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ORIGINAL ARTICLE *Reproductive endocrinology*

Gonadotrophin-induced paracrine regulation of human oocyte maturation by BDNF and GDNF secreted by granulosa cells

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BACKGROUND: In mammalian ovaries, diverse paracrine factors have been identified to mediate or modulate LH-induced changes during ovulation. Due to the difficulty in obtaining non-stimulated granulosa cells during IVF, little is known about the LH-induced paracrine factors in the human ovary. Based on earlier studies using murine ovarian cells showing the paracrine roles of brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) in promoting oocyte maturation, we investigated the expression of these ligands in human granulosa cells and their regulation of human oocyte development.

METHODS: Non-stimulated granulosa cells were obtained from non-stimulated IVM (*in vitro* maturation) patients after oocyte retrieval. Women undergoing non-stimulated IVM treatment at a mean age of 30.8 ± 1.3 ($n = 10$) were recruited for this study. Immature oocytes and granulosa cells were collected from IVF patients undergoing gonadotrophin stimulation and ICSI. Immunocytochemical analyses of granulosa cells were carried out to investigate expression profiles of BDNF and GDNF, together with real-time RT–PCR to analyze the gonadotrophin regulation of BDNF and GDNF transcript levels. In addition, immature oocytes were cultured to analyze the regulation of oocyte maturation by BDNF and GDNF.

RESULTS: BDNF and GDNF were found to be expressed in non-stimulated granulosa cells. After gonadotrophin (FSH and/or hCG) treatment, transcripts levels for BDNF and GDNF were significantly increased ($P < 0.05$). In cultured immature oocytes, treatment with BDNF or GDNF promoted total yields of metaphase II oocytes.

CONCLUSIONS: These findings demonstrate that FSH and hCG treatments augment the expression of BDNF and GDNF by granulosa cells and that these granulosa-cell-derived factors are candidate paracrine factors capable of promoting oocyte maturation.

Key words: *in vitro* maturation / human oocytes / granulosa cells / BDNF / GDNF

Introduction

Ovarian follicular development is a complex process mainly regulated by gonadotrophins. During ovulation, multiple intraovarian factors secreted by different ovarian cell compartments are essential in mediating or modulating gonadotrophin actions (Richards, 1994; Christenson and Stouffer, 1997). These factors play paracrine or autocrine roles in modulating different ovarian functions, including oocyte maturation, granulosa and theca cell luteinization, as well as follicle rupture. Because negligible LH receptors (LHRs) are present in oocytes, pituitary-secreted LH acts on granulosa cells and theca cells to trigger the final maturation of oocytes (Hsueh *et al.*, 1984). After resuming meiosis from the late prophase, oocytes undergo germinal vesicle breakdown (GVBD), followed by the extrusion of the first polar body in preparation for fertilization and early embryonic development (Tsafriri *et al.*, 1996). Recent studies based on animal

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models have demonstrated that several paracrine factors are important for oocyte maturation. Treatment of pre-ovulatory follicles with LH/hCG induced brain-derived neurotrophic factor (BDNF) and glial-cell-line-derived neurotrophic factor (GDNF) expression by granulosa cells, and these paracrine factors act on oocytes to promote oocyte maturation (Seifer *et al*., 2002; Kawamura *et al*., 2005). BDNF (Hyman *et al*., 1991) and GDNF (Lin *et al*., 1993) were previously known as neurotrophins and play important roles for neuronal survival. BDNF activates two receptors, including the high-affinity TrkB receptor and the pan-neurotrophin low-affinity receptor p75 (Klein *et al*., 1991). In the murine ovary, BDNF was found to be essential for the development of early follicles (Ojeda *et al*., 2000). In the pre-ovulatory period, BDNF is rapidly induced after the LH surge and is capable of promoting first polar body extrusion and cytoplasmic maturation of the oocytes for optimal development into blastocysts (Kawamura *et al*., 2005). In addition, recent studies indicate that treatment with GDNF also regulates oocyte maturation. GDNF acts through a two-component receptor system consisting of the ligand-specific binding subunit, GDNF family receptor-alpha1 (GFRA1) and the common signal transduction subunit ret proto-oncogene (Ret) (Jing *et al*., 1996).

