Aberrant GDF9 Expression and Activation Are Associated With Common Human Ovarian Disorders

Courtney M. Simpson, David M. Robertson, Sara L. Al-Musawi, Derek A. Heath, Kenneth P. McNatty, Lesley J. Ritter, David G. Mottershead, Robert B. Gilchrist, Craig A. Harrison,* and Peter G. Stanton*

Prince Henry's Institute of Medical Research (C.M.S., D.M.R., S.L.A.-M., C.A.H., P.G.S.), Clayton, Victoria 3168, Australia; Department of Biochemistry and Molecular Biology (C.M.S., C.A.H., P.G.S.), Monash University, Clayton, Victoria 3800, Australia; School of Biological Sciences (D.A.H., K.P.M.), Victoria University of Wellington, Wellington 6140, New Zealand; and Robinson Institute (L.J.R., D.G.M., R.B.G.), Research Centre for Reproductive Health, School of Paediatrics and Reproductive Health, University of Adelaide, South Australia 5005, Australia

Context: Growth differentiation factor 9 (GDF9) is a central regulator of folliculogenesis and ovulation rate. Fourteen mutations in human (h) GDF9 have been reported in women with premature ovarian failure or polycystic ovarian syndrome as well as in mothers of dizygotic twins, implicating GDF9 in the etiology of these conditions. We sought to determine how these mutations alter the biological activity of hGDF9.

Objective: The objective of the study was to determine whether aberrant GDF9 expression or activation is associated with common ovarian disorders.

Design: Homology modeling was used to predict the location of individual mutations within structurally important regions of the pro domains and mature domains of hGDF9. Each hGDF9 variant was generated by site-directed mutagenesis, expressed from human embryonic kidney 293T cells and assessed as to whether it resulted in defective production or the enhanced activation of mature hGDF9 in an in vitro granulosa cell proliferation bioassay.

Results: Mutations observed in mothers of dizygotic twins (P103S and P374L) completely abrogated GDF9 expression, suggesting that women heterozygous for these mutations would have a 50% reduction in GDF9 levels. Comparable declines in GDF9 in ewes result in a 2-fold increase in ovulation rate and fecundity. Remarkably, three prodomain mutations associated with premature ovarian failure (S186Y, V216M, and T238A) all resulted in the activation of hGDF9. Mechanistically, these mutations reduced the affinity of the prodomain for mature hGDF9, allowing the growth factor to more readily access its signaling receptors.

Conclusions: Together these findings indicate that alterations to hGDF9 synthesis and activity can contribute to the most common ovarian pathologies. **(*J Clin Endocrinol Metab* 99: E615–E624, 2014)**

Two common causes of infertility in women are the ovarian disorders, premature ovarian failure (POF), and polycystic ovarian syndrome (PCOS). POF is estimated to affect 1% of women under the age of 40 years (1) and is characterized by the premature depletion of ovarian follicles, leading to amenorrhea and associated changes in hormone levels (2, 3), which in turn lead to an increased risk of major diseases (4). PCOS is the most common female hormonal disorder, affecting 5%–10% of women of reproductive age, and is a major cause of anovulation,

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* C.A.H. and P.G.S. contributed equally to this work.

Abbreviations: BMP15, bone morphogenetic protein 15; CED, Camurati-Engelmann disease; DZ, dizygotic; GDF9, growth differentiation factor 9; h, human; HEK, human embryonic kidney; HRP, horseradish peroxidase; IMAC, immobilized metal affinity chromatography; mAb, monoclonal antibody; PCOS, polycystic ovarian syndrome; POF, premature ovarian failure; rm, recombinant mouse; RP-HPLC, reversed-phase HPLC.

menstrual irregularities, and infertility (5, 6). These women demonstrate an excessive formation of preantral and antral follicles, which lead to the development of ovarian cysts. In addition PCOS women have major metabolic disturbances, in particular insulin resistance, which are associated with significantly increased incidences of gestational and type 2 diabetes, cardiovascular disease, uterine cancer, and weight disorders (7–9). The causes of POF and PCOS remain unknown in most cases; however, genetic contributions are considered a significant etiological component (2).

Many studies have examined the possible involvement of growth differentiation factor 9 (GDF9) in ovarian disorders. GDF9, a member of the TGF-β superfamily, is an oocyte-secreted factor critical for mammalian ovarian folliculogenesis (10). GDF9 is synthesized as a precursor molecule consisting of an N-terminal prodomain and a C-terminal mature domain. Dimeric precursors are cleaved by proprotein convertases and the human variant of GDF9 is secreted from the oocyte in a latent form, noncovalently associated with its prodomain (11). After an unidentified activation process, human (h) GDF9 exerts its biological effects by interacting with two transmembrane serine/threonine kinase receptors (type I and type II) (12, 13). The type I receptor (activin receptor-like kinase 5, or possibly activin receptor-like kinase 4) acts downstream of the type II receptor (bone morphogenetic protein receptor II) and propagates the GDF9 signal by phosphorylating SMAD 2/3 transcription factors (14). *GDF9*$^{-/-}$ mice are infertile due to a block at the type 3b primary stage of folliculogenesis (10). These arrested follicles display abnormal granulosa cells that fail to acquire a theca layer (10). Because follicles grow from preantral to antral, GDF9 regulates the differentiation of granulosa cells adjacent to the oocyte into cumulus cells, which in turn are essential for ovulation, oocyte quality, and embryo development (15).

