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Impaired production of BMP-15 and GDF-9 mature proteins derived from proproteins WITH mutations in the proregion

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ABSTRACT

Mutations in the bone morphogenetic protein-15 (BMP-15) and growth and differentiation factor-9 (GDF-9) genes have been identified in women with primary ovarian insufficiency (POI) and mothers of dizygotic twins. Here, we show that biological activities of the conditioned media from human embryonic kidney 293F cells transfected with two representative BMP-15 and GDF-9 mutants identified in the affected women have significantly reduced biological activities compared with the corresponding wild-type. Moreover, this difference is due to decreased production of the mature proteins, attributed most likely to impaired posttranslational processing of the proprotein. As genetic studies of the BMP-15 and/or GDF-9 genes in ewes established that a reduction of these proteins is associated with an increased ovulation rate, it is conceivable that women affected with these mutations may have an increased probability of bearing dizygotic twins during active reproductive ages before diagnosis with POI at later ages due to an earlier exhaustion of ovarian reserve.

1. Introduction

Women under 40 years of age that experience more than 4–6 months of amenorrhea and present with elevated serum FSH levels are diagnosed with premature ovarian failure (POF) or premature menopause (Rebar and Connolly, 1990). However, POF differs from menopause, in that more than 50% of patients diagnosed with POF have new follicle growth and 5–10% deliver a child after diagnosis (Nelson et al., 1994; Rebar and Connolly, 1990; Rebar et al., 1982; Taylor et al., 1996), Hence, primary ovarian insufficiency (POI) has been proposed as a preferred term for this condition, as first introduced by Fuller Albright in 1942 (Albright et al., 1942). Since POI affects around 1% of women under 40 years of age (Conway, 2000; Coulam et al., 1986), it is important to uncover the pathogenesis of POI. Genetic linkage analysis of familial POI cases has demonstrated that some POI cases result from chromosomal and genetic abnormalities (Laissue et al., 2008; Simpson, 2008; Skillern and Rajkovic, 2008). However, the majority of POI cases are clinically idiopathic, though factors such as chemotherapy (Chemaitilly et al., 2006; Meirow, 2000; Sklar et al., 2006), pelvic surgery (Lass, 1999), autoimmune diseases (Bakalov et al., 2005; Hoek et al., 1997), infection and environmental factors (Jick et al., 1977) may play a role

Bone morphogenetic protein-15 (BMP-15) and growth differentiation factor-9 (GDF-9) have recently been recognized as candidate factors that may be involved in POI (Di Pasquale et al., 2004; Dixit et al., 2005, 2006; Kovanci et al., 2007; Laissue et al., 2006). Both BMP-15 and GDF-9 are TGF-β superfamily members and produced in oocytes within the ovary (Shimasaki et al., 2004). Like other members of the TGF-β superfamily, BMP-15 and GDF-9 are both produced as a proprotein, comprised of a signal peptide, a proregion and a mature region (Dube et al., 1998; Laitinen et al., 1998). After removal of the signal peptide, the proprotein dimerizes, for which the proregion is essential (Hogan, 1996). The dimerized proprotein then undergoes proteolytic cleavage at a conserved RXXR cleavage site, which separates the proregion from the bioactive mature region (Massagué et al., 1994). A major breakthrough in the field of these oocyte-specific factors occurred when naturally occurring mutations of the BMP-15 and GDF-9 genes were found in ewes (Galloway et al., 2000; Hanrahan et al., 2004; McNatty et al., 2005). Ewes homozygous for the BMP-15 or GDF-9 mutations are infertile due to an arrest in follicle growth at the primary stage, whereas heterozygous carriers are super-fertile displaying increased ovulation rates and litter size compared with wild-type ewes.

BMP-15 also plays an important role in ovarian function in women. The first identified human BMP-15 mutation associated with hypergonadotropic ovarian failure due to ovarian dysgenesis was a non-conserved substitution of a tyrosine with a cysteine at amino acid residue 235 of the proregion of BMP-15 (Di Pasquale

Abbreviations: BMP-15, bone morphogenetic protein-15; GDF-9, growth and differentiation factor-9; POI, primary ovarian insufficiency; POF, premature ovarian failure.

