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BMP15 mutations associated with Primary Ovarian Insufficiency cause a defective production of bioactive protein

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Abstract

BMP15 is selectively synthesized by oocytes as a pre-proprotein and is considered an ovarian follicle organizer whose adequate function is critical for female fertility. Missense mutations were reported in Primary Ovarian Insufficiency (POI) but their biological impact remained unexplored. Here, screening of 300 unrelated idiopathic overt POI women with primary or secondary amenorrhea led to the identification of 6 heterozygous BMP15 variations in 29 of them. All alterations are non-conservative and include one insertion of 3 nucleotides (p.L262_L263insL) and 5 missense substitutions. Except for the p.S5R located in the signal sequence, the other variants (p.R68W, p.R138H, p.L148P, p.A180T) localize in the proregion, which is essential for the processing and secretion of bioactive dimers. The mutations p.R68W, p.L148P and the novel p.R138H lead to marked reductions of mature protein production. Their biological effects, evaluated by a novel luciferase-reporter assay in a human granulosa cell line, were significantly reduced. Co-transfection experiments of defective mutants with equal amounts of wild-type BMP15 cDNA, thus reproducing the heterozygous state seen in patients, did not generate a complete recovery of wild-type activity. No or minor deleterious effects were detected for the variants p.L262_L263insL, p.A180T or p.S5R. In conclusion, heterozygous BMP15 mutations associated with the early onset of overt POI lead to defective secretion of bioactive dimers. These findings support the concept that an adequate amount of BMP15 secreted in the follicular fluid is critical for female fertility. We propose to consider the screening of BMP15 mutations among the analyses for the prediction of POI risk.

Keywords

Primary Ovarian Insufficiency; POI; BMP15; X chromosome; Female fertility; Premature Ovarian Failure; Ovary; Folliculogenesis

Introduction

Primary ovarian insufficiency (POI), which is probably a more appropriate term for premature ovarian failure (POF; MIM# 311360), is a disorder associated with female infertility affecting 1-2% of women under 40 years [Goswamy and Conway, 2005; Nelson et al., 2005; Beck-Peccoz and Persani, 2006; Welt, 2008]. This disease may be characterized by primary amenorrhea (PA) in major defects with prepubertal onset or secondary amenorrhea (SA) in women with post-pubertal onset. The cause of the ovarian defect remains obscure in most of the patients with spontaneous POI but the genetic contribution is considered a significant aetiological component. Several genetic mechanisms may be involved in POI pathogenesis but most of them are involved in less than 1% of the cases. *FMRI* premutation is known to affect about 10% of POI women and analysis of the expansion region in this gene is currently examined in POI patients for counseling in order to predict the familial risk of Fragile X mental retardation in males or POI in females [Bodega et al., 2006; De Caro et al., 2008].

We firstly examined the possible involvement of Bone Morphogenetic Protein-15 (*BMP15*; MIM# 300247) genetic defects in POI pathogenesis. *BMP15* is an oocyte-derived growth factor belonging to the Transforming Growth Factor-beta ($TGF-\beta$) superfamily which is involved in follicular development as critical regulator of many granulosa cell processes [Shimasaki et al., 2004; McNatty et al., 2004; Juengel and McNatty, 2005; Fabre et al., 2006]. *BMP15* gene is localized to Xp11.2 and it is expressed exclusively in the oocyte throughout folliculogenesis [Dube et al., 1998]. Similarly to other $TGF-\beta$ /*BMP* factors, *BMP15* is translated as a pre-proprotein which consist of a signal peptide, a large proregion and a mature peptide [Massagué, 1990; Chang et al., 2002]. After the removal of the signal peptide, the proprotein dimerizes first and then undergoes proteolytic cleavage at a conserved RXXR cleavage site [Shi and Massagué, 2003; Shimasaki et al., 2004; Juengel and McNatty, 2005; Moore and Shimasaki, 2005]. The proregion is known to have an important role in the processing of the pro-protein by driving the dimerization and the subsequent secretion of the active mature dimers. Furthermore, it has been shown that proper posttranslational processing of the *BMP15* proprotein represents a critical step of female fertility and that divergences in the *BMP15* pro-region may account for the species-specific differences in ovulation rate and ovarian reserve [Hashimoto et al., 2005]. The secreted bioactive mature *BMP15* dimers signal through serine/threonine kinase receptors. They, in turn, stimulate downstream regulatory SMAD proteins that translocate to the nucleus and regulate the transcription of target genes [Chang et al., 2002; Shi and Massagué, 2003]. *In vitro* studies identified granulosa cells (GCs) as the target of *BMP15* paracrine actions within the ovarian follicle [Otsuka et al., 2000; Shimasaki et al., 2004; Fabre et al., 2006] and *BMP15* was reported to bind *BMP Receptor I type B* (*BMPR1B*) and stimulate the activation of the SMAD1/5/8 pathway in rat primary granulosa cells and in the COV434 human granulosa cell line [Moore et al., 2003]. Animal models firstly showed that *BMP15* paracrine action in the ovary is required for proper folliculogenesis. Indeed, many natural mutations of *BMP15* gene, identified in several breeds of ewes, have profound effects on their fertility with homozygous carriers which are infertile and have a block of folliculogenesis at the primary stage, while the heterozygous animals exhibit higher ovulation rates and litter sizes than wild-type counterparts [Galloway et al., 2000; Juengel et al., 2002; Shimasaki et al., 2004; Hanrahan et al., 2004; Bodin et al., 2007]. The first

