CRTC2 and Nedd4 ligase involvement in FSH and TGFβ1 upregulation of connexin43 gap junction

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Abstract

The major mission of the ovarian follicle is the timely production of the mature fertilizable oocyte, and this is achieved by gonadotropin-regulated, gap junction-mediated cell–cell communication between the oocyte and surrounding nurturing granulosa cells. We have demonstrated that FSH and transforming growth factor beta 1 (TGFβ1) stimulate Gja1 gene-encoded connexin43 (Cx43) gap junction formation/function in rat ovarian granulosa cells is important for their induction of steroidogenesis; additionally, cAMP-protein kinase A (PKA)- and calcium-calcineurin-sensitive cAMP response element-binding (CREB) coactivator CRTC2 plays a crucial role during steroidogenesis. This study was to explore the potential molecular mechanism whereby FSH and TGFβ1 regulate Cx43 synthesis and degradation, particularly the involvement of CRTC2 and ubiquitin ligase Nedd4. Primary culture of granulosa cells from ovarian antral follicles of gonadotropin-primed immature rats was used. At 48 h post-treatment, FSH plus TGFβ1 increased Cx43 level and gap junction function in a PKA- and calcineurin-dependent manner, and TGFβ1 acting through its type I receptor modulated FSH action. Chromatin-immunoprecipitation analysis reveals FSH induced an early-phase (45 min) and FSH+TGFβ1 further elicited a late-phase (24 h) increase in CRTC2, CREB and CBP binding to the Gja1 promotor. Additionally, FSH+TGFβ1 increased the half-life of hyper-phosphorylated Cx43 (Cx43-P2). Also, the proteasome inhibitor MG132 prevented the brefeldin A (blocker of protein transport through Golgi)-reduced Cx43-P2 level and membrane Cx43 gap junction plaque. This is associated with FSH+TGFβ1-attenuated Cx43 interaction with Nedd4 and Cx43 ubiquitination. In all, this study uncovers that FSH and TGFβ1 upregulation of Cx43 gap junctions in ovarian granulosa cells critically involves enhancing CRTC2/CREB/CBP-mediated Cx43 expression and attenuating ubiquitin ligase Nedd4-mediated proteosomal degradation of Cx43 protein.

Key Words
- calcineurin
- connexin43
- CREB
- CRTC2
- FSH
- gap junction
- ovary granulosa cell
- Nedd4 ubiquitin ligase
- TGFβ1

Journal of Molecular Endocrinology (2015) 55, 263–275

http://jme.endocrinology-journals.org
DOI: 10.1530/JME-15-0076
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Published by Bioscientifica Ltd.
Printed in Great Britain

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Introduction

The ovarian follicle is a functional unit with gap junctions serving as a direct communication between the centrally located oocyte and its surrounding granulosa cells, and among granulosa cells (Mayerhofer & Garfield 1995, Granot & Dekel 1997, Kidder & Mhawi 2002). This cell-to-cell coupling occurs throughout all developmental stages of follicles (Mitchell & Burghardt 1986, Mayerhofer & Garfield 1995, Kidder & Mhawi 2002) and was reported to allow second messenger and nutritional building blocks like amino acids, glucose metabolites and nucleotides to be transferred from granulosa cells to the growing oocyte (Eppig 1991). Gap junctions between granulosa cells predominantly consist of connexin43 (Cx43) with large antral follicles having the strongest immunostaining intensity (Mayerhofer & Garfield 1995, Granot & Dekel 1997, Kidder & Mhawi 2002). Previous studies further implicate that Cx43 gap junction communication is critical for ovarian folliculogenesis, as embryonic ovaries of Cx43-deficient mice, when cultivated in normal adult female mice, had follicle development arrested at early preantral stages (Ackert et al. 2001, Gittens et al. 2003).

Cellular gap junctions can be acutely regulated in response to various stimuli, including changes in membrane voltage, cellular calcium concentration and pH and the phosphorylation status of connexin (Peracchia 2004, Márquez-Rosado et al. 2012). In addition, phosphorylation of Cx43 could occur at several stages of the connexin life cycle: connexon (hemi-channel) formation, trafficking from endoplasmic reticulum-Golgi to the plasma membrane, gap junction assembly and channel gating and connexin degradation (Laird 2005, Solan & Lampe 2009). Cx43 is a short-lived protein with a half-life of ~1–5 h in cultured cells, organs and living animals (Laird et al. 1991, Beardslee et al. 1998). The degradation of Cx43 gap junctions was reported to involve both proteasome (Laing & Beyer 1995) and lysosome (Berthoud et al. 2004).

