BIOLOGY OF REPRODUCTION **65,** 994–999 (2001)

Effect of Bone Morphogenetic Protein-7 on Folliculogenesis and Ovulation in the Rat[1]

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ABSTRACT

We have previously established the presence of a functional bone morphogenetic protein (BMP) system in the ovary by demonstrating the expression of BMP ligands and receptors as well as novel cellular functions. Specifically, BMP-4 and BMP-7 are expressed in theca cells, and their receptors by granulosa cells. These BMPs enhanced and attenuated the stimulatory action of FSH on estradiol and progesterone production, respectively. To investigate the underlying mechanism of the differential regulation, we analyzed mRNA levels for key regulators in the steroid biosynthetic pathways by RNase protection assay. BMP-7 enhanced P450 aromatase ($P450_{arom}$) but suppressed steroidogenic acute regulatory protein (StAR) mRNAs induced by FSH, whereas mRNAs encoding further-downstream steroidogenic enzymes, including P450 side-chain cleavage enzyme and 3β-hydroxysteroid dehydrogenase, were not significantly altered. These findings suggest that BMP-7 stimulation and inhibition of $P450_{arom}$ and StAR mRNA expression, respectively, may play a role in the mechanisms underlying the differential regulation of estradiol and progesterone production. To establish the physiological relevance of BMP functions, we investigated the in vivo effects of injections of recombinant BMP-7 into the ovarian bursa of rats. Ovaries treated with BMP-7 had decreased numbers of primordial follicles, yet had increased numbers of primary, preantral, and antral follicles, suggesting that BMP-7 may act to facilitate the transition of follicles from the primordial stage to the pool of primary, preantral, and antral follicles. In this regard, we have also found that BMP-7 caused an increase in DNA synthesis and proliferation of granulosa cells from small antral follicles in vitro. In contrast to the stimulatory activity, BMP-7 exhibited pronounced inhibitory effects on ovulation rate and serum progesterone levels. These findings establish important new biological activities of BMP-7 in the context of ovarian physiology, including folliculogenesis and ovulation.

follicular development, growth factors, ovary, ovulation, steroid hormones

INTRODUCTION

Folliculogenesis is a process in which less differentiated cells become committed to particular developmental pathways [1]. Follicles develop through primordial, primary, and preovulatory stages, after which ovulation occurs and the residual follicular cells luteinize to form corpora lutea. There are specific morphological and functional changes during this development [2, 3]. Regulation of the proliferation, cytodifferentiation, and atresia associated with folliculogenesis involves complex interactions between a host of factors originating from within the follicle itself and gonadotropins from the pituitary. The process of folliculogenesis can be divided into two distinct developmental stages; gonadotropin-independent and gonadotropin-dependent stages. Transition through the initial stages of follicle development (from primordial follicles to primary follicles) can be observed in hypophysectomized rats [4], and thus this process does not require pituitary gonadotropins. Follicle-stimulating hormone (FSH), however, is an essential factor in the regulation of follicle development from primary follicles through dominant preovulatory follicles, and no other ligand can replace FSH in this role. During the past decade, studies in ovarian function have focused on regulatory factors that are produced from the follicle. These studies have identified a number of factors that are produced by and function in oocytes, granulosa cells (GCs), and theca cells in a paracrine/autocrine manner. These factors act in the regulation of follicular growth and development, independent of the pituitary-derived gonadotropins, dependent on them, or both [5].

We have recently identified bone morphogenetic proteins (BMPs) as new factors that are produced in the ovary and exhibit regulatory activities in GC function [6]. In the study we demonstrated that BMP-4 and BMP-7 are produced by theca cells and can enhance and attenuate the stimulatory action of FSH on estradiol and progesterone production, respectively, in primary cultured rat GCs. The purpose of the present study is to identify the mechanisms of how BMP-7 exerts the differential regulation of FSH action in GCs and to further elucidate the biological role of BMP-7 in the regulation of folliculogenesis and ovulation.

MATERIALS AND METHODS

Reagents and Supplies

Recombinant human BMP-7 was a gift of Dr. Sampath (Creative Biomolecules, Hopkinton, MA). Human chorionic gonadotropin (hCG) and ovine FSH (NIDDK-oFSH-S20) were gifts of Dr. Parlow (the National Hormone and Pituitary Program, Torrance, CA). Medium 199 and McCoy 5a medium were purchased from Life Technologies Inc. (Rockville, MD). Diethylstilbestrol (DES), eCG, and heparin acrylic beads were from Sigma-Aldrich Co. (St. Louis, MO).

