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Identification and characterization of canine growth differentiation factor-9 and its splicing variant

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Abstract

Growth differentiation factor-9 (GDF-9), a member of the transforming growth factor- β (TGF- β) superfamily, is expressed exclusively in the oocyte within the ovary and plays essential roles in the ovarian function in mammals. However, a possible involvement of GDF-9 in canine ovarian physiology that has a unique ovulation process among mammals has not been studied.

Interestingly, we have isolated two types of cDNA clones generated by an alternative splicing from a canine ovarian total RNA. The predominant long form cDNA shares a common precursor structure with GDF-9s in other species whereas the minor short form cDNA has a 172 amino acid truncation in the proregion. Using a transient expression system, we found that the long form cDNA has a defect in mature protein production whereas the short form cDNA readily produces mature protein. However, mutations at one or two N-glycosylation sites in the mature domain of the short form GDF-9 caused a loss in mature protein production. These results suggest that the prodomain and N-linked glycosylation of the mature domain regulate proper processing and secretion of canine GDF-9. Based on the biological functions of GDF-9, these characteristics of canine GDF-9 could be causatively linked to the unique ovulation process in the Canidae.

Keywords

TGF- β ; Alternative splicing; GDF-9 processing; N-linked glycosylation; Ovary

1. Introduction

Members of the transforming growth factor- β (TGF- β) superfamily, including growth differentiation factor-9 (GDF-9), are translated as preproprotein precursors with an N-terminal signal peptide, followed by the prodomain and mature domain (Derynck and Miyazono, 2008). After the signal peptide is removed the remaining proproteins dimerize. Importantly, this process requires the proregion of the proproteins. The dimerized

proproteins then undergo proteolytic cleavage at conserved RXXR sequence to separate the proregion proteins from C-terminal bioactive mature proteins. A characteristic feature of most mature protein dimers of TGF- β superfamily members is a single disulfide-bridge formed by the fourth of seven conserved Cys residues of the two mature proteins. However, as GDF-9 and several other members of the superfamily lack the conserved fourth Cys in their mature regions they remain as non-covalently linked dimers (Liao et al., 2003). GDF-9 signals by binding to the hetero-oligomeric complex of two types of cell membrane serine/threonine kinase receptors, namely the activin receptor-like kinase (ALK)-5 type I receptor and bone morphogenetic protein (BMP) type II receptor. Binding activates the intracellular signaling effectors, Smad2/3, which regulate transcription of selected genes in response to the ligand activation (Mazerbourg et al., 2004; Kaivo-Oja et al., 2005).

GDF-9 is predominantly expressed in the ovary and known as an oocyte secretion factor that plays essential roles in normal ovarian function (Otsuka et al., 2011; Paulini and Melo, 2011). Targeted deletion of the *Gdf9* gene in mice by homologous recombination leads to female infertility as a result of arrested folliculogenesis at the primary stage (Dong et al., 1996). Ewes with naturally-occurring mutations in the *Gdf9* gene were identified by infertility resulting from arrest of follicle growth at the primary stage in homozygotes, whereas heterozygous carriers exhibit superfertility associated with an increased ovulation rate (Hanrahan et al., 2004). Naturally-occurring mutations in the *Gdf9* gene have also been identified in women with premature ovarian failure (POF) (Kovanci et al., 2007; Laissue et al., 2006; Zhao et al., 2007), and also occur in mothers of dizygotic twins (Montgomery et al., 2004; Palmer et al., 2006). Interestingly, most of those mutations in the human *Gdf9* gene are located in the proregion that is necessary for dimerization of the mature protein. As such, mutations in the proregion may influence proprotein dimerization, and thereby negatively impact production of functional dimeric mature proteins. Therefore, the mutations in the proregion of GDF-9 identified in women with POF or mothers of dizygotic twins may cause impaired processing of the proproteins by formation of mis-folded proprotein dimers. Indeed, in vitro transfection experiments with representative GDF-9 mutants identified in women with POF and/or mothers of dizygotic twins demonstrated impaired posttranslational processing of the proproteins (Inagaki and Shimasaki, 2010).

