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BASIC FIBROBLAST GROWTH FACTOR SENSE (FGF) AND ANTISENSE (*GFG*) RNA TRANSCRIPTS ARE EXPRESSED IN UNFERTILIZED HUMAN OOCYTES AND IN DIFFERENTIATED ADULT TISSUES

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SUMMARY: Basic fibroblast growth factor (bFGF) is a highly conserved and ubiquitously distributed mitogen. In amphibian oocytes bFGF mRNA is regulated post-transcriptionally by interaction with an antisense RNA transcript. We used reverse transcription-polymerase chain reaction (RT-PCR) and Northern hybridization to determine the presence of bFGF and its antisense RNA (*gfg*) in unfertilized human oocytes and postnatal differentiated tissues. BFGF and *gfg* transcripts were co-expressed in many tissues, with bFGF transcripts (7, 3.7 and 1.8 kb) being more abundant than the *gfg* transcript (1.5 kb) in 8 of 16 tissues examined. Sense and antisense expression was approximately equal in kidney and colon, while in heart, liver, skeletal muscle and testis *gfg* transcripts predominated. RT-PCR demonstrated the presence of bFGF and *gfg* transcripts in unfertilized oocytes where the antisense transcript was present in excess of the sense transcript. These findings suggest a role for *gfg* in regulation of bFGF expression.

Basic fibroblast growth factor (bFGF), a broad spectrum mitogen for cells of mesodermal origin, has been implicated as a paracrine regulator of mammalian ovarian function. BFGF has been purified to homogeneity from bovine corpus luteum (1), and the ovary been established as a site of bFGF expression (2-4) and action (5-7). Potential roles of bFGF in ovarian function include regulation of ovarian development (8), control of angiogenesis and granulosa cell proliferation in the developing follicle, regulation of plasminogen activator activity associated with ovulation, and early corpus luteum development (9). In the human ovary, bFGF mRNA expression has been detected in granulosa and cumulus cells (10), and a paracrine role for bFGF in follicular development and oocyte maturation has been proposed (7).

In amphibians, the oocyte is also a source of bFGF mRNA. BFGF mRNA transcripts are present in the developing oocyte of the African clawed toad *Xenopus laevis*, and play a vital role in oocyte maturation and embryonic mesoderm induction (reviewed in (11)). The *Xenopus* oocyte contains 3 maternal bFGF transcripts of 4.2, 2.3 and 1.5 kb in length (12). The 4.2 kb transcript encodes the *Xenopus* bFGF protein, which is highly homologous (>80% identity) to mammalian bFGFs. This transcript is present in the unfertilized oocyte, but disappears shortly after

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fertilization, and is subsequently re-expressed during the neurula stages. bFGF protein specified by this transcript in early cleavage stage embryos induces the formation of mesoderm from ectoderm. In contrast, the 1.5 kb bFGF mRNA is an antisense RNA transcribed from the opposite strand of the bFGF gene (13), and appears to be involved in regulation of the stability of the bFGF sense transcript. The sense and antisense transcripts share a 900 bp region of sequence homology at their 3' ends, and coexist as double-stranded RNA duplexes in the cytoplasm of the immature oocyte (13). Duplex formation targets the bFGF mRNA for modification by a dsRNA-specific deaminase (13) which is released from the nucleus at the time of germinal vesicle breakdown (GVB). Since the bFGF sense transcript disappears immediately after GVB it is believed that antisense-directed deaminase activity targets bFGF mRNA for rapid degradation (13).

The strong evolutionary conservation of the bFGF gene organization and sequence indicate the importance of this factor in normal cellular function, and support the possibility that its role in oocyte maturation and embryonic development has also been conserved. We have recently identified, cloned and sequenced a human bFGF antisense RNA transcript (*gfg*) with 75% homology to the *Xenopus* antisense bFGF transcript (14). Strand-specific cRNA probes detect a unique 1.5 kb mRNA transcript in human and rat cells. In order to address the possible functional conservation of the antisense bFGF transcript we have investigated the expression of bFGF sense and antisense transcripts in human oocytes and in differentiated adult tissues.