mutation of human *BMP15* gene was reported in two sisters with hypergonadotropic ovarian failure characterized by primary amenorrhea and ovarian dysgenesis (MIM# 300510) [Di Pasquale et al., 2004]. The mutant p.Y235C protein showed abnormal processing leading to the production of aberrant products with impaired activity and dominant negative effect on wild type action, thus compromising the proliferation of human granulosa cells. Subsequently, we have extended the genetic screening to a large series of unrelated women with primary or secondary amenorrhea due to idiopathic POI and identified new *BMP15* non-synonymous variants affecting 4.2% of 46,XX POI population [Di Pasquale et al., 2006]. In the same period, other groups from Europe and India, described additional variations in *BMP15* gene associated with POI phenotype [Dixit et al., 2006; Laissue et al., 2006; Ledig et al., 2008]. All these variations have been described in the heterozygous state and are located in the part of the gene encoding the pro-region. The data suggested the possibility that *BMP15* may represent the first identified ovarian determining gene on the X chromosome [Layman, 2006]. However, this possibility remained without answers as no functional studies have been so far reported for any of these recent variations. Here, we report the results of *BMP15* genetic screening carried out on 300 women affected by POI. In addition, we describe the post-translational processing, secretion and biological activity of all recombinant *BMP15* variants identified. For this, we have established an in-vitro luciferase reporter assay using COV434 cells, an immortalized human granulosa cell line that maintains the characteristics of a primary granulosa cell [Zhang et al., 2000]. This study gives a more definite picture of the impact of heterozygous *BMP15* mutations found in young POI women and provides evidences supporting the concept that defective *BMP15* protein production may contribute to ovarian insufficiency in humans.

Materials and Methods

Genetic analysis

A total of 300 unrelated Caucasian women with 46,XX karyotype and idiopathic overt POI, including 45 patients with primary amenorrhea and 255 women with secondary amenorrhea (age at POI: 29 ± 10 ($\pm$ SD) yr; median: 34; range 12-40) were collected from several clinical centers, following the previously described inclusion criteria [Di Pasquale et al., 2006]. Genetic investigation included two Caucasian control groups represented by 100 women with physiological menopause beyond 50 yr of age and 116 unrelated healthy subjects from the general population. Institutional ethical committees approved the study and informed consent for blood sampling and genetic investigations were obtained from all participants. The entire coding sequence and intron-exon junctions of *BMP15* gene (GenBank no. AF082349.1 and AF082350.1) were analyzed by denaturing high-performance liquid chromatography (DHPLC) of PCR-amplified fragments on an automatic instrument (WAVE apparatus, Transgenomics Inc.). In case of an aberrant elution profile compared to the wild-type, the DNA samples were analyzed by automated sequencing as previously described [Di Pasquale et al., 2006]. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence (GenBank no. NM_005448.1). The initiation codon is codon 1 (GenBank no. NP_005439.1).

