Identification and Regulation of Bone Morphogenetic Protein Antagonists Associated with Preantral Follicle Development in the Ovary

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The TGFβ superfamily comprises several bone morphogenetic proteins (BMP) capable of exerting gonadotropin-independent effects on the development of small preantral follicles. In embryonic tissues, BMP concentration gradients, partly formed by antagonistic factors, are essential for establishing phenotypic fate. By examining the expression of candidate genes whose protein products are known to interact with BMP ligands, we set out to determine which antagonists would most likely contribute toward regulation of paracrine signaling during early follicle development. Juvenile mouse ovaries of 4, 8, 12, and 21 d of age enriched with follicles at successive developmental stages were used to assess changes in candidate gene transcripts by quantitative RT-PCR. Although some antagonists were found to be positively associated with the emergence of developing follicles (Nog, Htra1, Fst, Bmpr, Vwc2), two (Sostdc1, Chrd) showed a corresponding reduction in expression. At each age, twisted gastrulation homolog 1 (Twsg1), Htra1, Nbl1, and Fst were consistently highly expressed and localization of these genes by in situ hybridization, and immunohistochemistry further highlighted a clear pattern of expression in granulosa cells of developing follicles. Moreover, with the exception of Nbl1, levels of these antagonists did not change in preantral follicles exposed to FSH in vitro, suggesting regulation by local factors. The presence of multiple antagonists in the juvenile ovary and their high level of expression in follicles imply the actions of certain growth factors are subject to local modulation and further highlights another important level of intraovarian regulation of follicle development. (Endocrinology 152: 3515–3526, 2011)
Disruptions in the function or expression of key components of TGFβ pathways have profound effects on the number and developmental potential of preantral follicles. For example, sheep and mice lacking functional BMP15 or GDF9, respectively, are sterile due to arrest in follicle development at the primary stage (15–17). Compound mutant mice with GC-specific deletions of BMP pathway components such as BMPR1A and BMPR1B, or intracellular signaling SMAD proteins develop GC tumors (18, 19). By comparison, mice with nonfunctional TGFβ signaling SMAD in GC exhibit impaired follicle development and reduced ovulation efficiency (20). Secretion of AMH by GC of developing follicles inhibits initiation of follicle growth in rodents (6), whereas BMP7 has a stimulatory effect (21). Other studies have demonstrated that the inhibition of endogenous BMP4 in mice through passive immunization caused a significant reduction in primordial follicle recruitment (22). Furthermore, culture of neonatal rat ovaries in the presence of antibodies to BMP4 results in widespread apoptosis and follicle loss, whereas elevated BMP4 exposure increases the proportion of growing follicles (23).

During development the level of exposure to specific growth factors regulates cell differentiation. For example, in Xenopus and Drosophila embryos, ectoderm cells require BMP signaling for their maintenance, but differentiation of these cells to become dorsal or ventral structures is mainly determined by the presence of extracellular BMP antagonists or binding proteins, which modify local concentrations of biologically active BMP (24, 25). Antagonism of BMP in combination with their limited diffusion in the extracellular matrix allows for local concentration gradients to be established, which are essential for normal embryogenesis (24, 26). Consequently, most loss-of-function mutants for the BMP antagonists are embryonic or perinatal lethal (27); hence, the role of these factors in normal adult physiology is not well defined.

Classical BMP antagonists share a unique cysteine knot structure, allowing them to specifically bind BMP (28). Other factors lacking a cysteine knot structure have been identified that can similarly bind BMP and other TGFβ ligands. Many BMP binding proteins antagonize signaling by physically preventing a receptor-ligand interaction (29) or by stabilizing the ligand, which can then be released in the presence of the appropriate enzyme (24, 25). Little is known about the expression of BMP antagonists in the postnatal or adult ovary. Some exceptions include gremlin 1 and 2, which are up-regulated by FSH in GC of large preantral/antral follicles (30, 31). Moreover, both of these factors, in addition to chordin and noggin, can neutralize BMP-regulated steroid production in follicle cells in vitro (30–33). In cumulus cells, expression of the BMP antago-

