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Human Growth Differentiation Factor 9 (GDF-9) and Its Novel Homolog GDF-9B Are Expressed in Oocytes during Early Folliculogenesis*

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ABSTRACT

Growth differentiation factor 9 (GDF-9) is a transforming growth factor-β family member that is required for normal folliculogenesis in female mice, but its role as a regulator of human fertility is still unclear. We determined here by *in situ* hybridization and immunohistochemical analyses the localization of the GDF-9 messenger ribonucleic acid (mRNA) and protein during human folliculogenesis. The GDF-9 transcripts were not detected in primordial follicles, but they are abundantly expressed in primary follicles in frozen sections of ovarian cortical tissue material obtained at laparoscopic surgery. We raised antipeptide antibodies against GDF-9 and showed by immunohistochemical studies on paraffin sections of whole human ovaries that the GDF-9 protein is most abundantly expressed in primary follicles. We recently demonstrated that a novel GDF-9-related factor, GDF-9B, is coexpressed with GDF-9 during murine folliculogenesis. We now isolated human GDF-9B complementary DNA and genomic clones and report the unusually restricted expression pattern of human GDF-9B. The human GDF-9B transcript can be detected only in the gonads by RT-PCR analysis, and *in situ* hybridization studies indicate that it is not expressed in small primary follicles but, rather, in the oocytes of late primary follicles. Functional studies using the *Xenopus laevis* embryo model indicate that unlike the transforming growth factor-β family members activin and bone morphogenetic protein-4, neither GDF-9 nor GDF-9B affects mesoderm induction, suggesting that they may use signaling pathways distinct from those well defined for activin and bone morphogenetic protein-4.

We conclude that 1) both GDF-9 mRNA and protein are abundantly expressed in oocytes of primary follicles in human ovary, suggesting that the GDF-9 transcript is translated at this early stage of folliculogenesis; 2) human GDF-9B is specifically expressed in gonads at low levels; and 3) the expression of GDF-9 mRNA begins slightly earlier than that of GDF-9B in the human oocytes during follicular development. Our results are consistent with the suggestion that GDF-9 and GDF-9B may regulate human folliculogenesis in a manner specific to the ovary. (*J Clin Endocrinol Metab* **84:** 2744–2750, 1999)

THE DEVELOPMENT of ovarian follicles from resting stage primordial follicles to the mature ovulating Graafian follicle is a biological process controlled in an endocrine manner by hypophyseal gonadotropins and regulated locally by ovarian factors (1). Infertility due to ovarian failure can be caused either by dysfunction of gonadotropin production or by defects involving ovarian function *per se*. Examples of the latter situation include defects in a number of genes specifically expressed in the gonads that have been recently shown to cause ovarian failure in human patients or genetically manipulated animals. In a subset of patients suffering from hereditary ovarian dysgenesis (XXGD, OMIM 233300), a missense mutation ($C_{566}\rightarrow T$) has been identified in the FSH receptor (FSHR) gene, which is expressed in the granulosa cells of developing follicles (2). A similar phenotype was obtained in mice homozygous for the targeted disruption of the FSHR gene (3). In studies using knockout mice, several genes have been shown to be essential for normal ovarian function (4). For example, both male and female Dazla knockout mice are infertile due to the loss of germ cells and the complete absence of gamete production (5). Also, mice mutated at the X chromosomal Zfx locus demonstrate a diminished number of germ cells as well as small animal size in both male and female mice (6).

Oocytes communicate with their surrounding somatic cells via paracrine signaling that is crucial for normal ovarian function (7). Growth differentiation factor 9 (GDF-9) is the first oocyte-derived growth factor found to be indispensable

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for fertility. In female mice disruption of the GDF-9 gene leads to a block in follicular development at the primary one-layer follicle stage, whereas male mice are fertile (8). Structurally, GDF-9 belongs to the transforming growth factor-β (TGFβ) growth factor family (9), which is a large group of polypeptide growth factors that have multiple roles in embryogenesis and in the control of cell growth and differentiation. During mouse folliculogenesis, GDF-9 expression begins at the primary follicular stage (10). Fitzpatrick *et al.* have shown that GDF-9 is also expressed in several nonovarian tissues in rodents and human (11). Human GDF-9 complementary DNA (cDNA) was initially cloned from ovarian cDNA library (10), and GDF-9 has been detected by RT-PCR from ribonucleic acid (RNA) derived from human preovulatory oocytes (12). However, localization of GDF-9 expression to specific cell types in the human has not been performed. We determined here by *in situ* hybridization and immunohistochemical analyses the localization of the GDF-9 messenger RNA (mRNA) and protein in human ovarian tissue and compare GDF-9 expression with that of human GDF-9B, a novel GDF-9-related gene that we recently identified in the mouse (13). This novel TGFβ family member is coexpressed with GDF-9 in oocytes during mouse folliculogenesis. Based on the mouse EST sequence, we cloned and characterized genomic and complementary DNA (cDNA) clones of the human ortholog of mouse GDF-9B and show here that the expression of human GDF-9B is gonad specific. We also determined by synthetic mRNA injection studies whether GDF-9 and GDF-9B share biological effects observed previously for TGFβ family members that induce mesoderm in *Xenopus laevis* embryos (14).

