Etv5, an ETS transcription factor, is expressed in granulosa and cumulus cells and serves as a transcriptional regulator of the cyclooxygenase-2

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Abstract

Etv4, *Etv1*, and *Etv5* are members of Etv4 subfamily of E26 transformation-specific (Ets) transcription factors that are known to influence a host of biological processes. We previously showed that *Etv5*, expressed in Sertoli cells, plays a crucial role in maintaining spermatogonial stem cell niche in the mouse testis. However, it is not yet known whether Etv4 family members are expressed in the ovary or play any role in ovarian functions. Here, we show that Etv5 and Etv4 are expressed in mouse ovaries in granulosa and cumulus cells during folliculogenesis. Both *Etv5* and *Etv4* mRNAs are also detected in cumulus–oocyte complexes (COCs) and denuded oocytes. Notably, Etv4 is highly expressed in the cumulus cells of ovulated COCs at 16-h post-human chorionic gonadotropin. Cyclooxygenase-2 (PTGS2), a rate-limiting enzyme for prostaglandin synthesis, is critical for oocyte maturation and ovulation. Since several putative Ets-binding sites are present in the PTGS2 promoter, we examined whether *Etv5* influences *Ptgs2* transcriptional activity. Indeed, we found that addition of *Etv5* increases the transcriptional activity of the 3·2-kb mouse *Ptgs2* promoter by 2·5-fold in luciferase reporter assays. Collectively, the results show that *Etv4* and *Etv5* are expressed in granulosa and cumulus cells during folliculogenesis and ovulation, suggesting that they influence cellular events in the ovary by regulating downstream genes such as *Ptgs2*.

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Introduction

Follicles are the major functional units of the ovary. During folliculogenesis, oocyte growth and maturation are coordinated with proliferation and differentiation of granulosa and theca cells. Once follicular growth is initiated, the oocyte grows to full size and granulosa cells (GCs) form multiple layers in response to various molecular signals. After the secondary follicle is formed, a theca cell layer forms around the follicle (Eppig 1991, Matzuk 2000). Follicle-stimulating hormone (Fshb) stimulates proliferation of GCs, aromatization of androgens to estrogens, and expression of luteinizing hormone (Lhb) receptor, while Lhb stimulates androgen production in theca cells. After ovulation, the collapsed follicles transform into corpora lutea, major endocrine units of pregnancy which produce progesterone (P_4), 17β-estradiol (E_2), and other hormones depending on species. The process of transformation of GCs into lutein cells is called luteinization (Johnson & Everitt 1995).

Follicular growth is regulated by various factors including gonadotropins, steroid hormones, growth factors, and prostaglandins (PGs) (Matzuk 2000). It is well established that a balance among Fshb, Lhb, and E_2 is important for folliculogenesis and ovulation. For ovulation, PGs, P_4, and certain proteases are implicated. For example, inactivation of cyclooxygenase-2 (PTGS2), rate-limiting enzyme in PG biosynthesis, and PTGER2, one of the Tbxa2r receptor subtypes, in mice results in defective ovulation (Matsumoto *et al.* 2001). P_4 receptor (PR) and a transcriptional cofactor *Nrip1* are also implicated in ovarian functions (Lydon *et al.* 1995, Leonardsson *et al.* 2002). Likewise, stromelysin and other proteases are implicated in ovulation (Hagglund *et al.* 1999).

Etv4 transcription factors belong to superfamily of E26 transformation-specific (Ets) transcription factors. The Ets transcription factors regulate expression of target genes by binding to a ∼10 bp element in the promoters of target genes, known as Ets-binding site (EBS; 5′-GGAA/T-3′) (Sharrocks 2001). More than 20 family members have been identified in mammals and are divided into subfamilies, such as Ets, translocation Ets leukemia (*Etv6*), *Etv4*, E74-like factor (*Spnb2*), and ETS-2 repressor factor (*Erf*), based on structural composition and homology within ETS domain (Oikawa & Yamada 2003). Ets factors have been linked to diverse biological processes. *Etv4* subfamily is composed of three members, *Etv4*, *Etv1*, and *Etv5*. They are highly conserved and are thus capable of inducing activation of target genes interchangeably via *Etv4* consensus element (Sharrocks 2001).

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Regulation of these factors at the transcription level is important for their availability in controlling target genes, while their activity may also be regulated by post-translational modification. Some of the known target genes of *Etv4* transcription factors are *Fshr*, *Ptgs2*, stromelysin, osteopontin, matrilysin, and urokinase plasminogen activator (Rorth *et al.* 1990, Crawford *et al.* 2001, Howe *et al.* 2001, Levallet *et al.* 2001, El-Tanani *et al.* 2004). While expression of *Etv4* factors in the ovary is not known, many of their target genes are associated with female reproductive events. Thus, it is plausible that the *Etv4* subfamily of Ets factors function as transcriptional activators of the above-mentioned downstream targets in reproduction.

The *Etv4* factors have been implicated in various cellular processes, including proliferation, differentiation, and tumorigenesis (Roehl & Nusslein-Volhard 2001, Oikawa & Yamada 2003). *Etv4* and *Etv1* are important for neuronal development (Arber *et al.* 2000, Laing *et al.* 2000, Vrieseling & Arber 2006). The role of *Etv5* in male reproduction has recently been revealed in the gene-targeted mouse model. Etv5$^{-/-}$ male mice exhibit a Sertoli cell-only syndrome (Chen *et al.* 2005). The cause of male infertility in Etv5$^{-/-}$ mice is a deficit in maintaining spermatogonial stem cell population. However, potential roles for *Etv5* in female reproduction are yet to be investigated.

