

# Aberrant Expression of Growth Differentiation Factor-9 in Oocytes of Women with Polycystic Ovary Syndrome

FABIO LOPES TEIXEIRA FILHO, EDMUND CHADA BARACAT, TAEK HOO LEE, CHANG SUK SUH, MOTOZUMI MATSUI, R. JEFFREY CHANG, SHUNICHI SHIMASAKI, AND GREGORY F. ERICKSON

Universidade Federal de São Paulo (F.L.T.F., E.C.B.), São Paulo, Brazil 01311-940; Kyungpook National University (T.H.L.), Taegu, Korea 700-721; Seoul National University College of Medicine (C.S.S.), Seoul, Korea 110-744; and Department of Reproductive Medicine (M.M., R.J.C., S.S., G.F.E.), University of California San Diego, La Jolla, California 92093-0674

Polycystic ovary syndrome (PCOS) is a major cause of female infertility. Despite substantial effort, the etiology and pathogenesis of PCOS and polycystic ovaries (PCO) in women remain unknown. Recent studies in laboratory animals have documented a link between dysfunction of two oocyte growth factors, growth differentiation factor-9 (GDF-9) and bone morphogenetic factor-15 (BMP-15), and aberrant folliculogenesis. Because aberrant follicle development is a hallmark of PCOS, we wondered whether the expression patterns of these growth factors might be disrupted in PCOS and PCO oocytes. To address this issue, we compared the pattern and level of expression of GDF-9 and BMP-15 mRNA in ovaries from normal cycling (n = 12), PCOS (n = 5), and PCO (n = 7) patients. *In situ* hybridization studies showed that the expression of GDF-9 and BMP-15 is restricted to the oocytes in all ovaries examined. Interestingly, a decreased level of GDF-9 signal was

observed in developing PCOS and PCO oocytes, compared with normal. This difference was evident throughout folliculogenesis, beginning at recruitment initiation and continuing through the small Graafian follicle stage. By contrast, there were no qualitative or quantitative changes in the expression of BMP-15 mRNA in PCOS oocytes during folliculogenesis. There were also no significant differences between normal and PCOS and PCOs in the levels of the mRNA encoding the housekeeping gene, cyclophilin. Together, these results indicate that the expression of GDF-9 mRNA is delayed and reduced in PCOS and PCO oocytes during their growth and differentiation phase. Because oocyte-derived GDF-9 is crucial for normal folliculogenesis and female fertility, we suggest that a dysregulation of oocyte GDF-9 expression may contribute to aberrant folliculogenesis in PCOS and PCO women. (*J Clin Endocrinol Metab* 87: 1337–1344, 2002)

POLYCYSTIC OVARY SYNDROME (PCOS) is one of the most common causes of anovulation, infertility, and menstrual irregularities in women, affecting between 5% and 10% of women of reproductive age worldwide (1, 2). It has been defined as a syndrome involving polycystic ovaries (PCOs), hyperandrogenism, and chronic anovulation with the exclusion of specific diseases of the ovaries, adrenals, and pituitary (3). This disorder is also a major risk factor for non-insulin-dependent diabetes mellitus, hypertension, and cardiovascular disease (4–6). Therefore, PCOS is a significant health issue for women beyond the reproductive endocrine abnormalities. Despite an enormous effort to define the cause of PCOS, the etiology and pathogenesis remain unclear, and there is no effective method to cure patients of PCOS. Consequently, understanding the mechanisms that cause PCOS is a major goal of medical research.

There is evidence suggesting that the mechanism of PCOS involves ovary dysfunction (7, 8). One important line of evidence is the observation by Hughesdon (9) that PCOS ovaries contain twice the normal number of growing follicles. This finding is particularly significant because it suggests that all steps in the process of folliculogenesis may be aberrant in PCOS. Another line of evidence is that PCOS follicles stop growing and developing when they reach 4–7 mm in diameter. The cessation of folliculogenesis at this stage re-

sults in the accumulation of large numbers of small antral follicles (so-called cysts) beneath the tunica albuginea, thereby generating the PCO phenotype (10, 11). The observation that theca interstitial cells associated with developing PCOS follicles exhibit abnormally high levels of androgen biosynthesis also supports a role of the ovary in PCOS (12–15). Collectively, this evidence has led to the hypothesis that there are abnormalities in folliculogenesis in PCOS from primordial follicle recruitment through the small Graafian follicle stage.