Materials and Methods

Human ovary and testis tissue samples

Human ovarian tissue samples were obtained from women (<35 yr of age) whose ovaries were removed because of cervical cancer without preoperative irradiation or as cortical biopsy material during laparoscopic surgery. Human testis samples were obtained from patients who had undergone orchidectomy due to prostate cancer. All tissue samples were collected by approval of the local ethical committee. For histological analyses the oophorectomy samples were formalin fixed and paraffin embedded, whereas ovarian biopsy and testis samples were directly embedded in OCT cryopreservation solution (Tissue-Tek, Miles, Inc., Elkhart, IN).

GDF-9 antibody and immunohistochemistry

To raise antibodies against GDF-9, rabbits were immunized with a peptide containing 13 amino acids corresponding to a C-terminal sequence of GDF-9 (EPDGSIAYKEYED) and an additional N-terminal cysteine residue allowing coupling to key hole limpet hemocyanin. The best immune serum, K3S1, was affinity purified using an affinity column containing the peptide coupled to epoxy-activated Sepharose 6B (Pharmacia Biotech, Uppsala, Sweden). Five-micron paraffin sections were deparaffinized, rehydrated, and treated in a microwave oven twice for 5 min each time in 0.01 mol/L citric acid monohydrate, pH 6.0. Sections were then incubated overnight at 4 C with the primary antibody diluted 1:100 in phosphate-buffered saline (PBS) containing 1% normal goat serum as a blocker. Sections were washed three times for 3 min each time in PBS and incubated for 30 min at room temperature with biotinylated goat antirabbit secondary antibody (Vector Laboratories, Inc., Burlingame, CA) diluted 1:200 in PBS containing 1% normal goat serum as a blocker. Sections were rinsed three times for 3 min each time in PBS and incubated with avidin-biotin-peroxidase complex (Vector Laboratories, Inc. at room temperature for 30 min (15). Finally, antigenic sites were visualized by using 3-amino-9-ethyl carbazole (Sigma Chemical Co., St. Louis, MO) in 50 mmol/L acetate buffer, pH 5, containing 0.03% H_2O_2. All experiments were controlled by incubating parallel sections without primary antibody or incubating the sections with primary antibody in the presence of the respective blocking peptide. The K3S1 antibody recognizes specifically oocyte GDF-9 in control mouse ovarian sections (our unpublished observations).

Cloning and characterization of the human GDF-9B gene

Primers were designed based on the mouse EST1 sequence (GenBank accession no. AA422665) to amplify part of the human GDF-9B. One primer pair (A/B, Table 1) amplified a 320-bp fragment from human genomic DNA and was cloned into pGEMT-Easy vector (Promega Corp., Madison, WI) for further analyses. Total human genomic PAC library (a gift from Pieter de Jong, Roswell Memorial Institute, Buffalo, NY) was screened by PCR using primers A and B. Fluorescent *in situ* hybridization (FISH) was performed on metaphase chromosomes derived from human peripheral blood lymphocytes. Identification of chromosomes was based on the banding pattern achieved using 5-bromodeoxyuridine incorporation (200 μg/mL) at the early replicating phase, as previously described (16, 17). Hybridizations with biotin 11-deoxy (d)-UTP (Sigma Chemical Co.)-labeled human GDF-9B gene-specific clones were carried out in 50% formamide and 10% dextran sulfate in 2 $\times$ SSC (standard saline citrate) as described previously (17–19). The slides were stained with Hoechst 33258 (1 μg/mL), exposed to UV light for 30 min, and counterstained with DAPI including antifading reagent (Vectachield, Vector Laboratories, Inc.). A multicolor image analysis was used for acquisition, display, and quantification of hybridization signals of metaphase chromosomes with the previously described system (20). The amplified 320-bp PCR product was labeled with [α-^{32}P]deoxy-CTP using the Prime-a-Gene kit (Promega Corp., Madison, WI) and hybridized onto high density filter containing human X chromosome specific cosmid library obtained from Resource Center of the German Human Genome Project via Max Planck Institute for Molecular Genetics (Berlin, Germany; http://resource.rzpd.de/cgi-resource/newlib). The GDF-9B-positive cosmids were digested with restriction enzymes *Eco*RI, *Bam*HI, *Kpn*I, and *Xba*I, and suitable sized fragments containing the human GDF-9B by hybridization were subcloned into pGEM7Zf(+) vector (Promega Corp.). The cloned fragments were sequenced with an ABI PRISM 377 DNA sequencer (Perkin Elmer Corp., PE Applied Biosystems, Foster City, CA).