Support for the involvement of GDF9 in ovarian pathologies came with the identification of two naturally occurring point mutations in sheep that cause sterility. The S395F mutation in Belclare sheep and S427R mutation in Thoka sheep are predicted to disrupt type I and type II receptor interactions, respectively (16, 17). Ewes homozygous for these inactivating mutations, or devoid of biologically active GDF9 (18, 19), are infertile with ovaries that resemble *GDF9*$^{-/-}$ mice. Interestingly, ewes heterozygous for these GDF9 mutations have increased ovulation rates and litter size (16, 17). This increase in ovulation rate can be attributed specifically to lower levels of biologically active GDF9 because partial neutralization of GDF9 by immunization in vivo also increases the ovulation rate (20). The exact mechanism as to how this occurs remains to be determined, but it is likely due to a greater level of precocious follicular development (21), with more follicles with granulosa cells acquiring LH receptivity and capable of ovulating at a smaller diameter (22, 23). Subsequent screening for *GDF9* mutations in women with POF and PCOS has identified numerous heterozygous missense mutations not found in control women (1, 3, 24, 25). Interestingly, mutations in *GDF9* are also found in mothers of dizygotic twins (26), implying that altered GDF9 activity may also increase the ovulation rate, as observed in ewes.

Most hGDF9 mutations (9 of 14) are located in the prodomain (Table 1); however, little is known about the effect of these mutations on GDF9 activity. Functional studies have been reported for only two of these variants, K67E and P103S (27), which caused a significant reduction in hGDF9 production and secretion. Homology modeling, based on the crystal structure of pro-TGF-β1, suggests that many of these mutations lie within structurally important regions of GDF9. As such, these mutations may alter posttranslational processing and production of hGDF9 and/or disrupt the stability of the latent complex. In this study, we examine the biological effect of hGDF9 mutations found in women with ovarian disorders and provide evidence that defective GDF9 production and/or activation may contribute to ovarian insufficiency in humans.

Materials and Methods

Reagents

The following reagents and antibodies were sourced: polyhistidine monoclonal antibody (mAb) (R&D Systems), GDF9 antibodies mAb53 and mAb72B (Oxford Brookes University, Oxford, UK), horseradish peroxidase (HRP)-conjugated anti-mouse IgG (GE Healthcare), Lumi-Light chemiluminesence Western blotting substrate (Roche), and DMEM, DMEM:F12, Opti-MEM, and SeeBlue Plus2 protein standards (Invitrogen).

GDF9 ELISA

A GDF9 ELISA was developed to measure the amount of GDF9 in human embryonic kidney (HEK)-293T conditioned medium. Maxi-sorp plates (PerkinElmer) coated with GDF9 mAb72B (500 ng per 50 μL/well in coating buffer; 50 mM Na_2CO_3, pH 9.6) raised against an N-terminal mature region peptide (KKPLGPASFNLSEYFC) were incubated overnight at room temperature and then blocked [1% BSA in Tris/HCl (pH 7.4), 1 h]. Conditioned media containing GDF9 variants (100 μL/well) were serially diluted in assay buffer (50 mM Tris HCl; 0.9% NaCl; 0.5% BSA; 0.01% Tween 40, pH 7.5) and added to 100 μL of the same buffer (final volume 200 μL/well) and incubated for 2 hours. Bound ligand was detected using biotinylated GDF9 mAb53 (100 μL/well) raised against a C-terminal hGDF9 peptide and cross-reacts with mouse GDF9 (28). Signal amplification/color reaction was achieved by the sequential addition of streptavidin-HRP (100 μL of 1:4000 dilution; Bio-

Table 1. Mutations of Human GDF9 Associated With POF, PCOS, and DZ Twinning

Nucleotide Variation	Amino Acid Variation	Protein Domain	Cases, n	Controls, n	Pathology	Reference	Effect of Mutation on Expression/Activity[a]
c.118T>G	Leu40Val	Pro	4/216	0/200	PCOS	(35)	Minor reduction in expression
c.133A>G	Met45Val	Pro	1/216	0/200	PCOS	(35)	Minor reduction in expression
c.199A>C	Lys67Glu	Pro	4/127	0/220	POF	(1)	Major reduction in expression
c.307C>T	Pro103Ser	Pro	1/62	0/60	POF	(24)	Abrogation of expression
			30/1693	13/1512	DZ twinning	(26)	
c.362C>T	Thr121Leu	Pro	4/1693	1/96	DZ twinning	(26)	Minor reduction in expression
c.436C>T	Arg146Cys	Pro	1/100	1/96	POF	(25)	Major reduction in expression
			2/216	0/200	PCOS	(35)	
c.557C>A	Ser186Tyr	Pro	1/203	0/54	POF	(3)	Minor reduction in expression but activation of latent protein
c.646G>A	Val216Met	Pro	2/127	0/220	POF	(1)	Major reduction in expression but activation of latent protein
c.712A>G	Thr238Ala	Pro	1/100	0/96	POF	(25)	Minor reduction in expression but activation of latent protein
c.1025A>T	Tyr342Phe	Mature	1/216	0/200	PCOS	(35)	Major reduction in expression
c.1121C>T	Pro374Leu	Mature	4/1693	0/1512	DZ twinning	(26)	Abrogation of expression
c.1275C>A	Ser425Arg	Mature	1/216	0/200	PCOS	(35)	Major reduction in expression
c.1283G>C	Ser428Thr	Mature	2/100	2/96	PCOS	(25)	Abrogation of expression
			2/216	0/200		(35)	
c.1360C>T	Arg454Cys	Mature	22/1693	12/1512	DZ twinning	(26)	Major reduction in expression

[a] See *Results* for details.

source) and tetramethylbenzidine (50 μL; Sigma-Aldrich) and stopped with 50 μL of 1 M H_2SO_4, with absorbance read at 450 nm. Recombinant mouse (rm) GDF9 (R&D Systems) was used as the standard. The sensitivity of the assay was 0.2 ng/mL.

