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ARTICLE

BMP15 c.-9C>G promoter sequence variant may contribute to the cause of non-syndromic premature ovarian failure

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Abstract *BMP15* has drawn particular attention in the pathophysiology of reproduction, as its mutations in mammalian species have been related to different reproductive phenotypes. In humans, *BMP15* coding regions have been sequenced in large panels of women with premature ovarian failure (POF), but only some mutations have been definitely validated as causing the phenotype. A functional association between the *BMP15* c.-9C>G promoter polymorphism and cause of POF have been reported. The aim of this study was to determine the potential functional effect of this sequence variant on specific *BMP15* promoter transactivation disturbances. Bioinformatics was used to identify transcription factor binding sites located on the promoter region of *BMP15*. Reverse transcription polymerase chain reaction was used to study specific gene expression in ovarian tissue. Luciferase reporter assays were used to establish transactivation disturbances caused by the *BMP15* c.-9C>G variant. The c.-9C>G variant was found to modify the PITX1 transcription factor binding site. *PITX1* and *BMP15* co-expressed in human and mouse ovarian tissue, and PITX1 transactivated both *BMP15* promoter versions (-9C and -9G). It was found that the *BMP15* c.-9G allele was related to *BMP15* increased transcription, supporting c.-9C>G as a causal agent of POF.

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Introduction

Premature ovarian failure (POF) is a frequent female reproductive disorder that affects 1–1.5% of women (Conway, 2000; Coulam et al., 1986; Luborsky et al., 2003). Clinically, POF is defined as at least 6 months of amenorrhoea occurring before the age of 40 years, associated with high FSH plasma levels (>40 mIU/ml). It can be found as an isolated phenotype (non-syndromic form) or accompanying further clinical features (syndromic presentation). Although the cause of POF remains elusive in most cases, some causative factors have been described, such as anticancer treatment, iatrogenic, autoimmune and metabolic disease, infection and genetic anomalies. Chromosomal abnormalities and point mutations in some autosomal and X-linked genes (e.g. *FSHR*, *LHR*, *NOBOX*, *SF1*, *FIGLA*, and *BMP15*) have been formally validated, by functional tests, as being causative of the phenotype (Aittomäki et al., 1995; Beau et al., 1998; Di Pasquale et al., 2004; Doherty et al., 2002; Laissue et al., 2008; Latronico et al., 1996; Lourenço et al., 2009; Quinn and Koopman, 2012; Rannikko et al., 2002; Rossetti et al., 2009; Touraine et al., 1999; Zhao et al., 2008).

Among the above, *BMP15* encodes a member of the transforming growth factor beta superfamily of growth factors, a group of proteins playing crucial roles during distinct developmental processes, including reproduction (Bragdon et al., 2011; Chang et al., 2002; Juengel and McNatty, 2005; Shimasaki et al., 2004). *BMP15* is specifically expressed in the ovary by oocytes from early follicular development stages. After a series of post-translational modifications, BMP15 proteins are secreted as homodimers (BMP15:BMP15) or heterodimers (BMP15:GDF9), when binding to their closely related paralog GDF9 (Aaltonen et al., 1999; Bodensteiner et al., 1999; Dube et al., 1998; Laitinen et al., 1998; Liao et al., 2003). Both BMP15:BMP15 and GDF9:GDF9 homodimers and BMP15:GDF9 heterodimers bind to granulosa cell serine/threonine kinase type I–II receptors which, in turn, activate the SMAD intracellular pathway (Moore et al., 2003; Pulkki et al., 2012). Signalling translocates then to the nucleus to regulate the expression of specific genes (Massagué et al., 2005). Functionally, *BMP15* expression in the oocyte stimulates granulosa cell proliferation and inhibits FSH action by suppressing *FSHR* expression, which is related to ovulation rate and fertility (Otsuka et al., 2000, 2011). It has been shown that GDF9 and BMP15 proteins activate the human primordial follicles development *in vitro*, with more beneficial effects of GDF9 (Kedem et al., 2011).

