

# Nerve Growth Factor-Dependent Activation of trkA Receptors in the Human Ovary Results in Synthesis of Follicle-Stimulating Hormone Receptors and Estrogen Secretion

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**Context:** Previous studies showed that nerve growth factor (NGF) induces the expression of functional FSH receptors (FSHR) in pre-antral follicles of the developing rat ovary.

**Objective:** The objective of this study was to determine whether NGF can affect granulosa cell (GC) function in human periovulatory follicles using intact human ovaries and isolated human GCs.

**Patients and Interventions:** Human GCs were obtained from *in vitro* fertilization patients and normal ovaries from women with elective pelvic surgery for nonovarian indications.

**Results:** In normal ovaries, NGF and trkA (NGF's high-affinity receptor) were detected by immunohistochemistry in GCs of preantral and antral follicles. NGF and trkA are also present in thecal cells of antral follicles. Both freshly collected and cultured GCs contained

immunoreactive NGF and trkA in addition to their respective mRNAs. Human GCs respond to NGF with increased estradiol (E<sub>2</sub>) secretion and a reduction in progesterone output. Exposure of human GCs to NGF increased FSHR mRNA content within 18 h of treatment, and this effect was blocked by the trk tyrosine kinase blocker K-252a. Also, cells preexposed to NGF released significantly more E<sub>2</sub> in response to hFSH than cells not pretreated with the neurotrophin, showing that the NGF-induced increase in FSHR gene expression results in the formation of functional FSHRs.

**Conclusions:** These results suggest that one of the functions of NGF in the preovulatory human ovary is to increase the secretion of E<sub>2</sub> while preventing early luteinization via an inhibitory effect on progesterone secretion. NGF stimulates E<sub>2</sub> secretion both directly and by increasing the formation of FSHRs.

IT IS NOW clear that the neurotrophin (NT) family of growth factors (1) is not only important for the differentiation and survival of neuronal cells (2, 3), but is also required for the differentiation and proliferative activity of nonneuronal cells (4), including the cardiovascular (5), immune (6), endocrine (7), and reproductive (8–10) systems.

All NTs and their respective receptors are expressed in the mammalian ovary (10). Studies in both rodents and humans have shown that this expression is initiated before the formation of the first primordial follicles (11–13), and that nerve growth factor (NGF), acting via trkA receptors, and brain-derived neurotrophic factor-NT4/5 signaling via trkB receptors are the NT systems most importantly involved in the control of early ovarian development in these species (14–16). In the peripubertal rat ovary, NGF is expressed in thecal

cells of antral follicles; at the time of the first preovulatory surge of gonadotropins, the abundance of both NGF and trkA mRNAs increases in these cells (17) as a consequence of an LH action mediated by IL-1 $\beta$  (17). It has been suggested that NGF signaling through its trkA receptor contributes to the rupture of the follicular wall at ovulation (17, 18).

Studies in cows and sheep (19, 20), two monoovulatory species, confirmed the presence of NGF and trkA in follicular cells. In the cow, trkA is expressed in both thecal and granulosa cells (GCs) of growing follicles (20), a distribution that contrasts with that in the rat, in which most trkA expression is seen in thecal-interstitial cells (17). Importantly, freshly isolated thecal cells from bovine antral follicles, but not cells maintained for several days in culture, responded to NGF with androgen and progesterone (P) release (20), suggesting that the steroidogenic effects of NGF in the ovary are only apparent before follicular cells become fully luteinized, either *in vitro* or after ovulation. The functional relationship between gonadotropins and NGF suggested by these observations was emphasized by studies of the sheep ovary showing that large follicles respond with increased NGF release to *in vitro* stimulation with a combination of LH and FSH (19).

These findings indicate that in both a polyovulatory spe-

Abbreviations: E<sub>2</sub>, Estradiol; FSHR, FSH receptor; GC, granulosa cell; h, human; hCG, human chorionic gonadotropin; IVF, *in vitro* fertilization; NGF, nerve growth factor; NGFAB, NGF antibody; NT, neurotrophin; nt, nucleotide; P, progesterone.

cies such as the rat and two monoovulatory species such as the cow and sheep, NGF is expressed in preovulatory follicles and contributes to events either leading to or associated with the ovulatory process. Whether NTs and, in particular, NGF affect human GC function is unknown. We used adult human ovaries and GCs isolated by aspiration of preovulatory follicles from women participating in an *in vitro* fertilization (IVF) program to address this issue. We performed experiments to determine whether NGF and its trkA receptor are expressed in the adult human ovary, to define their cellular sites of production, to examine the effect of NGF on estradiol ( $E_2$ ) and P secretion from GCs in culture, and to determine the effect of NGF on the formation of FSH receptors (FSHR).

## Subjects and Methods

### Source and collection of human GCs and ovaries

GCs were obtained from preovulatory follicles of 30 women participating in the IVF program of the Instituto de Investigaciones Materno Infantil, Facultad de Medicina, Universidad de Chile. The cells were collected at the time of oocyte collection via ultrasound-guided transvaginal aspiration. All procedures involving patients as well as written permission from the patients were approved by the ethics committee of the Instituto de Investigaciones Materno Infantil. The ovarian specimens used for immunohistochemical studies derived from 10 normal cycling women from 38–45 yr old, undergoing elective pelvic surgery for non-ovarian indications. These tissues were obtained as paraffin blocks from the Departamento de Patología, Facultad de Medicina, Hospital Clínico, Universidad de Chile. All procedures involving patients as well as written permission from the patients were approved by the ethics committee of the institution.

Synchronization of ovarian cycles was achieved by administering a GnRH agonist (Lupron, Abbott Laboratories, Chicago, IL; 14 IU, sc, for 7 d before menstruation, followed by additional 7 IU, sc, between d 10 and 12 of the menstrual cycle). Thereafter, ovulation was induced by the sequential administration of recombinant FSH (Puregon Pen, Organon Pharmaceuticals, West Orange, NJ; 200 IU, sc) daily for 3 d, followed by daily im administration of 3-hydroxy-3-methyl-glutaryl (Pergonal, Organon Pharmaceuticals; 150 IU). The progression of follicular development was followed by daily ultrasound evaluation, and the expected changes in plasma  $E_2$  levels were monitored in daily blood samples. When the development of at least three follicles 18 mm in diameter was achieved, a single injection of human chorionic gonadotropin (hCG; Pregnyl, Organon Pharmaceuticals; 10,000 IU, im) was administered, and GCs were aspirated 34 h later, at the time of oocyte collection.