In conventional IVF treatment, patients undergo controlled ovarian hyperstimulation (COH) to induce follicular growth. Following FSH and hCG administration, granulosa cells obtained after oocyte retrieval are all luteinized and not suitable to study gonadotrophin actions. In recent years, *in vitro* maturation (IVM) of oocytes has been developed as a new assisted reproductive technology for patients at high risk of developing OHSS, especially for women with polycystic ovary syndrome (PCOS) (Plachot, 1999). In IVM protocols without hormone stimulation, oocytes are aspirated when the leading follicle reaches a diameter between 10 and 14 mm (Trounson *et al*., 1994). After retrieval, oocytes are cultured for 28–36 h *in vitro* using maturation media supplemented with FSH. In this protocol, granulosa cells aspirated from the follicles are not exposed to the LH surge and remained unstimulated. We took advantage of the availability of these immature granulosa cells to study the LH regulation of paracrine factors secreted by granulosa cells. Here, we isolated non-stimulated granulosa cells and demonstrated their increased expression of BDNF and GDNF in response to LH/hCG treatment. Furthermore, we demonstrated that treatment with BDNF or GDNF promotes oocyte maturation by cultured human immature oocytes.

Materials and Methods

Patients and granulosa cell cultures

Human granulosa cells were collected from follicular aspirates of patients who underwent oocyte retrieval. Cells from two groups of patients were chosen for this study: (i) non-stimulated granulosa cells from natural cycle IVM patients ($n = 10$; mean age 30.8 ± 1.3, all patients had PCOS), (ii) luteinized granulosa cells from COH–IVF patients with male factor only ($n = 6$; mean age 32 ± 1.7) and (iii) surplus immature oocytes were obtained from COH–IVF patients undergoing ICSI ($n = 167$; mean age: 31 ± 0.3; average number of oocytes retrieved: 18 ± 0.5).

The Ethics Committee of the Peking University Third Hospital approved the study. Informed consent was obtained from all participants. Granulosa cells were collected from follicular fluid after oocyte retrieval. After centrifugation at 1000*g* for 10 min, follicular aspirates were centrifuged to obtain granulosa cells. To isolate granulosa cells from contaminating red blood cells, 50% Percoll gradients (Sigma-Aldrich, St. Louis, MO) were used to separate these cells following centrifugation for two times. Granulosa cells were further dispersed by washing with phosphate-buffered saline (PBS) before resuspension into McCoy's 5a media (Modified, Invitrogen, Carlsbad, CA, USA) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultured in 96-well plates (Nunclon, Roskilde, Denmark).

Immunofluorescence and confocal microscopy

To study the expression of LHR, estrogen receptor-α (ER), BDNF and GDNF by non-stimulated granulosa cells, cells were fixed in 4% paraformaldehyde (w/v) before repeated washing in PBS and then permeabilized for 20 min in PBS containing 0.2% Triton X-100 (Sigma) at room temperature. After rinsing several times in PBS, cells were blocked with 5% bovine serum albumin for 30 min at room temperature. Slides were incubated separately with the following antibodies for 18 h at 4°C: rabbit polyclonal antibody against LHR (1:200 dilutions, Santa Cruz Biotechnology), mouse monoclonal antibody against ER-α (1:150 dilution, Santa Cruz Biotechnology), rabbit polyclonal antibody against BDNF (1:500 dilution, Abcam) or rabbit polyclonal antibody against GDNF (1:350 dilution, Abcam). After three washes in PBS, slides were incubated with FITC-conjugated anti-rabbit or anti-mouse secondary antibodies (Jackson Labs, West Grove, PA) for 1 h at 37°C before washing in PBS again. Nuclei were stained with propidium iodide (Sigma) at a final concentration of 0.01 mg/ml for 10 min. Signals were captured under a laser scanning confocal microscope (Leica, Heidelberg, Germany). For some slides, the primary antibodies were replaced by preimmune mouse or rabbit immunoglobulin G (IgG) as negative controls.

