Published in final edited form as:
Reproduction. 2014 June ; 147(6): 865–874. doi:10.1530/REP-14-0005.

Roles of Gremlin1 and Gremlin2 in Regulating Ovarian Primordial to Primary Follicle Transition

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Abstract

A network of extracellular signaling factors has previously been shown to act in concert to control the ovarian primordial to primary follicle transition. The current study was designed to investigate the roles of the endogenous bone morphogenetic protein (BMP) inhibitors Gremlin1 (GREM1) and Gremlin2 (GREM2) in primordial follicle transition in the rat ovary. GREM1 and GREM2 treatment were found to reverse the effects of Anti-Müllerian Hormone (AMH) to inhibit follicle transition in a whole ovary culture system. GREM1 reversed the effect of bone morphogenetic protein 4 (BMP4) to stimulate primordial follicle transition. Immunohistochemical studies showed that GREM2, but not GREM1, was present in primordial follicles suggesting GREM2 may regulate primordial follicle transition *in vivo*. Co-immunoprecipitation studies indicated that GREM2 directly binds to AMH, as well as to BMP4. Transcriptome analyses of ovaries treated with GREM2 or GREM1 yielded negligible numbers of differentially expressed genes, suggesting the immediate effects of GREM2 or GREM1 appear to be at the level of protein-protein interactions, rather than direct actions on the cells. A number of other ovarian growth factors were found to influence the expression of *Grem2*. Observations suggest that *Grem2* is a part of the signaling network of growth factors that regulate the primordial to primary follicle transition. Insights into the regulatory networks affecting the pool of primordial follicles is important to understand the molecular basis for reproductive diseases such as primary ovarian insufficiency (POI).

INTRODUCTION

In the current study the effects of the bone morphogenetic protein (BMP) inhibitors Gremlin1 and Gremlin2 on ovarian primordial follicle development are investigated. Oocytes are stored in an arrested state in mammalian ovaries in structures called primordial follicles. Each primordial follicle contains an oocyte that is arrested in prophase I of meiosis, surrounded by a single layer of flattened (squamous) pre-granulosa cells (Hirshfield, 1991; Rajah *et al.*, 1992). During reproductive life a small number of primordial follicles continually leave the arrested state by undergoing the primordial to primary follicle

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DECLARATION OF INTEREST
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

transition. This transition involves an increase in oocyte size, the granulosa cells become cuboidal and proliferate, and recruitment of precursor theca cells occurs. Following follicle transition the follicles either undergo atresia and regress, or ovulate the oocyte from a large antral follicle (Peters *et al.*, 1975). When the pool of arrested primordial follicles becomes depleted reproduction ceases and women enter menopause.

Control of the primordial to primary follicle transition is vitally important to successful reproduction. A network of growth factors and other extracellular signaling factors act in concert to create the correct balance for promoting or inhibiting primordial follicle transition (Nilsson *et al.*, 2010; Pangas, 2007). In this way the available pool of primordial follicles is metered out so as to last for the duration of the expected reproductive lifespan (Pangas, 2012). If this balance is upset, then infertility and other reproductive pathologies can occur such as primary ovarian insufficiency (POI). In cases of POI the pool of primordial follicles is depleted early in life and women in their thirties or even twenties cease ovulating and enter menopause (Coulam *et al.*, 1986; Maclaran and Panay, 2011).

Growth factors that promote the primordial to primary follicle transition include kit ligand (*Kitl*) (Parrott and Skinner, 1999), fibroblast growth factor 2 (*Fgf2*) (Nilsson *et al.*, 2001; Nilsson and Skinner, 2004), neurotrophins (Dissen *et al.*, 2009; Dole *et al.*, 2008; Nilsson *et al.*, 2009), the transforming growth factor family members bone morphogenetic protein 4 (*Bmp4*) (Nilsson and Skinner, 2003) and bone morphogenetic protein 7 (*Bmp7*) (Lee *et al.*, 2001), and others (Fortune *et al.*, 2010; Skinner, 2005; Buratini and Price, 2011; Chaves *et al.*, 2012; Knight and Glister, 2006). Growth factors that inhibit the primordial follicle transition include *Cxcl12* (Holt *et al.*, 2006) and the transforming growth factor beta family member anti-Müllerian hormone (*Amh*) (Durlinger *et al.*, 2002; Durlinger *et al.*, 1999; Nilsson *et al.*, 2007). While many secreted growth factors have been tested experimentally for their ability to regulate primordial follicle transition, less work has been done to investigate the role played by endogenous inhibitors of secreted growth factors.

Gremlin1 (*Grem1*) and gremlin2 (*Grem2*, or Protein Related to DAN and Cerberus (PRDC)) are members of the DAN family of BMP inhibitors (Avsian-Kretchmer and Hsueh, 2004; Kattamuri *et al.*, 2012). These endogenously produced inhibitors have similar bioactivities and are known to bind to members of the BMP family of growth factors and prevent the growth factors from activating their receptors (Kosinski *et al.*, 2007; Segditsas *et al.*, 2008). Both *Grem1* and *Grem2* have been shown to be expressed in the ovary and to have roles in regulating later stages of follicle development (Sudo *et al.*, 2004; Pangas *et al.*, 2004; Myers *et al.*, 2011). However, neither *Grem1* nor *Grem2* are known to have roles in regulating the primordial to primary follicle transition.

The objective of the current study was to investigate whether *Grem1* and *Grem2* are part of the network of paracrine growth factors and inhibitors that regulate the primordial to primary follicle transition. Organ culture experiments using intact rat ovaries directly tested the effects of GREM1 and GREM2 proteins on primordial follicle transition. Immunohistochemistry was used to identify the follicle stages and cell types in the ovary that express GREM1 and GREM2. Coimmunoprecipitation experiments tested the ability of GREM2 to bind directly to BMP4 and AMH. A greater basic understanding of how the

network of paracrine signaling factors acts to regulate the recruitment of follicles from the arrested primordial follicle pool may help in understanding the causes of certain types of infertility in women, such as POI.

