CLINICAL STUDY

Mutations and sequence variants in *GDF9* and *BMP15* in patients with premature ovarian failure

Paul Laissue, Sophie Christin-Maitre¹, Philippe Touraine², Frederique Kuttann², Olli Ritvos³, Kristiina Aittomäki⁴, Nathalie Bourcigaux¹, Laetitia Jacquesson¹, Philippe Bouchard¹, Rene Frydman⁵, Didier Dewailly⁶, Anne-Céline Reyss⁶, Luke Jeffery³, Anne Bachelot², Nathalie Massin⁷, Marc Fellous and Reiner A Veitia

INSERM 709, Genomics and Epigenetics of Placental Pathology, Hôpital Cochin, Pavillon Baudelocque, 123 Bd de Port Royal, 75014, Paris, France, ¹Reproductive Endocrine Unit, Hôpital Saint-Antoine, Faculté Pierre et Marie Curie, UPR 1533, 75012 Paris, France, ²Faculty of Medicine, Université Paris V, Necker Hospital, APHP, 149 rue de Sèvres, 75743 Paris cedex 15, France, ³Program of Developmental and Reproductive Biology, Biomedicum Helsinki, University of Helsinki, Helsinki, Finland, ⁴Department of Clinical Genetics, Helsinki University Hospital, Helsinki, Finland, ⁵Department of Gynaecology and Obstetrics, Hôpital Antoine Béchère, 92141, Clamart cedex, France, ⁶Department of Endocrine Gynaecology and Reproductive Medicine, Hôpital Jeanne de Flandre, CHRU, 59037, Lille, France, and ⁷Department of Gynaecology and Obstetrics, Reproductive Unit, Intercommunal Hospital, 94110 Creteil, France

(Correspondence should be addressed to R. A. Veitia; Email: veitia@cochin.inserm.fr)

Abstract

Background and objective: Mutations in bone morphogenic protein 15 (*BMP15*) and growth/differentiation factor 9 (*GDF9*) lead to altered fertility in animal models. In the human, a heterozygous point mutation of *BMP15* has been associated with premature ovarian failure (POF).

Subject and methods: We have directly sequenced both genes in a cohort of 203 POF patients presenting with primary or secondary amenorrhea and high FSH levels and in a control population including 54 women with regular menstrual cycles who had at least one child.

Results: We have identified several heterozygous variants. One alteration in *GDF9* (S186Y) and one in *BMP15* (L148P) may have pathogenic effects as both positions are conserved in vertebrate species, ranging from the chicken to mammals. These variants were absent in the control samples. We also found synonymous and neutral substitutions.

Conclusions: We propose that although mutations in *BMP15* and *GDF9* are not a major cause of ovarian insufficiency, they may be involved in POF.

European Journal of Endocrinology 154 739–744

Introduction

Premature ovarian failure (POF) is defined as amenorrhea for at least 6 months occurring before the age of 40 years, associated with raised plasma gonadotrophin levels (follicle-stimulating hormone (FSH) greater than 40 mIU/ml) (1). POF has a prevalence of 1%. Ovarian failure can result from viral or auto-immune oophoritis, environmental toxins and iatrogenic causes such as pelvic surgery, radiation or chemotherapy. Deletions and translocations involving three extensive regions of the X chromosome (i.e. Xq13–22, Xq26–28 and Xp11.2–22.1) have been associated with POF (2–5). In Turner's syndrome, ovarian insufficiency is probably due to haploinsufficiency of pseudoautosomal loci of the X chromosome (6, 7). Mutations in autosomal genes (i.e. *FSHR*, *FOXL2*, *GALT*) have also been related to POF (8–11). Nevertheless, in most cases the etiology of the disease remains unknown.