Real-time RT–PCR analysis

To analyze transcript levels for BDNF and GDNF, SYBR Green-based quantitative RT–PCR was performed by using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). To study FSH and/or hCG regulation of BDNF and GDNF, non-stimulated granulosa cells isolated from follicular aspirates of non-stimulated IVM patients were treated with different gonadotrophins (20 ng/ml hCG or 20 ng/ml FSH or FSH combined with hCG; Fitzgerald, Chelmsford, MA, USA) for 3 h. In addition, luteinized granulosa cells from COH–IVF patients were obtained to study the transcript levels for BDNF and GDNF at 36 h following *in vivo* hCG injection. Cells were washed and collected before RNA extraction.

Total RNA was extracted using the RNeasy Plus Micro Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol and stored at −80°C. Carrier RNA was included in the extraction media to improve the recovery of total RNA from very small samples. In addition, gDNA Eliminator spin column was used to reduce the genomic DNA contamination. Equal amounts of mRNA extracted from granulosa cells of two patients were pooled in order to obtain enough cDNA for real-time RT–PCR assays. The concentration of RNA samples were adjusted based on absorbance at 260 nm prior to performing reverse transcription using SuperScript III First-Strand Synthesis System for Quantitive RT–PCR (Invitrogen). Real-time PCR was performed in 20-μl reaction volumes using a Power SYBR Green PCR Master Mix (Applied Biosystems) which contained Passive reference (ROX™ dye). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization, whereas omitting reverse transcriptase from cDNA synthesis was used for negative controls to exclude genomic DNA contamination. Real-time PCR results were analyzed using the ABI Prism 7000 SDS software (Applied Biosystems). For all transcripts, melting curve analysis and agarose gel electrophoresis were

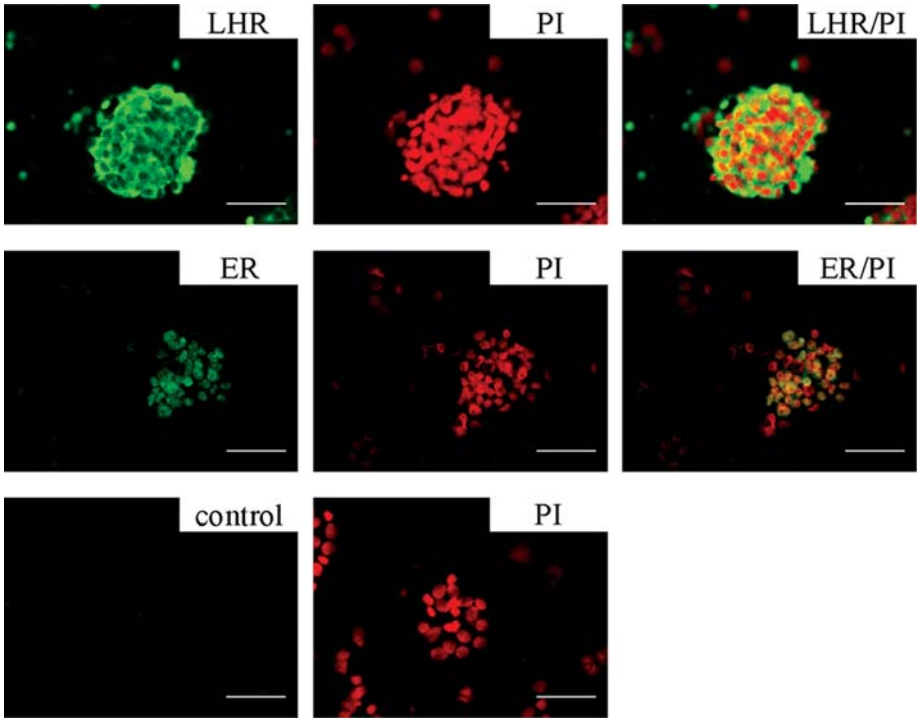

Figure 1 Expression of LH receptor (LHR) and estrogen receptor (ER) in human non-stimulated granulosa cells. Upper panel: localization of LHR in isolated human non-stimulated granulosa cells. Signals were detected in the cell surface consistent with the role of LHR as a transmembrane protein. Middle panel: immunofluorescence analysis of ER-α. ER-α was mainly located in the cell nucleus. Nuclei were stained with propidium iodide (PI). No signal was detected on control cells stained with rabbit or goat IgG.