RT-PCR cloning of human GDF-9B cDNA

A 5′-end cDNA representing the human GDF-9B gene was obtained by the rapid amplification of 5′-cDNA ends (RACE) technique, using the human testis Marathon-Ready cDNA kit (no. K7414–1, CLONTECH Laboratories, Inc. Palo Alto, CA). Gene-specific primer (C) was used in the first round PCR amplification with the AP1 primer in a 50-μL reaction [10 pmol of each primer, 0.2 mmol/L of each dNTP, 2.5 mmol/L $MgCl_2$, 1 $\times$ GeneAmp PCR buffer, and 2.5 U AmpliTaq DNA polymerase (Perkin Elmer Corp. PE Applied Biosystems)] in 30 cycles with an initial standard hot start procedure: 5 cycles of 95 C for 30 s and 72 C for 3 min, 5 cycles of 95 C for 30 s and 70 C for 3 min, followed by 25 cycles of 95 C for 30 s and 68 C for 3 min. One microliter of the first round PCR reaction was amplified with nested primers AP2 and D as described

TABLE 1. Primer sequences

A	5′-CGCTTCATGTGTCAGCAGCA-3′
B	5′-GAGCAATGATCCAGTGATCCCA-3′
C	5′-CGTTAGACATCAGGGAAGGTTTTGAG-3′
D	5′-TTCTGCACCCAGGGCTCCACATGG-3′
E	5′-AGAGCCACTGTGGTTTACCGCCATCA-3′
F	5′-GTACTGTTGCTGTCATCTGC-3′
G	5′-GGGCATGCTTCCCATCACCGTCACCGC-3′
H	5′-GGCTCAAGGTTTTAAGAGGACC-3′
I	5′-CTGCGGTACATGCTGGAGTTG-3′
J	5′-GAGCATGCTTTTCTCATGCGGCGCCGCCGCCAAGCTTG-CAGCATTGAATC-3′
K	5′-CTTCTAGATTATCTACATGTACAGGACTG-3′

above, and the 350-bp fragment of human GDF-9B obtained was directly sequenced.

Northern blot, RT-PCR, and in situ hybridization analyses

Northern blot analyses were performed as previously described (21). As probes for filter hybridizations we used a 798-bp human GDF-9B (primers E/F) and a 465-bp human GDF-9 (primers G/H) PCR fragment derived from genomic DNA and subcloned into pGEMT-Easy vector (Promega Corp.). The probes were labeled with [α-^{32}P]deoxy-CTP as described above. RT-PCR analyses were performed on Multiple Tissue cDNA panels I and II (K1420–1 and K1421–1, respectively, CLONTECH Laboratories, Inc.) using primers I and C to amplify a 340-bp cDNA fragment in a total volume of 50 μL [3 pmol of each primer, 0.08 mmol/L of each dNTP, 2.5 mmol/L $MgCl_2$, 1 × GeneAmp PCR buffer, and 0.75 U AmpliTaq DNA polymerase (Perkin Elmer Corp. PE Applied Biosystems)] in 40 cycles of 95 C for 30 s, 62 C for 45 s, and 72 C for 1.5 min preceded by initial denaturation at 95 C for 5 min and followed by a final extension at 72 C for 15 min. Twenty microliters of PCR reaction were analyzed on a 2% agarose gel, Southern blotted, and hybridized with the [α-^{32}P]deoxy-CTP-labeled 350-bp 5′-RACE fragment. The filter was washed with 1 × SSC-0.1% SDS and exposed overnight at room temperature. For *in situ* hybridization analyses, the [α-^{33}P]UTP-labeled antisense complementary RNA probes were *in vitro* transcribed from *Sph*I-linearized plasmids containing the GDF-9B and GDF-9 cDNAs described above. *In situ* RNA analyses were carried out on 9-μm cryostat sections as previously described (22). The slides were dipped in NTB-2 emulsion (Eastman Kodak Co., New Haven, CT) and exposed up to 57 days.

Xenopus laevis mesoderm induction assays

A 415-bp GDF-9B cDNA encoding the mature region of GDF-9B was synthesized by RT-PCR from mouse ovarian RNA using oligonucleotides J/K, cloned into pGEM-T vector (Promega Corp.), and sequenced. This GDF-9B cDNA was flanked with a *Sph*I site, allowing fusion to the mouse activin βA subunit proregion (A). For generating synthetic transcripts for embryo injections, a full-length mouse GDF-9 cDNA was subcloned into pSP64T3, and the mature region of mouse GDF-9B was subcloned into pRN3-AVg1 to replace the Vg1 mature region, resulting in pRN3-AGDF-9B. A similar AGDF-9 open reading frame (ORF) containing construct was generated. Capped transcripts were synthesized from linearized templates with SP6 or T3 RNA polymerase (Promega Corp.) using the Megascript kit (Ambion, Inc. Austin, TX). The translatability of the transcripts was tested using a reticulocyte lysate-based *in vitro* translation kit (Promega Corp.). Samples were analyzed by SDS-PAGE for visualization of the ^{35}S-labeled translated protein bands. *X. laevis* embryos were obtained as previously described (23). Embryos were injected at the four-cell stage into dorsal or ventral blastomeres with 0.02–2 ng GDF-9, AGDF-9, and AGDF-9B RNAs, and embryos were observed up to stage 40 to detect possible morphological alterations compared to the uninjected embryos. In some experiments animal caps were cut at stage 8 (midblastula stage), and these were allowed to grow in culture overnight. To control the responsiveness of the system to known dorsal and ventral mesoderm inducers, in some experiments the RNAs of activin A, AVg1, or bone morphogenetic protein-4 (BMP-4) were injected into parallel embryos (24–26).