MATERIALS AND METHODS

Chemicals. Deoxynucleotide triphosphates (dNTPs) and random hexanucleotide primers ($pd(N)_6$) and Percoll were purchased from Pharmacia Fine Chemicals, Dorval, Quebec, Canada. ^{32}P-α dCTP and ^{32}P-γ ATP were from Amersham, Oakville, Ontario, Canada. MMLV reverse transcriptase, restriction endonucleases and dithiothreitol (DTT) were obtained from BRL/Life Technologies, Burlington, Ontario, Canada. Taq DNA polymerase and RNasin were from Promega, Madison, WI. Culture media, fetal bovine serum (FBS) and all other culture reagents were from GIBCO Laboratories, Grand Island, N.Y. The bFGF cDNA probe, a 1.4 kb cDNA encompassing the 3 coding exons of rat bFGF, was generously provided by Dr. Andrew Baird. The 301 bp *gfg* cDNA probe was described previously (14). The human multiple tissue northern blots were purchased from Clontech, Palo Alto, CA. All other chemicals were from Sigma Chemical Company, St. Louis, Mo., unless otherwise noted.

Oocyte and granulosa cell collection. Patients attending the Grace Maternity Hospital In Vitro Fertilization Clinic were superovulated with human menopausal gonadotrophin (Pergonal, Serono), and follicular development was monitored by ultrasound and serum estradiol measurement. Ovulation was induced with hCG when the dominant follicles were >16-18 mm. Oocytes were collected 36 h later by transvaginal aspiration. Oocytes and associated cumulus cells were cultured in Ham's F10 supplemented with calcium lactate (1 mM), $NaHCO_3$ (20 mM), $KHCO_3$ (5 mM), $MgSO_4$ (1 mM), antibiotics (penicillin G, 75 mg/L; streptomycin, 50 mg/L) and 7.5% human serum albumin (HSA) for 6 h prior to *in vitro* fertilization. Cumulus and corona were removed by trituration 18 h after insemination and the eggs cultured for a further 24-72 h. Discarded eggs (those which showed no evidence of fertilization by 72 h post-insemination) were used for RT-PCR analysis. Granulosa cells were obtained from follicle washes after follicular aspiration. In some experiments, granulosa cells were purified by Percoll gradient centrifugation as described by Yan et al.(15). Briefly, follicular aspirates were diluted in Ham's F10 medium supplemented with 30 U/ml heparin and centrifuged at 500 xg for 20 minutes. The resulting cell pellet was resuspended in 2 ml of medium and layered on top of a 10 ml cushion of 70% Percoll. After centrifugation at 2000 xg for 30 minutes to remove contaminating blood cells the supernatant and 2 ml of the underlying Percoll cushion were collected to a fresh tube and centrifuged at 2000 xg for a further 30 minutes. Granulosa cells were collected at the interface, and washed 3 times in Ham's F10 medium before RNA extraction.

Reverse transcription-polymerase chain reaction. Oocytes and granulosa cells were dispersed in guanidium isothiocyanate and RNA was isolated as by sequential extraction in phenol and chloroform . In each experiment 3 to 6 oocytes were pooled for RNA extraction and RT-PCR amplification as previously described (14, 16). PCR reaction products were separated by electrophoresis on a 2% agarose gel at 80V for 1hr and the ethidium bromide-stained bands were visualized by UV transillumination.

Oligonucleotide primers and probes. Oligonucleotide primers were synthesized by the Regional DNA Synthesis Lab, Calgary, Alberta, Canada, and at the Marine Gene Probe Lab, Dalhousie University, Halifax, Nova Scotia, Canada. Primer sequences were selected using Amplify (William Engels, Genetics Department, University of Wisconsin, Madison, WI) on a MacIntosh IIsi. Primers for amplification of human bFGF sense transcripts (sP1 and sP2) were described previously (17) and generate a 352 bp RT-PCR product. PCR primers for amplification of the human bFGF antisense splice variant were asP1; 5'ATGTGGAAGTTTCCAGGAGGCCTGTCA-3' and asP2; 5'-GCCTAGCAACTCTGCTGGTGATGGGAG-3', which predict a 301 bp product (Fig. 1). The sequence of the P2A internal primer used for Southern hybridization was 5'-GAACCGCTGTGTCTCCAATATCTTCTTCAG-3'. Northern and Southern hybridizations were performed as previously described (14, 17).