To this end, we assessed the spatiotemporal expression profile of *Etv4*, *Etv1*, and *Etv5* in the mouse ovary during folliculogenesis and ovulation. Our data show for the first time that *Etv5* and *Etv4* are highly expressed in the GCs during follicular growth with different expression kinetics and that *Etv5* is capable of regulating activity of the *Ptgs2* promoter. Collectively, the results suggest that *Etv5* and *Etv4* are important transcriptional regulators during folliculogenesis and ovulation by regulating expression of target genes.

Materials and Methods

Animals and sample preparation

Five-week-old virgin CD-1 female mice were purchased from Orient-Bio (Gyunggi-do, Korea). Mice were maintained in accordance with the policies of the Konkuk University Institutional Animal Care and Use Committee. Mice were cared in a controlled barrier facility within College of Veterinary Medicine, Konkuk University. Temperature, humidity, and photoperiod (12-h light:12-h darkness cycle) were kept constant. Female mice were i.p. injected with 5 IU pregnant mare's serum gonadotropin (PMSG) (Sigma) to stimulate the growth of pre-ovulatory follicles. Forty-eight hours later, some PMSG-treated mice were i.p. injected with 5 IU human chorionic gonadotropin (hCG) (Sigma). Experimental groups are 0 h (PMSG only), 3 h (3-h post-hCG), 6 h (6-h post-hCG), 9 h (9-h post-hCG), 16 h (16-h post-hCG), and 24 h (24-h post-hCG). In some experiments, ovarian samples were collected from PMSG-injected mice at 0- (cycling mice), 3-, 6-, 12-, 24-, 36-, and 48-h post-injection. Five female mice were used in each experimental group. One ovary of each mouse was collected ($n=5$ each time point) for RNA extraction. The other ovaries were frozen for cryosectioning (below).

Materials

The mouse Ptgs2 (mPtgs2) promoter of 3·2 kb was a generous gift from Dr D DeWitt (Michigan State University). Anti-peptide polyclonal antibodies for Etv4 and Etv5 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Ptgs2 antibody was purchased from Cayman Chemical (Ann Arbor, MI, USA) and anti-Etv1 antibody was from Abcam (Cambridge, MA, USA).

Tissue preparation for cryosections

Some ovaries were frozen and embedded in Tissue Freezing Medium (Electron Microscopy Sciences, Washington, PA, USA). Cryosections of ovary were cut at 12 μm and mounted onto poly-L-lysine-coated slides (Polysciences, Inc., Washington, PA, USA).

Collection of cumulus–oocyte complexes (COCs) and denuded oocytes

Six-week-old CD-1 mice were injected with 5 IU PMSG followed by 5 IU hCG 48 h later to induce superovulation. At 16- or 24-h post-hCG, ampullae of oviducts were torn open for retrieval of ovulated COCs. Pre-ovulatory COCs were collected from 6-h post-hCG ovaries by ovary puncture. For denudation, cumulus cells were removed mechanically by a mouth-controlled pipette. Collected COCs or denuded oocytes were processed for RNA isolation or immunofluorescence staining. To isolate minute amounts of RNA from these samples, glycogen was used in a modified protocol.

RT-PCR

Total RNA extraction was performed by TRIzol Reagent (Sigma) according to the manufacturer's protocol. The resuspended RNA samples were treated with ribonuclease A (RNase A)-free DNase I (DNase I, Takara, Japan) for 1 h at 37 °C to remove residual genomic DNA. Concentration and purity of the samples were evaluated by the ratio of optical density $(OD)_{260}$:$(OD)_{280}$ by a spectrophotometer. Two micrograms of total RNA were incubated with 2 μl primer cocktail at 68 °C and subjected to reverse transcription (RT) using SuperScript III reverse transcriptase (Invitrogen) for cDNA synthesis. The samples were either used directly for PCR or stored in −20 °C. PCR was carried out using Prime Taq Premix (2×) (Genet Bio, Daejeon, Korea) and reactions were carried out in the PCR thermal cycler (Applied Biosystems, Foster City, CA, USA). Following initial denaturation at 95 °C for 5 min, PCR was performed at

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95 °C for 30 s, at specific annealing temperature (55–62 °C) for 30 s, and 72 °C for 30 s. Expression of the glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was compared as internal control. Primer sequences are given in Table 1.

Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was performed by real-time monitoring of increases in fluorescence of the SYBR Green dye (Molecular Probes, Eugene, OR, USA) as described (Wittwer *et al.* 1997, Morrison *et al.* 1998) using the ABI Prism 7500 Sequence Detection System (Applied Biosystems). For comparison of transcript levels between samples, a standard curve of cycle thresholds for serial dilutions of a cDNA sample was established and then used to calculate the relative abundance of each gene. Values were then normalized to the relative amounts of Gapdh cDNA, which were obtained from a similar standard curve. All PCRs were performed in triplicates. Melting curves were plotted to determine the identity of PCR product (Ririe *et al.* 1997).