Mutations in bone morphogenic protein 15 (*BMP15*) and its close autosomal paralogue growth/differentiation factor 9 (*GDF9*) are able to alter female sheep and mice fertility (12–15). Recently, a point mutation in *BMP15* has been associated with POF (16). A 4 bp deletion of *GDF9* apparently leading to a premature stop codon has been reported in two sisters with twins from one family (17). Furthermore, two missense mutations in *GDF9* have recently been associated with POF (18). *BMP15* and *GDF9* (respectively located on Xp11.2 and 5q31.1) encode soluble factors, which belong to the transforming growth factor superfamily (TGF β). TGF β proteins are involved in various developmental processes including reproductive functions (19–21). In mammals, *BMP15* and *GDF9* are specifically expressed in the oocytes in a similar spatio-temporal pattern throughout folliculogenesis (20, 22–24).

Both genes have two exons and encode preproteins composed of a signal peptide, a proregion and a

mature peptide. The signal peptide region is encoded by the first exon, the proregion by segments of both exons and the mature peptide region by the second exon. Their biological activity depends on the formation of homo and/or heterodimers after proteolytic processing (25–27).

Here we have screened a cohort of 203 POF patients for mutations in *GDF9* and *BMP15*. We analyzed the entire coding sequences of both genes by direct sequencing. The variants were screened in a panel of 54 women with normal fertility.

Materials and methods

Patients and control population

Women (203 subjects) were recruited from several clinical centres: Reproductive Endocrine Units from Hôpital Saint-Antoine, Hôpital Necker, Paris, France, Hôpital Jeanne de Flandres, Lille, France, a gynaecological unit in Hôpital Antoine Bécère, Clamart, France and the the Department of Obstetrics and Gynaecology of Helsinki University Hospital, Helsinki, Finland. Inclusion criteria were primary or secondary amenorrhea occurring before the age of 40 years, with an FSH serum level higher than 40 mIU/ml. The group of patients was composed of 83% of women of Caucasian origin, 14.9% of African origin and 2.1% of Asian origin (37 patients were Finnish). This study was approved by the institutional review board of the relevant hospitals and all participants gave their written informed consent. The control population included 54 women with regular menstrual cycles (28–32 days), having at least one child and no personal history of infertility or auto-immune disease. Among these women 40 were of Caucasian origin and 14 of African origin.

DNA extraction and sequencing

Genomic DNA was isolated from whole blood samples using the standard phenol–chloroform procedure. Both exons of *GDF9* and *BMP15* were amplified by PCR in all the patients ($n = 203$). The second exon was amplified in all control individuals ($n = 54$). PCR products were treated with shrimp alkaline phosphatase and exonuclease I as described by the manufacturer (USB, Cleveland, Ohio, USA) and directly sequenced using an ABI 3100 sequencer (Applied Biosystems, Foster City, CA, USA). The presence of each variant was confirmed by a new round of PCR amplification and sequencing.

In silico analysis

To determine the potential deleterious effect of the amino acid changes we used SIFT (<http://blocks.fhcr.org/sift/SIFT.html>) and PolyPhen softwares (<http://tux.embl-heidelberg.de/ramensky/index.shtml>).

The SIFT software uses the protein sequence similarity of different species and the hydrophobic characteristics of amino acids to calculate the probability of a deleterious effect of specific amino acid variants (28). Scores lower than 0.05 suggest a potential pathogenic amino acid substitution. The PolyPhen program offers information about the effect of the amino acid substitution by considering structural parameters and three-dimensional protein structures (29, 30). The GoCore program version 5.0.1 (<http://www.helsinki.fi/project/ritvos/GoCore/>) was used for performing multiple alignments of sequences from several vertebrate species including *H. sapiens*, *P. troglodytes*, *C. familiaris*, *R. norvegicus*, *M. musculus*, *S. scrofa*, *B. taurus*, *C. hircus*, *C. elaphus*, *O. aries*, *T. vulpecula*, *D. novemcinctus*, *O. garretti*, *M. mulatta*, *P. anubis*, *C. jacchus*, *M. domestica*, *G. gallus* and *E. rubripes*.