Materials and Methods

Animals and tissue collection

Ovaries [4, 8, 12, 21, 42 d post partum (dpp)] and organs (42 dpp) were collected from C57BL/6 mice (day of birth 0 dpp) and either snap frozen in liquid nitrogen for quantitative RT-PCR or formalin fixed, paraffin embedded, and sectioned at 5 μm for in situ hybridization (ISH) or immunohistochemistry. Oocytes and preantral follicles (14 dpp) and antral follicles (42 dpp) were mechanically isolated from ovaries and either processed for RNA isolation or cultured. Follicles were staged as described in Supplemental Materials and Methods, published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org. All mice were housed in accordance with the Animals (Scientific Procedures) Act of 1986 and associated Codes of Practice.

RT-PCR and quantitative PCR

Total RNA from organs was extracted with TriZol reagent according to the manufacturer’s protocol (Invitrogen Ltd., Life Technologies, Paisley, UK), checked for integrity on agarose gels, and assessed for purity and concentration by spectrophotometry (Nanodrop ND-1000; NanoDrop Technologies Inc., Wilmington, DE). RNA samples were adjusted to the same concentration and treated with deoxyribonuclease I (Invitrogen). For follicles and oocytes, RNA was isolated using RNeasy microcolumns (QIAGEN, Crawley, West Sussex, UK). cDNA was generated
from all deoxyribonuclease-treated RNA samples using random hexamer primers and SuperScript II reverse transcriptase in accordance with the manufacturer’s guidelines (Invitrogen). Primers were designed to detect mouse transcripts for the BMP antagonists listed in Supplemental Table 1. Control primers included internal reference genes (Hprt1 and Atp5b; PrimerDesign Ltd., Southampton, UK) and positive control genes specific for the oocyte (Ddx4, GCs (Amb), and theca (Cyp17a1). A presence/absence screen for all BMP antagonists was performed using cDNA from a range of adult tissues and isolated follicles and oocytes using KAPA 2G Fast Hot Start ReadyMix (Labtech International, East Sussex, UK). For quantitative PCR, master mixes containing 500 nM primers were prepared with Absolute QPCR SYBR Green Mix (Thermo Scientific, ABgene, Epsom, UK) and adjusted to 1× concentration with nuclease-free water. The same amount of cDNA from each sample (equivalent to 10 ng of reverse transcribed RNA) was added to each reaction in duplicate alongside a dilution series of external standards and no-template (H2O) controls. Standards were prepared from cDNA identical to real-time PCR products and purified using QIAquick PCR purification columns (QIAGEN). The concentration of each purified standard was determined (Nanodrop) and diluted greater than 10 orders of magnitude in nuclease-free water. Amplification was monitored with the DNA Engine Opticon 2 (Bio-Rad Laboratories Ltd., Hemel Hemstead, Herts, UK) consisting of an initial activation step at 95°C for 15 min followed by 40 cycles of denaturation (95°C), primer-specific annealing, extension (72°C), and an amplicon-specific fluorescence acquisition reading (Supplemental Table 1). The identity of each gene product was confirmed by DNA sequence analysis (Geneservice Ltd., Cambridge, UK), and a melting curve analysis followed by 40 cycles of denaturation (95°C), primer-specific annealing at 55°C, and extension (72°C) was used; all results shown are mean ± SEM for each group. The statistical analyses were performed using Prism 5 for Mac OS X (version 5.0b; GraphPad Inc., San Diego, CA).

**Preparation of cRNA probes and in situ hybridization**

Chemicals and reagents were purchased from Sigma (Sigma-Aldrich Company Ltd., Dorset, UK) except antidiogoxigenin (DIG)-alkaline phosphatase (AP), 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indoyl-phosphate, 4-toluidine salt solution. Amplification was monitored with the DNA Engine Opticon 2 (Bio-Rad Laboratories Ltd., Hemel Hemstead, Herts, UK) consisting of an initial activation step at 95°C for 15 min followed by 40 cycles of denaturation (95°C), primer-specific annealing, extension (72°C), and an amplicon-specific fluorescence acquisition reading (Supplemental Table 1). The identity of each gene product was confirmed by DNA sequence analysis (Geneservice Ltd., Cambridge, UK), and a melting curve analysis followed by 40 cycles of denaturation (95°C), primer-specific annealing at 55°C, and extension (72°C) was used; all results shown are mean ± SEM for each group. The statistical analyses were performed using Prism 5 for Mac OS X (version 5.0b; GraphPad Inc., San Diego, CA).

