Menopause: The Journal of The North American Menopause Society
Vol. 12, No. 6, pp. 749-754
DOI: 10.1097/01.gme.0000184424.96437.7a

Mutational screening of the coding region of growth differentiation factor 9 gene in Indian women with ovarian failure

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Abstract

Objective: To establish the risk associated with mutations in the coding region of *GDF9* gene in Indian women with ovarian failure.

Design: This case-control study was designed for mutational analysis of the *GDF9* coding region in a cohort of women with premature ovarian failure (n = 127), primary amenorrhea (n = 58), and secondary amenorrhea (n = 10) compared with controls (n = 220).

Results: This case-control study revealed eight mutations in the *GDF9* gene, including five novel mutations: c.1-8C>T, c.199A>C (p.Lys67Glu), c. 205C>T, c.646G>A (p.Val216Mat), and c.1353C>T, and three documented mutations: c.398-39C>G, c.447C>T, and c.546G>A. Missense mutation c.199A>C was present in 4 of 127 premature ovarian failure (POF) cases and 1 of 10 secondary amenorrhea cases. The c.646G>A mutation was present in two POF cases. Both missense mutations were absent in controls. Genotype distribution of c.447C>T was significantly different in POF cases than controls ($\chi^2 = 5.93$, $P = 0.05$). We chose two frequent single-nucleotide polymorphisms (c.398-39C>G, c.447C>T) for haplotyping and found that the C-T haplotype was significantly higher in patients ($P = 0.03$), whereas the C-C haplotype was representative of the control group.

Conclusions: We report two rare missense mutations, c.199A>C and c.646G>A, which are associated with ovarian failure. The presence of the c.447>T mutation might indicate a higher risk for POF. Haplotype C-T was significantly associated with ovarian failure, whereas the C-C haplotype was representative of the control group.

Key Words: Ovarian failure – *GDF9* – Mutation – Candidate gene.

Premature ovarian failure (POF, MIM#311360) is due to loss of functional follicles in women younger than 40 years with unexplained amenorrhea (of more than 6 months duration), high gonadotropin levels (follicle-stimulating hormone [FSH] > 40 IU/L), and low estrogen levels. It occurs in approximately 1% of women.[1] A wide spectrum of genetic and nongenetic mechanisms have been suggested as an explanation for the etiopathogenesis of ovarian failure. Some nongenetic mechanisms include immunological abnormalities, physical stress, nutrient deficiency, chemotherapy, and radiation effects. Genetic mechanisms involve chromosomal abnormalities, especially on the X-chromosome, and mutations in candidate genes. X-chromosomal abnormalities include many susceptible locus/genes, such as *FMR1*, *DIA*, *POF1*, *POF2*, *ZFX*, and *FMR2*.[2] Many case-control studies have been performed, in various populations for candidate genes of the hypothalamus-pituitary-ovarian axis, including *FSH*β, *LH*β, *FSHR*, and *LHR*.[3] It has

Received July 30, 2004; revised and accepted March 7, 2005.

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This study was supported by a grant from the Indian Council of Medical Research, New Delhi. H.D. was supported by fellowship from the Council of Scientific and Industrial Research (CSIR), Government of India.

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been reported that the Ala189Val substitution in the extracellular domain of FSH receptor (*FSHR* gene) is strongly associated with ovarian failure in a Finnish population.[4] Mutations in the *FSHR* gene are not commonly associated with ovarian failure in other populations.

The *FOXL2* gene has been studied in various populations as an important candidate gene for ovarian failure. FOXL2, a member of the winged helix/forkhead transcription factor family, is expressed predominantly in the developing human eyelid and mature ovary. Mutations were reported in *b*lepharophimosis/*p*tosis/*e*picanthus inversus *s*yndrome (BPES) type 1 and type 2 familial cases.[5] In type 1 BPES, females inherit ovarian failure in addition to eyelid defects, whereas only the eyelid abnormalities are seen in type 2 BPES. Truncated FOXL2 is associated with BPES type 1, whereas the expanded form of the protein is associated with BPES type 2.[5] Mutations in the *FOXL2* gene in patients with nonsyndromic POF have been found, but these are rare in diverse populations.