Results

Sequence analysis

The analysis of the coding sequence of *GDF9* revealed, in one case, a heterozygous transversion 557C > A (second exon). This leads to the change S186Y in the proregion. This variant was not found in any control ($n = 54$). Furthermore, we found two rather common synonymous substitutions, 447C > T and 546G > A (summary in Table 1).

We detected, in the first exon of *BMP15*, a heterozygous transition G308A (with respect to the sequence AH007120 in Genbank). This variant appeared in 99% of the patients and controls. In the second exon, six different variants were detected. In one patient, we found the heterozygous 443T > C transition, leading to an L148P change in the proregion. We did not find this variant in any control ($n = 54$). Furthermore, this patient presented an insertion of three base pairs (788insTCT) leading to the insertion of a leucine at position 263. Ins263L was found in nine other patients and in 16.6% (nine of 54) of the control sample. All the controls presenting these variants were of African origin. Among the patients showing this mutation 60% were of African origin. A transition 588G > A (A180T) was found in two patients at the heterozygous state. This variant was not present in any control. In addition, we detected three silent substitutions, 538G > A, 831T > C and 468G > A (Table 1).

Patients' phenotype

BMP15-L148P The patient was a 30-year-old African woman presenting with secondary amenorrhea. She was a second daughter of a non-consanguineous family. Puberty occurred when she was 13 years old, with normal secondary sex characteristics and breast development. The patient described initial

Table 1 Results of the sequence analysis of *BMP15* and *GDF9* in 203 POF patients. The overview includes base substitutions at the genomic level (numbering refers to the coding region), amino acid (AA) change, number of patients tested and allele frequency of each variant.

Gene	Sequence variation	AA change	Clinical information	No. of patients	Frequency in population
<i>BMP15</i>	788insTCT	Ins263L	POF	203	4.93% (10/203)
			Controls	54	16.6% (9/54)
	443T > C	L148P	POF	203	0.49% (1/203)
			Controls	54	0% (0/54)
	538G > A	A180T	POF	203	0.98% (2/203)
			Controls	54	0% (0/54)
	852C > T	S284S	POF	203	1.97% (4/203)
			Controls	54	0% (0/54)
	831T > C	T277T	POF	203	0.49% (1/203)
			Controls	54	0% (0/54)
<i>GDF9</i>	468G > A	V156V	POF	203	0.49% (1/203)
			Controls	54	0% (0/54)
	557C > A	S186Y	POF	203	0.49% (1/203)
			Controls	54	0% (0/54)
	447C > T	T149T	POF	203	75.9% (154/203)
			Controls	54	55.5% (30/54)
	546G > A	E182E	POF	203	23.15% (47/203)
			Controls	54	26% (14/54)

oligomenorrhea. When she was 19 years old, she received the oral contraceptive pill (OC) until she was 29 years old but she stopped it frequently. She then became pregnant twice but interrupted the pregnancy in both cases. When she definitely stopped taking the OC, secondary amenorrhea appeared. When she was referred to our department, the patient's height was 163 cm and her weight was 73 kg. Plasma FSH and luteinizing hormone (LH) were high: 120 mIU/l (normal (N), 4–8 mIU/l) and 100 mIU/l (N, 3–12 mIU/l) respectively. Plasma oestradiol and inhibin B were low: 30 pmol/l (N, 70–1000 pmol/l) and 15 pg/ml (N, 60–120 pg/ml) respectively. No anti-thyroid or anti-ovary antibodies could be detected. The karyotype was normal. Pelvic ultrasonography showed a small uterus (68 × 39 × 27 mm), an atrophic endometrium and very small ovaries in their surface (0.56 and 0.64 cm² for the right and left ovaries respectively (N, 2–6 cm²). No follicle was observed. The patient received an oral oestrogen-progestagen treatment. We did not have access to blood samples from any family members.