Production of GDF9 mutants

Mutations were introduced into full-length hGDF9 using the QuikChange Lightning site-directed mutagenesis kit (Stratagene) following the manufacturer's instructions. A pcDNA3.1 vector containing full-length hGDF9 cDNA was used as the template. For each construct, the mutated region was confirmed by DNA sequencing. Wild-type and mutant hGDF9 proteins were produced by transient transfection in HEK-293T cells using Lipofectamine 2000 (Invitrogen). Briefly, HEK-293T cells were plated at 9×10^5 cells/well in a six-well plate. Wild-type or mutant hGDF9 DNA (5 μg) was combined with Lipofectamine 2000 for 20 minutes according to the manufacturer's instructions. DNA-Lipofectamine complexes were added directly to cells and incubated in DMEM/10% fetal calf serum medium for 24 hours at 37°C in 5% CO_2 and then replaced with production media [DMEM:F12 medium containing L-glutamine, 0.02% BSA, 0.01% heparin (Sigma-Aldrich)] for 48 hours. Conditioned media were concentrated 10-fold using Nanosep microconcentrators (10 kDa; Pall Life Sciences). Cells were lysed (1% Triton X-100 in phosphate buffered saline, pH 7.4), and GDF9 expression in media and cell lysates were assessed by Western blotting of reduced samples on 4%–12% SDS-PAGE gels (BioRad Laboratories) and transferred onto ECL Hybond membranes (GE Healthcare). Blots were probed with GDF9 mAb53 (1:5000), followed by HRP-conjugated antimouse IgG (1:10 000) with detection using Lumi-light chemiluminesence reagents (Roche).

Purification of recombinant GDF9

A stable HEK-293E cell line expressing hGDF9 has been described previously (29). After 4 days in production media, conditioned medium from these cells was centrifuged, concentrated (Centricon Plus-70; Millipore), and then resuspended in 50 mM phosphate buffer and 0.5M NaCl (pH 8.0) prior to immobilized metal affinity chromatography (IMAC) using Ni-NTA agarose (Invitrogen). His-tagged hGDF9 was eluted from the Ni-NTA agarose with 50 mM phosphate buffer, 0.5 M NaCl, and 0.5 M imidazole (pH 8.0) prior to the final purification by reversed-phase HPLC (RP-HPLC) using a Jupiter C4 column (250 × 4.6 mm, 5 μm particle size; Phenomenex) and a gradient of 20%–70% acetonitrile in 0.1% heptafluorobutyric acid at 1 mL/min over 90 minutes. Western blotting and the GDF9 ELISA were used to assess the amount of purified protein.

Murine granulosa cell proliferation assay

Mice were maintained in accordance with the Australian Code of Practice for Care and Use of Animals for Scientific Purposes, with approval from the Adelaide University Animal Ethics Committee. This mitogen bioassay was performed as previously described (28, 30, 31). In brief, mural granulosa cells were recovered from 21- to 26-day-old 129/SV mice, 44–46 hours after administration of 5 IU of equine chorionic gonadotrophin (Folligon; Intervet). Cells were washed in B-TCM-199 medium with supplements (30), and assays were conducted with 25 000 granulosa cells per 125 μL/well with increasing concentrations (12.5–800 ng/mL) of wild-type or mutant hGDF9 at 37°C for 18 hours, followed by a 6-hour pulse of 15.4 kBq [^{3}H]thymidine (MP Biomedicals) under the same conditions. After the culture, mural granulosa cells were harvested, and the incorporated [^{3}H]thymidine was quantified as an indicator of the level of mural granulosa cell DNA synthesis and proliferation.

Molecular modeling

Phyre2 (Structural Bioinformatics Group, Imperial College, London, United Kingdom) (32) was used to model hGDF9 using pro-TGF-β1 [accession number 3RJR (33)] as the template. Subsequently the UCSF Chimera package (Resource for Biocomputing, Visualization, and Informatics, University of California, San

Francisco, San Francisco, California) (supported by National Institutes of Health P41 RR-01081) (34) was used to both overlay the hGDF9 promature sequence onto the template of a dimer of pro-TGF-β1 and to produce the molecular graphics images.

Statistical analysis

Significance ($P < .05$ or $P < .001$) was determined using one-way ANOVA for independent groups, using the Prism Program (version 5; GraphPad Software). Error bars shown represent ± SEM.

Results

Molecular modeling of human GDF9 variants

Previous genetic screening identified 14 heterozygous hGDF9 variants in patients with POF or PCOS or in mothers of dizygotic twins (Table 1) (1, 3, 24–26, 35). Because the prodomains and mature domains of all TGF-β proteins are predicted to have the same basic architecture, we generated a molecular model of pro-GDF9 (Figure 1) based on the pro-TGF-β1 crystal structure (33). We used this model to locate all the hGDF9 mutations except variant L40V, which is located in a region of the prodomain that has no corresponding sequence in TGF-β1. However, it is expected that L40V, similar to variants M45V and K67E, is situated within the α1-helix of the prodomain. For several TGF-β family members, this region of the prodomain has been demonstrated to interact directly with the mature domain, controlling the synthesis, secretion, and activity of the mature ligand (36). P103S resides in a region of the prodomain (between the α2-helix and β1-sheet) central to activation of other latent TGF-β proteins (33). Interestingly, the T238A variant in GDF9 is analogous in position to an activating mutation (H222D) in TGF-β1, which causes the rare bone disorder, Camurati-Engelmann disease (37). Other prodomain variants (T121L, R146C, S186Y, and V216M) are located in less well-characterized regions of the molecule. Within the mature domain, P374L, S425R, and S428T are situated on the outer convex surface of the finger regions and likely interact directly with the α2-helix of the prodomain during synthesis. Of the other mature domain variants, Y342F is in the disordered N terminus and R454C is located within the cysteine knot core of the molecule.