One possible cause of PCOS that has remained largely unexplored is an alteration in the growing primary oocyte. Compelling evidence that oocyte-derived growth factors, namely growth differentiation factor-9 (GDF-9) (16, 17) and bone morphogenetic factor-15 (BMP-15)/GDF-9B (18, 19), play a key role in folliculogenesis and female fertility has come from studies in laboratory animals (20–22). *In situ* hybridization studies in a variety of mammals (17, 23–25) have shown that the GDF-9 and BMP-15 genes are selectively expressed in developing oocytes during folliculogenesis. In GDF-9-deficient female mice, a wide range of reproductive abnormalities appear, including arrested follicle growth at the primary stage, reduced granulosa proliferation, inappropriate theca development, cyst formation, and infertility (26–28). A similar pattern of arrested follicle development and infertility has been reported in sheep homozygous for a BMP-15 mutation (29). The observation that GDF-9 (17, 30) and BMP-15 (30) mRNA are present in normal human oo-

Abbreviations: BMP-15, Bone morphogenetic factor-15; GDF-9, growth differentiation factor-9; PCO, polycystic ovaries; PCOS, polycystic ovary syndrome.

cytes fits the prediction that these growth factors may have functions in folliculogenesis and fertility in women.

Because altered folliculogenesis and cyst formation are hallmarks of PCOS, we wondered whether the pattern of expression of GDF-9 and BMP-15 might be altered in the PCOS and PCO oocytes during follicle development. Here, we provide evidence that the level of GDF-9, but not BMP-15, mRNA appears reduced in PCOS and PCO primary oocytes during their growth and differentiation phase.

## Materials and Methods

### Source of ovarian tissue

A total of 20 ovaries was used in these investigations. Normal ovaries were obtained from 12 regularly cycling women (aged 26–45 yr) at various stages of the menstrual cycle. The surgeries were for nonovarian gynecological reasons. None was receiving exogenous hormones. Ovaries were obtained from five patients (aged 19–29 yr) with PCOS as defined by chronic anovulation and hyperandrogenism (3). The clinical data of these PCOS subjects have been reported previously (31, 32). Briefly, the subjects had oligomenorrhea or amenorrhea and were hirsute. All had undergone laparotomy with the finding of PCO confirmed by histological examination. Serum hormone determinations revealed significant elevations of mean LH, total T, androstenedione, and dehydroepiandrosterone sulfate. Ovaries were obtained from seven patients with polycystic-appearing ovaries as diagnosed by the pathologist at the time of surgery. The PCO diagnosis was confirmed by a histological examination of the ovaries carried out in our laboratory (8). None of the clinical histories of the PCO patients was available. The protocol was approved by the Institutional Review Board at UCSD.

### In situ hybridization

Paraffin-embedded human ovarian tissue sections (10  $\mu$ m) were used for these experiments. The sections were taken randomly from the ovary pieces, and no effort was made to standardize where the sections were taken (*i.e.* maximal cross-sections or whole-thickness slices). The relevant *in situ* hybridization procedures have been described previously (33). GDF-9 and BMP-15 probes were designed to contain only the pro-region of the corresponding precursor to avoid cross-hybridization with each other and recognize all the transcripts expressed in the oocytes. Three plasmids constructed for the probe preparation are as follows: pHGDF-9–421 for GDF-9, a *SacI*-*Apal* segment (421 bp) of human GDF-9 cDNA (gifted by Dr. Se-Jin Lee) (16) was cloned into *SacI* and *Apal* sites of pBluescript II (SK+); pHBMP-15–404 for BMP-15, a DNA segment (404 bp) of human BMP-15 pro-domain (16) was amplified by RT-PCR from ovarian total RNA and cloned into a pCR II vector, (Invitrogen, Carlsbad, CA); pTRI-cyclophilin-human plasmid (Ambion, Inc., Austin, TX): a 103-bp cDNA insert of a highly conserved region of the human cyclophilin gene (34). Antisense and sense RNA probes were synthesized by transcription using <sup>35</sup>S-UTP and T7 or T3 polymerase after digestion with appropriate restriction enzymes. In each experiment, duplicate slides from all ovaries studied (normal, PCOS, PCO) were processed in the same *in situ* hybridization run using the same probe, conditions of hybridization, and length of autoradiographic exposure. The sections from all ovaries in all experiments were scored and the data were repeated at least three times with similar results.

### Morphometry

Follicles were classified into four groups as described (35): primordial (the oocyte was surrounded by a single layer of squamous granulosa cells); primary (the oocyte was surrounded by a single layer of mixed squamous and cuboidal or a single layer of cuboidal granulosa cells); secondary (the oocyte was surrounded by two to eight layers of granulosa cells but no antrum was present); and small Graafian (the follicle measured 0.5–7 mm in diameter with a fluid-filled antrum).