GDF9-S186Y The patient, of Caucasian origin, was referred to us at the age of 37 years for primary infertility. Menarche had occurred at 9.5 years of age with regular menstrual cycles every 21 days up to the age of 33 years, then her menses suddenly stopped. At that time, her FSH, LH and oestradiol serum levels were respectively 104 mIU/ml, 46 mIU/ml and <20 pg/ml. Anti-thyroid and anti-DNA serum antibodies were undetectable. No familial or personal history of pelvic surgery was recorded. Her height was 153 cm and her weight 60 kg. Her karyotype was 46XX. No other case of POF was identified in the

family. At the age of 35 years, a coelioscopy was performed showing atrophy of both ovaries. The ovary cortex biopsy was composed of interlacing bundles of stromal spindle cells and contained some white body remnants but there were no follicles on serial sections. After the initial diagnosis of POF, the patient received hormone replacement therapy. We did not have access to familial blood samples.

Discussion

BMP15 and *GDF9* play a key role in the molecular dialogue between the oocyte and the surrounding somatic cells, promoting granulosa cell mitosis and cumulus expansion by a paracrine effect (31, 32). They are synthesized as propeptides composed of a signal peptide, a proregion and a mature peptide. As for many TGFβ factors, the proregion of *BMP15* and *GDF9* might be necessary for a correct folding, dimerization and secretion of the mature protein (19). *In vivo*, the biological role of *BMP15* and *GDF9* in animal fertility has been demonstrated in mice and ruminants. Naturally occurring heterozygous mutations in *BMP15* and *GDF9* in sheep increase the ovulation quota leading to hyperfertility, whereas homozygous mutations produce infertile animals (12, 13). The biological effect of the mutations in mammals varies among species (12, 15). For example, *GDF9*^{-/-} mice present a phenotype that is similar to *BMP15* homozygous sheep mutants whereas *BMP15* knockout (KO) mice have normal ovaries and subfertility (15). The double gene KO leads to infertility while other combinations of alleles display different phenotypes (15). The difference in the effects of the mutations could be explained by the intrinsic characteristics of mono-ovulatory or

polyovulatory species. Moreover, the mutations in mice are loss-of-function whereas in sheep and human there are presumed to be dominant negative. Indeed, *in vitro* cell transfection assays using constructs corresponding to the natural mutations observed in sheep demonstrated that dominant negative interactions between these factors have an important effect on the secretion of the mature peptides (27). In the human, a heterozygous point mutation of *BMP15* that has been associated with POF (Y235C) is located in the proregion and *in vitro* experiments have shown a diminished stimulatory activity of granulosa cell mitosis by a potential dominant negative effect (16). Recently, two missense mutations (K67E and V216M) located in the *GDF9* proregion have been described in Indian POF patients and could be implicated in the ovarian phenotype (18). Sequence alignments have shown that the first variant is not likely to have any functional relevance since in the sheep and the chicken the K residue is replaced by an E. In the second case, the amino acid V is well conserved or replaced by a highly hydrophobic residue which argues in favour of a functional impact. The results using SIFT are in agreement.

In our cohort of 203 patients, we identified two novel heterozygous missense mutations, one in each gene, that could be implicated in the ovarian phenotype. Sequencing of *GDF9* revealed the mutation S186Y found in one patient with secondary amenorrhea. The absence of this variant in women with normal fertility suggests a potential pathogenic effect. This was further corroborated by the evolutionary conservation of the S186Y in 17 vertebrate species, ranging from chickens to mammals (Fig. 1). Accordingly, both the SIFT (score = 0.03) and PolyPhen predictions suggested a deleterious effect. We also found two

polymorphisms, 447C > T and 546G > A, in 154 (75.9%) and 47 (23.1%) respectively of our POF patients. These variants are common polymorphisms in diverse ethnical groups since they have also been described in Indian POF patients (18).

