Characterization of recombinant human growth differentiation factor-9 signaling in ovarian granulosa cells

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\textbf{Abstract}

Growth differentiation factor-9 (GDF9) is an oocyte secreted paracrine factor essential for mammalian ovarian folliculogenesis. Like other members of the transforming growth factor-\(\beta\) (TGF-\(\beta\)) superfamily, GDF9 is synthesized as a prepropeptide which needs processing by furin-like proteases to result in an active mature protein. We have previously characterized a preparation of unpurified recombinant mouse GDF9 which is bioactive as produced by human embryonic kidney 293T (HEK-293T) cells. However, we find that unpurified recombinant human GDF9 (hGDF9) produced by HEK-293T cells is not bioactive. Purified recombinant hGDF9 is bioactive and here we report the characterization of this protein. We find that the purified untagged mature region of hGDF9 is active in transcriptional reporter assays specific for Smad3/4 in human granulosa-luteal (hGL) cells. We also demonstrate the use of a BMP (Smad1/5) responsive (BRE-luciferase) adenovirus in primary cultures of hGL cells to detect BMP responses. Using this adenovirus we find that purified human GDF9 does not activate the Smad1/5 pathway. Purified hGDF9 mature region activated the Smad3 pathway also in the FSH responsive human granulosa tumor cell line KGN. Primary cultures of rat granulosa cells responded to purified hGDF9 with an increase in DNA synthesis as measured by \([3\mathrm{H}]\)-thymidine uptake. Here we also report that the inclusion of a C-terminal affinity purification tag destroys GDF9 bioactivity. This study is the first characterization of purified biologically active human GDF9 and as such is of importance for studies on human fertility, and efforts aimed at treating infertility conditions.

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\textbf{Keywords:} Growth differentiation factor-9 (GDF9); Granulosa cells; Signaling pathway; Human

\section{1. Introduction}

Growth differentiation factor-9 (GDF9), identified in the early 1990s (McPherron and Lee, 1993) as a member of the transforming growth factor-\(\beta\) (TGF-\(\beta\)) superfamily, is an oocyte derived factor expressed throughout the development of the maturing follicle (McGrath et al., 1995; Laitinen et al., 1998). The protein functions as a paracrine factor in the regulation of granulosa cell proliferation and differentiation (Elvin et al., 1999; Eppig, 2001; Gilchrist et al., 2006), and is essential for fertility, knockout mice displaying arrested follicular development at the primary follicle stage (Dong et al., 1996). In our previous studies we have studied the signaling of recombinant mouse GDF9 (mGDF9) produced by human embryonic kidney 293T (HEK-293T) cells. We have shown that mGDF9 induces Smad2 phosphorylation and inhibin production in rat diethylstilbestrol-treated granulosa cells (Roh et al., 2003) and in human granulosa-luteal (hGL) cells (Kaivo-Oja et al., 2003),
not requiring any purification steps to reveal these activities. Further, we have shown that the downstream signaling actions of mGDF9 are mediated by the type I receptor, ALK5, initiating the subsequent activation of Smad2 and Smad3, both in cultured rat granulosa cells (Mazerbourg et al., 2004) and in hGL cells (Kaivo-Oja et al., 2003, 2005). It has also been shown that mGDF9 uses the BMP type II receptor (BMPRII) as its other signaling receptor in rat granulosa cells (Vitt et al., 2002).

To date in vitro analyses of human GDF9 (hGDF9) have been restricted by the lack of a biologically active protein. However, the significance of GDF9 as a regulator of human fertility has been shown with mutation analyses. Mutations in the hGDF9 gene are associated with various reproductive abnormalities. It has been shown that aberrant expression of GDF9 is associated with polycystic ovary syndrome (PCOS) (Teixeira Filho et al., 2002) and screening of women with premature ovarian failure (POF) has revealed mutations in the GDF9 gene (Dixit et al., 2005; Laissue et al., 2006). In 2004 a rare deletion mutation in the hGDF9 gene was described in heterozygous sisters with spontaneous dizygotic twins (Montgomery et al., 2004) and more recently new variants in the hGDF9 gene that are significantly more common in mothers of dizygotic (DZ) twins than controls were described, suggesting that rare GDF9 variants contribute more common in mothers of dizygotic twins than controls were described, suggesting that rare GDF9 variants contribute to the likelihood of DZ twinning (Palmer et al., 2006). Although these studies reveal that abnormal expression of GDF9 can lead to adverse effects in fertility, the physiological role of GDF9 in humans is still not totally clear.