BMP15 has focused particular attention on the pathophysiology of reproduction, as its mutations in distinct mammalian species have been related to different reproductive phenotypes, ranging from complete infertility to hyperfertility (Otsuka et al., 2011; Pangas and Matzuk, 2004). In women with POF, *BMP15* coding regions have been directly sequenced in large panels. Strikingly, however, only some mutations have been definitely validated as causing the phenotype. This might have been caused by the complex gene expression in reproduction, as hundreds of genes are subtly regulated during folliculogenesis and ovulation (Matzuk and Lamb, 2002, 2008).

Sequence variants located in *BMP15* regulatory regions (especially in the minimal promoter) might affect its expression and contribute to pathogenic ovarian phenotypes. Interestingly, although the gene's complete promoter region has not been sequenced in large panels of patients, previous reports have suggested a functional association between the c.-9C>G sequence variant and ovarian phenotypes, including POF (Dixit et al., 2006; Gónzalez et al., 2008).

The aim of the present study was to determine (via in-silico and in-vitro assays) if the c.-9C>G variant might lead to *BMP15* promoter transactivation disturbances.

Materials and methods

In-silico *BMP15* promoter analysis

Potential transcription factor binding sites located in the *BMP15* promoter region were assessed using Genomatix Suite software (www.genomatix.de) (Germany) (Cartharius et al., 2005; Quandt et al., 1995). This software's MatInspector function was used for predicting transcription factor binding sites located 2 kb upstream of the gene's ATG initiation codon (genomic interval −2001 bp to +100 bp), using 0.75 core similarity threshold. ClustalW 2 software (www.ebi.ac.uk) was used for multiple alignment of the human *BMP15* promoter region with those from distinct mammalian species (*H. sapiens*- NG_012894.1, *P. troglodytes*- NC_006491.3, *P. abelii*- NC_012614.1, *M. mulata*- NC_007878.1, *O. aries*- NC_019484.1, *B. taurus*- AC_000187.1, *C. lupus familiaris*- NC_006621.3, *T. truncatus*- NW_004205773.1, *O. cuniculus*- NC_013685.1, *R. norvegicus*- NC_005120.3, *M. musculus*- NC_000086.7).

Plasmid constructs

As luciferase reporter we used a promoter region of 554 bp (from −555 bp to −1 bp, ENST00000252677) of the human *BMP15* gene. Briefly, using human genomic DNA extracted from whole blood samples (obtained from one of the four women who donated oocytes), this region was amplified by polymerase chain reaction using Pfx taq polymerase (Invitrogen Life Technologies, Grand Island, NY, USA). The c.-9G (BMP15-prom-G) and c.-9C (BMP15-prom-C) allele versions of the human *BMP15* promoter were generated by using reverse primers containing relevant (G or C) nucleotides at position -9. Forward and reverse primers included the *KpnI* and *XhoI* restriction sites at the 5′ and 3′ ends, respectively. Amplicons were subsequently cloned into the pGL4.22luc2CP/Puro plasmid (Promega, Madison, WI, USA) by standard digestion and ligation procedure, thereby leading to expression of the luciferase (Luc) reporter gene. These constructs, named BMP15-prom-G and BMP15-prom-C, were directly sequenced to discard those containing potential unexpected PCR-

induced mutations. A pCMV6-XL5 plasmid (OriGene, Rockville, MD, USA) containing the complete coding region of the human *PITX1* gene was kindly provided by Dr Christina Gurnett (Washington University) (Gurnett et al., 2008). This plasmid was used as a matrix for sub-cloning the *PITX1* coding region into the pcDNA 3.1/Zeo (+) vector (Invitrogen Life Technologies, Grand Island, NY, USA) (construct name: pcDNA-PITX1).

