Identification of a mutation in GDF9 as a novel cause of diminished ovarian reserve in young women

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Study question: Do any mutations in growth differentiation factor 9 (GDF9) have a role in diminished ovarian reserve (DOR) in young women?

Summary answer: The GDF9 p.R146C mutation may be a source of DOR in some young women.

What is known already: DOR affects 10% of women under 37 years of age and is associated with accelerated expenditure of follicles. GDF9 is an oocyte-secreted factor that plays a critical role in follicular development and female fertility. Several GDF9 variants have been linked to ovarian dysfunction.

Study design, size, duration: This case–control study included 139 women with DOR and 152 controls aged under 37 years.

Participants/materials, setting, methods: All women were recruited in a Chinese tertiary center and underwent DNA sequencing of GDF9 gene. We then determined the molecular and biological properties of mutant GDF9 proteins using protein expression, structural prediction and functional analyses.

Main results and the role of chance: We identified two mutations in the proregion of GDF9 gene: c.169T>G (p.D57Y) and c.436T>C (p.R146C). The p.R146C mutation was found in three women with DOR but was absent in the control population. This mutation was also associated with significant reductions in GDF9 mature protein secretion in cultured cells. Functional studies with human granulosa cells (GCs) showed that the p.R146C mutation reduced the abilities of GDF9 to stimulate GC proliferation and to activate the Smad2 pathway. Protein structure modeling predicted that p.R146C disrupted an α-helix in GDF9 protein. In contrast with p.R146C, the p.D57Y mutation, found in both the DOR and control groups (6 versus 2), had no obvious deleterious effects.

Limitations, reasons for caution: Larger studies in varying populations may validate the role of GDF9 mutation in young women with DOR.

Wider implications of the findings: These results may provide new insights into the pathophysiological mechanisms of early-onset DOR.

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Key words: GDF9 / diminished ovarian reserve / mutation / folliculogenesis / reproductive aging

† These authors contributed equally to this work.

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Introduction

Diminished ovarian reserve (DOR) is a disorder of ovarian function characterized by reduced numbers and/or quality of oocytes, which has been associated with female infertility, increased miscarriage rates and poor response to ovarian stimulation for IVF and embryo transfer (IVF-ET) (Broekmans et al., 2007, 2009). The prevalence of DOR has been estimated to be ~10% among young women (Nikolaou and Templeton, 2003), and its etiology remains unknown. Recent studies support a role for genetic factors in DOR (Matzuk and Lamb, 2002; Broekmans et al., 2007). Increasing evidence shows that variants of genes, such as fragile × mental retardation 1 (FMR1, MIM #309550) (Gleicher et al., 2009), steroidogenic factor 1 (SF1, MIM#184757) (Warman et al., 2010) and growth differentiation factor 9 (GDF9, MIM # 601918) (Wang et al., 2010c) may mediate the decline of ovarian function.

GDF9 was the first identified oocyte-secreted factor, and belongs to the transforming growth factor-β (TGF-β) superfamily. Like other members of the TGF-β superfamily, GDF9 is produced as a proprotein comprising a signal peptide, a proregion and a mature region (Laitinen et al., 1998). After removal of the signal peptide, the proprotein dimerizes and for this, the proregion is essential. The dimerized proprotein then undergoes proteolytic cleavage and releases the biologically mature protein (Shi and Massague, 2003). In mammals, oocytes preferentially express GDF9 within the ovary, where its signals to surrounding somatic cells are necessary for ovarian folliculogenesis (McGrath et al., 1995). The downstream signaling actions of GDF9 are mediated by the bone morphogenetic protein receptor type II (BMPRII) and type I (ALK5) receptors, which initiate the subsequent activation of Smad2/3 by phosphorylation in granulosa cells (GCs) (Shi and Massague, 2003). Phosphorylated Smad2/3 may regulate the expression of genes, such as Inhibin B, ptx3 and Has, and coordinate key events of the ovulatory process (Kaivo-Oja et al., 2003; Li et al., 2008). GDF9-deficient female mice are completely infertile because of multiple defects in the ovary, including blocks in folliculogenesis at the primary stage, degenerated oocytes, impaired GC differentiation and increased follicle-stimulating hormone (FSH) levels (Dong et al., 1996). In contrast, GDF9 knockout male mice are unaffected. In parallel, in vitro studies have revealed that recombinant GDF9 promotes the transition of pre-antral to antral follicles (Orisaka et al., 2006), enhances oocyte competence (Hussein et al., 2006) and stimulates GC differentiation via the Smad2/3 pathway (Elvin et al., 1999; Shi and Massague, 2003). Taken together, these data suggest that disruption of GDF9 gene may prevent folliculogenesis and oogenesis, resulting in ovarian failure. Consistent with these results, GDF9 mutations are related to abnormal reproductive phenotypes in women, including premature ovarian failure (Hannahran et al., 2004; Dixit et al., 2005; Chand et al., 2006), polycystic ovary syndrome (Wang et al., 2010a,b) and dizygotic twinning (Palmer et al., 2006). Our previous study found that GDF9 polymorphisms were closely associated with DOR and reduced reproductive potential (Wang et al., 2010c). In this study, we further screened the GDF9 gene to identify novel variants in a group of young women with DOR.