Pituitary secreted follicle-stimulating hormone (FSH) is the master hormone that stimulates ovarian antral follicle development acting mainly through regulating granulosa cell function, and this is vital to fertility control in the female (Richards et al. 2001). Earlier studies reported that FSH stimulates gap junction formation in rat granulosa cells (Burghardt & Matheson 1982) and increases intercellular communication and Cx43 mRNA level in a rat granulosa cell line (Sommersberg et al. 2000). This is consistent with the observation that the expression of Cx43 is increased during follicular growth and decreased after the ovulatory surge of the luteinizing hormone and during follicular atresia (Schreiber et al. 1993, Wiesen & Midgley 1993, 1994, Mayerhofer and Garfield 1995, Okuma et al. 1996, Granot & Dekel 1997).

It has been well recognized that FSH regulation of ovarian physiological functions requires intimate interaction with intra-ovarian factors, and the transforming growth factor TGFβ1 is implicated to play a significant role. Our studies as well as others revealed that TGFβ1 enhances FSH-induced advanced differentiation of ovarian granulosa cells, including increased synthesis of progesterone and estrogen and the expression of LH receptor, inhibin and key steroidogenic proteins, whereas TGFβ1 alone has no significant effect (Dodson & Schomberg 1987, Zhang et al. 1988, Gitay-Goren et al. 1993, Ke et al. 2004, Chen et al. 2007, Chen et al. 2008, Rosaiero et al. 2008, Zheng et al. 2008, Fang et al. 2012, Lai et al. 2014). In addition, our earlier study showed that TGFβ1 augments FSH-induced Cx43 gap junction formation and function in rat granulosa cells, and TGFβ1 alone has no significant effect (Ke et al. 2005). Furthermore, blockade of Cx43 gap junction formation dramatically attenuates FSH and TGFβ1-induced progesterone production (Ke et al. 2005), suggesting the upregulation of Cx43 gap junction function is crucial to FSH and TGFβ1 facilitation of steroidogenesis in ovarian granulosa cells.

FSH is known to activate the cAMP-protein kinase A (PKA) pathway and induces calcium signals in granulosa cells (Flores et al. 1990, Escamilla-Hernandez et al. 2008). Interestingly, a coactivator of cAMP response element-binding (CREB) protein, designated CREB-regulated transcription coactivator (CRTC2), was shown to serve as a coincidence sensor of cAMP and calcium signal, and this is attributed to CAMP-dependent PKA inhibition of salt-inducible kinase-induced phosphorylation inactivation of CRTC2 and calcium-dependent calcineurin-mediated dephosphorylation activation of CRTC2 (Screaton et al. 2004, Altarejos & Montminy. 2011). Our recent work revealed that FSH and TGFβ1 trigger CRTC2 activation through calcineurin- and PKA-mediated dephosphorylation in rat granulosa cells and that CRTC2 crucially mediates FSH and TGFβ1-induced expression of steroidogenic proteins (StAR protein, P450scc, 3β-HSD and aromatase enzyme) and thus the production of progesterone and estrogen (Fang et al. 2012, Lai et al. 2014).

Previously, our work revealed that TGFβ1 facilitates FSH induction of Cx43 gap junction formation and function in rat granulosa cells (Ke et al. 2005). And, FSH was reported to stimulate Cx43 gap junction formation
and activity in granulosa cells in a cAMP/PKA-dependent manner (Yogo et al. 2002, Yogo et al. 2006). Also, additional lines of evidence implicate that a Cx43-interacting protein, ubiquitin-ligase Nedd4, is involved in the regulation of internalization and degradation of Cx43 gap junction (Leykauf et al. 2006, Mollerup et al. 2011). Accordingly, we were interested to understand the role of CREB-coactivator CRTC2 and Nedd4 in FSH and TGFβ1 modulation of Cx43 gap junction formation/function and its stability in ovarian granulosa cells.