The sequence analysis of *BMP15* revealed that the heterozygous transition G308A (S103N, with respect to Genbank AH007120) that appeared in the first exon is in fact the most common allele. One patient carried a 443T > C transition at a heterozygous state in the second exon. This leads to a L148P change at the protein level. This mutation was located in the pro-region, closer to the N-terminus than that described by Di Pasquale *et al.* (16). The ovarian phenotype of the patient with the *BMP15* mutation was less severe than in the two cases previously described (16). A multiple sequence alignment showed that the amino acid L148P position was also conserved across vertebrates, from chicken to mammals, suggesting a crucial functional or structural role. As expected, both Polyphen and SIFT programs suggested a potential deleterious effect. The substitution L-to-P translates into an important change in hydrophobicity (L > P) and relevant modifications in terms of structure. The presence of a P might change the rigidity of the peptidic chain and could alter protein folding itself or its kinetics.

The mutation A180T, found in two patients, is not likely to be deleterious as suggested by the SIFT and PolyPhen programs. Although we did not find it in any control, the Thr appears in wild-type sequences from *C. hircus* and *O. aries*. The insertion of a leucine in position 263, found in ten patients, probably has a low biological impact, as suggested by its presence in 16.6% of the controls (nine of 54). Furthermore, it is present in rodents and the marsupial *T. vulpecula* (Fig. 1). It is interesting

Figure 1 Multiple protein sequence alignments of *BMP15* and *GDF9* between mammalian species displaying the regions of the second exon surrounding human mutations. Residues that differ from the wild-type (WT) human sequence are shaded in gray. Mutations found in the study are shaded in black. Dotted boxes highlight the positions of each variant.

to underline that 60% of patients and the totality of controls bearing this variant were of African origin. These results suggested that BMP15-Ins263L is a common variant in African populations.

It is possible that the adequate folding of the proregion could be necessary for a normal folding of the mature region. We hypothesize that the misfolded proregion of BMP15-L148P and GDF9-S186Y could affect the normal cleavage or inhibit/alter the folding of the mature region. This may lead to the production of abnormal dimers or could even inhibit the dimerization process. The importance of a normal structure of the BMP15 proregion has been recently demonstrated by Hashimoto *et al.* (33) who showed that the intrinsic molecular properties of the human BMP15 proregion are strong determinants for the correct processing of the mature protein (33). Furthermore, they highlighted the relevance of the post-translational processing of the BMP15 proregion (33).

We have concluded that although mutations in *BMP15* and *GDF9* are not a major cause of premature ovarian failure they can be involved in this disease. This is in line with the fact that Takebayashi *et al.* (34) failed to detect mutations in *GDF9* and the *BMP15* in a small cohort of 15 POF patients. Furthermore, our study corroborates the recent findings of Dixit *et al.* (18) showing a low *GDF9* mutation frequency in a large cohort of Indian POF cases. The new potentially deleterious mutation in *BMP15* and *GDF9* described in our POF patients deserve further functional *in vitro* studies. Nevertheless, these assays are particularly challenging since different studies using recombinant *GDF9* display contradicting results in terms of cumulus cell expansion and regulation of gene expression (35).

Acknowledgements

This study was supported by grants from the Groupe-ment d'Interet Scientifique-Institut Maladies rares and the Direction de la Recherche Clinique, Assistance publique, Hôpitaux de Paris (Programme hospitalier de recherche clinique AOR01066).