For in vitro analyses of human GDF9 function, both purified recombinant protein and functional bioassays to test the signaling pathways utilized are needed. Here we report that, in contrast to mGDF9, hGDF9 is produced in a latent form by HEK-293T cells, and that the position of an affinity purification tag crucially effects GDF9 bioactivity. In this study we have produced various epitope tagged forms of mouse and human GDF9 and purified the respective mature forms. We find that a C-terminal His6 tag destroys GDF9 bioactivity. Most importantly, the purified untagged mature region of hGDF9 was biologically active exhibiting the ability to activate a Smad3/4 specific transcriptional reporter in human granulosa-luteal cells, whereas no activation of the BMP pathway in these cells was observed. Purified hGDF9 mature region activated the Smad3 pathway also in the FSH responsive human granulosa tumor cell line KGN. Finally, in these studies which are the first to characterize a purified biologically active human GDF9, we demonstrate that hGDF9 stimulates DNA synthesis in primary cultures of rat granulosa cells. We believe these results are of particular importance for studies on human fertility, and efforts aimed at treating infertility conditions.

2. Materials and methods

2.1. Expression vector construction

Construction of the mGDF9wt vector has been previously described (Laitinen et al., 1998), in short the mouse GDF9 full-length cDNA was subcloned into the pEFIRE-P expression vector (Hobs et al., 1998). His6 tagged vectors were constructed by introducing the affinity tag into the mature region by PCR and by subcloning the pro- and the mature regions into the pEFIRE-P expression vector. C6H mGDF9 mature region was amplified with PCR with following primers: (5’ primer) 5’-agacctccccgagcgcgggggcgtggcagc-3’ and (3’ primer) 5’-tctgaattaggtcttggagttgacacg-3’. N6H mGDF9 mature region was amplified with PCR with following primers: (5’ primer) 5’-gcggcggtggcgtggcagcagacagggaccc-3’ and (3’ primer) 5’-tctgaattaggtcttggagttgacacg-3’. The mouse pro-region was digested out of the wild type construct and the pro-along with the tagged mature regions were subcloned into the pEFIRE-P expression vector. Human GDF9 DNA fragments were amplified from human genomic DNA with following primers: (5’ primer) 5’-gcggcggtggcgtggcagcagacagggaccc-3’ and (3’ primer) 5’-tctgaattaggtcttggagttgacacg-3’. The human pro-region was digested out of the wild type construct and the pro-along with the tagged mature regions were subcloned into pEFIRE-P expression vector.

2.2. Protein expression, purification and analysis

Development of a HEK-293T cell line expressing mGDF9 protein has been previously described (Kaivo-Oja et al., 2003). Cell lines expressing epitope tagged processed mouse and human GDF9 were developed by a similar protocol and were used as sources of recombinant GDF9 proteins. Levels of recombinant proteins in 293T cell growth media were compared in immunoblots using the known concentration of mGDF9 conditioned media (1 ng/μl) (Kaivo-Oja et al., 2003) as a standard. 6H-tagged recombinant proteins were purified with affinity chromatography. Briefly, 293T cell growth media containing the protein of interest was centrifuged and filtered through a 0.22-0.45 μm filter. The cleared media was loaded on a HiTrap Chelating column (GE Healthcare) charged with Ni2+-ions. First, the pro-region of mouse or human GDF9 was washed out of the column under denaturing conditions with urea and the respective mature region was eluted out of the column by increasing imidazole concentration. Finally, human C6H GDF9 and mouse N6H GDF9 were purified by high performance liquid chromatography (HPLC), a commercial service contracted from the Peptide and Protein Core Facility Laboratory, Haartman Institute, University of Helsinki. Purified fractions were analysed on silver gels and Western blots. Reduced protein fractions (with 10 mM DTT) were run in 15% SDS-PAGE gels, and stained with silver nitrate. Reduced fractions were run in 15% SDS-PAGE gels, and stained with silver nitrate and blotted onto a Hybond C nitrocellulose membrane as described previously (Kaivo-Oja et al., 2003). Blotted membranes were treated with GDF9 specific mAb-53 (1:10,000) (Gilchrist et al., 2004b) or anti-His primary antibody (1:1,100) (Amersham) and a secondary antibody, peroxidase-conjugated anti-IgG (Jackson ImmunoResearch Laboratories, Inc.; 1:20,000). Immunoreactive proteins were detected using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech) or alkaline phosphatase based detection (Bio-Rad).