The entire section of each ovary was scanned under bright field, and only those follicles sectioned through the oocyte nucleolus (the largest follicle cross-section) were analyzed. The intensity of the *in situ* hybridization signals was evaluated on a scale of 1 to 4 as previously described

(36, 37). The following rating was used: +, silver grains were sparse but clearly above background (see Fig. 2a); ++, silver grains were numerous but did not cover the whole area (see Fig. 2b); +++, silver grains were very numerous and began to merge in some places (see Fig. 2c); +++++, silver grains were very dense and formed a near uniform mass (see Fig. 2d). The data were confirmed independently by four different investigators. Follicles were classified as healthy if they showed no apoptosis (38), as defined by the absence of apoptotic bodies (39).

### Cyclophilin analysis

The estimate for the intensity of cyclophilin hybridization signals was obtained by measuring five random areas of tissue in each ovary section. Because corpora lutea were not present in PCOS/PCO ovaries, the cyclophilin signals in normal corpora lutea were not recorded. The average intensities were computed by a SigmaScan Pro Image Analysis program (version 5.0.0, SPSS, Inc., Chicago, IL). The cyclophilin results are presented as the mean  $\pm$  SE.

### Statistical analysis

Data were analyzed for statistical significance between ovaries by Mann-Whitney *U* test. When percentages of follicles with GDF-9 mRNA signal were compared, statistical significance was evaluated using  $\chi$ -square test or Fisher's exact probability test. Cyclophilin signals were analyzed by one-way ANOVA. Analyses were performed using Stat-View 5.0 software (Abacus Concept, Inc., Berkeley, CA). *P* values less than 0.05 were accepted as statistically significant.

## Results

Hybridization signals for GDF-9 and BMP-15 mRNA were readily detectable in the sections of all human ovaries examined (normal, PCOS, and PCO), and the signals were exclusively expressed in oocytes. Interestingly, there appeared to be a reduced expression of GDF-9 mRNA in PCOS ovaries when compared with normal (Fig. 1). This difference was not apparent for BMP-15 (Fig. 1). To obtain a clearer understanding of this difference, we performed a morphometric analysis of the levels of GDF-9 and BMP-15 mRNAs in the oocytes of normal and PCOS ovaries during the course of follicle development.

### GDF-9 mRNA in normal oocytes

We first characterized the pattern of GDF-9 mRNA expression in oocytes of normal ovaries for the purpose of establishing a standard to which the PCOS and PCO data could be meaningfully compared and interpreted. As seen in Table 1, a significant fraction (32%) of the oocytes in primordial follicles contained a positive albeit weak signal (Fig. 2a) for GDF-9. This finding is consistent with an early onset of oocyte GDF-9 gene expression when a normal human primordial follicle is released from growth arrest. Nearly all (96%) of the oocytes in normal primary follicles were positive for GDF-9 mRNA, and the hybridization signals were relatively strong (2+) in a few (8%) of these follicles (Table 1; Fig. 2b). In normal secondary follicles, the signal intensity for GDF-9 mRNA was generally higher than that observed in primary follicles, with 70% of the oocytes in secondary follicles showing a signal intensity of 2+ or greater (Table 2; Fig. 2c). We also identified three healthy small Graafian follicles with oocytes in normal ovaries. As seen in Fig. 2d, these oocytes showed strong (3+/4+) hybridization signals for GDF-9.

