**Keratinocyte growth factor and its receptor in human ovaries from fetuses, girls and women**

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**ABSTRACT:** Keratinocyte growth factor (KGF) promotes growth of rat pre-antral follicles. There is limited information regarding its presence or that of its unique receptor (KGFR) in human ovaries, specifically in pre-antral follicles. The aim of the study was to investigate the expression of KGF and KGFR in ovarian samples from human fetuses and girls/women. The samples were prepared for immunohistochemical study of the KGF protein and for in situ hybridization to localize mRNA transcripts of KGFR. Total RNA was extracted from frozen ovarian samples, and the expression of KGF mRNA transcripts was investigated by reverse transcriptase polymerase chain reaction. In both fetuses and girls/women, the protein for KGF was detected from primordial stages in oocytes, granulosa cells (GCs) and stroma cells. Its mRNA transcripts were also detected in all extracts. The mRNA transcripts for KGFR were detected mainly in stroma cells in ovarian samples from both sources; in 10% of the samples, follicular staining was noted also in oocytes and GCs. Further studies adding KGF to the culture medium are needed to elucidate its putative role in human primordial follicle activation.

**Key words:** primordial follicles / KGF / immunohistochemistry / in situ hybridization / RT–PCR

**Introduction**

Most follicles in ovaries of human adults as well as fetuses are ‘primordial’, characterized by a single flat layer of ovarian granulosa cells (GCs) surrounding the oocyte (Gosden, 1995; Gougeon, 1996; Abir et al., 2006). Primordial follicles are activated when their GCs become cuboidal, after which they are termed ‘primary follicles’. The GCs continue to proliferate until multiple GC layers surround the oocyte, forming ‘secondary follicles’ with a theca cell layer. Complex interactions between GCs and theca cells are responsible for the synthesis of steroid hormones, such as 17ß estradiol (E₂). ‘Antral follicles’ containing a fluid-filled cavity represent the final developmental stage. Follicle-stimulating hormone (FSH) regulates folliculogenesis from the second stage onwards (Gougeon, 1996; Abir et al., 2006), but the signals that trigger the growth of primordial follicles are still unknown. In this paper, the follicles preceding the antral stage (primordial, primary and secondary) will be collectively termed ‘pre-antral’.

Major advances in anticancer treatment have resulted in a significant increase in survival of young female patients (Abir et al., 2006, 2008a).

One of the long-term side effects of the anticancer therapy is infertility due to follicular depletion. The options for fertility preservation in cancer patients are limited, consisting mainly of cryopreservation of ovarian tissue containing primordial follicles before initiation of therapy. Although reimplantation of frozen–thawed ovarian tissue of ovarian tissue containing primordial follicles before initiation of therapy. Although reimplantation of frozen–thawed ovarian tissue has so far led to nine pregnancies, including five live births (Demmeester et al., 2007; Abir et al., 2008a; Andersen et al., 2008), in some cancers, transplantation of ovarian tissue carries a risk of reseeding the malignancy (Abir et al., 2006). This danger could be eliminated by in vitro maturation of primordial follicles, followed by routine in vitro fertilization (Abir et al., 2006; Telfer et al., 2008). However, the development of this system is currently hindered by an uncertainty regarding factors that activate primordial follicular growth.

Keratinocyte growth factor (KGF) also known as fibroblast growth factor (FGF)-7 (Rubin et al., 1995) is a paracrine growth and differentiation factor that is known to promote epithelial cell proliferation, differentiation and migration as well as DNA synthesis (Rubin et al., 1995; De Giorgi et al., 2007). Evidence from rats suggests that KGF may play a role in promoting growth of primordial as well as secondary follicles (McGee et al., 1999; Kezele et al., 2005) and in decreasing...
apoptosis (McGee et al., 1999). KGF was also expressed in stroma cells surrounding primordial follicles (Kezele et al., 2005).