Immunofluorescence staining and confocal laser microscopy

Cryosections were fixed with ice-cold methanol for 20 min at room temperature and washed thrice with PBS for 5 min each. Freshly isolated COCs were fixed with 4% paraformaldehyde in PBS. The sections were then permeabilized with PBS containing 0·1% Tween 20 for 20 min and washed with PBS. The sections were blocked in 2% BSA in PBS for 1 h followed by specific primary antibodies in 2% BSA in PBS for 40 min at room temperature. The slides were rinsed thrice with 2% BSA in PBS. The sections were incubated with Alexa Fluor 488-conjugated secondary antibodies (Molecular Probes) at 1:250 in 2% BSA in PBS for 30 min in dark and washed twice. The sections were counterstained with TO-PRO-3-iodide in PBS (1:500) for 20 min and rinsed thrice in PBS. Cover slips were mounted onto sections with Antifade mounting medium (Invitrogen) and sealed with nail polish. Images were obtained using the Olympus Fluoview FV1000 Confocal Microscope (Olympus, Tokyo, Japan) and analyzed using the software Fluoview version 1.5, a platform associated with the confocal microscope.

Western blot

Protein extracts from ovaries were prepared in solubilization buffer (50 mm Tris–Cl (pH 7·5), 150 mm NaCl, 1% Nonidet P-40 (NP-40), 10% glycerol, 1 mm EGTA) containing an aliquot of Complete protease inhibitor cocktail (Roche). Tissues were homogenized with Polytron homogenizer (Brinkmann, Westbury, NY, USA) and centrifuged at 12 800 ***g***. The supernatants were subjected to Bradford assays (Bio-Rad Laboratories) for quantitation. Approximately 60–100 μg proteins were loaded onto 7·5% SDS-PAGE gels. After transferring to nitrocellulose membranes, the membranes were subjected to western blotting with specific antibodies. Intensity of anti-β-actin is compared among loaded samples. Chemiluminescence signal was detected by LAS3000 (Fuji Film, Japan).

Transfection and luciferase reporter assays

293T cells were obtained from American Type Culture Collection (Rockville, MD, USA). The cells were grown in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% FBS (Sigma) and penicillin (100 U/ml)–streptomycin (100 μg/ml) in a 5% CO_2 atmosphere. The cells were split into six-well plates a day before transfection. The cells were transfected with a mixture containing FuGENE6 transfection reagent (Roche), 0·6 μg/ml mPtgs2-luciferase reporter vector, 0·2 μg/ml renilla luciferase vector, 0·6 μg/ml empty, Etv5, or Etv1 expression vector under the control of CMV promoter in serum-free DMEM for 5 h. All transfection reactions were normalized to a total of 2·0 μg/ml plasmid DNA with

Table 1 Primers used for RT-PCR and quantitative RT-PCR in this study

Gene name	Primer sequence	Product size (bp)	GenBank Accession no.
Etv4_1	(F) TGAAAGGCGGATACTTGGAC (R) GTCCGGTACCTGAGCTTCTG	203	NM_008815
Etv4_2[a]	(F) CCACCAGGATCAAGAAGGAA (R) TTGTCTGGGGGAGTCATAGG	131	NM_008815
Etv1	(F) TGAAAATGGAGCCCCAATCA (R) GCTGGTGTTCCTGAAGGCTTC	139	NM_007960
Etv5	(F) AGGACCCCAGGCTGTACTTT (R) TGGCCGATTCTTCTGGATAC	260	NM_023794
Ptgs2	(F) TTGCATTCTTTGCCCAGCAC (R) TCCACTCCATGGCCCAGTC	96	NM_011198
Gapdh	(F) TGCCCCCATGTTTGTGATG (R) TGTGGTCATGAGCCCTTCC	249	NM_001037921

[a]Used in RT-PCR and qRT-PCR in Fig. 1.

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pCDNA3. The transfection mixture was replaced with complete media. After 24 h, the cells were harvested in lysis buffer (0·05% Tris/MES, (pH 7·8), 1% Triton X-100). Relative light units from luciferase activity were determined using Veritas (Turner Biosystems, Sunnyvale, CA, USA) and normalized to the renilla luciferase activity using Dual Luciferase Assay Kit (Promega). Data are presented as fold activation relative to vehicle-treated cells in the pCDNA-transfected group and represented the mean from at least three independent transfection experiments. A statistical significance was examined by a student's *t*-test (two tails) using Microsoft Excel program. All error bars represent S.D. from the mean.

Results

Etv4, Etv1, and Etv5 are expressed during follicular growth and ovulation

RNA samples of PMSG/hCG-treated ovaries were first validated by the expression of a known marker gene of hormone responsiveness, *Ptgs2*. *Ptgs2* is a crucial factor for ovulation and is generally induced around 3-h post-hCG (Lim *et al*. 1997, Joyce *et al*. 2001, Segi *et al*. 2003). As shown in Fig. 1A, induction of *Ptgs2* at 3-h post-hCG is consistent with previous reports (Joyce *et al*. 2001). Thus, these ovarian