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Details of the ISH procedure are given in the Supplemental Material. Briefly, sections of ovary were deparaffinized and hydrated with decreasing concentrations of ethanol. Antigen retrieval was performed by boiling slides in citrate buffer [10 mM citric acid (pH 6.0)] and nonspecific binding blocked using CAS-Block (30 min; Invitrogen). Primary antibodies used were rat anti-twisted gastrulation homolog 1 (TWSG1; 6.25 μg/ml; R&D Systems Inc., Abingdon, UK), rabbit anti-Ampulla serine peptidase 1 (HTRA1; 6.25 μg/ml; Abcam, Cambridge, UK), goat anti-neuronal nitric oxide synthase, suppressor of tumorigenicity 1 (NBL1; 2 μg/ml; Santa Cruz Biotechnology Inc., Santa Cruz, CA), rabbit anti-SMAD1/5/8 (0.5 μg/ml; Santa Cruz Biotechnology), phospho-SMAD1/5/1 (1:25; Cell Signaling Technology Inc., Danvers, MA) overnight at 4°C. After washes in PBS with 0.5% Tween 20, sections were incubated in the appropriate secondary antibody: antirat Alexa488, antirabbit Alexa555, or antigoat Alexa555 (1:200; all Invitrogen) for 60 min. Sections were mounted in Prolong Gold medium containing 4’,6’-diamino-2-phenylindole (DAPI, Invitrogen), and digital images were recorded using a Leica inverted SP5 confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany).

**Immunohistochemistry**

Sections were deparaffinized and rehydrated. Antigen retrieval was performed by boiling slides in citrate buffer [10 mM citric acid (pH 6.0)] and nonspecific binding blocked using CAS-Block (30 min; Invitrogen). Primary antibodies used were rat anti-twisted gastrulation homolog 1 (TWSG1; 6.25 μg/ml; R&D Systems Inc., Abingdon, UK), rabbit anti-Ampulla serine peptidase 1 (HTRA1; 6.25 μg/ml; Abcam, Cambridge, UK), goat anti-neuronal nitric oxide synthase, suppressor of tumorigenicity 1 (NBL1; 2 μg/ml; Santa Cruz Biotechnology Inc., Santa Cruz, CA), rabbit anti-SMAD1/5/8 (0.5 μg/ml; Santa Cruz Biotechnology), phospho-SMAD1/5/1 (1:25; Cell Signaling Technology Inc., Danvers, MA) overnight at 4°C. After washes in PBS with 0.5% Tween 20, sections were incubated in the appropriate secondary antibody: antirat Alexa488, antirabbit Alexa555, or antigoat Alexa555 (1:200; all Invitrogen) for 60 min. Sections were mounted in Prolong Gold medium containing 4’,6’-diamino-2-phenylindole (DAPI, Invitrogen), and digital images were recorded using a Leica inverted SP5 confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany).

**Preantral follicle culture**

Isolated follicles were cultured in MEM-α (Invitrogen) supplemented with 0.1% (wt/vol) BSA; 75 μg/ml penicillin (Sigma); 100 μg/ml streptomycin sulfate (Sigma); and a solution of insulin (5 μg/ml), transferrin (5 μg/ml), and sodium selenite (5 ng/ml; all Sigma) in 96-well plates. Single follicles were placed in individual wells containing 100 μl medium supplemented with 0 (PBS vehicle), 1.0, or 10 ng/ml recombinant human (rh) FSH (National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases, Torrance, CA). At least six to eight follicles were cultured per treatment group for each plate, derived from an individual ovary. Cultures were maintained under humid conditions at 37°C with 5% CO₂ for up to 72 h, with 75% of the medium being replaced after 48 h. Follicles were photographed every 24 h, and measurements of the vertical and horizontal diameters were used to calculate the mean diameter.
and spherical volume for each follicle. Only follicles with a clearly identifiable, centrally placed round oocyte were included in the analysis. At 8, 24, and 72 h, follicles were grouped by treatment and plate, immediately frozen in liquid nitrogen, and processed for RNA extraction, cDNA synthesis, and quantitative PCR as described above.