KGF has its own unique high affinity tyrosine kinase receptor, KGFR (FGFR2b and FGFR2-IIIb), a splicing variant of the same gene as that of the FGF receptor 2 (FGFR2, Bek) (Rubin et al., 1995; Finch and Rubin, 2004; De Giorgi et al., 2007). The extracellular domain of both receptors contains three immunoglobulin (Ig)-like loops, and their only sequence difference lies in the carboxy (C)-terminal of the Ig-like loop closest to the intramembranal part (receptor stem), where amino acid homology is only 47%. KGF also interacts with heparin and heparan sulfate proteoglycans at low affinity but high density, and heparan sulfate modulates interactions between KGF and its receptor by activating its optimal orientation (De Giorgi et al., 2007).

In a previous report from our laboratory, we identified the protein for FGFR2 in human oocytes of pre-antral follicles from fetuses and girls/women, as well as in GCs of girls/women (Ben-Haroush et al., 2005). These findings suggest the possible presence of the KGFR protein as well. However, no direct information on the expression of KGF and its receptor in human pre-antral follicles is currently available. The aim of the present study was to determine if the findings pointing to KGF as a potential activating agent of pre-antral follicular growth in rats (McGee et al., 1999; Kezele et al., 2005) could be extended to the human ovary. Specifically, we sought to localize the protein for KGF by immunohistochemistry (IMH), to detect its mRNA transcripts by reversed transcriptase polymerase chain reaction (RT–PCR) and to localize the mRNA transcripts for KGFR by in situ hybridization (ISH) in ovaries from fetuses and girls/women.

Materials and Methods

Human ovaries from fetuses, girls and women

Ovarian samples were obtained from 16 aborted human fetuses aged 21–35 gestational weeks (GWs): nine fetuses had anatomical abnormalities, five had chromosomal aberrations, one had a genetic disorder (achondroplasia) and one was normal. In addition, small ovarian biopsy samples were donated by 23 girls/women aged 5–39 years or their guardians. All had undergone gynecological laparoscopies. Seventeen had various forms of cancer, and their operation was performed for cryopreservation of ovarian tissue before commencement of chemotherapy (Abir et al., 2005, 2008b; Harel et al., 2006; Pinkas et al., 2008). Two sections per sample were utilized to identify the mRNA transcripts for KGFR. The antisense oligonucleotide probe was 30 bases in length and custom-designed by Biognostik (Biognostik, Gottingen, Germany). The antisense phosphodiester DNA oligonucleotide probes were provided by the company with specific TnSeq Custom Design Hybriprobe kit (1 pmol/l in deionized sterile water, Biognostik), and were labeled at the 5’ and 3’ ends with fluorescein-isothiocyanate. Specific antisense probes were designed for the human KGFR gene (www.ncbi.nlm.nih.gov/, Accession Number U11184, KGFR exon K, specific for non-soluble KGFR). Negative controls consisted of random sequences of oligonucleotides (control HybrProbes) of comparable length to the targeted gene. Three positive control probes (polydeoxymidine tissue control and two housekeeping genes: β-actin and α-tubulin) were also provided by the company and used in all experiments.

Cryopreservation of ovarian tissue

Tissue slices were placed in cryogenic vials (Nalge Nunc International, Roskilde, Denmark) filled with a solution of 1.5 M dimethylsulfoxide (Sigma, St Louis, MO, USA) (Abir et al., 2005; Harel et al., 2006; Pinkas et al., 2008). Prior to freezing, the samples were kept on ice for 30 min to achieve equilibrium. All samples were frozen in a programmable freezer (Kryo 360-1/7, Planer Biomed, Sunbury on Thames, UK) and immediately placed in liquid nitrogen. The slices were cryopreserved—stored for 3 months to 2 years until RNA extraction.

Histological preparation

Our histological preparation method has been described in detail elsewhere (Abir et al., 2005, 2008b; Harel et al., 2006; Pinkas et al., 2008). In addition to the human samples, a mouse uterus was prepared to serve as a positive control (see ‘IMH for KGF’ section). The fixed specimens were dehydrated in a graded series of ethanol followed by paraffin embedding and sectioning. Unstained sections were placed on OptiPlus positive-charged microscope slides (BioGenex Laboratories, San Ramon, CA, USA) for IMH and ISH.

