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The spatiotemporal expression pattern of the bone morphogenetic protein family in rat ovary cell types during the estrous cycle

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Abstract

In the mammalian ovary, great interest in the expression and function of the bone morphogenetic protein (BMP) family has been recently generated from evidence of their critical role in determining folliculogenesis and female fertility. Despite extensive work, there is a need to understand the cellular sites of expression of these important regulatory molecules, and how their gene expression changes within the basic ovary cell types through the cycle. Here we have performed a detailed *in situ* hybridization analysis of the spatial and temporal expression patterns of the BMP ligands (BMP-2, -3, -3b, -4, -6, -7, -15), receptors (BMPR-IA, -IB, -II), and BMP antagonist, follistatin, in rat ovaries over the normal estrous cycle. We have found that: i) all of the mRNAs are expressed in a cell-specific manner in the major classes of ovary cell types (oocyte, granulosa, theca interstitial, theca externa, corpora lutea, secondary interstitial, vascular and ovary surface epithelium); and ii) most undergo dynamic changes during follicular and corpora luteal morphogenesis and histogenesis. The general principle to emerge from these studies is that the developmental programs of folliculogenesis (recruitment, selection, atresia), ovulation, and luteogenesis (luteinization, luteolysis) are accompanied by rather dramatic spatial and temporal changes in the expression patterns of these BMP genes. These results lead us to hypothesize previously unanticipated roles for the BMP family in determining fundamental developmental events that ensure the proper timing and developmental events required for the generation of the estrous cycle.

Background

The adult mammalian ovary is a complex tissue composed of four fundamental cell types: the oocytes, granulosa, interstitial, and corpora luteal cells. Within this basic quartet are many other cell types such as the ovary surface epithelium and blood vessels in addition to the classic subtypes of granulosa (membrana, periantral, cumulus, and corona radiata), theca (interna and externa), and luteal cells (granulosa and theca lutein). The coordinated control of proliferation, differentiation, and apoptosis in these cell types is the underlying basis for primate and

non-primate menstrual and estrous cycles, respectively. The process by which each cell type obtains its state of differentiation is the subject of intense study. The role of hormones in this process is clear; however, new results indicate that autocrine/paracrine or growth factor mechanisms also have important roles.

The bone morphogenetic protein (BMP) family including ligands, receptors, and binding proteins, has emerged as a central player in ovary physiology and female fertility [1]. The BMPs represent a large subclass of the transforming

growth factor- β (TGF- β) superfamily of ligands whose biological responses are mediated by Ser/Thr kinase receptors via the Smad-signaling molecules [2]. The mRNAs encoding several BMP family members have been identified in the murine ovary, including the ligands, BMP-2 [3], BMP-3 [3], BMP-3b [3], BMP-4 [4], BMP-6 [5], BMP-7 [4], BMP-15 [6–8], the BMP receptors, BMPR-IA, BMPR-IB and BMPR-II [4] and the BMP binding protein, follistatin (FS) [9]. In the murine, BMP-4 and -7 mRNA have been identified in the theca interstitial cells [4], BMP-6 and -15 in oocytes [5–8], BMPR-IA, BMPR-IB and BMPR-II in oocytes and granulosa cells (GCs) [4], and FS in granulosa and lutein cells [9,10].

The direct involvement of the BMP system in regulating ovary function has been established. *In vitro* experiments with rat GCs have demonstrated that BMP-4 and -7 inhibit and stimulate FSH-induced progesterone and estradiol production, respectively [4]. In related studies BMP-6 and BMP-15 were found to block FSH action through inhibiting FSH-dependent adenylate cyclase activity and FSH receptor expression, respectively [11,12]. The importance of these findings is underscored by the fact that FSH action is obligatory for normal folliculogenesis and female fertility. In addition, BMP-7 and -15, but not BMP-6 have stimulatory effects on GC DNA synthesis, indicating they may play a mitogenic role in follicle growth [8,12,13]. The importance of this finding is underscored by the fact that the control of GC proliferation is crucial for follicle dominance and atresia. Equally important, other studies have shown that loss-of-function and naturally occurring mutations in BMP ligands and receptors are associated with dysregulation of folliculogenesis and ovulation [14–19]. Of particular interest is the finding that ewes having a mutation in the *bmp15* gene in both alleles are infertile whereas the heterozygotes are superfertile [16]. Thus, the *bmp15* gene can control ovulation quota through a dosage-sensitive mechanism. Evidence that a mutation at the Ser/Thr kinase domain of BMPR-IB leads to high fecundity in ewes [17–19] provides further support that BMP signaling pathways play a key role in specifying ovary function.

Although it is becoming clear that intrinsic BMPs are important players in the ovary, the cellular localization of BMPs in the ovary is poorly understood, and a proper understanding of how the cell-specific expression of the BMP system changes during the cycle is still lacking. As part of our ongoing investigations of the roles of BMPs in ovary physiology, we have analyzed the spatiotemporal expression patterns of the BMP system in the rat ovary cell types during the normal estrous cycle.

Materials and Methods

Animals

Adult female Sprague-Dawley rats (2–3 months of age) were housed (four animals per cage) in isolation under controlled lighting conditions (14 h of light, 10 hr of darkness; lights on from 0500 to 1900 h) at 23°C and provided formulated rat chow and water *ad libitum*. Vaginal smears were taken daily, and rats exhibiting two or more consecutive 4-day estrous cycles were used. The animals (two at each time of the cycle) were sacrificed on proestrus (P1000 and 2000 h), estrus (E0200 and E1000 h), diestrus I (DI, 1100 h) and diestrus II (DII, 1100 h). The rats were handled in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and the protocols were approved by the University of California, San Diego Animal Subjects Committee.

Plasmid construction for synthesizing riboprobes

Probes for *in situ* hybridization studies were made by RT-PCR. Total RNA from adult rat ovaries was prepared and single-stranded cDNA was synthesized by reverse transcriptase and then subjected to PCR as described previously [20]. To design primers for PCR, DNA sequence information was first collected from GenBank and primers for PCR were designed based on standard guidelines. All sequence information was derived from rat cDNA clones except for BMP-2, -7, BMPR-IB and BMPR-II whose cDNA sequences in rat have not been deposited into the GenBank. However, because cDNA sequences in human and mouse are available from the GenBank, we designed the PCR primers by choosing the homologous DNA sequence regions between human and mouse homologues. Specifically, these primers are derived from the cDNA clones at the following nucleotide numbers: #511 – #528 and #1066 – #1083 (accession number of the cDNA clone is M22489) for BMP-2 [21]; #923 – #942 and #1341 – #1360 (accession number D63860) for BMP-3 (Kangawa, unpublished); #878 – #897 and #1292 – #1311 (accession number D49494) for BMP-3b [3]; #737 – #757 and #1181 – #1200 (accession number Z22607) for BMP-4 [22]; #6 – #25 and #475 – #494 (accession number U66298) for BMP-6 (Knittel et al., unpublished); #497 – 514 and #865 – #882 (accession number X56906) for BMP-7 [23]; #441 – #460 and #876 – #895 (accession number D38082) for BMPR-IA [24]; #528 – #547 and #965 – #984 (accession number U89326) for BMPR-IB (Astrom et al., unpublished); #525 – #544 and #895 – #904 (accession number AF003942) for BMPR-II [25]. These primers were selected from different exons of the corresponding genes to discriminate PCR products that might arise from possible chromosome DNA contaminants. All PCR products were cloned into pBluescript SK+ and their DNA sequences confirmed. The plasmid containing FS was constructed by sub-cloning of the XbaI-PstI

fragment derived for rat FS cDNA clone [9], pROF304 into pBluescript SK+.

In situ hybridization

Ovaries were fixed in 4% paraformaldehyde at 4°C, washed in PBS, dehydrated and embedded in paraffin. Eight consecutive sections (8 µm) were cut from each ovary and mounted onto poly-L-lysine-coated glass slides. The sections were digested with proteinase K, acetylated, washed and dehydrated as previously described [10,26]. Anti-sense and sense cRNA probes were prepared by means of *in vitro* transcription using T3 or T7 RNA polymerase. Hybridization was carried out with the ³⁵S-labeled RNA probe (4–6 × 10⁶ cpm/ml) in a solution containing 50% (vol/vol) deionized formamide, 0.3 M NaCl, 10 mM Tris (pH 8.2), 1 mM EDTA, 0.05% yeast tRNA, 10 mM dithiothreitol, 1 × Denhardt's solution and 10% dextran sulfate. Hybridization solution (20 µl) was placed over each section and covered with a 60 × 22 mm acid washed, siliconized coverslip. Coverslips were sealed with liquid DPX. Sections were hybridized for 16 h at 58–60°C in a humidified chamber. After hybridization, the sections were treated with ribonuclease A and washed in 15 mM NaCl/1.5 mM sodium citrate at 60–62°C for 30 min. Dehydrated slides were exposed to X-ray film for several days. After adequate X-ray film images were obtained, the ovary sections were treated with xylene, rinsed in 100% ethanol, air dried, and then coated with Kodak NTB-2 liquid autoradiograph emulsion. Slides were exposed for four weeks at 4°C in a linedesiccated dark box. After exposure, the slides were developed (Kodak D19, 3.5 min, 14°C), rinsed briefly in distilled water and fixed. After washing in distilled water for 1 h, slides were lightly counterstained with hematoxylin and eosin.

After an autoradiography and counterstaining, the sections were analyzed microscopically. The *in situ* experiments were performed two times for each BMP ligand and receptor. The intensity of the hybridization signals were determined as previously reported [26–28]. A subjective comparison of eight sections from each ovary from six animals hybridized with the same concentrations of sense and antisense cRNAs and exposed for the same time (4 weeks) was made. The hybridization signal was estimated on a scale of 1+ to 4+ as originally described by Meunier [29]: +, silver grains sparse, but positive hybridization; ++, silver grains are numerous but do not cover the cell type in question; +++, silver grains are very numerous and begin to merge in some places; +++++, silver grains are very dense and form a near uniform mass above the cell type in question; +/-, there was heterogeneity in the hybridization signal, e.g., some of the histological units contained a signal, others did not; -, no detectable hybridization signal. The slides were analyzed simultaneously by two investigators and the results confirmed independently by

another researcher. In each experiment, tissues hybridized with the sense probe were used as the negative control. In all cases, the sense probes showed no specific hybridization to the adult ovaries above background (see for example Fig. 1C).

Results

Expression of BMP-2 mRNA

In adult rat ovaries, BMP-2 mRNA is highly expressed in the GC of atretic follicles and at relatively high levels in the GC of Graafian follicles (GF) and some corpora lutea (CL) (Table 1, Fig. 1A,1B). BMP-2 mRNA is also expressed at lower levels in the secondary interstitial cells (SIC) and theca interstitial cells (TIC) but undetectable in other ovary cell types (Table 1). In the SIC, BMP-2 mRNA was constitutively expressed at low to moderate levels over the cycle. A weak variable expression was sometimes seen in the TIC (Table 1).