In recent studies, the *INH*α gene has been credited to be a very important candidate gene responsible for ovarian failure. Strong associations with the c.769G>A (p.Ala253Thr) mutation in its mature peptide region have been documented among different world populations, including an Indian population.[6-8] Inhibins are produced by granulosa cells surrounding oocytes in the ovary. Inhibins regulate FSH production by gonadotrophs in the anterior pituitary through a negative feedback mechanism. Lower serum levels of inhibin and higher levels of FSH in POF cases further strengthen the physiological role of inhibins in the negative feedback control of FSH.[9] A strong association of *INH*α gene mutation with ovarian failure strongly suggests the need for further studies of candidate genes involved in the negative feedback control network of FSH regulation by inhibin. Inhibin production is positively regulated by two oocyte-secreted growth factors, namely GDF9 (growth differentiation factor 9) and BMP15 (bone morphogenetic protein 15).[10] A mutation in any of the two factors can decrease inhibin production and lead to ovarian failure. This study presents a mutational analysis of the *GDF9* gene in Indian women with ovarian failure.

GDF9 is a member of the transforming growth factor β (TGFβ) superfamily and is exclusively expressed in oocytes. GDF9 is involved in oocyte control of cumulus expansion, regulation of several key enzymes of granulosa cells, and granulosa cell proliferation and differentiation.[11,12] *GDF9*-knockout female mice demonstrate infertility and lack of ovarian development.[13] The first mutational screening of the *GDF9* gene was reported in Japanese women with ovarian failure, although no mutations were found.[14] Here we report the mutational screening results of the *GDF9* gene, which revealed two rare missense mutations in propeptide region associated with ovarian failure. Haplotype analysis and statistical observations showed that the *GDF9* gene is an important candidate gene for ovarian failure.

METHODS

Patient and control recruitment

A total of 195 patients with ovarian failure were recruited, including women with nonfamilial POF (n = 116), familial POF (n = 11), nonfamilial primary amenorrhea (n = 53), familial primary amenorrhea (n = 5), and secondary amenorrhea (n = 10). Patients were recruited at the Infertility Institute and Research Centre, Hyderabad, and the Institute of Reproductive Medicine, Kolkata. The diagnostic criteria for POF include at least 6 months of amenorrhea before the age of 40 years and a high FSH serum level (40 IU/L). Primary amenorrhea is defined as a the complete absence of menses or only induced menses. Secondary amenorrhea is defined as a cessation of menses with a history of menses before the age of 40 years. All patients were assessed clinically, with complete medical and gynecological history including history of menses, age at menopause, serum FSH levels (assessed three times at 1-month intervals), and serum luteinizing hormone (LH) levels; no patient had a history of autoimmune disease. Patient consent forms were collected by dedicated clinic members. Karyotyping with high-resolution GTG banding was performed for all patients to test for cytogenetic anomalies. Patients with chromosomal abnormalities were excluded from the study. Controls (n = 220) were healthy women with a regular menstrual history, normal FSH level, and a history of successful pregnancies. Control recruitment was population based to support the study.

DNA extraction and karyotyping

A 5-mL aliquot of peripheral blood was collected in EDTA vacutainers for genomic DNA isolation, and another 5 mL of peripheral blood was collected in heparin vacutainers for cytogenetic analysis. DNA was extracted using the Nucleon BACC2 DNA extraction kit (Amersham Pharmacia Biotech) according to the manufacturer's protocol. Chromosomal analysis was performed on phytohemagglutinin-stimulated peripheral lymphocyte cultures using standard cytogenetic methods.

Polymerase chain reaction

The *GDF9* gene comprises two exons 397 bp and 968 bp in length. Primers and polymerase chain reaction (PCR) conditions followed those described by Takebayashi et al.[14] The first exon was amplified using GDF9F1 and GDF9R1 primers. The first half of the second exon was amplified using GDF9F2 and GDF9R2 primers, and the second half of the second exon was amplified using GDF9F3 and GDF9R3 primers.

DNA sequencing

All PCR products were obtained from the above primers amplifying the coding region of the *GDF9* gene. Sequencing was performed using the Big Dye terminator sequencing protocol, supported by Applied Biosystems using an ABI prism 3730 DNA analyzer.

Statistical analysis

The STAT-SAK program was used to perform Fisher's exact and χ^2 tests, and to determine odds ratios and CIs. SNPAlyzer software was used for case-control haplotype analysis and Hardy-Weinberg disequilibrium.