Cell culture, transfection and in-vitro luciferase assays

COS-7 cells (which do not endogenously express *PITX1*) were seeded at 120,000 cells per well in 24-well culture dishes in Dulbecco's phosphate-buffered saline medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were co-transfected using Fugene (Promega, Madison, WI, USA) reagent, with 500 ng of BMP15-prom-G or BMP15-prom-C constructs, and 50 ng of pcDNA 3.1-PITX1-WT plasmid. Negative controls involved co-transfection with empty vectors: pGL4-empty/pcDNA-PITX1, BMP15-prom-C/pcDNA-empty and PGL4-BMP15-prom-G/pcDNA-empty. A Renilla luciferase plasmid (Promega, Madison, WI, USA) was co-transfected in all experiments (30 ng per well) to monitor transfection efficiency. The Dual-Luciferase Reporter Assay System (DLR) (Promega, Madison, WI, USA) was used for assessing BMP15-prom-G and BMP15-prom-C promoter transcription activity 48 h after transfection. Each experiment was carried out at least twice each time with six replicates. The firefly activity observed for each replicate was divided by the recorded Renilla luciferase vector activity. All luciferase results were reported as relative luciferase units (RLU). A Student's *t*-test was used for estimating statistical significance.

BMP15 and *PITX1* expression

Human oocytes were recovered from four healthy donors having regular menstrual cycles who were attending the Colombian Fertility and Sterility Center (Bogotá, Colombia). All participants provided written informed consent. Briefly, after mild ovarian stimulation, metaphase II oocytes from follicles displaying cumulus expansion cells and corona radiata were isolated by using hyluronidase (Hyase-10X, Vitrolife, Inc, San Diego, CA, USA). Oocytes were pooled and stored in Trizol reagent (Invitrogen Life Technologies, Grand Island, NY, USA). Adult 8-week-old female Balb/c mice were used for dissecting complete ovaries. An RNA Mini Kit (Invitrogen Life Technologies, Grand Island, NY, USA) was used to isolate total RNA from human oocytes and mouse ovaries. cDNA was synthesized using Superscript III Reverse Transcriptase (Invitrogen Life Technologies, Grand Island, NY, USA). Human *PITX1*-cDNA was amplified by standard PCR, using primers (5′-GAGGGAGGAGATCGCCGTGTGGACC-3′ and 5′-CCTCAACGCGTGCCAGTACAACAGCTGA-3′) located on contiguous exons, which are separated by a 1954 bp intron. Mouse *Pitx1*-cDNA was amplified using primers located on exons 2 and 3. Human and mouse *BMP15* RT-PCR was carried out using primers located on the second and third exons. All amplicons were directly sequenced with internal primers and compared with wild type sequences (*BMP15*: ENSG00000130385, *Bmp15*: ENSMUSG00000023279, *PITX1*: ENSG00000069011, *Pitx1*: ENSMUSG00000021506). All the experimental steps of this study were approved by the Universidad del Rosario's Ethics Committee, and was conducted in line with the Declaration of Helsinki (approval date: 1 February 2012. Institional Review Board reference number: CEI-ABN026-000033).

Results

In-silico *BMP15* promoter analysis and ovarian *PITX1* expression

Genomatix software predicted 493 transcription factor binding sites, having over 0.80 matrix similarity (a perfect match with the matrix would be a score of 1.00), located in the *BMP15*-5′UTR region (Supplementary Table S1). This software determined that PITX1 would bind exclusively to the 5′aacaCTAAgcctttcaa-3′ region, which includes the -9C position (capital letters are defined as the matrix core sequence, the underlined letter is the -9C position) (Figure 1). Multiple sequence comparison among mammalian species revealed strict conservation of a cytosine at position -9 (Figure 1). Reverse transcription polymerase chain reaction