It is known that the reproductive system in domestic bitches is unique among other species (Concannon, 2011). The bitches are a polyovulatory species and have a monoestrus cycle that results in non-seasonal breeding. The luteal phase of the non-pregnant cycle is similar in duration to that of pregnancy. Ovulation is spontaneous and occurs within 60 h after the preovulatory Luteinizing hormone (LH) surge. The interval between the LH surge and the ovulation is long compared with that of other mammals. In most mammals, oocytes are ovulated at the metaphase II stage. However in canines as in foxes, oocytes are ovulated at prophase of the first meiotic division during the germinal vesicle stage (Pearson and Enders, 1943). As such, canine oocytes mature in the oviduct 48–60 h after ovulation.

GDF-9 plays an important role in regulating several aspects of granulosa cell function during the preovulatory stage of follicle development. Mouse oocytes are incapable of synthesizing cholesterol, thus they require cumulus cells to provide them with newly synthesized cholesterol. Mechanistically, oocyte-derived GDF-9 seems to promote cholesterol biosynthesis in cumulus cells (Su et al., 2008). In addition, GDF-9 inhibits follicle stimulating hormone (FSH)-induced steroidogenesis while promoting cumulus cell progesterone production by stimulating the expression of an intrinsic prostaglandin-E2/EP2 receptor signaling pathway (Elvin et al., 2000). GDF-9 also enhances cumulus cell expansion in the presence of FSH (Elvin et al., 1999a), but not absence of FSH (Dragovic et al., 2005), which may relate to GDF-9 enhancement of hyaluronan synthase 2 (HAS-2) and

cyclooxygenase 2 (COX-2) mRNAs (Elvin et al., 1999a). Thus, GDF-9 regulates diverse processes and gene expression during the preovulatory stage.

GDF-9 has the ability to regulate determinative developmental events in folliculogenesis during preovulatory stage. This led us to the hypothesis that GDF-9 is a key contributing factor in bitches to their unique ovulation process. To test this hypothesis, we began an investigation of GDF-9's role(s) in canine ovaries. Here, we present the results of our initial study identifying and characterizing two splicing variant forms of canine GDF-9 cDNAs, their deduced amino acid sequences, posttranslational modifications and characteristics of the posttranslational processing.

2. Materials and methods

2.1. Reverse transcriptase-polymerase chain reaction (RT-PCR) and Northern blotting

Canine ovaries at anestrus stage (Three 2-year-old Beagles, weighing 9–10 kg) were obtained from Kitasato University Veterinary Teaching Hospital. The experimental procedures and the care of animals were performed in accordance with the requirements of the Institutional Animal Care Committee at Kitasato University, in compliance with NIH guidelines. Total RNA was isolated from canine ovaries using ISOGEN (Nippon Gene Co., Tokyo, Japan) based on the acid guanidinium thiocyanate–phenol–chloroform extraction method.

Five micrograms of total RNA were reverse-transcribed using Superscript II enzyme and oligo dT primer (Invitrogen, Carlsbad, CA, USA) in a total volume of 20 μ l. PCR amplifications using high fidelity polymerase (Expand HiFi, Roche Diagnostics, Indianapolis, IN, USA) were carried out under standard conditions for 30 cycles with denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, polymerization at 72 °C for 1 min, and final polymerization at 72 °C for 10 min. The specific primers for canine GDF-9 gene were 5'-ACTGAATTCCAGGCCATGGCACTTCTCAGCAACT-3' and 5'-TCTCTCGAGACGACAAGTGCACTTAGTGGCTATC-3'. Primer design was assisted by a BLAST search of the canine genome using sheep GDF-9 (NM_001142888) as a query. A sequence with 82% homology to the sheep counterpart was detected on chromosome 11.