To prepare GCs for culture, the follicular fluid was centrifuged for 10 min at  $1200 \times g$ . The pellets were resuspended in 3 ml Ham's F-12/DMEM (Sigma-Aldrich Corp., St. Louis, MO), and contaminating thecal/interstitial cells and erythrocytes were removed by layering the crude GC suspension over 3 ml of a 50% Percoll (Sigma-Aldrich Corp.) solution in saline, followed by centrifugation at  $1200 \times g$  for 45 min. The purified GCs were aspirated from the top of the Percoll solution and resuspended in Ham's F-12/DMEM containing 10% (vol/vol) fetal bovine serum (HyClone, Logan, UT). The cells were subsequently washed twice with the same culture medium. Their viability was determined by trypan blue. All subsequent studies to measure either steroid production or mRNA abundance were performed using pools of human GCs from all follicles obtained from each patient.

### RT-PCR

Total RNA from human GCs incubated with or without NGF (50 ng/ml) was extracted using TRIzol (Invitrogen Life Technologies, Inc., Carlsbad, CA). Five hundred nanograms of the RNA was then reversed transcribed in a 20- $\mu$ l volume using SuperScript II (Invitrogen Life Technologies, Inc.). The primers used to amplify segments of human NGF and trkA receptor mRNA were previously reported (12, 21). The forward primer for NGF (5'-TAA AAA GCG GCG ACT CCG TT-3')

corresponds to nucleotides (nt) 405–424 in human (h) NGF mRNA (accession no. BC032517); the reverse primer (5'-ATT CGC CCC TGT GGA AGA TG-3') is complementary to nt 552–571 in the mRNA. The forward trkA primer (5'-CCA TCG TGA AGA GTG GTC TC-3') corresponds to nt 289–308 in htrkA mRNA (accession no. BC062580); the reverse primer (5'-GGT GAC ATT GGC CAG GGT CA-3') is complementary to nt 745–764. These primers amplify an NGF cDNA of 167 bp and a trkA cDNA of 475 bp. The PCR consisted of 35 cycles (NGF) and 40 cycles (trkA) of denaturation at 94 C for 30 sec, annealing at 62 C for 1 min, and primer extension at 72 C for 1 min, followed by a final extension of 7 min at 72 C. The PCR products were size fractionated on a 1% agarose gel, and the signals were visualized using a UV transilluminator with Doc-it software Image Acquisition and 1 D Analysis (UVP, Inc., Laboratory Products, Upland, CA). In both cases, the PCR also contained primers to amplify a segment of the  $\beta$ -actin gene. Under the *in vitro* conditions employed,  $\beta$ -actin mRNA levels remained unchanged regardless of treatment (data not shown). The  $\beta$ -actin forward primer used (5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3') corresponds to nt 543–572 in human  $\beta$ -actin mRNA (accession no. BC013380); the antisense primer (5'-CTA GAA GCA TTG CGG TGG ACG ATG GAG GG-3') is complementary to nt 1174–1203 in the mRNA. The resulting PCR product is 661 bp in length.

### Immunohistochemistry

GC. Cells aspirated from 10 women were used in these studies. After isolation, they were seeded on silanized slides and allowed to adhere for 10 min. Thereafter, the cells were fixed in methanol for 15 min at 4 C, washed with 0.01 M PBS (pH 7.3), and incubated in a peroxidase-blocking reagent (DakoCytomation, Carpinteria, CA) for 10 min at room temperature to inhibit endogenous peroxidases. Next, they were incubated in 10% skim milk (10 min, room temperature) to block nonspecific binding. Incubation with NGF or trkA antibodies was carried out overnight at 4 C. The rabbit polyclonal antibodies to NGF (provided by Dr. H. F. Urbanski, Oregon Regional Primate Research Center) were used at a 1:100 dilution in PBS containing 1% BSA. The use of these antibodies for the immunohistochemical detection of NGF has been previously reported (17, 22, 23). The rabbit polyclonal antibodies to trkA (sc-14024; 1:100 dilution) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The next day, the slides were incubated with biotinylated goat anti-rabbit  $\gamma$ -globulin (Lab Vision Co., Fremont, CA) for 10 min at room temperature, followed by streptavidin-peroxidase (Lab Vision), then the immunoreaction was developed with 3,3'-diaminobenzidine. The slides were dehydrated in ethanol, cleared in xylene, coverslipped, and examined using a Zeiss Axioskop microscope (New York, NY) equipped with a digital camera (Coolpix 995, Nikon, Tokyo, Japan).

Ovaries. Formalin-fixed, paraffin-embedded ovaries from 10 women were sectioned at 4–6  $\mu$ m. Immunohistochemical identification of NGF- and TrkA-containing cells was carried out, in duplicate for each protein of interest, for each woman. The slides were washed twice in water before antigen retrieval [40 min at 90 C in 10 mM sodium citrate buffer (pH 6.0)]. Thereafter, the immunoreactions were performed as outlined above for GCs, with the NGF or trkA antibodies diluted 1:500. The sections were counterstained with 10% hematoxylin before dehydration and coverslipping. To confirm the specificity of the staining, selected slides containing cells or tissue sections were incubated in the absence of primary or secondary antibodies or with preadsorbed primary antibodies. The preadsorption procedure consisted of incubating the antisera with either NGF protein or trkA antigenic peptide (at 10  $\mu$ g/ml each), overnight at 4 C.

Cell culture. To determine the effect of NGF on steroid secretion, freshly isolated and purified human GCs were plated into 24-well culture plates (at  $1 \times 10^5$  cells/well) in Ham's F-12/DMEM supplemented with 10% fetal bovine serum (HyClone), 50 mg/liter penicillin/streptomycin, and 80 mg/liter gentamicin. When the cells reached approximately 80% confluence (usually in 48 h), the medium was replaced with serum-free Ham's F-12/DMEM in the presence of the same antibiotics, and the cells were exposed to different concentrations of NGF (10, 50, and 100 ng/ml; Sigma-Aldrich Corp.) for 18 h with testosterone ( $2.2 \times 10^{-8}$  M) as aromatase substrate. To neutralize the biological effects of NGF, other wells

were treated with NGF (10 or 100 ng/ml) and the polyclonal antibodies to NGF mentioned above (diluted 1:1000) for 18 h. The culture media were stored at  $-20^{\circ}\text{C}$  until assayed for  $E_2$  and P.