used to verify the specificity of amplification. The following primers designed from human GenBank sequences were used: GAPDH: sense, 5′-CTC ATG ACC ACA GT C CAT GC-3′, antisense, 5′-TTC AGC TCT GGG ATG ACC TT-3′ and BDNF (Pattyn *et al*., 2003, 2006): sense, 5′-AGT GCC GAA CTA CCC AGT CGT A-3′, antisense, 5′-CTT ATG AAT CGC CAG CCA ATT C-3′. GDNF primer was ordered from QuantiTect Primer Assay, Qiagen (Hs_GDNF_1_SG QuantiTect Primer Assay QT00043330).

IVM of the immature oocyte

To measure whether treatments with BDNF and GDNF promote oocyte maturation, immature oocytes were collected from IVF patients who underwent stimulation and ICSI. Oocytes were retrieved with ultrasound guidance at 36 h after hCG injection. Retrieved oocytes with surrounding cumulus cells were cultured in human tubal fluid (HTF) medium (Global Media) supplemented with 10% HSA before culturing in a 5% CO_2 incubator at 37°C. Two hours later, the oocytes were denuded following treatment with 80 IU/ml hyaluronidase (Sigma-Aldrich) and mechanical pipetting. Denuded oocytes were observed using an inverted microscope to measure oocyte maturity. Oocytes showing the extrusion of the first polar body [metaphase II (MII) stage] were immediately used for ICSI. Oocytes without the first polar body were immature and defined as GV or MI stage depending on whether the visible GV was present. When most of the retrieved oocytes matured with the expected normal rates for IVF/ICSI patients, their immature oocytes were clinically useless and adopted for scientific research after informed consent. These extra immature oocytes were cultured in HTF medium with or without different doses of BDNF or GDNF for 24 h to measure the maturation rates.

Statistical analysis

All data are presented as mean ± SE of at least three independent experiments. Results were analyzed by one-way analysis of variance or chi-square test, and P values < 0.05 were considered to be statistically significant.

Results

Expression of LHR, ER-α, BDNF and GDNF in human non-stimulated granulosa cells

To confirm the identity of isolated granulosa cells, we analyzed the expression of LHR and ER-α using immunofluorescence (Fig. 1). Both of these products have been found in granulosa cells (Hurst *et al*., 1995). After isolation, non-stimulated granulosa cells obtained from IVM patients were round or cuboid in shape with distinct nuclei. Under confocal microscopy, signals for LHR and ER-α were found in the plasma membrane and nucleus, respectively.

In murine ovary, BDNF and GDNF are secreted by granulosa cells and act as paracrine factors on oocytes (Kawamura *et al*., 2005, 2008). To verify the paracrine effects of these factors in the human ovary, we also identified the expression of BDNF and GDNF in human granulosa cells obtained from IVM patients. Immunofluorescence analyses indicated the expression of these two factors in human granulosa cells (Fig. 2).