IMH for KGF

Our IMH method has been employed in several earlier studies (Abir et al., 2005, 2008b; Harel et al., 2006; Pinkas et al., 2008). Two sections per sample were utilized to identify the protein for KGF. Sections from a mouse uterus were used as positive controls (according to instructions from Santa Cruz Biotechnology, Santa Cruz, CA, USA, which supplied the primary antibody). Unfortunately, there are no commercially available antibodies for the identification of KGFR. To enhance antigen retrieval, all the slides were microwaved with citrate buffer (CheMate buffer, DAKO-Cytomation, Glostrup, Denmark), and to block endogenous peroxidase activity, the slides were quenched in 3% hydrogen peroxide (H2O2, Vitanmed, Binyamina, Israel).

We used a goat polyclonal antibody as the primary antibody against KGF, which was reported to be suitable for IMH by the manufacturer (Santa Cruz Biotechnology, catalogue number: sc-27126). The samples were incubated with the primary antibody, diluted 1:30, 1:50. A negative control solution was prepared by the absorption of the primary antibody for KGF with its corresponding blocking peptide (Santa Cruz Biotechnology, Santa Cruz Biotechnology, catalogue number: sc-27126P). The negative control was placed on sections adjacent to those with the primary antibody.

Thereafter, the sections were incubated with secondary antibodies from an LSAB + System, horseradish-peroxidase (HRP) kit (DAKO-Cytomation) (Harel et al., 2006; Pinkas et al., 2008). Finally, the sections were exposed to 3-amino-9-ethylcarbazole (AEC) substrate-chromogen (DAKO-Cytomation) containing H2O2 (red-brown staining = antigen detection), and counterstained with Mayer’s hematoxylin (Pioneer Research Chemicals Ltd, Colchester Essex, UK) (blue staining).

Non-radioactive ISH for KGFR

Our ISH protocol has been described in detail elsewhere (Abir et al., 2008b; Pinkas et al., 2008). It was used in the present study to detect the mRNA transcripts for KGFR. The antisense oligonucleotide probe was 30 bases in length and custom-designed by Biognostik (Biognostik, Gottingen, Germany). The antisense phosphodiester DNA oligonucleotide probes were provided by the company with specific TnSeq Custom Design Hybriprobe kit (1 pmol/l in deionized sterile water, Biognostik), and were labeled at the 5’ and 3’ ends with fluorescein-isothiocyanate. Specific antisense probes were designed for the human KGFR gene (www.ncbi.nlm.nih.gov/, Accession Number U11184, KGFR exon K, specific for non-soluble KGFR). Negative controls consisted of random sequences of oligonucleotides (control HybrProbes) of comparable length to the targeted gene. Three positive control probes (polydeoxymidine tissue control and two housekeeping genes: β-actin and α-tubulin) were also provided by the company and used in all experiments.

Two sections per sample were utilized to identify the mRNA transcripts for KGFR. The probes were applied on the sections, with negative controls being placed on adjacent sections. In addition, 10 separate sections were utilized for each of the three positive controls. All the probes were diluted in Hybribuffer (40 U/ml = 120 µl/ml, Biognostik), and the slides were incubated overnight at 30°C. Hybridization was terminated.
by stringency rinses with saline sodium citrate (SSC) buffer (diluted in distilled water from a 20× SSC buffer solution; Sigma) at room temperature and at 39°C. Thereafter, the slides were incubated with Tris-blocking buffer (TNB, Perkin-Elmer, Boston, MA, USA), further incubated with an anti-fluorescein–HRP conjugate (Perkin-Elmer) (diluted 1:25 with TNB), and then exposed to tyramide signal amplification plus fluorescein (Perkin-Elmer). Finally, the sections were rinsed and incubated overnight with AEC (red-brown staining = presence of KGFR). The next day, the sections were counterstained with Mayer’s hematoxylin (Pioneer Research Chemicals Ltd) (blue staining).