Results

Distribution of BMP antagonists in the adult mouse

Using RT-PCR, an initial presence/absence screen of adult tissues established the general tissue distribution of BMP antagonists in the mouse (Fig. 1). Several antagonists were widely distributed across a range of tissues including Ctgf, Dand5, Htra1, Nbl1, and Twsg1, whereas others exhibited more variable tissue-specific expression such as Chrd, Fst, Grem1, Grem2, Sost, and Vwc2. Cerberus (Cer1) was undetectable by RT-PCR in any tissue under the current conditions but was weakly detectable when the cycle number was increased to 36 times (not shown). Of all 16 antagonists profiled, nine were consistently detectable in the adult ovary (Fig 1).

Quantitative expression of BMP antagonists in the juvenile mouse ovary

Comparing transcript abundance in whole ovary (Fig. 2), Twsg1, Htra1, Nbl1, and Fst were consistently the most highly expressed antagonists examined at each age. Bmpr1, Ctgf, Dand5, Fstl3, Grem2, and Vwc2 were expressed at a moderate level. By comparison, Chrd, Nog, Grem1, and Sostdc1 were generally expressed at a low level, whereas Cer1 and Sost were undetectable in the juvenile ovary.

When expression levels across the age groups were compared with that of d 4 levels in terms of fold change (Fig. 3), a significant increase in expression was observed for Bmpr1, Htra1, Nog, and Vwc2, whereas Fst was elevated in d 21 relative to d 8 and 12. The increased expression of these factors therefore coincides with the emergence of larger follicles and/or associated structures (see Supplemental Fig. 1). Ctgf and Grem1 exhibited similar patterns of elevated expression with age (P = 0.056 and P = 0.03, respectively, ANOVA), but there was no significant difference in expression between specific days (post hoc test). By comparison, Dand5, Fstl3, Grem2, and Twsg1 were all consistently expressed throughout each of the age groups, suggesting either relatively constant follicular expression or expression from an extrafollicular compartment. Chrd and Sostdc1 were the only two factors that were significantly reduced in expression from d 4. Similarly, Nbl1 exhibited a trend toward a reduction in expression with increasing age (P = 0.052, ANOVA).

Expression of BMP antagonists in isolated preantral follicles

To determine whether follicular BMP antagonist expression contributes toward overall ovarian expression, samples of oocytes and individual follicles from the transitional to large antral stage were collected and analyzed by RT-PCR (Fig. 4). Consistent with overall low expression in whole ovaries, several factors including Cer1, Chrd, Sost, and Sostdc1 were undetectable in oocytes and preantral and antral follicles. Nog and Grem1 were barely detectable in antral follicle samples only. Bmpr1 was absent in oocytes and was only weakly detectable in preantral follicles from the primary plus stage. Ctgf, Fst, Grem2, and Nbl1 mRNA was also absent in oocytes but detectable in all follicles from the transitional stage, suggesting GC as the main source of expression. Dand5 was as well as follicles from the primary plus stage was weakly detectable in oocytes. Fstl3 was weakly detectable in oocytes and all follicle types examined. Htra1 and Twsg1 mRNA was detectable in all oocyte and follicle samples. By comparison, Vwc2 expression was weak in oocytes and secondary plus staged follicles only.
In situ localization of BMP antagonists in the juvenile ovary

To further qualify transcript expression and to establish the precise location of selected BMP antagonists in the ovary, cRNA probes were prepared for four of the most abundantly expressed BMP antagonists including Twsrg1, Htra1, Fst, and Nbl1 (Fig. 2). Fst was also chosen as a control because the expression of this factor is well documented, and similarly, Gdf9 was included as an oocyte-specific methodological control. In general, expression patterns demonstrated by ISH (Fig. 5) were found to closely resemble profiles of expression as determined by quantitative PCR (Fig. 3); factors that showed a significant increase in expression with age by quantitative PCR were easily detectable in large preantral and small antral follicle compartments found in d 21 ovaries by ISH, whereas those factors expressed at a more constant level across all age groups by quantitative PCR were detectable in follicles as well as other somatic cell compartments by ISH.