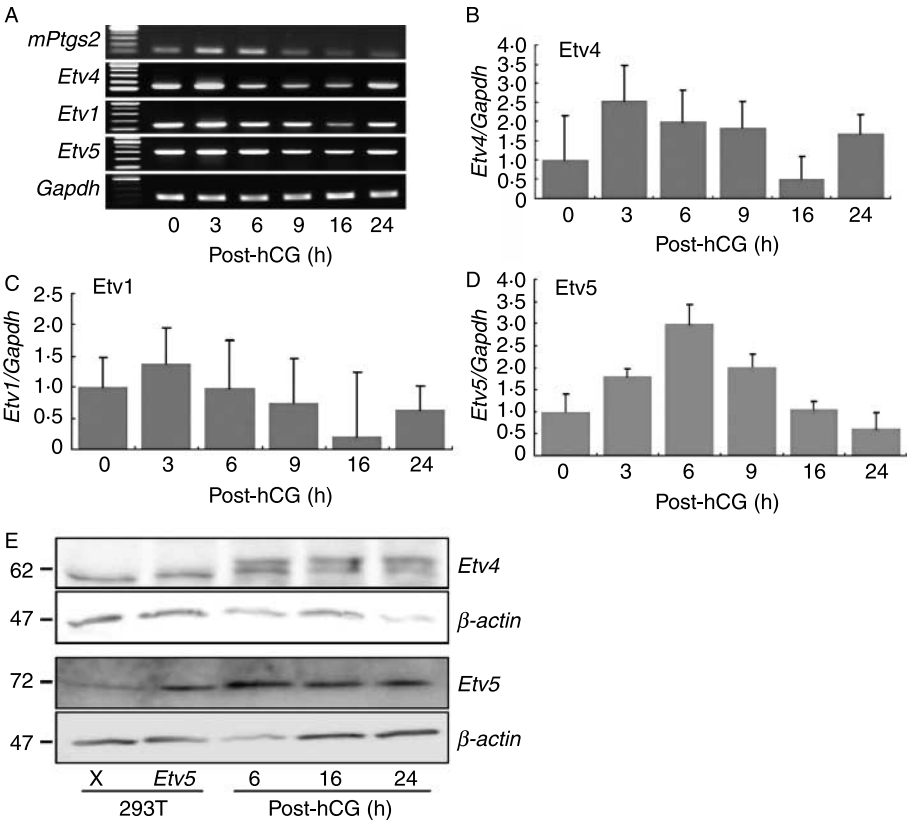

Figure 1 Expression of *Etv4* transcription factors in the ovaries of PMSG/hCG-injected mice. Total RNA samples were obtained from whole ovaries of hormone-treated mice at 0-, 3-, 6-, 9-, 16-, and 24-h post-hCG. RNA samples were subjected to reverse transcription (RT) ($n=5$ each group). (A) RT-PCR analysis of *Ptgs2*, *Etv4*, *Etv1*, and *Etv5* in one representative set of RT samples. *Ptgs2* was used as a marker to show hormone responsiveness. Primer sequences are shown in Table 1. (B–D) Quantitative RT(qRT)-PCR analysis of *Etv4*, *Etv1*, and *Etv5* using three sets of samples showing good hormone responsiveness judging from *Ptgs2* expression. qRT-PCR was performed by monitoring in real time the increases in fluorescence of the SYBR green dye as described, using ABI Prism 7500 Sequence Detection System. For comparison of transcript levels between samples, a standard curve of cycle thresholds for several serial dilutions of a cDNA sample was established and then used to calculate the relative abundance of each gene. Values were then normalized to the relative amounts of Gapdh cDNA, which were obtained from a similar standard curve. All PCRs were performed in triplicate. Error bars represent S.D. values. (E) Western blot analysis of Etv4 and Etv5 protein. Immunoreactive Etv4 and Etv5 at predicted sizes are detected in ovary lysates obtained from PMSG/hCG-treated mice at indicated post-hCG time points. 293T transfected with full-length Etv5 serves as a positive control. β-actin serves as loading control. All primary antibodies were used at 1:1000. This experiment was repeated twice with independent samples.

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RNA samples show proper hormone responsiveness. We then used these samples to examine mRNA expression of *Etv4*, *Etv1*, and *Etv5*. As shown in Fig. 1A, these three genes were all expressed in the ovary.

To quantify mRNA expression of *Etv4*, *Etv1*, and *Etv5* in the ovaries of PMSG/hCG-treated mice, we performed quantitative real-time PCR using three sets of ovarian cDNA samples (Fig. 1B–D). The expression of *Etv4* is slightly increased at 3-h post-hCG and decreased at 16 h (Fig. 1B). *Etv1* is maintained at low levels during follicular growth and ovulation (Fig. 1C). *Etv5* is expressed at all time points but its mRNA level is the highest around 6- to 9-h post-hCG (Fig. 1D).

To examine whether *Etv5* and *Etv4* transcripts are translated in mouse ovaries, we performed western blot analysis using ovary lysates obtained from hormone-treated mice. As shown in Fig. 1E, steady-state expression of ~70 kDa ETV5 protein was detected in all ovary samples. Immunoreactive ETV4 protein, in doublets, was also detected in the ovaries of hormone-treated mice. This doublet feature of immunoreactive ETV4 was previously reported by others (Kathuria *et al.* 2004).

Expression of Etv4 transcription factors in the ovaries of PMSG-injected mice

The above results show that *Etv4*, *Etv1*, and *Etv5* are all expressed in ovaries during pre-ovulatory and ovulatory follicular growth. We then examined whether expression of these factors fluctuate during earlier phases of folliculogenesis in the ovaries of PMSG-injected mice. As shown in Fig. 2, *Etv4*, *Etv1*, and *Etv5* all maintain steady-state levels of mRNA expression in groups of 0- to 48-h post-PMSG. Immunoreactive ETV5 and ETV4 protein are detected at all time points in western blot analyses.