Animals

Female Sprague-Dawley rats (23–25 days old) were purchased from the Charles River Laboratory (Wilmington, MA). Animals were housed under controlled lighting, humidity, and temperature, and were given standard rat chow and water ad libitum. All animal protocols were approved by the University of California at San Diego Institutional Animal Care and Use Committee.

[1]This work was supported in part by NICHD/NIH through cooperative agreement U54HD12303 as part of the Specialized Cooperative Centers Program in Reproduction Research, The Lalor Foundation, and University of California San Diego Academic Senate grant RY440M. R.K.M. was supported by NIH Training Grant T32 HD07203-17.

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Received: 22 March 2001.
First decision: 10 April 2001.
Accepted: 7 May 2001.

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

Preparation of Primary Granulosa Cells

Twenty-three-day-old female rats weighing from 55 to 60 g were implanted with silastic capsules containing 10 mg of DES to increase GC number [7]. Four days after the implantation, rats were killed by CO_2. Ovaries were removed and the GCs were extracted by puncturing ovaries into Medium 199 containing 25 mM Hepes (pH 7.2) and 0.1% BSA. Collected GCs were centrifuged (500 × *g* for 5 min), dispersed by repeated washing, and suspended in McCoy 5a medium containing 100 U/ml penicillin, 100 mg/ml streptomycin sulfate, and 2 mM L-glutamine (Life Technologies Inc.). After staining with trypan-blue (Life Technologies Inc.), viable GC number was counted using a hemocytometer. GCs were cultured at 37°C in the same media in an atmosphere of 5% CO_2 in air.

RNA Extraction

GCs (2×10^6 viable cells) were cultured in a six-well plate with 2 ml of McCoy 5a medium containing 10 ng/ml FSH or 100 ng/ml BMP-7 (or both) in the presence of 10^{-7} M androstenedione. After a 48-h culture, total cellular RNA was isolated by the guanidium acid-isothiocyanate-phenol-chlorform method using TRIzol (Life Technologies Inc.), quantified by measuring absorbance at 260 nm and stored at −80°C until assay. The quality of each RNA sample was assessed on a 1% denaturing agarose gel.

Riboprobe Preparation

Template cDNAs for the synthesis of riboprobes encoding P450 aromatase ($P450_{arom}$), steroidogenic acute regulatory protein (StAR), P450 side-chain cleavage enzyme ($P450_{scc}$), 3β-hydroxysteroid dehydrogenase (3β-HSD) and ribosomal protein-L19 (RPL19) were constructed through reverse-transcription-polymerase chain reaction (RT-PCR) using rat ovarian RNA. Briefly, DNA segments (90–220 base pairs) of each $P450_{arom}$, StAR, $P450_{scc}$, 3β-HSD, and RPL19 cDNA were amplified by PCR and cloned into *Eco*RV sites of pBluescript SK+ (Stratagene Inc., La Jolla, CA). DNA sequences of all these inserts were confirmed. Probe labeling was performed with an RNA transcription kit (Stratagene) using [α-^{32}P]UTP (650 Ci/mmol; ICN Biomedicals, Irvine, CA).

RNase Protection Assay

RNase protection assay was performed using a commercially available kit (Ambion Inc., Austin, TX) as described previously [8]. Briefly, an aliquot of α-^{32}P-labeled antisense probes (3.0×10^5 cpm each) was added to 3 μg of total RNA sample and precipitated by ethanol. The pellet was then dissolved in hybridization buffer (80% formamide, 100 mM sodium citrate pH 6.4, 0.3 M sodium acetate pH 6.4, 1 mM EDTA), denatured in boiling water, and incubated overnight at 45°C. After hybridization, RNase A/T1 mixture in RNase digestion buffer was added and incubated for 30 min in RNase inactivation/precipitation mixture and centrifuged. The pellet was dissolved in gel loading buffer and loaded onto a 6% polyacrylamide/7 M urea sequencing gel to resolve the protected RNA fragment. The gel was then directly submitted to phosphorimaging analysis to determine the intensity of each protected band using a Bio-Rad PhosphorImager (Bio-Rad, Hercules, CA). The relative integrated density of each band was normalized to that of RPL19. Three separate experiments with triplicate determinations for each treatment were performed for the assay.

