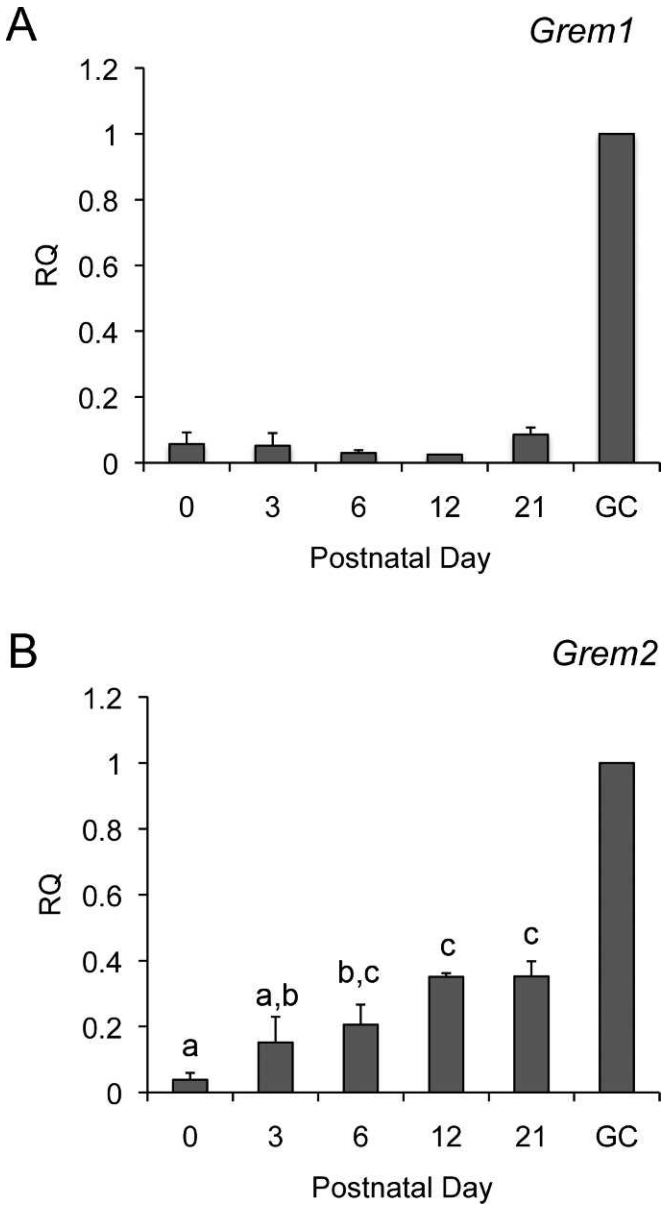

FIG. 4. *Grem1* and *Grem2* are coexpressed in the postnatal ovary. Relative quantity (RQ) of *Grem1* (**A**) and *Grem2* (**B**) in the postnatal ovary determined by quantitative PCR. Ovaries were collected at birth (Postnatal Day 0) and Days 3, 6, 12, and 21 (n = 3 ovaries each age). Data are shown relative to the transcript levels in a sample of preovulatory granulosa cells (GC) collected following superovulation at Day 21. Statistical analysis by one-way analysis of variance followed by the Fisher least significant difference test was performed on all samples, excluding the calibrator (GC) sample. Bars with different letters have statistically different means (e.g., "a" is different from "c" but not "a,b").

DISCUSSION

In the present study, we investigated the role of gremlin in ovarian development and function. Among many of the factors reported to be key for germ cell development, the BMPs and their associated signaling pathway are necessary for primordial germ cell specification, maintenance, and function [29–31, 36–44]. We hypothesized that in the absence of gremlin, BMP signaling would be disrupted and that primordial germ cell specification or development may be affected. Our findings revealed that while newborn *Grem1*$^{-/-}$ ovaries had similar architecture to control ovaries, they contained fewer oocytes. Decreases in germ cell numbers prior to birth may be due to defects in PGC specification, migration, proliferation, or apoptosis. In vitro organ culture systems used to study PGC numbers and motility have been reported for the human [45] and the mouse [46], though there have been contrasting findings between species. Although BMP4 was reported to increase PGC numbers in the mouse [46], it had the opposite effect in the human and negatively regulated PGC numbers via apoptotic mechanisms [45]. Studies are ongoing to determine the mechanism(s) behind the germ cell loss in the *Grem1*$^{-/-}$ mouse model.

In addition, we found that *Grem1*$^{-/-}$ newborn ovaries had proportionally more oocytes found as germ cell cysts and fewer as primordial follicles than would be expected. In the mouse ovary, germ cell cyst breakdown to form primordial follicles occurs within the first few days of birth [47, 48]. While BMPs, in particular BMP4 and BMP7, have been reported to be important paracrine factors in the primordial to primary follicle transition [49–51], defined roles from germ cell cyst to

primordial follicle are not known. Our results suggest that gremlin may also have a role in limiting BMP function during primordial follicle formation in the neonatal mouse ovary. An alternative possibility is that loss of *Grem1* indirectly results in alterations in the activity or expression of other factors known or suspected to be involved in the formation of primordial follicles, such as estrogen, activin, or notch [52–54]. Thus, additional studies will be necessary to determine which of these pathways are disrupted.