For Northern blotting, total RNA (5 μ g) was electrophoresed on an agarose-formaldehyde gel and transferred to a nylon filter. Hybridization with digoxigenin (DIG)-labeled cRNA probes for the prodomain region of canine GDF-9 and detection were performed by using a DIG RNA labeling and detection kit (Roche Diagnostics, Indianapolis, IN, USA) as previously described elsewhere (Hashimoto et al., 2006). The DNA fragments used as probes (284 bp–641 bp, indicated in Fig. 1) were amplified by the primer set (5'-GGGATCCCTAAATCCAACAG-3' and 5'-TCTCGTCACCAGTGTAGTTG-3').

DNA sequence of the plasmids was confirmed by ABI PRISM 3730 Genetic Analyzer using a Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA, USA).

2.2. Construction of expression vectors

Full-length canine GDF-9 cDNAs were amplified by RT-PCR using primers described above. The products were sub-cloned in frame at the upstream site of the FLAG epitope tag present in pCMV-Tag4A (Stratagene, San Diego, CA). The acquired plasmids were designated as pcG9 LP (long precursor) or pcG9 SP (short precursor). For the generation of human GDF-9 protein, previously described full-length human GDF-9 cDNA was used (Liao et al., 2003). The cDNA was amplified using the primers (5'-TTTGAATTCTCCCAAGCCATGGCACGTCCCAA-3' and 5'-

ACACTCGAGACGACAGGTGCACTTTGTAGCTA-3'), then sub-cloned in frame upstream of the FLAG epitope tag of pCMV-Tag4A. The resulting plasmid was designated as phG9.

Mutations in the N-linked glycosylation sites of GDF-9 mature protein were created by standard PCR-based site-direct mutagenesis. The N at position 19 or/and 92 of the mature region was substituted with a Q (N19Q and N92Q). The primer sets used were as follows: 5'-GCTTCTTTCCAGCTGAGTGAATACTTGA-3' and 5'-TTCACCTCAGCTGGAAAGAAGCGGGAGCC-3' (for canine GDF-9 SP N19Q), 5'-GAGAAGCTCCAGTCGTCAGTGCCAAGAC-3' and 5'-CACTGACGACTGGAGCTTCTCATGGATG-3' (for canine GDF-9 SP N92Q) and 5'-GCTTCCTTCCAGCTGAGTGAATACTTC-3' and 5'-TTCACCTCAGCTGGAAAGGAAGCTGGGCC-3' (for human GDF-9 N19Q). Canine and human GDF-9 cDNAs containing mutations were sub-cloned into pCMV-Tag4A as described above. These plasmids were designated as pcG9 SP N19Q, pcG9 SP N92Q, pcG9 SP N19Q/N92Q and phG9 N19Q.

To gain the sequence of feline (*Felis catus*), raccoon dog (*Nyctereutes procyonoides*) and red fox (*Vulpes vulpes*) GDF-9, putative GDF-9 fragments were amplified by PCR from genomic DNA using a primer set (5'-TGAATGACACAAGTGCTCAGGC-3' and 5'-CACTGAGAGGGCTGTATTTG-3'), then sub-cloned into a pGEM-T vector (Promega Madison, WI, USA).

2.3. Production and analysis of recombinant proteins

Human embryonic kidney (HEK) 293 cells (RIKEN Cell Bank, Tsukuba, Japan) were maintained in DMEM/F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (growth medium). Cells were seeded in 24-well-plates at a density of 3×10^5 cells/well and transfected with expression plasmids using FuGene6 transfection reagent (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions. Twenty-four hours after transfection, culture media were replaced with serum-free media (200 µl/well) and cells cultured for 3–5 days before harvest of media for protein analysis. Conditioned media were precipitated by acetone. Cells were lysed in RIPA buffer containing protease inhibitors. Samples were then subjected to immunoblotting analysis as described previously (Hashimoto et al., 2006) under reducing conditions using the anti-FLAG M2 antibody (Stratagene, San Diego, CA, USA).

To evaluate N-linked glycosylation of proteins, conditioned media from cells transfected with pcG9 SP or phG9 were precipitated with acetone at -20 °C and treated by PNGase F (New England BioLabs, Ipswich, MA, USA) according to the manufacturer's instructions.