Thymidine Uptake and GC Proliferation Analyses

GCs (2×10^5 viable cells) were cultured in 1.5-ml polypropylene tubes containing 200 μl (final volume) of culture media. After a 24-h preculture period GCs were incubated with 0.5 μCi/tube of [methyl-^{3}H]thymidine (Amersham Pharmacia Biotech, Piscataway, NJ) either alone or together with increasing doses of BMP-7. After another 24 h of culture, the cells were washed with PBS, centrifuged (2000 × *g* for 30 min), and incubated with 10% ice-cold trichloroacetic acid for 30 min at 4°C. The cell pellet was solubilized in 0.2 M NaOH and its radioactivity was measured using a scintillation counter. Viable GC number was also counted by trypan-blue selection using hemocytometry from cells cultured for 24 h with or without BMP-7 (300 ng/ml). The data are shown as mean ± SEM of at least three separate experiments, with triplicate determinations for each treatment.

In Vivo BMP-7 Studies

Because BMP-7 is a heparin binding protein, we first coupled BMP-7 with heparin acrylic beads by overnight incubation at 4°C in order to increase the half-life of the BMP-7 in vivo as reported previously [9]. Heparin beads alone were also used as a control. At age 28 days, rats were divided into three groups. Rats in group 1 (n = 8) served as a nonoperated control group, rats in group 2 (n = 6) were treated with the heparin vehicle alone, and rats in group 3 (n = 8) were treated with BMP-7 in the heparin vehicle. Prior to administration of BMP-7 and the vehicle, rats in groups 2 and 3 were anesthetized with isoflurane and a small incision was made in the left dorsal lumbar region to expose the left ovary. Either BMP-7 (1 μg in a 20-μl heparin vehicle) or heparin vehicle (20 μl) alone was injected with a 28-gauge needle into the ovarian bursa through the periovarian fat tissue after the ligature of the uterotubal junction [10]. Successful intrabursal injection was confirmed by swelling. One microgram was chosen as a dose of BMP-7 on the basis of previous studies using ovarian bursal injections of various agents including inhibin, activin, and insulin-like growth factor binding protein-3 [11, 12]. The contralateral right ovary was left intact as an internal control. The control rats (group 1) were not operated on. Within an hour following surgical procedures, rats in all groups were given an i.p. injection of eCG (20 IU in 500 μl saline). After 48 h, all groups were given an i.p. injection of 10 IU hCG in 500 μl saline [13]. Animals were killed 18 h later with CO_2. The oviduct containing ovulated oocytes was separated from the ovary and the expanded ampullar portion of the oviduct was dissected. Oocytes encased in cumulus GCs were then collected into media containing hyaluronidase (Sigma-Aldrich), which facilitates separation of the oocytes from the complex with cumulus GCs. The oocytes were counted manually using a microscope. Ovulated oocytes were expressed as the mean of the percentage of oocytes ovulated from the left (treated) ovary compared with the total number of ovulated oocytes (left and right ovaries).

Immediately after the oviduct was dissected, ovaries were removed for histological analysis, fixed in 10% neutral buffered formalin, and embedded in paraffin wax. Blood was also collected for steroid analysis either by cardiac puncture or by collecting truncal blood following decapitation. Serum was separated from blood and stored at −20°C until assay. For radioimmunoassay, steroids were extracted from serum in hexane/ethyl acetate (3:1) and redissolved in assay buffer (0.1 M phosphate buffer containing 0.1% BSA and 0.1% sodium azide). Progesterone and estradiol levels were measured by standard radioimmunoassay procedures.

Histological Classification and Quantification of Ovarian Follicles

Whole ovaries from five rats, in which the left ovary was treated with BMP-7 and the right ovary was left intact as a control, were collected and embedded in paraffin blocks for histological quantification of follicles at specific stages of folliculogenesis. Serial sections were cut on a microtome at a thickness of 10 μm, which was found to be optimal for quantifying ovarian follicles [14], and stained with hematoxylin-eosin. All follicles in each section were analyzed and counted according to the criteria described below. To ensure that each follicle was counted only once, follicles were counted only when the nucleolus of the oocyte was visible on the section. Primordial follicles were defined as having a small oocyte with either a single layer of squamous GCs or a mixture of squamous and cuboidal GCs. Primary follicles were defined as having a single layer of only cuboidal GCs surrounding the oocyte. Follicles with multiple layers of GCs, but no clear antrum, were classified as preantral follicles, and antral follicles were distinguished by the presence of an antrum.

