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ARTICLE

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

Dora Janeth Fonseca [a,b], Oscar Ortega-Recalde [a], Clara Esteban-Perez [c], Harold Moreno-Ortiz [c], Liliana Catherine Patiño [a], Olga María Bermúdez [a], Angela María Ortiz [a], Carlos M. Restrepo [a,b], Elkin Lucena [a], Paul Laissue [a,b,*]

[a] *Unidad de Genética, Grupo GENIUROS, Escuela de Medicina y Ciencias de la Salud, Universidad del Rosario, Bogotá, Colombia*; [b] *Departamento de Fertilidad Humana, Genética Molecular de Colombia, Bogotá, Colombia*; [c] *Department of Reproductive Genetics, Fertility and Sterility Colombian Center, Bogotá, Colombia*

* Corresponding author. *E-mail address:* paul.laissue@urosario.edu.co (P Laissue).

Professor Paul Laissue obtained his MD from the Universidad del Rosario (Bogotá, Colombia). He subsequently obtained a Masters degree in Human Genetics (Paris 7 University), a Masters degree in Physiopathology of Reproduction (Paris 5 University) and a PhD in Human Genetics (Paris 7 University). He is currently Scientific Director of the Genetics Unit of the Universidad del Rosario in Bogotá. The aim of his scientific work is related to understanding the molecular basis of human infertility and other complex diseases.

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-

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