BIOLOGY OF REPRODUCTION **69,** 1265–1272 (2003)
Published online before print 11 June 2003.
DOI 10.1095/biolreprod.103.018671

Bone Morphogenetic Protein-4 Acts as an Ovarian Follicle Survival Factor and Promotes Primordial Follicle Development[1]

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ABSTRACT

The growth and development of follicles within the ovary are highly dependent on autocrine and paracrine signaling involving growth factors from granulosa cells, theca cells, stromal interstitial cells, and the oocytes. The growth factor bone morphogenetic protein-4 (BMP-4) and its receptor (BMPR-IB) have been detected in ovaries, and a mutation in BMPR-IB has been associated with abnormal ovulation rate. The objective of the current study was to examine the role that BMP-4 plays in the early stages of primordial follicle development. Ovaries from 4-day-old rats were placed into a whole-ovary organ culture system for 2 wk to investigate the effect that treatment with exogenous BMP-4 has on early follicle development. BMP-4-treated ovaries had a significantly higher proportion of developing primary follicles and fewer arrested primordial follicles than did untreated controls. This indicates that BMP-4 promotes primordial follicle development and the primordial-to-primary follicle transition. Ovaries were also treated with neutralizing antibody against BMP-4 to determine effects of removing endogenously produced BMP-4. Interestingly, ovaries treated with BMP-4 antibody were markedly smaller than controls. This was associated with a progressive loss of oocytes and primordial follicles, a progressive increase in cellular apoptosis, and an accompanying loss of normal ovarian tissue morphology over time. Immunocytochemistry localized BMP-4 protein to isolated stromal cell populations, selected stromal cells (i.e., pretheca cells) associated with developing primordial follicles, and the basement membrane of follicles. Ovaries were treated with BMP-4 and RNA collected after organ culture to determine whether BMP-4 signaling affects expression of other growth factors. Kit ligand and basic fibroblast growth factor expression was unchanged, but TGFα expression was decreased in whole ovaries. Taken together, these data suggest that BMP-4 plays an important role in promoting the survival and development of primordial follicles in the neonatal ovary.

follicle, oocyte development, ovary

INTRODUCTION

Normal ovarian function is essential for the reproductive success of females. The growth and development of the follicles within the ovary are highly dependent on interactions between ovarian epithelial cells and mesenchymal cells. Paracrine signaling between the epithelial-derived granulosa cells and surrounding mesenchymal-derived theca or interstitial cells is important for follicle development in both antral stages of follicle development [1–5] and in the earliest stage of primordial follicle development [6–12]. An understanding of the signaling pathways involved in the control of follicle development leads to a better understanding of the control of reproduction and may lead to treatments for some forms of infertility. The current study is designed to further investigate the cell-cell interactions in primordial follicle development.

The paracrine growth factors bone morphogenetic protein-4 (BMP-4) and BMP-7, and their receptors, BMPR-II, BMPR-IB, and BMPR-IA, have been detected in ovaries [13]. A mutation in BMPR-IB has been associated with an increased ovulation rate in ewes [14–16]. In mice, a null mutation knock-out of the BMPR-IB gene results in a failure of cumulus granulosa cell expansion at the time of ovulation as well as decreased granulosa cell aromatase production [17]. In rat ovaries, high levels of BMP-4 and BMP-7 were expressed in the theca cells of antral follicles while BMPR-IB was highly expressed in granulosa cells and oocytes [13]. In granulosa cells from antral follicles, BMP-4 and BMP-7 have been shown to inhibit progesterone and promote estrogen secretion [13, 14, 16]. BMP-7 has been demonstrated to promote the initiation of development of primordial follicles to primary and preantral follicles [18]. While some functions of BMP-4 have been reported for antral follicles, it has not yet been determined if BMP-4 exerts effects on the initiation of follicular development or growth.

Bone morphogenic proteins (BMPs) are members of the transforming growth factor beta (TGFβ) superfamily of growth factors, which includes TGFβ1, TGFβ2, TGFβ3, growth-differentiation factors (GDFs) and Müllerian inhibitory substance (MIS) [19–21]. BMPs will bind to plasma membrane receptors BMPR-IB or BMPR-IA, that then heterodimerize with BMPR-II [16, 19]. Expression of BMP-2 has been demonstrated in human ovaries [22]. Cultured human granulosa-luteal cells are able to respond to BMP-2 stimulation by increasing estradiol production [23] and the expression of the inhibin β(B) subunit [24]. Granulosa cells are not a source of BMP-2 protein [25]. BMP-3 is present in ovaries as well as in cultured granulosa-luteal cells. BMP-3 expression levels can be regulated in granulosa-luteal cells by hCG treatment [22, 25]. BMP-6 and BMP-15 are produced by the oocytes of ovarian follicles [26–28]. They act on granulosa cells to modulate the effects of FSH on steroid hormone production [29–31]. In addition, BMP-15 stimulates granulosa cell proliferation [29]. BMP-4 was first characterized for its role in bone and cartilage metabolism [32–34]. It has subsequently been implicated in regulating embryonic mesoderm formation [35], formation of primordial germ cells [36, 37], limb bud regulation [38], and morphogenesis in several organ systems during development, including lung [39], liver [40], tooth [41], and fa-

[1]This work was supported by NIH grants to M.K.S.
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Received: 24 April 2003.
First decision: 12 May 2003.
Accepted: 28 May 2003.