These results indicate that GDF-9 mRNA is normally ex-

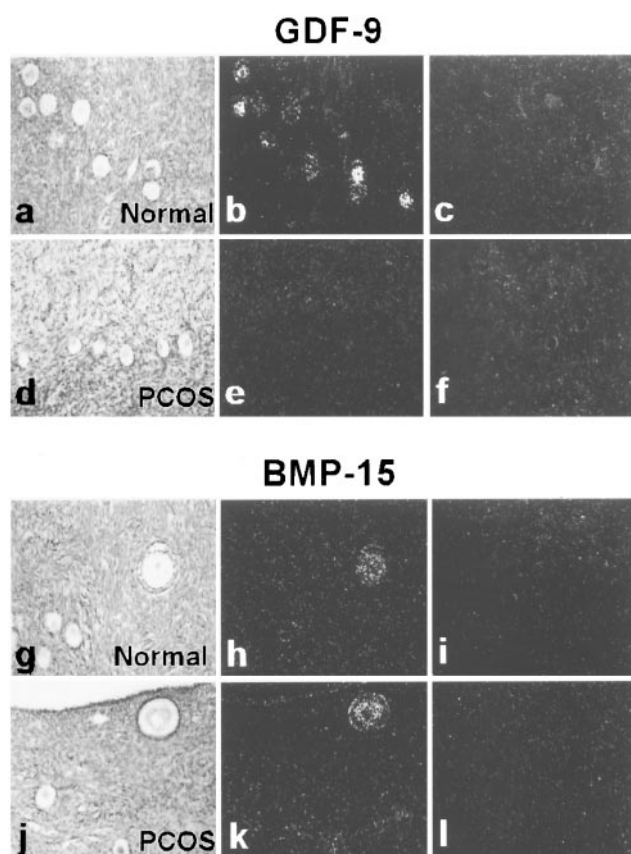


FIG. 1. *In situ* hybridization of GDF-9 (top) and BMP-15 (bottom) mRNAs in sections of normal and PCOS ovaries hybridized with the indicated antisense and sense probes. Bright field (a, d, g, and j) and dark field (b, c, e, f, h, i, k, and l) photomicrographs. White dots in the dark field micrographs represent silver grains in the photographic emulsion. Note positive hybridization signals (high concentration of silver grains) over some oocytes. Hybridization with the sense probes (c, f, i, and l) showed only a low level of nonspecific background labeling. Original magnification of the photographs,  $\times 10$ .

pressed in about one-third of the primordial follicles present in ovaries of cycling women. During folliculogenesis, the levels of oocyte GDF-9 mRNA increase progressively, with near maximum amounts being observed in oocytes of fully grown secondary follicles. GDF-9 mRNA levels remain high in oocytes of healthy small Graafian follicles.

#### GDF-9 mRNA in PCOS and PCO oocytes

Using this normal pattern of oocyte GDF-9 expression as a standard, we compared the levels of oocyte GDF-9 mRNA during folliculogenesis in PCOS and PCO. In contrast to normal, GDF-9 mRNA was not detected in PCOS and PCO primordial follicles (Table 1; Fig. 2e). In PCOS/PCO primary follicles, we observed GDF-9 transcripts in only a small fraction of the oocytes (8–12% in PCOS/PCO *vs.* 96% in normal), and when present, the signals were lower ( $P < 0.01$ ) than in normal ovaries (Table 1; Fig. 2f). A dramatic increase in the percentage of PCOS/PCO oocytes expressing detectable GDF-9 mRNA was observed when the follicles reached the secondary stage, but the hybridization signals were low ( $P < 0.01$ ), compared with normal (Table 1; Fig. 2g). As seen in Table 1, a significant fraction of the PCOS secondary follicles

were still negative for GDF-9 mRNA (43–60% in PCOS/PCO and 12% in normal). An examination of PCOS oocytes in six healthy small Graafian follicles revealed hybridization signals for GDF-9 (Fig. 2h), but the intensity appeared low ( $1+/2+$ ).

Because the PCOS and polycystic ovaries were archival, we were concerned that the low levels of oocyte GDF-9 mRNA may have suffered from long-term storage. To test this, we examined GDF-9 expression in the oocytes of a set of fresh Stein-Leventhal PCOS ovaries. Both the expression pattern and levels of GDF-9 mRNA in the fresh Stein-Leventhal PCOS oocytes were indistinguishable from those observed in the archival PCOS and PCO oocytes.

In summary, these findings demonstrate that in maturing PCOS and PCO oocytes, GDF-9 mRNA levels are lower than normal at all stages of folliculogenesis (Table 1; Fig. 3).

#### BMP-15 mRNA in normal and PCOS oocytes

We also examined whether PCOS oocytes displayed alterations in BMP-15 mRNA expression. BMP-15 mRNA was not detected in the primordial follicles of either normal or PCOS ovaries (Table 2; Fig. 2, i and m). Similarly, the vast majority ( $>90\%$ ) of oocytes in the normal and PCOS primary follicles were negative for BMP-15 mRNA (Table 2; Fig. 2, j and n); however, we did detect a weak hybridization signal in a few primary follicles (Table 2). This suggests that the onset of BMP-15 expression in maturing human oocytes correlates with the primary-to-secondary follicle transition stage. In secondary follicles, the hybridization signal for BMP-15 mRNA increased, and similar labeling patterns were seen in normal and PCOS oocytes (Table 2; Fig. 2, k and o). The oocytes in healthy small Graafian follicles strongly expressed BMP-15 mRNA ( $3+/4+$ ), and the levels appeared comparable in normal and PCOS ovaries (Fig. 2, l and p). Taken together, these data revealed no significant difference in the expression pattern and levels of BMP-15 mRNA between PCOS and normal oocytes during folliculogenesis.