To determine if the delay in primordial follicle development affects later follicular development, we transplanted neonatal *Grem1*$^{-/-}$ ovaries under the kidney capsule of ovariectomized adult mice. Ovarian transplants from control and *Grem1*$^{-/-}$ mice exhibited follicles at all stages of development. An intriguing observation from our ovarian transplantation experiments was the appearance of large luteinized structures in 40% of the mutant ovaries that were morphologically similar to corpora lutea. These structures were not observed in any of the control ovaries, suggesting that later aspects of antral follicle development were altered in the absence of *Grem1*. BMPs are known regulators of granulosa cell differentiation [6]; however, the precise roles of BMPs remain poorly understood. Whether the luteinized structures in *Grem1*$^{-/-}$ transplanted ovaries represent pre- or postovulatory structures remains to be determined. Interestingly, in a rare 3-wk-old female *Grem1*$^{-/-}$ mouse that survived (Supplemental Fig. S2), we observed follicles with declining AMH expression, which is associated with the onset of FSH sensitivity and a hallmark of granulosa cell maturation [35] but not as prominent in aged-matched controls. These data suggest that in the absence of *Grem1*, granulosa cell maturity is possibly accelerated, perhaps leading to premature granulosa cell differentiation. Roles for TGFB family members have been implicated in the timing of luteinization [8, 55] and observed in mouse models with targeted deletions of TGFB family transcription factors *Smad4* or *Smad2/3* [23, 56].

To study the role of gremlin in adult fertility, we produced granulosa cell-specific knockout mice using cre recombinase under the control of the *Amhr2* promoter. *Amhr2cre* is useful for recombination in somatic cells of growing follicles [22, 23, 57] and thus will represent a different developmental time frame to that observed *Grem1*$^{-/-}$ neonates and neonatal transplants. Over a 9-mo period, *Grem1* cKO mice delivered healthy pups in litter sizes comparable to control mice. No adverse phenotypes were observed in *Grem1* cKO mice, and ovarian architecture was normal. The phenotypic differences observed between our two *Grem1*$^{-/-}$ models and the *Grem1* cKO model may reflect the different developmental stages at which gremlin was ablated or that ablation in the *Grem1* cKO did not reduce gremlin levels to a point that uncovered the phenotype. Alternatively, the lack of a notable phenotype in the conditional ablation of gremlin may reflect a modest or nonessential role for this BMP antagonist during the later stages of follicular development.

A feature of BMP signaling is the various regulatory mechanisms that exist to control BMP-mediated actions. Most of these signals are inhibitory [58] and include spatial and temporal control of extracellular antagonists, receptor specificity, and downstream effectors and inhibitors of SMAD proteins [59]. Some of the most studied BMP antagonists include noggin, chordin, sclerostin, gremlin, BAMBI (BMP and activin membrane bound inhibitor), SMAD6, SMAD7, DAN (differential screening-selected gene aberrant in neuroblastoma), Cerberus, and gremlin 2 (also known as PRDC [protein related to DAN and cerberus]) [3]. These proteins play the important role of modulating BMP activities within a variety of systems, some of which include early embryo development [60], neural development [61], prostate development [62], inflammation of cardiac tissues [63], and bone development [64]. Noggin has been recently shown to interact with *Grem1* during skeletal development [65]. The lack of full loss of oocytes at birth, or effects on postnatal folliculogenesis in our gremlin mouse models, could suggest compensatory mechanisms by other antagonists in the ovary. In particular, the closely related *Grem2* is coexpressed in the ovary with *Grem1* [16, 17]. We hypothesize that functional redundancies for gremlin also occur in the ovary.

The precise roles of the BMPs during folliculogenesis are not entirely clear, although they have been proposed for multiple developmental stages (Supplemental Fig. S4). To investigate the role of the BMP2, BMP4, and BMP7 antagonist gremlin in the neonatal and adult ovary, we employed three models representing different developmental stage of folliculogenesis (Supplemental Fig. S4). Thus, variations in the phenotype between global and conditional knockouts may partly reflect differences in timing of the deletion. G*rem1*$^{-/-}$ ovaries contain decreased oocyte numbers and delayed primordial follicle development. This may result from defective embryonic development of the reproductive tract, indirect disruption of other signaling pathways, or a direct consequence of additional BMP signaling in the embryonic ovary. This phenotype would not be evident in the *Grem1* cKO because of the timing of recombination of floxed alleles in the conditional knockout. However, progression of folliculogenesis beyond the primordial stage appears normal in both *Grem1*$^{-/-}$ and *Grem1* cKO mice, indicating that *Grem1* is not required for preantral and antral stage follicle development or that expression of other BMP antagonists, such as *Grem2*, may compensate for its loss. BMP antagonists are known to act cooperatively to limit BMP activity; therefore, combinatorial knockouts of BMP antagonists will be required to address these questions and fully uncover their function(s) during folliculogenesis.

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