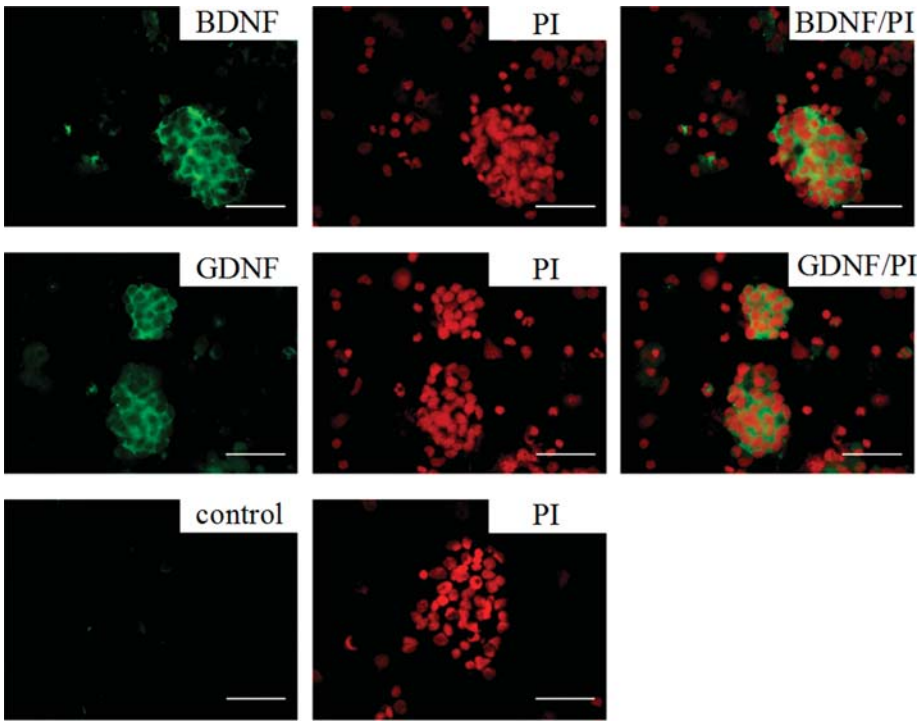

Figure 2 Expression of BDNF and GDNF in isolated non-stimulated granulosa cells. Upper and middle panels: detection of BDNF and GDNF antigens using immuno-histochemical staining. The two factors were both found in non-stimulated granulosa cells isolated from women undergoing IVM treatment. The two lower panels depict cells stained with non-immune IgG to serve as controls.

Stimulatory effects of gonadotrophin treatment on BDNF and GDNF mRNA expression in cultured non-stimulated granulosa cells

To study the possible effects of FSH or hCG on BDNF and GDNF expression, we isolated human non-stimulated granulosa cells from non-stimulated IVM patients and treated them with FSH and/or hCG. After culturing for 3 h, the transcripts for BDNF and GDNF were detected using real-time RT–PCR. As shown in Fig. 3, although the stimulatory effects of FSH on BDNF and GDNF expression were not statistical significant, treatment with hCG-increased BDNF and GDNF mRNA levels by 3.7- and 3.0-fold, respectively ($P < 0.05$). Treatment with both FSH and hCG resulted in a stimulation of 3.5- and 3.9-fold, respectively ($P < 0.05$).

BDNF and GDNF transcript levels in stimulated or non-stimulated granulosa cells

To compare changes of BDNF and GDNF transcript levels from patients with or without gonadotrophin stimulation, granulosa cells from IVM and IVF patients were isolated for real-time RT–PCR analysis (Fig. 4). Although mRNA levels for BDNF in granulosa cells derived from women at 36 h after hCG injection were increased, the changes were not significant ($P > 0.05$). For GDNF mRNA, there was a significant increase in patients at 36 h after hCG treatment ($P < 0.05$).

Effects of treatment with BDNF and GDNF on nuclear maturation of denuded immature oocytes

Earlier studies using mouse oocytes demonstrated the ability of BDNF and GDNF to promote nuclear maturation of oocytes (Seifer *et al.*, 2003; Kawamura *et al.*, 2005, 2008). Based on these findings, we tested the ability of BDNF and GDNF to regulate human oocyte maturation. For each factor, two different doses were chosen (50 or 100 ng/ml) and both showed similar effects (data not shown). As shown in Table IA, treatment with BDNF or GDNF had a negligible effect on the promotion of GVBD (Table IA). Due to the limited number of oocytes available, the effects of factors on first polar body extrusion of oocytes were not statistically evident in either the GV starting or the MI starting group ($P > 0.05$) (Table IA and B). However, based on statistical analyses, both BDNF and GDNF significantly promoted the total MII oocyte yields (MII oocytes/immature oocytes) after treatment with each factor ($P < 0.05$) (Table IC).