#### Cyclophilin analysis

To provide further validation that the mRNA in the archival ovary samples had not suffered from long-term storage, we analyzed the intensity of the hybridization signals for a housekeeping gene, cyclophilin. Relatively high levels of cyclophilin mRNA were distributed rather uniformly throughout all the ovary samples examined, and the intensity of the cyclophilin hybridization signals were not significantly different among the normal, PCOS, and polycystic ovaries examined (Fig. 4).

#### Discussion

A key question in reproductive medicine concerns the nature of the abnormalities or defects that lead to PCOS and PCOs in women. The diversity in the endocrine profiles of women with PCOS and PCOs have led to the search for some unifying principle to explain the etiology and pathogenesis of this syndrome. Here, we found that a reduced level of GDF-9, but not BMP-15, mRNA is evident in growing oocytes in PCOS and polycystic ovaries from primordial follicle re-



**TABLE 1.** Quantification of the hybridization signal of GDF-9 mRNA in oocytes of developing follicles in Normal, PCOS, and PCO ovaries

Follicle type	Hybridization signal intensity: follicle no. (%)				
	ND	+1	+2	+3	+4
Primordial					
Normal <sup>a</sup> (n = 10) <sup>a</sup>	13 (68%)	6 (32%)	0	0	0
PCOS <sup>b</sup> (n = 5)	22 (100%)	0	0	0	0
PCO <sup>b</sup> (n = 2)	2 (100%)	0	0	0	0
Primary					
Normal <sup>c</sup> (n = 12)	3 (4%)	66 (88%)	6 (8%)	0	0
PCOS <sup>d</sup> (n = 5)	58 (92%)	5 (8%)	0	0	0
PCO <sup>d</sup> (n = 7)	21 (88%)	3 (12%)	0	0	0
Secondary					
Normal <sup>a</sup> (n = 10)	2 (12%)	2 (12%)	7 (41%)	5 (29%)	1 (6%)
PCOS <sup>b</sup> (n = 4)	6 (43%)	5 (36%)	3 (21%)	0	0
PCO <sup>b</sup> (n = 5)	6 (60%)	3 (30%)	1 (10%)	0	0
Graafian					
Normal <sup>b</sup> (n = 3)	0	0	1 (33%)	1 (33%)	1 (33%)
PCOS (n = 4)	0	2 (33%)	4 (67%)	0	0
PCO <sup>b</sup> (n = 1)	0	0	1 (100%)	0	0

The superscripts a, b, c, and d indicate significant differences in signal intensity at  $P < 0.01$  and  $P < 0.001$ , respectively. ND, Nondetectable.

<sup>a</sup> Number of patients whose ovaries contributed to the data.

<sup>b</sup> Statistical analysis could not be performed because the sample number was too small.

cruitment through the small Graafian follicle stage. On the basis of the data, we propose that a reduction in the levels of GDF-9 in growing primary oocytes may be a common denominator of PCOS and polycystic ovaries in women. It remains to be determined whether the reduced GDF-9 mRNA levels cause the follicular arrest in PCOS/PCO or whether they are simply an epiphenomenon of another primary abnormality. Nonetheless, because GDF-9 is required for proper folliculogenesis and female fertility, it is intriguing to hypothesize that aberrant expression of oocyte GDF-9 mRNA may contribute to the pathogenesis of follicle growth and development in PCOS and PCOs.

Previous studies have reported the expression of GDF-9 and BMP-15 mRNA in normal human oocytes in primary follicles (17, 30); however, until the present study, there had been no study of whether these growth factors are expressed at other stages of follicle development. With regard to GDF-9, we have found expression in normal human oocytes starting in the early stages of primordial follicle recruitment, increasing progressively to relatively high levels through the gonadotropin-independent preantral stages, and maintained at high levels in fully grown oocytes in small healthy Graafian (class 5) follicles. Therefore, as in the case of laboratory animals (17, 25), a major activity occurring within the human oocyte during normal folliculogenesis is the synthesis of GDF-9 mRNA. Because GDF-9 is obligatory for normal folliculogenesis and female fertility in mice (26), it will be interesting to determine whether GDF-9 functions as a critical regulator of follicle growth and development in women.

Consistent with the report of Aaltonen *et al.* (30), we found that BMP-15 transcripts first appear in normal human oocytes at the transition of the primary to the secondary follicle stage. This can be contrasted with the GDF-9 results in which the messenger RNA appears well before the appearance of a second layer of granulosa cells. We do not know whether this difference is attributable to a differential regulation of GDF-9 and BMP-15 mRNA expression during human oogenesis or decreased BMP-15 mRNA synthesis and/or

mRNA stabilization. Our observations indicate that normal human oocytes exhibit relatively high levels of BMP-15 mRNA through the rest of preantral follicle development and up to at least the small Graafian follicle stage. This is consistent with our previous study with rat oocytes *in vivo* showing a similar qualitative and quantitative pattern of BMP-15 mRNA and protein expression during the growth and differentiation phases (40). The challenge is to identify the physiological significance for the oocyte BMP-15 after the primary/secondary follicle transition. In this regard, it is notable that studies with mutant sheep have established the concept that oocyte BMP-15 is an important factor in determining folliculogenesis and ovulation quota *in vivo* (29). It remains to be determined whether this interesting concept operates in women.