ISSN: 0006-3363. http://www.biolreprod.org

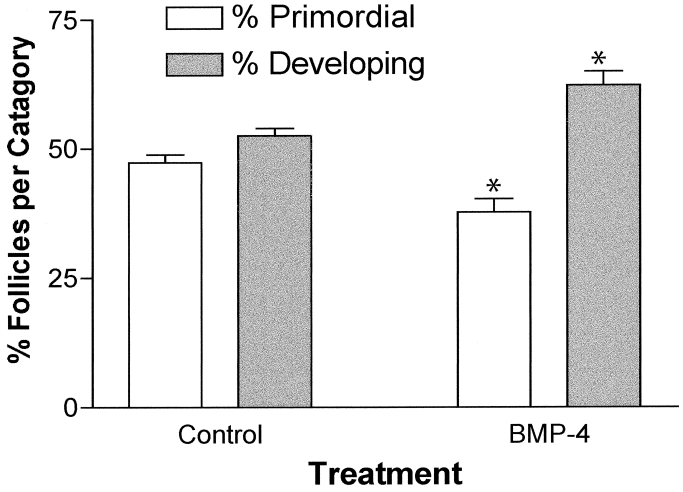

FIG. 1. Effect of BMP-4 treatment on primordial-to-primary follicle transition in cultured ovaries. Ovaries from 4-day-old rats were placed into culture for 14 days. Cultured ovaries were incubated in the absence (control) or presence of 100 ng/ml BMP-4 (BMP-4). After culture, all ovaries were fixed, stained, and subjected to morphological analysis. The follicles per ovary cross-section were categorized as being either primordial or developing (which includes all follicles having undergone the primordial-to-primary transition). Data are presented as the mean ± SEM from three separate experiments with n = 3 for controls and n = 5 for BMP-4-treated ovaries. An asterisk indicates a value significantly different than that of the similarly colored bar in the control group ($P \leq 0.04$ by Student *t*-test).

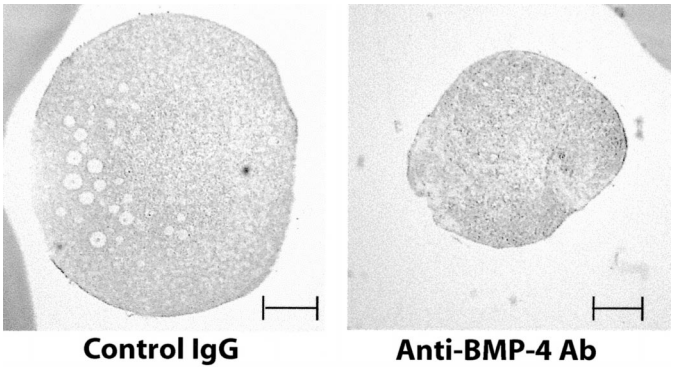

FIG. 2. Effect of BMP-4 neutralizing antibody treatment on ovarian and follicular growth in culture. Ovaries from 4-day-old rats were placed into culture for 14 days and treated either with BMP-4 antibody (20 μg/ml) or nonspecific control IgG (20 μg/ml). After culture, ovaries were fixed, sectioned, and stained. Left panel, control IgG treated. Right panel, anti-BMP-4 treated. Images are representative of three separate experiments. Microscope magnification ×100 and bar length = 200 μm.

cial mesenchyme [42]. During tissue morphogenesis, the actions of BMP-4 are often opposed and controlled in combination with fibroblast growth factor-8 (FGF-8) or FGF-10 [39–41].

The object of the current study was to investigate the actions of BMP-4 on early follicle development. Whether BMP-4 influences the expression of other paracrine growth factors is also examined.

MATERIALS AND METHODS

Organ Cultures

Postnatal Day 4 rat ovaries were dissected from freshly killed rat pups. Whole ovaries were cultured as previously described [10] on floating filters (0.4-μm Millicell-CM; Millipore, Bedford, MD) in 0.5 ml Dulbecco modified Eagle medium (DMEM)-Ham F-12 medium (1:1, vol/vol) containing 0.1% bovine serum albumin (BSA; Sigma, St. Louis, MO), 0.1% Albumax (Gibco BRL, Gaithersburg, MD), 27.5 μg/ml transferrin, 1 μg/ml insulin (human recombinant, Sigma), and 0.05 mg/ml L-ascorbic acid (Sigma) in a four-well culture plate (Nunc plate; Applied Scientific, South San Francisco, CA). Ovaries were randomly assigned to treatment groups with 2–3 ovaries per floating filter. Treatments during organ culture included anti-human BMP-4 neutralizing antibody (R&D Systems, Inc., Minneapolis, MN) at 20 μg/ml and human recombinant BMP-4 (R&D Systems, Inc.) at 100 ng/ml. Anti-human BMP-4 shows 5% cross-reactivity to rhBMP-2, rmBMP RIA, and rmBMP RIB according to manufacturer's specifications. Medium was supplemented with penicillin and streptomycin to prevent bacterial contamination. After culture, ovaries were fixed, sectioned, and stained with hematoxylin/eosin for use in morphological analysis. Alternatively, if mRNA levels were to be measured from cultured ovaries, then after culture, two ovaries were pooled and homogenized in 500 μM Trizol (Gibco BRL) and stored at −20° until RNA isolation.