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et al., 2004). The two patients with the BMP-15 mutation were sisters and heterozygous carriers. Laparoscopic examination indicated that the ovaries of these patients are grossly "streak", like that observed in the homozygous, but not heterozygous, BMP-15 mutant ewes. As it was shown that the mutant protein antagonizes the mitotic properties of wild-type BMP-15, the simplest interpretation of this finding is that the infertility in those patients could be attributed to the antagonistic nature of the mutant. The same group extended the study to conduct genetic screening in unrelated POI patients, and demonstrated that mutations in the BMP-15 gene are associated with a high incidence of POI (Di Pasquale et al., 2006). Furthermore, they showed that three mutations (BMP-15^{R68W}, BMP-15^{L148P}, and BMP-15^{R138H}) identified in women with overt POI lead to decreased production of mature protein in an *in vitro* assay (Rossetti et al., 2009). Other groups have also investigated whether BMP-15 mutations are involved in POI (Dixit et al., 2006; Laissue et al., 2006; Ledig et al., 2008; Takebayashi et al., 2000), with two studies (Dixit et al., 2006; Laissue et al., 2006) identifying additional BMP-15 mutations that occur with a higher frequency in POI patients than in normal women. Mutations in the BMP-15 gene have also been identified in mothers of dizygotic twins (Zhao et al., 2008); however, it remains to be determined whether these variants are mutations associated with the pathogenesis of POI and/or dizygotic twins or rare polymorphisms, thus caution is recommended in the interpretation of BMP-15 mutations identified in POI cases (Ledig et al., 2008).

Screening for mutations in the GDF-9 gene in POI patients has identified several missense mutations not found in control women (Dixit et al., 2005; Kovanci et al., 2007; Laissue et al., 2006; Zhao et al., 2007). Moreover, mutations in the GDF-9 gene have also been identified as being significantly more common in mothers of dizygotic twins compared with controls (Zhao et al., 2007). However, the nature and biological impact of these mutations on the GDF-9 gene are unknown because no structure/function studies have been performed.

It is notable that in the majority of BMP-15 and GDF-9 mutations identified in POI patients and/or mothers of dizygotic twins, the mutation site is located in the proregion of the proprotein. Thus, if the processing of these mutant proproteins occurred normally, the resulting mature proteins should be indistinguishable from the wild-type, and no functional defects in the mutants would be expected. Therefore, we hypothesize that BMP-15 and GDF-9 mutations described in POI patients and/or mothers of dizygotic twins may result from altered posttranslational processing of these proteins.

To test our hypothesis, we have chosen two representative BMP-15 and GDF-9 mutants identified in women with POI and/or mothers of dizygotic twins (Dixit et al., 2005, 2006; Kovanci et al., 2007). They are BMP-15^{R76C}, BMP-15^{R206H}, GDF-9^{K67E} and GDF-9^{P103S}, that occur with a high incidence (n = 3/133, 1/133, 4/127 and 1/61, respectively) in POI patients, and were not identified in normal women (n = 0/197, 0/197, 0/220 and 0/60, respectively) (Dixit et al., 2005, 2006; Kovanci et al., 2007). These mutations are predicted to be deleterious and thus may have pathogenic effects. Moreover, GDF-9^{P103S} was also identified in mothers of dizygotic twins with a significantly higher frequency than in controls (0.0119 *vs* 0.0048, $p < 0.02886$) (Palmer et al., 2006). In the current study, we have explored whether and to what extent these mutant proteins affect BMP-15 and GDF-9 biology.

2. Materials and methods

2.1. Reagents and supplies

Female Sprague–Dawley rats were purchased from Charles River Laboratories (Wilmington, MA). A human granulosa cell line (COV-434) and a mouse embryo teratocarcinoma epithelial cell line (P19) were generously provided by Drs. Peter Schrier and Sylvia Evans, respectively. Phospho Smad1/5/8, Phospho Smad2, Smad2/3 and Smad5 antibodies were obtained from Cell Signaling Technology (Beverly, MA).

2.2. Construction of expression plasmids

We previously described the generation of phBMP-15F and phGDF-9F plasmids that have a Flag epitope (F) at the C-terminus (Liao et al., 2004; Otsuka et al., 2000). These plasmids were used as templates to construct expression plasmids of phBMP-$15F^{R76C}$, phBMP-$15F^{R206H}$, phGDF-$9F^{K67E}$, and phGDF-$9F^{P103S}$ by a site-directed mutagenesis technique (Hashimoto et al., 2005; Liao et al., 2003, 2004). Comparison with previously reported data, in regard to the biological activities of rhBMP-15 and rhGDF-9, indicates that the incorporation of the flag epitope tag at the C-terminus does not alter the biological activities of untagged rhBMP-15 and rhGDF-9 (Li et al., 2009; Liao et al., 2004; Mottershead et al., 2008; Otsuka et al., 2000).