References

- Coulam CB. Premature gonadal failure. *Fertility and Sterility* 1982 **38** 645–655.
- Tharapel AT, Anderson KP, Simpson JL, Martens PR, Wilroy RS Jr, Llerena JC Jr & Schwartz CE. Deletion (X)(q26.1–>q28) in a proband and her mother: molecular characterization and phenotypic-karyotypic deductions. *American Journal of Human Genetics* 1993 **52A** 63–71.
- Davison RM, Fox M & Conway GS. Mapping of the POF1 locus and identification of putative genes for premature ovarian failure. *Molecular Human Reproduction* 2000 **6** 314–318.
- Marozzi A, Manfredini E, Tibiletti MG, Furlan D, Villa N, Vegetti W, Crosignani P, Ginelli E, Meneveri R & Dalpra L. Molecular definition of Xq common-deleted region in patients affected by premature ovarian failure. *Human Genetics* 2000 **107** 304–311.
- Zinn AR, Tonk VS, Chen Z, Flejter WL, Gardner HA, Guerra R, Kushner H, Schwartz S, Sybert VP, Van Dyke DL & Ross JL. Evidence for a Turner syndrome locus or loci at Xp11.2–p22.1. *American Journal of Human Genetics* 1998 **63** 1757–1766.
- Zinn AR & Ross JL. Turner syndrome and haploinsufficiency. *Current Opinion in Genetic Development* 1998 **8** 322–327.
- Sybert VP & McCauley E. Turner's syndrome. *New England Journal of Medicine* 2004 **351** 1227–1238.
- Aittomaki K, Lucena JL, Pakarinen P, Sistonen P, Tapanainen J, Gromoll J, Kaskikari R, Sankila EM, Lehtvaslaiho H, Engel AR, Nieschlag E, Huhtaniemi I & de la Chapelle A. Mutation in the follicle-stimulating hormone receptor gene causes hereditary hypergonadotropic ovarian failure. *Cell* 1995 **82** 959–968.
- Touraine P, Beau I, Gougeon A, Meduri G, Desroches A, Pichard C, Detoeuf M, Paniel B, Prieur M, Zorn JR, Milgrom E, Kuttann F & Misrahi M. New natural inactivating mutations of the follicle-stimulating hormone receptor: correlations between receptor function and phenotype. *Molecular Endocrinology* 1999 **13** 1844–1854.
- Crisponi L, Deiana M, Loi A, Chiappe F, Uda M, Amati P, Bisceglia L, Zelante L, Nagaraja R, Porcu S, Ristaldi MS, Marzella R, Rocchi M, Nicolino M, Lienhardt-Roussie AR, Nivelon A, Verloes A, Schlessinger D, Gasparini P, Bonneau D, Cao A & Pilia G. The putative forkhead transcription factor FOXL2 is mutated in blepharophimosis/ptosis/epicanthus inversus syndrome. *Nature Genetics* 2001 **27** 159–166.
- Goswami D & Conway GS. Premature ovarian failure. *Human Reproduction Update* 2005 **11** 391–410.
- Galloway SM, McNatty KP, Cambridge LM, Laitinen MP, Juengel JL, Jokiranta TS, McLaren RJ, Luiro K, Dodds KG, Montgomery GW, Beattie AE, Davis GH & Ritvos O. Mutations in an oocyte-derived growth factor gene (*BMP15*) cause increased ovulation rate and infertility in a dosage-sensitive manner. *Nature Genetics* 2000 **25** 279–283.
- Hanrahan JP, Gregan SM, Mulsant P, Mullen M, Davis GH, Powell R & Galloway SM. Mutations in the genes for oocyte-derived growth factors *GDF9* and *BMP15* are associated with both increased ovulation rate and sterility in Cambridge and Belclare sheep (*Ovis aries*). *Biology of Reproduction* 2004 **70** 900–909.
- Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N & Matzuk MM. Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature* 1996 **383** 531–535.
- Yan C, Wang P, DeMayo J, DeMayo FJ, Elvin JA, Carino C, Prasad SV, Skinner SS, Dunbar BS, Dube JL, Celeste AJ & Matzuk MM. Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. *Molecular Endocrinology* 2001 **15** 854–866.
- Di Pasquale E, Beck-Peccoz P & Persani L. Hypergonadotropic ovarian failure associated with an inherited mutation of human bone morphogenetic protein-15 (*BMP15*) gene. *American Journal of Human Genetics* 2004 **75** 106–111.
- Montgomery GW, Zhao ZZ, Marsh AJ, Mayne R, Treloar SA, James M, Martin NG, Boomsma DI & Duffy DL. A deletion mutation in *GDF9* in sisters with spontaneous DZ twins. *Twin Research* 2004 **7** 556–563.
- Dixit H, Rao LK, Padmalatha V, Kanakavalli M, Deenadayal M, Gupta N, Chakravarty B & Singh L. Mutational screening of the coding region of growth differentiation factor 9 gene in Indian women with ovarian failure. *Menopause* 2005 **12** 749–754.
- Chang H, Brown CW & Matzuk MM. Genetic analysis of the mammalian transforming growth factor-beta superfamily. *Endocrine Reviews* 2002 **23** 787–823.
- Shimasaki S, Moore RK, Otsuka F & Erickson GF. The bone morphogenetic protein system in mammalian reproduction. *Endocrine Reviews* 2004 **25** 72–101.
- Juengel JL & McNatty KP. The role of proteins of the transforming growth factor-beta superfamily in the intraovarian regulation of follicular development. *Human Reproduction Update* 2005 **11** 143–160.