Results

GDF-9 mRNA and protein are expressed in human oocytes

To study the distribution of GDF-9 transcripts in human ovary, *in situ* hybridization analysis was carried out on frozen sections of ovarian cortical tissue obtained as biopsy material samples from women undergoing laparoscopic surgery. The results indicate that GDF-9 mRNAs are clearly expressed in oocytes of primary follicles (Fig. 1). As these tissue samples very seldom contained secondary or tertiary follicles, the expression of GDF-9 mRNA could not be well studied during these later stages of follicular development. For immunolocalization studies, we raised an antipeptide antibody, K3S1, against a C-terminal epitope of mature GDF-9 protein (conserved in the mouse, rat, and human sequences) to investigate whether the GDF-9 transcript in oocytes is translated. Immunohistochemical analyses of sections of paraffin-embedded whole human ovaries indicated that the K3S1 antibody stains strongly oocytes of primary follicles (Fig. 2, A and B). No staining was observed when sections were incubated with only the secondary antibody (Fig. 2C), and the staining seen with K3S1 was abolished in the presence of the blocking peptide (data not shown).

Human GDF-9B gene

We previously identified by searching the database a 406-bp mouse EST sequence (mouse EST1, GenBank accession no. AA422665) that showed significant homology to GDF-9 (13). Based on this mouse EST sequence, several primers were designed, and one such primer pair (A/B) amplified a 320-bp fragment from human genomic DNA. The PCR fragment was sequenced and verified to contain a novel GDF-9-like sequence, designated as human GDF-9B. Genomic clones for the human GDF-9B gene were first obtained by screening a total human genomic P1 artificial chromosome (PAC) library by PCR with primers A and B. Only one PAC clone, PAC 72O6, was positive for this gene and was hybridized by FISH to chromosome Xp11.2.

To obtain the coding sequence and characterize the exon-

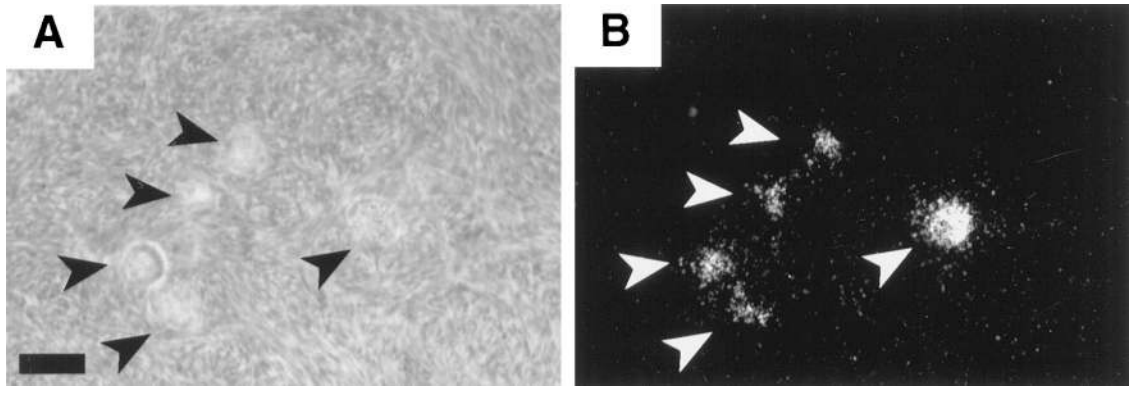

FIG. 1. *In situ* hybridization analysis of GDF-9 mRNAs in human ovarian biopsy samples derived from a 26-yr-old female [brightfield (A) and darkfield (B) micrographs]. *Black bar*, 50 μm.