In the GC, BMP-2 mRNA is expressed in a precise manner, indicating that gene activity is regulated during follicle development. A weak hybridization signal was first detected in some GC of early primary follicles, consistent with BMP-2 gene activation during recruitment. As folliculogenesis progressed, BMP-2 mRNA expression increased rapidly reaching maximal levels in the GC of secondary follicles. The expression of GC BMP-2 mRNA was heterogeneous. In preantral follicles, BMP-2 mRNA expression was confined mainly to the membrana GC with little or no expression in the corona radiata GC next to the oocyte (Fig. 1D,1E). This indicates that the GC become functionally diverse very early in follicle development, i.e. about the time the follicle has two layers of GC. As the follicle undergoes its development to the preovulatory stage (i.e. from selection at E 0200 h through ovulation at E 0200 h the next cycle), the membrana, but not the cumulus and periantral GC, express high levels of BMP-2 mRNA (Fig. 1F,1G). These findings indicate that the oocyte may regulate GC BMP-2 gene expression by a morphogen gradient mechanism. Finally the highest levels of BMP-2 mRNA in the ovary were seen in the GC of atretic follicles (Fig. 1H,1I).

BMP-2 mRNA expression in the dominant follicle was rapidly down-regulated following ovulation. As such, there was no detectable BMP-2 mRNA in the new CL-I during luteinization i.e. E 2000 h to DI 1100 h (Fig. 1J,1K). The message was re-expressed at DII 1100 h (luteolysis) with strong hybridization for BMP-2 appearing in clusters of endothelial cells scattered throughout the CL-I (Fig. 1A,1B,1L,1M). This correlation suggests a possible function for endothelial-derived BMP-2 in luteolysis (Fig. 1N).

Figure 1

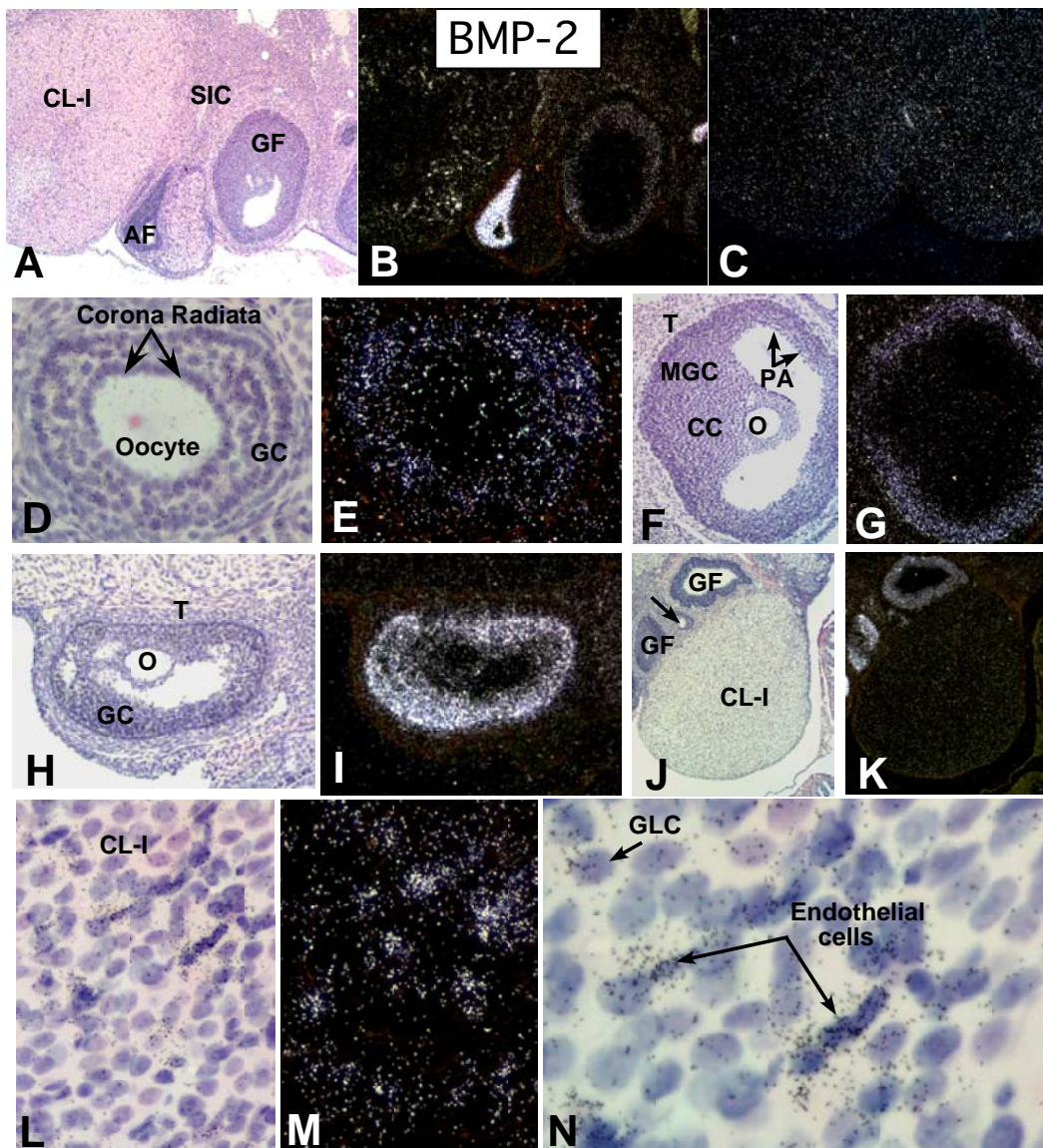


Figure 1

In situ hybridization of BMP-2 mRNA in the ovaries of adult cycling rats. Brightfield (A, D, F, H, J, L, N) and Darkfield (B, C, E, G, I, K, M). **A, B:** Sections from a DII 1100 h ovary (4X); new corpus luteum (CL-I); atretic follicle (AF); secondary interstitial cells (SIC); healthy Graafian follicle (GF); **C:** Negative control hybridized with sense BMP-2 cRNA probe; **D, E:** Secondary follicle with 3–5 layers of GCs at DII 1100 h (20X). Note positive membrana GC at periphery. **F, G:** Dominant follicle at DII 1100 h (10X); Positive membrana GC (MGC); Negative oocyte (O), cumulus GC (CC), periantral GC (PA) and theca (T); **H, I:** Atretic follicle at P 1000 h showing strongly positive GC (10X); **J, K:** Negative newly formed CL-I at E 0200 h (4X); Positive GF and preantral follicle (arrow); **L, M:** New CL-I after initiation of luteolysis at DII 1100 h showing clusters of positive cells (20X); **N:** Higher magnification (40X) of CL-I in L showing positive endothelial and granulosa lutein cells (GLC).

Table 1: Cellular localization and relative intensity of mRNAs for BMPs in ovaries of adult cycling rats.

TISSUE	BMP-2	BMP-3	BMP-3b	BMP-4	BMP-6	BMP-7	BMP-15	BMPR-IA	BMPR-IB	BMPR-II	FS
Oocyte											
Primordial	-	-	-	-	-	-	-	+	++/+	+/-	-
Primary	-	-	-	-	-	-	+	++	+++/>++	+	-
Secondary	-	-	-	-	+++/>+	-	+++/>++	+++	+++	+/-	-
Tertiary	-	-	-	-	+++	-	+++	+++	+++	+/-	-
Dominant	-	-	-	-	+++	-	+++/>+	++	+++	+/-	-
Atretic	-	-	-	-	+++/>++	-	+++/>+	+++/>++	++	+/-	-
Granulosa Cells											
Primordial	-	-	-	-	-	-	-	+/-	+/-	-	-
Primary	+/-	-	-	-	-	-	-	+	+/-	+	-
Secondary	+++/>-	-	-	-	+++/>-	-	-	+++/>+	+++	+++/>++	++
Tertiary	+++/>-	-	-	-	+++/>-	-	-	++	+++	+++	+++
Dominant	+++/>-	-	-	-	+/-	-	-	++	+++	+++	+++
Atretic	+++	-	-	-	+++	-	-	++	+++	+++	+/-
Theca-interstitial cells											
Primordial	-	-	-	-	-	-	-	-	-	-	-
Primary	-	-	-	+	-	-	-	-	-	-	-
Secondary	+/-	-	-	+++/>-	-	+++/>-	-	+	+	-	-
Tertiary	+/-	+/-	+	+++/>-	-	+++/>-	-	+++/>+	+	-	-
Dominant	+/-	+/-	+++/>-	+++/>-	-	+++/>-	-	+++/>+	++	-	+/-
Atretic	+/-	-	+/-	+/-	-	+/-	-	+	+	-	-
Theca externa	-	-	+++/>-	+++/>-	-	-	-	+	+++/>-	-	-
Corpora lutea – Healthy											
Granulosa Lutein	+/-	-	-	+/-	+++/>+	-	-	+++/>+	+	++	+++
Theca Lutein	+/-	+/-	+/-	+/-	-	+++/>-	-	+++/>+	+	+/-	+++
Vascular Endothelial	-	-	-	-	-	-	-	+/-	+/-	-	-
Theca Externa	-	-	+++/>+	+++	-	-	-	+/-	+++/>+	-	-
Corpora lutea – Luteolytic	+++/>-	+/-	-	++	+/-	-	-	+	+++/>-	+/-	-/>+++
Vascular System											
Endothelium	-	-	-	+++	+++/>-	-	-	+/-	-	+/-	-
Smooth Muscle	-	-	-	-	-	-	-	+/-	-	-	-
Tunica Adventitia	-	-	-	-	-	-	-	+/-	++	-	-
Secondary-interstitial cells	+++/>+	-	-	-	-	+/-	-	+	+++/>+	-	-/>+++
Surface epithelium	-	-	+++/>-	+++/>-	+++	-	-	+/-	-	-	-
Sex Cords	-	-	+++	+++	-	+++	-	+	+++	+/-	-

The intensities of the hybridization signals indicated above represent a subjective consensus of all sections examined from all the ovaries collected from all of the animals over the estrous cycle (see Materials and Methods). The hybridization signals were estimated on a scale of - to 4+ as described in Materials and Methods. The slashes represent the variability in signal intensity that was observed for any given histological unit. The nature of this variability, whether it be temporal (cycle-dependent or developmental), or spatial (heterogeneity within or between the histological unit), is fully described in the Results section.

Expression of BMP-3 and -3b mRNA

The hybridization signal for BMP-3 was relatively weak in the ovaries examined (data not shown). BMP-3 mRNA expression was present in the TIC of healthy Graafian follicles and in the theca lutein cells (TLC) of the CL-1 at DI

1100 h (Table 1). This is consistent with a role for theca-derived BMP-3 in regulating the dominant follicle and CL.