METHODS

Animals and Ovary Culture

Four-day old female Sprague-Dawley rats (Harlan Laboratories, Inc., USA) were euthanized according to Washington State University IACUC committee approved protocol (IACUC # 02568) and their ovaries removed and cultured whole as described previously (Dole *et al.*, 2008). Four-day old rat ovaries contain primarily primordial follicles. Whole ovaries were cultured on floating filters (.4 μm Millicell-CM, Millipore, Bedford, MD, USA) in .5 ml Dulbecco's modified Eagle's medium (DMEM)-Ham's F-12 medium (1:1, vol/vol) containing 0.1% BSA (Sigma), 0.1% Albumax (Gibco BRL, Gaithersburg, MD, USA), 27.5 μg/ml transferrin, and 0.05 mg/ml L-ascorbic acid (Sigma) in a four-well culture plate (Nunc plate, Applied Scientific, South San Francisco, CA, USA) for two or ten days. The medium was supplemented with penicillin (400 units/ml), streptomycin (400 μg/ml) and gentamycin (100 μg/ml) to prevent bacterial contamination. Ovaries were randomly assigned to treatment groups with 1–3 ovaries per floating filter per well. Wells were treated every two days with the following recombinant proteins alone or in combination: mouse gremlin (GREM)(50ng/ml; R&D Systems, cat# 956-GR), mouse gremlin-2 (GREM2; PRDC) (100ng/ml; R&D Systems, cat# 2069-PR), human anti-Müllerian hormone (AMH)(50ng/ml; R&D Systems, cat# 1737-MS), mouse kit ligand (KITLG)(50ng/ml; R&D Systems, cat# 455-MC) and rat fibroblast growth factor 2 (FGF2)(50ng/ml; R&D Systems, cat# 3339-FB). GREM1 and GREM2 dosages were chosen based on effective dosages for inhibiting BMP stimulatory activity in cell culture, as provided by the manufacturer. Preliminary studies using 4-day old rat whole ovary culture showed no difference in the effect of GREM1 on follicle development between 50ng/ml and 150ng/ml. After 10 days of culture ovaries were fixed in Bouin's fixative (Sigma) for one hour. Ovaries were then embedded in paraffin, sectioned at 3 μm and stained with hematoxylin/eosin for use in morphological analysis. Ovaries from 4-day old rats cultured for 10 days have follicles in pre-antral stages having up to three granulosa cell layers. Most follicles are morphologically normal (Supplemental Figure S1)

Morphological Analysis

For each ovary the number of oocytes at each developmental stage was counted and the counts for each stage were averaged across the two consecutive histological sections that had the largest ovarian cross section. With two adjacent thin sections counted, it is expected that the sections are essentially equivalent, with the same follicles counted in most cases. If this is not the case, then these counts are discarded. The oocyte nucleus had to be visible for an oocyte to be counted. Normally, between 70 and 150 follicles were present in each ovarian cross-section. From five to twelve ovaries were evaluated per treatment group. Oocytes were classified as primordial (stage 0) or as one of the developing pre-antral stages (stages 1–4) as described previously (Parrott and Skinner, 1999; Kezele and Skinner, 2003). Primordial follicles consist of an oocyte partially or completely surrounded by a single layer

of squamous pre-granulosa cells. Follicles with only one cuboidal granulosa cell visible, the rest being squamous, were also classified as primordial (Gougeon and Busso, 2000; Gougeon and Chainy, 1987; de Bruin *et al.*, 2002; Meredith *et al.*, 2000). Developing (stages 1–4) follicles contain successively more cuboidal granulosa cells in layers around the oocyte (Nilsson *et al.*, 2001; Parrott and Skinner, 1999). The proportion (*i.e*. percentage) of developing follicles out of total follicles per cross section was calculated for each ovary. Data were expressed and graphed as proportion of developing follicles relative to controls, with the control means set equal to 1.0. This normalization to controls accounts for variations between litters in response to treatment and for minor differences between individuals reading the slides in categorizing follicles into different developmental stages, as differences between treatment groups were consistent. One individual performed counts for GREM1 experiments. Three individuals performed counts for GREM2 experiments, with the three periodically all counting the same ovary and comparing results.

RNA Collection

For ovary culture experiments in which ovarian RNA was collected, 2–3 ovaries per well were cultured for two days either untreated (controls) or were treated with GREM1 (50ng/ml) or GREM2 (100ng/ml). After two days of culture there are no morphological differences between control and growth factor-treated ovaries (Nilsson *et al.*, 2006). Therefore, measurements of whole-ovary gene expression will reflect differences in RNA transcription rather than differing proportions of cell types due to differential cell proliferation between treatments. RNA was isolated from whole rat ovaries after homogenization in one ml Trizol™ reagent (Sigma-Aldritch, USA), according to manufacturer's instructions. Two or three ovaries from the same culture well (from different rat pups out of the same litter) and receiving the same treatment were pooled and homogenized together. Homogenized samples were stored at −70C until the time of RNA isolation.

Immunohistochemistry

Rat ovaries were fixed in 10% neutral buffered formalin, paraffin embedded and sectioned using standard procedures. Ovary sections were immunostained as described previously (Nilsson *et al.*, 2002) for the presence of GREM1 or GREM2 using an anti-Gremlin primary antibody (Mouse Gremlin antibody; R&D Systems cat# AF956) or anti-Grem2 primary antibody (human Grem2 H-58; Santa Cruz Biotechnology cat# sc-135103). Briefly, sections were de-paraffinized, rehydrated through a graded ethanol series, boiled in 10 mM sodium citrate buffer, washed with 0.1%Triton-X solution, and then blocked with 10% rabbit serum or 10% goat serum for anti-Gremlin or anti-Grem2 experiments, respectively (normal rabbit serum or normal goat serum; Vector Laboratories Inc., Burlingame, CA, USA) for 20 min prior to incubation with primary antibody for 12 h. Anti-GREM1 antibody and anti-GREM2 were used at 200 ng/ml and non-specific IgG at 200 ng/ml. The sections were then washed with PBS and incubated with 1:400 diluted biotin-conjugated secondary antibodies for 45 min (Biotinylated anti-goat (BA-5000) or Biotinylated anti-rabbit (BA-1000); Vector laboratories, USA) and then again washed several times before applying streptavidin peroxidase (Histostain-Plus Streptavidin-Peroxidase; Invitrogen USA, cat# 50–420Z). A DAB color reaction was performed as per manufacturers instructions (Peroxidase substrate

kit DAB; Vector laboratories, cat# SK-4100) to localize sites of GREM and GREM2 in tissues.

Microarray Transcriptome Analysis

The mRNA processing and hybridization were performed at the Genomics Core Laboratory, Center for Reproductive Biology, Washington State University, Pullman, WA using standard Affymetrix reagents and protocol. Briefly, mRNA was transcribed into cDNA with random primers and cRNA was transcribed and from that single-stranded sense DNA was synthesized which was fragmented and labeled with biotin. Biotin-labeled fragmented ssDNA was then hybridized to the Rat Gene 1.0 ST microarrays containing more than 27,000 transcripts (Affymetrix, Santa Clara, CA, USA). Hybridized chips were scanned on Affymetrix Scanner 3000. CEL files containing raw data were then pre-processed and analyzed with Partek Genomic Suite 6.5 beta software (Partek Incorporated, St. Louis, MO) using an RMA and GCcontent adjusted algorithm. The signals from an average of 28 different probes for each transcript were compared to give a single value. Lists of differentially expressed genes for each treatment were generated using the following cut off criteria: signal ratio Control/Treatment > 1.20 change, mean difference for unlogged signals between control and treatment > 10, t-test p-values < 0.05, and correcting for organ culture date batch effects. CEL files (MIAME compliant raw data) from this study have been deposited with the NCBI gene expression and hybridization array data repository (GEO, http://www.ncbi.nlm.nih.gov/geo, #GSE pending) and can be also accessed through www.skinner.wsu.edu.

Previous studies have demonstrated that microarray data are validated with quantitative PCR data (Kezele *et al.*, 2005; Shima *et al.*, 2004). Due to the presence of an average of 28 different olionucleotide probes for each specific gene being used on the microarray, versus only a single primer set for a gene in quantitative PCR, the microarray is more effective at eliminating false positive and provides a more robust quantification of changes in gene expression.