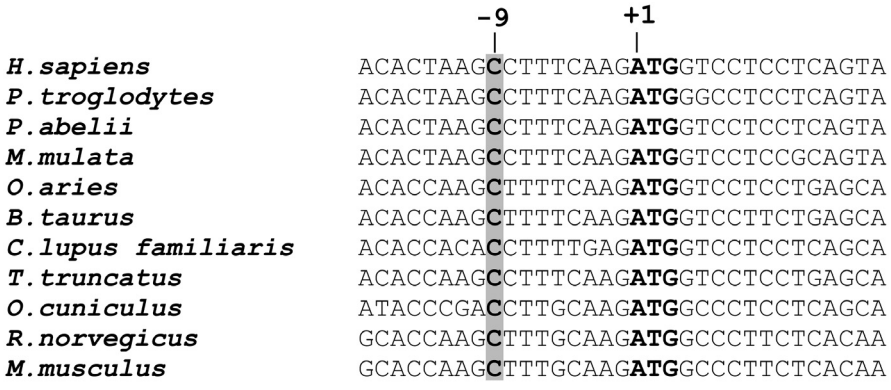

Figure 1 Multiple alignment of *BMP15* promoter sequences of vertebrate species. Adenine of the first ATG codon (methionine) was considered as +1. The -9C position is shadowed in grey.

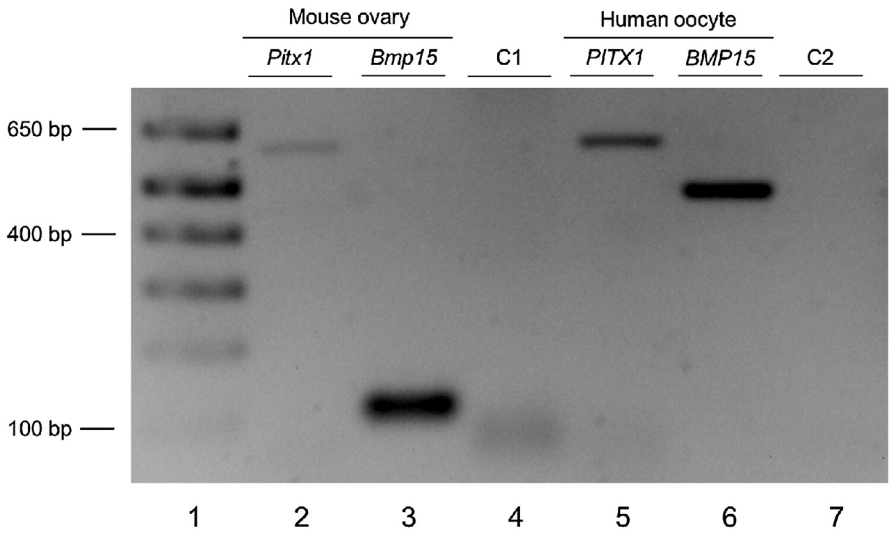

Figure 2 *BMP15* and *PITX1* RT-PCR from human oocytes and mouse ovaries. Lane 1: DNA ladder; lane 2: *Pitx1* expression in mouse ovary; lane 3: *Bmp15* expression in mouse ovary; lane 4: *Pitx1* negative control (C1); Lane 5: *PITX* expression in human oocytes; lane 6: *BMP15* expression in adult human oocytes; lane 7 *PITX1* negative control (C2). bp, base pairs.

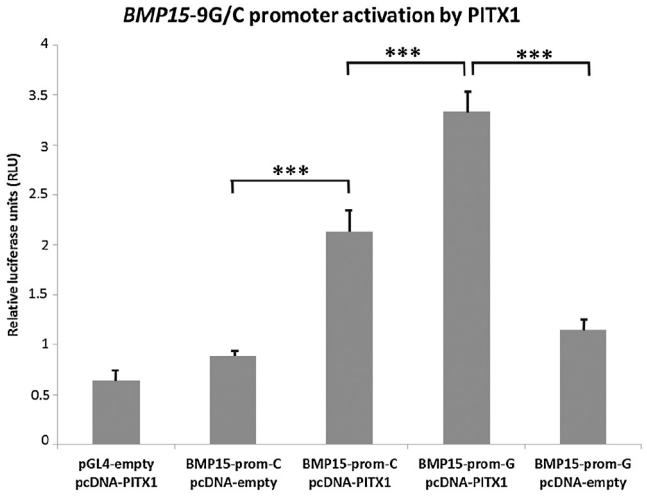

Figure 3 Luciferase reporter assays showing PITX1 transactivation properties on both BMP15-9G and BMP15-9G C promoter alleles. Luciferase activity is reported as relative luciferase units (RLU). ***, $P < 0.001$.