RESULTS AND DISCUSSION

Organization of the human bFGF/gfg gene locus. The intron-exon splice boundaries of the bFGF sense and antisense transcripts, and the regions recognized by the PCR primers are indicated in Fig. 1. The sense transcript-specific primers (sP1 and sP2) target exons 1 and 3 of the bFGF coding region, and generate an RT-PCR amplification product of 352 bp (16). The antisense transcript specific primers (asP1 and asP2) recognize two 3' coding exons of the human bFGF antisense gene, and generate a 301 bp RT-PCR product designated *gfg-1*(14). The human antisense cDNA product has 73% sequence identity with exons 3 and 4 of the *X. laevis* antisense transcript. Exons 1 and 2 of the human bFGF antisense gene have yet to be characterized. The P2A oligonucleotide probe spans the exon splice site of the antisense transcript, and was used as an internal probe to confirm the identity of the *gfg* RT-PCR product. The 301 bp *gfg-1* cDNA (GenBank Accession number L31408) was used to generate probes for Northern hybridization studies as previously described (14).

Expression of bFGF and gfg in human tissues. Expression of bFGF sense and antisense transcripts was first examined by Northern hybridization in human tissues. Multiple tissue northern blots containing 2 μg of polyA+ selected RNA were probed sequentially with probes for bFGF, *gfg* and glyceraldehyde phosphate dehydrogenase (GPDH) as previously described (14). RNA transcript sizes were determined by comparison with cRNA markers of known size. As shown in Fig. 2, the bFGF coding sequence double-stranded bFGF cDNA probe detected 3 predominant mRNA transcripts of 7, 3.7 and 1.8 kb at various levels of intensity in most tissues examined. Intermediate size (2-2.5 kb) transcripts detected in placenta, skeletal muscle and pancreas may result from partial degradation of the 7 and 3.7 kb transcripts.

After stripping, the blot was reprobed with the 301 bp *gfg*-1 cDNA probe, which detected a discrete 1.5 kb transcript in nearly all tissues examined, but most abundantly in heart, liver, skeletal muscle, testis and kidney (Fig. 2, *middle panel*). The relative levels of bFGF and *gfg* are summarized in Fig. 3. Total bFGF transcript levels were calculated as the sum of the 7, and 3.7 kb and 1.8 kb transcripts, since these appear to be derived by differential polyadenylation site

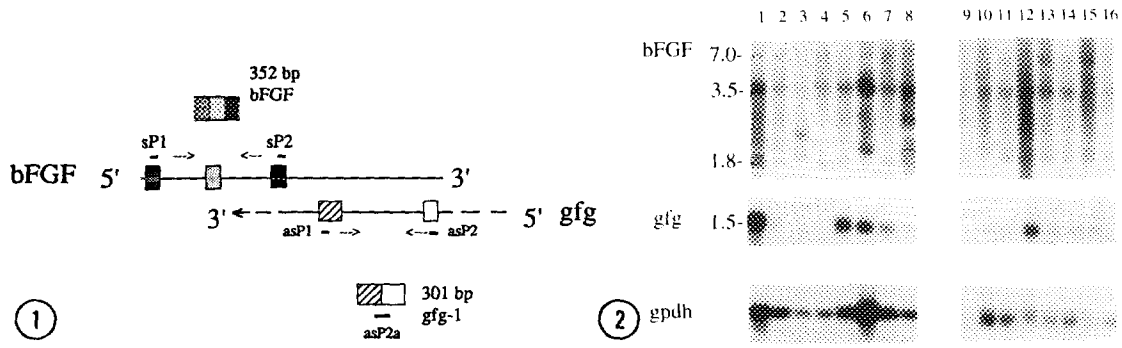

Fig. 1. Schematic representation of the human bFGF sense and antisense (*gfg*) genes. PCR primer pairs specific for bFGF (sP1/sP2) and *gfg* (asP1/asP2) and the expected RT-PCR amplification products are shown. Boxes denote bFGF and *gfg* coding exons.