Expression of Twsrg1 mRNA appeared to be almost ubiquitous in the ovary with a much stronger hybridization signal apparent in the GC of follicles of all stages of development including primordial (Fig. 5, A and B). A strong signal was also detectable in theca cells of large antral follicles in the adult ovary as well as in the ovarian surface epithelium. A weaker hybridization signal was detectable in oocytes, stromal and interstitial cells, and some cells of the corpus luteum.

Htra1 antisense hybridization (Fig. 5, C and D) was undetectable in primordial follicles but was weakly detectable in GC of follicles at the primary stage. From the primary stage, signal intensity remained relatively constant in GCs until the large preantral stage, whereafter signal strength increased considerably and became most intense in small antral sized follicles. In large antral follicles, Htra1 expression was almost absent from mural GC but remained in cumulus cells and those in close proximity to the antrum. Htra1 was also expressed in the corpus luteum.

Hybridization signal for Nbl1 (Fig. 5, E and F) was apparent in most cell types in ovary at d 21, which was often difficult to discriminate from background levels due to the relatively low level of specific staining. Overall, signal strength appeared stronger in GC and ovarian surface epithelium relative to all other cell types. It was not possible to clearly identify Nbl1 expression in primordial and primary follicles; however, in the d 42 ovary, staining intensity was clearly strongest in the GC of large antral follicles. Staining was absent from corpora lutea.

For Fst antisense hybridization (Fig. 5, G and H), a clear pattern of staining was observed in GC of developing follicles. No hybridization signal was detectable in primordial staged follicles, but a very weak signal could be observed in follicles at the primary stage. Hybridization intensity became stronger with increasing follicle size and was particularly strong in large preantral and small antral follicles. There was no evidence of Fst expression in any other ovarian cell type.

To confirm that the above hybridizations were not due to an unaccountable somatic cell artifact, a probe directed against Gdf9 mRNA was also hybridized to sections of ovary (Fig. 5, I and J). Gdf9 was expressed in oocytes of primary staged follicles and all subsequent stages. No specific staining was detectable in sections hybridized under similar conditions with a DIG-labeled bacterial control probe (Fig. 5, K and L).

Immunohistochemical localization of ovarian BMP antagonists and intracellular SMAD

Sections of mouse ovary (21 dpp) were stained with antibodies against TWSG1, HTRA1, and NBL1 to relate protein expression with patterns of mRNA data and determine their compartmental relationship with the SMAD...
proteins (SMAD1/5/8) used for BMP signaling (Fig. 6). TWSG1 was present in most cell types but appeared strongest in oocytes and GC of all follicle stages in a pattern similar to ISH. HTRA1 protein was also detectable in GC of follicles, the expression of which was particularly strong in follicles that had developed more than one layer of GC. NBL1 protein expression appeared ubiquitous in the ovary. By comparison, the nonphosphorylated form of SMAD1/5/8 was found in the GC compartment only. Unlike the antagonists, SMAD1/5/8 staining was absent in primordial follicles but was manifest from the transitional stage onward. Nuclear phospho-SMAD1 was generally found in GC of large preantral and small antral follicles, often in close proximity to the oocyte. GDF9, which was

![FIG. 3. Relative expression of BMP antagonists in the juvenile mouse ovary. Each transcript was normalized to Hprt1, and fold changes were calculated relative to 4 dpp expression as described in Materials and Methods. Values on the y-axis are means ± SEM (n = 4–6 ovaries per group). Numbers on the x-axis refer to age group (days postpartum). Groups with different letters indicate statistical differences (P < 0.05). The absence of a letter over a bar indicates no significant difference when compared with any other group (Bonferroni post hoc test).](https://academic.oup.com/endo/article-abstract/152/9/3515/2457278)