RNA extraction

The frozen ovarian fragments were partially thawed at 37°C for removal from the freezing solution and placed in TRizol Reagent (Pioneer Research Chemicals) (Abir et al., 2005; Harel et al., 2006; Pinkas et al., 2008). Because the amount of ovarian tissue per individual fetus was occasionally very small, in some cases, samples from fetuses of the same gestational age were pooled. All samples were homogenized, chloroform (Biolab, Jerusalem, Israel) was then added and centrifugation at 4°C was performed, followed by phenol–chloroform–isoamyl alcohol (Sigma) addition to the supernatants, and further centrifugation at 4°C. The RNA fractions were mixed with isopropanol (Biolab) and kept overnight at −20°C, followed by centrifugation at 4°C to obtain pellets. Finally, ethanol (75%) was added for stabilization. Total RNA was later suspended in RNase-free diethylpyrocarbonate-treated water. The concentration of each sample was measured using a spectrophotometer (Cary UV 100; Varian, Mulgrave, Australia), and samples were stored at −80°C until RT–PCR was performed.

Reversed transcriptase polymerase chain reaction

Primers for the KGF (Accession Number NM_002009) transcripts were designed to span an intron so that the genomic DNA contamination could be detected. The forward primer (5′-CAGTGGCGAGTTGGAA TTGTG-3′) was designed in exon 3 at nucleotide 750–769 and the reverse primer (5′-CTGCTGAACTGTTCTTCTT-3′) was designed in exon 4 at the nucleotide 1046–1065. A single round PCR assay was carried out for 35 cycles at 57°C annealing temperature. β-Actin gene served as a positive control (274 bp) for the RT–PCR assay (Pinkas et al., 2008).

A total of 1 μg RNA for each sample was used for cDNA synthesis in the presence (RT+) or absence (RT−) of reverse transcriptase, as described previously (Abir et al., 2005). cDNA amplification was performed essentially as previously described (Harel et al., 2006). The final 40 μl PCR mixture for each sample contained 2 μl reverse-transcribed cDNA, 4 μl 10× PCR buffer, 100 mmol/l each dNTP, 2 U of Taq polymerase (Roach, GMP Grade, Germany) and 0.4 μmol/l of each primer. Ten microliters of amplified products with an expected DNA length of 316 bp were electrophoresed on 2% agarose gel along with a 100 bp DNA ladder (Fermentas, Ontario, Canada) as a fragment size reference and stained with ethidium bromide.

Four ovarian RNA samples from 7 human fetuses aged 22–35 GW and 10 ovarian RNA samples from women aged 21–38 years were tested for the presence of KGF transcripts by RT–PCR assay.

Results

Table I summarizes the IMH and ISH findings for the expression KGF and KGFR, respectively, in ovarian cells from fetuses and girls/women. A total of 2007 fetal follicles were stained for the IMH and ISH studies together, including 1880 (93.7%) primordial, 122 (6.1%) primary and 5 (0.2%) secondary, in addition to a total of 808 follicles from girls/women, including 646 (80%) primordial, 166 (20.5%) primary and 12 (1.5%) secondary. No antral follicles were identified in any of the samples.

### IMH detection of KGF

Figure 1A and B shows the characteristic cellular localization of KGF by IMH in human ovarian tissue from fetuses and girls/women. Positive staining was identified from primordial stages onwards. Oocytes from all samples stained positively: full cytoplasmatic staining in all the fetal samples and in 66% (30 samples) of the samples from girls/women with partial cytoplasmatic staining in the rest (34%, 16 samples). Positive nuclear oocyte staining was identified in a portion of the oocytes from both sources (fetuses and girls/women). Positive staining was identified in a portion of the GCs from all fetal samples and in 60% (28 samples) of the samples from girls/women; the remainder (40%, 18 samples) showed no GC staining. KGF expression was also detected in a portion of the stroma cells of all samples from both ovarian sources (fetuses and girls/women). There were no further associations of the results by ovarian source, fetal abnormality, age or follicular class. The IMH negative controls (Fig. 1C) did not stain positively (blue). The positive controls were strongly stained in the uterine endometrium and, to a lesser extent, in its myometrium (Fig. 1D).