Development of GDF9 ELISA

To determine the effect of the 14 GDF9 mutations on the synthesis and secretion of the mature growth factor, a specific and sensitive GDF9 ELISA was required. By combining mAb72B and mAb53 as capture and detection antibodies, respectively, rmGDF9 was detected at concentrations below 0.25 ng/mL (Figure 2). We then tested two preparations of hGDF9 (wild type and L40V) conditioned media and showed that they were detected in the same manner as rmGDF9. The cross-reactivity of other representative members of the TGF-β superfamily [human bone morphogenetic protein 15 (BMP15), activin A, and TGF-β3] was less than 0.1% (Figure 2).

Characterization of the synthesis and secretion of GDF9 variants

To evaluate the biological impact of the 14 hGDF9 variants on synthesis and secretion, mutations were introduced into hGDF9 cDNA by in vitro site-directed mutagenesis. HEK-293T cells were then transiently transfected with expression vectors for wild-type hGDF9 or the variant forms. The conditioned media and cell lysates were collected for Western blot analysis (Figure 3, A and B). Using GDF9 mAb53, hGDF9 variants were detected in monomeric precursor (60 kDa) and mature (20 kDa) forms, although some were expressed at low levels (Figure 3A). Interestingly, the ELISA results indicated that variants P103S, P374L, and S428T abrogated mature hGDF9 expression, whereas variants K67E, V216M, Y324F, S425R, and R454C were poorly expressed (~10 ng/mL; Figure 3C). All variants were expressed at significantly ($P < .05$) reduced levels compared with wild-type hGDF9 (30 ng/mL). An analysis of cell lysates indicated that the variation in hGDF9 production was not due to a loss of hGDF9 precursor expression because it was present at similar levels in cell lysates for all variants tested (Figure 3B).

POF variants located at the C terminus of the prodomain activate latent GDF9

We have previously shown that hGDF9 is secreted in a latent form, which can promote granulosa cell proliferation only when the prodomain is removed (11, 29). We therefore examined whether any of the 14 mutations affected the stability of the latent hGDF9 complex. Mural granulosa cells were treated with conditioned media containing either wild-type hGDF9 or variant forms. Wild-type hGDF9 and most variants were unable to stimulate proliferation of granulosa cells, as determined by ^{3}H-thymidine incorporation (Figure 4A). Remarkably, three variants (S186Y, V216M, and T238A) significantly increased granulosa cell proliferation in a dose-dependent manner (Figure 4A). Because each of these activating mutations lies within the prodomain, we predicted they would affect the stability of the latent hGDF9 complex, rather than increase the activity of mature hGDF9. To test this, wild-type and T238A prodomain variants were gen-