In silico analysis

To predict aminoacidic variants effect on protein structure and function, we used several bioinformatic tools available on the Internet. SIFT (<http://blocks.fhcrc.org/sift/SIFT.html>) calculates the probability of a deleterious change using protein sequence similarity among species and the hydrophobic characteristics of single amino acids. PolyPhen (<http://genetics.bwh.harvard.edu/pph/>) considers structural parameters and three-dimensional protein structures. NNpredict

(<http://alexander.compbio.ucsf.edu/~nomi/nnpredict.html>) predicts the secondary structure based on the aminoacidic composition of the protein. Protscale (www.expasy.ch) provides the protein hydrophobicity profile. NetNGlyc (www.cbs.dtu.dk) server predicts N-Glycosylation sites on the basis of the sequence context of Asn-Xaa-Ser/Thr sequons.

Construction of BMP15 expression plasmids

BMP15 gene variants were introduced by site-directed mutagenesis into the pCDNAhumanBMP15wt-MycHis vector, containing a full-length human BMP15 wild-type cDNA [Di Pasquale et al., 2004]. Mutagenesis reaction for each variant was performed using *Pfu Ultra* Taq DNA polymerase (Stratagene) and specific couples of primers (Supp. Table S1). For luciferase assays, plasmid variants were obtained from amplification of BMP15 sequence from corresponding pCDNAhumanBMP15-MycHis mutated vector with specific primers introducing restriction sequences for ClaI and XbaI, at 5' and 3' ends respectively (Supp. Table S1). Variant sequences were then subcloned in pCS2++ empty vector.

Production of recombinant human BMP15-MycHis chimeras

Human embryonic kidney 293T (HEK293T) cells were grown in Dulbecco's modified Eagle's medium (GIBCO) with 10% (v/v) fetal calf serum (FCS - GIBCO), 5000 units/ml penicillin, 5 mg/ml streptomycin (SIGMA) at 37°C with 5% CO₂. After stable transfection with 10 µg of pcDNAhBMP15-MycHis using Lipofectamine Transfection Reagent (Invitrogen), clones were selected with 600 µg/ml of Zeocin (Invitrogen) as previously described [Di Pasquale et al., 2004]. Positive clones were screened for BMP15 expression by Real Time-PCR, as previously described [Bodin et al., 2007]. Selected clones were seeded at a density of 2×10^5 cells/cm² and maintained in medium containing 10% serum. At 70% confluence, cell culture media were switched to 1% serum and the conditioned media were harvested after 72h. The expression of a constitutive protein such as α -tubulin was checked by means of a monoclonal antibody (Sigma) to assess that culture media (6 ml per dish) were obtained from an equivalent amount of cells (see Fig. 1B). Human recombinant BMP15 proteins were purified from equal amounts of concentrated conditioned media (Centricon 10,000 Da, Becton) and from cells extracts using nickel resin columns (Probond, Invitrogen). The amount of recovered protein was evaluated by colorimetric assay (BCA kit, Pierce).

In vitro molecular studies

Equal amount of purified proteins were analyzed by SDS-PAGE or denaturant nonreducing condition Western blot, using 10 and 20 µg of sample respectively. In both cases the detection was performed using the ECL Plus kit (Amersham) after incubation with the monoclonal anti-Myc antibody (Invitrogen) and the peroxidase-conjugated secondary antibody (anti-mouse IgG-horseradish peroxidase, Chemicon), as previously described [Bodin et al., 2007]. Purified products from the medium and cell extracts of not transfected HEK293T cells were used as negative control.

Real-Time PCR

Total RNA was extracted from stable transfected HEK293T cells using the Trizol reagent (Invitrogen). One microgram of total RNA was treated with DNase I amplification grade (Invitrogen) before the reverse transcription reaction using the SuperScript II first-strand cDNA synthesis kit (Invitrogen). BMP15 expression was screened in several independent clones for each variant. Real-time PCR were performed using Syber Green (Applied Biosystems), 100 ng of template (cDNAss), and 10µM of primers sense and antisense, as previously described [Bodin et al., 2007]. Amplification was performed on an ABI Prism

7900HT apparatus (Applied Biosystems) and a dissociation curve was added at the end of the polymerization. The raw data were analyzed with the SDS 2.1 software (Applied Biosystems). The quantitative relative study (ΔC_t) was performed using the HEK293T cell sample as the calibrator and comparing the BMP15 transcript levels in mutant and wild-type cell clones. Hypoxanthine-guanine phosphoribosyl transferase gene amplification was used for normalization.