### ISH detection of KGFR

Figure 2A and B shows the characteristic cellular localization of KGFR mRNA in human ovarian tissue from fetuses and girls/women.

Table I: Protein (IMH) expression of KGF and mRNA (ISH) expression of its receptor (KGFR) in ovaries from fetuses and girls/women

<table>
<thead>
<tr>
<th>Antigen Source</th>
<th>Protein IMH</th>
<th>mRNA ISH</th>
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<tbody>
<tr>
<td>Oocyte</td>
<td>++</td>
<td>±</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>+</td>
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</tr>
<tr>
<td>Nucleus</td>
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<td>±</td>
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<td>GCs</td>
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<tr>
<td>Stroma</td>
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<tr>
<td>Adult</td>
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<td>Oocyte</td>
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<td>Cytoplasm</td>
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<td>Stroma</td>
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GCs, granulosa cells; Adult, girls/women.

Staining intensities: − = no staining, + = positive staining (full or partial cytoplasmic staining—see Results section for clarifications). ± = positive staining in a portion of the cells.
Positive staining was most prominent in a portion of the stroma cells of all samples from both sources. In 10% of the samples (three from fetuses and five from women/girls), there was also follicular staining: in the cytoplasm (from both ovarian sources) and nuclei (only in fetal samples) of the oocytes and in a portion of the GCs, without any follicular staining in the majority of the samples (90%, 29 from fetuses and 41 from women/girls). There were no further associations of the results with ovarian source, fetal abnormality, age or follicular class. The ISH negative controls (Fig. 2C) did not stain positively (blue). The ISH positive controls stained in all the ovarian cells (Fig. 2D).

Detection of KGF transcripts by RT–PCR

Figure 3 shows a representative RT–PCR gel illustrating KGF gene expression in fetal and adult ovaries. The transcripts for KGF were present in all fetal and adult samples. The control β-actin gene was positive in all samples tested (Fig. 3). None of the negative controls processed without reverse transcriptase (RT-) yielded an amplification product.

Discussion

Although KGF has been detected in follicular fluid of human antral follicles (Osuga et al., 2001) and in ovarian surface epithelium and ovarian cancer specimens (Parrott et al., 2000), our study is the first to provide information regarding the presence of the KGF system in human pre-antral follicles from fetuses and girls/women. The protein for KGF was detected already from primordial stages in oocytes and GCs as well as in stroma cells of all samples, and its mRNA transcripts were present in the ovarian extracts. The mRNA transcripts for KGFR were detected mainly in stroma cells, with positive staining only in a minority of the oocytes and GCs from both ovarian sources.

In the rat ovary, the protein for KGF was found only in stroma cells surrounding primordial follicles (Kezele et al., 2005). The discrepancy in cellular localization of the protein between rats and humans can be attributed to differences between species. When rat primordial follicles were incubated with KGF, more than 65% developed to primary stages. KGF also induced the growth of rat secondary follicles, as indicated by an increase in their protein content and diameter concomitant with a ≤40% decrease in apoptosis (McGee et al., 1999). In bovine antral follicles, KGF mRNA was expressed in theca...
cells and KGFR mRNA in GCs, in direct correlation with follicular size (Parrott and Skinner, 1998).

Recently, the protein for FGFR2 was identified in oocytes of pre-antral follicles from human fetuses, girls/women and in GCs of girls/women (Ben-Haroush et al., 2005). In that study, the antibody used for IMH was produced against a peptide mapped within the C-terminal in the cytoplasmic domain, where the amino acid sequence of FGFR2 is identical to that of KGFR (Rubin et al., 1995; De Giorgi et al., 2007). Therefore, it is possible that the procedure may have also picked up the KGFR protein, although this remains unclear. Moreover, despite the mRNA expression of KGFR in follicles of a minority of the human ovarian samples in the present study, we cannot be certain that our previous detection of the FGFR2 protein in human GCs of primordial follicles (Ben-Haroush et al., 2005) also indicated the presence of the KGFR protein.