All data shown in this study are representative examples of three replicate experiments following the transient transfection step.

3. Results

3.1. Cloning of canine GDF-9 cDNA and its splicing variant

To characterize the primary amino acid sequence of canine GDF-9, we cloned cDNAs from canine ovarian RNA by RT-PCR using primers encompassing the entire cDNA coding region. As shown in Fig. 2A, a very strong band that migrated slightly faster than a 1500 bp marker was obtained. This PCR product was cloned and sequenced (Fig. 1), which corresponded to the canine GDF-9 sequence registered in Gen-Bank (accession No. GQ983543). The obtained nucleotide sequence covers the entire open reading frame

encoding 447 amino acids (Fig. 1). The coding region of canine GDF-9 contains a typical signal peptide-like sequence that is rich in hydrophobic amino acids in the middle region and ending with a small amino acid (Gly) at the N-terminal 24 amino acid sequence (Nielsen et al., 1997), followed by the proprotein with six theoretical N-glycosylation sites. A tetra amino acid sequence rich in Arg residues (RHRR), a potential recognition site for the proprotein convertase to separate the prodomain and the mature domain, is located at the 308th to 311th amino acids in the proprotein. The deduced amino acid sequence of canine GDF-9 showed high amino acid sequence homology with the that of other animal species, including the pig, sheep, mouse and human (Table 1). Interestingly, we detected another very faint PCR product migrating at between 800 and 900 bp markers in the PCR products (indicated by an arrow in Fig. 2A). The DNA sequence of this faint band revealed that it contained canine GDF-9 with a deletion of a segment encoding the 126th through 297th amino acids in the prodomain with no frame shift (Fig. 1, GenBank accession No. JQ180447). Although the corresponding short form of GDF-9 mRNA was not detectable in canine ovarian RNA by Northern blotting analysis (Fig. 2B) it appears to be an alternative splice variant as consensus bi-nucleotide sequences for the donor (GT) and acceptor (AG) splicing sites were confirmed (Fig. 2C).

3.2. Recombinant expression of canine GDF-9s

To characterize and compare the production, posttranslational processing and secretion of both forms of canine GDF-9 proteins, we transiently transfected HEK293 cells with expression plasmids encoding canine GDF-9 with the long precursor (LP) and short precursor (SP) tagged with a FLAG-epitope at the C-terminus (Fig. 3A).

The mature protein from the GDF-9 SP was readily detected in the conditioned medium by Western blotting (Fig. 3B). In contrast, we failed repeatedly to detect the mature protein from the GDF-9 LP in the conditioned medium. To ensure the successful transfection and production of canine GDF-9 LP, we analyzed the lysates of transfected cells for Western blotting. As expected, the GDF-9 LP protein was detected in transfected cells at similar levels to those obtained from the GDF-9 SP (Fig. 3B). As a comparison, we have tested human GDF-9 with the same transfection protocol and found similar success to the canine GDF-9 SP (Fig. 3C). These findings suggested that the canine GDF-9 SP is predominantly processed post-translationally as compared with the LP.

We also examined the effect of furin, a proprotein convertase, on the posttranslational processing of canine (Fig. 3B) and human (Fig. 3C) GDF-9. The intensity of the mature protein bands slightly increased in the conditioned medium of all samples after treatment with furin. However, furin co-expression was not sufficient for the processing of the canine GDF-9 LP.

3.3. Effect of the N-linked glycans on the posttranslational processing of canine GDF-9

There is a difference in the number of potential N-linked glycosylation site in the mature GDF-9 proteins between the dog and other animals; while other animals, namely, the pig, sheep, cow, mouse, rat and human have one N-glycosylation site, dogs have two sites (Fig. 1). As shown in Fig. 4A, canine and human GDF-9 mature proteins migrate at 24 and 20 kDa, respectively. Therefore, we predicted that canine GDF-9 mature protein is N-glycosylated at both sites whereas human GDF-9 mature protein is singly glycosylated. The PNGase F treatment of both proteins resulted in the disappearance of 24 and 20 kDa bands from the canine and human GDF-9, respectively and instead the appearance of 17 kDa bands for both species. These results suggested that both N-glycosylation sites in canine GDF-9 mature protein are indeed glycosylated.