Materials and Methods

Patients and sample collection

A cohort of 139 patients, aged under 37 years old and with a proven diagnosis of DOR, were recruited from the Centre of Reproductive Medicine, Women’s Hospital, School of Medicine, Zhejiang University. The criteria for DOR included AMH, FSH and estradiol (E2) levels as well as antral follicle counts (AFC) (Sills et al., 2009; Ferraretti et al., 2011; May-Panloup et al., 2012). Patients were considered to have DOR if they had serum AMH levels <1.1 ng/ml and/or an AFC ≤5 combined with serum FSH levels >10 IU/l and/or E2 >80 pg/ml on Day 3 of a spontaneous cycle. The control (normal ovarian reserve) group included 152 infertile women who underwent IVF-ET treatment and had serum AMH levels >2 ng/ml and/or an AFC ≥6 and serum FSH levels <10 IU/l and E2 below 80 pg/ml. Women with known conditions affecting ovarian reserve, including ovarian surgery, polycystic ovarian syndrome, polycystic ovaries, endometriosis or BMI >25 were excluded. Sample size was determined by using the PS software (http://biostat.mc.vanderbilt.edu.twik/bin/view/Main/PowerSampleSize) based on our pilot study and an α of 0.05 and power of 80%. All the participants were genetically unrelated ethnic Han Chinese and were from southeast of China. The ethnic origin was obtained by a combination of self-assessment and questionnaire. The clinical characteristics of women in control and DOR groups are shown in Table I. Ethical approval for this project was granted by the Ethics Committee of Women’s Hospital, School of Medicine, Zhejiang University. Written informed consent was obtained from each subject before sample collection.

Mutational analysis

Genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) using the QIAamp DNA Blood kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. The two coding exons of GDF9 were amplified with PCR, and the primers are listed in Table II. The PCR products were sequenced with the BigDye Terminator Kit (Applied Biosystems, Foster City, CA, USA) via the ABI Prism Sequencer 3100 (Applied Biosystems) in both forward and reverse directions with the same primers as listed in Table II. The genetic variation nomenclature refers to the cDNA sequence NM_005260.3 and protein sequence NP_005251.1 (NCBI). Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon (codon 1) in the reference sequence.

<table>
<thead>
<tr>
<th>Table I Clinical characteristics of patients.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>-------------------------</td>
</tr>
<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
</tr>
<tr>
<td>Day-3 FSH (IU/l)</td>
</tr>
<tr>
<td>Day-3 LH (IU/l)</td>
</tr>
<tr>
<td>Day-3 E₂ (pg/ml)</td>
</tr>
<tr>
<td>Day-3 T (nmol/l)</td>
</tr>
<tr>
<td>AMH (ng/ml)</td>
</tr>
<tr>
<td>AFC</td>
</tr>
<tr>
<td>No. of follicles &gt; 14 mm</td>
</tr>
<tr>
<td>No. of MII oocytes</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
</tr>
<tr>
<td>Good quality embryos (%)</td>
</tr>
<tr>
<td>Pregnancy rate (%)</td>
</tr>
</tbody>
</table>

AFC, antral follicle count; AMH, anti-Müllerian hormone; DOR, diminished ovarian reserve; E₂, estradiol; n.s., non-significant; T, testosterone.
**Table II** Primers and products size.