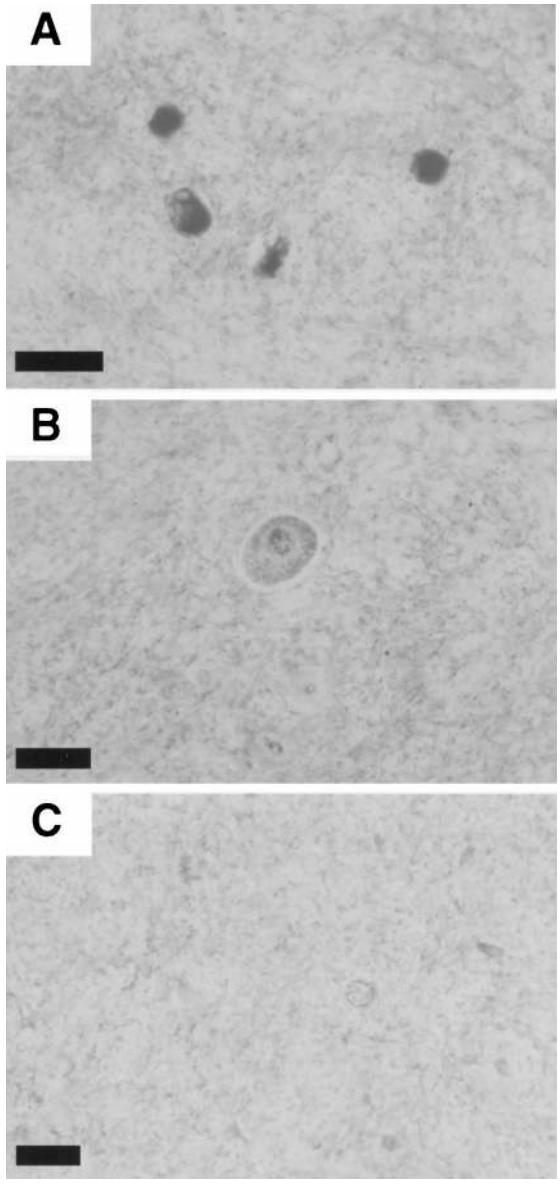

FIG. 2. Expression of GDF-9 protein in human ovary. In A and B, immunohistochemistry reveals GDF-9 protein localization to oocytes of primary follicles in human ovary, whereas no staining is observed in a control panel (C). *Black bars*, 50 μm.

intron structure of the human GDF-9B gene, two approaches, RACE and genomic cloning, were used in parallel. We screened a human X chromosome-specific arrayed cosmid library by hybridization using the above-mentioned PCR fragment as a probe and obtained a total of three cosmid clones (cos1, cos2, and cos3) for subcloning and sequencing (Fig. 3A). By double digests, using various restriction enzymes, Southern blotting, and hybridization analyses, we were able to identify overlapping subclones of these cosmids that represented human GDF-9B gene (Fig. 3A). 5′-RACE was carried out using gene-specific primers designed to the 5′-end of the exon 2 and human testis cDNA as a template. We obtained a 350-bp PCR fragment that represented part of the both exons compared with the genomic sequence, and thus unraveled the exon-intron boundaries of the GDF-9B gene (Fig. 3, A and B). The ATG codon encoding the first methione and the upstream 5′-untranslated region sequence, including an in-frame STOP codon 18 codons upstream of the first ATG codon, were deduced from the genomic sequence. A purine is present in position −3 upstream of the putative translation initiator ATG, consistent with Kozak's consensus sequence (27). We sequenced a total of about 6-kb genomic DNA that contained two exons, separated by a 4643-bp intron, and some 5′- and 3′-untranslated region sequences flanking the protein coding regions (EMBL accession no. AJ132405; Fig. 3A). The first 109 amino acids are encoded by the exon 1 and the remaining 283 amino acids by the exon 2, showing similar exon-intron structure as GDF-9. The exon-intron boundaries follow the GT-AG rule (Fig. 3B) (28). The human GDF-9B ORF contains an 18-amino acid signal peptide predicted using the Signal P program (Signal P V1.1 server at http://genome.cbs. dtu.dk/services/SignalP/) (29), followed by a 249-amino acid proregion and a 125-amino acid mature region that is likely to be released proteolytically from the proregion.

Human GDF-9B expression is restricted to gonads

The expression of human GDF-9B was studied initially by Northern blot hybridization analysis using mRNAs derived from several human tissues [heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon (mucosal lining), and peripheral blood leukocytes]. However, GDF-9B was not detected in any of these tissues, whereas GDF-9 mRNA expression could be seen in ovary and testis (data not shown), consistent with previously reported data (11). As GDF-9B transcripts were clearly less abundantly expressed than GDF-9 mRNAs, we chose RT-PCR analysis as a more sensitive method to screen the tissue distribution of human GDF-9B mRNAs. RT-PCR followed by Southern blotting and hybridization with a 5′-RACE fragment as a probe indicated that GDF-9B is expressed only in ovary and testis in the human (Fig. 4A). Glyceraldehyde-3-phosphate dehydrogenase gene expression as a control was uniformly observed in all tissues analyzed (data not shown).