Histology

The number of follicles at each developmental stage was counted in two serial sections and averaged from the largest cross-section through the center of the ovary. The oocyte nucleus had to be visible in a follicle in order to be counted. Normally 150–200 follicles were present in a cross-section. It has previously been demonstrated that total follicle number per section does not change after 2 wk of culture compared with freshly isolated 4-day-old ovaries [7]. Follicles were classified as either primordial (stage 0), or as one of the developing preantral stages (stages 1–4) as described previously [7]. Briefly, primordial follicles consist of an oocyte partially or completely encapsulated by flattened squamous pregranulosa cells. Developing (stage 1–4) follicles contain successively more cuboidal granulosa cells in layers around the oocyte [7, 10].

TUNEL Assay

TUNEL assays to indicate apoptotic cells in histological sections were performed as per manufacturer's protocols (Roche Applied Science, Indianapolis, IN; Cat. No. 1 684 795) using the In Situ Cell Death Detection Kit, Fluorescein.

Immunocytochemistry

Localization of BMP-4 protein was determined by immunocytochemical analysis. Four-day-old rat ovaries were cultured for 2 wk and then fixed in Bouin solution (0.9% picric acid, 9% formaldehyde, 5% acetic acid) for 1–2 h. Ovaries were paraffin embedded and sectioned at 3–5 μm. Ovaries were deparaffinized in xylenes and hydrated through an ethanol series of 100%, 90%, and 70%. Antigens were exposed by boiling sections for 5 min in 0.01 M sodium citrate buffer, pH 6.0. A solution of 10% goat serum in phosphate buffered saline (PBS) was used as a blocking agent prior to incubating sections with primary antibody overnight at 4°C. Slides were incubated with monoclonal mouse anti-human BMP-4 antibody (R&D Systems, Inc.) at 25 μg/ml overnight at 4°C. Secondary antibody (biotinylated goat anti-mouse IgG; Vector Laboratories, Inc., Burlingame, CA) was detected by using the Vectastain kit (Vector) and diaminobenzadine (Vector). Negative controls were incubated in the presence of non-immune mouse IgG as a primary antibody at 25 μg/ml.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from cultured ovaries homogenized in Trizol reagent as per the protocol provided (Gibco BRL). RNA was stored at −70°C until use. The RNA samples were reverse transcribed to cDNA as described previously [43]. Steady-state levels of kit ligand (KL), basic fibroblast growth factor (bFGF), transforming growth factor alpha (TGFα), and cyclophilin (i.e., 1B15) mRNAs were analyzed using a specific quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay for each gene [43]. These quantitative RT-PCR assays have previously been described in detail [9]. The primers used were KL, 5′-GGA CAA GTT TTC GAA TAT TTC TGA AGG CTT GAG TAA TTA TTG-3′ (5′-primer; 42 mer) and 5′-AGG CCC CAA AAG CAA ACC CGA TCA CAA GAG-3′ (3′-primer; 30 mer), which generated a specific 452-base pair (bp) KL PCR product; bFGF, 5′-GTT GGT ATG TGG CAC TGA AAC-3′ (5′-primer, 21 mer) and 5′-TGG GTC ACA ACC AAG CTA TA-3′ (3′-primer, 20 mer), which generated a specific 271-