A strong expression of BMP-3b mRNA was seen in some ovaries (Table 1). During folliculogenesis, the theca cells (T) of the theca interna (TI) and externa (TE) of dominant

follicles strongly expressed BMP-3b mRNA (Fig. 2A,2B,2C). In the TI, the expression appeared mainly in the TIC located nearest the TE (Fig. 2C). After ovulation the signal was reduced. In the CL-I, BMP-3b mRNA was expressed at low to moderate levels in the TLC and TE cells during luteinization (Fig. 2D,2E). Little or no BMP-3b mRNA was evident in degenerating CL (Table 1, Fig. 2A,2B). Interestingly, there was high expression in the ovary surface epithelium (OSE) (Fig. 2F), and in the epithelial cells of the sex cords (SC) located in the hilum of the ovary (Fig. 2G). In atretic follicles BMP-3b mRNA expression was undetectable (Fig. 2H,2I).

Expression of BMP-4 mRNA

BMP-4 mRNA was expressed in a variety of ovary cell types with the highest levels appearing in the theca, CL, OSE, SC and endothelium of some blood vessels (Table 1, Fig. 3A,3B,3C). BMP-4 mRNA was constitutively expressed at high levels in both the SC (Fig. 3C) and the endothelium lining the large sinusoids in the mesovarium (Fig. 3D). A weak hybridization signal was occasionally seen in some oocytes of primary follicles, but it was rare.

In the theca, the levels of BMP-4 mRNA change during folliculogenesis. A weak BMP-4 signal was first detectable in primary follicles, being localized to a few stroma fibroblasts near the basal lamina. This finding is consistent with the intriguing idea that the theca may begin to develop very early in preantral folliculogenesis, i.e. during the initial steps of primary follicle development. In secondary follicles, BMP-4 mRNA was present at moderate levels in an outer layer of flattened cells (the TE) and in a subset of TIC adjacent to these cells (Fig. 3E,3F,3G). BMP-4 mRNA was expressed at high levels in the theca of developing dominant follicles, again being localized to the TE and adjacent TIC (Fig. 3H,3I,3J). Unlike healthy follicles, BMP-4 mRNA was very low or undetectable in atretic follicles. High levels of BMP-4 mRNA continue to be expressed in the TE of the CL throughout luteogenesis, i.e. luteinization (E 0200 h to DI 1100 h), and luteolysis (DII 1100 h to E 0200 h the next cycle) (Fig. 3K,3L). A potentially important finding is that the OSE covering the ovulated follicle and newly formed CL-I expressed high levels of BMP-4 mRNA while little or no BMP-4 mRNA was detected in other areas of the surface epithelium (Fig. 3M,3N,3O). This is consistent with a role for BMP-4 in OSE regeneration following ovulation.

Expression of BMP-6 mRNA

BMP-6 mRNA was present in the oocytes, GC, CL, blood vessels and the OSE (Table 1, Fig. 4A,4B). In the vascular system, BMP-6 mRNA was continuously expressed at relatively high levels in the endothelial cells of some large arteries and veins in the medulla (Fig. 4C). Thus BMP-6, like BMP-4, could have a role in ovarian blood circulation.

BMP-6 mRNA showed relatively high expression in the OSE throughout the cycle, suggesting a general, non-regulated role for BMP-6 in the OSE and/or adjacent tissues (Fig. 4A,4B).

The expression of BMP-6 mRNA in oocytes was developmentally regulated. There was no detectable BMP-6 mRNA in primordial and primary follicles (Table 1); however a weak hybridization signal was detected in oocytes of early secondary follicles when the second layer of GCs was forming (Fig. 4D). Thereafter, expression increased sharply and was maximal in oocytes after the fourth layer of GC had developed (Fig. 4E,4F). High levels of BMP-6 mRNA were present in oocytes of all Graafian follicles, both healthy and atretic (Fig. 4A,4B,4E,4F,4G,4H,4I,4J). The developmental changes in oocyte BMP-6 mRNA expression showed no obvious cyclic variation.

Like oocytes, BMP-6 mRNA was first detected in the GC at the early secondary stage. Expression increased as the follicle grew and was maximal after the fourth layer of GC had formed (Fig. 4D,4E,4F). In the preantral follicles, only the membrana GC expressed BMP-6 mRNA, consistent with a role of the oocyte in regulating GC BMP-6 expression (Fig. 4E,4F). BMP-6 mRNA was not detectable in the GC of dominant follicles (Fig. 4G,4H). This loss of BMP-6 mRNA became evident soon after selection at E 0200 h, and continued through dominant follicle development. This suggests the inhibition of GC BMP-6 gene activity may be required for the formation of dominant follicles. The finding that BMP-6 mRNA is strongly expressed in GC during atresia (Fig. 4I,4J) is consistent with this idea. It is notable that BMP-6 mRNA was expressed in the TE of the CL-I from ovulation throughout luteolysis (Fig. 4A,4B,4K,4L).

Expression of BMP-7 mRNA

As with BMP-4, relatively high expression of BMP-7 mRNA was identified in the theca, CL and SC (Table 1 and Fig. 5A,5B,5C). A specific pattern of BMP-7 expression was identified in the theca during folliculogenesis and luteogenesis. The theca of developing follicles first exhibited BMP-7 mRNA when the second layer of GC appeared, i.e. at the primary/secondary follicle transition (Table 1). Subsequent follicle growth was accompanied by increased BMP-7 mRNA expression which reached high levels in the theca of dominant follicles (Fig. 5D,5E). An intriguing feature of theca BMP-7 mRNA expression is that it restricted to the inner most layer, being present in a subset of fibroblast-like cells juxtaposed to the basal lamina and a sub-population of adjacent TIC (Fig. 5D,5E,5F). Notably, this pattern of theca BMP-7 mRNA expression is just the opposite of BMP-4 mRNA whose expression is limited to the outer half of the theca. These observations are consistent with the novel concept that the TIC of the TI represent

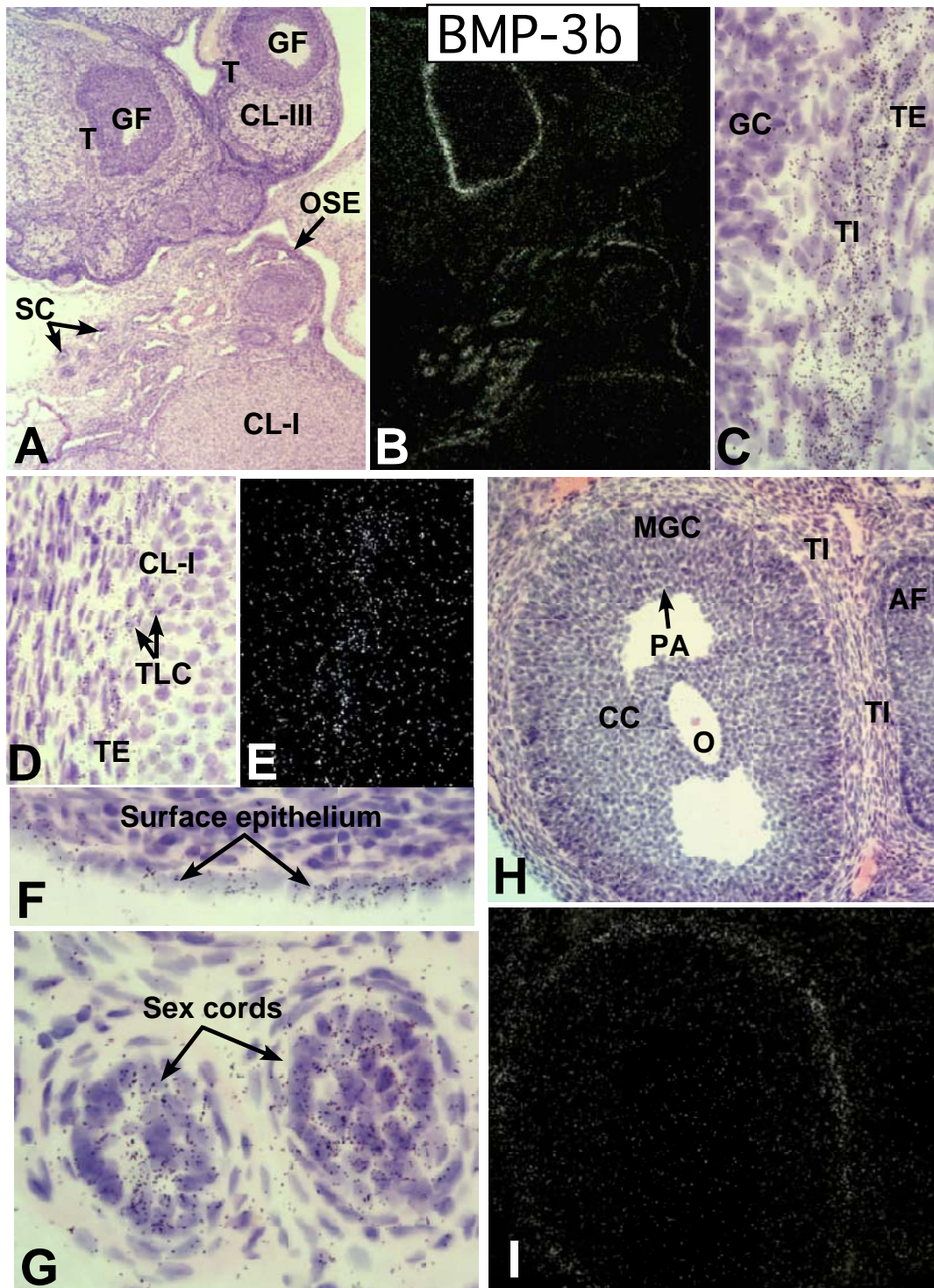


Figure 2

In situ hybridization of BMP-3b mRNA in ovaries of adult cycling rats. All photographs are from ovaries at DI 1100 h. Brightfield (A, C, D, F, G, H) and Darkfield (B, E, I). **A, B:** Low power photomicrograph showing positive theca (T), ovary surface epithelium (OSE), sex cords (SC), and CL-I (4X); **C:** Higher power (40X) of theca in B showing strongly positive theca interna (TI) and theca externa (TE); **D, E:** New CL-I showing positive signal in some theca lutein (TLC) and TE cells (20X); **F:** Positive OSE (40X); **G:** Positive SC (40X); **H, I:** Positive TI in healthy GF. Note negative TI of atretic AF follicle (10X).