![FIG. 4. RT-PCR screen of BMP antagonists in isolated oocytes and preantral follicles. Due to limited starting material, all samples were cycled 34 times. Samples were staged according to morphology and size whereby sample O represents 16 oocytes (mean diameter 64 μm), sample t represents nine transitional staged follicles (mean diameter 50 μm), sample 1+ represents six primary plus staged follicles (mean diameter 76 μm), sample 2 represents six secondary staged follicles (mean diameter 90 μm), sample 2+ represents six secondary plus staged follicles (mean diameter 110 μm), sample Sa represents one small antral follicle (diameter 280 μm), and sample La represents one large antral follicle (diameter 470 μm). All preantral follicle samples and oocytes were isolated from a 14-dpp mouse. Antral follicles were isolated from a 42-dpp mouse. Ddx4 [DEAD (Asp-Glu-Ala-Asp) box polypeptide 4/vasa/mouse vasa homolog] and Gdf9 (growth differentiation factor 9) are included as oocyte-specific markers, whereas Amh (anti-Mullerian hormone) and Cyp17a1 (cytochrome P450, family 17, subfamily A, polypeptide 1) are specific for granulosa and theca cells respectively. The -ve samples contain no cDNA.)
Regulation of BMP antagonists in preantral follicles by FSH

Because preantral follicles are known to respond to FSH and because Twsg1, Htra1, Nbl1, and Fst were all abundantly expressed in GC of the same stage follicles, we investigated the potential of FSH to regulate these factors in vitro (Fig. 7). Expression of Gdf9, a marker of oocyte
viability, was unchanged after 24 h of culture in the absence of FSH (Fig. 7A). To assess acute changes after FSH exposure, groups of follicles were assayed after 8 and 24 h in 10.0 ng/ml rhFSH. No differences were found for any genes assayed after 8 h (data not shown). A small but significant increase in Kitl1 was evident after 24 h. Conversely, a decrease in Nbl1 expression was found (P < 0.05), whereas Twsig1, Htra1 and Fst expression remained unchanged (Fig. 7C). Preantral follicles cultured in the presence of either 1.0 or 10.0 ng/ml rhFSH grew at an increased rate compared with controls (0 ng/ml) so that after 48 h, follicles exposed to exogenous rhFSH were larger (P < 0.01) (Fig. 7B). When follicles were collected at 72 h, kit ligand 1 (Kitl1), a factor known to be regulated by FSH was increased significantly in rhFSH-treated samples relative to controls confirming the bioactivity of rhFSH in this model (Fig. 7D). In the same samples, Twsig1, Htra1, Nbl1, and Fst expression did not differ from control levels at either concentration of rhFSH (Fig. 7D).

**Discussion**

Many studies have highlighted a role for intraovarian BMP as central mediators of follicle development (reviewed in Ref. 1). However, a major component of BMP regulation is via interaction with extracellular antagonists (27). We therefore used the juvenile mouse ovary model to investigate changes in transcript levels for members of the BMP antagonist family during preantral follicle development. This study shows that a number of endogenous antagonists with known effects on BMP are expressed from a very early stage in follicle development (summarized in Table 1). We further investigated four factors that are highly expressed in the ovary: Twsig1, Htra1, Nbl1, and Fst. The presence of these antagonists in unilaminar follicles suggests regulation by intraovarian factors, and the lack of response to FSH indirectly supports this. An intriguing feature of these factors is their known ability to bind BMP2 and BMP4 as well as other TGFβ members (37–40). We have also shown expression of other known BMP antagonists, some for the first time in ovary, identifying new potential regulatory factors.

Of all antagonists examined, Twsig1 was one of the most abundantly expressed and its transcript was localized to GC and oocytes of follicles at all preantral stages including primordial. As an antagonist, TWSG1 can either bind directly with BMP2 and BMP4 or it can associate with both BMP/chordin to prevent receptor interaction by forming a more stable trimolecular complex (41, 42). The latter state facilitates diffusion through the extracellular
shown to bind several other TGF and vitro matrix (45). Like TWSG1, HTRA1 also has a high binding as a regulatory component of the ovarian extracellular in a screen of factors involved in ovulation, was implicated sesses the ability to cleave IGF binding proteins (46) and, vivo (45). HTRA1 is a unique serine protease that pos-

pregnant mare serum gonadotropin administration by FSH in preantral follicles in our culture system or by 

are a major source of with previous studies that have also demonstrated that GC in large preantral and small antral follicles. This concurs stage of follicle development, increasing to a maximal level 

chordin levels were low in whole ovary and almost un-

However, this is unlikely to operate in the ovary because 

domain gradients in developing tissues (43). 