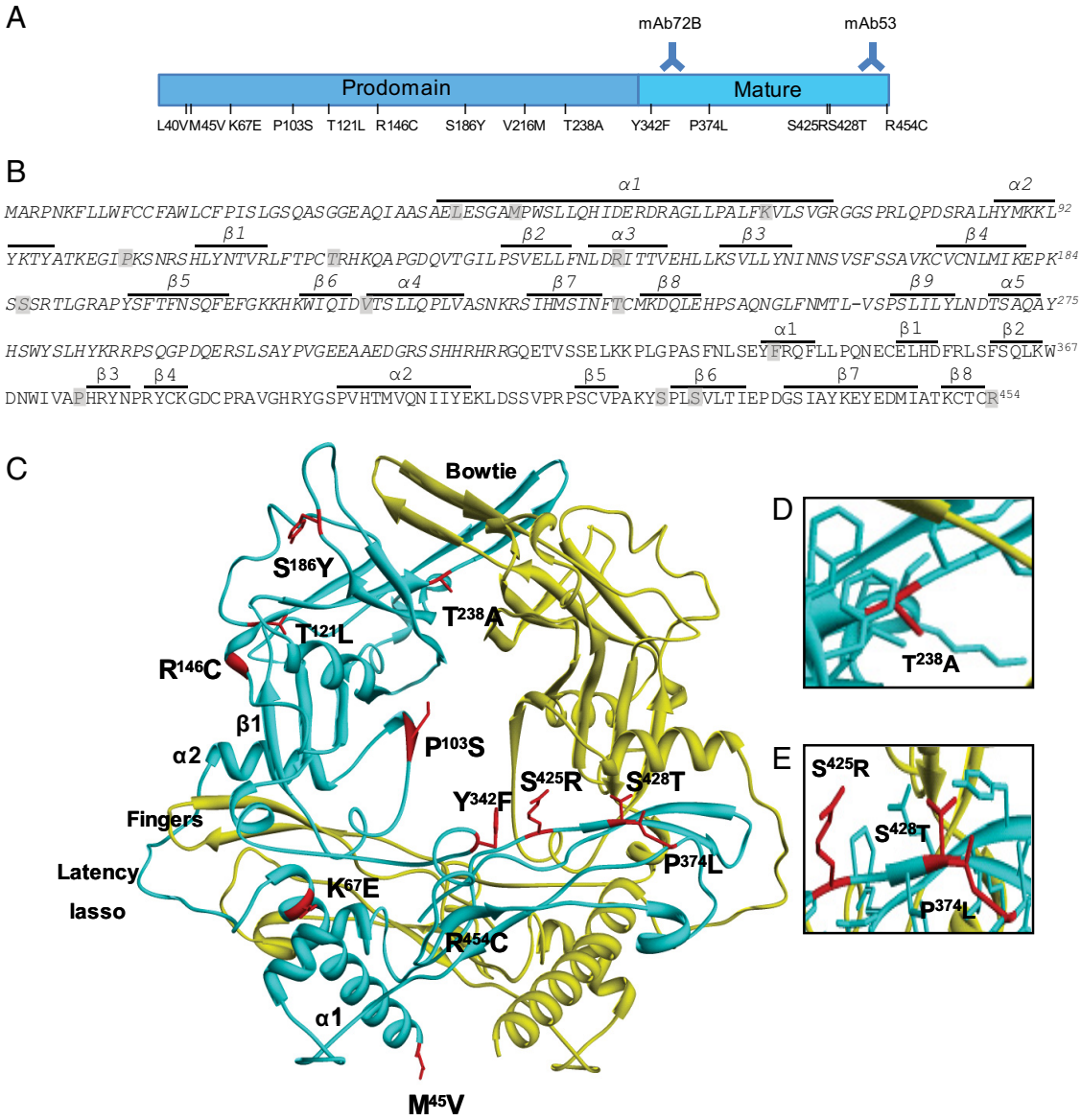

Figure 1. Homology modeling of promature GDF9. A, Schematic showing the prodomains and mature domains of human GDF9 with the positions of the mutations and the epitopes recognized by the monoclonal antibodies. B, Sequence of human GDF9 with the prodomain, indicated by italics. Secondary structure elements (β-sheets and α-helices) as determined by QuickPhyre (Quick Protein Homology/Analogy Recognition Engine; Structural Bioinformatics Group, Imperial College, London, United Kingdom) are depicted above the sequence. Residues associated with POF, DZ twinning, and PCOS are indicated (shaded gray). C, Ribbon schematic of a homology model of the GDF9 homodimer. GDF9 promature subunits are colored blue and yellow. Key features of the GDF9 promature subunits have been highlighted (bowtie, latency lasso, fingers, α1- and α2-helices, and β1-sheet). GDF9 variants are shown in red. The location of V216M cannot be seen due to its positioning on the model. The location of the POF mutant T238A (D) and the mature domain mutants P374L, S425R, and S428T (E) have been indicated as side chains, highlighted in red.

erated (in the absence of the mature domain) and expressed transiently in HEK-293T cells. Mural granulosa cells were then treated with mature rmGDF9 (100 ng/mL) and increasing doses of the prodomain variants. Although the wild-type prodomain blocked GDF9-stimulated granulosa cell proliferation, the T238A mutant prodomain had no effect (Figure 4B), indicating that this mutation decreases the affinity of the prodomain for mature GDF9, enabling the growth factor to more readily access its signaling receptors. To verify that the activating mutations did not affect mature hGDF9 activity per se, we used a purification process previously shown to activate latent hGDF9 (29). Briefly, with a His6 tag at the N terminus of the prodomain, we used $Ni2^{+}$-based IMAC followed by RP-HPLC to purify the mature domains of wild-type GDF9 and the S186Y, V216M, and T238A variants away from their prodomains. After purification all three variants demonstrated similar activity when compared with wild-type hGDF9 (Figure 4C). Together these results indicate that the S186Y, V216M, and T238A variants cause hGDF9 to be secreted in an active form, although they do not alter affinity for receptors.

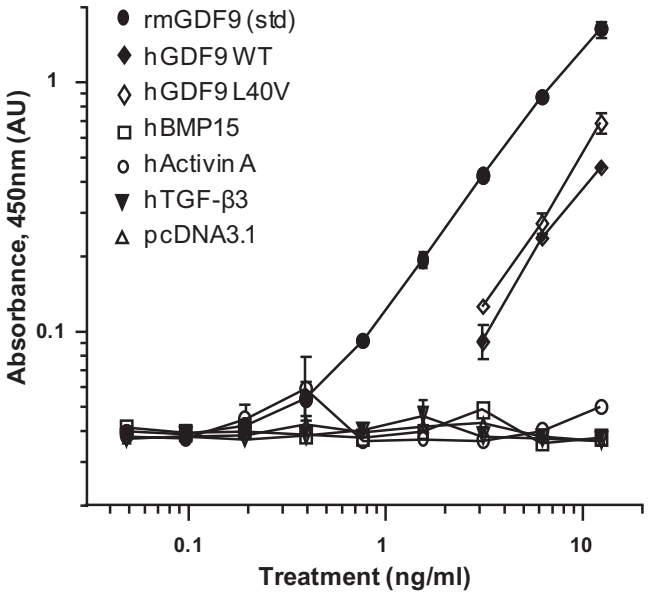

Figure 2. GDF9 ELISA. A GDF9 ELISA was developed to measure the amount of GDF9 in HEK-293T conditioned medium. Recombinant mouse GDF9 (●) was used as a standard, and the specificity of the assay was assessed using a range of TGF-β family members; wild-type human GDF9 (♦), human GDF9 L40V (◊), human BMP15 (□), human activin A (○), and human TGF-β3 (▼). Dilutions of concentrated media from cells transfected with empty vector, pcDNA3.1 (r), were included as controls. The ELISA has a specificity of less than 0.1%, with a sensitivity of 0.2 ng/mL. Values represent mean ± SEM in duplicate, from a representative experiment.