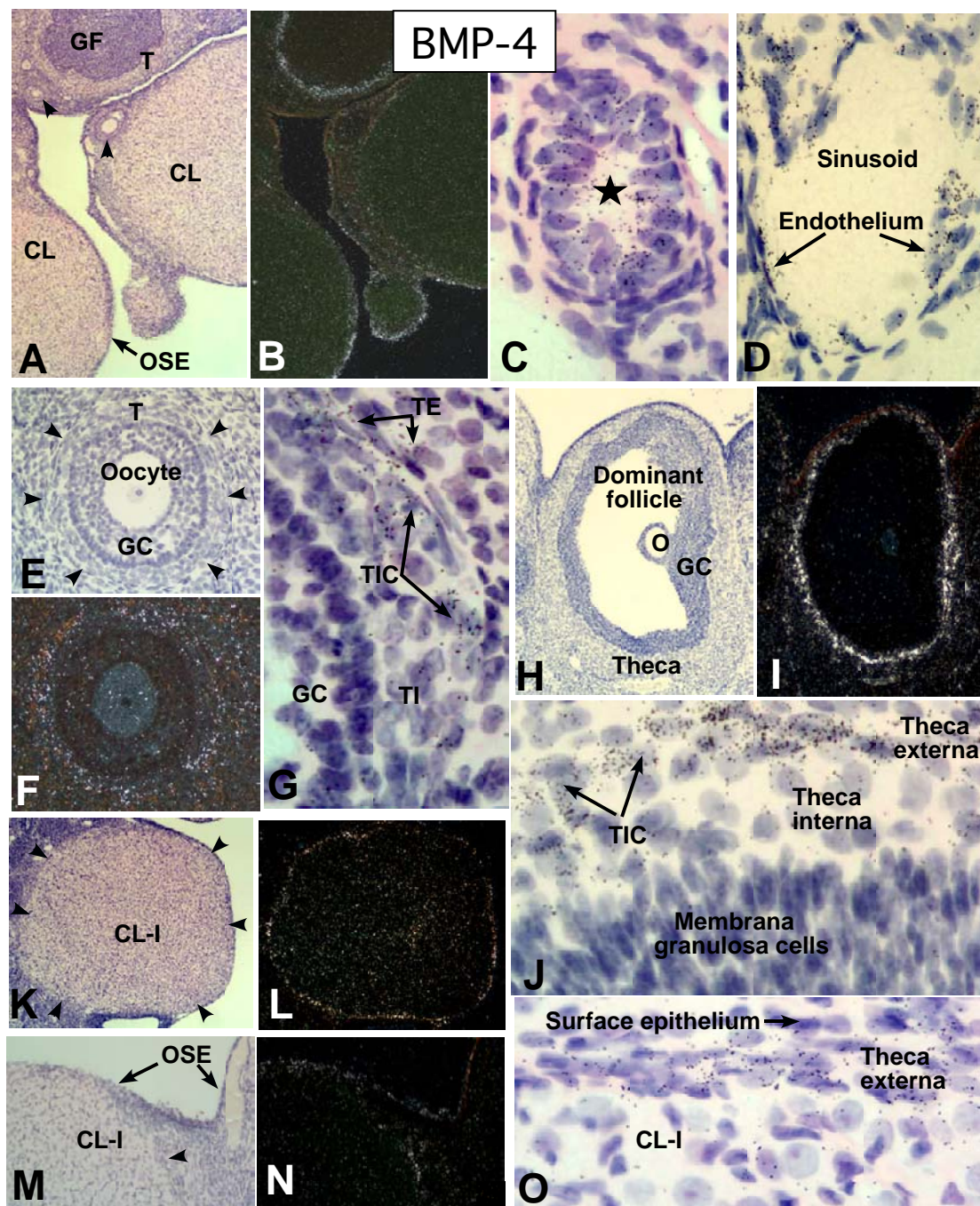


Figure 3

In situ hybridization of BMP-4 mRNA in ovaries of adult cycling rats. Brightfield (A, C, D, E, G, H, J, K, M, O) and Darkfield (B, F, I, L, N). **A, B:** Sections from P 1000 h ovaries (4X). Positive OSE, and T; weakly positive CL; negative preantral follicles (arrow heads); **C:** Positive SC () at P 1000 h (40X); **D:** Positive endothelial cells of large sinusoid (40X); **E, F:** Secondary follicle at P 1000 h (20X) showing positive cells (arrow heads) at periphery of T. **G:** High power (40X) of E showing positive theca interstitial cells (TIC) (arrows) in outer half of TI and positive cells (arrows) in the TE. **H, I:** Dominant follicle at P 2000 h showing positive theca (10X); **J:** Higher magnification (40X) of H showing positive TE and adjacent TIC in the dominant follicle. **K, L:** Older CL-I (10X) at E 1000 h of the next cycle showing positive TE (arrow heads); **M, N:** CL-I at P 1000 h (20X); showing positive TE (arrow head) and OSE only over the new CL; **O:** Higher magnification of CL-I showing positive OSE and underlying TE (40X).

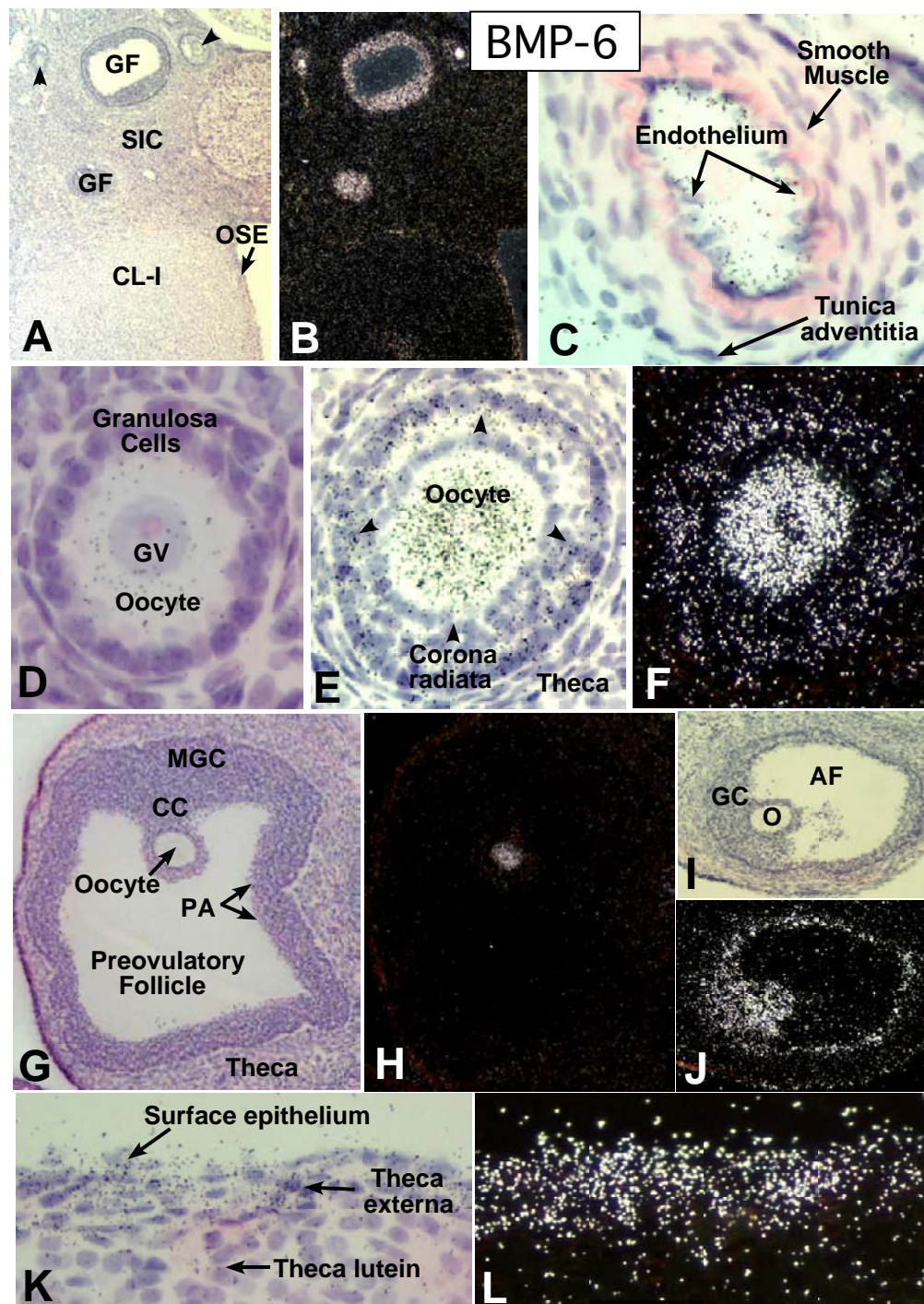


Figure 4

In situ hybridization of BMP-6 mRNA in ovaries of adult cycling rats. Brightfield (A, C, D, E, G, I, K) and Darkfield (B, F, H, J, L). **A, B:** Sections from DI I 100 h ovary (4X); positive GC in GF; preantral follicles with positive oocytes and GC (arrow heads); positive CL-I and positive OSE. **C:** Artery in mesovarium at P 1000 h (40X); positive endothelium. **D:** Early secondary follicle at E 0200 h (40X) with weakly positive oocyte; germinal vesicle (GV). **E, F:** Early secondary follicle at E 1000 h (20X); positive oocyte; positive peripheral membrana GC (arrow heads); negative corona radiata. **G, H:** Ovulating follicle at E 1000 h (4X) showing positive oocyte and negative MGC, CC, and PA cells. **I, J:** Atretic follicle (AF) at E 1000 h (10X) with positive oocyte (O) and GC. **K, L:** New CL-I at DI I 100 h (20X) showing positive TE and OSE.

two classes based on the BMP mRNAs that they expressed. As atresia occurs, BMP-7 mRNA is rapidly decreased to low or undetectable levels, indicating that BMP-7 is a marker for healthy follicles.

Following ovulation, BMP-7 mRNA was expressed in moderate amounts in groups of TLC of the newly formed CL-I from E 1000 h to DI 1100 h (Fig. 5A,5B,5G), however after the initiation of luteolysis at DII 1100 h, BMP-7 mRNA becomes undetectable. Thus, BMP-7 transcripts are down-regulated in both the luteolytic CL and apoptotic follicles.

Expression of BMP-15 mRNA

BMP-15 mRNA expression was strictly germ cell specific and was developmentally regulated during normal folliculogenesis over the estrous cycle (Table 1, Fig. 6A,6B). BMP-15 mRNA was absent in oocytes of primordial follicles. When the squamous GC start to become cuboidal a weak hybridization signal was detected in the oocyte (Fig. 6C), indicating BMP-15 gene activation is closely correlated with recruitment. Low levels of expression were seen throughout the growth of the primary follicle, but began to increase when a second layer of GC was forming (Fig. 6D). As follicle development proceeded, the expression of BMP-15 mRNA increased dramatically and very high near maximal levels were seen after the third layer of GC had formed (Fig. 6E). Expression was maintained at high levels from this stage through ovulation (Fig. 6F). It is notable that the BMP-15 mRNA levels appeared to decrease during atresia (Table 1, Fig. 6G). The stage in the cycle did not appear to affect the BMP-15 expression.

Expression of BMPR-IA mRNA

The BMPR-IA mRNA was ubiquitously expressed in the adult rat ovary, indicating a broad function in a variety of ovary cell types during the cycle (Table 1, Fig. 7A,7B). The highest expression was in the oocytes, GC, theca, and CL, with the greatest concentrations appearing in developing oocytes (Table 1). Constitutively low to moderate expression was seen in the vascular system, SIC, SE, SC, and TE (Table 1).

The potential importance of oocyte BMPR-IA mRNA is illustrated by its continuous expression throughout the course of oogenesis as it occurs within the adult ovary. A weak hybridization signal could be identified in primordial follicles (Fig. 7C). In the early stages of primary follicle development the levels of BMPR-IA mRNA in the oocyte increased and were maximal in late preantral follicles (Fig. 7D, 7F). During the development of the dominant follicle, BMPR-IA mRNA was abundantly expressed but the signal appeared weaker than in the secondary follicles (Fig. 7G,7H). In atretic follicles, BMPR-IA mRNA was highly expressed (Fig. 7I,7J).