Based on information from RT-PCR of isolated follicles and oocytes.

Based on information from quantitative PCR assays on juvenile ovaries.

Based on information from ISH.

Like Htra1, the pattern of Fst expression was mainly re-

stricted to GC through the same stages of follicle develop-

ment, consistent with previous studies (48–50). Follis-

statin expression in antral-derived GC in vitro can be 

regulated and augmented by factors such as TGFβ1, ac-

tivin, and FSH (51–53). The ability of FSH to induce fol-

listatin expression may depend on follicle stage or be short 

lived [−2 h in cultured rat GC; (53)], which may account 

for the lack of significant response observed in cultured preantral follicles. Fst and Htra1 mRNA were detectable 

in GC from the transitional/primary stage, and both were 

also relatively abundant in d 4 ovaries that are densely 

populated with primordial follicles. In fact, embryonic 

expression of follistatin is important for normal ovarian de-

velopment (54).

Consistent with our results, Nbl1 has previously been shown to be widely expressed in adult tissues. As with other highly expressed antagonists in the ovary, the phys-

iological targets for NBL1 include GDF5 and BMP2 and 

BMP4 (37, 55). However, little is known about the func-

tion of this gene in adult reproductive tissues. In the ju-

venile ovary, Nbl1 was expressed at relatively high levels, 

and its presence in most somatic cells implicates it as a 

potentially important regulator of local BMP. Despite 

this, null mutant mice have no obvious phenotypic abnor-

malities and are viable and fertile (37). DAN domain fam-

ily, member 5 is closely related to NBL1, and RT-PCR 

results indicate the transcript is also detectable in most 

tissues. The similar tissue distributions, albeit at different 

levels, and the lack of obvious phenotype in Nbl1 mutants,
raises the possibility that some redundancy operates with these genes.

In our screen of adult organs, gremlin 1 expression was strongest in the ovary relative to other tissues. However, we were unable to detect Grem1 at any appreciable level in juvenile ovaries until mice were 21 d old, suggesting that it may be either associated with larger follicles or is developmentally regulated. In support of the latter, a study by Pangas et al. (30) demonstrated that gremlin 1 expression is undetectable in small preantral follicles and can be induced by GDF9 and BMP4 in cultured GC derived from large antral follicles. Gremlin 1 is an important ovarian modulator of BMP4 signaling in large follicles (30, 32), yet it is not clear how this factor interacts with other BMP during early follicle development. On the other hand, gremlin 2, which shares a high degree of homology with gremlin 1, has been shown to be up-regulated in GC by FSH, and in vitro was found to inhibit the downstream luciferase reporter activity of BMP2 and BMP4 and to a lesser extent BMP6 and BMP7 (31). The same authors were able to detect the Grem2 transcript from the small antral stage by ISH. Using RT-PCR on isolated follicles, we were able to detect Grem2 from the earlier transitional stage, perhaps reflecting the difference in sensitivity of the two techniques. Collectively these studies indicate that both gremlin 1 and gremlin 2 are important regulators of BMP in the ovary, although the more abundant expression of Grem2 in the juvenile ovary suggests they may have distinct but overlapping roles at different stages of follicle development.

Connective-tissue growth factor (CTGF) is another factor well documented in the ovary and known to be regulated by FSH in GC (56). CTGF is growth factor with a wide range of functions in many different tissues that can also bind and inhibit the actions of BMP4 and enhance the actions of TGFβ1 (57). We found Ctgf mRNA was first expressed in transitional/primary stage follicles with abundant expression in preantral follicles. This agrees with other studies in rat, which have also shown that Ctgf can be induced by GDF9, activin, and TGFβ1 in nondifferentiated GC (56, 58).