2.3. Reagents and growth factors

TGFβ1 was purchased from R&D Systems (Minneapolis, MN). E. coli produced BMP2 was a kind gift from Dr. Peter Mace (University of Otago, NZ). Fetal calf serum (FCS) was purchased from Euroclone Ltd. (Devon, UK). DMEM and Ham’s F-12 were purchased from Invitrogen Life Technologies, Inc. (Gaithersburg, MD). Heparin (Fragmin) was purchased from Pharmacia & Upjohn (Stockholm, Sweden). BSA was purchased from Roche (Mannheim, Germany). Peroxidase-conjugated rabbit antimouse IgG was purchased from Jackson ImmunoResearch Laboratories, Inc; (West Grove, PA).

2.4. Reporter gene constructs

The pGL3CAGA12-luciferase reporter plasmid was provided by Dr. C.H. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden) (Dennler et al., 1998). The pGL3BRE-luciferase reporter plasmid (Korchynskyi and ten Dijke, 2002) was provided by Dr. P. ten Dijke (The Netherlands Cancer Institute).
2.5. hGL cell cultures

hGL cells were obtained with informed consent from women undergoing in vitro fertilization (IVF) treatments. For each experiment, cells from one to six patients were pooled, enzymatically dispersed, and separated from red blood cells by centrifugation through Ficoll-Paque as previously described (Erama et al., 1993). Thereafter, hGL cells were counted and plated at a density of 3–4 × 10^4 cells/well on 24-well plates (Cellstar, Greiner Bio-one, Frickenhausen, Germany; final concentration, 3–4 × 10^4 cells/ml). hGL cells were cultured in DMEM supplemented with 10% FCS, 2 mM L-glutamine, and antibiotics (100 ìg/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin-B; GIBCO). Cells were cultured 1–2 days before adenovirus infections and 2–3 days before ligand stimulation experiments.

2.6. Adenovirus infections

The recombinant adenovirus Ad-CAGA12-luciferase (Dooley et al., 2003) was provided by Dr. P. ten Dijke (The Netherlands Cancer Institute). Reporter BRE-Luc adenovirus was generated using AdEasy system accordingly to provided protocol (He et al., 1998). Briefly, the cassette that contains the BRE enhancer, minimal MLP promoter, luciferase CDS and polya signal (Korchynskyi and ten Dijke, 2002) was recloned from pGL3 plasmid (Promega) into the pShuttle vector and the construct obtained was recombined with the Easy-1 adenoviral backbone in BJ1583 cells. The linearized cosmid was transfected into 293 cells and amplified as an adenovirus. Viruses were amplified and titrated in transcomplemental 293A cells and purified with cesium chloride gradient ultracentrifugation as described previously (He et al., 1998). The use of recombinant adenoviruses in hGL cultures has been previously optimized (Bondestam et al., 2002). The hGL cells were infected by incubating the cells with viruses at 37 °C in serum-free DMEM supplemented with L-glutamine and antibiotics for 45 min, and DMEM containing 2% FCS was added on top to stop the infection. The cells were then incubated for 24 h before continuing the luciferase-assay experiments.