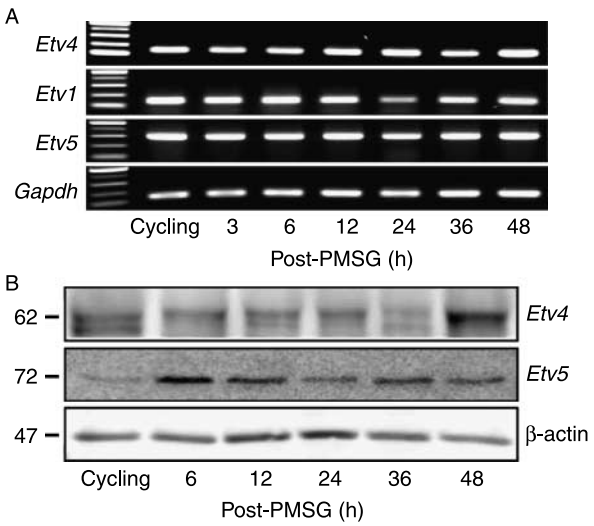

Figure 2 Expression of Etv4 transcription factors in the ovaries of PMSG-treated mice. (A) Total RNA samples were obtained from whole ovaries of PMSG-treated mice at 0, 3, 6, 12, 24, 36, and 48 h. RNA samples were subjected to reverse transcription (RT). All three genes maintain steady-state levels of expression. (B) Western blot analysis of immunoreactive Etv4 and Etv5 at indicated time points. β-actin serves as loading control. All primary antibodies were used at 1:1000. These experiments were repeated twice with independent samples.

Etv4 transcription factors exhibit cell type-specific expression in the mouse ovary

To examine cell type-specific localization of *Etv4*, *Etv1*, and *Etv5*, we performed immunofluorescence staining in the ovarian cryosections at 48-h post-hCG and 6-h post-hCG (following PMSG). As shown in Fig. 3, *Etv4* was predominantly localized in GC layers in early and late follicles at both time points. Immunoreactive ETV4 is also detected in nuclei of oocytes within follicles. *Etv1* expression was not noted in any major cell types in the ovary. On a brain section, anti-ETV1 antibody shows weak positive staining in the cell bodies (data not shown), thus negative staining of ETV1 in the mouse ovary does not seem to be due to poor antibody reactivity. Immunoreactive ETV5 was also found in GCs of follicles at various stages. The result suggests that both *Etv4* and *Etv5* functions in GCs during folliculogenesis.

Expression of Etv4 and Etv5 in the ovulated COCs

To examine expression of *Etv4* and *Etv5* in the ovulated eggs, COCs were collected from ampullae of superovulated mice. For each sample, 20–30 COCs or oocytes were used and *Gapdh* expression was compared among samples. As shown in Fig. 4, both *Etv4* and *Etv5* were localized in cumulus cells. Etv4 is localized in most of the cumulus cells with high abundance (Fig. 4A). Green fluorescence in ooplasm is likely an artefact, as rabbit IgG used as a negative control also gave similar pattern of staining in the ooplasm (data not shown).

COCs and denuded oocytes were collected by ovary puncture (6-h post-hCG) or oviduct tearing (16- and 24-h post-hCG) for RT-PCR analysis of *Etv4* and *Etv5*. *Etv5* exhibits steady-state expression in COCs and oocytes (Fig. 4B). By contrast, *Etv4* message is induced at the highest level in the ovulated COCs (16-h post-hCG). This expression subsides by 24 h. Collectively, these results show that *Etv4* and *Etv5* are expressed in cumulus cells of ovulated oocytes with different time kinetics and that they are potential transcriptional regulators of genes associated with folliculogenesis and ovulation.

Etv5 increases the promoter activity of the mPtgs2 promoter

As mentioned above, one of the potential target genes of *Etv4* transcription factors is *Ptgs2*. It is known previously that the human *Ptgs2* promoter of about 1·5 kb responds to addition of *Etv4* or *Etv5*, showing heightened reporter activity (Howe *et al.* 2001). By MatInspector database (Cartharius *et al.* 2005), we found that there are at least 19 potential EBS with a core sequence of GGAA/T within the mPtgs2 promoter of 3·2 kb (data not shown). Two of these sites are classified as potential