Interactions between several growth factors, rather than a single factor, are probably responsible for the development of pre-antral follicles, especially at the primordial stages (Abir et al., 2006). Others reported that the combination of stem cell factor (SCF), a known activator of primordial follicles in rats (Parrott and Skinner, 1999), with KGF did not result in higher growth levels than the addition of each factor alone (Kezele et al., 2005). Nevertheless, the addition of an anti-KGF antibody together with SCF inhibited SCF-induced follicular growth; KGF induced an increase in SCF mRNA expression; and SCF induced an increase in KGF mRNA expression. In cultured rat secondary follicles, KGF increased \( \alpha \)-inhibin protein expression (McGee et al., 1999). Moreover, there was more growth in the presence of the combination of KGF and FSH than KGF alone, as indicated by the increase in follicular protein, DNA content, diameter and \( \alpha \)-inhibin protein expression.

KGF is also a paracrine mediator for E2 activity in the female reproductive tract (Rubin et al., 1995). E2 as well as human chorionic gonadotrophin (hCG) were found to stimulate KGF gene expression in bovine antral theca cells, and KGF inhibited steroidogenesis and hCG-induced progesterone production in bovine and rat antral GCs (Parrott and Skinner, 1998). Further studies are required to determine if KGF plays similar roles in humans as in rats (Parrott and Skinner, 1998; McGee et al., 1999; Kezele et al., 2005) and cows (Parrott and Skinner, 1998).

In a study in cultured human follicles, we recently found that FGF-2, which is closely related to KGF, promoted E2 production but contributed marginally to primordial follicle activation (Garor et al., 2008). These

Figure 2 In situ hybridization (ISH) photographs of KGFR expression. (A) Section of human ovary from 33-GW-old fetus. Note the primordial follicles, with weak red-brown staining indicating KGFR mRNA expression in the oocytes (as full cytoplasmic staining with and without nuclear staining) and in a portion of the GCs and stroma cells. Original magnification × 400. (B) Section of human ovary from 22-year-old woman. Note the primordial follicle, with red-brown staining indicating KGFR mRNA expression in the oocyte (as partial cytoplasmic staining without nuclear staining), in the GCs and in a portion of the stroma cells. Original magnification × 400. (C) Negative control of the same ovary as in (B). Note the large primary follicle, the overall blue staining and the lack of red-brown staining. Original magnification × 400. (D) Positive control of human ovary from 6-year-old girl. Note the primordial follicles, with the red-brown staining indicating \( \beta \)-actin expression in the oocytes (partial cytoplasmic staining without nuclear staining), in the GCs and in a portion of the stroma cells. Original magnification × 400.
results disagreed with earlier studies in animal models wherein FGF-2 was found to stimulate significant growth of primordial follicles in the rat (Nilsson et al., 2001) and goat (Matos et al., 2007). Thus, definite conclusions regarding the possible role of KGF in human primordial follicles can be drawn only after KGF is included in the culture medium. Be that as it may, our detection of KGF both on the protein (in all ovarian cells) and mRNA levels suggests that KGF may have other, as yet unknown, functions in the human ovary, possibly related to steroidogenesis (Parrott and Skinner, 1998) or induction of ovarian surface epithelium cancer (Parrott et al., 2000). Additional studies in humans are needed to clarify the role of KGF, other growth factors and their synergistic interactions in the human ovary, particularly in pre-antral follicles.

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**References**


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**Figure 3** Representative RT-PCR gel illustrating KGF gene expression in fetal and adult ovaries. (A) KGF (316 bp). (B) β-Actin (274 bp). Lane 1: 100 bp DNA ladder; Lane 2: ovary from 28-year-old woman; Lane 3: ovary from 21-year-old woman; Lane 4: ovary from 37-year-old woman; Lane 5: ovary from 35-GW-old fetus; Lane 6: ovary from 25-GW-old fetus; Lane 7: pooled ovarian tissue from two 25-GW-old fetuses; Lane 8: pooled ovarian tissue from three 22–23-GW-old fetuses; Lane 9: (RT-) control.


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