- 22 Laitinen M, Vuojolainen K, Jaatinen R, Ketola I, Aaltonen J, Lehtonen E, Heikinheimo M & Ritvos O. A novel growth differentiation factor-9 (GDF-9) related factor is co-expressed with GDF-9 in mouse oocytes during folliculogenesis. *Mechanics of Development* 1998 **78** 135–140.
- 23 Dube JL, Wang P, Elvin J, Lyons KM, Celeste AJ & Matzuk MM. The bone morphogenetic protein 15 gene is X-linked and expressed in oocytes. *Molecular Endocrinology* 1998 **12** 1809–1817.
- 24 McGrath SA, Esqueda AF & Lee SJ. Oocyte-specific expression of growth/differentiation factor-9. *Molecular Endocrinology* 1995 **9** 131–136.
- 25 Massague J, Attisano L & Wrana JL. The TGF-beta family and its composite receptors. *Trends in Cell Biology* 1994 **4** 172–178.
- 26 Liao WX, Moore RK, Otsuka F & Shimasaki S. Effect of intracellular interactions on the processing and secretion of bone morphogenetic protein-15 (BMP-15) and growth and differentiation factor-9. Implication of the aberrant ovarian phenotype of BMP-15 mutant sheep. *Journal of Biological Chemistry* 2003 **278** 3713–3719.
- 27 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. *Journal of Biological Chemistry* 2004 **279** 17391–17396.
- 28 Ng PC & Henikoff S. SIFT: predicting amino acid changes that affect protein function. *Nucleic Acids Research* 2003 **31** 3812–3814.
- 29 Sunyaev S, Ramensky V & Bork P. Towards a structural basis of human non-synonymous single nucleotide polymorphisms. *Trends in Genetics* 2000 **16** 198–200.
- 30 Ramensky V, Bork P & Sunyaev S. Human non-synonymous SNPs: server and survey. *Nucleic Acids Research* 2002 **30** 3894–3900.
- 31 Gilchrist RB, Ritter LJ, Cranfield M, Jeffery LA, Amato F, Scott SJ, Myllymaa S, Kaivo-Oja N, Lankinen H, Mottershead DG, Groome NP & Ritvos O. Immunoneutralization of growth differentiation factor 9 reveals it partially accounts for mouse oocyte mitogenic activity. *Biology of Reproduction* 2004 **71** 732–739.
- 32 Otsuka F & Shimasaki S. A negative feedback system between oocyte bone morphogenetic protein 15 and granulosa cell kit ligand: its role in regulating granulosa cell mitosis. *PNAS* 2002 **99** 8060–8065.
- 33 Hashimoto O, Moore RK & Shimasaki S. Posttranslational processing of mouse and human BMP-15: potential implication in the determination of ovulation quota. *PNAS* 2005 **102** 5426–5431.
- 34 Takebayashi K, Takakura K, Wang H, Kimura F, Kasahara K & Noda Y. Mutation analysis of the growth differentiation factor-9 and 9B genes with premature ovarian failure and polycystic ovary syndrome. *Fertility and Sterility* 2000 **74** 976–979.
- 35 Pangas SA & Matzuk MM. The art and artifact of GDF9 activity: cumulus expansion and the cumulus expansion-enabling factor. *Biology of Reproduction* 2005 **73** 582–585.

Received 14 November 2005

Accepted 7 February 2006