In the GC (Fig. 7C,7D,7G,7H) the pattern of BMPR-IA mRNA expression during folliculogenesis was similar to that found in developing oocytes. In CL, moderate amounts of BMPR-IA mRNA were expressed as it underwent luteinization and luteolysis. In the theca, BMPR-IA mRNA expression was not notable, except for a stage-specific increase among the early tertiary follicles at P 1000 h (Fig. 7E,7F).

Expression of BMPR-IB mRNA

The expression of BMPR-IB mRNA was strong in oocytes, GC, CL, and SC (Table 1, Fig. 8A,8B,8C). There was also expression in the theca, vascular, and SIC tissues, but at lower levels. In blood vessels, the BMPR-IB mRNA was moderately expressed in the tunica adventitia of the ovarian arteries, suggesting a role for the BMPs in the ovary arterial system (Fig. 8D). In the theca, some TIC and TE cells showed BMPR-IB expression with the highest levels appearing in dominant follicles at proestrus (Table 1, Fig. 8A,8B,8H,8I).

As with BMPR-IA, the message for BMPR-IB was clearly visible in oocytes of primordial and primary follicles (Fig. 8E). At the primary/secondary follicle transition, a sharp increase in oocyte BMPR-IB mRNA expression occurred (Fig. 8G). High expression was observed in all dominant follicles. In the GC there were also clear follicle stage-specific shifts in BMPR-IB mRNA expression. BMPR-IB mRNA was first detected in some cuboidal GC of newly recruited primary follicles (Fig. 8E,8F). The formation of a second layer of GC at the primary/secondary follicle transition was marked by a sharp increase in BMPR-IB mRNA expression in all the GC (Fig. 8G). The entire mass of GC in developing dominant follicles strongly expressed BMPR-IB mRNA (Fig. 8A,8B,8H,8I). Cells in atretic follicles (GC and oocyte) continued to show high expression of BMPR-IB mRNA (Fig. 8J,8K).

There was a marked decrease in BMPR-IB mRNA following ovulation, whereby little or no expression was evident in the CL-I during the luteinization i.e. E 0200 h to DI 1100 h (Table 1). However, when luteolysis was initiated at DII 1100 h, the BMPR-IB mRNA is expressed in the TE (Fig. 8L,8M). Then at P 2000 h, there was strong BMPR-IB expression in both the TE and in groups of endothelial cells scattered throughout the luteolytic CL-I (Fig. 8N,8O).

Expression of BMPR-II mRNA

Over the cycle, the GC expressed the highest levels of BMPR-II mRNA, with the CL, SC, and the vascular endothelium showing low variable levels of expression (Table 1, Fig. 9A,9B). Although the oocytes of most follicles showed no hybridization signal above background, a weak expression was consistently seen in some oocytes of

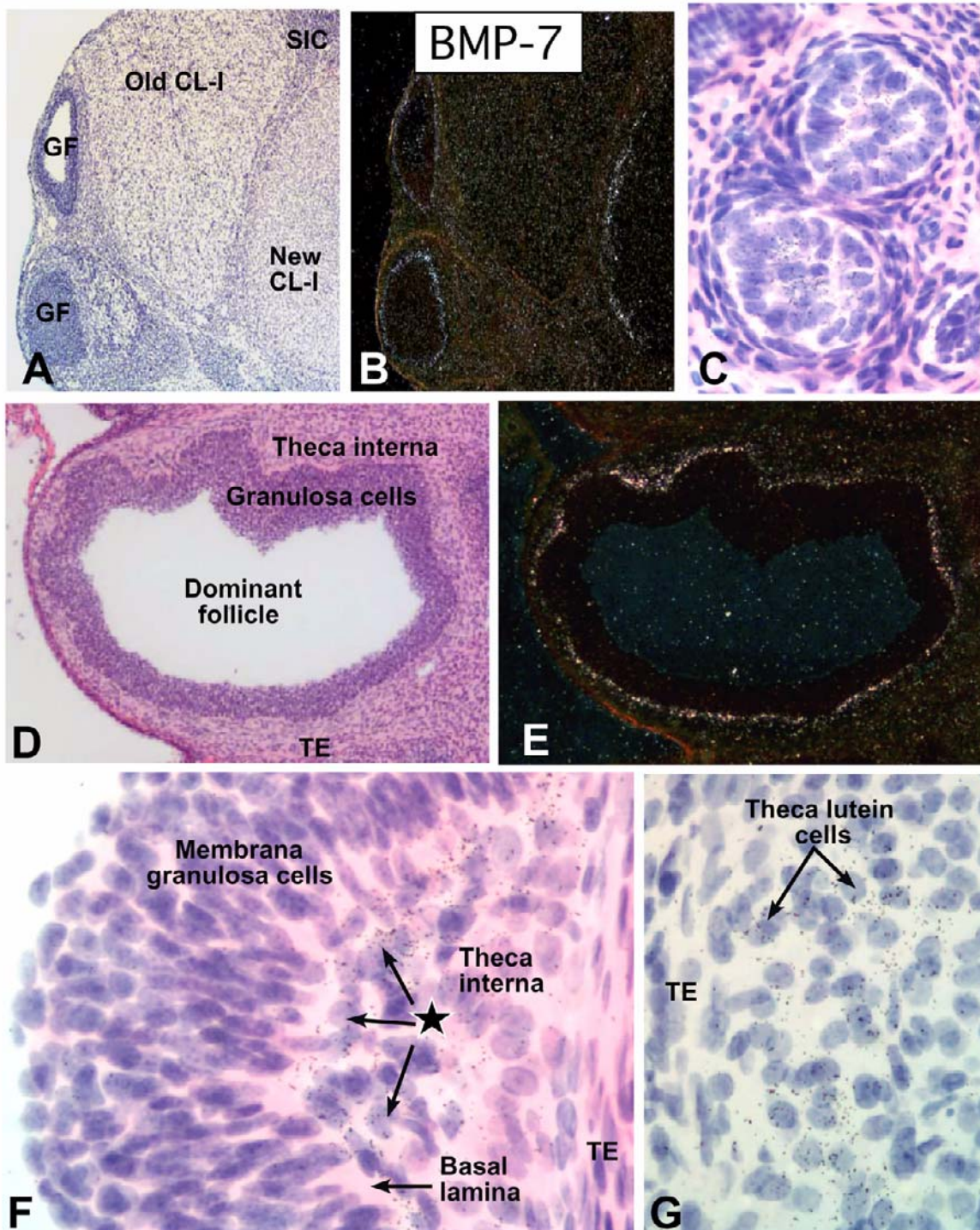


Figure 5

In situ hybridization of BMP-7 mRNA in ovaries of adult cycling rats. Brightfield (A, C, D, F, G) and Darkfield (B, E). **A, B:** Sections of DI 1100 h ovaries (4X); positive Graafian follicle (GF); positive new CL-I; negative old CL-I and SIC; **C:** Positive sex cords at E 0200 h (40X); **D, E:** Dominant follicle at P 2000 h (4X) showing positive theca interna. **F:** Higher magnification (40X) of D showing positive fibroblast-like cells beneath the basal lamina and adjacent positive TIC (). **G:** CL-I at DI 1100 h showing positive theca lutein cells (40X).

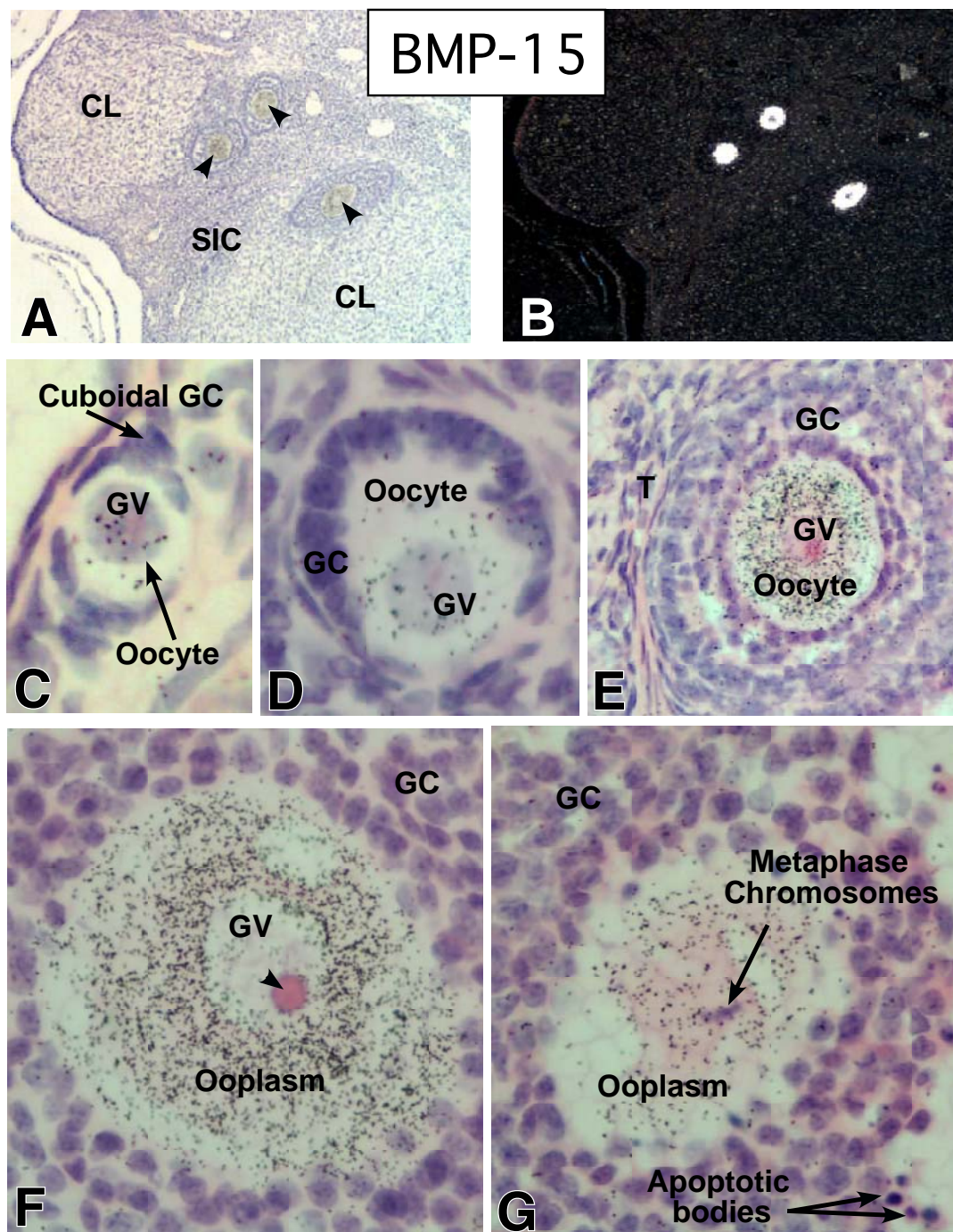


Figure 6

In situ hybridization of BMP-15 mRNA in ovaries of adult cycling rats. Brightfield (A, C, D, E, F, G) and Darkfield (B). **A, B:** Sections from a DII 1100 h ovary (4X); Positive oocytes in preantral follicles (arrow heads); **C:** Early primary follicle at DI 1100 h showing one cuboidal GC and a weakly positive oocyte: note most of the signal is located within the GV or nucleus (40X). **D:** Preantral follicle at P 1000 h (40X) in the process of forming a second layer of GC. Note a weakly positive oocyte; **E:** Mid secondary follicle at DII 1100 h showing a strongly positive full-grown oocyte surrounded by three to four layers of GC (20X); **F:** Mid cross section of a strongly positive oocyte of a dominant follicle at P 1000 h (40X). Hybridization signals are seen predominantly in the ooplasm, with some signal associated with the outer region of the nucleolus (arrow head); **G:** An atretic follicle at E 0200 h (40X) with a moderately positive oocyte that has resumed meiosis

