Growth differentiation factor 3 is induced by bone morphogenetic protein 6 (BMP-6) and BMP-7 and increases luteinizing hormone receptor messenger RNA expression in human granulosa cells

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Objective: To examine the relevance of growth differentiation factor 3 (GDF-3) and bone morphogenetic protein (BMP) cytokines in human ovary.
Design: Molecular studies.
Setting: Research laboratory.
Patient(s): Eight women undergoing salpingo-oophorectomy and 30 women undergoing ovarian stimulation for in vitro fertilization.
Intervention(s): Localizing GDF-3 protein in human ovaries; granulosa cells (GC) cultured with GDF-3, BMP-6, or BMP-7 followed by RNA extraction.
Main Outcome Measure(s): The localization of GDF-3 protein in normal human ovaries via immunohistochemical analysis, GDF-3 messenger RNA (mRNA) expression evaluation via quantitative real-time reverse transcription and polymerase chain reaction (RT-PCR), and evaluation of the effect of GDF-3 on leuteinizing hormone (LH) receptor mRNA expression via quantitative real-time RT-PCR.
Result(s): In the ovary, BMP cytokines, of the transforming growth factor beta (TGF-β) superfamily, are known as a luteinization inhibitor by suppressing LH receptor expression in GC. Growth differentiation factor 3, a TGF-β superfamily cytokine, is recognized as an inhibitor of BMP cytokines in other cells. Immunohistochemical analysis showed that GDF-3 was strongly detected in the GC of antral follicles. An in vitro assay revealed that BMP-6 or BMP-7 induced GDF-3 mRNA in GC. Also, GDF-3 increased LH receptor mRNA expression and inhibited the effect of BMP-7, which suppressed the LH receptor mRNA expression in GC.
Conclusion(s): GDF-3, induced with BMP-6 and BMP-7, might play a role in folliculogenesis by inhibiting the effect of BMP cytokines. (Fertil Steril® 2012;97:979–83. ©2012 by American Society for Reproductive Medicine.)
Key Words: BMP, GDF-3, LH receptor, ovary

Follicular growth, selection, and ovulation are essential processes for the achievement of pregnancy. Once follicle growth progresses beyond the secondary stage, granulosa cells (GC) express follicle-stimulating hormone (FSH) receptor and proliferate under the stimulation of FSH (1). When the follicle approaches ovulation, luteinizing hormone (LH) receptor is increasingly expressed in GC. The increase in LH receptors makes GC sensitive to the LH surge that cues luteinization of these cells. Any perturbation of these events, such as premature luteinization or luteinization failure, has the potential to impair reproduction (2, 3).

Bone morphogenetic protein (BMP) cytokines, members of the transforming growth factor beta (TGF-β) superfamily, are required for follicular growth in many species. Oocytes express BMP-6 and BMP-15 and growth differentiation factor 9 (GDF-9); GC express BMP-2 and BMP-6; and theca cells express BMP-4 and BMP-7 (4, 5). The BMP cytokines contribute to folliculogenesis by inhibiting premature luteinization

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by suppressing LH receptors (4). It is interesting that mice or sheep with abnormalities in BMP signaling exhibit a precocious maturation of ovarian follicles (2–4). In conditional knockout mice against SMAD-4, which is a necessary factor for TGF-β ligand signaling, GC express a higher amount of LH receptors (3). Therefore, malfunction of the BMP cytokines, which inhibit premature luteinization by suppressing the LH receptor, may lead to inadequate follicular development and impaired fertility (4).

In normal ovaries, follicles still possess BMP cytokines in granulosa and theca cells as late as the preovulatory stage (4, 6, 7). Therefore, it is plausible that some mechanisms exist to suppress the antiluteinizing effect of BMP cytokines, especially in preovulatory follicles. But the precise mechanisms remain to be elucidated.

Growth differentiation factor 3, a member of TGF-β superfamily, is known to inhibit BMP cytokines in frog embryos (8, 9), human embryonic stem (ES) cells (9, 10), and mouse ES cells (9). In ES cells, GDF-3 inhibits BMP cytokines' signaling to maintain pluripotency of cells (9, 10). However, the role of GDF-3 in the ovary is still unclear. We hypothesized that GDF-3 might inhibit the effect of BMP cytokines, which are known as a luteinization inhibitor, to lead to follicular maturation. We investigated the localization of GDF-3 in the human ovary and examined its function, especially from the point of LH receptor mRNA expression in GC.