To investigate whether an extra N-glycosylation site in canine GDF-9 mature protein is common in the Canidae, we performed PCR cloning of the *Gdf9* gene from genomic DNA of two other Canidae species, the red fox and raccoon dog. As shown in Fig. 4B, red fox and raccoon dogs also have two N-glycosylation sites. However, cat GDF-9 has only one N-glycosylation site. The amino acid sequence homology of canine GDF-9 mature protein to foxes, raccoon dogs and cats was 99.0%, 99.0% and 89.3%, respectively.

To explore the effect of N-linked glycans on the posttranslational processing of canine GDF-9, we mutated the N-linked glycosylation sites at the mature domain of canine and human GDF-9 and compared their production, processing and secretion by the transient expression protocol described above (Fig. 5). Canine GDF-9 SP proteins with mutations at one or both N-linked glycosylation sites were detected in cell lysates and migrated faster than the wild type GDF-9 SP at a level dependent on the number of mutation sites (Fig. 5, lower panel). However, the mature protein of GDF-9 SP mutants was not detected in conditioned media (Fig. 5, upper panel). This is in contrast to the mature protein of mutated human GDF-9 that was detected in conditioned medium. These results suggest that N-linked glycosylation of the mature region may be critical for proper processing and secretion in canine GDF-9.

4. Discussion

In the present study, we cloned two different sizes of canine GDF-9 cDNAs from the ovary. The DNA sequence of the longer cDNA clone shows similarity with GDF-9s of other species. However, the shorter cDNA clone is unique and has a truncated DNA segment encoding the 126th to 297th amino acids located in the proregion of the precursor. Comparison of their DNA sequences suggests that the shorter cDNA is a splice variant. To our knowledge, in TGF- β superfamily members, only TGF- β 2 reportedly has a splice variant, which contains an additional exon (84 nucleotides) inserted in the proregion (also called latency-associated proprotein region) (Koishi et al., 2000; Konrad et al., 2006). In contrast, the splice variant of canine GDF-9 has a long truncation (172 amino acids) in the proregion. The mature proteins of TGF- β superfamily members including GDF-9, GDF-8 (myostatin), GDF-11 (BMP-11), BMP-15 and TGF- β 1-3 are known to form noncovalent complexes with their respective cleaved prodomains (Böttinger et al., 1996; Ge et al., 2005; Lee and McPherron, 2001; McIntosh et al., 2008). Because of the deletion of 172 amino acids in the proregion, the mature protein generated from the canine GDF-9 SP may exist without forming complexes with the proregion protein.

In transfection assays using HEK 293 cells, the canine GDF-9 LP proprotein was produced, yet the mature protein was undetectable in both conditioned medium and cell lysates (Fig. 3). Also, co-expression of Furin had a minimal effect on the proteolytic cleavage. These results suggest that the proregion of canine GDF-9 LP prevents posttranslational processing of the proprotein to release the mature protein. A similar observation was previously reported with recombinant Nodal, another TGF- β family member (Constom and Robertson, 1999). In their study, the Nodal prodomain prevented secretion of not only mature Nodal protein but also mature proteins of dorsalin, another member of the TGF- β superfamily, and BMP-4 in media of cells transfected with the respective chimeric constructs. Fusing the prodomain of dorsalin to the mature domain of Nodal resulted in the successful secretion of mature Nodal protein.