RESULTS

We performed a sequence analysis of the *GDF9* coding region in women with nonfamilial POF (n = 116), familial POF (n = 11), nonfamilial primary amenorrhea (n = 53), familial primary amenorrhea (n = 5), secondary amenorrhea (n = 10), and in controls (n = 220). Patient and control populations were in Hardy-Weinberg equilibrium for genetic variations. All POF cases had a mean age of attaining amenorrhea of 26 years (range 14-39 years), mean FSH level of 59.3 IU/L, and mean LH level of 33.7 IU/L compared with a mean age of controls of 37 years (range 30-45 years). Our results revealed eight mutations, including five novel mutations: c.1-8C>T (promoter region), c.199A>C (p.Lys67Glu), c205C>T (silent), c.646G>A (p.Val216Mat), and c.1353C>T (silent), and three documented mutations: c.398-39C>G (intronic), c.447C>T (silent), and c.546G>A (silent). All mutations were confirmed by repeating the sequencing three times, including sequencing the amplification product in the reverse direction. Details of mutations are given in Table 1. Interestingly, both novel missense mutations c.199A>C (p.Lys67Glu) and c.646G>A (p.Val216Mat) were rare and associated with ovarian failure, and were completely absent in 220 controls. Mutation c.199A>C was present in 4 of 127 POF cases and 1 of 10 secondary amenorrhea cases. Mutation c.646G>A was present in 2 of 127 POF cases. Clinical details of patients carrying these missense mutations are given in Table 2. POF cases carrying the above-mentioned missense mutations showed higher levels of FSH and LH compared with mean levels of these hormones in all POF cases. Both missense mutations are present in propeptide region of GDF9 protein, and therefore may affect the processing of propeptide cleavage. The occurrence of silent mutation c.447C>T was higher in POF cases than in controls. We used the χ^2 test to determine the independence of genotype frequencies of two frequent mutations, c.398-39C>G (intronic) and c.447C>T (silent), as shown in Table 3. χ^2 analysis showed a significant difference in genotype distribution of c.447C>T mutation in POF cases ($\chi^2 = 5.93$, $P = 0.005$). We performed haplotype analysis for two frequent single-nucleotide polymorphisms, c.398-39C>G and c.447C>T, using SNPAlyzer software. We found that the C-T haplotype was significantly higher in patients ($P = 0.03$), whereas the C-C haplotype was representative of the control group ($P = 0.004$), as shown in Table 4.

DISCUSSION

A genetic explanation of the etiology of POF is still unavailable. In recent studies, missense mutation c.769G>A (p.Ala257Thr) in the mature peptide region of the *INH*α gene is strongly associated with ovarian failure in various populations, including Indian populations.[6-8] A strong association with the *INH*α

TABLE 1. *Summary of GDF9 gene mutation analysis*

db SNP ref. ID	SNP position	Nucleotide change	Amino acid change	Protein domain	POF	PA	SA	Control
Novel	Promoter	c.1-8C>T	—	—	1/127	1/58	0/10	4/220
Novel	Exon 1	c.199A>C	p.Lys67Glu	Propeptide	4/127	0/58	1/10	0/220
Novel	Exon 1	c.205C>T	Silent	—	0/127	0/58	1/10	0/220
rs254285	Intron	c.398-39C>G	—	—	31/127	10/58	2/10	40/220
rs254286	Exon 2	c.447C>T	Silent	—	108/127	48/58	9/10	171/220
rs10491279	Exon 2	c.546G>A	Silent	—	4/127	1/58	0/10	12/220
Novel	Exon 2	c.646G>A	p.Val216Mat	Propeptide	2/127	0/58	0/10	0/220
Novel	Exon 2	c.1353C>T	Silent	—	1/127	2/58	0/10	9/220

SNP, single nucleotide polymorphism; POF, premature ovarian failure; PA, primary amenorrhoea; SA, secondary amenorrhoea.

TABLE 2. *Clinical details of the patients with missense mutations*

Patient ID	Age at menopause (y)	Mutation	Phenotype	FSH level (IU/L)	LH level (IU/L)	TSH level (mIU/L)
38	18	c.199A>C	Sec. amenorrhea	27	19	2.6
120	33	c.199A>C	POF	68	45	2.8
170	25	c.199A>C	POF	58	32	1.3
237	17	c.199A>C	POF	82	51	NA
265	28	c.199A>C	POF	88	37	4.0
60	27	c.646G>A	POF	56	41	NA
164	28	c.646G>A	POF	110	36	2.0

FSH, follicle-stimulating hormone; LH, luteinizing hormone; TSH, thyroid-stimulating hormone; POF, premature ovarian failure.

gene suggests the possible involvement of a regulatory network component that contributes to the negative feedback control of FSH by inhibins. Two oocyte-derived growth factors, namely GDF9 and BMP15, enhance inhibin production by granulosa cells.[10] Mutations in GDF9 or BMP15 growth factors can cause inhibin production, perturb oocyte granulosa cell microenvironments, and obstruct follicle development. The key roles of these growth factors suggest their possible involvement in the etiology of ovarian failure. Many mutation studies of *GDF9* and *BMP15* genes have been performed in sheep manifesting higher ovulation rates and infertility problems. A mutational screening of human *GDF9* gene in a Japanese population with ovarian failure found no mutations.[14] We studied the mutational status of the *GDF9* gene in Indian women and report two rare missense mutations, c.199A>C and c.646G>A, associated with ovarian failure.