Transient transfections and Luciferase Assays

The bioassay was performed using a BMP responsive element (BRE)-luciferase reporter, that was previously demonstrated to display a specific and dose dependent activation with various BMPs [Zilberberg et al., 2007]. Indeed, we were able to reproduce a dose-response curve to rhBMP-4 (R&D Systems) also in our experimental conditions (see Supp. Figure S1). For transient transfection, COV434 granulosa cells were seeded at a density of 7×10^5 cells/well in 12-well plates in DMEM supplemented with 10% FCS. When subconfluent, cells were cotransfected with the wild-type or mutant pCS2 expression vector (400ng/well) and the BRE-luciferase reporter (100ng/well), by using Fugene HD (Roche) and the manufacturer's protocol. The pCS2++ empty vector was used as negative control. pRL-tk plasmid containing the *Renilla* luciferase gene (Promega) cotransfection (10ng/well) was used as an internal control. Approximately 24h after transfection, the medium was replaced with 1% serum medium with 10 ng/ml of rhBMP-4 added only in positive control wells. After treating for 16h, cells were rinsed with ice-cold PBS and then lysed with 200 μ l of Passive Lysis Buffer 1X (Promega). Cell lysates from each well were centrifuged at 12,000 rpm for 2 minutes to pellet the cell debris and 20 μ l of the supernatants were then assayed for luciferase activity using the Dual Luciferase reporter Assay kit (Promega). Luminescence in relative light units (RLU) was measured for 10s in a Fluoroskan Ascent instrument (Labsystems). Firefly luciferase activity was first normalized to the level of *Renilla* luciferase activity. Results were then calculated as fold induction relative to pCS2-BMP15 wild-type expression vector.

Statistics

Reporter luciferase assays were performed at least four times in triplicate wells. All values represent the mean $\pm$ SD. A Student's *t* test was used for statistical evaluation and a $P < 0.05$ was considered statistically significant.

Results

Genetic analyses

The genetic screening revealed the presence of 6 different heterozygous BMP15 variants in a cohort of 300 patients with overt POI (Table 1). All alterations are non-conservative and include one insertion of a leucine at the aminoacidic position 262 (p.ins262L) and five missense substitutions: p.S5R, p.R68W, p.R138H, p.L148P and p.A180T. Only the p.R138H is novel, whereas the others had been previously reported by us or other groups in patients of African, Caucasian or Indian origin [Di Pasquale et al., 2006;Dixit et al., 2006;Laissue et al., 2006;Ledig et al., 2008]. Only the p.S5R is located in the signal sequence, all other variations occur in the part of gene encoding the proregion. Except for the p.ins262L, none was detected in 116 unrelated healthy subjects and in 100 control women with physiological menopause. Thus, the missense variations were exclusively associated with primary or secondary amenorrhea with an onset between 14-30 years.

In silico analyses

By three different bioinformatic tools (Table 2), the p.S5R, p.A180T substitutions and the p.L262_L263insL insertion are not likely to be deleterious. The p.R68W and p.R138H substitutions are predicted, at least in 2 out of 3 analyses, to cause significant modifications that probably damage the secondary protein structure. Whereas the p.L148P missense variant appears as the most deleterious, modifying the hydrophobicity in the pro-region that may alter protein folding (Supp. Figure S2).