GDF-9B is expressed in human oocytes

To study in more detail the distribution of GDF-9B transcripts in human ovary, *in situ* hybridization analysis was carried out on ovarian sections obtained as biopsy samples from women undergoing laparoscopic surgery. GDF-9B expression was detected in the oocytes of large primary follicles (Fig. 4B), whereas no hybridization could be seen in small primary follicles in which GDF-9 transcripts and protein were clearly present. Compared to GDF-9 expression, GDF-9B transcription appears to begin slightly later during human folliculogenesis. Although GDF-9B transcripts were detected in human testis by RT-PCR experiments, we could not localize GDF-9B transcripts to a specific cell type in human testis by *in situ* hybridization (data not shown).

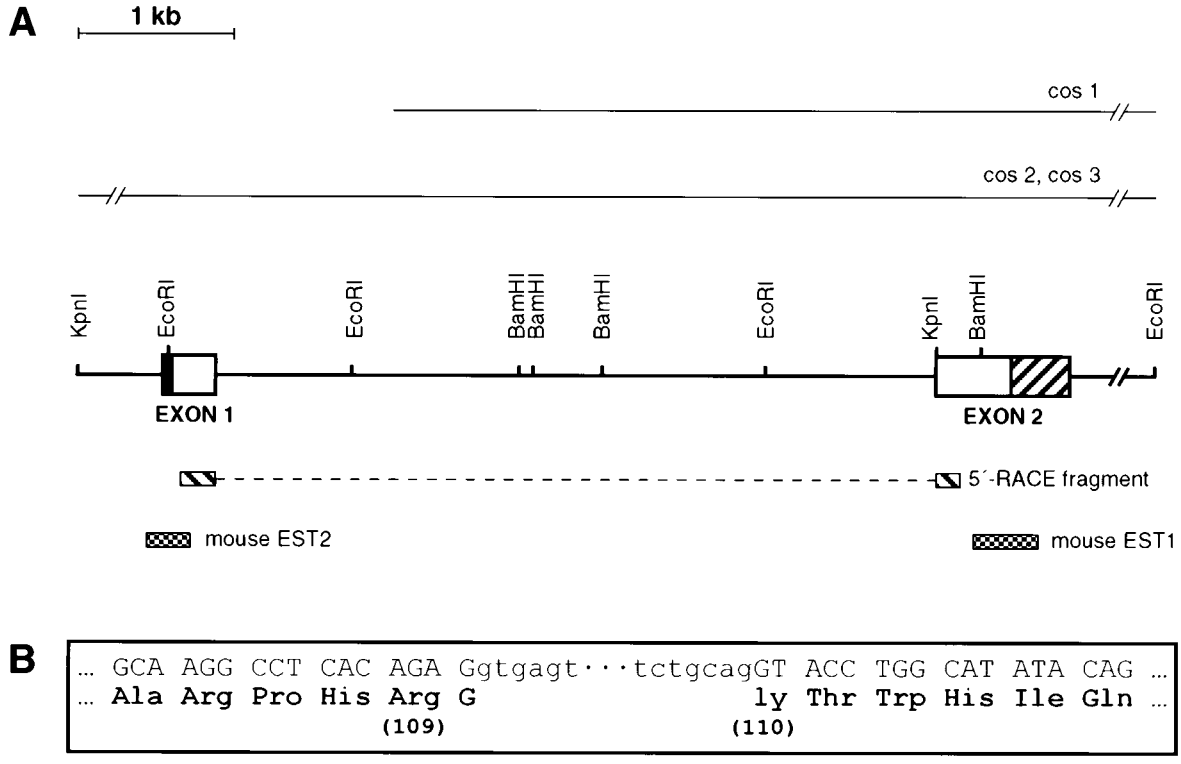

FIG. 3. Genomic structure (A) and the exon-intron boundary of the human GDF-9B gene (B). In A, the *black box* indicates signal peptide followed by proregion, shown as a *white box*, and mature region, shown as *oblique lines*. In addition, the 5′-RACE product and two mouse EST clones are presented. In B, the ORF is shown in *capital letters*, and intronic sequences are shown in *lowercase letters*.

GDF-9 and GDF-9B do not induce mesoderm in X. laevis embryos

To assess the signaling pathways used by GDF-9 and GDF-9B, we used a *X. laevis* assay in which a number of members of the TGFβ family induce mesoderm during early embryogenesis. For example, whereas injection of activin mRNA induces dorsal mesoderm, *e.g.* muscle (26), injection of BMP-4 mRNA induces ventral mesoderm, *e.g.* blood (24). These different activities result from activation of different receptors, which transmit their signals by activating either the Smad1 (BMP-4) or Smad2 (activin) transducing molecules (14). Injection of synthetic mRNA for GDF-9 did not have an effect on embryonic development at any of the concentrations tested, whereas in parallel injections, activin and BMP-4 induced dorsal and ventral mesoderms, respectively. One explanation for this lack of activity is that GDF-9 is not efficiently processed into bioactive forms, as previously demonstrated for *X. laevis* Vg1 (25). Activated Vg1 was produced by fusing the COOH-terminal domain to the proregion of either BMP-4 (BVg1) or activin (AVg1) and was shown to have potent mesoderm-inducing activity (25, 30, 31). We therefore injected AGDF-9 and AGDF-9B synthetic RNA into *X. laevis* embryos, and again, no effect on embryogenesis was detected, whereas AVg1 efficiently induced dorsal mesoderm in control embryos. Control experiments confirmed that all of the synthetic RNAs used were efficiently translated in a reticulocyte lysate *in vitro* translation system.