Discussion

Our data demonstrated that treatment with gonadotrophins stimulated the expression of two putative intraovarian paracrine factors, BDNF and GDNF, in human non-stimulated granulosa cells. Real-time RT–PCR indicated significant increases in BDNF and GDNF transcript levels in cultured granulosa cells shortly following hCG treatment. When comparing the expression levels of these two factors

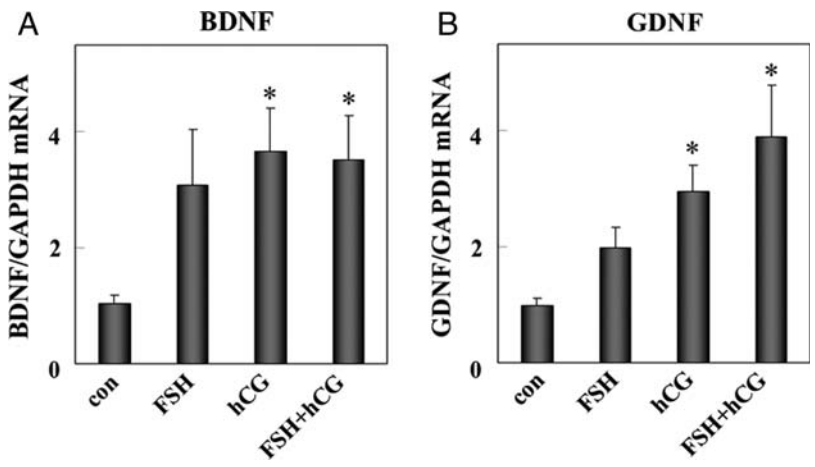

Figure 3 Gonadotrophin stimulation of BDNF and GDNF expression in cultured non-stimulated granulosa cells. Transcript levels for BDNF (**A**) and GDNF (**B**) in cultured cells at 3 h after hormone treatment were quantified by real-time RT–PCR. All data were normalized based on GAPDH levels. Mean ± SE of five independent experiments. *$P < 0.05$ when compared with the untreated group.

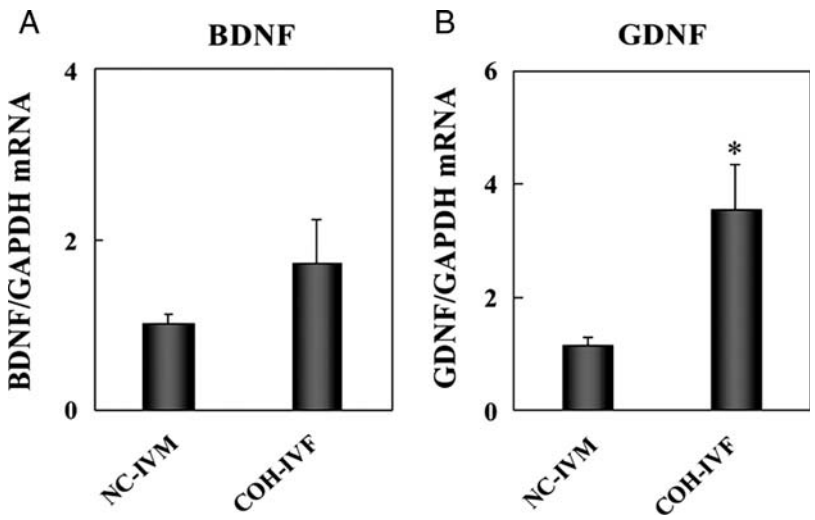

Figure 4 Expression of BDNF and GDNF transcripts in granulosa cells isolated from non-stimulated IVM and gonadotrophin-treated IVF patients. Levels of BDNF (**A**) and GDNF (**B**) transcripts were determined in human non-stimulated (NC-IVM) and stimulated (COH–IVF) granulosa cells. Samples were isolated from women who underwent IVM or IVF treatments and transcript levels were determined using real-time RT–PCR. Levels of GAPDH served as internal controls. Mean ± SE of six independent observations. *$P < 0.05$ when compared with non-stimulated cells.

between isolated non-stimulated granulosa cells from IVM patients and stimulated granulosa cells from IVF patients, the mRNA levels of GDNF were found to increase at the ovulatory period. In addition, treatment with BDNF and GDNF promoted the oocyte maturation rate for cultured human immature oocytes.