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>Amplification site</th>
<th>Amplification length (bp)</th>
</tr>
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<tbody>
<tr>
<td>F1: 5'-CTTCAGCTAGGCATCTTTGAGGCG-3</td>
<td>GDF-9; exon 1</td>
<td>742</td>
</tr>
<tr>
<td>R1: 5'-GCCACCTTAAACCAATGAGAGCG-3</td>
<td>GDF-9; exon 2(A)</td>
<td>816</td>
</tr>
<tr>
<td>F2: 5'-CATGGGAGACTCTTGTCTCTAC-3</td>
<td>GDF-9; exon 2(B)</td>
<td>844</td>
</tr>
<tr>
<td>R2: 5'-GGATGCTCCAGCTGCGTCTTTCATG-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F3: 5'-CTTTTATGGGGCTCCAAACAAGAGAG-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R3: 5'-GATCAACCTACACAGGCTCCTC-3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Plasmid constructions**

A full-length human GDF9 cDNA was amplified by RT–PCR from male testis total RNA and subcloned into the pcDNA4Myc-His expression vector. A previous study showed that incorporation of a C-terminal His tag destroyed GDF9 bioactivity (Mottershead et al., 2008). In the present study, we included a translational stop codon in the reverse PCR primer to terminate translation of the His-tag in the plasmids. The variants were introduced using Quick Change Site-directed Mutagenesis Kit (Agilent Technologies, Inc., Santa Clara, CA, USA) into wild-type GDF9 expression constructs as templates. All plasmids were confirmed by direct sequencing.

**Transfection and protein analysis**

Human embryonic kidney 293T (HEK293T) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS) for 24 h before transfection. Cells were seeded into 6-well plates (3.0 × 10^5/well). Plasmid constructs encoding either wild-type or mutant GDF9 were transfected into HEK293T cells using FuGene 6 (Roche Applied Science, Indianapolis, IN, USA). The positive clones were selected with 600 μg/ml Zeocin and confirmed by RT–PCR for GDF9 gene. The selected clones were maintained in the medium containing 10% FBS. At 80% confluence, cells were switched to a serum-free medium (conditioned medium). The conditioned medium was evaluated with the BCA Kit (Pierce, Thermo Fisher Scientific, Rockford, IL, USA) and analyzed by SDS-PAGE immunoblotting using an anti-GDF9 antibody. Non-transfected HEK293T cells were used as controls.

**GC proliferation assay**

Human GCs were harvested from pre-ovulatory follicles of women undergoing IVF-ET. GCs were cultured in DMEM/F12 medium (Gibco) containing 10% FBS. For growth and assays, GCs were cultured in 96-well plates in the presence or absence of the indicated concentrations of conditioned media collected from the HEK293T cells which had been transfected with either wild-type or mutant plasmids. Cell lysates were then subjected to western blot analysis using an anti-Phospho Smad2 antibody or anti-total Smad2 antibody (Cell Signaling Technology, Danvers, MA, USA) and visualized with ECL detection reagent.

**Structure model of GDF9 mutant**

Sorting intolerant from tolerant program (SIFT; http://blocks.fhcrc.org/sift/SIFT.html) was used to assess the possible functional consequences of mutations. The tertiary structure modeling of wild type and mutant GDF9 was performed by I-TASSER (Zhang, 2008) and visualized using RasMol 2.7.5 (Sayle and Milner-White, 1995). The GDF9 protein structures were presented using ribbon diagrams.

**Statistical analyses**

Statistical analysis was performed using SPSS 15.0 for WINDOWS. Independent samples t-test was used to evaluate the statistical significance between two groups. One-way analysis of variance (ANOVA) and Turkey’s post hoc tests were used to evaluate the statistical significance of the difference between more than two groups. χ² test was used to compare fertilization, good quality embryo, pregnancy rates and genotype frequencies between two groups. To account for multiple testing, the Bonferroni correction was applied. P-values of <0.05 were considered statistically significant.

**Results**

**Evaluation of general clinical characteristics**

A total of 139 patients with DOR and 152 controls were included in the study (Table I). AMH levels and AFC in the DOR group were 0.67 ± 0.39 ng/ml and 3.99 ± 0.91, respectively and significantly lower than in the control group, 4.37 ± 1.59 ng/ml and 10.13 ± 2.70, respectively.

**Identification of GDF9 heterozygous mutations in DOR women**

After sequencing the GDF9 genes of PBMCs from 139 patients with DOR, we identified four SNPs in the GDF9 gene: c.447C>T, c.546G>A, c.169G>T (p.D57Y) and c.436C>T (p.R146C) (Table II). The c.447C>T and c.546G>A have been reported in our previous study (Wang et al., 2010c). The c.169G>T and c.436C>T are heterozygous variants in the prorogregion detected in the present study. The c.436C>T changes arginine to cysteine (p.R146C), and was present in three patients with DOR, while absent in controls (Table III). The c.169G>T results in tyrosine being substituted for aspartic acid (p.D57Y) and was present in six
Table III

GDF9 sequencing results in 139 women with DOR and 152 controls.