In vitro molecular characterization of synthesis, processing and secretion of BMP15 variants

In order to evaluate the biological impact of the 6 variations on the molecular processing, dimerization and secretion of the BMP15 protein, we stably transfected HEK293T cells with expression vectors for wild-type human BMP15 or its variant forms (p.ins262L, p.A180T, p.L148P, p.R138H, p.R68W and p.S5R), all tagged on the C-terminus with the Myc epitope and a polyhistidine sequence. The conditioned media obtained from equivalent amounts of cultured cells were purified using affinity chromatography and equal amounts of purified products were analyzed by SDS-PAGE Western blot. We documented a marked reduction of mature rhBMP15 protein secreted in the case of p.R68W, p.R138H and p.L148P mutations in comparison with the wild-type and less deleterious variations (Fig. 1A). Only faint or diminished bands corresponding to the secreted and bioactive mature forms were seen in these mutants lanes even when a double amount of purified proteins was loaded on the gel (Fig. 1C). Moreover, the p.L148P precursor band migrates at a lower molecular weight than the wild type and the other protein forms. In denaturant non-reducing Western blot experiments, the media conditioned by the 3 most deleterious mutations show important reductions of the secreted mature dimer and of the monomeric peptide without evidence of abnormal processing products (Fig. 1D). By densitometric analysis of serial amounts of wild-type mature peptide bands, we estimated the reductions in mature BMP15 protein secretion of 3-, 8- and 20-folds for p.R138H, p.R68W and p.L148P, respectively (Fig. 1E). These results were confirmed using at least 3 independent protein preparations run in separate experiments. Finally, we evaluated BMP15 mRNA expression by real-time PCR experiments to exclude the possibility of a mechanism of impaired transcription or diminished mRNA stability in different HEK293T stable clones. The quantitative analysis confirms similar BMP15 transcript levels in selected mutant clones and wild-type (Supp. Figures S3-S5).

Functional analysis of the in vitro biological activity of BMP15 variants using luciferase reporter assay

We investigated human BMP15 wild-type biological activity in comparison with those of the mutant forms of the protein by means of a reporter assay based on their ability to stimulate a BMP inducible promoter driving the luciferase expression. Transient transfection of the BRE-luciferase reporter construct in COV434 cells shows specific activation of the BMP-signaling pathway by exogenous stimulation with 10 ng/ml of rhBMP-4 agonist and by human BMP15 wild-type endogenously synthesized within the same cells after transfection (Fig. 2A). Co-transfection experiments of BMP15 carrying the p.A180T and p.L262_L263insL variants with the BRE-Luc vector showed a conserved stimulatory activity (Fig. 2B). On the contrary, a slight but significant decrease (-15% vs wild-type) of luciferase activity was detected for the p.S5R variant. Larger reductions (-25 to -33% vs wild-type) were measured in the case of p.R68W, p.R138H and p.L148P mutations. The activities of these mutants are significantly different from those detected with either 200 or 400 ng of wild-type constructs. When mutant plasmids were co-transfected with equal amount of the wild-type cDNA in experiments that aim to reproduce in-vitro the heterozygous status seen in POI patients, the impaired luciferase activity was entirely

rescued in the case of p.S5R, but only partially in the case of the more deleterious mutations (p.L148P, p.R138H and p.R68W) (Fig. 2B).

Discussion

In this study we report the screening of *BMP15* gene in 300 unrelated Caucasian women affected with idiopathic overt POI and provide the molecular and functional in-vitro characterization of the detected variants. *In silico* predictions indicate deleterious effects on protein function for at least 3 of these variants (p.R68W, p.R138H, p.L148P). *In vitro* studies are consistent with such predictions and demonstrate a defective mature protein production in the presence of these mutations. This may either results from an impaired processing or decreased precursor stability. These defects are reflected also by the results of the bioassay consistent with a defective stimulation of BMP pathway in granulosa cells. Though a significant reduction of luciferase stimulation was seen also in the case of p.S5R variant, the whole of *in silico* and *in vitro* studies suggest a minor functional role, if any, for this and remaining variants, p.A180T and p.ins262L. Both of these latter variations are indeed likely to represent polymorphisms deprived of a functional significance, as they have been found by us or other Authors [Morón et al., 2007] also in women with known physiological menopause beyond 50 years of age.

The most deleterious effects appear to be caused by the p.L148P substitution which falls adjacent to the predicted glycosylation site at Asparagine 147, one of the 5 predicted sites of N-glycosylation in the *BMP15* precursor [Dube et al., 1998]. N-glycosylation occurs in the Asn-X-Ser stretch. The NetNGlyc online tool predicts that the Leucine to Proline substitution at residue 148 would prevent the N-linked glycosylation by rendering hardly accessible the Asparagine 147. Defective glycosylation would also explain the decrease in molecular weight of the p.L148P precursor.