Co-Immunoprecipitation

Co-immunoprecipitation experiments were performed to determine what growth factors would bind to GREM2. Recombinant proteins GREM2, BMP4 (R&D Systems, cat# 314-BP) and AMH were placed alone or in combination in one ml of binding buffer (0.5% BSA in PBS) for 1 hr at room temperature (RT) with rocking. Pull-down antibodies were added to each reaction tube [anti-GREM2 (1000ng/ml; Santa Cruz Biotechnology cat# sc-135103), anti-BMP4 (500ng/ml; R&D Systems cat# MAB757), or anti-AMH (1000ng/ml; Santa Cruz Biotechnology cat# sc-34833)] and incubated at 4° overnight with rocking. Antibody-bound protein complexes were removed from solution by adding 20μL of Protein A/G Agarose beads (pre-blocked with binding buffer)(Santa Cruz Biotechnology cat# sc-2003) to each reaction tube and incubating with rocking for 1 hour, then centrifuging 5 min. at 1000x g and discarding supernatant. Beads were rinsed 4 times in blocking buffer then resuspended in 40μL SDS sample buffer for western blotting and boiled to remove proteins from beads. Reaction samples were stored at −20° until the time of western blot.

Western blots were performed in duplicate to qualitatively evaluate growth factor binding using 15% acrylamide/0.4% bis-acrylamide resolving gels and a 5% acrylamide/0.17% bis-acrylamide stacking gels, then nitrocellulose transfer membranes. GREM2, BMP4 and AMH proteins were loaded as positive controls. Primary antibodies were anti-GREM2 (400ng/ml; Santa Cruz Biotechnology cat# sc-135103), anti-BMP4 (5000ng/ml; R&D Systems cat# MAB757), or anti-AMH (2000ng/ml; Santa Cruz Biotechnology cat# sc-34883). Secondary horseradish peroxidase-conjugated antibodies were anti-rabbit IgG-HRP to detect anti-GREM2 antibody (200ng/ml; Santa Cruz Biotechnology cat# sc-2004), anti-mouse IgG-HRP to detect anti-BMP4 antibody (1:1000 dilution; Cell Signaling Technology cat#7076), anti-goat IgG-HRP to detect anti-AMH antibody (1:1000 dilution; R&D Systems cat# HAF017). Blots were visualized on film using the SuperSignal West Pico Chemoluminescent Substrate kit (Thermo Scientific, Pierce Biotechnology, cat# 34077).

Statistical Analysis

Treatment groups are compared using analysis of variance (ANOVA) followed by post-hoc tests where appropriate. The data for GREM1 cultures and GREM2 cultures are both normally distributed, and so parametric ANOVA is appropriate. The GREM2 treatment groups have unequal variances (AMH is small), so consideration must be made for that. For GREM1, it was decided beforehand that our comparisons of interest were between untreated control *vs.* GREM1, control *vs.* BMP4, control *vs.* AMH, BMP4 *vs.* GREM1+BMP4, and AMH *vs.* GREM1+AMH. Results of both Dunnet's and the conservative Bonferroni's tests are now presented. For GREM2, it was similarly decided beforehand that our comparisons of interest were between untreated control *vs.* GREM2, control *vs.* AMH, and AMH *vs.* GREM2+AMH. Since the variance for the AMH treated group is small in GREM2 experiments, but similar to that of other treatments in the GREM1 experiments, one might conclude that the population variance for AMH is normal, and so regular ANOVA and post-tests would be appropriate for GREM2 studies. However, this may not be the case, so the results of alternate analyses appropriate for unequal variances (Kruskal-Wallace test, Dunn's post-hoc and Student's t-test with Welch's correction) are also presented. Groups were considered statistically significant with $p \leq 0.05$. Statistics were calculated using Graph Pad Prism version 5.0b for Macintosh, Graph Pad Software, San Diego, CA, USA.

RESULTS

Ovaries were isolated from four-day old rats for use in organ culture experiments to test the effects of gremlin-1 (GREM1) and gremlin-2 (GREM2) on primordial to primary follicle transition. Four-day old rat ovaries contain predominantly primordial follicles. Whole ovaries were cultured intact for ten days as described in the Methods in the presence or absence of GREM1, GREM2, bone morphogenetic protein 4 (BMP4) or anti-Müllerian hormone (AMH). Following culture, ovarian sections were obtained and evaluated morphologically to determine the numbers of primordial and developing follicles per section (Figure 1 and 2).

Treatment of ovaries with GREM1 alone did not result in any change in the proportion of developing follicles compared to controls (Figure 1). Using both Dunnet's and the conservative Bonferronis's post-hoc tests, treatment with BMP4 increased the proportion of developing follicles indicating an increase in primordial to primary follicle transition. AMH treatment resulted in a decrease in developing follicles as expected. Co-treatment of ovaries with both GREM1 and BMP4 reversed the effects of BMP4. Similarly, co-treatment with both GREM1 and AMH reversed the effects of AMH on follicle transition. These results indicate that GREM1 is inhibitory to the actions of BMP4, and also inhibitory to AMH.

Although the biological activities of GREM1 and GREM2 have been shown to be similar (Kosinski *et al.*, 2007; Segditsas *et al.*, 2008), experiments were performed in which cultured ovaries were treated with GREM2 and/or AMH. Both Dunnet's and the non-parametric Dunn's post-hoc tests showed GREM2 and AMH-treated groups to have a decrease in developing follicles compared to the control. In contrast, the conservative Bonferroni test did not identify any groups as significantly different from each other. However, a t-test using Welch's correction for unequal variances showed a significant difference between the AMH-treated group and the group co-treated with AMH and GREM2. Both parametric (ANOVA) and non-parametric (Kruskal-Wallace) analyses were performed. Although the data for GREM2 culture experiments is normally distributed, consideration must be made for the possibility of unequal variances between these treatment groups. Together, these data suggest that treatment of ovaries with GREM2 resulted in a modest but statistically significant decrease in developing follicles, (Figure 2). A preliminary organ culture experiment demonstrated GREM2 inhibited BMP4 actions similarly to GREM1 (data not shown) as would be expected from previous literature (Kattamuri *et al.*, 2012; Kosinski *et al.*, 2007). As expected, treatment with AMH resulted in a decrease in developing follicles. Interestingly, co-treatment with both GREM2 and AMH reversed the effect of AMH treatment. These results suggest that GREM2 is inhibitory to the actions of AMH.

Immunohistochemical experiments were performed to localize the expression of GREM1 and GREM2 in rat ovaries (Figure 3 and 4). GREM1 protein is detectable in the granulosa cells and oocytes of large developing follicles (Figure 3). However, expression of GREM1 in primordial follicles was not detected. This suggests that GREM1 may not participate in primordial follicle transition *in vivo*. GREM2 protein is present in the granulosa cells of both developing and primordial follicles (Figure 4). GREM2 also appears to be present in the nuclei of some oocytes. Therefore GREM2 is expressed in primordial follicles, while both GREM2 and GREM1 are expressed in later-stage developing follicles having several layers of granulosa cells.