<table>
<thead>
<tr>
<th>Location</th>
<th>Amino acid variation</th>
<th>dbSNP ID</th>
<th>MAF</th>
<th>Genomic position</th>
<th>Patients with DOR</th>
<th>Controls</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>chr5:132198477</td>
<td>c.169G&gt;T</td>
<td>Exon1</td>
<td>Wild</td>
<td>0.472</td>
<td>133 (95.7) 0 (0) 6 (4.3)</td>
<td>150 2 (1.3) 21 (1.3)</td>
<td>0.026</td>
</tr>
<tr>
<td>chr5:132198210</td>
<td>c.436C&gt;T</td>
<td>Exon2</td>
<td>Wild</td>
<td>0.276</td>
<td>150 2 (1.3) 21 (1.3)</td>
<td>0 (0) 0 (0) 0 (0)</td>
<td>0.276</td>
</tr>
<tr>
<td>chr5:132198199</td>
<td>c.447C&gt;T</td>
<td>Exon2</td>
<td>Wild</td>
<td>0.083</td>
<td>150 2 (1.3) 21 (1.3)</td>
<td>0 (0) 0 (0) 0 (0)</td>
<td>0.083</td>
</tr>
<tr>
<td>chr5:132198100</td>
<td>c.546G&gt;A</td>
<td>Exon2</td>
<td>Wild</td>
<td>1.000</td>
<td>150 2 (1.3) 21 (1.3)</td>
<td>0 (0) 0 (0) 0 (0)</td>
<td>1.000</td>
</tr>
</tbody>
</table>

DOR, diminished ovarian reserve; NP, not present; N, number of women; SIFT, sorting intolerant from tolerant. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference cDNA sequence NM_005260.3 and protein sequence NP_005251.1. The initiation codon is codon 1.

aGenomic position is based on Genome Reference Consortium Human Build 37, Hg19 chromosomal.
bMAF, minor allele frequency, is based on 1000 genomes.
cData were analyzed by χ² test and adjusted by Bonferroni correction.

To evaluate the biological impact of GDF9 mutations on GDF9 protein production and secretion, we stably transfected HEK293T cells with expression plasmids encoding wild-type GDF9 or its variants (p.R146C or p.D57Y) and analyzed GDF9 proteins in the conditioned media using SDS-PAGE western blots. The proproteins and mature proteins of GDF9 from the cells transfected with wild-type or mutations (p.R146C or p.D57Y) were detected in conditioned media (Fig. 2). The levels of mature GDF9 protein in the conditioned media from the cells transfected with p.R146C were remarkably reduced (P < 0.01) compared with that from cells transfected with wild-type GDF9 (Fig. 2). The levels of GDF9 mature protein from the cells transfected with p.D57Y were not different from that those with wild type (Fig. 2).

Impaired bioactivity of GDF9 protein with the p.R146C mutation

GCs are the primary target cells of GDF9. We examined whether the bioactivity of mutant GDF9 was different from that of wild-type GDF9. Using Alamar Blue assays, we found that wild-type GDF9 at 30–1000 ng/ml promoted the proliferation of GCs in a dose-dependent manner (Fig. 3A). However, the proliferation rate of GCs in the presence of conditioned medium containing GDF9 with the p.R146C mutation was significantly lower than that in the wild-type group (Fig. 3A; ANOVA followed by Turkey’s post hoc tests). The proliferation rate of GCs treated with GDF9 with p.D57Y mutation was not significantly different from that of the wild-type group. The results indicate that the p.R146C mutation reduces the bioactivity of GDF9.

Attenuated activation of Smad signaling in GCs treated with GDF9 with p.R146C

We analyzed the effect of mutations on GDF9 signal transduction by examining the phosphorylation of Smad2 protein in human GCs. Treatment of GCs with wild-type or mutant GDF9 up-regulated total Smad2 protein expression in a dose-dependent fashion, and, there was no significant difference in expression levels among the three groups (Fig. 3B). However, the level of phosphorylated Smad2 in the p.R146C mutant group was significantly lower than that in wild-type or p.D57Y mutant groups (P < 0.01, ANOVA followed by Turkey’s post hoc tests) (Fig. 3B and C).