It is known that the proregion of the TGF- β superfamily members is required to produce functional mature protein dimers (Hogan, 1996). However, in the case of canine GDF-9, the presence of the proregion prevented posttranslational processing, whereas truncation of a large portion of the proregion promoted proper processing. An important question is whether

the same regulation occurs in the oocyte of bitch ovaries. If the outcomes from the experiments using HEK293 cells are similar to those *in vivo*, then functional GDF-9 mature proteins may not be produced. In this regard, our previous study demonstrated that mouse BMP-15 proprotein is resistant to the post-translational processing (Hashimoto et al., 2005). Specifically, recombinant mouse BMP-15 proprotein produced in HEK 293 cells was not cleaved even in the presence of furin, whereas recombinant human BMP-15 and chimeric BMP-15 composed of the human proregion and mouse mature region were readily processed. Importantly, mouse BMP-15 mature protein was not detected *in vivo* in oocytes until 3 h before ovulation (Yoshino et al., 2006). Therefore, it is of particular interest to investigate whether levels of two mRNA variants and/or mature GDF-9 proteins vary *in vivo* at different stages of folliculogenesis and during the ovulation process in dogs.

Canine GDF-9 has two N-linked glycans in the mature protein in contrast to only one in other mammals. We have also confirmed the presence of an extra N-glycosylation site in raccoon dogs and red fox GDF-9. Thus, there is a parallelism between the unique ovulation process in dogs, raccoons, and red foxes and possession of two glycosylation sites in GDF-9 in these three species. N-linked glycans play a role in regulating posttranslational processing leading to the mature protein production and secretion. For example, mutations at the N-linked glycosylation sites of proregions of TGF- β s and inhibin α subunit as well as that of the mature region of inhibin α subunit caused a corresponding reduction of mature protein secretion (Brunner et al., 1992; Antenos et al., 2007). Consistent with these studies, mutations at the N-glycosylation sites in canine GDF-9 mature protein resulted in the complete loss of GDF-9 mature protein in the conditioned medium of transfected cells (Fig. 5). It is known that N-linked glycans play roles in key protein modifications for eukaryotic secretory proteins. These modifications encourage the recruitment of molecular chaperones and sorting receptors, which recognize the specific glycoforms. Mutations in the sugar chains result in the protein being eliminated from the secretory pathway. Therefore, N-linked glycans of eukaryotic secretory proteins act as quality control protein tags. Taken together, canine GDF-9 may be highly controlled for its protein quality by N-linked glycans in addition to the observed alternative splicing event.

As described above, GDF-9 can promote the expression of COX-2 and HAS that are necessary for cumulus expansion and ovulation (Elvin et al., 1999b; Elvin et al., 2000). Experiments using a mutant mouse model revealed that GDF-9 together with BMP-15 regulates LH induced oocyte meiotic resumption by modification of cumulus cell function after the preovulatory LH surge (Su et al., 2004). Therefore, these biological functions of GDF-9 in the ovary prompt us to hypothesize that the defect in posttranslational processing of canine GDF-9 LP that is predominantly expressed in the canine ovary may lead to reduced or nonexistent availability of functional GDF-9, resulting in the long interval between the LH surge and ovulation and/or the delayed oocyte maturation compared with other mammalian species. Furthermore, given that human GDF-9 mutations present in mothers of dizygotic twins exhibit impaired posttranslational processing along with a reduced secretion of the mature protein (Palmer et al., 2006; Inagaki and Shimasaki, 2010), impaired processing of canine GDF-9 LP may be linked to the polyovular nature of the Canidae.

In summary, we have isolated 2 forms of canine GDF-9 cDNA from the canine ovary. The predominant long form cDNA exhibited a defect in mature protein production whereas the minor short form cDNA that has a 172 amino acid truncation at the proregion readily produced the mature protein in an *in vitro* transfection system. However, mutations at one or two N-glycosylation sites in the mature protein domain of short form GDF-9 caused a loss of mature protein production. These findings suggest that bioavailability of functional mature GDF-9 in the dog is highly controlled by the posttranslational processing and

modifications. It will be of interest in future studies to determine whether these characteristics of canine GDF-9 are causatively related to the unique ovulation process in the Canidae.