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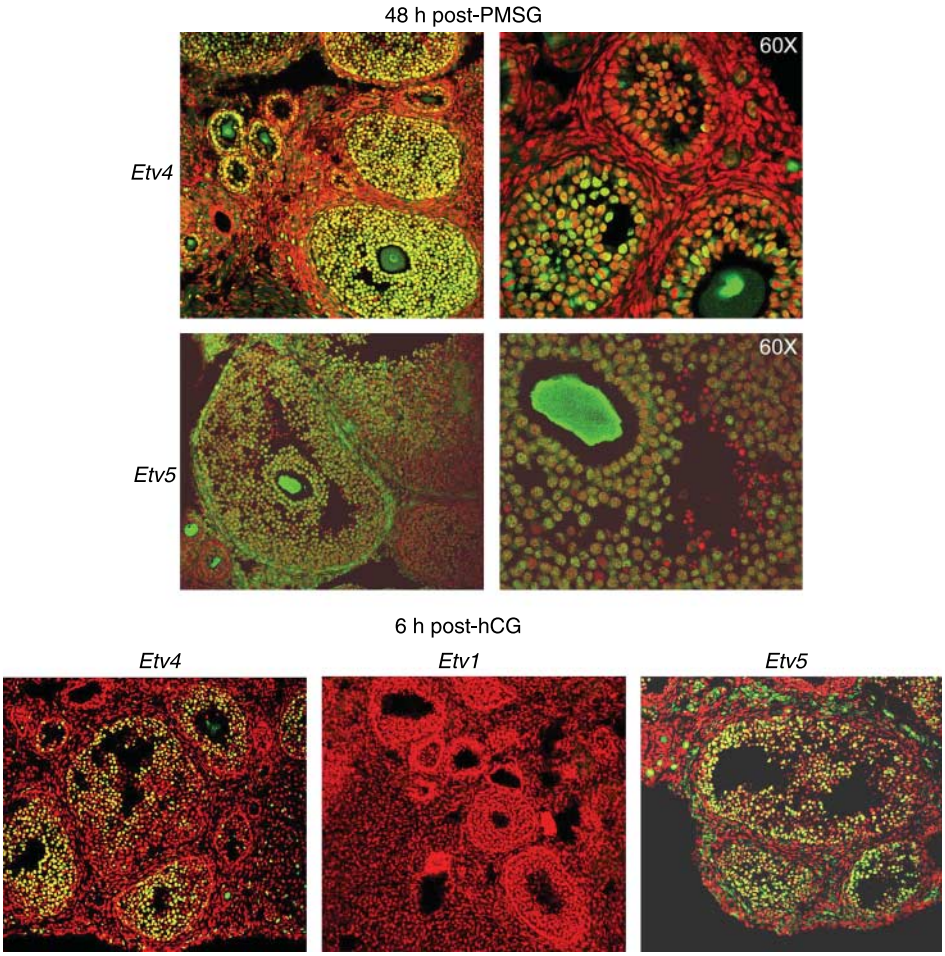

Figure 3 Cell type-specific expression of *Etv4* transcription factors in the mouse ovary. Immunolocalization of *Etv4*, *Etv1*, and *Etv4* on cryosections was performed with specific antibodies and visualized by confocal laser microscopy. Rabbit anti-ETV4, rabbit anti-ETV1, and goat anti-ETV5 antibodies were used and followed by Alexa Fluor 488-conjugated secondary antibodies. Thus, green fluorescence indicates the site of protein expression. Red fluorescence is from a nuclear dye TO-PRO-3-iodide. Yellow fluorescence is an overlay of protein localization (green) and nuclear staining (red). *Etv4* and *Etv5* expression is noted in GC layers strongly. *Etv1* was not detected, although weak nuclear staining was detected in some cell bodies of a brain section which was used as a positive control (data not shown). Anti-ETV5 and anti-ETV4 antibodies (Santa Cruz) were used at 4 μg/ml. Anti-ETV1 antibody (Abcam) was used at 1:500.

binding sites for *Etv4* factors. From the expression profiles above, we hypothesized that *Etv4* factors expressed in the GCs is one of the transcriptional regulators of *Ptgs2* required for ovulation. Thus, we performed luciferase reporter assays in 293T cells to examine whether the addition of *Etv4* transcription factors increases transcriptional activity of the mPtgs2 promoter. As shown in Fig. 5B, the addition of *Etv5* increases basal activity of the 3·2 kb mPtgs2 promoter close to 2·5-fold. *Etv1* did not have such effect. As shown in Fig. 5C and as described previously (Lim *et al.* 1997, Takahashi *et al.* 2006), *Ptgs2* is expressed in granulosa and cumulus cells of ovulated oocytes (Fig. 5C). Distinct perinuclear staining of *Ptgs2* is visible in these cells. Thus, *Etv5* and *Ptgs2* are indeed present in the same cell type making direct transcriptional interaction possible.

Discussion

Follicular development and ovulation require well-coordinated signaling by multiple intra-ovarian and extra-ovarian factors. These factors include gonadotropins, steroid hormones, growth factors, and cell cycle molecules. While certain endocrine factors have been obvious targets of research on ovarian functions for a long time, the list of genes involved in ovarian physiology is expanding by the

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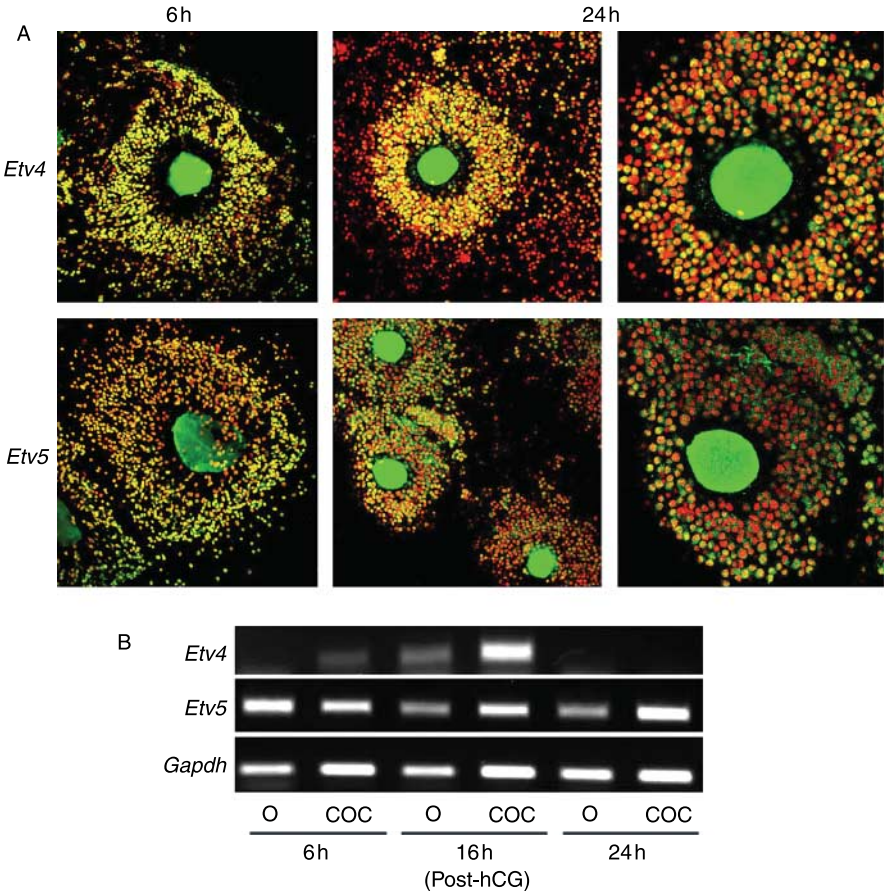