Because measurement of FSHR mRNA abundance showed that NGF treatment increased FSHR gene expression (see below), we performed experiments to determine whether this increase resulted in the formation of biologically competent FSHRs. Freshly isolated human GCs ( $1 \times 10^5$  cells/well) were plated into 24-well culture plates as described above, changed to serum-free medium after 48 h, and treated for 18 h in the presence or absence of NGF (50 ng/ml). After two washes, the culture medium was replaced with fresh medium without NGF, but containing  $2.2 \times 10^{-8}$  M testosterone and hFSH (10 mIU/ml; donated by the Human Reproduction Program of the World Health Organization). Twenty-four hours later, the media were collected and stored at  $-20^{\circ}\text{C}$  until assayed for  $E_2$ .

**Steroid assays.**  $E_2$  and P were measured using a luminescence immunoassay (Vitros ECI, Johnson & Johnson, Raritan, NJ). The sensitivity of the  $E_2$  assay was 10 pmol/liter, and the sensitivity of the P assay was 0.25 nmol/liter. The intra- and interassay coefficients of variation for the  $E_2$  assay were 5.6 and 6.7%, respectively. For the P assay, the intra- and interassay coefficients of variation were 6.8 and 8.5%, respectively.

### Measurement of FSHR mRNA

**Semiquantitative RT-PCR.** The primers used to amplify a segment of the hFSHR gene were those previously described by Sudo *et al.* (24). The forward primer (5'-TTT GTG GTC ATC TGT GGC TGC-3') corresponds to nt 1690–1710 in the hFSHR mRNA (accession no. M65085), and the reverse primer (5'-CAA AGG CAA GGA CTG AAT TAT CAT T-3') is complementary to nt 2185–2209. These primers amplify a 520-bp segment of the mRNA. The PCR consisted of 35 cycles of denaturation at  $94^{\circ}\text{C}$  for 30 sec, annealing at  $62^{\circ}\text{C}$  for 1 min, and primer extension at  $72^{\circ}\text{C}$  for 1 min, ending with an extension period of 7 min at  $72^{\circ}\text{C}$ . The PCR included primers to simultaneously amplify a segment of  $\beta$ -actin mRNA as an internal control. Because the size of the FSHR PCR product is too close to the size of the  $\beta$ -actin cDNA previously used in the PCR amplifications of NGF and *trkA*, we used a  $\beta$ -actin primer set that amplifies a 262-bp DNA segment instead of a 661-bp fragment. The forward primer (5'-CCC AGG CAC CAG GGC GTG AT-3') corresponds to nt 186–205 in human  $\beta$ -actin mRNA, and the antisense primer (5'-TCA AAC ATG ATC TGG GTC AT-3') is complementary to nt 429–448. The PCR products were separated on a 1% agarose gel and analyzed as outlined above, and their identities were verified by automatic sequencing using an Applied Biosystems DNA Sequencer model 3730 (Applied Biosystems, Foster City, CA).

**Real-time PCR.** Human GCs ( $5 \times 10^5$  cells/well) were cultured as described above and incubated with increasing concentrations of NGF (10, 50, and 100 ng/ml) for 18 h. To determine whether NGF affects the expression of FSHR by activating its high-affinity receptor *trkA*, an inhibitor of *trkA* receptor, K252a (25), was used at a 100-nM dose. Five hundred nanograms of total RNA was reversed transcribed as outlined above, and the resulting cDNAs were amplified by real-time PCR using a procedure we have previously described in detail (26). Each sample was run in triplicate. The FSHR primers used were a sense primer (5'-AAC ACC CAT CCA AGG AAT GG-3') corresponding to nt 2056–2075 in hFSHR mRNA, and an antisense primer (5'-GGGCTA AATGAC TTA GAG GGA CAA-3') complementary to nt 2123–2146. The internal fluorescence oligodeoxynucleotide probe (5'-TCT TCA GCT CCC AGA GTC ACC AGT GGT TC-3'; PerkinElmer Applied Biosystems, Foster City, CA) corresponds to nt 2083–2111 in hFSHR mRNA and was covalently linked to the fluorescent dye 6-carboxyfluorescein at the 5' end and the quencher dye tetramethylrhodamine at the 3' end. All primers were selected with the assistance of the program Primer Express (PerkinElmer Applied Biosystems). The 18S ribosomal RNA was used as a normalizing unit for each reaction, using a set of primers purchased as a kit (TaqMan Ribosomal RNA Control Reagents Kit, PerkinElmer Applied Biosystems). The real-time PCRs were carried out in a volume of 10  $\mu\text{l}$  containing 2  $\mu\text{l}$  diluted RT reaction, 5  $\mu\text{l}$  TaqMan Universal PCR Master Mix (PerkinElmer Applied Biosystems), 250 nM of each fluorescent FSHR and 18S probe, 300 nM of each FSHR primer, and 10 nM of each 18S ribosomal primer. The PCR consisted of an initial annealing

period of 2 min at  $50^{\circ}\text{C}$ , followed by 10-min denaturing at  $95^{\circ}\text{C}$ , 40 cycles of 15-sec denaturing at  $95^{\circ}\text{C}$ , and 1-min annealing/extension at  $60^{\circ}\text{C}$ . The identity of the FSHR product amplified by real-time was verified by sequencing.

### Statistical analysis

For statistical analysis, differences in  $E_2$  and P secretion among several groups and FSHR mRNA levels between groups were analyzed by ANOVA, followed by Tukey's test. To compare the effectiveness of the treatment with NGF antibody (NGFAB), the statistical difference between the group treated with NGF plus NGFAB (six patients) and the group treated with NGF alone from the same six patients was assessed with Kruskal-Wallis test.  $P < 0.05$  was considered significant. Results are expressed as the mean  $\pm$  SEM.

## Results

### Immunoreactive NGF and *trkA* receptors are present in follicles of normal human ovary and isolated human GCs

Immunohistochemical staining of adult human ovaries using polyclonal antibodies to either NGF or *trkA* demonstrated the presence of both proteins in GCs of preantral and antral follicles (Fig. 1, NGF in A and D, *trkA* in B and E). Adjacent sections incubated in the absence of first antibodies showed no specific staining (Fig. 1, NGF in C and *trkA* in F). Likewise, sections incubated with antibodies preadsorbed with the corresponding antigenic peptides had no staining (for NGF, see Fig. 1C; for *trkA*, see Fig. 1F). Although NGF immunostaining was present in some interstitial cells between preantral (Fig. 1A, arrows) and antral follicles (Fig. 1D, arrows), *trkA*-immunoreactive material was only seen in thecal cells of antral follicles (Fig. 1E, arrows, compare with preantral follicles in B). Freshly fixed human GCs also showed NGF and *trkA* immunostaining (Fig. 1, G and H), and the negative control is shown in Fig. 1. Immunonegative cells may represent granulosa lacking NGF or *trkA* expression, or contaminating cells (lymphocytes, erythrocytes, etc.).