Oocyte development and ovulation are mainly stimulated by pituitary-derived gonadotrophins. During follicular development, the FSH receptor is expressed in granulosa cells at all follicle stages, but LHRs are expressed only in theca cells and mature granulosa cells. Here, we took advantage of the IVM protocol to obtain human non-stimulated granulosa cells collected from patients not receiving exogenous gonadotrophin treatment. The cells were separated after oocyte retrieval at a time when the cohort follicles did not undergo atresia just before the selection of follicle dominance was established (Jurema and Nogueira, 2006). Strong plasma membrane staining of the LHR on isolated granulosa cells is consistent with that of the previous reports (Takao *et al*., 1997). In addition, the expression of the ER was also detected. Previous reports have investigated changes in the expression of ER-α in the human ovary during folliculogenesis. Primordial and pre-antral follicles contain negligible levels of ER, whereas granulosa cells of antral follicles express the ER before the LH surge but it is decreased after the LH surge (Iwai *et al*., 1990). Our data are consistent with the expression of these receptors in granulosa cells before the LH surge.

Table I BDNF and GDNF stimulation of oocyte maturation by cultured denuded immature oocytes.

A					B			C	
GV starting	**GV**	**GVBD**	**MI**	**MII**	**MI starting**	**MI**	**MII**	**Immature oocytes (GV and MI)**	**MII**
Control ($n = 62$)	9 (14.52%)	85.48%	34 (54.84%)	19 (30.65%)	Control ($n = 60$)	17 (28.33%)	43 (71.67%)	Control ($n = 122$)	62 (50.82%)
BDNF ($n = 60$)	9 (15.00%)	85.00%	22 (36.67%)	29 (48.33%)	BDNF ($n = 61$)	9 (14.75%)	52 (85.25%)	BDNF ($n = 121$)	81 (66.94%)*
GDNF ($n = 63$)	11 (17.46%)	82.54%	24 (38.10%)	28 (44.44%)	GDNF ($n = 60$)	10 (16.67%)	50 (83.33%)	GDNF ($n = 123$)	78 (63.41%)*

The extra immature oocytes divided at GV and MI stages were collected from 167 IVF patients undergoing ICSI (an average of 2.2 extra oocytes was supplied per donor). After culturing for 24 h with or without growth factors, the percentage of oocytes showing GVBD and first polar body extrusion was determined. (A) Effects of treatment with BDNF and GDNF on oocyte maturation starting from GV stage oocytes. (B) Effects of treatment with BDNF and GDNF on oocyte maturation from MI stage oocytes. (C) Effects of BDNF and GDNF on final MII yields from all of the collected immature oocytes.
*$P < 0.05$ when compared with untreated group.

Although LH plays a crucial role in ovulation and luteinization, due to the restricted expression of its receptor on mature granulosa cells and theca cells, intraovarian factors secreted by somatic cells are required to mediate oocyte functions under LH action. Studies based on animal models have demonstrated diverse paracrine factors, including epidermal growth factor-like growth factors (amphiregulin, epiregulin and beta-cellulin), INSL3 (insulin-like factor 3), BDNF and GDNF (McGee *et al.*, 1996; Kawamura *et al.*, 2004; Park *et al.*, 2004) in the regulation of oocyte functions. Murine studies have indicated that BDNF and GDNF produced by ovarian granulosa cells could act on their specific receptors TrkB and GFRA1-Ret receptor complex expressed in the oocyte. BDNF has been detected in human follicular fluid and synthesized by cumulus cells obtained from IVF treatment (Seifer *et al.*, 2002; Feng *et al.*, 2003). Seifer *et al.* (2006) also evaluated the presence or absence of neurotrophins and their respective receptors within adult human pre-ovulatory follicles. Mural and cumulus granulosa cells were positive for BDNF, NT-4/5, NT-3 and NGF (nerve growth factor) while unfertilized oocytes were positive for TrkB, TrkC and TrkA receptors. Our data, showing that non-stimulated granulosa cells produce BDNF and GDNF consistent with earlier results, may indicate their paracrine roles in the human ovary.