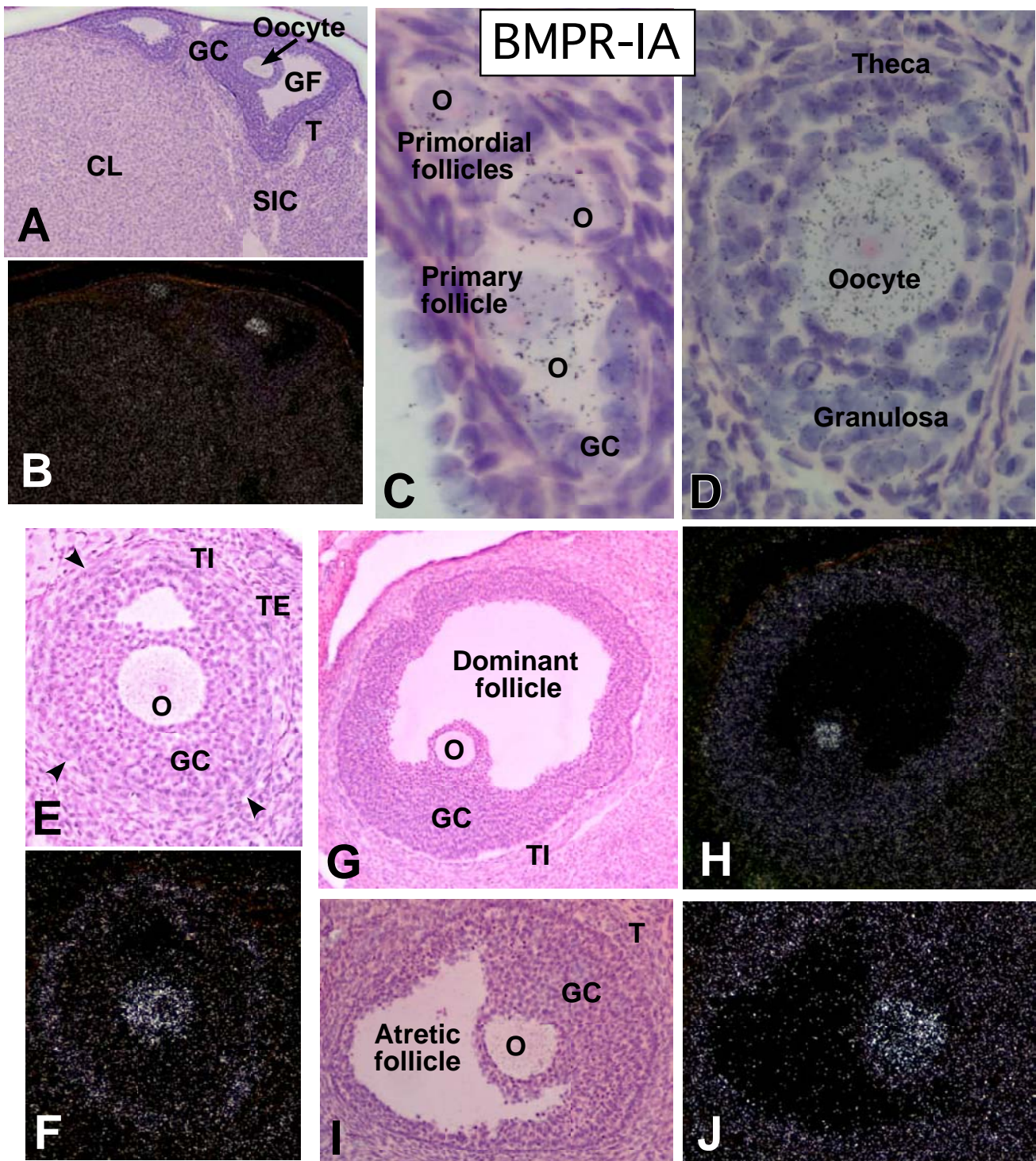


Figure 7

In situ hybridization of BMPR-IA mRNA in ovaries of adult cycling rats. Brightfield (A, C, D, E, G, I) and Darkfield (B, F, H, J). **A, B:** Sections from DII 1100 h ovaries (4X). Positive oocyte; **C:** Positive oocytes (O) and GC in primordial and primary follicles at P 1000 h (40X); **D:** Positive secondary follicle with three layers of GC at DII 1100 h (20X); **E, F:** Positive oocyte of an early tertiary follicle at P 1000 h. Note expression in TI (arrowheads) (10X); **G, H:** Preovulatory follicle at E 1000 h showing positive oocyte (O); Note weak signal in GC and TI (4X); **I, J:** Atretic follicle at E 1000 h showing positive O and GC (10X).

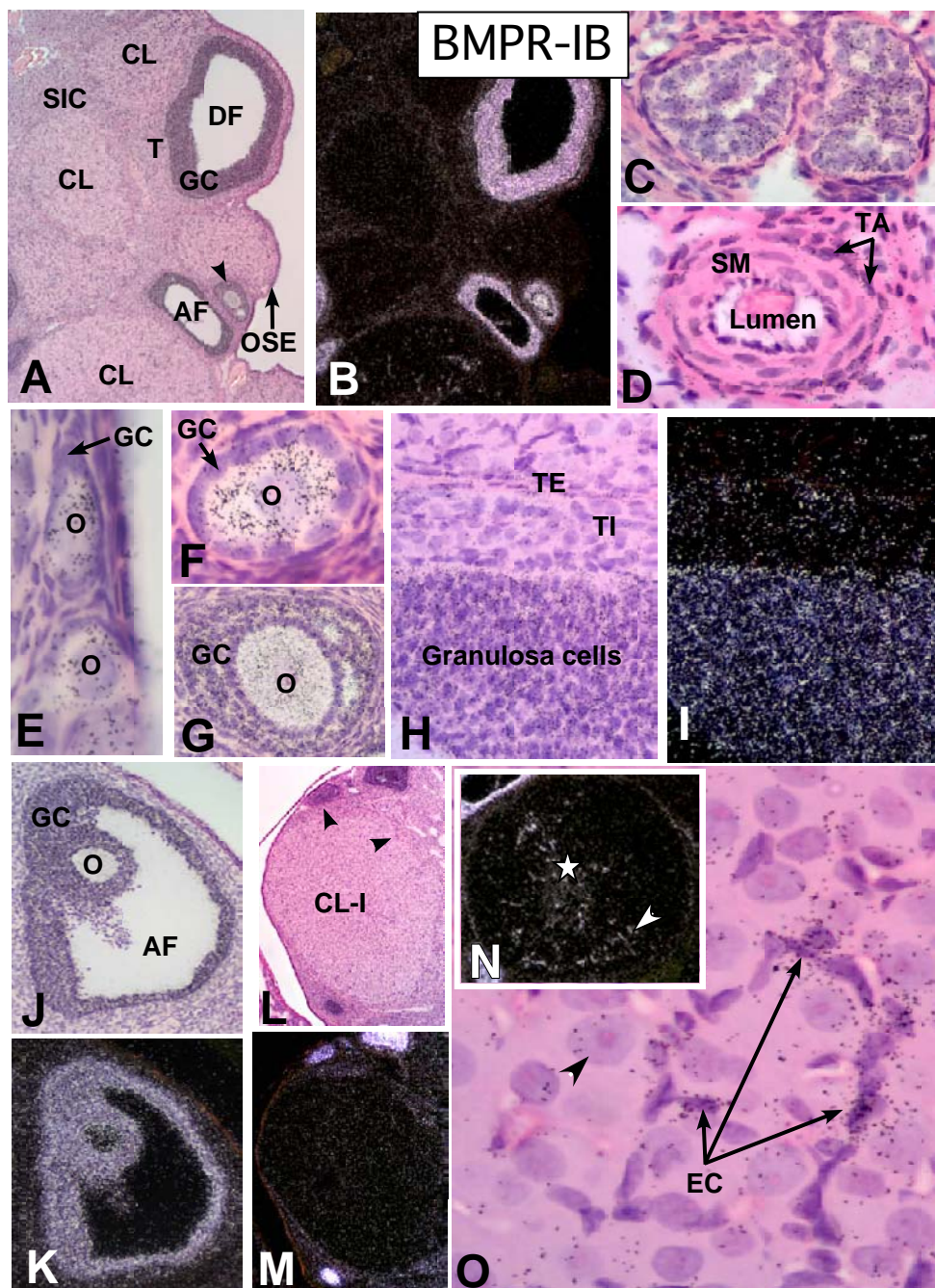


Figure 8

In situ hybridization of BMPR-IB mRNA in ovaries of adult cycling rats. Brightfield (A, C, D, E, F, G, H, J, L, O) and Darkfield (B, I, K, M, N). **A, B:** Sections from a P 2000 h ovary (4X); positive GC in dominant (DF), AF and preantral follicle (arrow head); Note positive oocyte. **C:** Positive sex cords at P 1000 h (40X); **D:** Artery at P 2000 h (40X) showing positive tunica adventita (TA) next to smooth muscle (SM); **E:** Positive oocytes (O) in primordial and early primary follicles at DI I 100 h (40X); **F:** Positive O in late primary follicle with single layer of weakly positive GC at P 2000 h (40X); **G:** Strongly positive O and GC in secondary follicle at E 1000 h (20X); **H, I:** Higher magnification (20X) of wall of DF at P 1000 h showing positive GC, TE, and a number of TIC in the outer zone of TI; **J, K:** Positive AF at P 1000 h (10X); **L, M:** CL-I at DII I 100 h showing positive TE (arrow heads); **N:** CL-I at P 2000 h showing positive TE at periphery (arrow head) and clusters of positive cells scattered within the CL (); **O:** Higher magnification (40X) of N showing positive endothelial (EC) and a few adjacent weakly positive granulosa lutein cells (arrow head).

primary and early secondary follicles (Table 1, Fig. 9C). There was no detectable BMPR-II mRNA expression in GC of the primordial follicles; however, a low expression was detected in primary follicles after recruitment (Fig. 9C). During preantral follicle growth, expression increased reaching high maximal levels in the GC of secondary follicles (Fig. 9A,9B). High expression was seen in all the GC of the dominant follicle, except at ovulation when there appeared to be a decrease in BMPR-II mRNA within the cumulus GC (Fig. 9D,9E). Ovulation was accompanied by a rapid decrease in the levels of BMPR-II mRNA. During the life of the CL, low to moderate expression was seen in the lutein, but not the endothelial cells (Fig. 9H). BMPR-II mRNA was strongly expressed in all GC during atresia (Fig. 9F,9G).