Using these normal oocyte data as a standard, we found that the expression of GDF-9, but not BMP-15, mRNA appears aberrant in PCOS and PCO primary oocytes during their growth and differentiation phase. This observation is supported by the following data. First, GDF-9 mRNA expression in the vast majority of PCOS/PCO oocytes did not apparently begin until follicle development had reached the primary/secondary transition stage. This situation differed markedly from normal in which oocyte GDF-9 mRNA was present in about a third of the primordial follicles, evidently the newly recruited, and in almost all (96%) of the primary follicles. And second, when transcripts are detected, the intensity of the GDF-9 hybridization signals in PCOS and PCO oocytes appeared weaker, compared with normal oocytes in follicles at comparable stages of development. These changes were selective for GDF-9 because the pattern and levels of oocyte BMP-15 mRNA expression appear normal in PCOS follicles. Collectively, these data support the possibility that there may be some defect in the control mechanism governing GDF-9 mRNA expression in developing oocytes in women with PCOS and PCOs. It should be stressed that the question of whether the changes in GDF-9 mRNA are coupled with changes in GDF-9 protein synthesis or activity in

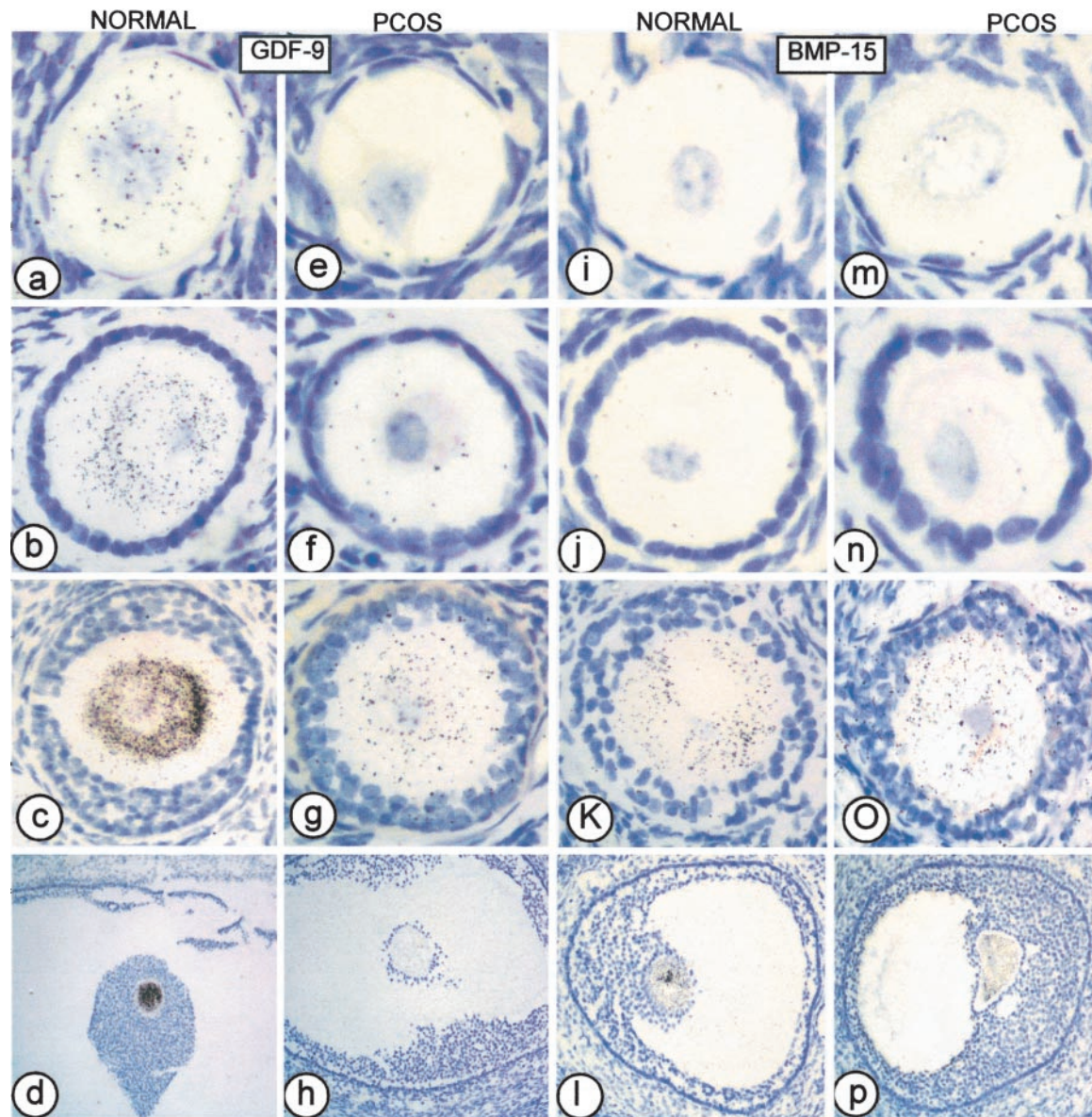


FIG. 2. Comparison of the *in situ* hybridization signals for GDF-9 (left two panels) and BMP-15 (right two panels) in human oocytes of primordial (row 1), primary (row 2), secondary (row 3), small Graafian follicles (row 4) in normal and PCOS ovaries. All photos are bright field micrographs hybridized with the indicated antisense probes. Black dots represent silver grains in the autoradiographic emulsion. Note the positive hybridization signals over the oocytes. The scoring system used to quantify the relative signal intensities can be seen in the micrographs in column 1 (GDF-9 normal): a, +; b, ++; c, +++; d, +++++. Original magnifications: rows 1 and 2,  $\times 40$ ; row 3,  $\times 20$ ; row 4,  $\times 10$ .