Statistical Analysis

Differences between groups were analyzed for statistical significance using either ANOVA followed by the Fisher PLSD for posthoc analysis or unpaired *t*-tests (StatView 5.0 software; Abacus Concepts, Inc., Berkeley, CA). P values < 0.05 were accepted as statistically significant.

RESULTS

The steady state mRNA levels for key regulators of steroidogenesis were measured by RNase protection assay. For this assay we cultured rat GCs in serum-free medium for 48 h in the presence or absence of BMP-7, FSH, or both followed by the extraction of total cellular RNA. As shown in Figure 1, BMP-7 without FSH had no effect on the mRNA levels of $P450_{arom}$, StAR, $P450_{scc}$, and 3β-HSD. This result is supported by our previous result that BMP-7 does not alter the production of progesterone and estradiol

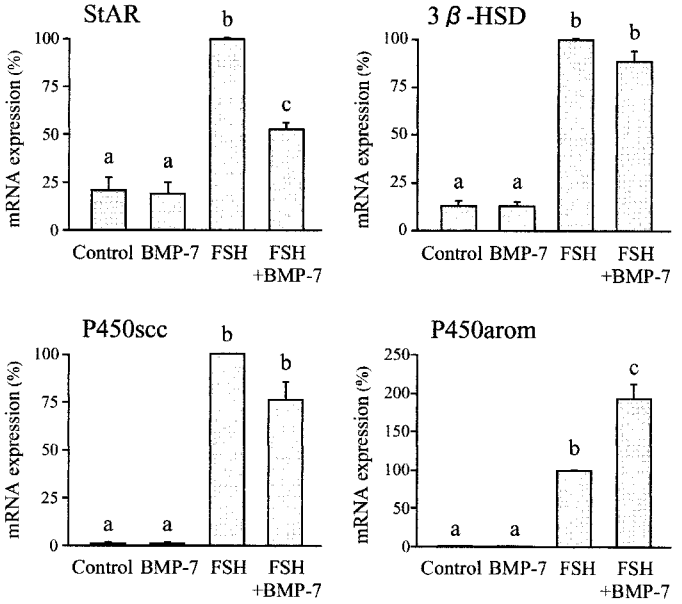

FIG. 1. Effects of FSH, BMP-7, or both, on the mRNA levels of StAR, $P450_{scc}$, 3β-HSD, and $P450_{arom}$. GCs (2×10^6 cells) were cultured either alone or together with FSH (10 ng/ml), BMP-7 (100 ng/ml), or both, for 48 h, after which total RNA was extracted and then subjected to an RNase protection assay as described in *Materials and Methods*. The intensity of each protected RNA signal was normalized to that of RPL19 mRNA. The data show the results from three separate experiments. Bars with different letters indicate that group means are significantly different at $P < 0.05$.

by GCs [6]. Treatment of GCs with FSH (10 ng/ml) alone increased the mRNA accumulation of all these factors, which is consistent with our previous report [15]. BMP-7 (100 ng/ml) amplified FSH-induced $P450_{arom}$ mRNA levels by twofold (Fig. 1), and this amplification is likely to be the cause of BMP-7 enhancement of FSH-induced estradiol production in vitro that was demonstrated previously [6]. In contrast, the level of StAR mRNA induced by FSH was suppressed by half by cotreatment with BMP-7, whereas $P450_{scc}$ and 3β-HSD mRNA levels were not significantly altered by BMP-7 (Fig. 1).

To investigate the physiological role of BMP-7 in the ovary, we injected BMP-7 into the left ovarian bursa in vivo (group 3). All follicles in each 10-μm histological section from both the BMP-7 treated and untreated control ovaries were counted according to the criteria described in *Materials and Methods*. A total of 10 150 follicles were identified and quantified in the ovaries of the five BMP-7 treated rats. There was no significant difference in number of total follicles between the untreated right ovary (1051 ± 209) and the BMP-7 treated left ovary (978 ± 188). However, the distribution of follicles at specific stages of folliculogenesis in the control and BMP-7 treated ovaries appeared to be different when the follicle numbers at each stage are presented as a percentage of total follicles (Fig. 2A). The actual number of primordial follicles in the untreated ovaries was 661.4 ± 191.2, whereas that in BMP-7 treated ovaries was 416.0 ± 165.6. In contrast, the BMP-7 treated ovaries had increased numbers of primary follicles (from 117.6 ± 22.7 follicles in the untreated ovary to 197.0 ± 31.4 follicles in the treated ovary), preantral follicles (from 242.6 ± 66.3 follicles in the untreated ovary to 311.6 ± 64.9 follicles in the treated ovary), and antral follicles (from 30.8 ± 5.0 follicles in the untreated ovary to 53.6 ± 7.9 follicles in the treated ovary). Consequently, the number of primordial follicles in BMP-7 treated ovaries was