2.3. Transfection and cell culture

The recombinant proteins were produced using FreeStyle™ 293 Expression System (Invitrogen, Carlsbad, CA). Briefly, human embryonic kidney 293F cells were cultured in 30 ml FreeStyle serum-free medium (Invitrogen) in a 125 ml Erlenmyer flask on an orbital shaker rotating at 135 rpm in a 37 °C CO_2 incubator with a humidified atmosphere. The cells were then seeded in 160 μl of the medium in a 48-well plate at a density of 10^6 cells/ml and transfected with 160 ng of each plasmid using 293fectin™ transfection reagent (Invitrogen). The transfected cells were cultured on the orbital shaker rotating at 135 rpm in the incubator for 1–4 days, then the conditioned media were collected and the cells lysed for further analysis.

2.4. Bioactivity studies

For the bioactivity studies, primary granulosa cells were harvested from ovaries of Sprague–Dawley rats (23 days old) that had been implanted subcutaneously with a silastic capsule containing 10 mg of diethylstilbestrol for 4 days to increase granulosa cell number. All animal protocols were approved by the University of California San Diego Institutional Animal Care and Use Committee. Primary granulosa cells (2×10^5 viable cells) were cultured in a 96-well plate containing 200 μl of serum-free McCoy's 5A medium as described previously (Otsuka et al., 2000) in the presence or in the absence of 50 μl of conditioned media collected from 293F cells cultured for 4 days after the transfection with either phBMP-15F, phBMP-$15F^{R76C}$, phBMP-$15F^{R206H}$, phGDF-9F, phGDF-$9F^{K67E}$, or phGDF-$9F^{P103S}$. After 24 h culture, the granulosa cells were incubated with 5-bromo-2′-deoxyuridine (BrdU; 10 μM) for additional 24 h at 37 °C. BrdU incorporation was quantified by a BrdU ELISA assay kit purchased from Roche Applied Science (Indianapolis, IN) according to the manufacturer's instruction.

For analysis of Smad phosphorylation, COV434 and P19 cells were cultured in a 48-well plate in DMEM/F12 containing 10% fetal bovine serum, 100 U/ml penicillin, 1000 mg/ml streptomycin and 2 mM glutamine. When the cells reached approximately 80% confluency the medium was replaced with 200 μl of serum-free DMEM/F12. After a 3 h preculture, 50 μl of conditioned medium from the transfected 293F cells was added and cells were incubated for 1 h. Cells were then washed once in chilled PBS and solubilized in a lysis buffer. The cell lysates were subjected to Western blotting analysis using anti-phospho Smad1/5/8, anti-phospho Smad2, anti-total Smad5 or anti-total Smad2/3 antibodies as reported previously (McMahon et al., 2008). The relative integrated density of each protein band was digitized by NIH image J 1.40 (National Institutes of Health, Bethesda, MD).

2.5. RNA extraction and quantitative real-time PCR

The 293F cells were seeded in 800 μl of medium in a 12-well plate at a density of 10^6 cells/ml and transfected with 800 ng of each plasmid. After culture for 48 h, the cells were collected by centrifugation and total cellular RNA extracted as described previously (Liao et al., 2003). PCR primer pairs were selected from different exons of the corresponding genes to discriminate PCR products that might arise from possible chromosome DNA contaminants. Specifically, they were derived from the cDNA clones at the following nucleotide numbers: BMP-15, 131 bp (GenBank, accession nos. NM_005448, 252–271 and 361–382); GDF-9, 94 bp (NM_005260, 581–600 and 655–674); and housekeeping gene, ribosomal protein L19, 190 bp (NM_000981, 401–420 and 571–590). For the quantification of mRNA expression, real-time PCR was performed using LightCycler-FastStart DNA master SYBR Green I system (Roche Diagnostic Co., Tokyo, Japan) with annealing at 60 °C and the PCR reaction containing 4 mM $MgCl_2$ following the manufacturer's protocol. Accumulated levels of fluorescence during amplification were analyzed by the second-derivative method after melting-curve analysis (Roche Diagnostic), with expression levels of target genes in each sample standardized to RPL19 expression.

2.6. Protein analysis

For analysis of intracellular proteins, cells were washed once in chilled PBS and solubilized in lysis buffer containing Protease-Arrest, 2% SDS and 4% β-mercaptoethanol. For analysis of proteins secretion, conditioned media were