RT-PCR of total RNA isolated from either freshly collected human GCs or human GCs cultured for 2–3 d revealed the presence of both NGF and *trkA* mRNAs in all samples (Fig. 2). As previously seen in bovine ovaries (20), *trkA* mRNA content decreased substantially in cultured cells, compared with freshly collected human GCs, in the absence of changes in NGF expression (Fig. 2).

### NGF induces $E_2$ secretion and inhibits P secretion in human GCs

The presence of NGF and its high-affinity *trkA* receptor in human GCs *in situ* and in GCs isolated from periovulatory follicles suggested that NGF might affect steroid secretion acting directly within this ovarian compartment. Isolated GCs cultured for 18 h in the presence of two different doses of NGF (10 and 100 ng/ml) responded to the neurotrophin with a significant ( $P < 0.05$ ) increase in  $E_2$  secretion (Fig. 3). The basal  $E_2$  level was  $753 \pm 141$  pmol/liter. A reduction ( $P < 0.05$ ) in P output was also observed (Fig. 3). The basal P level was  $1181 \pm 359$  nmol/liter. The change in  $E_2$  release was already apparent at the 10 ng/ml dose of NGF and was not further increased by the 100 ng/ml dose. Concomitant incubation with polyclonal antibodies to NGF to immunoneutralize NGF actions prevented both effects of NGF on

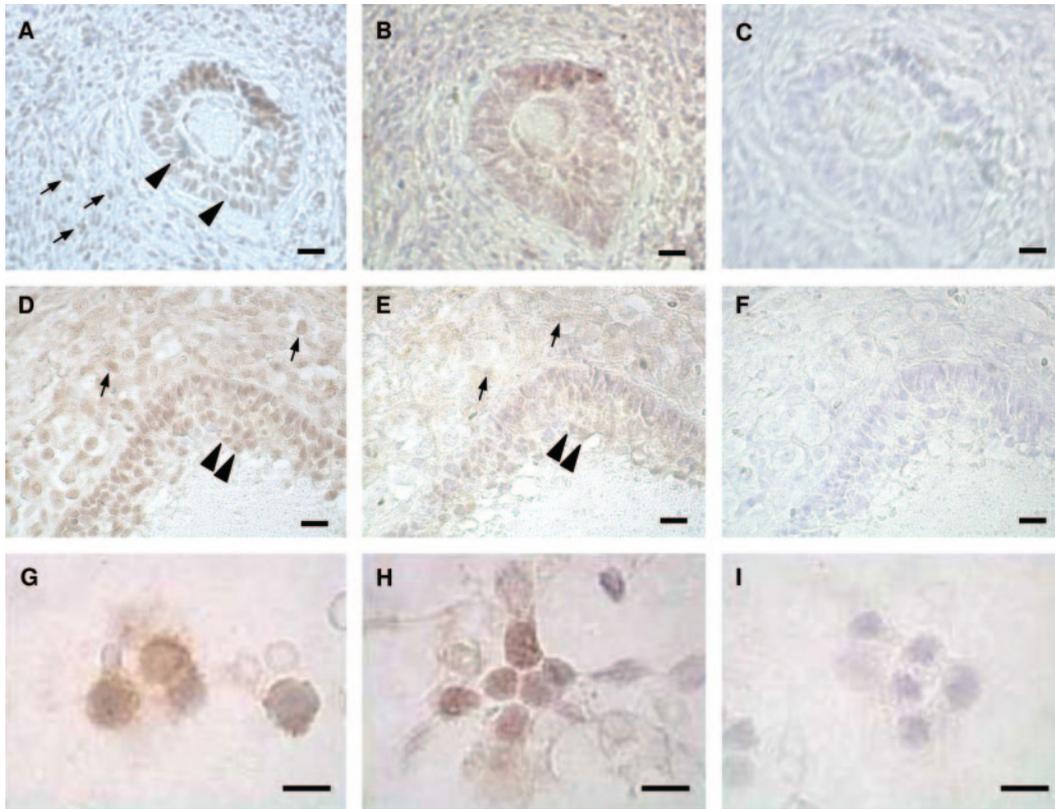


FIG. 1. Detection of NGF and trkA immunoreactivity in GCs of the intact adult human ovary and cultured human GCs derived from gonadotropin-induced preovulatory follicles. A, NGF immunoreactivity is abundant in GCs of preantral follicles (*arrowheads*), but can also be seen in surrounding stromal cells (*arrows*). B, GCs of preantral follicles contain lower, but detectable, levels of trkA protein. C, Lack of staining in sections incubated with preadsorbed antibodies to NGF. D, NGF immunoreactivity is present in both GCs (*arrowheads*) and thecal cells of antral follicles (*arrows*). E, TrkA-immunoreactive material is also seen in GCs of antral follicles (*arrowheads*) and scattered surrounding theca cells (*arrows*). F, Control section incubated with preadsorbed antibodies to trkA. G–I, Detection of NGF (G) and trkA immunoreactivity (H) in freshly collected hCGs, and lack of staining in control cells incubated in the absence of primary antibodies (I). The cells were aspirated at the time of oocyte collection for IVF, purified by centrifugation through a 50% Percoll solution, and seeded onto coverslips before fixation in methanol. NGF and trkA immunohistochemistry was carried out as described in *Subjects and Methods*. Bars: A–F, 20  $\mu\text{m}$ ; G–I, 10  $\mu\text{m}$ .

steroid secretion (Fig. 3). In additional experiments, we counted the number of cells per well after exposure to NGF and also measured protein content per well. In addition, we stained the cultured cells with antibodies to Ki67, a marker for proliferation. In no case were significant differences detected. It is, however, still possible that NGF may stimulate proliferation in less dense cultures and after an exposure longer than 18 h.