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Abbreviations

ALK	<i>activin</i> receptor-like kinase
BLAST	<i>basic local alignment search tool</i>
BMP	bone morphogenetic protein
CM	conditioned medium
COX	cyclooxygenase
DIG	digoxigenin
FSH	follicle stimulating hormone
GDF	growth differentiation factor
HAS	hyaluronan synthase
LH	luteinizing hormone
RIPA	radio immunoprecipitation assay
POF	premature ovarian failure
RT-PCR	reverse transcriptase-polymerase chain reaction
TGF-β	transforming growth factor- β

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1  ATGGCACTTCTCAGCAACTTCTCTTGGTTTTCTGCTTAACGTTGCTGTTTTCT 60
   M A I L S N F F L W F F C L T L L C F P
61  ATTAGTCTTGGTCTCAAGTTTCGAGAGGAGAGCTCGGGCGCGCTGGAGCTGAGTCG 120
   I S L G S Q V S R G E A R A A A G A E S
121  GAATCTGTGGCTGAGCTTGGTATTGCTGGCGCTCCAGTGAAGAGACAGATCGGC 180
   E S V A E P W S L L R P P D G R D R S G
181  TTCCTTTCTCTCTTTCAAGTTCTATATATGGGCAAGTGGCAGCGCTAGAGCTTG 240
   F L S P L F K V L Y D G Q G G T P R A L
241  CGCTACATGAAGAGGCTCTAAGGCTGTGCTACCAAGAGGGGATCCCTAAATCCAAC 300
   R Y M K R L Y K A C A T K E G I P K S N
301  AGAAGCCCTCTTACAACACTGTTGACTCTTACGTCCTGTGCCAGCACACGAGGCT 360
   R S P L Y N T V R L F T S C A Q H T Q A
361  CCTGGGACACGGCGACAGGCTCTGTTCACTGTGGACCTGCTCTTAACCTGGATCTG 420
   P G D Q A T G S V P P P V D L L F N L D R
421  GTTCTCCCGTTGAACACTTACTGAAGTCGGGTTTGTGTACTTTTCAAGTAATTCATT 480
   V P P V E H L L K S G L L Y S F S N S I
481  TCTTTCCCTCTGCGTTAAATGCTGTGCACCTGGTGATCAAGAGCCAGAGTTTTCT 540
   S F P S A V K C L C H L V I K E P E F S
541  AGCTGGACCCCGAGAGCTCATCTTATTGACCTTAACTCAGAGTTGAATTGAAA 600
   S W T P Q R A P S L L T F N S Q F E L K
601  AAGAAATACAAATGTTGAGTTGATGTGACCACTGCTCTCCGGCTCTAGTGGCTTG 660
   K K Y K W F E V D V T T A L R P L V A L
661  CACAAGGAAGTCTTCACTGTCTGTAATTTTACCTGTGTGAGAAACAGCAGGAGAT 720
   H K R S L H M S V N F C V R N Q Q Q D
721  CCTGTGGACAGGATGGCGCTGGAATAGGACGCTGCTGTAACCCCTCTGCTTTTA 780
   P V A Q D G P W H R L L V P P S L L L
781  TATCTGAATGACACAAGTGTCTCAGGCTCATCAGAGTGGATTCCCTCACTATAAAGG 840
   Y L N D S A Q A H H R W D S L H Y K R
841  AGATCTTCAAGAGGCTGACCAAGAGGAGTCTGTCTGCTGCTCCATAGAGAGGAA 900
   R S S Q R A D Q K G G L S A C P I G E E
901  TCGACTGAAGGTGAAGATCTTCCGTCACCGGAGAGTCAGGACACGTCAGCTTGAA 960
   S T E G G R S S R B G Q D T V S L E
961  CTGCACAAGCTCTGGCTCCGCTCTTTCAATCTGAGTGAATACTTGAACACTTTCTT 1020
   L H K P L A P A S F N S E Y L K H F L
1021  TTCCCCAACACAGTGTGAGCTCCAGACTTCAGGCTGAGCTTTAGTCAGCTGAAGTGG 1080
   F P Q H E C E L H D F R L S F S Q L K W
1081  GACAAGTGAATGTCGGCCCGCAGACATCAACCTCGATCTGTAAGGGGACTGTCCA 1140
   D N W I V A P H R Y N P R Y C K G D C P
1141  AGGGCAGTTGACATCGATATGGCTCGCCAGTCACACCATGGTGAGAACATCATCCAT 1200
   R A V G H R Y G S P V H T M V Q N I I H
1201  GAGAAGCTCAACTCGTGCAGTCCAGACATCGTGTACTGCAATACAGCCCTCTC 1260
   E K L N S V P R P S C V P A K Y S P L
1261  AGTGTTTTGGACATTGAGCCAGATGGCTCAATTGCTTACAAGAAATATGAAGATATGATA 1320
   S V L T I E P D G S I A Y K E Y E D M I
1321  GCCACTAAGTGCACTTGTCTGTTAA 1320
   A T K C T C R *