Expression of FS mRNA

The message for the BMP antagonist, FS, was highly expressed during the estrous cycle, being present in the GC, CL, and SIC (Table 1, Fig. 10A,10B). It is notable that a striking change in FS mRNA expression occurred in the SIC during the preovulatory period. In all the SIC, FS mRNA was absent during most of the cycle, but at P 2000 h, it was very strongly expressed (Fig. 10C,10D,10E). This observation suggests a burst of FS gene activity that may be associated with the preovulatory surge of LH during proestrus.

Folliculogenesis and luteogenesis were accompanied by striking changes in FS expression some of which were cycle dependent. During the preantral stages of folliculogenesis, FS mRNA was poorly expressed in the GC. By comparison FS mRNA was highly expressed in all the GC of the dominant follicles over the cycle (Table 1, Fig. 10A,10B). Then, during atresia, FS mRNA was low or absent in the GC (Table 1). Thus, FS gene activity and follicle dominance appear to be physiologically coupled. After ovulation, the CL showed high levels of FS mRNA expression during luteinization at E 0200 h and E 1000 h and moderate levels at DI 1100 h (Fig. 10F,10G,10H,10I,10J,10K). The high expression of FS mRNA correlates with a high expression of progesterone by the new CL-I. Interestingly, FS mRNA fell to non-detectable levels in the CL-I when luteolysis occurred at DII 1100 h (Fig. 10L,10M). FS mRNA remained undetectable in the CL until P 2000 h when high expression was induced in a subset of lutein cells (Fig. 10N,10O). This high expression in the luteolytic CL-I persisted through estrous of the next cycle.

Discussion

To better understand the physiological role of the BMP family in ovarian function, the spatiotemporal expression patterns of a number of relevant BMP ligands, receptors, and a binding protein were determined by in situ hybrid-

ization in rat ovaries over the estrous cycle. The general principle to emerge from our data is that the developmental programs of folliculogenesis (recruitment, selection, and atresia), ovulation and luteogenesis (luteinization and luteolysis) are accompanied by precise cell-specific changes in the expression of the genes encoding the BMP family. Therefore, we hypothesize the regulated expression of BMP gene activity forms a part of the controlling network that ensures the proper timing of the developmental events that generates a normal estrous cycle.

The finding that the genes encoding the three types of BMP receptors are expressed in primordial follicles indicates that these fundamental reproductive units are targets for the BMPs. In this regard, we have shown recently that BMP-7 can promote primordial follicle growth [13]. Thus, it is reasonable to propose that one function of the BMP receptors in the primordial follicle might be to mediate the activation of recruitment by endogenous BMP-7. As there is no evidence that primordial follicles express BMP ligands, the mechanism by which this activation occurs would be paracrine, most likely involving BMP-7 from adjacent tissues such as the theca, secondary interstitial, or sex cords.

Unlike lower vertebrates, the developing mammalian follicle is composed of more than one layer of GC. One of the most important concepts generated in the ovary BMP field is that oocyte derived GDF-9 [14] plays a role in the mechanisms governing the development of the second layer of GC i.e. the primary/secondary follicle transition. The question of how GDF-9 controls this step has yet to be answered. Our data show that the primary/secondary transition stage is accompanied by some rather dramatic changes in BMP expression including increased expression of: i) BMP-6, BMP-15, and BMPR-IB in the oocyte; ii) BMP-2, BMP-6, and BMPR-IA, -IB and -II in the GC; and iii) BMP-4 and BMP-7 in the theca. Because folliculogenesis in the murine proceeds to the preovulatory stage in the absence of BMP-6 [30], BMP-15 [15], and BMPR-IB [31], one can assume that the marked increases in these regulatory molecules are not essential for the development of a second layer of GC. The question of whether the increased expression of BMPR-II and BMP-2, -4, and -7 contribute to this important developmental event remains to be answered. One interesting implication of our BMP-2 data is that the GC have already attained different states of differentiation by the early stages of secondary follicle development. Based on previous work [32,33], it is not unreasonable to propose that oocyte morphogens may have control over GC fate beginning as early as the primary/secondary follicle transition. It will be interesting to investigate how this phenomenon fits into the mechanisms underlying the primary/secondary follicle transition.

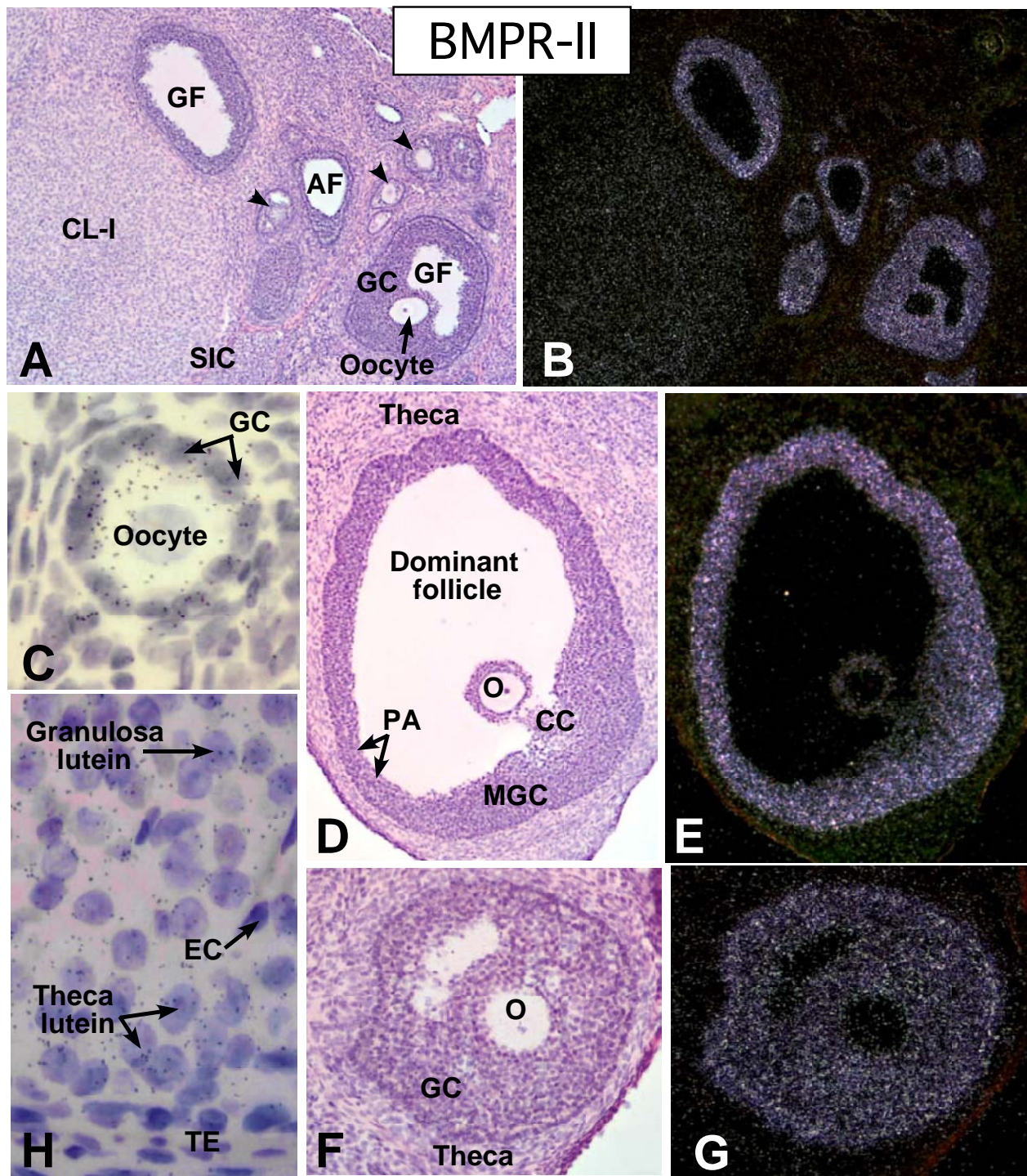


Figure 9

In situ hybridization of BMPR-II mRNA in ovaries of adult cycling rats. Brightfield (A, C, D, F, H) and Darkfield (B, E, G). **A, B:** Sections from DI 1100 h ovaries (4X). Positive new CL-I; positive GC in GF, AF, and preantral follicles (arrow heads); negative secondary interstitial cells (SIC); **C:** Early secondary follicle showing positive GC surrounding weakly positive oocyte (40X); **D, E:** Dominant preovulatory follicle at E 0200 h (4X); positive MGC and negative oocyte (O) surrounded by weakly positive CC. **F, G:** Atretic follicle at P 2000 h (10X) showing positive GC. **H:** Higher magnification of new CL-I in panel A (40X); moderately positive lutein cells; negative endothelial cells (EC) and theca externa (TE).