Since co-treatment of ovaries with both GREM2 and AMH reversed the effects of AMH on primordial to primary follicle transition, co-immunoprecipitation experiments were performed to test whether GREM2 bound to and inhibited directly AMH. Qualitative western blots were used to evaluate coimmunoprecipitation and growth factor binding. Successful immunoprecipitation and western blot detection of GREM2 (lane 5), AMH (lane 10) and BMP4 (lane 14) were demonstrated. Control experiments were performed to show that GREM2 bound to its known binding partner BMP4 (Figure 5). When GREM2 and

BMP4 are incubated together, precipitation with anti-BMP4 antibody also coprecipitates GREM2 allowing detection of GREM2 by western blot (Figure 5, lane 1). Similarly, incubation of GREM2 with BMP4 followed by precipitation with anti-GREM2 antibody results in detection of BMP4 (Figure 5, lane 12). When a negative control was performed where GREM2 and BMP4 are incubated together with no precipitating antibody, then neither GREM2 nor BMP4 are detected by western blot (Figure 5, lanes 3 and 13). The anti-BMP4 antibody does not bind and precipitate GREM2 (Figure 5, lane 6). The anti-GREM2 antibody does bind and precipitate a small amount of BMP4, but this small band is less than the BMP4 band seen when BMP4 co-precipitates with GREM2 using the anti-GREM2 antibody (Figure 5, lane 15 *vs.* lane 12). Observations indicate that GREM2 binds to BMP4 as expected.

When GREM2 and AMH are incubated together, precipitation with anti-AMH antibody also co-precipitates GREM2 (Figure 5, lane 2). Similarly, incubation of GREM2 with AMH followed by precipitation with anti-GREM2 antibody results in detection of AMH (Figure 5, lane 8). This suggests that GREM2 binds to AMH. When GREM2 and AMH are co-incubated with no precipitating antibody, then no GREM2 is detected by western blot (Figure 5, lane 4), but some AMH is detectable (Figure 5, lane 9). However, this was less AMH than detected when GREM2 and AMH are incubated together followed by co-precipitation with anti-GREM2 antibody (Figure 5, lanes 8 *vs.* 9). The anti-AMH antibody does not non-specifically bind GREM2 (Figure 5, lane 7). The anti-GREM2 antibody may bind a trace amount of AMH (Figure 5, lane 11), or this faint band may reflect a small amount of non-specific AMH protein carry-over to the western blot, similarly to lane 9. In summary, GREM2 and AMH are seen to bind when precipitated by anti-AMH antibody. When GREM2-AMH complexes are precipitated by anti-GREM2 antibody results are supportive of GREM2-AMH binding. Observations suggest that GREM2 binds to AMH. Coimmunoprecipitation experiments to test whether GREM1 bound to AMH were attempted, but issues of cross-reactivity of antibodies to these proteins rendered the results uninterpretable (data not shown).

In order to investigate the effects that GREM2 and GREM1 have on gene expression in the ovary, RNA was collected from whole cultured ovaries from four-day old rats treated for two days with GREM2, GREM1, or left untreated as controls. A 2-day culture was used to reduce the confounding factor of morphological changes that are seen in the ovary after a 10-day culture. RNA was subjected to microarray analysis using Affymetrix Rat Gene 1.0 ST arrays (see Methods). GREM2 treatment resulted in identification of 57 differentially expressed transcripts compared to controls, of which 32 were annotated as genes. GREM1 treatment resulted in identification of 29 differentially expressed transcripts compared to controls, of which 8 were annotated as genes (Supplemental Table S1). These differentially expressed genes were distributed among several functional categories, with no functions being obviously overrepresented. With the exception of a couple EST, the altered expression was >1.2 and <1.5 fold change (increases and decreases) such that negligible effects on gene expression were observed (Supplemental Table S1).

Microarray data from a previous study (Nilsson *et al.*, 2010) were interrogated to further examine the expression of *Grem1* and *Grem2* during early follicle development. In this

study of the network of genes regulating primordial follicle transition, ovaries from four-day old rats were treated with several different growth factors for two days and then RNA was collected for microarray analysis (Nilsson *et al.*, 2010). The relative expression of *Grem1* and *Grem2* in response to the different growth factor treatments is presented in Figure 6. The expression levels of *Grem1* were near the background and detection limit for the microarray, while expression levels of *Grem2* were well above background. This suggests that *Grem1* mRNA expression is quite low compared to *Grem2*. Since four-day old ovaries cultured for two days contain only primordial and early-stage follicles, this is consistent with only *Grem2* acting to regulate the primordial to primary follicle transition. It can also be seen that treatment with these growth factors that are known to regulate the primordial follicle transition, including AMH and BMP4, often results in significant increases in *Grem2* expression compared to untreated controls. This further suggests that *Grem2* is a part of the network of growth factor signaling controlling early follicle development, perhaps playing an autoregulatory feedback role to prevent excessive signaling (Glister *et al.*, 2011).

DISCUSSION

The roles that *Grem1* and *Grem2* might play in regulating the primordial to primary follicle transition were investigated using the rat as a model. Ovary organ culture experiments showed that GREM1 was able to reverse the stimulatory effect that BMP4 had on primordial follicle transition (Figure 1). This is to be expected, as GREM1 is known to bind and inhibit BMPs (Rosen, 2006; Avsian-Kretchmer and Hsueh, 2004; Kattamuri *et al.*, 2012). Interestingly, GREM1 treatment was also able to reverse the inhibitory effect that AMH had on primordial follicle transition. This raises the possibility that GREM1 is able to directly bind and inhibit AMH, which like BMPs is a member of the transforming growth factor beta family of proteins.

GREM2 was also able to reverse the inhibitory effect that AMH had on primordial follicle transition, similarly to GREM1 (Figure 2). GREM2 treatment alone had an inhibitory effect on follicle transition, but interestingly this effect was not additive with that of AMH. Rather, GREM2 reversed the effect of AMH. One potential explanation for the inhibitory effect seen when ovaries were treated with GREM2 alone is that GREM2 binds and inhibits the BMPs that are produced endogenously in the cultured ovaries. Preliminary organ culture experiments supported the ability of GREM2 to inhibit BMP4 stimulation of follicle transition (data not shown) as would be expected from previous literature (Avsian-Kretchmer and Hsueh, 2004; Kosinski *et al.*, 2007; Kattamuri *et al.*, 2012) BMP4 and BMP7 are known stimulators of primordial follicle transition (Nilsson and Skinner, 2003; Lee *et al.*, 2001), so the well-established function of GREM2 to bind BMPs (Kattamuri *et al.*, 2012) could result in a suppression of follicle transition. However, if GREM2 also directly binds and inhibits AMH, then for those ovaries treated with both GREM2 and AMH the exogenous AMH could occupy the available GREM2, and release the endogenous BMPs from inhibition. Alternatively, GREM2 treatment may change signaling cascades in other ways that result in a net decrease in follicle transition. Further studies will be needed to determine the exact mechanism by which GREM2 treatment alone decreases primordial to primary follicle transition.