#### NGF increases FSHR expression in human GCs

A previous study demonstrated that NGF induces an increase in newly synthesized FSH receptors in 2-d-old rat ovaries (26). To determine whether NGF is also able to up-regulate FSHR expression in human GCs of periovulatory follicles, we treated purified human GCs for 18 h with different doses of NGF (10, 50, and 100 ng/ml). At the end of the incubation, total RNA was extracted, and FSHR mRNA was measured by semiquantitative PCR. The results showed that the 10 ng/ml dose was already effective, and that the 50 ng/ml dose produced a maximum increase in FSHR mRNA abundance (data not shown). The gel shown in Fig. 4A depicts the effect of the 50 ng/ml dose on FSHR mRNA content. To more precisely quantify this result, we measured FSHR

mRNA abundance by real-time PCR in RNA extracted from human GCs derived from several patients. As shown in Fig. 4B, NGF induced a significant ( $P < 0.01$ ) increase in FSHR mRNA levels, and this increase was obliterated by concomitant treatment of the cells with the trk tyrosine kinase inhibitor K252a. Thus, NGF induces FSHR gene expression in human GCs via trkA receptor signaling.

#### NGF induces the formation of biologically active FSHRs in human GCs *in vitro*

To determine whether the increase in FSHR gene expression induced by NGF is accompanied by formation of biologically active FSHRs, we treated GCs with NGF (50 ng/ml) for 18 h. After the culture medium was changed, the cells were exposed to FSH for an additional 24-h period. Under these conditions, only the cells primed with NGF responded to FSH with a significant increase in  $E_2$  release (Fig. 5). This increase was greater ( $P < 0.05$ ) than the response of cells treated with NGF only. The increased  $E_2$  levels detected in cells treated with only NGF may be due to a residual effect of NGF remaining in the culture wells after the culture medium was changed or may be a direct effect of NGF on the

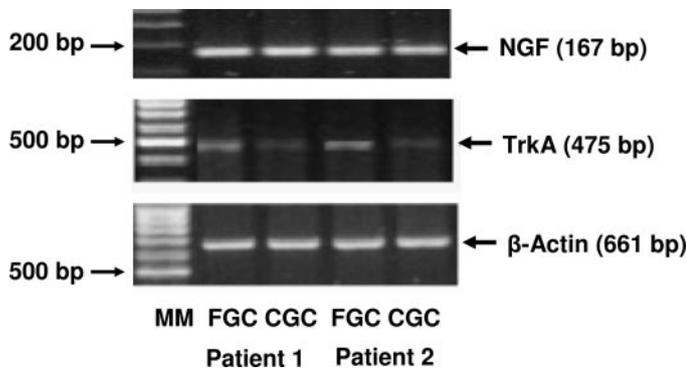


FIG. 2. Human GCs express the mRNAs encoding both NGF and its high-affinity receptor *trkA*, as determined by RT-PCR. The ethidium bromide-stained gels show the presence of PCR products amplified from reverse transcribed total RNA extracted from human GCs and amplified with primers recognizing specific regions of NGF (*upper panel*) and *trkA* (*middle panel*) mRNAs. Notice that although NGF mRNA content was similar in freshly collected cells (FGC) and cultured cells (CGC; 48 h in Ham's F-12/DMEM supplemented with 10% fetal bovine serum, followed by 18 h in serum-free medium), *trkA* mRNA levels declined markedly in cultured cells. Amplification of a segment from  $\beta$ -actin mRNA (*lower panel*) demonstrated that these differences were not due to procedural variability among the different samples. Each lane shows the PCR products amplified from total RNA derived from freshly collected and cultured GCs from one of two patients, representative of a total of 10 patients.

aromatase enzyme. Cells not primed with NGF failed to respond to FSH with a significant increase in  $E_2$  output (Fig. 5).

### Discussion

In this study we describe the presence of NGF and its high-affinity *trkA* receptor in GCs of the human ovary both *in situ* and after isolation from gonadotropin-induced preovulatory follicles. We also show that NGF acts on isolated human GCs to diminish the biochemical differentiation of the cells into luteal cells (by decreasing P secretion) while enhancing the steroidogenic activity characteristic of preovulatory follicles (by promoting the production of  $E_2$ ). The ability of NGF to modify human GC steroidogenic function means that NGF appears to act at two different, but complementary, levels to stimulate  $E_2$  release, directly and indirectly by inducing up-regulation of FSHRs. Our results show that this latter effect requires the activation of *trkA*, the high-affinity tyrosine kinase NGF receptor.

In a previous report we demonstrated the ability of NGF to induce FSHR expression in preantral follicles of the rat ovary (26) and concluded that NT was important for the acquisition of these receptors in early development, before the ovary becomes subjected to gonadotropin control. The present results indicate that NGF exerts a similar effect on preovulatory human GCs and suggest that this function has been conserved through evolution. They also suggest that NGF is important for maintaining GCs engaged in estrogen production and away from premature luteinization. We do not know the signaling mechanisms underlying these effects of NGF, but it would not be unreasonable to expect an involvement of the ERK1/ERK2 signaling pathway, not only because stimulation of *trkA* receptors results in activation of this pathway (27, 28), but also because ERK1/ERK2 are in-