MATERIALS AND METHODS

The experimental procedures were approved by the institutional review board, and signed, informed consent for use of the samples was obtained from each patient. Except where otherwise indicated, all reagents were purchased from Sigma. Recombinant human GDF-3 and BMP-7 were purchased from R&D Systems. An antibody against GDF-3 was purchased from Lifespan Biosciences.

Collection of Ovarian Tissues and Immunohistochemistry

Tissue specimens of human ovaries were obtained after signed, informed consent from eight women (age range: 28 to 40 years) who underwent salpingo-oophorectomy for the treatment of uterine cervical cancer. All patients had normal ovarian cycles before surgery, and no histologic abnormalities or malignant lesions were observed in the ovarian tissues. Ovarian tissues were fixed in neutral-buffered formalin and embedded in paraffin blocks, and 6-μm sections were prepared. Antigen retrieval was performed using sodium citrate buffer (10 mM, pH 6.0) (11). The sections were stained with 10 μg/mL anti-GDF-3 antibody or rabbit IgG as a negative control by use of an Envision+ System/HRP rabbit kit (DAB+; Dako Japan). In some experiments, GDF-3 antibody, which had been preabsorbed with recombinant GDF-3 (50 μg/mL), was used as a preabsorption control.

Cell Culture of Human Granulosa Cells (GC)

Granulosa cells were obtained from 30 patients undergoing ovarian stimulation for in vitro fertilization (IVF). The method to purify and culture human GC was described previously elsewhere (12). The collected human GC were cultured in Dulbecco's modified eagle medium/nutrient mixture F-12 (DMEM/F-12) containing 5% fetal bovine serum (FBS) and antibiotics in 12-well plates at a density of 2×10^5 cells/mL. To investigate the regulation of GDF-3, GC were cultured with BMP-6 (100 ng/mL) or BMP-7 (100 ng/mL) for 24 hours. To evaluate the effects of GDF-3, human GC were cultured with or without GDF-3 (60–100 ng/mL) for 24 hours in the presence or absence of BMP-7 (10–100 ng/mL). The GDF-3 was used 30 minutes before stimulation with BMP-7.

Reverse Transcription (RT) and Quantitative Real-Time Polymerase Chain Reaction (PCR) Analysis

Total RNA was extracted from GC by use of the RNeasy mini-kit (Qiagen). Reverse transcription (RT) was performed using Rever Tra Dash (Toyobo). One microgram of total RNA was reverse transcribed in a 20-μL volume. For the quantification of various mRNA levels, real-time polymerase chain reaction (PCR) was performed using a LightCycler (Roche Diagnostic GmbH), according to the manufacturer's instructions. The PCR primers were selected from different exons of the corresponding genes to discriminate the PCR products that might arise from possible chromosomal DNA contaminants. The primer sequences and real-time PCR conditions were previously described elsewhere (12), with the exception of the primer sequence of GDF-3 (NM_020634: 152-171 and 410-391). Relative expression of each mRNA was normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. All results were shown as mean $\pm$ standard deviation (SD) of data from at least three separate experiments. Data were analyzed by one-way analysis of variance (ANOVA) with post hoc test for multiple comparisons. $P < .05$ was considered statistically significant.

RESULTS

The Localization of GDF-3 in Human Ovaries

The localization of GDF-3 protein in human ovaries was examined by immunohistochemistry using normal human ovaries. The intensity of the staining of GDF-3 in GC was negative in primordial and primary follicles; and weak in GC of secondary follicles (Fig. 1A, 1C, and 1D), while GDF-3 protein was detected in the oocytes of primordial, primary, and secondary follicles. As shown in Figure 1E and G, GDF-3 was strongly detected in the GC of antral follicles. With preabsorption GDF-3 antibody, which had been preabsorbed with recombinant GDF-3, the intensity was decreased (see Fig. 1I).

Regulation of GDF-3 in GC

To investigate the regulation of GDF-3 gene expression, human GC were cultured with BMP-6 or BMP-7. As shown in Figure 2, there was an approximately fourfold to fivefold ($n = 5$, $P < .01$) increase in GDF-3 mRNA expression with BMP-6 or BMP-7 stimulation compared with control samples.