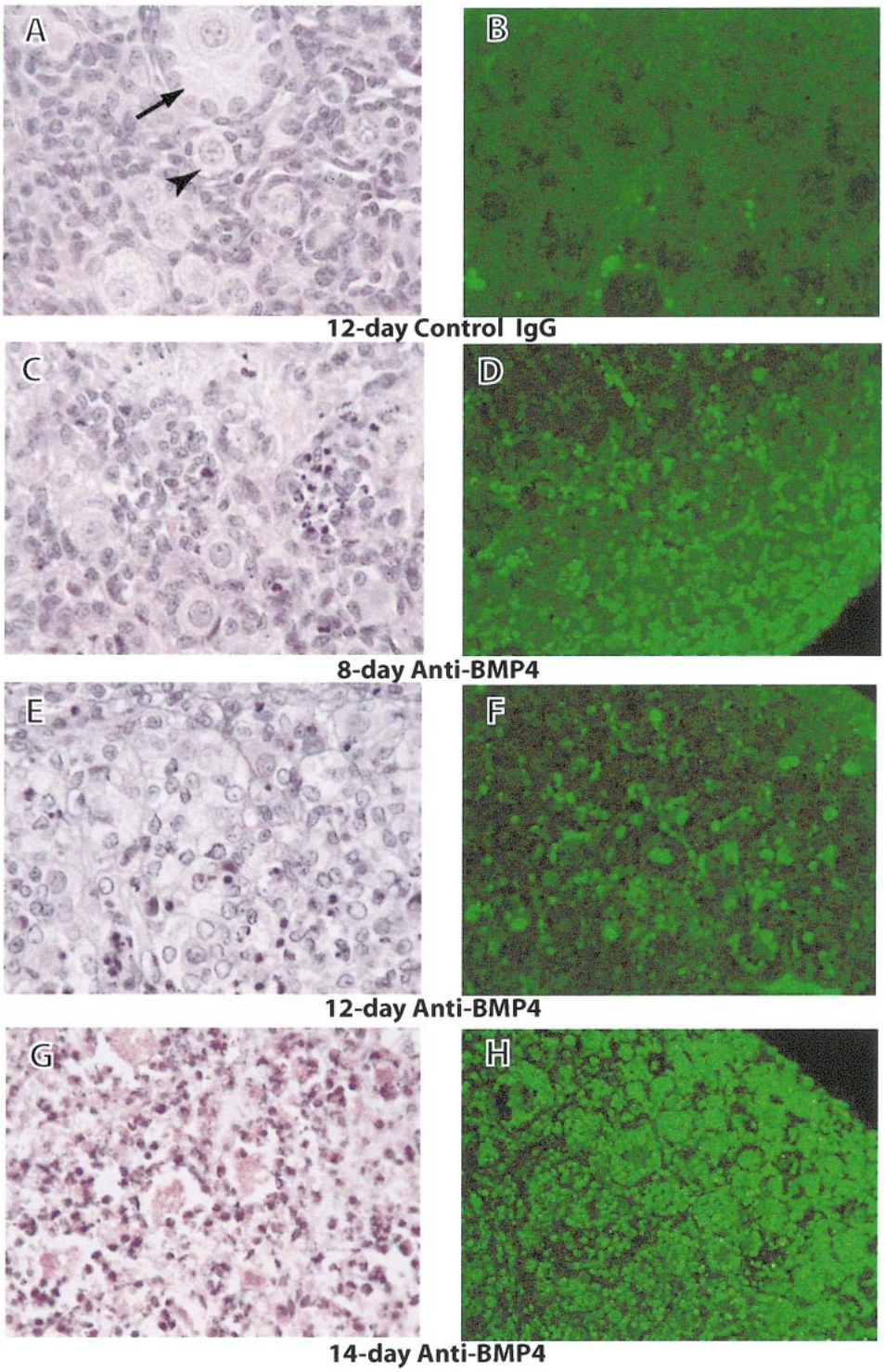

FIG. 3. Effect of BMP-4 neutralizing antibody treatment on ovarian morphology and cell apoptosis in cultured ovaries. Ovaries from 4-day-old rats were placed into culture for 8, 12, or 14 days and treated either with BMP-4 antibody (20 μg/ml) or nonspecific control IgG (20 μg/ml). After culture, ovaries were fixed and sectioned. Some sections were stained with hematoxylin and eosin (**A**, **C**, **E**, and **G**). Parallel sections were subjected to TUNEL assay. Apoptotic cell nuclei appear green (**B**, **D**, **F**, and **H**). **A**, **B**) Control ovaries treated with nonspecific IgG for 12 days. Control ovaries treated for 14 days appeared similar. Arrow indicates a developing primary follicle. Arrowhead indicates an arrested primordial follicle. Ovaries were treated with BMP-4 antibody for 8 days (**C**, **D**), 12 days (**E**, **F**), or 14 days (**G**, **H**). Images are representative of three separate experiments. Microscope magnification ×400.

bp bFGF PCR product; TGFα, 5′-TAA TGA CTG CCC AGA TTC CC-3′ (5′ primer, 20 mer) and 5′-GAT GAT GAG GAC AGC CAG GG-3′ (3′ primer, 20 mer), which generated a specific 218-bp TGFα PCR product; and 1B15, 5′-ACA CGC CAT AAT GGC ACT GGT GGC AAG TCC ATC-3′ (5′ primer, 33 mer) and 5′-ATT TGC CAT GGA CAA GAT GCC AGG ACC TGT ATG-3′ (3′ primer, 33 mer), which generated a specific 1B15 105-bp product. Amplification was performed using the following conditions: 0.4 μM each primer, 16 μM dNTPs, and 1.25 U AmpliTaq polymerase in 50 μl GeneAmp PCR buffer (containing 1.5 mM $MgCl_2$, Perkin Elmer). Each PCR amplification consisted of an initial denaturing reaction (5 min, 95°C), 26–30 cycles of denaturing (1 min, 95°C), annealing (2 min, 55°C [KL, TGFα] or 2 min, 54°C [bFGF] or 1 min, 60°C [1B15]), and elongation (3 min [KL, TGFα] or 2 min [bFGF, 1B15], 72°C) reactions, and a final elongation reaction (10 min, 72°C). At least 0.1 μCi of ^{32}P-labeled dCTP (Redivue, Amersham Life Sciences, Arlington Heights, IL) was included in each sample during amplification for detection purposes. Specific PCR products were quantified by electrophoresis of all samples on 4%–5% polyacrylamide gels, exposing the gels to a phosphor screen for 8–24 h, followed by quantifying the specific bands on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Each gene was assayed in separate PCR reactions from the same RT. PCR of unknown samples was performed in parallel with known amounts of each gene inserted into a plasmid to use as a standard curve. Absolute quantities of each cDNA per well were read from the standard curves and then normalized per well for IB15. The optimal number of cycles for amplification was determined for each assay in order to achieve maximum sensitivity while maintaining linearity (i.e., logarithmic phase of PCR reactions). KL and bFGF PCR products were