Fig. 2. Co-expression of bFGF and *gfg* mRNA transcripts in human tissues. Northern blots containing 2 μg of polyA+ RNA from normal human tissues were probed sequentially for bFGF, *gfg* and glyceraldehyde phosphate dehydrogenase (GPDH) expression. *lane 1*, heart; *lane 2*, brain; *lane 3*, placenta; *lane 4*, lung; *lane 5*, liver; *lane 6*, skeletal muscle; *lane 7*, kidney; *lane 8*, pancreas; *lane 9*, spleen; *lane 10*, thymus; *lane 11*, prostate; *lane 12*, testis; *lane 13*, ovary; *lane 14*, small intestine; *lane 15*, colon; *lane 16*, peripheral blood leukocytes.

usage (18). The derivation of the atypical size transcripts was unknown and so these were excluded from the present analysis. The ratio of sense to antisense bFGF RNA was markedly tissue dependent as shown in Fig. 3. BFGF sense transcripts predominated in brain, placenta, lung, pancreas, thymus, prostate, ovary and small intestine, while antisense transcripts were more abundant than sense transcripts in heart, liver, skeletal muscle and testis. Sense and antisense transcripts were approximately equally abundant in kidney and colon. Transcript levels were too low to compare reliably in spleen or peripheral blood leukocytes. The tissue-specific expression of sense and antisense transcripts reported here is in agreement with the recent report by Zuniga and co-workers (19) of bFGF mRNA isoform expression in avian tissues.

We have previously reported that the antisense transcript does not correspond to any of the multiple bFGF transcripts detected in rat tissues with strand-specific cRNA probes or double stranded bFGF coding exon probes (14). This is confirmed in human tissues by the densitometric scan (Fig. 4) which clearly demonstrates that the antisense transcript is smaller than the 1.8 kb sense transcript detected with the bFGF cDNA probe. The double-stranded *gfg*-1 cDNA probe used in this experiment also hybridized weakly to the 7 kb but not to the 3.7 or 1.8 kb bFGF sense transcripts. This is not surprising since the *gfg*-1 probe contains sequences in common between the coding exons of the antisense transcript and the distal 3' untranslated tail of the bFGF sense RNA. Failure to detect either the 3.7 or 1.8 kb transcripts with the *gfg*-1 probe indicates that these shorter bFGF transcripts are probably derived by differential polyadenylation splice site usage as previously suggested (18). The antisense orientation of the 1.5 kb transcript in human tissues has been confirmed by Northern hybridization with strand-specific cRNA probes (14).

Expression of bFGF and gfg in human granulosa cells and oocytes. Total RNA prepared from human granulosa cells and unfertilized oocytes was subjected to RT-PCR amplification with

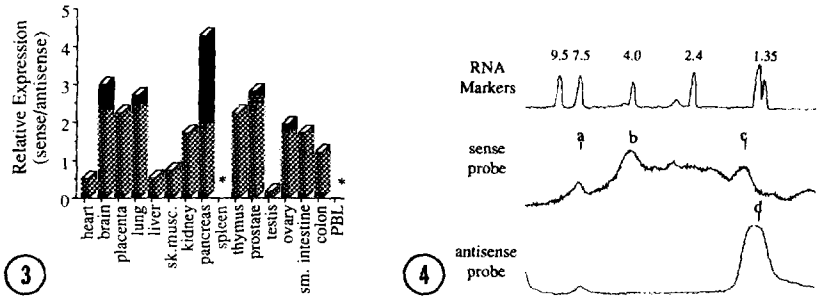

Fig. 3. Ratio of sense/antisense bFGF mRNA expression in human tissues. Relative abundance of bFGF (sense) and *gfg* (antisense) mRNAs was determined by densitometric scanning of a range of exposures of the Northern blots shown in Fig. 2.
*, not determined. (Expression was too low to reliably estimate the level of either transcript in the spleen or peripheral blood leukocytes (PBL)).