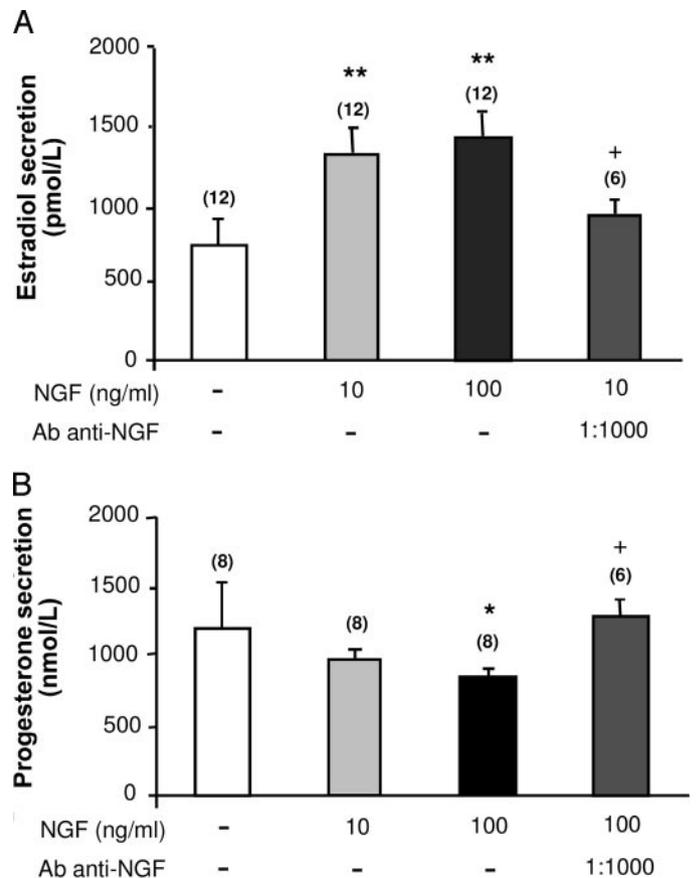


FIG. 3. NGF stimulates  $E_2$  secretion, but inhibits P output, from human GCs in culture. A, Stimulatory effect of NGF on  $E_2$  secretion and immunoneutralization of this effect by antibodies to NGF. B, Inhibitory effect of NGF on P secretion and blockade of this effect by immunoneutralization of NGF actions. GCs aspirated from preovulatory follicles at the time of oocyte collection for IVF were plated in 24-well plates at  $1 \times 10^5$  cells/well and cultured as described in *Subjects and Methods*. After reaching approximately 80% confluence, they were cultured for an additional 18 h in serum-free medium in the presence of NGF (10 or 100 ng/ml) and  $2.2 \times 10^{-8}$  M testosterone. At the end of this period, the medium was collected for  $E_2$  and P assays. Other cultures were treated with both NGF and neutralizing polyclonal antibodies against NGF (diluted 1:1000). The concentration of NGF used was changed according to maximal effect in the steroid release (10 ng/ml NGF in the case of  $E_2$  and 100 ng/ml NGF for P). Results are expressed as picomoles per liter for  $E_2$  and nanomoles per liter for P. Bars are the means, and vertical lines are the SEM. The cells used in these experiments were derived from 12 patients. Each treatment was applied in duplicate to cells from each patient. To compare the effectiveness of the treatment with NGF plus NGFAb, we analyzed the statistical difference between the cells treated with NGF plus NGFAb from six patients and cells from the same six patients, incubated with NGF alone. The cells of this last group are included in a bigger group of 12 patients for  $E_2$  release and eight patients for P release. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  (vs. untreated control); +,  $P < 0.01$  (vs. the six patients of the NGF-treated group).

involved in the suppression of luteinization caused by another growth factor, bone morphogenetic protein-7, in human GCs in culture (29).

An aspect of the study that remains unresolved is the relationship between these effects of NGF on steroid secretion and the preovulatory surge of gonadotropins. The surge

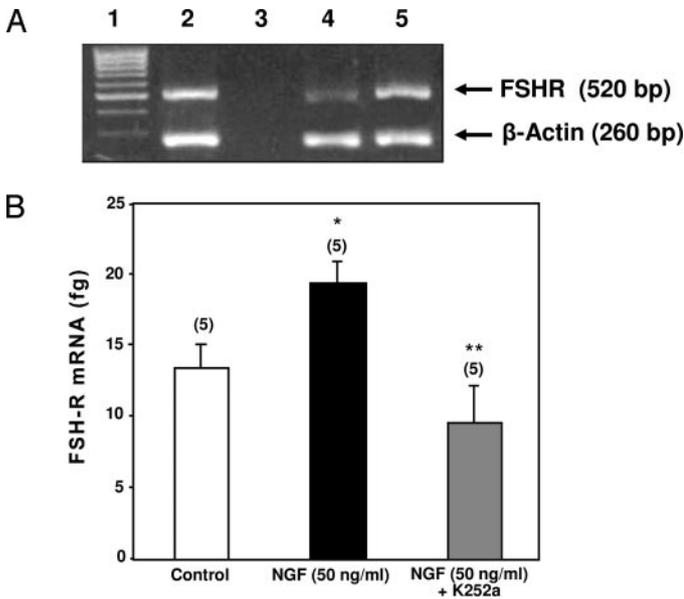


FIG. 4. NGF increases FSHR mRNA abundance in cultured human GCs via *trk* receptor activation. A, Representative gel demonstrating the ability of NGF to increase FSHR mRNA levels in cultured human GCs. The cells were treated with NGF (50 ng/ml) for 18 h in serum-free medium. Lane 1, 100-bp DNA molecular weight marker; lane 2, positive control (human pheochromocytoma); lane 3, no reverse transcriptase; lane 4, FSHR mRNA levels in untreated cells; lane 5, FSHR mRNA content in cells treated with NGF (50 ng/ml). B, The increase in FSHR mRNA content induced by NGF requires activation of *trk* receptors. The cells were treated with NGF at 50 ng/ml and with K252a, a blocker of *trk* tyrosine kinase activity, at 100 nM for 18 h. The resulting changes in FSHR mRNA steady-state levels were quantified by real-time PCR. Numbers on top of bars are the number of independent observations per group. Bars represent the mean of five independent observations (*i.e.* pools of human GCs from five different patients). AU, Arbitrary units. \*,  $P < 0.05$  vs. untreated control group; \*\*,  $P < 0.01$  vs. NGF-treated group.

normally decreases estrogen production and increases P output, but paradoxically it also increases NGF and *trkA* expression (17). One potential explanation is that as the LH surge progresses, it reduces the NGF effect by setting in motion parallel desensitizing/modulatory intracellular pathways. The identity of such modulatory pathways remains unknown. The divergent actions of NGF on  $E_2$  and P secretion that we observed using GCs in culture might be diminished or terminated after the gonadotropin surge *in vivo*, but our results indicate that if one exposes luteinizing GCs *in vitro* to NGF, the neurotrophin is able to partially reverse the luteinizing effect of hCG and recreate a preovulatory/presurge condition.

An intriguing difference in the cellular localization of NGF and *trkA* exists between rats, a polyovulatory species, and cows and humans, which are monoovulatory. In the rat, both NGF and *trkA* mRNA as well as their respective protein products are almost exclusively present in thecal-interstitial cells (17). In contrast, *trkA* mRNA is present in both cell compartments of the cow ovary, with a greater abundance in GCs (20); the NGF gene, in contrast, has a distribution similar to that of the rat, because it is exclusively expressed in thecal-interstitial cells. In all follicles of the bovine ovary, GCs