Immunohistochemical experiments showed that both GREM1 and GREM2 proteins are present in the granulosa cells of later-stage developing follicles that have multiple layers of granulosa cells (Figure 3 and Figure 4). These results are in agreement with findings from previous *in-situ* hybridization studies that showed *Grem1* and *Grem2* expression to be localized to the granulosa cells of developing follicles (Sudo *et al.*, 2004; Pangas *et al.*, 2004). In a study by Fenwick et al. (Fenwick *et al.*, 2011) GREM2 mRNA was detected at much higher levels in preantral follicles than was *Grem1* mRNA by PCR. This is consistent with the mRNA expression results from Nilsson et al., 2010 (Figure 6) that again suggest that in ovaries containing only primordial and preantral stage follicles mRNA expression of *Grem2* is higher than that of *Grem1*. Neither *Grem1* nor *Grem2* mRNA were detected in mouse oocytes (Fenwick *et al.*, 2011), while in the current study gremlin proteins were detectible in some oocytes. This might reflect species differences or paracrine regulation. In the current study GREM2, but not GREM1, was also detected in primordial follicles. This suggests that while either GREM1 or GREM2 can affect the regulation of the primordial follicle transition in an experimental organ culture system, only GREM2 is present locally to act on primordial follicles *in vivo*. This does not exclude the possibility that GREM1 from later-stage developing follicles acts on distant primordial follicles, but such later stage follicles are not present in the neonatal ovaries used in these experiments.

Since GREM2 was shown to reverse the effects of AMH on the primordial to primary follicle transition, co-immunoprecipitation experiments were performed to test if GREM2 binds directly to AMH, as well as to its known binding partner BMP4 (Kattamuri *et al.*, 2012). Combined results indicate that GREM2 binds AMH, as well as BMP4 (Figure 5). This explains how GREM2 treatment alone can inhibit the primordial to primary follicle transition in cultured ovaries, and yet GREM2 treatment in combination with AMH will reverse the inhibitory effect of AMH. Further studies are needed to successfully investigate if GREM1 will also directly bind and inhibit AMH.

Microarray analyses of mRNA isolated from ovaries treated with GREM2 or with GREM1 yielded negligible numbers of differentially expressed genes compared to controls with a 1.2 – 1.5 fold change in expression for all except several EST, and no annotated genes >1.5 fold (Supplemental Table S1). Furthermore, no particular physiological processes or functional categories were identified as being over-represented among these differentially expressed genes. This suggests that the immediate effects of GREM2 (or GREM1) appear to be at the level of protein-protein interactions rather than by induction of changes in gene expression.

In summary, these results show that Grem2 is a part of the signaling network of growth factors and other genes that regulate the primordial to primary follicle transition (Figure 7). While both GREM1 and GREM2 are able to affect follicle transition in cultured ovaries, only GREM2 is normally present in primordial and early-stage developing follicles. GREM2 acts as an inhibitor of BMPs and AMH through direct protein-protein interactions (Figure 7). An improved understanding of the regulatory network controlling the primordial to primary follicle transition is important for understanding the basis for reproductive diseases affecting the pool of primordial follicles, such as primary ovarian insufficiency (POI).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Ms. Heather Johnson for assistance in preparation of the manuscript.

FUNDING

This work was supported by an NIH grant to MKS.

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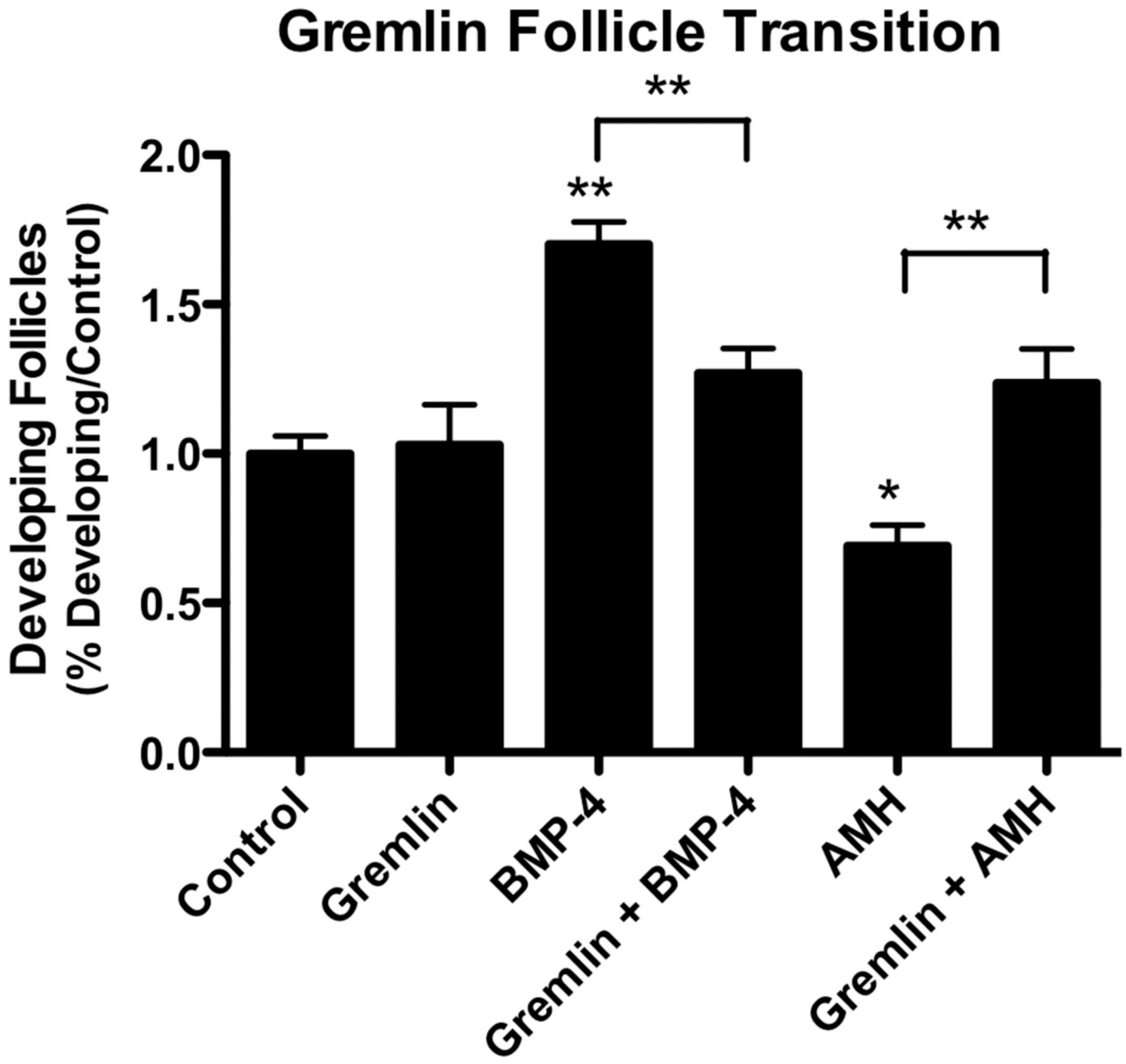


Figure 1.

Primordial to primary follicle transition after GREM1 treatment. The follicles per ovarian cross-section were counted and categorized as either primordial or as developing (i.e. after primordial to primary follicle transition) after organ culture. Data are expressed as the proportion of developing follicles for each treatment divided by the proportion of developing follicles in the control group. N = 5–12 ovaries per treatment, from 6 experiments performed in replicate. (*) = $p<0.05$ and (**) = $p<0.01$ compared to control by Dunnett's post-hoc test after significant ANOVA result. (##) = $p<0.005$ by Student's t-test.