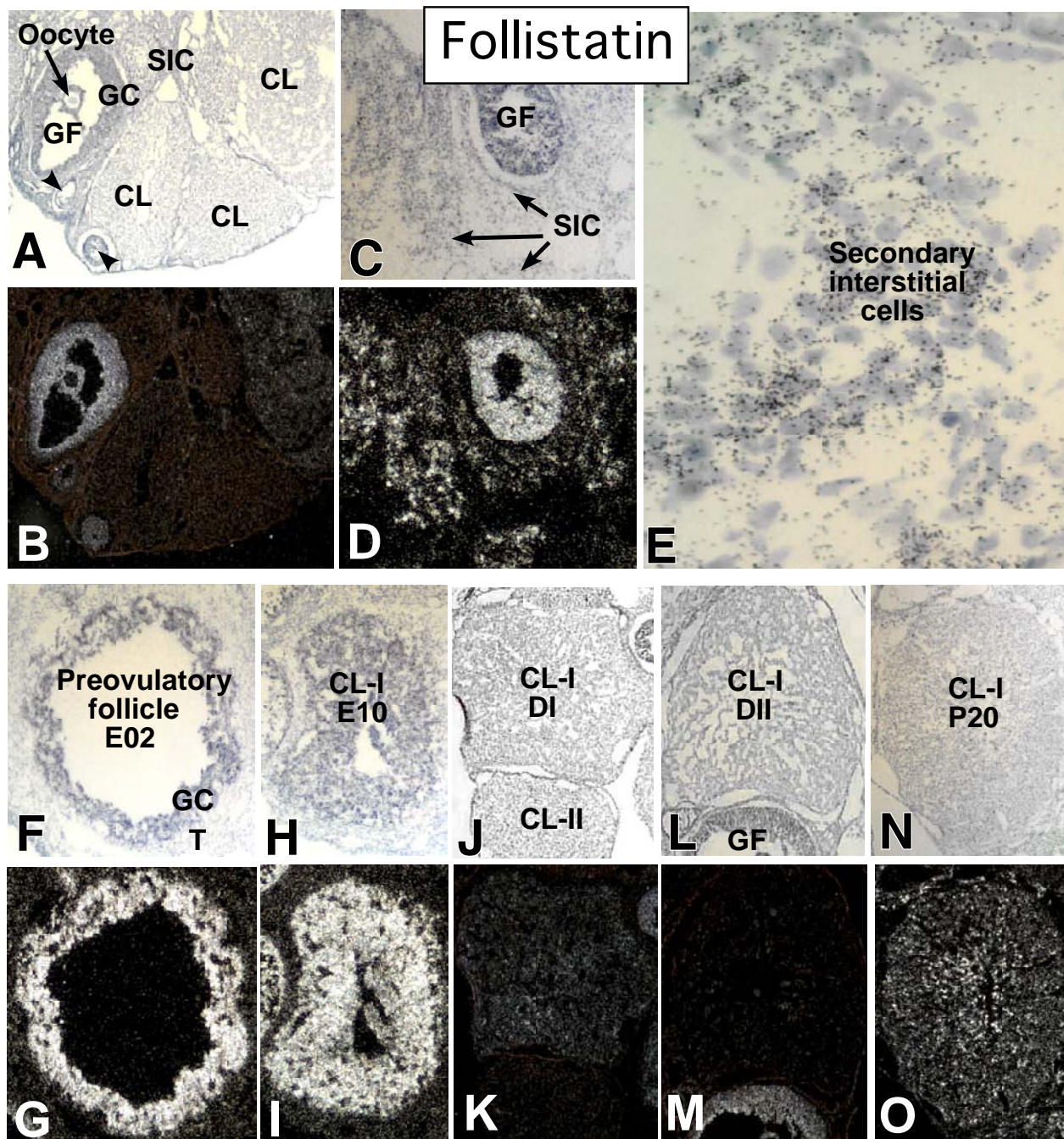


Figure 10

In situ hybridization of FS mRNA in ovaries of adult cycling rats. Brightfield (A, C, E, F, H, J, L, N) and Darkfield (B, D, G, I, K, M, O). **A, B:** Sections from a DI 1100 h ovary (4X) showing strongly positive GC in GF and weakly positive GC in preantral follicles (arrow heads); **C, D:** Positive SIC at P 2000 h (4X); **E:** Higher magnification of panel C showing high density of silver grains (black dots) over the SIC (40X); **F to O:** FS expression in CL-I during luteogenesis (4X) i.e. luteinization (E 0200 h, E 1000 h, DI 1100 h) and luteolysis (DII 1100 h, P 2000 h).

Our results demonstrate for the first time that the theca interna of healthy follicles is composed of two distinct populations of theca interstitial cells (TIC). One group, which expresses BMP-4, is present as an outer layer of cells juxtaposed to the theca externa (TE), and another, which expresses BMP-7, is present at the proximal side of the theca interna near the basal lamina of the follicle. These differences can be found throughout folliculogenesis, beginning during the secondary stage. This, together with the failure to detect BMP-4 and 7 in the theca of atretic follicles, supports the idea that the cellular sites and coordinate expression of these BMP genes may have important regulatory roles in maintaining folliculogenesis. In this regard, a recent study has demonstrated the ability of BMP-4 to inhibit cyclic AMP-stimulated androgen production by the cultured human theca cell line, presumably by activating BMP receptors (BMPR-IA, -IB, and -II) which are expressed in these cells [34]. Because theca derived androgen is required for follicle estrogen biosynthesis and has been implicated in ovary pathology [35], it will be interesting to determine how the expression and functions of these TIC-specific BMPs are integrated into the overall processes of physiology and pathophysiology. Besides the TI, a high level of BMP expression (BMP-3b and 4) is found within the smooth muscle cells of the TE during the process of follicle formation. Very little is known about the role of the TE in folliculogenesis: however this new finding raises the possibility that BMP-3b and -4 could have autocrine actions on TE histogenesis and/or paracrine actions on theca/follicle morphogenesis.

The rapid cessation of mitosis and the expression of apoptosis in the GC is the sinequanon of follicle atresia. Our results suggest a possible role for BMPs in follicle atresia. The supporting data are as follow. First, a striking feature of atresia in the rat ovary is the cessation of BMP expression (BMP-3b, -4, and -7) within the theca compartment. This observation is particularly interesting with respect to BMP-7. In previous work, we demonstrated that BMP-7 could stimulate DNA synthesis in rat GC [13], as it does in human osteoblasts [36]. Equally relevant, several reports have demonstrated the role for BMP-7 in preventing apoptosis in other cell systems [37,38]. Together, these data suggest that theca-derived BMP-7 might be a follicle survival factor through its ability to enhance GC proliferation and suppress apoptosis. Second, atresia is accompanied by a relatively high level of expression of BMP-2 and -6 and the BMPR-IA, -IB, and -II. Significantly, this occurred in the absence of the BMP antagonist, FS. Thus, it is possible that GC BMP receptor signaling pathways are under maximal stimulation by BMP-2 and -6 during atresia. How this phenomenon contributes to atresia is unknown, but our finding [12] that BMP-6 inhibits FSH signaling is relevant because there is compelling evidence

that continuous FSH stimulation is essential for follicle survival.

The expression of the family of BMPs (ligands, receptors and a binding protein) in the fundamental cell types of the dominant follicles (DF) argues for a physiological role of an intrinsic BMP in directing growth and cell fates in the DF. This is supported by two main lines of evidence. First, we found that the genes encoding the BMP receptors (BMPR-IA, -IB, -II) are highly expressed in the DF throughout its course of development. And second, the BMP ligands (BMP-4, -6, -7, and -15) act on the GC to control the level of mitosis and FSH receptor signaling [1]. In this regard, one particularly interesting observation is the dramatic loss of BMP-6 mRNA in the GC when the DF is selected at E 0200 h. Because BMP-6 can prevent FSH action [12], the rapid loss of BMP-6 expression may be required for FSH to exert its crucial functions during DF-development. The secondary rise of FSH on estrous morning is obligatory for DF selection, and we have preliminary data showing that FSH induces the loss of BMP-6 mRNA in GC. Thus, the secondary rise of FSH may down regulate BMP-6 expression in the DF. It is notable that the absence of GC BMP-6 is a marker for the DF. Finally, with regard to FSH action, FS has been shown to neutralize the bioactivity of the FSH inhibitor, BMP-15 [39]. It is possible, therefore, that the strong expression of FS by the DF may serve to modulate BMP-15 action such that sufficient FSH receptors are expressed in the GC to permit the development of a preovulatory follicle.

A role for the BMPs in regulating luteogenesis is strongly suggested our data. Some key findings are as follows. First, the classical view of CL development is that luteinization is inhibited by oocyte-derived luteinization inhibitors [32,40,41], two of which have been identified as BMP-6 and -15 [8,12]. Therefore, the loss of oocyte BMP-6 and -15 at ovulation undoubtedly plays an especially prominent role in activating luteinization. Second, the fact that CL BMP-2 expression is suppressed and activated during luteinization and luteolysis, respectively, suggests that BMP-2 might be a novel luteinization inhibitor. Further work is necessary to investigate this possibility. Third, FS is strongly expressed during luteinization, but is undetectable at luteolysis. Thus, it is conceivable that the cycle-specific expression of FS within the CL may control the level of progesterone production through anti-BMP mechanisms. And fourth, a role of BMPR-IB in luteogenesis was suggested by our finding that luteinization and luteolysis, respectively are characterized by the absence and presence of BMPR-IB expression in the CL. A functional link between the expression of BMPR-IB and luteolysis comes from loss-of-function studies showing that the CL of the cycle continues to secrete progesterone in the absence of BMPR-IB [31]. This work, together with our

finding that BMPR-IB is selectively expressed in the TE of the CL at luteolysis, supports the novel hypothesis that the regulation of inducible and TE specific BMPR-IB gene expression may control luteolysis.

The observation that the ovarian sex cords (SC) express an intrinsic BMP system replete with ligands (BMP-3b, -4, -7) and receptors (BMPR-IA, -IB, -II) is novel. The SC are vestiges of the cranial portion of the mesonephric tubules and ducts, and in the adult ovary appear as a cluster of blind tubules in the hilar region. Although little is known about the physiology and biology of ovarian SC, they have been linked to the genesis of ovary cancer through distinct TGF- β signaling pathways [42,43]. Significantly, the importance of FS in the genesis of this cancer has been established [44]. Given the evidence that BMPs are potent growth factors and that FS can modulate their bioactivity, it will be interesting to investigate the possibility that this intrinsic BMP system may be involved to pathogenesis of SC tumors. Because SC were occasionally found juxtaposed to developing follicles, blood vessels and secondary interstitial cells, one cannot exclude the possibility that the SC-derived BMPs may have paracrine functions.

A functional role for the BMPs in the ovary surface epithelium (OSE) is suggested by the strong expression of BMP-3b, -4, and -6. Based on our data, BMP-3b and -6 appear to be constitutively expressed throughout the epithelium over the cycle, a finding consistent with a maintenance-type role for these OSE BMPs. An interesting finding is the cell-specific induction of BMP-4 gene expression in a subset of epithelial cells covering the ovulated follicle and the newly formed CL-I. Although the function of this inducible and cell-specific gene expression is unclear, it is tightly correlated with the replenishment of the surface epithelium that is exfoliated from the surface of ovulating follicle. Thus, the importance of this highly specific expression of BMP-4 may relate to the regeneration of the OSE during postovulatory repair. Although BMP-4 expression appears tightly regulated over the cycle, it remains to be determined how the regulation is controlled. Because the OSE does not appear to express BMP receptors, the OSE-derived BMP-4 probably acts as a paracrine growth factor in the underlying stroma. It is noteworthy that epidemiological studies have shown that the risk of epithelial ovarian cancer is decreased by factors that suppress ovulation, i.e., pregnancy, breast-feeding, and oral contraceptive pill. [45,46]. Given the relationship between LH-dependent ovulation and the induction of BMP-4 expression, it will be important to explore a possible link between the risk of ovary cancer and the ovulation-inducible and OSE cell-specific BMP-4 gene expression.

It is clear from this work that the regulation of inducible and cell-specific expression of the BMP family is a general

property of the mammalian ovary. Consequently, the potential for independent and coordinated contributions of different BMPs to control growth, differentiation, and apoptosis is widely distributed throughout all the histological units of the ovary. The current challenges are to understand how specific hormone/growth factor interactions regulate BMP expression in the fundamental ovary cell types, and how these interactions are integrated into the morphological and histological events that generate the estrous cycle. The clinical relevance is emphasized by our previous finding that the expression of GDF-9 is dysregulated in oocytes of women with Polycystic Ovary Syndrome [47].

Acknowledgements

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