2.7. Transient transfections and luciferase assays

KGN cells were cultured in DMEM/F12 1:1 supplemented with 10% FCS, 2 mM L-glutamine, 100 ìU/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO₂. The KGN cells were plated at low confluency on 24-well plates and grown overnight before transfections. Transfections were performed in 0.5 mL medium with 100 ng/well CAGA12-luciferase reporter construct (Dennler et al., 1998) or the BMP response element (BRE)-luciferase reporter construct (Korchynskyi and ten Dijke, 2002), and 10 ng/well β-galactosidase reporter plasmid using the PEI transfection reagent (Polysciences Inc.). Twenty-four hours later KGN cells were starved 4–8 h in 0.2% FCS DMEM and treated with TGFβ, BMP2, or GDF9 in 0.2% FCS/DMEM for 24 h. Ad-CAGA12-luc or Ad-BRE-luc infected hGL cells were treated with TGFβ, BMP2 or GDF9 in 2% FCS/DMEM for 24 h. The cells were then lysed into 1× passive lysis buffer, and luciferase activity was measured with luciferase assay reagent (Promega Corp., Madison, WI) and normalized to β-galactosidase activity. Data are the mean ± S.E.M. of triplicate determinations from representative experiments, relative to an adjusted value of 1.0 for the mean of the control wells.

2.8. Measurement of DNA synthesis

Determination of the effects of GDF9 on [³H]-thymidine incorporation of rat granulosa cells was performed as described (McNatty et al., 2005). All experiments involving rats were approved by the Wallaceville Animal Ethics committee. Briefly, granulosa cells were collected from all surface-visible follicles approximately 46 h after i.p. administration of 20 IU eCG (Intervet Ltd., Auckland NZ) to 23–26 day old Sprague–Dawley rats. Isolated cells (20,000 cells per well) were incubated in M199 (Earle’s salts; Sigma) with 100 ìU/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), 2 mM GlutaMax-1 (Invitrogen), 0.23 mM sodium pyruvate (Sigma) 0.3 mg/ml polyvinyl alcohol (Sigma) with 250 proliferation conditioned media containing untagged mGDF9 (total volume of conditioned media was 40% in all comparison wells) or purified untagged hGDF9 or C6H tagged hGDF9 at varying concentrations (0–1000 ng/ml). After 18 h of culture, methyl-[³H]-thymidine (PerkinElmer) was added to each well and cells were harvested for determination of [³H]-thymidine incorporation 6 h later. In each assay, each treatment was applied to 4 replicate wells. The bioassay was repeated with 3 separate pools of granulosa cells. The average cpm for each treatment was calculated as described for each pool of granulosa cells (McNatty et al., 2005). Differences between controls and treatments or between treatments were analyzed using the paired t-test function of Microsoft Excel 2003. Data were transformed (natural log) prior to analyses.

3. Results

3.1. Mouse GDF9 is produced by HEK-293T cells in a biologically active form whereas human GDF9 is produced in a latent form

In contrast to mouse GDF9 (mGDF9), human GDF9 (hGDF9) is not produced by HEK-293T cells in an active form.
(Fig. 1A). In these initial studies, Smad3 activation was monitored by transducing human granulosa-luteal (hGL) cells with an adenovirus encoding the CAGA-luciferase reporter (Kaivo-Oja et al., 2005), which contains repeats of the Smad3/4 response element (GTCT/AGAC) in front of the luciferase cDNA (Dennler et al., 1998). This lack of bioactivity in the case of hGDF9 was not due to a problem in the production of the protein or in processing of the precursor (Fig. 1C). These results led us to produce various His6 tagged forms of mouse and human GDF9 to enable the purification of these proteins and the characterization of the bioactivity of the human protein (Fig. 1B). The constructs differ only in the location of the His6 epitope tag, fused to the carboxy
terminus of the protein in the case of C6H GDF9, and near the amino terminus of the processed mature region in the case of N6H GDF9 (inserted between the 4th and 5th amino acids of the mature region to minimize problems in processing (Wolfraim et al., 2002)). The different recombinant proteins derived from the expression constructs shown in Fig. 1B were produced in stable HEK-293T cell lines. The various forms of recombinant GDF9 can be detected in medium conditioned by these cell lines utilizing the monoclonal antibody mAb-53, which is specific for an epitope conserved within the mammalian GDF9 proteins (Gilchrist et al., 2004b). The His6 tagged forms of mouse and human GDF9 were expressed and processed similarly to the wild type proteins when produced by HEK-293T cells (Fig. 1C).