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Fig. 1.
Nucleotide sequence and the deduced amino acid sequence of canine GDF-9 cDNA. The underline shows the putative signal sequence. Theoretical N-glycosylation sites are heavily underlined. The predicted cleavage site indicated with a broken line. An asterisk indicates the terminal codon. The sequence deleted in proregion by alternative splicing is indicated by blue.

Fig. 2. Expression of GDF-9 in the canine ovary. (A) RT-PCR analysis of mRNA expression of GDF-9 in canine ovaries. Arrow indicated the cDNA of 830 bp. (B) Northern blot analysis of the ovaries using DIG-labeled cRNA probes for the prodomain region of canine GDF-9. The positions of the 18S and 28S rRNA are shown in the left. (C) Schematic drawing of the alternative splicing of canine GDF-9 gene. The donor and acceptor sites in the intron are indicated by red.

Fig. 3.

Expression of canine GDF-9 in HEK293 cells. (A) Schematic drawing of the dog wild and variant GDF-9 expression plasmids. (B) Conditioned media (CM, left panel) and cell lysates (Cell, right panel) were from 293 cells transiently transfected with canine GDF-9 expression plasmids. The samples were subjected to SDS-PAGE immunoblotting analysis using an anti-FLAG antibody. (C) Expression of human GDF-9 by HEK293 cells. Conditioned media (CM, left panel) and cell lysates (Cell, right panel) were from 293 cells transiently transfected with human GDF-9 expression plasmids. The samples were subjected to SDS-PAGE immunoblotting analysis using anti-FLAG antibody on the same blot. Migration of GDF-9 proteins is indicated by the bars to the right of the figure. Shaded bars indicate the GDF-9 mature region and open bars indicate the GDF-9 proregion. Vector, pCMV-tag4A. Fu, Furin.

Fig. 4.

N-linked glycosylation of canine GDF-9. (A) Conditioned media of 293 cells expressing either canine GDF-9 or human GDF-9 were treated with PNGase F (1–4 U/reaction). The disappearance of the 23.7 kDa band of canine GDF-9 by PNGase F indicates that this band represents the N-glycosylated form of the molecule. The asterisk indicates cross-reactivity of PNGase F under these immunoblotting conditions. (B) Comparison of the deduced amino acid sequence of GDF-9s. The part of mature polypeptides of dog, red fox, raccoon dog and cat GDF-9 are aligned. The theoretical N-glycosylation site is underlined.

Fig. 5.

Effect of N-linked glycosylation in canine GDF-9 mature protein. Conditioned media and cell lysates from 293 cells transiently transfected with mutant canine and human GDF-9 constructs were subjected to SDS-PAGE immunoblotting analysis using an anti-FLAG antibody. CM, Conditioned media. Cell, cell lysates.

Table 1

Homology of the canine GDF-9 protein with various species GDF-9.

Species	Accession No.	Proregion	Mature region
Pig	NM_001001909	72.85%	92.59%
Sheep	NM_001142888	74.83%	89.63%
Mouse	BC052667	63.58%	85.93%
Human	NM_005260	72.97%	91.85%