Fig. 4. The antisense transcript does not correspond to any of the multiple bFGF transcripts detected with the bFGF coding sequence cDNA probe. Human heart RNA was probed sequentially with cDNA probes for bFGF (coding exons 1-3; sense probe) and *gfg* (antisense probe). The resulting autoradiograms were scanned and analyzed with the NIH Image program. The positions of the RNA size markers are indicated in the top panel. The RNA transcripts detected with the sense probe were 7 kb (a), 3.7 kb (b) and 1.8 kb (c). The antisense transcript (d) was estimated to be 1.5 kb in size.

oligonucleotide primers specific for the bFGF sense or antisense transcripts, as previously described. As shown in Fig. 5, PCR of reverse transcribed granulosa cell RNA generated the predicted 352 bp bFGF cDNA product with the sense-specific primers, and the 301 bp *gfg* product with the antisense-specific primers. Negative control reactions, which contained all components except input RNA, did not produce any detectable cDNA product. RT-PCR of total RNA from unfertilized human oocytes detected an abundant *gfg* product, as well as a less abundant bFGF product (Fig. 6A). The identity of the two cDNA products was confirmed by sequential restriction enzyme analysis (not shown) and subsequently by Southern hybridization with ^{32}P-labeled internal probes specific for the bFGF and *gfg* products (Fig. 6 B, C).

In the developing *X. laevis* oocyte the antisense bFGF transcript is present in 20-fold excess over the sense transcript suggesting that all of the sense bFGF transcripts in the unfertilized oocyte may exist as heteroduplexes with the antisense transcript (13). Several independent RT-PCR amplifications on different pools of oocyte RNA confirmed the pattern shown in Fig. 6a, suggesting that in human oocytes, as in *X. laevis*, *gfg* transcripts are present in excess of bFGF transcripts.

The present study demonstrates for the first time the presence of bFGF sense and antisense RNA transcripts in the unfertilized human oocyte, and supports the possibility that bFGF and *gfg* play a role in human oocyte maturation and embryonic development. Normal embryonic development is critically dependent on mesoderm induction and dorsal-ventral pattern formation driven by growth factor signals from the underlying endoderm and bFGF is an essential component of the induction process. In amphibian embryos bFGF mRNA stability is regulated postranscriptionally by interaction with its antisense RNA (13). The present demonstration that maternal bFGF sense and antisense transcripts are present in unfertilized human oocytes supports the possibility that this functional relationship is conserved in higher vertebrates. The co-

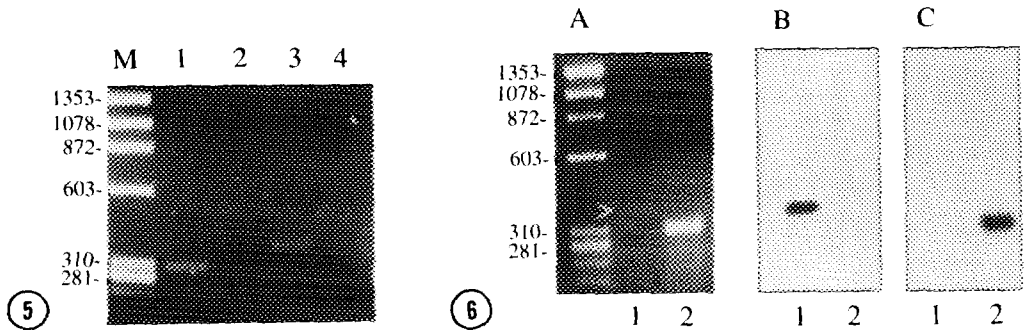

Fig. 5. Detection of *gfg* and bFGF mRNA transcripts in human granulosa cells. Total RNA (300 ng) was reverse transcribed and amplified for 30 cycles using primer pairs specific for *gfg* (lane 1) or bFGF (lane 3). Negative control reactions (lanes 2 and 4) contained all components except RNA. Product sizes were estimated by comparison with ϕX Hae III markers (M).

Fig. 6. Detection of bFGF and *gfg* mRNA transcripts in human oocytes. Total RNA from pooled oocytes was reverse transcribed and amplified for 30 cycles in the presence of primers for bFGF (lane 1) and *gfg* (lane 2). **A:** Ethidium bromide-stained gel of reaction products. The arrowhead points to the faint bFGF product. **B:** Southern hybridization of the gel shown in (A) with ^{32}P-labeled bFGF probe. **C:** Southern hybridization with the ^{32}P-labeled*gfg*-specific probe.