3.2. C-terminally tagged mouse and human GDF9 are inactive

The placement of a His6 tag at the C-terminus of the mouse and human mature regions of GDF9 enabled the purification of these proteins via Ni2+ based IMAC chromatography (Fig. 2A–C). Bioactivity of the conditioned media and the purified GDF9 mature regions was assayed on primary cultures of hGL cells (Kaivo-Oja et al., 2005). The hGL cells were treated with either purified mouse (Fig. 2D) or human (Fig. 2E and F) GDF9 C6H, or with untagged mouse GDF9 conditioned medium as a control. Both mouse and human GDF9 were inactive as the purified C6H-tagged forms. We found that although wild type mGDF9 is produced in a bioactive form, the bioactivity is lost when a His6 tag is placed at the C-terminus of the mature region, demonstrated by the lack of bioactivity of conditioned medium containing mGDF9 C6H (Fig. 2D). Furthermore, human GDF9 C6H was additionally purified via high performance liquid chromatography (HPLC) (Fig. 2C) resulting in the removal of higher molecular weight impurities. However, the purified hGDF9 C6H still did not exhibit detectable bioactivity (Fig. 2F).

3.3. N-terminally tagged mature mouse GDF9 is active

Since the addition of a His6-tag on the C-terminus of the mGDF9 mature region resulted in the loss of biological activity (Fig. 2D), we assessed the effect of the placement of a His6-tag at the N-terminus of the mature region of GDF9. The tag was inserted between the 4th and 5th amino acid after the furin consensus sequence with the aim of minimizing the impact of the tag on the processing of the protein (Wolfraim et al., 2002). The N6H(4/5) mGDF9 mature region was purified by Ni2+ based IMAC chromatography using a similar protocol to the C6H tagged mouse and human GDF9 proteins (Fig. 3). A HPLC step...
was used to finally purify the N6H(4/5) mGF9 mature region which appears as a single major 21 kDa band on a silver stained SDS-PAGE gel or Western blot (Fig. 3B and D). The bioactivity of the N6H mGDF9 conditioned media was tested and it was as active as the respective wild type mGDF9 conditioned media (data not shown). The bioactivity of the partially purified (Fig. 3E) and the HPLC purified (Fig. 3F) N6H mGDF9 was tested on hGL cells and the protein was found to activate the Smad3 signaling pathway in a dose dependent manner. The analogous human N6H(4/5) GDF9 protein was also produced in 293T cells, and although the protein was produced and processed we were not successful in our attempts to purify the protein via

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**Fig. 4.** Purified hGDF9wt activates the Smad3 signaling pathway in ovarian granulosa cells in a dose dependent manner and the BMP signaling pathway is not activated. Purified untagged human GDF9 protein (reduced with 10 mM DTT) analyzed on a (A) SDS-PAGE silver gel and on a (B) Western blot immunostained with GDF9 mAb-53. (C) hGL cells transduced with an adenovirus encoding the Smad3/4 (CAGA-luc) reporter were incubated for 24 h in the absence (Cont) or presence of mGDF9wt CM (170 ng/ml) or in the presence of various concentrations of purified hGDF9. (D) KGN cells transfected with the Smad3/4 responsive (CAGA-luc) reporter were incubated for 24 h in the absence (Cont) or presence of TGFβ (3.5 ng/ml), mGDF9wt CM (200 ng/ml), or in the presence of various concentrations of purified hGDF9. (E) hGL cells transduced with an adenovirus encoding the Smad1/5 (BRE-luc) reporter were incubated for 24 h in the absence (Cont) or presence of BMP2 (85 ng/ml), TGFβ (3.5 ng/ml), Activin A (Act, 85 ng/ml) or various concentrations of purified hGDF9. (F) KGN cells transfected with the Smad1/5 (BRE-luc) reporter were incubated for 24 h in the absence (Cont) or presence of BMP2 (100 ng/ml), TGFβ (4 ng/ml), Activin A (Act, 100 ng/ml) or various concentrations of purified hGDF9. The mean luciferase values for the controls and samples with low or undetectable activity were (C) Cont 0.23; (D) Cont 0.003; (E) Cont 0.01, TGFβ 0.009, Act 0.004, purified hGDF9 at 85, 170 and 340 ng/ml, 0.011, 0.011 and 0.009, respectively; (F) Cont 0.002, TGFβ 0.001, Act 0.001, purified hGDF9 at 100, 200 and 400 ng/ml, 0.001, 0.001 and 0.001, respectively.
Ni2+ based IMAC (data not shown). In the case of the N6H(4/5) hGDF9 protein, the N-terminal His6 tag is possibly hidden by the pro-region and not available for binding to the IMAC resin.