Discussion

We report here that human GDF-9 transcripts are expressed in oocytes during early folliculogenesis. Furthermore, we are able to show by immunohistochemical analysis of ovarian tissue sections using GDF-9-specific antibody that the GDF-9 transcript is translated to the respective protein at early stages of follicular development. The newly characterized X chromosomal GDF-9 homolog, GDF-9B, is expressed only in the gonads at low levels and thus exhibits an even more restricted expression pattern than GDF-9.

The human GDF-9B gene belongs to the TGFβ family of growth factors and is most closely related to GDF-9. The exon-intron structure of GDF-9B is similar to that of GDF-9; two exons are separated by a single intron in both genes, the intron being 4.6 kb in GDF-9B and 1.6 kb in human GDF-9. The partial testicular cDNA of human GDF-9B obtained here in parallel with the genomic sequence unequivocally reveals the exonic structure of human GDF-9B gene. The human GDF-9B gene maps to chromosome Xp11.2, in contrast to the autosomal GDF-9 (chromosome 5, Lawrence Berkeley National Laboratory, P1 clone 1076B9, GenBank accession no. AC004500; and our FISH data not shown). During the preparation of this manuscript, a human polypeptide sequence deduced from a genomic DNA clone and named BMP-15 was reported (32). This sequence shows identity with GDF-9B, except for three amino acids in the proregion of the predicted protein encoded by exon 1. However, no data on the human BMP-15 transcripts were presented in that paper.

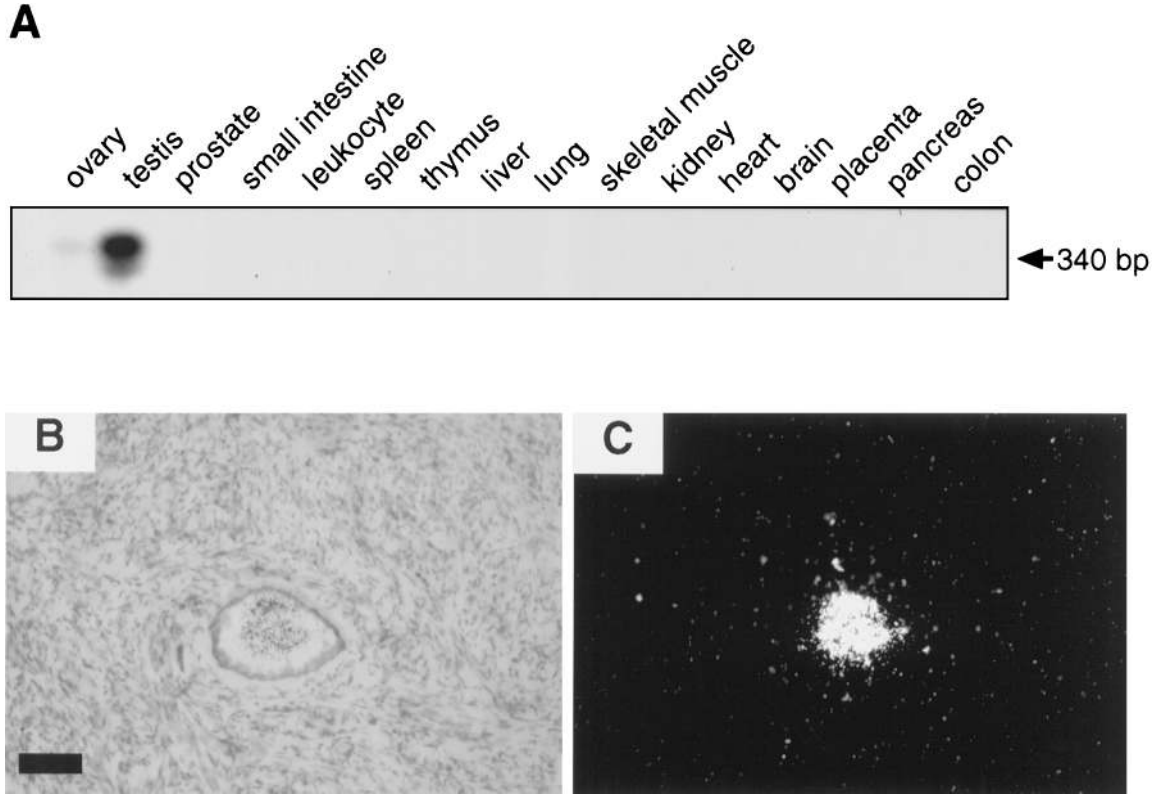

FIG. 4. Tissue distribution of human GDF-9B transcripts. A, An autoradiograph of a multiple tissue human cDNA panel amplified by PCR, Southern blotted and hybridized using the 5′-RACE product as a probe. *In situ* hybridization localization of the GDF-9B transcript to the oocyte. Brightfield (B) and darkfield (C) micrographs of a ovarian section derived from a 27-yr-old female are shown. *Black bar*, 50 μm.