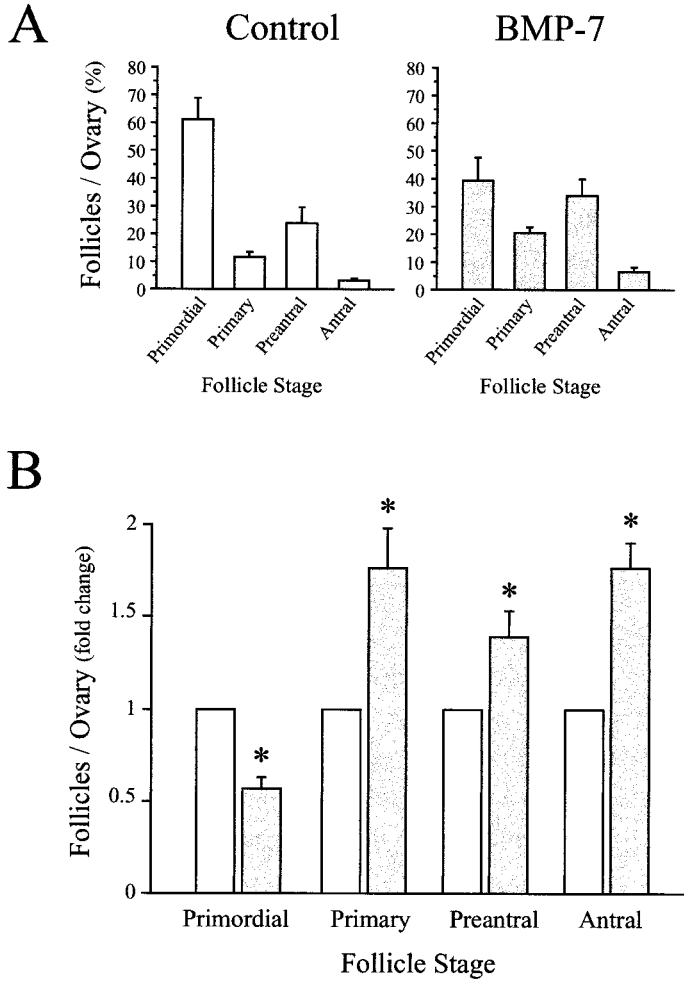

FIG. 2. Effect of in vivo administration of BMP-7 on numbers of follicles at specific stages of folliculogenesis. **A**) Distribution of specific follicle types in untreated (control) and BMP-7 treated ovaries. The number of follicles at indicated stages according to the classification criteria described in *Materials and Methods* are expressed as a percentage of total follicles. **B**) Comparison in fold change of indicated follicle numbers in BMP-7 treated ovaries (shaded bars) and the untreated contralateral ovaries (open bars). Asterisks denote significant differences at $P < 0.05$.

only 56.5 ± 0.07% of those present in the contralateral untreated ovary, whereas the number of primary, preantral, and antral follicles in the treated ovaries were 1.8 ± 0.22-, 1.4 ± 0.14-, and 1.8 ± 0.14-fold higher, respectively, than the corresponding follicles in the control ovaries (Fig. 2B).

To evaluate the effect of BMP-7 on ovulation rate, we counted the number of oocytes ovulated from the left (treated) and right (untreated) ovaries into each oviduct following ovulation induction by the administration of hCG. In the untreated control rats (group 1), the left and right ovaries ovulated statistically equal numbers of oocytes (the left ovary ovulated 49.5 ± 1.9% of the total ovulated oocytes) as expected, with the left ovary ovulating 30.5 ± 5.9 oocytes and the right ovary ovulating 32.0 ± 3.4 oocytes (Fig. 3). In rats in which the left ovary was treated with the heparin vehicle alone (group 2), there were also no significant differences between the oocytes ovulated from each ovary, with the left ovaries ovulating 44.8 ± 3.5% of the total ovulated oocytes (the left ovary ovulated 21.2 ± 6.5 oocytes, and the right ovary ovulated 25.6 ± 5.5 oocytes). In ovaries that were treated with BMP-7 (group 3), however, there was a significant decrease in the number of ovu-