showed that *PITX1* and *BMP15* were co-expressed in adult human oocytes as well as in adult mouse ovaries (**Figure 2**). Direct sequencing of *PITX1* and *BMP15* RT-PCR amplicons showed that they corresponded to these genes' cDNA wild-type sequences.

Luciferase reporter assays

Luciferase reporter experiments demonstrated that PITX1 was able to activate both promoter versions of *BMP15* (BMP15-prom-G and BMP15-prom-C) (**Figure 3**). The transcription activity of PITX1 with the BMP15-prom-G construct was 1.6 fold higher than that observed with the BMP15-prom-C promoter ($P = 1.4 \times 10^{-4}$) (**Figure 3**).

Discussion

The crucial role of *BMP15* in the biology of reproduction was proposed more than 20 years ago, when KO models of mice, as well as natural mutations in sheep, revealed specific ovarian phenotypes. *Bmp15* and *Gdf9* homozygous KO mice displayed subfertility and infertility, respectively, whereas the double KO ($Bmp15^{-/-}$ and $Gdf9^{+/-}$) had a more pronounced ovarian phenotype than $Bmp15^{-/-}$ animals (Dong et al., 1996; Yan et al., 2001). To date, *BMP15* and *GDF9* are the principal genes for which mutations have been related to hyperfertility and infertility phenotypes in higher mammals (Abir and Fisch, 2011; Galloway et al., 2000; Hanrahan et al., 2004; Laissue et al., 2008). For instance, heterozygous and homozygous mutations in sheep were related to hyperfertility and infertility, respectively. Concerning human disease, Di Pasquale et al. (2004) reported the first heterozygous *BMP15* mutation (p.Cys235Tyr) conducive to POF. This mutation, which is located in the protein's pro-domain, causes a decrease of granulosa cell proliferation via a dominant negative effect.

Diverse hypothetical mechanisms underlying BMP15/GDF9 proteins' intriguing functional behaviour have been proposed (Hashimoto et al., 2005; Liao et al., 2003, 2004; Yan et al., 2001; Yoshino et al., 2006). Recently, this complex molecular puzzle was partially elucidated, as it has been shown that BMP15:GDF9 heterodimers in mice and humans act via a unique protein complex formed by the BMPR2 serine threonine kinase type 2 receptor, the ALK4/5/7 type 1 receptor and the ALK6 co-receptor (Peng et al., 2013). Furthermore, BMP15:GDF9 biological activity in mice and humans has been found to be significantly higher than that of BMP15 or GDF9 homodimers (Peng et al., 2013).

The *BMP15* coding regions have been sequenced in more than 1200 women with POF to date. Only four mutations (p.Arg68Trp, p.Arg138His, p.Leu148Pro, p.Tyr235Cys) have been validated (by in-vitro functional tests) as causative agents (Di Pasquale et al., 2004; Rossetti et al., 2009). Although these

findings have been relatively unexpected, owing to the crucial ovarian function of *BMP15* as observed in animal models, they may be explained by the complex gene expression and regulation in mammalian reproduction. Indeed, sex determination, gametogenesis, follicular development and ovulation are subtly modulated by hundreds of genes belonging to several physiological and molecular overlapping pathways (Matzuk and Burns, 2012; Matzuk and Lamb, 2002, 2008). This scenario implies that a significant number of genes must have fine transcriptional regulation during such multistep processes.