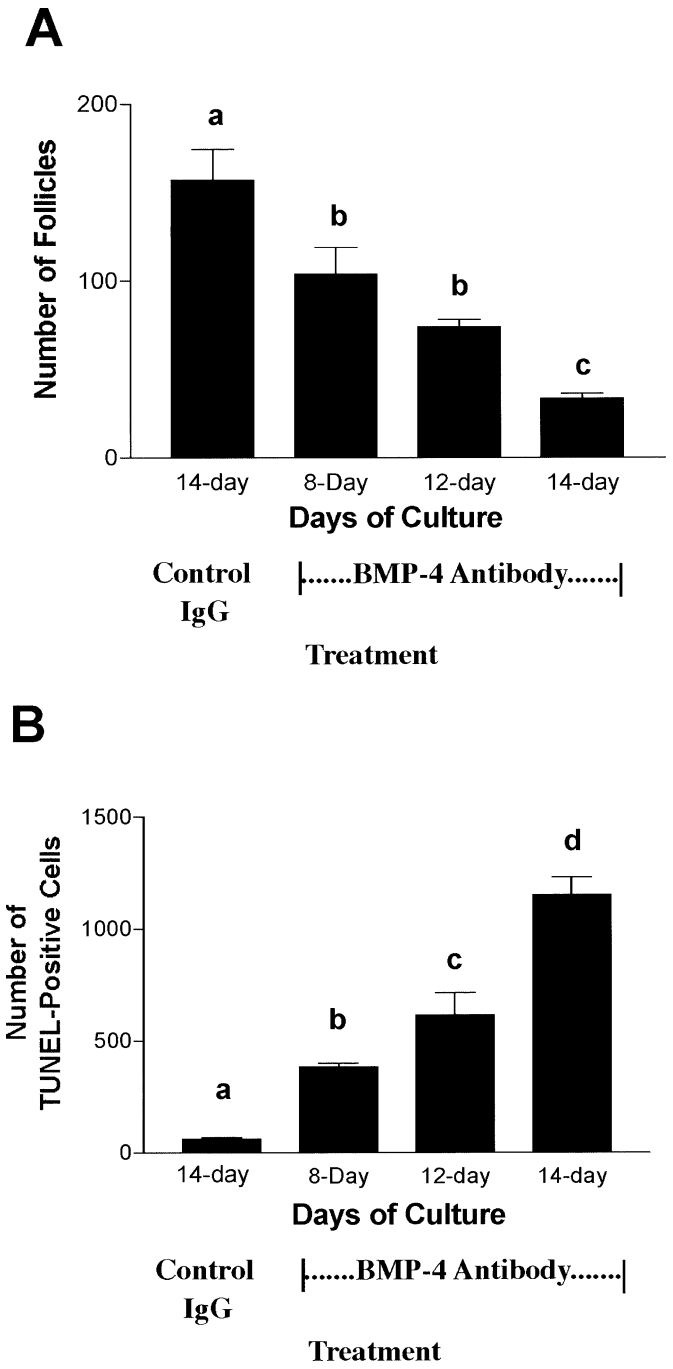

FIG. 4. Effect of BMP-4 neutralizing antibody treatment on follicle number and apoptosis. Ovaries from 4-day-old rats were placed into culture for 8, 12, or 14 days and treated either with BMP-4 antibody (20 μg/ml) or nonspecific control IgG (20 μg/ml). After culture, ovaries were fixed and sectioned. **A**) The number of ovarian follicles per ovary section was determined by morphological analysis of stained sections. **B**) The number of apoptotic cells per section was determined by counting the number of TUNEL-positive cells after TUNEL assay. Control values are from ovaries treated with control IgG for 12 or 14 days. Data are presented as the mean ± SEM. Different letter superscripts are significantly ($P \leq 0.05$) different according to Tukey HSD analysis performed after a significant ANOVA result. Data are from three separate experiments performed in replicate.

amplified for 30 cycles, TGFα PCR products were amplified for 27 cycles, and 1B15 PCR products were amplified for 26 cycles. The sensitivity of each quantitative PCR assay was below 1 fg, which corresponds to less than 125 fg target mRNA/μg total RNA.

Statistics

Pairs comparisons are performed using a Student *t*-test. Multiple comparison tests are performed using Tukey-Kramer Honestly Significant Difference (HSD) after a significant result was obtained using ANOVA. Groups were considered significantly different if $P \leq 0.05$. All statistics were calculated with the help of GraphPad Prism, version 3.0a, for Macintosh (GraphPad Software, San Diego, CA).

RESULTS

Organ culture experiments were performed to determine the actions of BMP-4 on the primordial to primary follicle transition in rat ovaries. There was no difference in the total number (primordial plus developing) of follicles present in BMP-4-treated (137 ± 15.0; mean ± SEM) and untreated (134 ± 11.3) ovaries. In untreated ovaries, 53% ± 2.5% of follicles were in developing stages after 2 wk of culture. The proportion of developing follicles was significantly higher ($P \leq 0.04$) in BMP-4 treated ovaries at 62% (Fig. 1). The proportion of primordial follicles was correspondingly decreased with exogenous BMP-4 treatment. Observations indicate that BMP-4 promotes the primordial to primary follicle transition.

Cultured ovaries from 4-day-old rats were treated with neutralizing antibody to BMP-4 to determine if endogenously produced BMP-4 was important for ovarian growth and folliculogenesis. After 2 wk of culture, the ovaries treated with anti-BMP-4 antibody were qualitatively smaller (Fig. 2) and had a less organized histological appearance (Fig. 3) compared with control ovaries treated with nonspecific IgG. This indicates that BMP-4 produced within the ovary is important for ovarian growth and development.