localization of sense and antisense bFGF transcripts in normal adult tissues and the predominance of bFGF sense transcripts in brain, in which bFGF is relatively abundant, suggest a functional relationship between the sense and antisense mRNAs. However, some fundamental questions about bFGF sense/antisense interactions clearly remain to be answered. Although duplex formation has been reported to target the bFGF mRNA for deamination and rapid degradation after germinal vesicle breakdown in amphibian oocytes (13), this is confounded by a recent report that oocyte cytoplasm contains a factor which protects double-stranded RNA from the action of dsRNA-deaminase (20). Duplex formation does not appear to prevent translation, as demonstrated by the presence of immunoreactive bFGF present in oocytes and stage 17 embryos (12) and by in vitro translation of bFGF from synthetic dsRNA hybrids (13). The role(s) of sense-antisense pairing may vary at different stages of oocyte development. Double-stranded RNAs are resistant to degradation by RNase A or T (21, 22). Since elements in the 3' untranslated region (UTR) of many mRNAs regulate transcript stability, sense-antisense overlap in this region may stabilize these transcripts by masking target sequences in the 3' UTR. However, the ultimate consequences of this interaction on bFGF mRNA stability and translation in vivo have yet to be determined. Interaction of bFGF sense and antisense transcripts may be critically important in determining the spatial and temporal pattern of bFGF activity during mesoderm induction as well as in differentiated tissues postnatally.

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REFERENCES

1. Gospodarowicz, D., Cheng, J., Lui, G.-M., Baird, A., Esch, F. and Bohlen, P. (1985) Endocrinology 117, 2383.
2. Neufeld, G., Ferrara, N., Schweigerer, L., Mitchell, R. and Gospodarowicz, D. (1987) Endocrinology 121, 597-603.
3. Koos, R. and Olson, C. (1989) Mol Endocrinol 3, 2041-2048.
4. Shimasaki, S., Emoto, N., Koba, A., Mercado, M., Shibata, F., Cooksey, K., Baird, A. and Ling, N. (1988) Biochem. Biophys. Res. Commun. 157, 256-263.
5. Baird, A. and Hsueh, A. J. W. (1986) Reg. Peptides 16, 243-250.
6. Adashi, E., Resnick, C., Croft, C., May, J. and Gospodarowicz, D. (1988) Mol. Cell. Endocrinol. 55, 7-14.
7. Lapolt, P. S., Yamoto, M., Veljkovic, M., Sincich, C., Ny, T., Tsafriri, A. and Hsueh, A. J. W. (1990) Endocrinology 127, 2357-2363.
8. Gospodarowicz, D., Plouet, J. and Fujii, D. K. (1989) Endocrinology 125, 1266-1276.
9. Gospodarowicz, D. and Ferrara, N. (1989) J Steroid Biochem 32, 183-191.
10. Watson, R., Anthony, F., Pickett, M., Lambden, P., Masson, G. and Thomas, E. (1992) Biochem. Biophys. Res. Commun. 187, 1227-1231.
11. Kimelman, D. and Kirschner, M. (1990) Devel. Biol. 125, 153-162.
12. Kimelman, D., Abraham, J., Haaparanta, T., Palisi, T. and Kirschner, M. (1988) Science 242, 1053-1056.
13. Kimelman, D. and Kirschner, M. W. (1989) Cell 59, 687-696.
14. Murphy, P.R. and Knee, R. (1994) Mol. Endocrinol. 8, 852-859.
15. Yan, Z., Hunter, V., Weed, J., Hutchinson, S., Lyles, R., and Terranova, P. (1993) Fertil. Steril. 59, 332-338.
16. Murphy, P.,R. Sato, Y. and Knee, R. (1992) Mol. Endocrinol. 6, 877-884.
17. Murphy, P. R., Guo, J. Z. and Friesen, H. G. (1990) Mol. Endocrinol. 4, 196-200.
18. Bost, L. M. and Hjelmeland, L. M. (1993) Growth Factors 9, 195-203.
19. Zuniga, A., Borja, M., Meijers, C. and Zeller, R. (1993) Devel. Biol. 157, 110-118.
20. Saccomanno, l. and Bass, B. L. (1994) Mol. Cell. Biol. 14, 5425-5432.
21. Bass, B. L. and Weintraub, H. (1987) Cell 48, 607-613.
22. Bass, B. and Weintraub, H. (1988) Cell 55, 1089-1098.