Structural model of GDF9

To investigate whether p.R146C and p.D57Y mutations might alter the GDF9 protein structure, we predicted the structure model of GDF9 using I-TASSER. No significant differences were observed in the
predicted structure between wild-type and p.D57Y mutant GDF9 (Fig. 4A and B). In contrast, the three-dimensional model of p.R146C revealed that the p.R146C mutation might result in the loss of an α-helix (Fig. 4C and D). Instead, a β-sheet was formed (Fig. 4B and C). This disruption of secondary structure is likely to disrupt protein folding and secretion.

**Discussion**

In this study, we report two heterozygous mutations (p.D57Y and p.R146C) in GDF9. The p.R146C mutation was suspected as a cause of DOR in some young patients, because several findings in the present study support its possible contribution to the pathogenesis of GDF9 mutation and diminished ovarian reserve.
DOR. These findings include the absence of p.R146C in matched controls, reduced mature protein production and bioactivity by the p.R146C mutation, and disruption in the three-dimensional protein structure of GDF9 by the p.R146C mutation.

All TGF-β superfamily members require proregion domains to form a dimeric proprotein, which can then be processed by specific cleavage enzymes to separate the proregion dimer from the functional mature protein dimer. In the present study, we demonstrated that the GDF9 p.R146C mutation was absent in the control cohort of women, and may have deleterious effects on the secretion of mature GDF9 protein. The substitution of a positively charged arginine with uncharged cysteine in this proregion maybe a steric hindrance to forming the correct structure of proprotein dimers, thus resulting in reduced production of the functional dimeric mature protein. The additional cysteine in GDF9 p.R146C is likely to form another disulfide bond with one of the pre-existing cysteine residues, resulting in a misfolded proprotein. Arginine at position 146 is highly conserved in most mammals including mouse, rat, sheep, goat, cow, pig, chimpanzee and human, so alteration of arginine 146 may have possible functional consequences. Indeed, GDF9 molecular modeling validates that the p.R146C mutation affects the tertiary structure by disrupting the α-helix.

A key factor in determining ovarian lifespan is the number of primordial follicles at birth and the rate of loss from this finite pool during mid-adult life. GDF9 is essential for ovarian function and maintenance as it controls multiple ovarian processes. Not only does it act directly on folliculogenesis (Juengel and McNatty, 2005), but also it regulates ovarian hormone secretion such as inhibin B (Kaivo-Oja et al., 2003). Decreased protein production and impaired bioactivity in GDF9 p.R146C mutation may have a role in deterioration of ovarian function. There appear to be four major consequences of DGF9 disruptions: (i) follicle viability and apoptosis of primordial follicles; (ii) quality of oocytes; (iii) proliferation and survival of GCs and (iv) ovarian endocrine homeostasis. Indeed, a recent study has linked the GDF9 expression levels in the follicular fluid to oocyte quality and age in women (Han et al. 2011). These may explain the poor response to ovarian stimulation and low-quality oocytes in the three DOR patients with the p.R146C mutation during IVF treatment in the present study.

### Table IV Clinical characteristics of women carrying GDF9 mutations.

<table>
<thead>
<tr>
<th>GDF9 mutation</th>
<th>Patient ID</th>
<th>Age (years)</th>
<th>Phenotype</th>
<th>bFSH (IU/l)</th>
<th>E&lt;sub&gt;2&lt;/sub&gt; (pg/ml)</th>
<th>AFC</th>
<th>AMH (ng/ml)</th>
<th>No. of follicles ≥ 14 mm</th>
<th>No. of good quality embryos</th>
<th>Pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>p.D57Y (c.169G&gt;T)</td>
<td>51</td>
<td>28</td>
<td>DOR</td>
<td>9.11</td>
<td>87.27</td>
<td>4</td>
<td>1.02</td>
<td>3</td>
<td>1</td>
<td>No</td>
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<tr>
<td></td>
<td>191</td>
<td>25</td>
<td>DOR</td>
<td>10.39</td>
<td>67.1</td>
<td>4</td>
<td>0.81</td>
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<td></td>
<td>205</td>
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<td>DOR</td>
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<td>97.1</td>
<td>10</td>
<td>0.84</td>
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<td>289</td>
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<td>DOR</td>
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<td>121.3</td>
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<td>303</td>
<td>28</td>
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<td>108.9</td>
<td>4</td>
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</table>

AFC, antral follicle count; AMH, anti-Müllerian hormone; DOR, diminished ovarian reserve.