Experiments were performed to better characterize the cellular aberrations of ovarian growth that occur when endogenous BMP-4 activity is blocked. Ovaries from 4-day-old rats were cultured in the absence or presence of BMP-4 neutralizing antibody for 8, 12, or 14 days (Fig. 3). Ovaries from each time point were fixed, sectioned, stained, and the number of follicles per section counted. There was a significant ($P \leq 0.01$) decrease in the number of follicles per section in BMP-4 antibody-treated ovaries compared with untreated controls (Fig. 4). The number of follicles decreased further as the ovaries were cultured longer in the presence of BMP-4 antibody (Fig. 4). This suggests that BMP-4 is important for the survival of follicle cells. To see if cellular apoptosis was involved in the loss of follicles from ovaries incubated with BMP-4 antibody, TUNEL assays were performed on ovaries cultured with or without BMP-4 neutralizing antibody for 8, 12, or 14 days. The number of TUNEL-positive cells was counted in ovary sections from each treatment group. There was a significant ($P \leq 0.01$) increase in the number of TUNEL-positive cells as the time of incubation with BMP-4 antibody increased from 8 to 12 to 14 days (Fig. 4). These data suggest that cell apoptosis is involved in the loss of follicles that occurs when the action of endogenous BMP-4 is blocked. The decrease in follicle number and increase in cell apoptosis occurred in parallel with a progressively more disorganized morphology of the ovary tissue and the appearance of more cells with pyknotic nuclei (Fig. 3).

Immunocytochemical analysis was performed to localize the expression of BMP-4 protein in neonatal rat ovaries. BMP-4 neutralizing antibody was used as a primary antibody on untreated control ovaries after culture. BMP-4 protein was found to localize to isolated populations of stromal tissue in the ovaries (Fig. 5A). BMP-4 staining was also detectable in stromal cells (i.e., precursor theca cells) as-

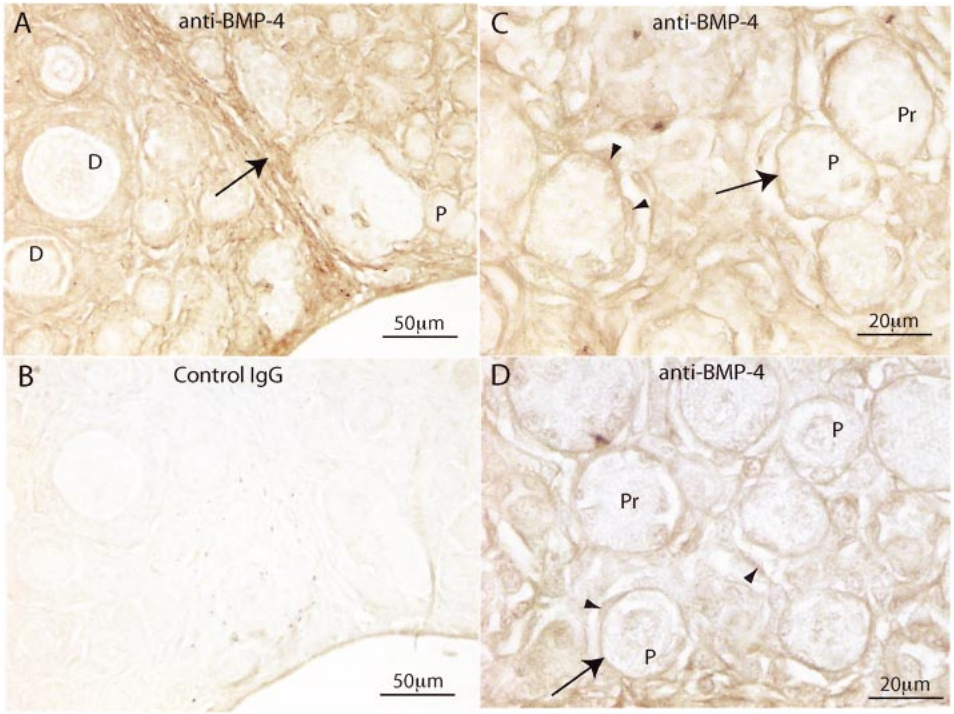

FIG. 5. Immunohistochemical localization of BMP-4 protein in cultured ovaries. The presence of BMP-4 protein is indicated by a dark brown stain in ovary sections. **A**) Ovary section showing BMP-4 staining at high intensity in a section of stromal tissue (arrow). **B**) Control ovary section stained using nonimmune IgG as a primary antibody. **C**, **D**) High-magnification images of ovary sections showing BMP-4 protein localization to follicle basement membranes (arrows) and to interstitial cells around follicles (arrowheads). Images are representative of two experiments. P, primordial follicle; Pr, primary developing follicle; D, developing follicle beyond primary stage.

sociated with developing primordial follicles and in the basement membranes around follicles (Fig. 5).

KL, bFGF, and TGFα are paracrine growth factors involved in the growth and regulation of various cell types in the ovary [3, 7, 10, 44–51]. Experiments were performed to determine if BMP-4 treatment affected expression of mRNA for KL, bFGF, or TGFα in cultured ovaries from 4-day-old rats. RNA was isolated from ovaries incubated in the absence or presence of BMP-4 for 3 days. Quantitative RT-PCR was used to determine that BMP-4 treatment did not alter either KL or bFGF mRNA expression (Fig. 6). However, BMP-4 treatment did decrease TGFα mRNA expression compared with untreated controls (Fig. 6).