3.4. Purified human GDF9 activates the Smad3 signaling pathway in human ovarian granulosa cells, whereas the Smad1/5 signaling pathway is not activated

Human untagged mature GDF9 protein produced in mammalian cells and purified to homogeneity (a gift from BioTechVisions, Ltd.) appears in silver staining (Fig. 4A) and Western blotting (Fig. 4B) as a major single 21 kDa band representing the pure mature region of hGDF9. The purified hGDF9 activated the Smad3 signaling pathway in hGL cells in a dose dependent manner (Fig. 4C), similarly as we have previously reported for unpurified mGDF9 (Kaivo-Oja et al., 2005). As the hGL cells have undergone a process of terminal differentiation in response to a luteinizing surge of gonadotropins, we wanted to further characterize the purified human GDF9 on granulosa cells which have not become terminally differentiated. The human granulosa tumor cell line KGN was chosen as it is FSH responsive and as such has been used as a model system for human granulosa cell responses (Nishi et al., 2001). It can be seen in Fig. 4D that the KGN cell line responds to purified human GDF9, as measured via Smad3 activation, in a similar manner as the hGL cells (Fig. 4C). Since we observed activation of the Smad3 pathway by purified hGDF9 in human ovarian granulosa cells, we wanted to determine whether the protein would also activate the BMP signaling pathway (Smad1/5) in such cells. As hGL cells are refractory to transfection by liposome-based reagents (Bondestam et al., 2002) we made an adenovirus incorporating the BRE-luciferase reporter and used adenoviral gene transduction to test various ligands in an analogous manner as with the adenoviral CAGA-luciferase reporter (Fig. 4C). BMP2 activated the BRE-luciferase reporter in human granulosa-luteal cells whereas TGFβ, activin A or purified hGDF9 did not (Fig. 4E). Further, the same result was obtained in the KGN cell line, BMP2 stimulating the BRE-luciferase reporter (introduced in this case as a plasmid via liposome based transfection), whereas TGFβ, activin A or purified hGDF9 did not (Fig. 4F). The lack of activation of the BRE-luciferase reporter in human ovarian granulosa cells demonstrates that although hGDF9 uses a hybrid of the TGFβ/activin and BMP pathways at the receptor level, the downstream signaling events are mediated by the TGFβ/activin (Smad2/3) pathway.

3.5. Purified human GDF9 stimulates rat granulosa cell [³H]-thymidine incorporation

The effects of the purified C6H tagged and untagged forms of hGDF9 on rat granulosa cell DNA synthesis as monitored by [³H]-thymidine incorporation were tested. Both unpurified mGDF9 and purified hGDF9 were potent stimulators of rat granulosa cell DNA synthesis (Fig. 5A and B) with increased (P < 0.05) [³H]-thymidine incorporation observed at doses ≥100 ng/ml for both unpurified mGDF9 and purified untagged hGDF9. The purified C6H tagged hGDF9 protein was completely inactive in the rat granulosa cell [³H]-thymidine incorporation assay (Fig. 5C), clearly demonstrating that the stimulation of DNA synthesis by the purified hGDF9 sample (Fig. 5B) was due to the presence of the hGDF9 protein.