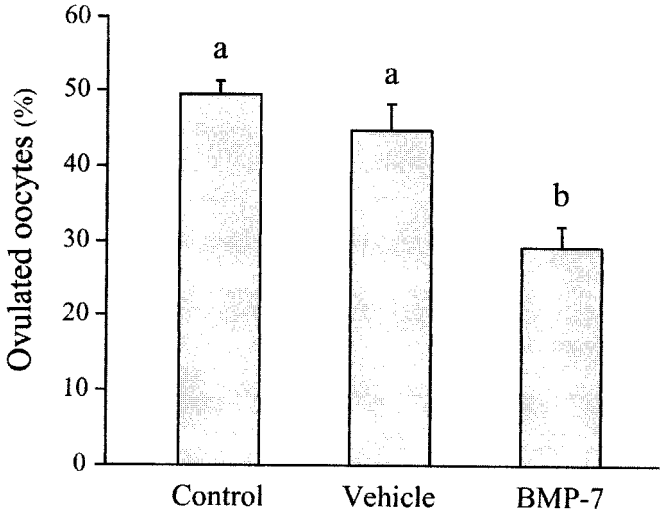

FIG. 3. Effect of in vivo administration of BMP-7 on ovulation. Oocytes ovulated by the left ovary treated without (control) or with heparin vehicle or BMP-7 expressed as a percentage of the total oocytes ovulated by both ovaries. Bars with different letters indicate that group means are significantly different at $P < 0.05$.

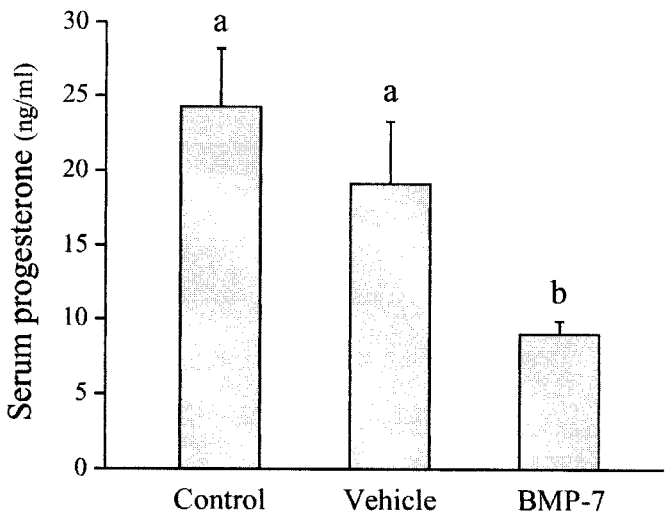

FIG. 4. Effect of in vivo administration of BMP-7 on serum progesterone levels. Serum progesterone levels were measured from rats in which the left ovary was treated in vivo with BMP-7, the heparin vehicle, or no treatment. Bars with different letters indicate that group means are significantly different at $P < 0.05$.

lated oocytes compared with untreated ovaries, with BMP-7 treated ovaries contributing only 29.3 ± 2.7% of the total ovulated oocytes (the left ovary ovulated only 11.7 ± 4.6 oocytes, whereas the right ovary ovulated 28 ± 5.1 oocytes). The fact that the number of oocytes ovulated from the right (untreated) ovary in group 3 is not significantly different from those in groups 1 and 2 indicates that the BMP-7 injected into the left ovary in group 3 did not affect the function of the right ovary.

To determine the effects of BMP-7 on progesterone and estradiol production in vivo, we measured levels of these steroids in serum at the time the animals were killed, following ovulation. In the nontreated control rats (group 1), the serum progesterone level was 24.3 ± 3.6 ng/ml (Fig. 4). There was no significant change in progesterone in the animals that received an intrabursal injection of the heparin vehicle alone (19.2 ± 4.1 ng/ml) (group 2). However, the administration of BMP-7 into the ovarian bursa of the left ovary (group 3) caused a significant drop in serum progesterone to 9.0 ± 0.9 ng/ml, which is only 37.4% of the values observed in the control animals (groups 1 and 2). Levels of serum estradiol were low in all groups (less than 50 pg/ml), and there were no significant changes observed (data not shown).