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Figure 2 Effects of mutations on the production and secretion of GDF9 in HEK293 cells transfected with wild-type or mutant GDF9 genes. (A) Wild-type (WT) and mutant forms of GDF9 were expressed in HEK293T cells. Immunoblotting was performed to examine proprotein and mature protein of GDF9 in conditioned medium using an anti-GDF9 antibody. Coomassie-brilliant blue-stained gel was used as a loading control. NT, non-transfected. (B) Quantitative analysis of GDF9 proteins. *P < 0.01, compared with the wild-type GDF9. N, number of the repeated experiments.
Defects of the GDF9 mature protein observed in p.R146C carriers were similar to another GDF9 mutation found in ewes (Hanrahan et al., 2004). Hanrahan et al. showed that reduction of GDF9 products caused by the heterozygous mutation were associated with increased ovulation rates and smaller corpora lutea. This phenotype was also reproduced by immunizing wild-type ewes against GDF9 (Juengel et al., 2004). These results imply that low levels of GDF9 mature protein may promote earlier acquisition of LH receptors and induce precocious follicle maturation and multifetal pregnancy. Consistently, mutations in GDF9 are significantly more frequent in the mothers of dizygotic twins in humans (Palmer et al., 2006). Mothers of dizygotic twins reach menopause significantly earlier than those of monozygotic twins (Martin et al., 1997). Therefore, it is not difficult to imagine that the three cases with the GDF9 heterozygous mutations in the present study may also experience multiple ovulation at an early age, resulting in early exhaustion of ovarian reserve. Further study is needed to verify this hypothesis.

We did not detect any deleterious effects of the GDF9 p.D57Y mutation. This may be due to the fact the affected amino acid is non-conserved and that the mutation occurs in control subjects. Two studies also report this variant in healthy Chinese women (Wang et al., 2010a,b). Our study suggests that p.D57Y may be a common SNP unique in Chinese women. This requires verification in larger, varied population.

There are some limitations in the present study. Selection bias may have occurred because all patients and controls were restricted to

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**Figure 3** Effect of mutations on protein functions. (A) The proliferation of GCs, in the presence of conditioned media containing wild-type, p.R146C mutant or p.D57Y mutant GDF9, was measured by Alamar Blue assay with indicated concentrations of different recombinant proteins. (B) Activation of the Smad2 pathway by recombinant proteins of GDF9. GCs were incubated with different concentrations of wild type, p.D57Y or p.R146C mutant GDF9. Protein lysates were analyzed by SDS-PAGE immunoblot with anti-phospho-Smad2 and anti-total-Smad2 antibodies. GAPDH was used as a loading control. (C) The curves represent quantitative analysis of the Smad2 phosphorylation. *P < 0.01, compared with the wild-type group at the same concentrations (ANOVA followed by Turkey’s post hoc tests). N, number of the repeated experiments.
infertile women. The allele frequency of these observed variants should be evaluated in ethnically matched healthy women. Also, as we focussed on mutational screening of GDF9-coding regions, but additional studies may be expanded to the complete genomic sequence including the 5′ and 3′-UTR regions. Several GDF9 mutations in the 5′ and 3′-UTR regions have been reported in ovarian dysfunction (Palmer et al., 2006). Such investigations may find novel mutations related to DOR.

To our knowledge, this is the first study to evaluate mutational consequences in the GDF9 gene of young women with DOR. Our data suggest that the GDF9 p.R146C mutation may be one of the causes of DOR. However, because we identified mutations in a minor subset of DOR patients, we could not exclude the possibility of other mutations exerting their effects in DOR patients. Larger-scale investigations will be necessary to elucidate the precise etiology of DOR in young women.

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**Authors’ roles**

T.T.W. and Z.H.K. participated, together with H.F.H. and J.Z.S., in the design of the study. T.T.W., Z.H.K., Y.S., L.T.C., X.J.C., C.F., D.Z. and R.J.Z. carried out the experiments. Data analysis was performed by T.T.W., Z.H.K., Y.T.W. and Y.Z. The manuscript was written by T.T.W., Z.H.K. and J.Z.S. H.F.H. critically read the manuscript. All authors read and approved the final manuscript.

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Conflict of interest
The authors declare that there is no conflict of interest.

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