In this study we have shown moderate expression of the antagonist Bmp6 in ovary. BMP binding endothelial regulator (BMPER) can bind BMP ligands, including BMP4, and has a very strong affinity for chordin. In other model systems, this affinity between BMPER and chordin is believed to be sufficiently strong enough to establish morphogenetic fields by the attraction and relocation of chordin complexes (43), but our finding of low levels of Chrd in ovary does not support this mode of action. The expression and role of BMPER has yet to be determined in ovarian physiology.

Historically noggin was one of the first BMP antagonists to be characterized due to its ability to influence patterning during embryogenesis (59). In various cultured cell lines, noggin protein reportedly binds BMP2, BMP4, BMP5, BMP6, and BMP7 as well as GDF5 and GDF6, and this ability to bind a broad range of ligands means it is often used as a tool to experimentally block BMP activity (27). In sheep GC, noggin has been shown to neutralize the effect of BMP2 and BMP4 on reducing progesterone production in vitro, although noggin was undetectable in sheep ovary (33), supporting our findings of low expression of this antagonist in juvenile mouse ovaries. Similarly, chordin was able to neutralize the ability of BMP4 and BMP7 to suppress androgen production in bovine theca cells (32). Our finding that noggin and chordin are absent in mouse follicles suggests that although they are effective inhibitors of BMP, they may not have a biological role in ovarian physiology. Similarly, the lack of expression of Sostdc1, Cer1, and Sost in adult ovary, together with the fertility and viability of null mutants for Sostdc1 and Cer1 (60, 61), suggests that these antagonists play little or no role in the regulation of follicle development in the mouse. However, they may still be important factors in other species. For instance, there is a case report of a woman with a mutation in the Nog gene who suffered premature ovarian failure (62), and splice variants of Chrd with biological activity have been reported for the adult human ovary (63). Moreover, because many of the BMP antagonists can be regulated by the ligand they antagonize (64), this raises the possibility that variation in antagonist expression between species may reflect the reported variation in expression of BMP in different species (65).

Based on their expression patterns, this study highlights antagonists that are most likely involved in regulating BMP members in the ovary, particularly during the early stages of follicle development. Factors such as Tws51, Htra1, and Fst predominate in GC from an early stage, and a common feature of these antagonists is their known affinity for BMP4. This may be physiologically relevant in ovary because evidence from previous studies supports a role for this BMP in the initiation and survival of primordial follicles (22, 23). Considerable progress has been made in understanding the role of TGFβ members in mammalian follicle development by studying animals that either have mutations in, or lack specific ligands, receptors, or downstream signaling molecules (35). The presence of antagonists highlighted in this study provides a framework for addressing a further extracellular level of regulation of BMP signaling that may be essential for normal follicle development.
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References

7. Erickson GF, Shimasaki S 2003 The spatiotemporal expression pattern of the bone morphogenetic protein family in rat oavy cell types during the estrous cycle. Reprod Biol Endocrinol 1:9
12. Guéripel X, Brun V, Gougeon A 2006 Oocyte bone morphogenetic protein 15, but not growth differentiation factor 9, is increased during gonadotropin-induced follicular development in the immature mouse and is associated with cumulus oophorus expansion. Biol Reprod 75:836–843
32. Glister C, Richards SL, Knight PG 2003 Bone morphogenetic proteins (BMP) -4, -6, and -7 potently suppress basal and luteinizing...
hormone-induced androgen production by bovine theca interna cells in primary culture: could ovarian hyperandrogenic dysfunction be caused by a defect in thecal BMP signal? Endocrinology 146: 1883–1892


34. Sugura K, Su YQ, Li Q, Wigglesworth K, Matzuk MM, Eppig JJ 2009 Fibroblast growth factors and epithelial growth factor cooperate with oocyte-derived members of the TGFβ superfamily to regulate Spry2 mRNA levels in mouse cumulus cells. Biol Reprod 81: 833–841

35. Edson MA, Nagaraja AK, Matzuk MM 2009 The mammalian ovary from genesis to revelation. Endocr Rev 30:624–712


42. Scott IC, Blitz IL, Pappano WN, Maas SA, Cho KW, Greenspan DS 2001 Homologues of Twisted gastrulation are extracellular cofactors in antagonism of BMP signalling. Nature 410:475–478