Therefore, we hypothesized that the *BMP15* c.-9C>G (rs3810682) promoter polymorphism might modify the transactivation properties of a specific transcription factor contributing towards POF molecular aetiology. Such sequence variant has aroused particular interest, as previous studies have suggested its potential implication in pathogenic reproductive phenotypes, including POF. Indeed, a mutational screening of the *BMP15* open reading frame (which included a short 5′exon 1-UTR region and intron-exon boundaries) in a panel of 202 Indian women with POF revealed that the c.-9G allele was part of a haplotype associated with the phenotype (Dixit et al., 2006). A study of 398 polycystic ovary syndrome (PCOS) women showed that, although the c.-9C>G variant was not related to disease pathogenesis, it might have been associated with specific clinical features, such as infertility (Gónzalez et al., 2008). Additionally, the c.-9C/G alleles have been related to differential ovarian response to recombinant FSH (rFSH) during assisted reproduction techniques, as the G allele has been linked to high responders (Abir and Fisch, 2011; Hanevik et al., 2011; Morón et al., 2006).

In the present study, an in-silico approach was taken, which identified that the PITX1 (pituitary homebox 1 protein) factor binds to a sequence located between −14 to −8 of the *BMP15* promoter (**Supplementary Table S1** and **Figure 1**). The strict conservation of this region among mammalian species argued in favour of a putative regulatory functional role (**Figure 1**). This sequence has previously been described as a canonical PITX1 binding motif present in specific pituitary gene promoters (Tremblay et al., 1998). PITX1 is a bicoid-related homeodomain transcription factor present in the anterior pituitary gland where it cooperates in activating hormone-encoding genes (e.g. *PRL*, *βTSH*, *POMC*, *βLH*, βFSH) (Quentien et al., 2006; Tremblay et al., 1998). It was shown that PITX1 and BMP15 are co-expressed in both human adult oocytes and in adult mouse ovaries (**Figure 2**). Functional in-vitro experiments showed that both *BMP15* promoter constructs (BMP15-prom-G and BMP15-prom-C) were activated by PITX1 (**Figure 3**). A statistically significant 1.6 fold increase in *BMP15* transcription activity conferred by the BMP15-prom-G construct was found. This feature might lead to alterations in granulosa cell proliferation rate, which may contribute towards POF molecular aetiology. High ovarian BMP15 levels in humans might lead to enhanced reduction of FSHR mRNA expression, similar to that observed in transgenic mice (TG-bmp15) overexpressing *Bmp15* (McMahon et al., 2008). Interestingly, these animals had a low and high number of primary and secondary follicles, respectively. These findings were associated with high granulosa cell mitosis in primary follicles and increased secondary follicle atresia. TG-bmp15 animals had premature menopause secondary to accelerated follicle development and atresia. It is worth noting that (as in other complex diseases) the *BMP15* c.-9G allele might be part of a complex functional assortment of coding and non-coding genomic variants leading to the phenotype. Such assumption would seem rational since some women carrying this variant do not present hypofertility phenotypes.

In conclusion, in the present study, it was established that the c.-9C>G variant modifies the PITX1 transcription factor binding site, and that *PITX1* and *BMP15* are co-expressed in human and mouse ovarian tissue. We showed that PITX1 transactivates both *BMP15* promoter versions (-9C and -9G) and that the *BMP15* c.-9G allele is related to *BMP15* increased transcription.

It was also found that PITX1 regulates *BMP15* transcription and argues in favour of c.-9C>G contributing towards POF aetiology. We estimate that further sequence analysis of the *BMP15* promoter region in POF women, from other ethnic populations, would be useful to identify haplotypes (including the -9C>G variant) reinforcing the role of this SNP during POF molecular aetiology. Additional functional studies are necessary to establish whether the -9C>G polymorphism is related to differential molar concentrations of BMP15/GDF9 homo and heterodimers.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.rbmo.2014.07.018.

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