DISCUSSION

Signaling between cell types in the ovary is vital for normal ovarian function. In the Booroola Merino sheep, a defect in BMP signaling leads to an increase in ovulation rate [14]. One proposed mechanism for this increased ovulation rate is that interfering with the normal inhibitory effects of BMP-4 alters differentiation and luteinization of granulosa cells and leads to precocious maturation of ovulatory follicles [16]. However, an alternate possibility is that increased ovulation rates may be due to an increase in the rate that follicles in the arrested primordial follicle pool undergo the primordial-to-primary follicle transition and begin developing. If it were the case that the primordial-to-primary follicle transition rate was higher in BMP-4 mutants, it would suggest that the normal function of BMP-4 was to inhibit primordial follicle transition. In the current study, experiments were performed to determine if BMP-4 influences the rate of the primordial-to-primary follicle transition. A whole-ovary organ culture system was used to examine the actions of exogenous BMP-4 on early follicle development. A neutralizing antibody against BMP-4 was used to determine what effect removing endogenously produced BMP-4 has on early follicle development.

Ovaries treated with exogenous BMP-4 exhibit an increase in the number of developing follicles compared with untreated controls (Fig. 1). This result contradicts the idea that BMP-4 signaling inhibits early follicle development. Rather, the data suggest that BMP-4 promotes the primordial-to-primary follicle transition. In this way, BMP-4 is similar to BMP-7, which exhibits a similar expression pattern in ovaries and also promotes the primordial-to-primary follicle transition [13]. BMP-4 now joins the growing list of paracrine growth factors that have been shown to regulate the primordial-to-primary follicle transition and early follicle development. KL and leukemia inhibitory factor (LIF) are factors present in the pregranulosa cells surrounding the oocytes of primordial follicles [7, 12]. KL promotes the primordial follicle transition, recruitment of thecal cells from the surrounding stroma, and thecal cell proliferation [7–9]. LIF also promotes the primordial follicle transition and upregulates KL expression [12]. Basic fibroblast growth factor (bFGF) is present in the oocytes of primordial follicles and can increase proliferation of granulosa, theca, and ovarian stromal cells [10, 52, 53]. Nerve growth factor is a neurotrophin that has been demonstrated to promote the primordial follicle transition [54]. The oocyte-derived factor growth and differentiation factor-9 (GDF-9) has been demonstrated to be important for further development and progression of primary follicles [55, 56]. A negative regulator of early follicle development is MIS. MIS is produced by secondary stage developing preantral follicles and can inhibit the primordial-to-primary follicle transition [57, 58].

Ovaries treated with BMP-4 neutralizing antibody and cultured were markedly smaller than ovaries treated with a nonspecific control IgG (Fig. 2). This appears to be associated with a progressive loss of oocytes and follicles, a progressive increase in cellular apoptosis, and an accompanying loss of normal ovarian tissue morphology over time (Figs. 3 and 4). These data suggest that BMP-4 plays an important role in the survival of both follicular and nonfollicular cell types in the neonatal ovary. BMP-4 receptor