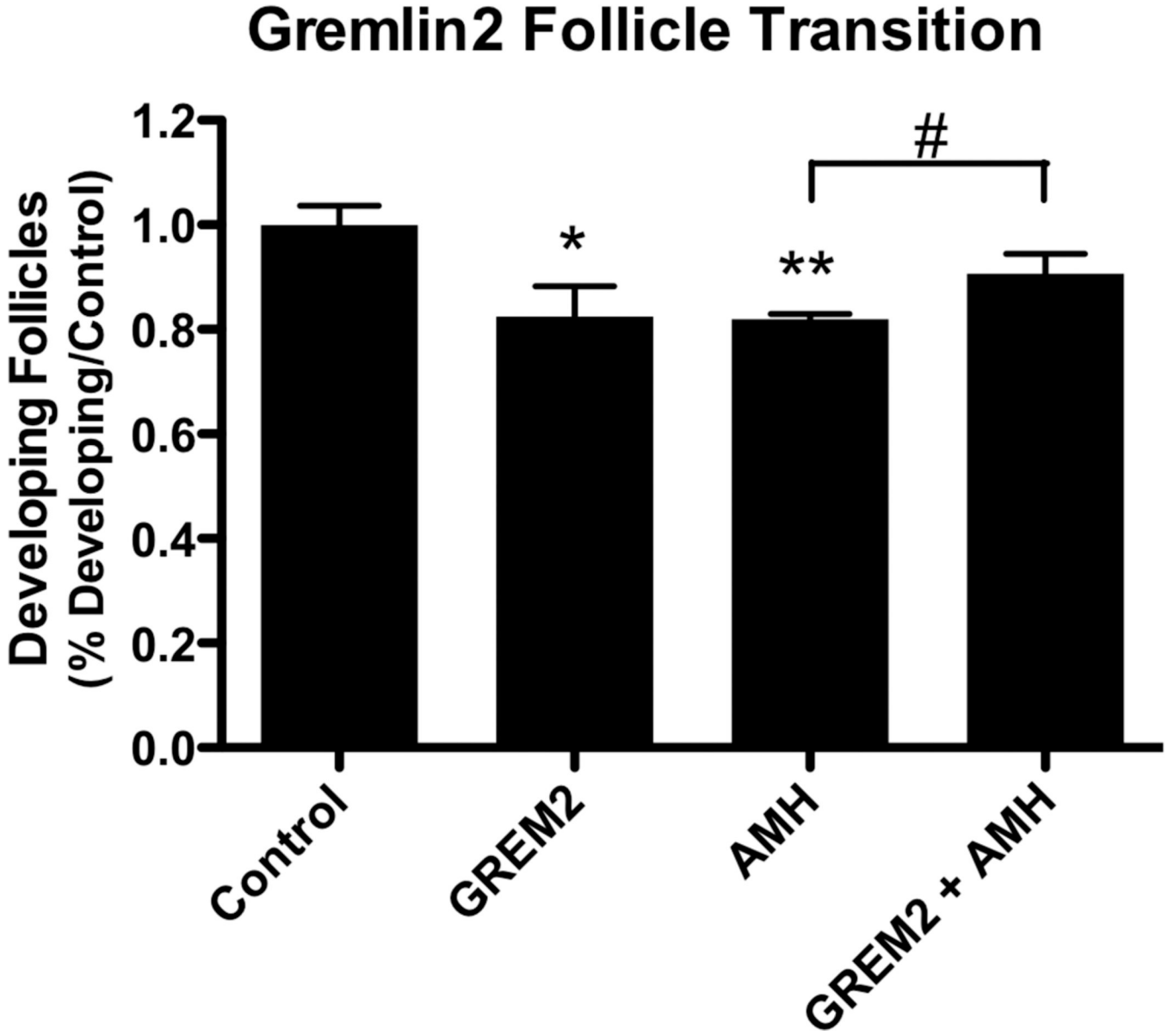


Figure 2.

Primordial to primary follicle transition after GREM2 treatment. The follicles per ovarian cross-section were counted and categorized as either primordial or as developing (i.e. after primordial to primary follicle transition) after organ culture. Data are expressed as the proportion of developing follicles for each treatment divided by the proportion of developing follicles in the control group. N = 6–11 ovaries per treatment, from 5 experiments performed in replicate. (*) = $p<0.05$ and (**) = $p<0.01$ compared to control by Dunnetts's post-hoc test after significant ANOVA test result. # = $p \leq 0.05$ by Student's t-test with Welch's correction for unequal variances.

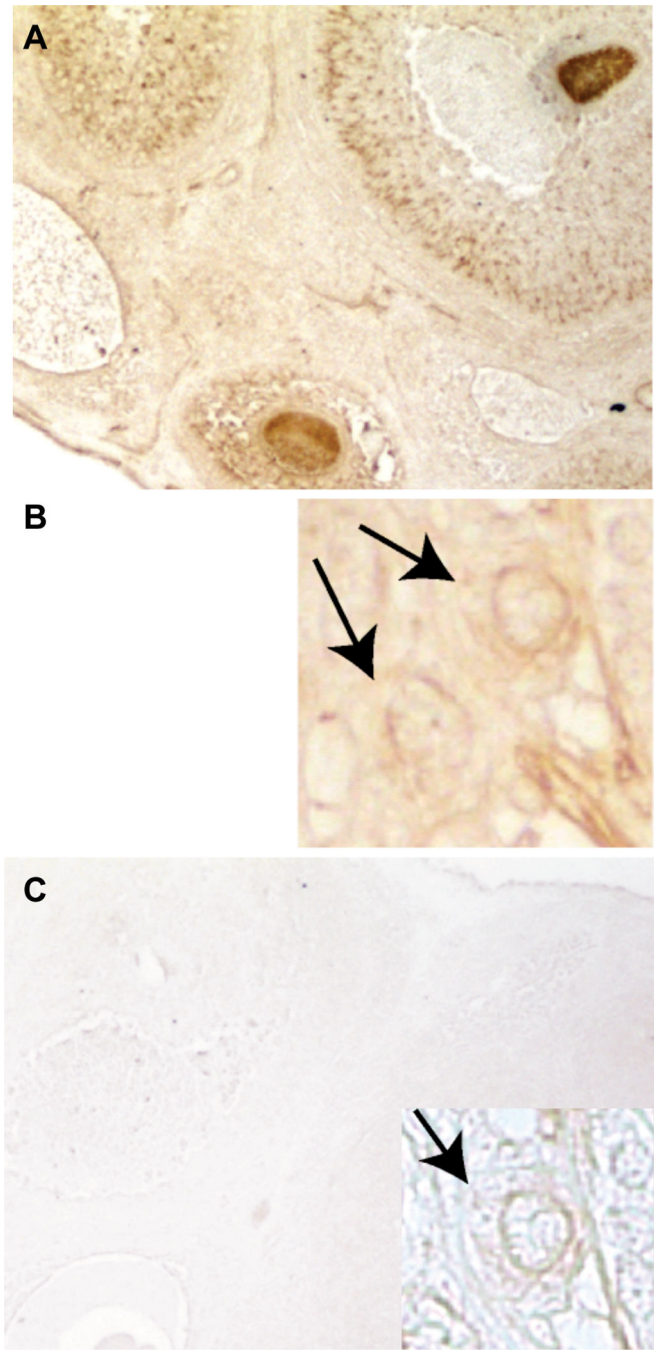


Figure 3.