Mutations located within the mature domain of GDF9 affect bioactivity

Of the five GDF9 mutations found in the mature domain, two (P374L and S428T) abrogated hGDF9 expression (Figure 3A). To assess the effect of the remaining mutations on biological activity, the mature domains of variants Y342F, S425R, and R454C were separated from their prodomains by IMAC and RP-HPLC. Treatment of mural granulosa cells with wild-type mature hGDF9 resulted in a dose-dependent stimulation of cellular DNA synthesis (EC_{50} 10 ng/mL), as assessed by [^{3}H]thymidine incorporation (Figure 5). Interestingly, the extent of granulosa cell proliferation in response to wild-type hGDF9 was 3-fold lower than that observed with the three GDF9 variants (EC_{50} 3 ng/mL). Thus, these mutations may increase the affinity of hGDF9 for its signaling receptors.

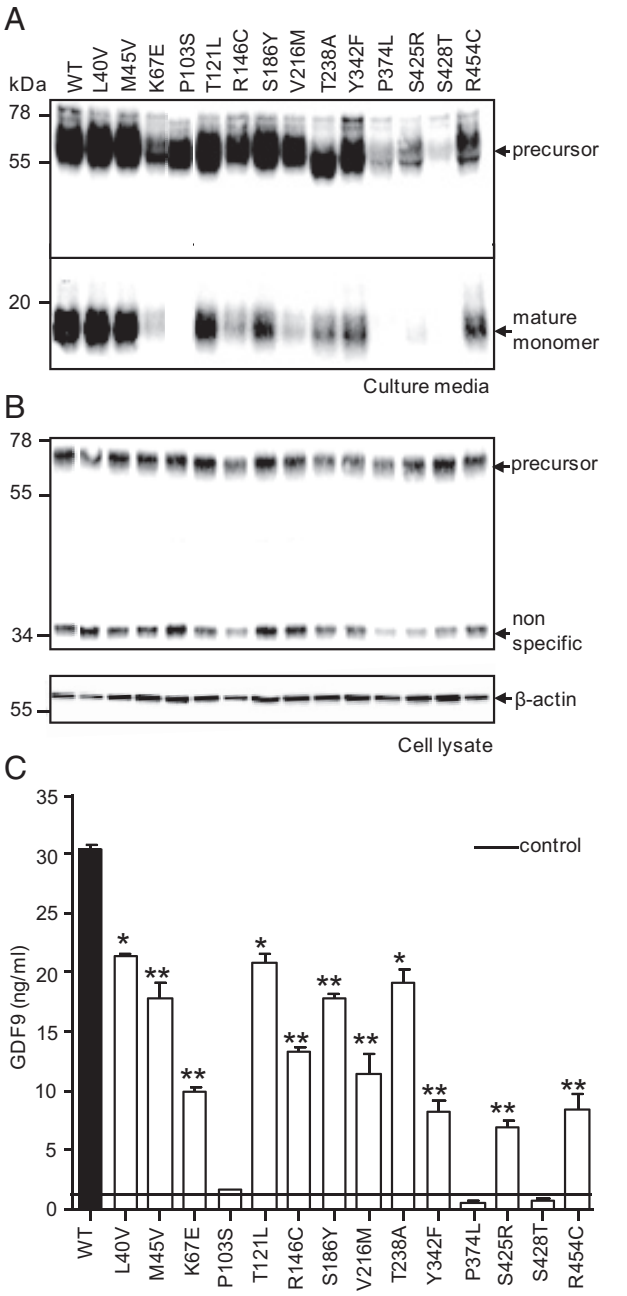

Figure 3. Effects of naturally occurring GDF9 mutations on growth factor synthesis and secretion. Mutations were substituted into human GDF9 using in vitro site-directed mutagenesis. To determine the effects of the mutations on GDF9 production, conditioned media (A) and cell lysates (B) from HEK-293T cells transfected with either wild-type (WT; lane 1) or mutant constructs (lanes 2–15) were analyzed by Western blot. Total protein loaded was the same for all variants as determined by a Bradford protein assay. Samples were detected under reducing conditions using GDF9 mAb53 specific for the mature domain. The 60-kDa GDF9 precursor and 20-kDa mature monomer are shown (note that the bottom portion of the Western blot was exposed for a longer time to best show the differences in the expression of mature GDF9). C, Total protein in conditioned medium was measured by a Bradford protein assay and shown to be the same for each GDF9 variant (200 μg/mL). Subsequently, GDF9 levels in conditioned medium were determined by an ELISA for each variant in triplicate, and values represent mean ± SEM from a representative experiment. *, $P < .05$; **, $P < .0001$.