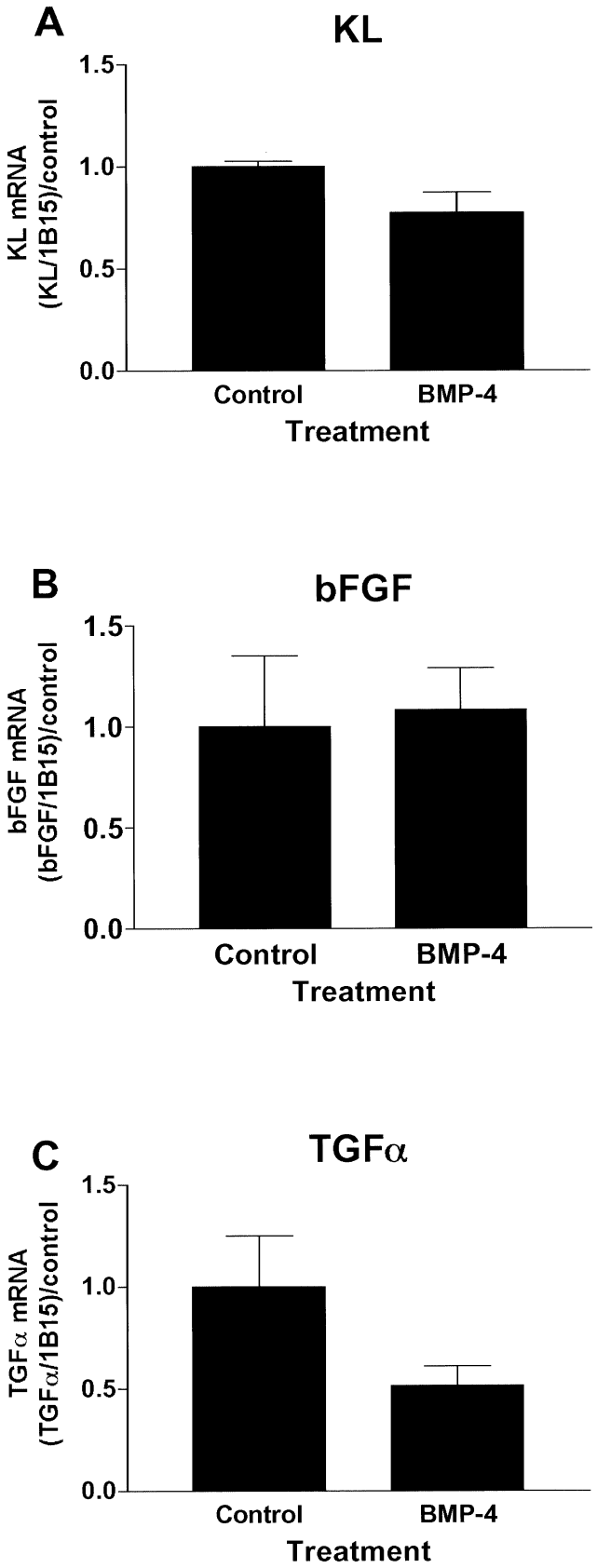

FIG. 6. Kit ligand (KL), basic fibroblast growth factor (bFGF), and transforming growth factor-alpha (TGFα) mRNA expression in cultured ovaries in response to BMP-4 treatment. Ovaries from 4-day-old rats were placed into culture and treated with 100 ng/ml BMP-4 or were not treated for 3 days. RT-QPCR was performed on RNA from whole ovaries to determine levels of KL, bFGF, and TGFα mRNA expression. Data are expressed as KL, bFGF, or TGFα mRNA/1B15 mRNA (pg/well) normalized to untreated control values. **A**) KL mRNA expression. **B**) bFGF mRNA expression. **C**) TGFα mRNA expression. Data are from separate experiments performed in replicate, with the mean ± SEM presented.

mRNAs for BMPR-II, BMPR-IA, and BMPR-IB have been detected in both oocytes and granulosa cells of rat ovaries [13]. In the current study, BMP-4 protein was detected in bands of stromal/interstitial cells and in the basement membranes and stromal cells surrounding follicles (Fig. 5). Stromal interstitial cells thus could use BMP-4 to signal in a paracrine manner to granulosa cells and oocytes in nearby follicles. This BMP-4 signal may act to promote cell survival in oocytes and/or granulosa cells. Loss of BMP-4 signaling would then result in granulosa cell apoptosis, which, because of granulosa-oocyte communication, could cause oocyte apoptosis. Alternatively, loss of BMP-4 signaling to BMP receptors in the oocyte could directly cause oocyte apoptosis. In either case, a treatment such as BMP-4 neutralizing antibody that interferes with BMP-4 signaling would be expected to result in a loss of follicles and an increase in apoptosis.

Treatment of neonatal rat ovaries with anti-BMP-4 antibody resulted in apoptosis of ovarian stromal-interstitial cells as well as apoptosis of follicular granulosa and oocytes. This appears to be due to autocrine actions of BMP-4 as a cell survival factor. Examination of the in situ hybridization results of Shimasaki et al. [13] reveal that BMPR-IA and BMPR-1B mRNA are expressed at a higher level in ovarian stroma than the background level seen in corpora lutea. These observations indicate the presence of BMP-4 receptors in selected stromal-interstitial cells. Alternatively, signaling from the cells of the follicles may be important for survival of the stromal-interstitial tissue. As follicles degenerate, this signal would be lost and stromal apoptosis would increase. However, it must be noted that the existence of intact follicles is not absolutely required for stromal cell survival. Mice with mutations in genes, such as the c-kit receptor, lack germ cells in their ovaries and have no intact follicles, but still have viable stromal tissue. Therefore, oocytes and follicles are not essential for stromal tissue viability [59]. Further studies will be needed to characterize the stromal/epithelial interactions required for early follicular development and neonatal ovarian growth.

BMP-4 signaling may affect the expression of other paracrine growth factors in the ovary. In the current study, neonatal ovaries were treated with BMP-4 and the expression of KL, bFGF, and TGFα were examined. These growth factors have previously been shown to regulate early follicle development and/or ovarian cell proliferation [7, 8, 10, 44–51]. KL and bFGF have themselves both been shown to promote the primordial-to-primary follicle transition [7, 10]. TGFα has been shown to regulate ovarian surface cells [47, 50] as well as to be present in primordial follicles [51] and to promote growth in granulosa and theca cells [48]. KL and bFGF expression were unaffected by BMP-4 treatment, but TGFα expression decreased in the ovary. Therefore, the actions of BMP-4 as a cell survival factor are likely in part mediated indirectly through growth factors such as TGFα. TGFα might act on pregranulosa and surrounding stromal cells as a proliferative factor rather than influence apoptotic pathways.

In summary, BMP-4 was found to promote the primordial-to-primary follicle transition in neonatal rat ovaries. BMP-4 neutralizing antibody treatment interfered with en-

dogenously produced BMP-4 and promoted a progressive loss of oocytes and follicles, a progressive increase in cell apoptosis, and an accompanying loss of normal ovarian tissue morphology over time. BMP-4 protein was localized to isolated ovarian stromal tissue and to the basement membranes and stromal cells (i.e., precursor theca cells) surrounding follicles. Together, these data suggest that BMP-4 acts as a paracrine signaling molecule derived from surrounding stromal-interstitial cells and acts on the oocyte and/or granulosa cells of primordial follicles to promote cell survival in developing follicles.

ACKNOWLEDGMENTS

We would like to thank Dr. Ingrid Sadler and Ms. Jacquilyn Ague for their invaluable technical assistance. Thanks also to Ms. Jill Griffin for assistance in preparing the manuscript.

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