Figure 4 Expression of *Etv4* and *Etv5* in the ovulated cumulus–oocyte complexes. Cumulus–oocyte complexes (COCs) were collected from ampullae of superovulated mice. (A) *Etv4* and *Etv5* expression at 6- and 16-h post-hCG was confirmed with specific antibody in ovulated COCs. Green fluorescence indicates immunoreactive protein and red fluorescence indicates nuclear staining. Both *Etv5* and *Etv4* are localized in cumulus cells. Strong green staining in the ooplasm is likely an artefact, since staining with rabbit IgG also generated similar staining pattern in the ooplasm (data not shown). (B) RT-PCR analyses were performed in RNA sampled obtained from denuded oocytes (O) and COCs (COC) at indicated time points. These experiments were repeated twice with independent samples.

availability of diverse gene-targeted mouse models and other advanced bioinformatic techniques.

The hypothesis we tested herein is that *Etv5* and related factor *Etv4* exhibit specific expression patterns in the mouse ovary during folliculogenesis and ovulation. We formulated this notion from our previous work showing the crucial function of *Etv5* in maintaining spermatogonial stem cell niche in the mouse testis (Chen *et al*. 2005). Since developmental process and cellular components of male and female gonads share common as well as distinct characteristics, it is plausible that *Etv5* serves an important function in female mice as in male. In the mouse testis, *Etv5* is predominantly expressed in Sertoli cells. It is believed that *Etv5* in Sertoli cells induces certain secreted factors, affecting stem cell niche at the basal side of Sertoli cells (Chen *et al*. 2005). As we have shown herein, *Etv5* and *Etv4* are expressed primarily in the granulosa and cumulus cells, the counterpart of Sertoli cells in female mice. Spatiotemporal expression patterns of *Etv5* and *Etv4* in the mouse ovary suggest that these transcription factors may play important roles in regulating genes involved in events of follicular growth and ovulation. Preliminary work with *Etv5*-deficient female mice show ovulation defects (Lim *et al*. unpublished observations) but the underlying mechanism is still under investigation. Our data showing cell type-specific localization of *Etv5* will help reveal the mechanism of infertility in ETV5-deficient female mice.

Our results show that *Etv5* expression is maintained during all stages of follicular growth and ovulation. This is reminiscent of expression pattern of *Etv5* in the mouse testis, where *Etv5* is found in Sertoli cells at all times with the onset of puberty (Chen *et al*. 2005). By contrast, expression of *Etv4* exhibits notable fluctuation during follicular growth. By 9-h

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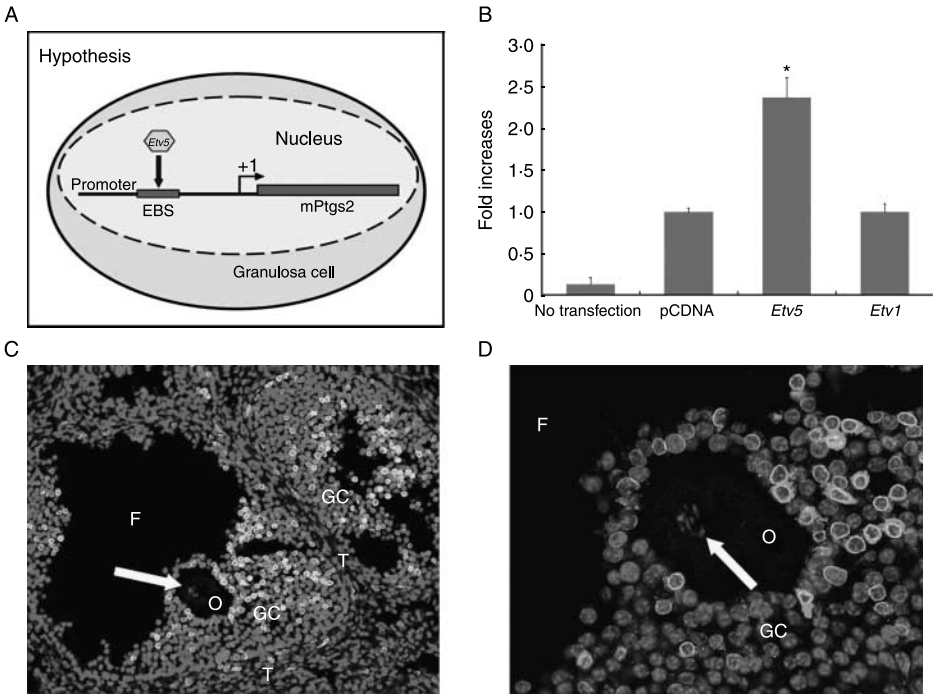