Discussion

Genetic screening of mothers of dizygotic (DZ) twins or women with ovarian disorders such as POF and PCOS has identified 14 mutations in hGDF9, with most these (9 of 14) located in the prodomain. Based on our understanding of the synthesis of TGF-β superfamily members, we hypothesized that these mutations would affect the expres-

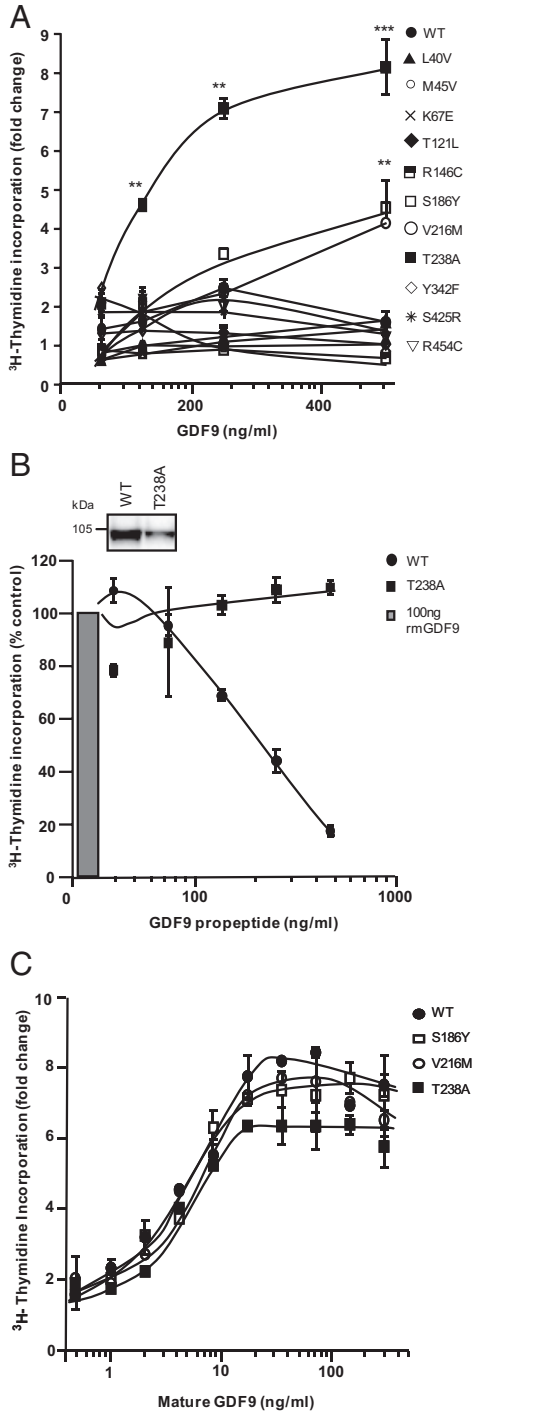

Figure 4. Mutations located in the prodomain disrupt the latent human GDF9 complex. A, Mural granulosa cells were cultured with increasing concentrations of wild-type or mutant conditioned media (62.5–500 ng/mL). After 24 hours of culture, the labeled thymidine incorporated into cells was counted. B, The ability of wild-type and mutant GDF9 prodomain variants (62.5–1000 ng/mL) to bind to mature rmGDF9 (100 ng/mL) and block GDF9 signaling was assessed in a mural granulosa cell assay. C, Wild-type and GDF9 variants were purified via a two-step procedure using IMAC followed by RP-HPLC. Fractions containing only mature GDF9 were pooled. Mural granulosa cells were cultured with increasing concentrations of RP-HPLC purified wild-type, S186Y, V216M, or T238A mature GDF9 (0.048–250 ng/mL). Points represent mean ± SEM from a representative experiment. *, $P < .05$; **, $P < .01$.

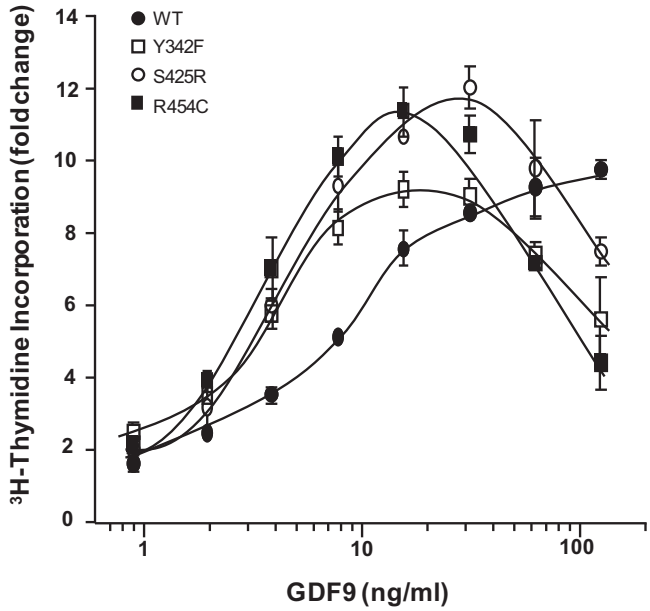

Figure 5. Mature domain mutants increase GDF9 bioactivity. To produce large amounts of mature GDF9, wild-type and mutant GDF9 variants were purified using IMAC and RP-HPLC. Mural granulosa cells were cultured with increasing concentrations of mature human wild-type or mutant GDF9 (1.9–250 ng/mL). Points represent mean ± SEM from a representative experiment.

sion and activity of GDF9 (36, 38). Our in vitro studies are consistent with this prediction because we identified mutations that did the following: 1) disrupted secretion of mature hGDF9; 2) affected the stability of the latent complex, resulting in the activation of hGDF9; or 3) enhanced mature hGDF9 bioactivity. Some of these findings are also consistent with the observed phenotypes of animals with altered ovarian function due to inactivating mutations in *GDF9*.