The reduced amount of secreted products observed in the case of defective POI mutations reproduce a defect similar to that described in the case of natural ovine *BMP15* mutations, that are instead associated with increased ovulation rate in the heterozygous state [Galloway et al., 2000, 2002; Juengel et al., 2002; Hanrahan et al., 2004; Bodin et al., 2007]. As in sheep, increased ovulation rate in humans might be uncovered by an augmented risk of multiple or dizygotic twinning (DZ). We cannot confirm such hypothesis as in our population no pregnancies were recorded in the women with secondary amenorrhea and *BMP15* mutations. However, the existence of a relationship between twinning and POI is plausible as mothers of DZ twins reach menopause significantly earlier than mothers of monozygotic twins, such difference being due to a higher numbers of mothers of DZ twins reporting POI [Martin et al., 1997]. Accordingly, mutations in *GDF9*, the *BMP15* homologous, are significantly more frequent in mothers of DZ twins [Hoekstra et al., 2008]. In a very recent study involving more than a thousand Caucasian women, the Authors failed to detect a significant association between *BMP15* variations and DZ twins [Zhao et al., 2008]. However, the age at menopause of the women involved in this study was unknown, and this model of increased rate of ovulation and follicle expenditure remains intriguing and without definitive answers.

No clear cut genotype-phenotype correlation can be seen in the case of *BMP15* mutations. The original p.Y235C mutation was indeed associated with ovarian dysgenesis and primary amenorrhea and was found to produce aberrant protein products and dominant negative effects [Di Pasquale et al., 2004]. The *BMP15* variants here reported are instead associated with defective mature protein production and have been detected in patients with both primary and secondary amenorrhea. The most deleterious mutation p.L148P was found in women with POI onset at 20 and 30 years of age, but POI occurred before 20 years of age in

the carriers of p.R68W and p.R138H mutations. The lack of a correlation between the functional defects observed *in vitro* and the age of POI onset may support a model of complex pathogenesis [Toniolo, 2006], in which BMP15 variations may predispose to POI and contribute in association with other alterations to generate the ovarian defect. Following this idea we screened additional candidates in these patients, but we could not find any alteration in the coding sequence of genes involved in the BMP pathway, such as GDF9 and BMPRI1B, or in other candidates, such as FMR1, FSHR, FOXL2 and NOBOX (data not shown).

Taken together, the present results support a model in which the deleterious mutations (p.R68W, p.R138H and p.L148P) may contribute to POI pathogenesis through a defective production of BMP15 protein. This model is consistent with the finding of a nonsense heterozygous mutation causing an early truncation of BMP15 protein (p.Glu211X) in an Indian woman with POI [Dixit et al., 2006]. BMP15 was indeed described to protect granulosa cells from apoptosis and augment follicular developmental competence [Hussein et al., 2005, 2006]. It is therefore likely that human BMP15 mutations leading to defective protein secretion in the follicular fluid might predispose to augmented atresia and diminished ovarian follicle viability.

BMP15 gene maps within the Xp Turner locus [Zinn et al., 1998] and Xp deletions highlight the involvement of the proximal part of Xp in ovarian function [Ogata et al., 2001; Lachlan et al., 2006]. In our experimental conditions, half amounts of wild-type cDNA alone or in the presence of the null C53Y mutation exhibited a significant decrease in luciferase activity. Moreover, when half amounts of variant human cDNAs were co-expressed with equal amounts of wild-type counterpart the impaired bioactivity of more deleterious mutations was only partially rescued. It is thus conceivable that BMP15 insufficiency may be critical during the early phases of the folliculogenesis when a double dose of BMP15 would be required.