The GDF9 member of the TGFβ family was the first oocyte-derived growth factor shown to be required for normal growth and maturation of ovarian somatic cells. Its production is restricted to oocytes in the human ovary.[15] GDF9 plays a very important paracrine role in the maintenance, differentiation, and proliferation of granulosa cells surrounding the oocyte in the ovary. Ovarian GDF9 mRNA is first expressed at the primary follicle stage and persists even after ovulation in mammals.[16]

Like other TGFβ family members, GDF9 is also synthesized as preproprotein and processed by the cleavage of a signal peptide. After cleaving the peptide, GDF9 proprotein can noncovalently bind with either GDF9 propeptide or BMP15 propeptide, resulting in homodimer or heterodimer formation. GDF9 proprotein (like other TGFβ members) contains a proteolytic cleavage site between the prodomain and mature peptide. These propeptide regions are cleaved by specific endoproteases, resulting in dimers of mature peptides. Mature peptide dimers bind to specific receptors located on granulosa cells of follicles and activate the Smad-signaling pathway. GDF9 ligand binds and activates its type II receptor BMPRII (bone morphogenetic protein receptor II), which later interacts with type I receptor ALK5.[17,18] The activated ALK5 receptor initiates intracellular signal transmission into the nucleus of granulosa cells via the Smad2-mediated pathway.

Naturally occurring mutations of the *GDF9* gene uncovered in sheep were used for functional analysis to reveal the mechanism behind impaired activity of

TABLE 3. *Genotype frequencies of GDF9 sequence variants in patients and controls*

SNP and genotype	No. (%) of subjects with genotype			
	POF	PA	SA	Controls
c.398-39C>G	$\chi2 = 1.919$ $P = 0.16$	$\chi2 = 0.275$ $P = 0.87$	$\chi2 = 0.02$ $P = 0.88$	
CC	96/127 (75.6%)	48/58 (82.76%)	8/10 (80%)	180/220 (81.8%)
CG	31/127 (24.4%)	10/58 (17.24%)	2/10 (20%)	40/220 (18.2%)
GG	0/127	0/58	0/10	0/220
c.447C>T	$\chi2 = 5.93$ $P = 0.05$[a]	$\chi2 = 4.1$ $P = 0.13$	$\chi2 = 2.586$ $P = 0.27$	
CC	19/127 (14.96%)	10/58 (18.96%)	1/10 (10%)	49/220 (22.3%)
CT	47/127 (37.0%)	19/58 (32.76%)	3/10 (30%)	93/220 (42.72%)
TT	61/127 (48.03%)	29/58 (50%)	6/10 (60%)	78/220 (35.45%)

SNP, single nucleotide polymorphism; POF, premature ovarian failure; PA, primary amenorrhea; SA, secondary amenorrhea.
[a]Significant *P* value.

TABLE 4. *Haplotype frequencies in patients and controls*

Haplotype	Overall frequency	POF	PA	SA	Control	$\chi 2$	*P* value
C-C	0.2940	0.2126	0.2672	0.1500	0.3618	22.24	0.004[a]
G-C	0.0928	0.1220	0.0689	0.1000	0.0722	5.62	0.114
C-T	0.6130	0.6653	0.6638	0.7500	0.5472	11.45	0.03[a]

POF, premature ovarian failure; PA, primary amenorrhea; SA, secondary amenorrhea.
[a]Significant *P* value.

GDF9 and its other partner, BMP15.[19,20] Liao et al.[19] demonstrated that the proprotein heterodimer is significantly less susceptible to proteolytic cleavage than the individual homodimers of GDF9/BMP15. They hypothesized that mutations in *BMP15* and/or *GDF9* can lead to ovarian failure due to impaired prodomain cleavage. Mutations in *GDF9* or *BMP15* do not primarily inhibit homo/heterodimer formation, but result in unstable heterodimers that undergo rapid proteolytic degradation. There is also increasing evidence that germline mutations in these genes also lead to protein misfolding during protein synthesis itself. *BMP15* mutation in sheep can also affect GDF9 biosynthesis in a dominant-negative fashion. Studies of recombinant human GDF9 and BMP15 carrying naturally occurring mutations in sheep showed that mutations in *GDF9* or *BMP15* severely impaired secretion.[20] Functional characterization of naturally occurring mutations in both *GDF9* and *BMP15* suggest impaired secretion of mature dimer as a primary mechanism, but this hypothesis needs to be further elucidated.