In order to identify a possible mechanism to explain the increase in the numbers of primary, preantral, and antral follicles, we evaluated the ability of BMP-7 to stimulate GC proliferation and DNA synthesis in vitro. For these studies, primary rat GCs from small antral follicles were cultured for 24 h with ^{3}H-labeled thymidine in the presence or absence of increasing doses of BMP-7, and the incorporated ^{3}H into the cells was measured (Fig. 5A). Addition of BMP-7 substantially increased (by threefold) the incorporation of thymidine into GCs with the maximum effective dose of 300 ng/ml. To examine whether the observed thymidine incorporation is correlated with cell proliferation, numbers of GCs were counted after 24 h of incubation with 300 ng/ml of BMP-7. Treatment with the saturating dose of BMP-7 increased the number of GCs to ~1.5-fold above the control level (Fig. 5B).

DISCUSSION

In a previous study, we demonstrated that BMP-7 mRNA is expressed by ovarian theca cells and provided evidence that it acts in a paracrine manner regulating GC function [6]. In vitro, BMP-7 reduced FSH-induced progesterone production, and simultaneously enhanced FSH-induced estradiol production by rat primary GCs. We also demonstrated that GCs express mRNA encoding the BMP receptors of types IA, IB, and II [6], which have been shown to be functional receptors for BMP-7 in a number of cell types [16]. In the present study we sought to identify the cellular mechanisms of the biological activity of BMP-7 in GCs and to determine the effects of in vivo BMP-7 administration on ovarian function.

In order to identify the mechanisms of BMP-7 regulation on GC steroidogenesis, we examined the steady-state

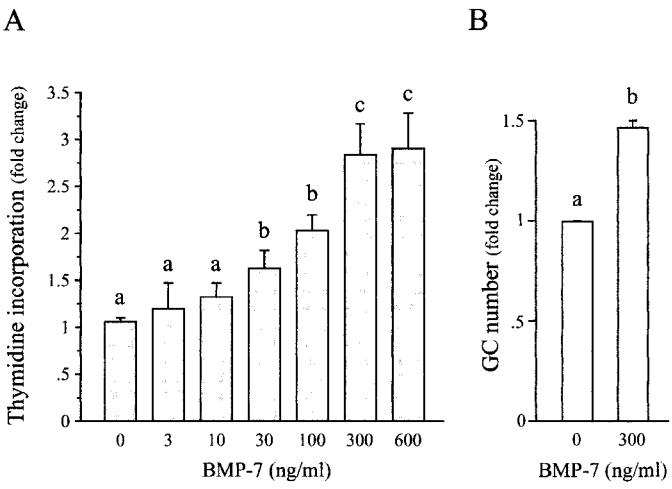

FIG. 5. Effect of BMP-7 on GC mitosis. **A**) GCs (2×10^5 cells) were treated for 24 h with [methyl-^{3}H]thymidine and indicated concentrations of BMP-7. The labeled thymidine incorporated into the cells was counted. **B**) GC number was also counted after a 24-h culture with or without BMP-7. Data are mean ± SEM. Bars with different letters indicate that group means are significantly different at $P < 0.05$.

mRNA levels of steroidogenic factors including StAR, $P450_{scc}$, 3β-HSD, and $P450_{arom}$. In these studies we found that coculture of BMP-7 with FSH caused a pronounced decrease in StAR mRNA and, conversely, a marked increase in $P450_{arom}$ mRNA levels. There were no significant changes in $P450_{scc}$ and 3β-HSD mRNA levels caused by BMP-7. These findings suggest that the divergent effects of BMP-7 on estradiol and progesterone production are caused mainly by a combination of an enhanced conversion of androstendione to estradiol (the $P450_{arom}$ effect) and a reduced efficiency of cholesterol transport from the outer to the inner mitochondrial membrane (the StAR effect) where the side-chain cleavage enzyme is located.