Ovarian immunohistochemical localization of GREM1 in 19-day old ovaries. A) GREM1 is expressed in the granulosa cells of developing follicles with several layers of granulosa cells. B) Higher magnification image showing primordial follicles (arrows) having no distinct GREM1 expression. C) Negative control using non-specific IgG as a primary antibody and inset at higher magnification with arrow indicating a primordial follicle.

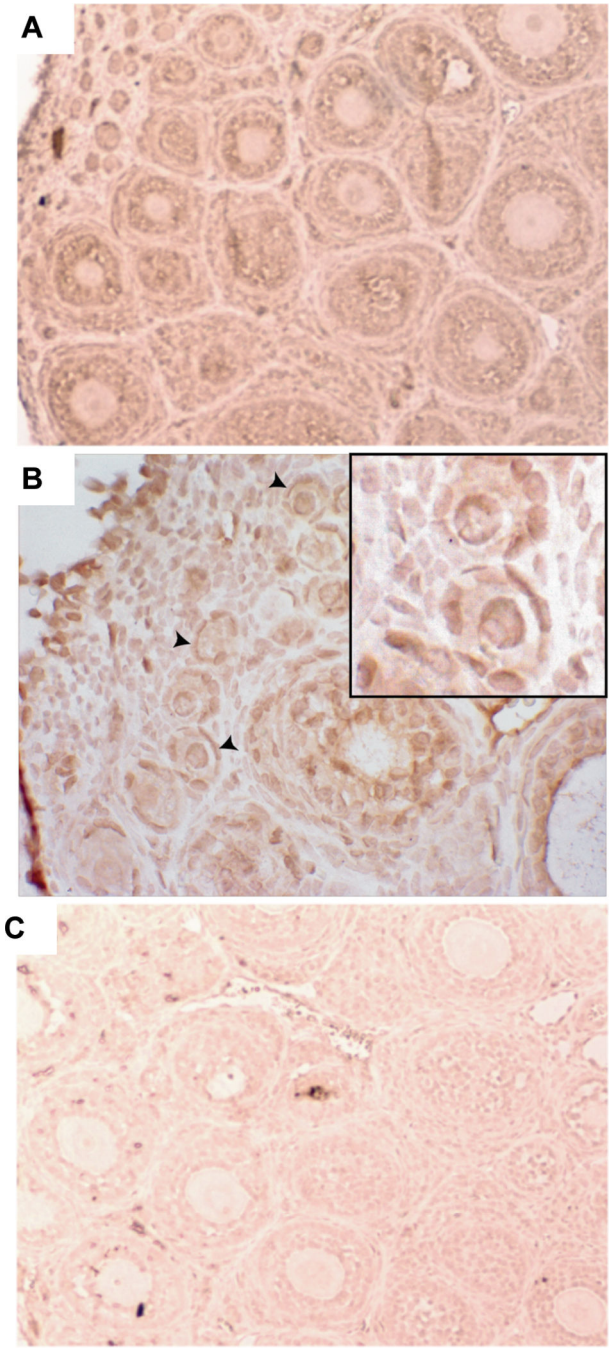
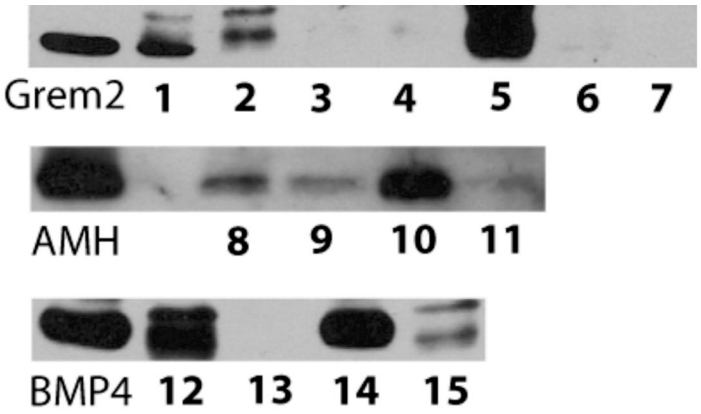


Figure 4.

Ovarian immunohistochemical localization of GREM2 in 14-day old ovaries. A) GREM2 is prominently expressed in the granulosa cells of developing follicles with several layers of granulosa cells. B) Higher magnification image showing GREM2 expression in the flattened granulosa cells (arrowheads) of primordial follicles. (Inset) Higher magnification view showing of GREM2 expression in a primordial (lower) and transitional follicle. C) Negative control using non-specific IgG as a primary antibody.

A



B

Lane	Co-IP Proteins	Precipitating Ab	Western Detection Ab against:
1	GREM2+BMP4	Anti-BMP4	GREM2
2	GREM2+AMH	Anti-AMH	GREM2
3	GREM2+BMP4	None	GREM2
4	GREM2+AMH	None	GREM2
5	GREM2	Anti-GREM2	GREM2
6	GREM2	Anti-BMP4	GREM2
7	GREM2	Anti-AMH	GREM2
8	GREM2+AMH	Anti-GREM2	AMH
9	GREM2+AMH	None	AMH
10	AMH	Anti-AMH	AMH
11	AMH	Anti-GREM2	AMH
12	GREM2+BMP4	Anti-GREM2	BMP4
13	GREM2+BMP4	None	BMP4
14	BMP4	Anti-BMP4	BMP4
15	BMP4	Anti-GREM2	BMP4

Figure 5.

GREM2 co-immunoprecipitation. A) Western blots of samples after co-IP detecting GREM2, AMH and BMP4. Lane numbers are explained in B) describing for each sample the proteins combined, the antibody used for co-immunoprecipitation and the antibody used for protein detection in the western blot.