PCOS and PCO has yet to be answered. Nonetheless, it seems from our data that dysregulated expression of GDF-9 represents a common difference between normal and PCOS and PCO oocytes. The challenge now is to understand the cause of the aberrant expression of oocyte GDF-9 and its relationship to the pathology of PCOS and polycystic ovaries.

Little is known about the mechanisms controlling gene expression during mammalian oogenesis. Nonetheless, it is known that the mammalian oocyte, arrested in the dictyate stage of meiosis I, undergoes dramatic growth and cytodifferentiation during folliculogenesis. The growth phase is characterized by dramatic increases in the synthesis and accumulation of total RNA, including poly(A<sup>+</sup>) mRNAs (41–44). Some of these transcripts are translated during the

growth phase including zona pellucida 3 mRNA (45), GDF-9 (17, 24), and BMP-15 (40), but others like tissue-type plasminogen activator are not translated until meiotic maturation is initiated in the full-grown oocyte during ovulation (46). Thus, the amount of oocyte GDF-9 mRNA in the growing primary oocyte can be controlled at several levels including transcription, stability, and translation. It will be important to identify what molecular mechanisms (inhibition of synthesis, degradation/deadenylation, and/or increased translation) are responsible for the selective decrease in GDF-9 mRNA in growing primary oocytes in PCOS/PCO ovaries.

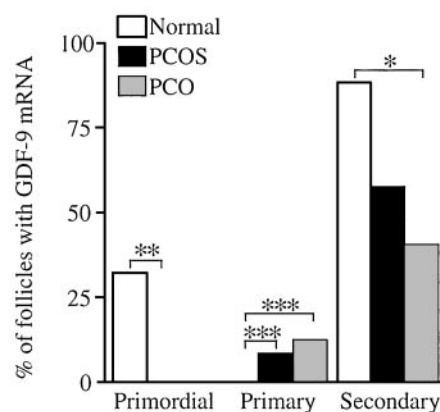
Although the biological consequences of this reduction in GDF-9 mRNA in developing PCOS and PCO oocytes are



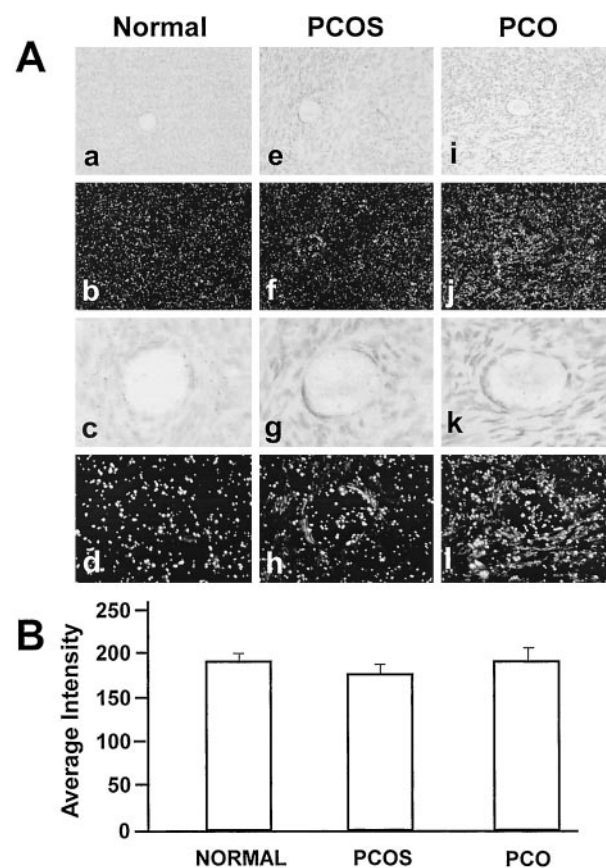
**TABLE 2.** Quantification of the hybridization signal of BMP-15 mRNA in oocytes of developing follicles of Normal and PCOS ovaries