Female *GDF9*-knockout mice show a lack of ovarian development and infertility.[16] *GDF9*-null mice show formation of primordial and primary one-layer follicles but arrest of follicular development beyond the primary one-layer follicle stage. In null mice, oocyte growth and zona pellucida formation proceed normally, but other aspects of oocyte differentiation are compromised. GDF9 antagonizes FSH-induced differentiation of granulosa cells, estrogen production, progesterone production, and LH receptor formation by cultured granulosa cells.[21] Despite this antagonistic effect, GDF9 synergistically stimulates inhibin production and FSH in a stage-dependent manner, probably by posttranscriptional actions.[10]

Mutations in the *GDF9* gene may adversely affect granulosa cell proliferation and differentiation, inhibin production, and subsequent FSH level modulation. The mechanism of GDF9 paracrine action determines its importance as a significant candidate gene for POF. It has been established that women with POF contain normal primordial follicles but lack mature follicles. *In vivo* doses of recombinant GDF9 in immature female rats stimulated progression of primordial and primary follicles to preantral follicles as well as thecal layer development.[22] Treatment with recombinant GDF9 can induce proliferation and differentiation of follicles, and therefore may be beneficial for women with POF. This concept urges a need to investigate the germ-line mutational status of *GDF9* in various populations.

The first mutational screening of *GDF9* in Japanese women with ovarian disorders did not reveal any genetic variation in the *GDF9* coding region.[14] Mutational screening of the *GDF9* coding region in Indian women with ovarian failure revealed eight polymorphisms, including the five novel mutations listed in Table 1. The different mutational status of Japanese and Indian populations is probably indicative of ethnic differences. Here we report two rare missense mutations (c.199A>C, c.646G>A) present in the propeptide region of GDF9 protein and associated with ovarian failure with complete absence in controls. Mutation c.199A>C altered lysine, a basic amino acid, into glutamate, an acidic amino acid at the 67 position. Mutation c.646G>A altered valine, a hydrophobic nonsulfur amino acid, into methionine, a slightly polar sulfur-containing amino acid at the 216 position. Moreover, valine^{216} is highly conserved across species including sheep, chicken, mouse, rat, and bovine. Haplotype analysis suggested a significant association of the C-T haplotype with ovarian failure, whereas the C-C haplotype was representative of controls. We hypothesize that *GDF9* may have a spectrum of mutations in various world populations rather than a common causative mutation. Our study signifies an imperative mutational screening of a crucial candidate gene that should also be studied in other world populations. Our study will also lead to functional studies of missense mutations in human cell lines similar to those performed in sheep.

CONCLUSIONS

We report eight polymorphisms, including five novel mutations. The two rare missense mutations that we report, c.199A>C and c.646G>A, are associated with ovarian failure and may impair secretion and interfere

with propeptide processing from the mature peptide region. The presence of the c.447>T mutation might indicate a higher risk for POF. Haplotype C-T was significantly associated with ovarian failure, whereas the C-C haplotype was representative of the control group. Mutational screening of the *GDF9* coding region should be performed in other populations to elucidate these findings.

Acknowledgements: The authors thank Mrs. D.V. Jayanthi of the Infertility Institute and Research Centre, Secunderabad, for helping with patient recruitment, and Dr. Sujatha for helping with the recruitment of controls.