In vivo injection of BMP-7 into the ovarian bursa caused significant differences at each stage of follicular development when compared with control ovaries. Ovaries injected with BMP-7 had increased numbers of primary, preantral, and antral follicles but had decreased numbers of primordial follicles and ovulated oocytes. The increase in primary, preantral, and antral follicles and the corresponding decrease in primordial follicles suggest that BMP-7 may act to facilitate the transition of primordial follicles into the pool of growing follicles. The regulation and control mechanisms governing the early phases of follicular development is poorly understood; however, recent studies suggest that ovarian factors play important roles in this process. The first stage of follicular development is the recruitment of primordial follicles, a process that occurs independent of gonadotropins because follicular recruitment is unaffected by administration of gonadotropins [17] and takes place in hypophysectomized animals [4]. Our data suggest that BMP-7 may be one of the factors that promote this process. To investigate the mechanism in which BMP-7 may be acting in this context, we evaluated the potential of BMP-7 to stimulate mitosis of GCs in vitro. From these experiments we found that BMP-7, independent of FSH, caused a marked increase in GC thymidine uptake and GC proliferation. Accordingly, the stimulation of early folliculogenesis by BMP-7 could be mediated through its ability to promote GC division. It is possible that BMP-7 secreted from theca cells of larger follicles can act to stimulate the growth of adjacent primordial follicles.

In striking contrast to the stimulatory activity that BMP-7 had on folliculogenesis, BMP-7 exhibited a marked inhibitory effect on ovulation. The ovary that was treated with BMP-7 ovulated significantly less oocytes than the contralateral control ovary. These also had significantly lower circulating progesterone levels than control animals that received either an injection of the heparin vehicle or no injection. This finding is supported by our previous studies in which we demonstrated that BMP-7 inhibits FSH-induced progesterone production (by ~60%) and delays the timing of the FSH induction (by ~12 h) in rat GCs in vitro [6]. This decrease in progesterone production could be due to the decrease in the expression of StAR mRNA by GCs, as presented in the current study. Progesterone has been implicated as a necessary factor in the ovulation process [13, 18–22]. In the rat, the serum progesterone concentration increases significantly within 60 min of hCG injection [23] through several biosynthetic steps and enzymes [24, 25] from low-density lipoproteins [26]. It should also be noted that decreased circulating progesterone levels can be correlated with decreased ovulatory activity [27–29]. Furthermore, it has been demonstrated that blocking progesterone activity with a progesterone receptor antagonist causes an inhibition of ovulation in rats [28, 30]. It is therefore conceivable that BMP-7 inhibition of progesterone production by the GCs of antral follicles may be a mechanism by which BMP-7 inhibits ovulation. In the present study, circulating estradiol levels were low and there was no observed significant difference between BMP-7 treated and control animals. This observation is supported by previous reports that administration of surge doses of hCG/LH or eCG to induce ovulation in rats causes a dramatic decrease in circulating estrogen levels to negligible amounts following ovulation [31, 32].

The present studies taken together provide evidence that BMP-7 exhibits pronounced biological activity in the ovary, influencing multiple stages of folliculogenesis from initial recruitment through ovulation. The biological activities of BMP-7 suggest that it is involved in promoting follicular growth and development while simultaneously inhibiting ovulation and luteinization. The ability of BMP-7 to stimulate GC proliferation and aromatase activity (by increasing $P450_{arom}$ mRNA) is consistent with a follicle promoting factor, whereas the ability of BMP-7 to inhibit progesterone production (and related synthetic enzyme mRNAs) and to inhibit ovulation is consistent with an antiluteinization role. The present in vivo data demonstrating increased numbers of primary, preantral, and antral follicles and decreased numbers of ovulated oocytes in BMP-7 treated ovaries support this theory.

Recent research in ovarian physiology has demonstrated that there are many complex regulatory systems within the ovary that are necessary for normal ovarian function. Members of the transforming growth factor β (TGFβ) superfamily have been identified as important components of this intraovarian system. Namely, GDF-9 has been shown to be necessary for folliculogenesis, as GDF-9 knockout mice are infertile with a block at the primary follicle stage [33]; and sheep homozygous for a naturally occurring mutation in BMP-15, named Inverdale ewes (Fec^X), are also infertile with a block at the primary follicle stage [34]. In comparison, mice deficient in BMP-7 by targeted deletion die shortly after birth due to severe bilateral renal dysplasia [35]. Therefore, loss-of-function studies of BMP-7 in folliculogenesis are not possible using these animals. However, our data suggest that BMP-7 may be necessary for normal folliculogenesis and ovulation. Further studies of the cellular mechanisms, specific contributions, and interactions of individual members of the TGFβ superfamily will be necessary for understanding the local control of ovarian function.

ACKNOWLEDGMENTS

We appreciate Ms. Quing Li for excellent technical assistance, Dr. Gregory Erickson for constructive discussion, Dr. Kuber Sampath for a gift of recombinant human BMP-7, and Andi Hartgrove for typing.

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