In conclusion, molecular and functional findings support the concept that an adequate amount of BMP15 secreted in the follicular fluid is critical for female fertility. Heterozygous BMP15 mutations leading to defective production of bioactive protein may represent a novel mechanism contributing to the early onset of primary ovarian insufficiency in humans. Since defective mutations are found in 6/300 POI women (2%), we propose to consider the screening of BMP15 mutations among the analyses for the prediction of POI risk.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

A: SDS-PAGE protein analysis. 10 micrograms of purified protein from conditioned media of the wild-type (wt), mutants, and not-transfected (nt) cells were analyzed. The BMP15 mature peptide is visible in the wt and p.S5R, p.A180T and p.262insL (p.L262_L263insL) lanes whereas only a small amount of it is detectable in the p.R68W, p.R138H and p.L148P mutant lanes. **B:** Analysis of α -tubulin expression in total cell lysates showing that control and conditioned media were obtained from a similar amount of cultured cells. **C:** SDS-PAGE protein analysis. 20 micrograms of proteins purified from the mutant media are compared with 10 micrograms of wt counterpart. Decrement in mature peptide bands of mutant proteins does not depend on the amount of protein loaded. **D:** Denaturant non-reducing Western blot analysis. 20 micrograms of proteins loaded. Faint bands corresponding to the mature peptide and precursor, as dimer or monomer, are present in the mutants conditioned media. No abnormal products of processing are revealed. **E:**

Densitometric analysis (Scion Image software, IBM) of BMP15 mature peptide bands. Decreasing amounts of the wild-type preparation (10, 5, 2, 1, 0.5 micrograms) are compared with a fixed quantity of mutated proteins (10 micrograms). A reduction in mature BMP15 mutant protein secretion of 3-fold, 8-fold and 20-fold is present respectively for the p.R138H, p.R68W and p.L148P substitutions.

Figure 2.

In vitro reporter luciferase assay. COV434 granulosa cells were transiently cotransfected with 400 ng of the different BMP15 vectors and 100 ng of the BRE-luciferase reporter vector. Results are expressed as the mean ($\pm$ SD) of 12 determinations in four separate experiments. **A:** The cell system shows specific activation of the BMP-signaling pathway by incubation with 10 ng/ml of rhBMP-4 agonist or transfection with wild-type BMP15 vector. The defective p.C53Y mutation is responsible for the FecXL phenotype in Lacaune sheep [Bodin et al., 2007] and the vector carrying this mutation was associated with a complete loss of luciferase activity. Co-transfection with equal amounts of wild-type (200 ng) and p.C53Y mutation (200 ng) restores luciferase activity to 50% of the wild-type alone (400 ng). **B:** In presence of p.S5R, p.R68W, p.R138H and p.L148P mutations, the activity on the BMP pathway resulted significantly different from the stimulus produced by 200 or 400 ng of wild-type cDNA ($P<0.05$, Student's t test). This impairment is only partially rescued

when 200 ng of p.R68W, p.R138H and p.L148P mutant plasmids were co-transfected with equal amounts of the wild-type.

Table 1

BMP15 sequence variations* identified by genetic analysis in 300 POI women.

BMP15 GENE VARIANT	BMP15 PROTEIN VARIANT	PROTEIN DOMAIN	POI patients (n=300)		CONTROLS		AGE AT MENOPAUSE (years)
			PA n=45	SA n=255	A n=100	B n=116	
c.788_789insTCT	p.L262_I263insL	proregion	4	13	5	--	14-54
c.538G>A	p.A180T	proregion	--	6	--	--	21-29
c.443T>C	p.L148P	proregion	--	2	--	--	20-30
c.413G>A	p.R138H	proregion	1	--	--	--	---
c.202C>T	p.R68W	proregion	--	2	--	--	16
c.13A>C	p.S5R	signal peptide	--	1	--	--	30

45 women with primary amenorrhea, PA, and 255 women with secondary amenorrhea, SA. Control group A was represented by 100 women with physiological menopause. Control group B included 116 healthy subjects from the general population.

* Mutation nomenclature was based on GenBank reference sequences: NM_005448.1 for cDNA and NP_005439.1 for protein. Numbering uses +1 as the A of the initiation codon.

Table 2

In silico analysis of BMP15 variants.

Software	p.L262_L263insL	p.A180T	p.L148P	p.R138H	p.R68W	p.S5R
SIFT	-	tolerated	affects protein function	affects protein function	affects protein function	affects protein function *
NNpredict	normal secondary structure	altered secondary structure	altered secondary structure	altered secondary structure	altered secondary structure	altered secondary structure
PolyPhen	-	benign	possibly damaging	benign	probably damaging	benign

* Prediction statistically not significant