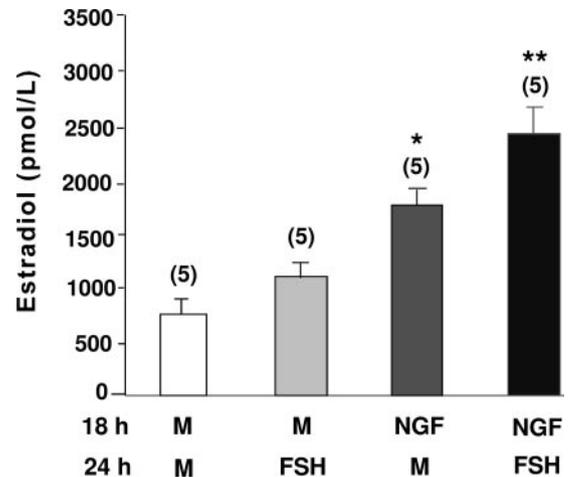


FIG. 5. Exposure of human GCs to NGF results in the formation of biologically active FSHRs. After purification, the cells were seeded in serum-containing medium. Forty-eight hours later, they were switched to serum-free medium containing NGF (50 ng/ml). After 18 h, the medium was replaced with medium with or without hFSH (10 IU/liter) in the presence of testosterone ( $2.2 \times 10^{-8}$  M) as a substrate for  $E_2$  synthesis, and the incubation was continued for an additional 24 h. The results are expressed as the mean  $\pm$  SEM. Numbers on top of bars are the number of independent observations per group. The cells used in this experiment were derived from five patients. \*,  $P < 0.01$  vs. groups without NGF treatment; \*\*,  $P < 0.001$  vs. groups without NGF treatment). The NGF plus FSH group has a  $P < 0.05$  vs. group primed with NGF, but not treated with FSH. M, Culture medium alone.

contain an immunoreactive NGF-like material that appears to be a cross-reacting protein species because the cells do not express NGF mRNA, assessed by either *in situ* hybridization or ribonuclease protection assay (20). The present results show that human GCs not only contain NGF-immunoreactive material, but also express NGF mRNA. Adding complexity to these species-related differences in NGF and *trkA* cellular localization are the findings of *trkA* immunoreactivity and mRNA in GCs of preantral follicles of the mouse ovary (16) and an apparent lack of *trkA* mRNA in GCs from rhesus monkeys (our unpublished observations). Although the study of mouse ovaries (16) did not examine the presence of NGF and its *trkA* receptor in periovulatory follicles, it appears that, with the exception of the rat ovary, most other species examined to date, including humans, express *trkA* receptors in GCs. The functional significance of these differences is unclear, because one of the important biological actions of NGF, namely, the induction of FSHR, is maintained in rats (26) and humans (this study). Moreover, the ovaries from NGF knockout mice show a profound reduction in FSHR mRNA content (16), highlighting the importance of NGF in maintaining GC responsiveness to FSH.

It is likely that NGF and its *trkA* receptor contribute to the regulation of other functions of ovarian follicles via paracrine and autocrine interactions that can take place separately within the GC and thecal-interstitial cell compartments or involve communication between these two cell compartments of the developing follicle. Examples of such interactions can be found in the ability of NGF to stimulate androgen and prostaglandin  $E_2$  production in thecal cells from bovine antral follicles (20), to inhibit gap junctional commu-

nication between thecal cells of rat ovaries (18), and to induce the formation of functional FSHR in GCs of rat ovaries (26).

The present study shows that, as previously seen in cows, *trkA* receptors are present in both preantral and antral follicles of the human ovary. It is, therefore, plausible that upon NGF binding, *trkA* receptors initiate a signaling cascade that promotes  $E_2$  production and facilitates the acquisition of FSHR to gradually enhance the response of the growing follicle to gonadotropins. A potential involvement of *trkA* receptors in ovulation was initially suggested by experiments in rats, which demonstrated that *trkA* gene expression in the thecal-interstitial compartment of periovulatory follicles increases more than 100-fold during the hours preceding the first ovulation (17). The ability of hCG to rapidly increase *trkA* receptor expression in ovarian cell dispersates indicated that a significant fraction of the preovulatory increase in *trkA* mRNA abundance is an LH-dependent phenomenon. It is, therefore, likely that the elevated *trkA* mRNA levels detected in freshly isolated human GCs were caused by the gonadotropin treatment given to the patients to generate ovulatory follicles. The decline in *trkA* mRNA levels observed when human GCs were placed in culture supports this concept. A similar decline was observed in cultured thecal cells isolated from bovine ovaries (20).

A major concept in mammalian ovarian physiology is that GCs differentiate into luteal cells after ovulation, producing high levels of P (30). This process is accelerated when GCs from periovulatory follicles are placed in culture, so that within a few days, the cells differentiate into luteal cells (31). Our results show that exposing human GCs to NGF causes the cells to produce more  $E_2$  and less P, suggesting that a function of NGF in the GC compartment of preovulatory follicles is to maintain a follicular phenotype and prevent the untimely differentiation of GCs into their luteal counterparts. Also supporting this concept is the observation that exposure of human GCs to NGF results in the formation of biologically active FSHRs, capable of stimulating  $E_2$  production. Previous studies of small preantral follicles of the rat ovary showed that NGF induces FSHR through a signaling pathway independent of cAMP (26). Should a similar mechanism operate in human periovulatory follicles, it would indicate that the gonadotropin-dependent activation of a cAMP-dependent pathway that leads to ovulation (32) also sets in motion an NGF-dependent, cAMP-independent pathway.

Together, our findings indicate that NGF is an intragonadal molecule involved in promoting follicular steroidogenesis and GC responsiveness to gonadotropins in the human ovary and identify human GCs of the preovulatory ovary as one of the nonneuronal, endocrine targets of NGF actions.