A significant finding from this study was the activation of latent hGDF9 caused by the mutations S186Y, V216M, and T238A. Women with these mutations reached menopause between the ages of 27 and 33 years and had elevated FSH and LH levels and atrophic ovaries devoid of follicles (1, 3, 25). Activating mutations are extremely rare in TGF-β proteins, having previously been described only for TGF-β1 in the bone disorder, Camurati-Engelmann disease (CED) (37, 39). Similar to the hGDF9 variants presented here, the TGF-β1 CED mutations (R156C, E169K, R218C, R218H, H222D, and C225R) result in reduced expression of mature TGF-β1 but a concomitant increase in the activation of this normally latent growth factor (36). Interestingly, the location of the T238A hGDF9 mutation in the prodomain dimer interface is identical with that of the CED-causing mutation, H222D, in TGF-β1. The H222D mutation disrupts a pH-regulated salt bridge between Glu169 and His222, leading to instability of the pro-TGF-β1 complex and a 5-fold increase in TGF-β1 activation (36). The T238A mutation in the

hGDF9 prodomain appears to have a similar effect on the stability of the pro-GDF9 complex because we observed a significant increase in the activity of this variant. The V216M mutation, which may alter hydrophobic interactions within the core of the prodomain, and the S186Y mutation, located in a disordered region of the prodomain, also activate hGDF9, although to a lesser extent than the T238A mutation. These data suggest that mutations in the latency-associated prodomain that facilitate GDF9 signaling may contribute to premature ovarian failure. The physiological mechanisms underpinning this require further study; however, it seems reasonable to hypothesize that, based on the potent granulosa cell mitogenic activity of GDF9 (31), increased activity of GDF9 in these women may increase the proportion of growing follicles, leading to a premature depletion of ovarian reserve.

Interestingly, the converse (ie, a decrease in GDF9 production/signaling) also seems sufficient to disrupt ovarian function. Western blot and an ELISA analysis of all 14 GDF9 mutations resulted in a significant reduction in growth factor secretion. Variants P103S, P374L, and S428T were the most disruptive, although, like other mutations, they did not affect precursor expression within the cell. This type of disconnect between synthesis and secretion of a TGF-β protein is normally associated with the inability of the prodomain to template the correct folding of the mature growth factor (36, 38, 40). Therefore, it is not surprising that two of the most deleterious mutations, P374L and S428T, are located in the region of mature GDF9 (outer convex surface of the fingers), which contacts the prodomain during synthesis (38). The reason variant P103S abrogates GDF9 expression is less obvious because it lies in a region of the prodomain that does not directly contact the mature domain; however, Pro103 is highly conserved across species and may be required for maintaining the integrity of the hydrophobic core of the molecule.

It is anticipated that women heterozygous for the most deleterious mutations would have a 50% reduction in their GDF9 levels. Because two of these mutations (P103S and P374L) are significantly more frequent in mothers of DZ twins than controls (26), this suggests that reduced GDF9 signaling may contribute to enhanced ovulation rate in humans. Although this may seem counterintuitive, there is indeed substantial precedence for the same phenomenon in monoovular animals carrying inactivating mutations in either GDF9 or its homolog BMP15. Sheep heterozygous for an inactivating mutation in GDF9 (S395F) have increased fecundity (16). It is postulated that reduced GDF9 signaling impairs granulosa cell proliferation but enhances LH/FSH sensitivity of follicles in the ovaries of these heterozygous ewes (41), with the consequence that numerous follicles are selected and ovulate at a smaller size (42). Several studies have indicated that mothers of DZ twins have an early menopause (43), implying a direct relationship between twinning and POF. GDF9 could be an important mediator of this relationship because the P103S mutation associated with DZ twinning has also been identified in one woman in whom POF manifested at 22 years of age (24). The concept that GDF9 influences multiple aspects of ovarian function is further supported by the observation that the S428T mutation, which also abrogates GDF9 expression in vitro, is associated with POF and PCOS but not twinning (25, 35). Interestingly the S428T mutation has a direct correlate in Thoka sheep (S427R), which results in sterility in homozygous ewes (17).

Other prodomain mutations result in either modest (L40V, M45V, and T121L) or significant (K67E and R146C) decreases in GDF9 secretion. Variants L40V, M45V, and K67E are located in the α1-helix of the prodomain and, as such, may disrupt binding to the mature domain during synthesis. Mutant T121L lies in a disordered region between the first and second β-sheets of the prodomain but is adjacent to an important cysteine residue, whereas mutant R146C introduces a free cysteine residue, which is predicted to disrupt prodomain folding. Interestingly, the final three mutations in the mature domain (Y342F, S425R, and R454C) result in a greater than 3-fold decrease in GDF9 expression, but an apparent increase in activity relative to wild-type GDF9, when the prodomain is removed. The reason for the enhanced activity of these variants is not obvious because none of the mutations are predicted to affect type I or type II receptor binding.

This study provides molecular and functional in vitro characterization of hGDF9 mutations identified in mothers of DZ twins and women with POF or PCOS. Mutations in hGDF9 resulted in a disruption in secretion, a reduced stability of the latent complex, and/or an increase in activity. The fact that there was no clear-cut genotype-phenotype correlation among GDF9 mutations (ie, mutations that either decrease expression or activate hGDF9 are associated with POF) supports the concept that any changes in GDF9 protein levels are capable of disrupting ovarian function and female fertility.

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Address all correspondence and requests for reprints to: Peter G. Stanton, PhD, Prince Henry's Institute of Medical Research, 246 Clayton Road, Clayton, Victoria 3168, Australia. E-mail: peter.stanton@princehenrys.org.

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