Our studies for determining the tissue distribution of human GDF-9B/BMP-15 transcripts were problematic, because GDF-9B/BMP-15 transcripts were not detectable by Northern blot analysis in any tissue studied, even when using a sensitive, single stranded, DNA probe (data not shown). However, using RT-PCR followed by Southern blotting and hybridization, we were able to show that GDF-9B/BMP-15 is expressed in both ovary and testis. Our inability to detect human GDF-9B/BMP-15 transcripts on Northern blots can be explained by the fact that in the human ovary samples, most RNA species are derived from the stromal cells, and only a small fraction of mRNAs is of oocyte origin. No expression of GDF-9B/BMP-15 transcripts was observed in extragonadal tissues. Therefore, the expression of GDF-9B/BMP-15 seems to be even more gonad specific than GDF-9 expression, as GDF-9 transcripts are present also in human pituitary, uterus, and bone marrow in addition to gonads (11). The expression level of human GDF-9B/BMP-15 in gonads is apparently not as strong as that of GDF-9. The low abundance of GDF-9B/BMP-15 transcripts is also reflected by the number of ESTs identified for this gene in public databases; no human ESTs and only two mouse ESTs encoding for GDF-9B/BMP-15 were recognized. In contrast, a number of human and mouse ESTs representing GDF-9 were found in sequence databases. To localize human GDF-9 and GDF-9B/BMP-15 transcripts more specifically in the gonads, *in situ* hybridizations on tissue sections were performed. In human ovary, GDF-9 transcripts were already observed in small primary stage follicles, whereas the expression of GDF-9B/BMP-15 was confined to the late primary stage follicles, and no signal was observed in small primary or primordial follicles. Thus, it seems that GDF-9 expression precedes that of GDF-9B/BMP-15 during human folliculogenesis. As our ovarian biopsy samples represent the cortical region of the ovary, and they rarely contain follicles larger than the primary stage, the expression of these genes could not be studied in secondary or antral follicular stages. The expression patterns of these genes suggest that GDF-9 and GDF-9B/BMP-15 might be involved in the regulation of human ovarian function.

The significance of GDF-9 and GDF-9B/BMP-15 in human fertility is not yet known, but GDF-9 knockout mouse studies suggest that GDF-9 is likely to be important in human early folliculogenesis also. The infertility of GDF-9-deficient mice indicates that GDF-9B/BMP-15, which is still expressed in oocytes in these mice (32), is not able to rescue the biological function of GDF-9. Similar gene deletion studies may shed light on the biological function of GDF-9B/BMP-15 as well. We here evaluated using the *X. laevis* embryo model, whether GDF-9 and GDF-9B/BMP-15 share functional features with mesoderm-inducing members of the TGFβ gene family. Our results suggest that GDF-9 and GDF-9B/BMP-15 may not activate the receptor system used by either activin or BMP-4, and that all of the necessary components of the GDF-9 and GDF-9B/BMP-15 receptor signaling system are not likely to be present in the early *X. laevis* embryo. These experiments suggest that GDF-9 and GDF-9B/BMP-15 may exhibit ovary-specific effects and underline the importance of studying

their biology in ovarian organ and cell culture systems. The first such studies recently performed in a rat model by Hayashi *et al.* showed that recombinant rat GDF-9 enhances the growth and differentiation of cultured early ovarian follicles (33). Similar approaches need to be taken with GDF-9B/BMP-15 to understand its biological function during folliculogenesis. As a genetic approach to evaluate the role of these oocyte genes in human ovarian failure, we have initiated mutation-screening efforts of GDF-9 and GDF-9B/BMP-15 genes in a series of Finnish ovarian dysgenesis (XXGD) patients who do not have a mutation in the FSHR gene (2). Interestingly, a recent study by Zinn *et al.* (34) provides evidence that certain components of the Turner syndrome phenotype, including ovarian dysfunction, map to Xp11.2-p22.1 by deletion mapping of nonmosaic Turner patients. As GDF-9B/BMP-15 is expressed in the oocytes, it is possible that GDF-9B/BMP-15 might contribute to ovarian dysgenesis in the Turner syndrome phenotype.

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Note Added in Proof

Using the human sequence described here, we have subsequently cloned full-length cDNAs encoding the mouse and the rat GDF-9B (Jaatinen *et al.*, Mol Cell Endocrinol., July 1999 issue). The numbering of the respective cosmid clones used in this work is as follows: cos1, cosLLNLc110C2430; cos2, cos2LLNLc110E1332; and cos3, cosLLNLc110P2427.