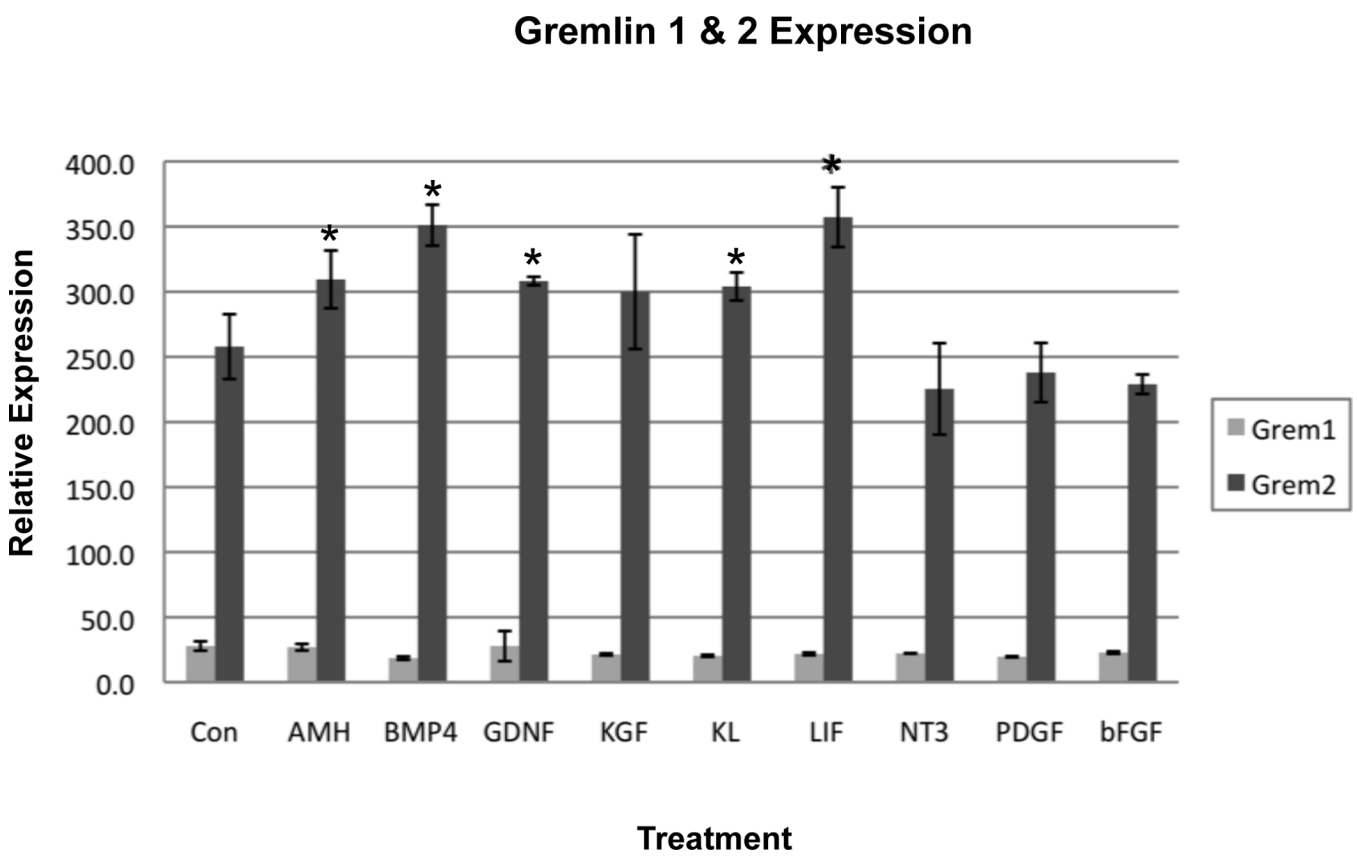


Figure 6.

Expression of Grem1 and Grem2 mRNA in 4-day old rat ovaries cultured for two days with the growth factor treatments indicated. Messenger RNA expression is presented as measured by microarray analysis. (*) = expression significantly ($p<0.05$) different than untreated controls by Students t-test. Modified from Nilsson et al, 2010.

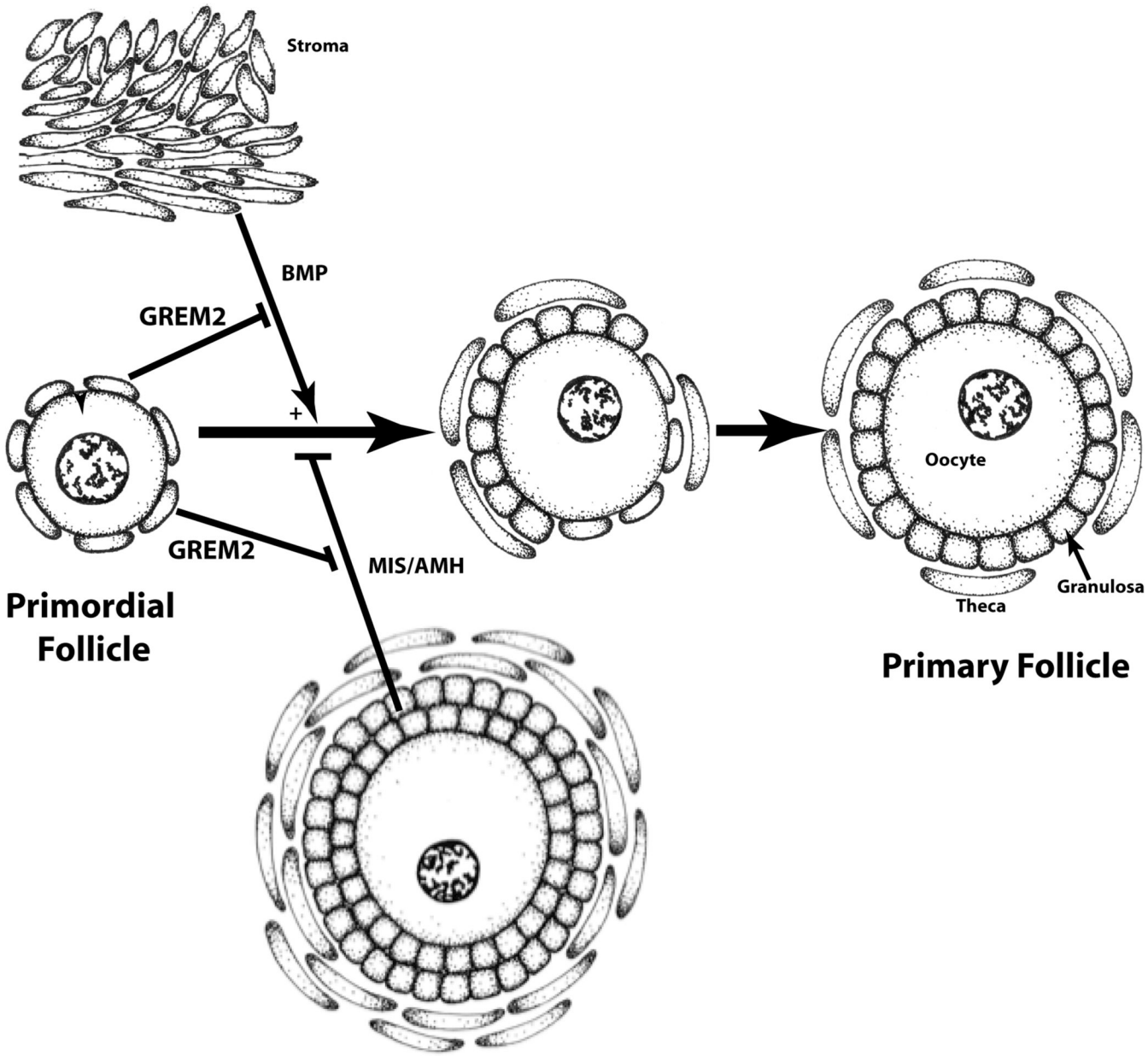


Figure 7.

Model of GREM2 actions affecting primordial to primary follicle transition. GREM2 is secreted by granulosa cells of primordial and developing follicles. GREM2 acts to bind and inhibit the actions of BMP4 from stroma and AMH from granulosa of developing follicles.