Follicle type	Hybridization signal intensity: follicle no. (%)				
	ND	+1	+2	+3	+4
Primordial					
Normal (n = 8) <sup>a</sup>	8 (100%)	0	0	0	0
PCOS (n = 5)	15 (100%)	0	0	0	0
Primary					
Normal (n = 12)	94 (95%)	5 (5%)	0	0	0
PCOS (n = 5)	53 (91%)	5 (9%)	0	0	0
Secondary					
Normal (n = 6)	4 (31%)	4 (31%)	2 (15%)	2 (15%)	1 (8%)
PCOS (n = 4)	6 (50%)	1 (8%)	3 (25%)	1 (8%)	1 (8%)
Graafian					
Normal <sup>b</sup> (n = 3)	0	0	0	2 (67%)	1 (33%)
PCOS <sup>b</sup> (n = 2)	0	0	0	2 (100%)	0

ND, Nondetectable.

<sup>a</sup> Number of patients whose ovaries contributed to the data.<sup>b</sup> Statistical analysis could not be performed because the sample number was too small.**FIG. 3.** Histogram of the percentage of preantral follicles (primordial, primary, secondary) in normal, PCOS, and polycystic ovaries that show a positive hybridization signal for GDF-9. Data shown are means calculated from data presented in Table 1: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

unknown, the ability of GDF-9 to directly affect a wide variety of functional activities during folliculogenesis has been demonstrated by studies in rodents. For example, GDF-9 alone can stimulate preantral follicle growth *in vitro* (47) and *in vivo* (48), stimulate preantral granulosa cell mitosis (49), increase inhibin- $\alpha$  production (47), and inhibit kit ligand expression (49). These results illustrate that GDF-9 can also inhibit FSH-dependent granulosa responses, including E2, progesterone, and LH receptor expression (50). Furthermore, GDF-9 can regulate the processes of granulosa differentiation in preovulatory follicles including the induction of cumulus expansion, the stimulation of steroid acute regulatory protein, hyaluron synthetase 2, and cyclooxygenase-2 and the inhibition on LH receptor expression (51). The mechanism by which GDF-9 exerts these regulatory functions in differentiated granulosa cells is through the expression of an intrinsic PGE<sub>2</sub> ligand receptor signaling pathway, which acts as an autocrine/paracrine mediator of GDF-9 action (52). Finally, there is evidence in rats that GDF-9 can modulate steroid biosynthesis in cultured theca interstitial cells (53), emphasizing a potential role for GDF-9 in the mechanism of androgen production. Thus, the emerging concept is that oo-

**FIG. 4.** *In situ* hybridization signals for cyclophilin mRNA in sections of normal, PCOS, and polycystic ovaries after hybridization with the antisense probe. A, Representative photomicrographs of hybridization signals in the ovary cortex. a, e, i, Bright field low power ( $\times 10$ ); c, g, and k, bright field high power ( $\times 20$ ); b, f, j, d, h, and i, dark field of same photographs. B, Quantitative analysis of cyclophilin hybridization signals. Data are mean  $\pm$  SE.

cyte GDF-9 controls a multiplicity of activities that support the growth and differentiation of follicle cells from relatively early progenitors to mature cells of preovulatory follicles. In view of its importance in regulating follicle growth and development in laboratory animals, profiling the biological functions of GDF-9 in normal human ovaries should help us

understand the possible role of a dysregulation of GDF-9 expression in the pathogenesis of PCOS and polycystic ovaries in women.

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Address all correspondence and requests for reprints to: Gregory F. Erickson, Ph.D., Department of Reproductive Medicine, University of California San Diego, 9500 Gilman Drive, 2058 CMME, La Jolla, California 92093-0674. E-mail: gerickson@ucsd.edu.

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