REFERENCES

1. Coulam CB, Adamson SC, Annegers JF. Incidence of premature ovarian failure. *Obstet Gynecol* 1986;67:604-606.
2. Schlessinger D, Herrera L, Crisponi L, et al. Genes and translocations involved in POF. *Am J Med Genet* 2002;111:328-333.
3. Achermann JC, Ozisik G, Meeks JJ, Jameson JL. Genetic causes of human reproductive disease. *J Clin Endocrinol Metab* 2002; 87:2447-2454.
4. Aittomaki K, Lucena JLD, Pakarinen P, et al. Mutation in the follicle stimulating hormone receptor gene causes hereditary hypergonadotropic ovarian failure. *Cell* 1995;82:959-968.
5. Crisponi L, Deiana M, Loi A, et al. The putative forkhead transcription factor FOXL2 is mutated in blepharophimosis/ptosis/epicanthus/inversus syndrome. *Nat Genet* 2001;27:159-166.
6. Shelling AN, Burton KA, Chand AL, et al. Inhibin: a candidate gene for premature ovarian failure. *Hum Reprod* 2000;15:2644-2649.
7. Marozzi A, Porta C, Vegetti W, et al. Mutation analysis of the inhibin alpha gene in a cohort of Italian women affected by ovarian failure. *Hum Reprod* 2002;17:1741-1745.
8. Dixit H, Deenadayal M, Singh L. Mutational analysis of the mature peptide region of inhibin genes in Indian women with ovarian failure. *Hum Reprod* 2004;19:1760-1764.
9. Welt CK, Hall JE, Adams JM, Taylor AE. Relationship of estradiol and inhibin to the FSH variability in hypergonadotropic hypogonadism or premature ovarian failure. *J Clin Endocrinol Metab* 2004;DOI:10.1210/jc.2004-1319.
10. Roh JS, Bondestam J, Mazerbourg S, et al. Growth differentiation factor-9 stimulates inhibin production and activates Smad2 in cultured rat granulosa cells. *Endocrinology* 2003;144:172-178.
11. Elvin JA, Clark AT, Wang P, Wolfman NM, Matzuk MM. Paracrine actions of growth differentiation factor-9 in the mammalian ovary. *Mol Endocrinol* 1999;13:1035-1048.
12. Latham KE, Wigglesworth K, McMenamin M, Eppig JJ. Stage-dependent effects of oocytes and growth differentiation factor 9 on mouse granulosa cell development: advance programming and subsequent control of the transition from preantral secondary follicles to early antral tertiary follicles. *Biol Reprod* 2004;70: 1253-1262.
13. Dong JW, Albertini DF, Nishimori K, Kumar TR, Lu NF, Matzuk MM. Growth differentiation factor-9 is required during early ovarian growth folliculogenesis. *Nature* 1996;383: 531-535.
14. Takebayashi K, Takakura K, Wang HQ, Kimura F, Kasahara K, Noda Y. Mutation analysis of the growth differentiation factor-9 and -9B genes in patients with premature ovarian failure and polycystic ovary syndrome. *Fertil Steril* 2000;74:976-979.
15. Fitzpatrick SL, Sindoni DM, Shughrue PJ, Lane MV, Merchenthaler IJ, Frail DE. Expression of growth differentiation factor-9 messenger ribonucleic acid in ovarian and non-ovarian rodent and human tissues. *Endocrinology* 1998;139:2571-2578.
16. Aaltonen J, Laitinen MP, Vuojolainen K, et al. Human growth differentiation factor 9 (GDF-9) and its novel homolog GDF-9B are expressed in oocytes during early folliculogenesis. *J Clin Endocrinol Metab* 1999;84:2744-2750.
17. Vitt UA, Mazerbourg S, Klein C, Hsueh AJW. Bone morphogenetic protein receptor type II is a receptor for growth differentiation factor-9. *Biol Reprod* 2002;67:473-480.
18. Mazerbourg S, Klein C, Roh J, et al. Growth differentiation factor-9 signaling is mediated by the type I receptor, activin receptor-like kinase 5. *Mol Endocrinol* 2004;18:653-665.
19. Liao WX, Moore RK, Otsuka F, Shimasaki S. Effect of intracellular interaction on the processing and secretion of Bone morphogenetic protein-15 (BMP-15) and growth and differentiation factor-9. *J Biol Chem* 2003;279:3713-3719.
20. Liao WX, Moore RK, Shimasaki S. Functional and molecular characterization of naturally occurring mutations in the oocyte-secreted factors bone morphogenetic protein-15 and growth and differentiation factor-9. *J Biol Chem* 2004;279:17391-17395.
21. Vitt UA, Hayashi M, Klein C, Hsueh AJW. Growth differentiation factor-9 stimulates proliferation but suppresses the follicle-stimulating hormone-induced differentiation of cultured granulosa cells from small antral and preovulatory rat follicles. *Biol Reprod* 2000;62:370-377.
22. Vitt UA, McGee EA, Hayashi M, Hsueh AJW. *In vivo* treatment with GDF-9 stimulates primordial and primary follicle progression and theca cell marker CYP17 in ovaries of immature rats. *Endocrinology* 2000;141:3814-3820.