Although previous studies have focused on factors present in the human follicular fluid (Glorioso *et al.*, 1986; Gruemmer *et al.*, 2005; Yding Andersen *et al.*, 2008) or secreted by cultured human granulosa-luteal cells (Akayama *et al.*, 2005; Hwu *et al.*, 2009; Peluso *et al.*, 2009), few studies deal with factors secreted during the pre-ovulatory period. Based on cultured granulosa cells isolated from IVM patients, we found a major stimulation of both BDNF and GDNF after treatment with gonadotrophin (FSH and LH), similar to the regulation patterns seen in mice *in vivo* (Kawamura *et al.*, 2005, 2008). Perlman *et al.* reported gene expression profiles of human granulosa cells from normal cycling IVM patients using oligonucleotide gene chips (Perlman *et al.*, 2006). Transcript levels of 74 genes were modified significantly at 2 h after FSH stimulation. Among these genes, BDNF transcripts were found to show a 2.7-fold increase in expression levels. In our tests, the BDNF and GDNF expression obtained from granulosa cells of PCOS women did not show a significant increase after FSH treatment but they were significantly induced by hCG or hCG combined with FSH.

Unlike non-stimulated granulosa cells, luteinized human granulosa cells have been widely used to investigate the luteinization mechanism and associated ovarian processes (Matsubara *et al.*, 2000; Iwase *et al.*, 2009). We compared the expression levels of BDNF and GDNF between non-stimulated and stimulated granulosa cells and found a significantly increased expression of GDNF, which may indicate a potential role of GDNF in luteal phase. A recent study focused on the daily variation of serum BDNF in women during normal menstrual cycles and found that luteal BDNF levels were significantly higher than follicular levels in fertile women (Pluchino *et al.*, 2009). Our data, showing a rapid increase of BDNF expression after hCG induction in vitro and a relatively stable expression level in granulosa cells at 36 h after hCG administration *in vivo*, indicate a time-course expression of BDNF under LH action.

Previous findings have shown that both BDNF and GDNF are capable of promoting first polar body extrusion in rodent and porcine ovaries (Seifer *et al.*, 2002; Feng *et al.*, 2003; Linher *et al.*, 2007). Consistent with previous results, the present findings demonstrated that treatment with BDNF and GDNF had negligible effects on GVBD but promoted the yields of MII oocytes maybe through facilitating first polar body extrusion. More recent reports studied BDNF actions using human COCs and parthenogenetic activation and showed that BDNF signalling within COCs could play a role in oocyte maturation and early embryogenesis (Anderson *et al.*, 2010). In addition, BDNF was found to condition the oocytes for optimal fertilization and development into preimplantation embryos in mice (Kawamura *et al.*, 2005). In contrast, GDNF showed no effect on the induction of the cytoplasmic maturation of oocytes, whereas GDNF synthesized by oviduct and uterus could facilitate early embryonic development (Kawamura *et al.*, 2005, 2008). Due to the limitation of human embryos, the present study did not address the issue of early embryonic development.

The present findings demonstrated two intraovarian paracrine factors that are important for the nuclear maturation of human oocytes. During IVM treatment, numerous studies have been carried out to optimize culture conditions (Chian and Tan, 2002; Jurema and Nogueira, 2006) by supplementing culture media with diverse gonadotrophins, growth factors, steroids, serum and proteins from various sources. Because culture conditions in vitro can affect early

embryo gene expression and development (Ho *et al*., 1994), understanding the activities of ovarian paracrine factors could lead to new approaches in infertility treatment, and facilitate formulation of optimal and secure culture conditions for IVM and IVF.

Authors' roles

P.Z., J.Q., E.-K.D. and A.J.W.H. designed research; P.Z., S.H., Y.Z., S.L. and L.-Y.Y. performed research; P.Z., L.S., E.-K.D. and A.J.W.H. analyzed data; and P.Z., J.Q., E.-K.D. and A.J.W.H. wrote the paper.

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