4. Discussion

It is now well-established that during folliculogenesis there is a dynamic interplay between the oocyte and the surrounding somatic cells that mutually influences growth and differentiation of the somatic granulosa cells and the oocyte, and is essential for normal fertility (Eppig, 2001; Gilchrist et al., 2004a). Studies using mice (Dong et al., 1996; Carabatsos et al., 1998) and
A previous study by Hayashi et al. (1999) demonstrated that the active form of the protein was not active in conditioned media, as is the unmodified human protein. The N6H(4/5) mGDF9 protein does not require any purification steps to reveal the Smad activation activity. In contrast, we show in the present study that hGDF9, which is 90% identical to mGDF9 in the mature portion of the molecule, is produced by HEK-293T cells in an inactive form needing purification before the bioactivity of the protein can be detected. Although the reason for this species-specific difference between recombinant mouse and human GDF9 is not yet clear, we have shown in the current study by SDSPAGE immunoblotting that this lack of bioactivity in the case of hGDF9 is not due to a problem in the production of the protein or in processing of the precursor in HEK-293T cells.

The main purpose of this study was to produce and purify biologically active human GDF9, and to achieve this aim we chose to utilize the His6 tag which enables the purification of the tagged protein via Ni²⁺ based IMAC chromatography. We report that the position of the affinity purification tag crucially affects GDF9 bioactivity. We initially placed the His6 tag at the carboxy terminus of both the mouse and human GDF9 proteins. This resulted in the loss of biological activity in the case of mGDF9 C6H conditioned media, and likewise the purified form of the C6H tagged mGDF9 was inactive. As is the case for the unpurified human GDF9 wt form, the C6H tagged human GDF9 conditioned media and the purified protein were inactive. Next, the His6 tag was placed at the amino terminus of the mouse and human GDF9 mature regions. The tag was inserted between the 4th and 5th amino acid after the furin consensus sequence with the mature portion of the molecule, is produced by HEK-293T cells.

In conclusion, the present study demonstrates that the purified untagged mature region of hGDF9 became available to us and hence we tested the protein in transcriptional reporter assays specific for Smad3/4 activation in human ovarian granulosa cells. The purified human GDF9 mature region activated Smad3 in both our human granulosa cell models, i.e. hGL and KGN cells. KGN cells are a FSH responsive human granulosa tumor cell line (Nishi et al., 2001), hence the activation of Smad3 in these cells by hGDF9 demonstrates that the similar bioactivity observed in hGL cells is not an artifact of luteinization. No Smad1/5 activation (characteristic of the BMP signaling pathway) was detected in either hGL or KGN cells after treatment with hGDF9, indicating that the biological activity of hGDF9 is restricted to activation of the TGFβ/activin signaling pathway. To give our results a broader relevance we studied the effect of hGDF9 on granulosa cell proliferation in primary rat granulosa cell cultures. The ability of unpurified rodent GDF9 to act on granulosa cell proliferation has been studied (Gilchrist et al., 2001, 2004a,b, 2006; Hickey et al., 2005), and consistent with previous results we find that purified human GDF9 is a potent stimulator of mitogenesis in rat granulosa cells. The effect of a C-terminal epitope tag on the ability of hGDF9 to induce granulosa cell mitogenesis was clear. The HPLC purified C6H hGDF9 was not able to activate Smad3 and it did not have significant mitogenic activity. We tested if the purified C6H hGDF9 would be antagonising the bioactivity of the purified hGDF9, but no effect was seen (data not shown). Recently Gilchrist et al (Gilchrist et al., 2006) demonstrated by using mural oocyte-granulosa cell co-cultures that oocyte paracrine factors primarily utilize a similar signaling pathway (TGFβ/activin) to that used by GDF9 for transmitting their mitogenic actions on granulosa cells. As the C6H tag on the hGDF9 protein impairs its ability to signal through Smad3, it is not surprising that it does not induce mitogenesis in ovarian granulosa cells.
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