Figure 5 Activation of the mPtgs2 Promoter by *Etv5*. (A) A hypothesis. mPtgs2 has potential Ets-binding sites in the promoter. *mPtgs2* and *Etv5* are both expressed in granulosa and cumulus cells. (B) In 293T cells, 3·2 kb mPtgs2 promoter with luciferase reporter was transfected along with full-length mouse *Etv5* or *Etv1* cDNA. Addition of Etv5, but not Etv1, increases mPtgs2 promoter activity up to 2·5-fold. Error bars represent S.D. and statistical significance was examined by a student *t*-test. $*P<0{\cdot}01$. This experiment was repeated four times with similar results. (C and D) Immunofluorescence staining of mPtgs2 in the ovarian section at 6-h post-hCG. Distinct perinuclear staining of mPtgs2 is noted in GC layers and cumulus cells surrounding an oocyte (O). Arrows indicate chromosomes (red) in the oocyte.

post-hCG, the levels of *Etv4* are built up in the ovary, and with ovulation, its expression is downregulated (Fig. 1). High *Etv4* expression is now reflected in the cumulus cells of ovulated COCs (Fig. 4). This result is suggestive of *Etv4*'s role in pre-ovulatory GCs and post-ovulatory cumulus cells within a narrow time frame. Dynamic expression of *Etv4* suggests that it may play a role in oocyte–cumulus cell crosstalk prior to ovulation. While this dynamic expression pattern of *Etv4* during follicular growth is interesting, *Etv4*-deficient female mice do not seem to have any problem with their reproductive performance (Laing *et al.* 2000). Thus, *Etv4* may not be essential in ovarian functions.

We show that the promoter activity of *mPtgs2* can be increased by *Etv5*, but not by *Etv1*. *Etv5* and *Ptgs2* are both expressed in pre-ovulatory GCs and cumulus cells in the ovulated COCs, increasing the likelihood that *Ptgs2* is under direct transcriptional regulation by *Etv5*. Whether *Etv4* also regulate *Ptgs2* transcription is yet to be investigated. In addition, since there are several putative binding sites for *Etv4* transcription factors within the 3·2 kb of the *mPtgs2* promoter, identification of actual binding site(s) for *Etv5* should ensue.

Three members of the *Etv4* subfamily, *Etv4*, *Etv1*, and *Etv5*, share high sequence homology and have well-conserved functional domains. The protein sequence of these three members are more than 95% identical within the Ets domain, 85% within N-terminal transactivation domain, and approximately 50% overall (de Launoit *et al.* 2000). While gene targeting experiments revealed specific neuronal functions for Etv4 and Etv1, they are also associated with oncogenesis. For example, overexpression of Etv4 is observed in metastatic mammary adenocarcinomas in MMTV-*neu* transgenic mice (Trimble *et al.* 1993). In these mice, all three members of *Etv4* subfamily are coordinately overexpressed in mammary tumors (Shepherd *et al.* 2001). Indeed, *Etv4* factors are overexpressed human breast cancer cells (Baert *et al.* 2002). Furthermore, *Etv4* is overexpressed in ovarian carcinoma in humans (Davidson *et al.* 2003), while upregulation of *Etv5* is associated with degree of myometrial infiltration in endometrial carcinoma (Planaguma *et al.* 2005). Thus, *Etv4* transcriptional factors seem to bear oncogenic features in female reproductive organs. Expression of *Etv4* factors are correlated with tumor invasiveness and thus their functions is assumed to be associated with regulation of certain proteases required for tumor invasion (Horiuchi *et al.* 2003, Yamamoto *et al.* 2004).

Our previous work on the expression of *Etv5*, *Etv1*, and *Etv4* in mouse uterus during periimplantation (Koo *et al.*

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2005), we showed that expression of *Etv5* is associated with early events of implantation. *Etv1* is expressed highly in the developing vasculature of post-implantation uterus. Thus, it is possible that *Etv4* transcription factors play distinct functions in the female reproductive organs potentially in hormone-regulated manners. It will be important to scrutinize steroid hormone-induced expression patterns of *Etv5*, *Etv1*, and *Etv4*, to gain further insights into the roles of these factors in ovulation and other female reproductive functions.

Collectively, the present investigation provides new information that *Etv4* subfamily of Ets transcription factors exhibit specific spatiotemporal expression in the mouse ovary. These transcription factors may play important roles in regulating genes involved in proliferation and differentiation of GC layers and subsequent ovulation. Our preliminary work shows that *Etv5* and *Etv4* are expressed also in cumulus cells in humans (unpublished observations), and the role for these factors in functions of human cumulus cells will be important to explore. Further investigation is required to reveal mechanism of *Etv5* action in ovulation using *Etv5*-deficient mice. Identification of other target genes for *Etv5* and *Etv4* will also provide clues to understand mechanisms of action of these transcription factors in follicular growth and ovulation.

Declaration of Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Author contribution statement

Study concept and design: K M, H S and H J L; Statistical analysis: J E; Acquisition of data: J E and K H; Analyses and interpretation of data: J E, K H, K M, H S and H J L; Writing the manuscript: J E and H J L.

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