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### References

1. Thoenen H 1991 The changing scene of neurotrophic factors. *Trends Neurosci* 14:165–170
2. Davies AM 2000 Neurotrophins: neurotrophic modulation of neurite growth. *Curr Biol* 10:R198–R200
3. Levi-Montalcini R 1987 The nerve growth factor 35 years later. *Science* 237:1154–1162
4. Tessarollo L 1998 Pleiotrophic functions of neurotrophins in development. *Cytokine Growth Factor Rev* 9:125–137
5. Donovan MJ, Hahn R, Tessarollo L, Hempstead BL 1996 Identification of an essential nonneuronal function of neurotrophin 3 in mammalian cardiac development. *Nat Genet* 14:210–213
6. García-Suárez O, Germanà A, Hannestad J, Ciriaco E, Laurá R, Naves J, Esteban I, Silos-Santiago I, Vega JA 2000 *TrkA* is necessary for the normal development of the murine thymus. *J Neuroimmunol* 108:11–21
7. Pierucci D, Cicconi S, Bonini P, Ferrelli F, Pastore D, Matteucci C, Marselli L, Marchetti P, Ris F, Halban P, Oberholzer J, Federici M, Cozzolino F, Lauro R, Borboni P, Marlier LN 2001 NGF-withdrawal induces apoptosis in pancreatic  $\beta$  cells in vitro. *Diabetologia* 44:1281
8. Kawamura K, Kawamura N, Mulders SM, Sollewijn Gelpke MD, Hsueh AJ 2005 Ovarian brain-derived neurotrophic factor (BDNF) promotes the development of oocytes into preimplantation embryos. *Proc Natl Acad Sci USA* 102:9206–9211
9. Cupp AS, Tessarollo L, Skinner MK 2005 Testis developmental phenotypes in neurotrophin receptor *trkA* and *trkC* null mutations: role in formation of seminiferous cords and germ cell survival. *Biol Reprod* 66:1838–1845
10. Dissen GA, Paredes A, Romero C, Dees WL, Ojeda SR 2004 Neural and neurotrophic control of ovarian development. In: Leung P, Adashi E, eds. *The ovary*, 2nd Ed. San Diego: Academic Press; 3–23
11. Dissen GA, Newman Hirshfield A, Malamed S, Ojeda SR 1995 Expression of neurotrophins and their receptors in the mammalian ovary is developmentally regulated: changes at the time of folliculogenesis. *Endocrinology* 136:4681–4692
12. Anderson RA, Robson LLL, Brooks J, Spears N 2002 Neurotrophins and their receptors are expressed in the human fetal ovary. *J Clin Endocrinol Metab* 87:890–897
13. Abir R, Fisch B, Barnnet M, Jin G, Ben-Haroush A, Felz C, Kessler-Ickson G, Feldberg D, Nitke S, Ao A 2005 Presence of NGF and its receptors in ovaries from human fetuses and adults. *Mol Hum Reprod* 11:229–236
14. Spears N, Molinek MD, Robinson LL, Fulton N, Cameron H, Shimoda K, Telfer EE, Anderson RA, Price DJ 2003 The role of neurotrophin receptors in female germ-cell survival in mouse and human. *Development* 130:5481–5491
15. Paredes A, Romero C, Dissen GA, DeChiara TM, Reichardt L, Cornea A, Ojeda SR, Xu B 2004 *TrkB* receptors are required for follicular growth and oocyte survival in the mammalian ovary. *Dev Biol* 267:430–449
16. Dissen GA, Romero C, Newman Hirshfield A, Ojeda SR 2001 Nerve growth factor is required for early follicular development in the mammalian ovary. *Endocrinology* 142:2078–2086
17. Dissen GA, Hill DF, Costa ME, Dees WL, Lara HE, Ojeda SR 1996 A role for *trkA* nerve growth factor receptors in mammalian ovulation. *Endocrinology* 137:198–209
18. Mayerhofer A, Dissen GA, Parrott JA, Hill DF, Mayerhofer D, Garfield RE, Costa ME, Skinner MK, Ojeda SR 1996 Involvement of nerve growth factor in the ovulatory cascade: *TrkA* receptor activation inhibits gap-junctional communication between theca cells. *Endocrinology* 137:5662–5670
19. Mattioli M, Barboni B, Gioia L, Lucidi P 1999 Nerve growth factor production in sheep antral follicles. *Dom Anim Endocrinol* 17:361–371
20. Dissen GA, Parrott JA, Skinner MK, Hill DF, Costa ME, Ojeda SR 2000 Direct effects of nerve growth factor on theca cells from antral ovarian follicles. *Endocrinology* 141:4736–4750
21. Davidson B, Lazarovici P, Ezersky A, Nesland JM, Berner A, Risberg B, Tropé CG, Kristensen GB, Goscinski M, Van de Putte G, Reich R 2001 Expression levels of the nerve growth factor receptors *TrkA* and *p75* in effusions and solid tumors of serous ovarian carcinoma patients. *Clin Cancer Res* 7:3457–3464
22. Lara HE, McDonald JK, Ojeda SR 1990 Involvement of nerve growth factor in female sexual development. *Endocrinology* 126:364–375

23. **Lara HE, Dissen GA, Leyton V, Paredes A, Fuenzalida H, Fiedler L, Ojeda SR** 2000 An increased intraovarian synthesis of nerve growth factor and its low affinity receptor is a principal component of steroid-induced polycystic ovary in the rat. *Endocrinology* 141:1059–1072
24. **Sudo S, Kudo M, Wada S, Sato O, Hsueh AJ, Fujimoto S** 2002 Genetic and functional analyses of polymorphisms in the human FSH receptor gene. *Mol Hum Reprod* 8:893–899
25. **Nye SH, Squinto SP, Glass DJ, Stitt TN, Hantzopoulos P, Macchi MJ, Lindsay NS, Ip NY, Yancopoulos GD** 1992 K-252a and staurosporine selectively block autophosphorylation of neurotrophin receptors and neurotrophin-mediated responses. *Mol Biol Cell* 3:677–686
26. **Romero C, Paredes A, Dissen GA, Ojeda SR** 2002 Nerve growth factor induces the expression of functional FSH receptors in newly formed follicles of the rat ovary. *Endocrinology* 143:1485–1494
27. **Jiang H, Movsesyan V, Fink Jr DW, Fasler M, Whalin M, Katagiri Y, Monshipouri M, Dickens G, Lelkes PI, Guroff G, Lazarovici P** 1997 Expression of human p140trk receptors in p140trk-deficient, PC12/endothelial cells results in nerve growth factor-induced signal transduction and DNA synthesis. *J Cell Biochem* 66:229–244
28. **Patapoutain A, Reichardt LF** 2001 Trk receptors: mediators of neurotrophin action. *Curr Opin Neurobiol* 11:272–280
29. **Moore RK, Otsuka F, Shimasaki S** 2001 Role of ERK1/2 in the differential synthesis of progesterone and estradiol by granulosa cells. *Biochem Biophys Res Commun* 289:796–800
30. **Espey LL, Lipner H** 1994 Ovulation. In: Knobil E, Neill JD, eds. *Physiology of reproduction*, 2nd Ed. New York: Raven Press; 725–780
31. **Hsueh AJW, Adashi EY, Jones PBC, Welsh THJ** 1984 Hormonal regulation of the differentiation of cultured ovarian granulosa cells. *Endocr Rev* 5:76–127
32. **Richards JS, Russell DL, Robker RL, Dajee M, Alliston TN** 1998 Molecular mechanisms of ovulation and luteinization. *Mol Cell Endocrinol* 145:47–54