Materials and methods

Materials

Ovine FSH (oFSH-19-SIAFP) and equine chorionic gonadotropin (eCG) were purchased from the National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases, and Dr A F Parlow (Harbor-UCLA Medical Center, Torrance, CA, USA). Recombinant human TGFβ1 was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Penicillin and streptomycin were obtained from Gibco Life Technologies, Inc. Engelbreth-Holm-Swarm sarcoma tumors-derived (EHS) matrigel, rabbit polyclonal antibody against Cx43, mouse monoclonal antibodies against ubiquitin and β-actin, Lucifer Yellow, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (IgG) antibody, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), brefeldin A (BFA), E64 and MG132 were purchased from Sigma Chemical Co. Mouse monoclonal antibody against Cx43 was obtained from BD Transduction Laboratories (Lexington, KY, USA). Myristoylated protein kinase A inhibitor (PKAI) and calcineurin auto-inhibitory peptide (CNI) and cycloheximide (CHX) were purchased from Calbiochem (San Diego, CA, USA). Goat polyclonal antibody against CRTC2, rabbit polyclonal antibody against CBP and protein-A/G plus agarose beads were obtained from Santa Cruz Biotechnology. Rabbit polyclonal antibodies against Nedd4 and CREB were from Cell Signaling Technology, Inc. (Beverly, MA, USA).

Animals

Immature Sprague Dawley-derived rats (21 days) were obtained from the Animal Center at National Yang-Ming University (Taipei, Taiwan). Rats were maintained under controlled temperature (22–25°C) and light conditions (12 h light:12 h darkness cycle). Food (Lab Diet from PMI Feeds, Inc., St Louis, MO, USA) and water were available and animals were allowed to feed ad libitum. This study was conducted in accordance with both the United States National Research Council’s Guide for the Care and Use of Laboratory Animals and National Yang-Ming University’s institutional guidelines.

Cell culture and treatment

Isolation and culture of rat ovarian mural granulosa cells were conducted as previously described (Ke et al. 2005). In brief, immature rats (24–25 days) were injected once subcutaneously with 15 IU eCG to induce the development of multiple follicles to antral follicle stage. Rats were sacrificed 48 h later and ovarian granulosa cells were isolated from mid- to large-sized antral follicles and inoculated into EHS matrigel-coated culture wares in growth medium (DMEM/F12, 1:1, containing 2 μg/ml bovine insulin, 0.1% fatty acid-free BSA, 100 U/ml penicillin and 100 μg/ml streptomycin) and allowed to attach for 24 h at 37°C, 5% CO2—95% air. Cells were then washed and cultured in incubation medium (DMEM/F12, 1:1, containing 0.1% lactalbumin hydrolysate, 100 U/ml penicillin and 100 μg/ml streptomycin) for an additional 20 h before the beginning of treatment. Granulosa cells were treated as described below and in the figure legends. This granulosa cell model does not undergo spontaneous luteinization as control cells under cultivation period remain flattened and secrete very low levels of progesterone (see Fang et al. 2012, Lai et al. 2013) and is ideal for studying the regulation of granulosa cell differentiation.

Immunoblotting analysis of Cx43

Granulosa cells (~6×10^6) were cultured in matrigel-coated 60 mm dishes for 2 days and treated as described in Fig. 1. At the end of culture, cell lysates were prepared in lysis buffer (radioimmunoprecipitation assay/RIPA buffer containing a protease inhibitor cocktail and 5 mM of phosphatase inhibitors, Na orthovanadate and NaF) by sonication using ultrasonicator (Misonix Models XL-2020) and analyzed for Cx43 protein by immunoblotting using mouse monoclonal antibody (BD Transduction) as previously described (Ke et al. 2005). Relative quantification of chemiluminescent signals on x-ray film was analyzed using a two-dimensional laser scanning densitometer (Molecular Dynamics, Sunnyvale, CA, USA). In this study, gel electrophoresis was run for a longer time length than that in our earlier work (Ke et al. 2005), and we
found that Cx43-P2 contains two dominant bands; thus, quantification of the density of Cx43-P2 is the sum of these two dominant bands. This was further characterized by using the alkaline phosphatase digestion method, and the result is shown in Supplementary Figure 1, see section on supplementary data given at the end of this article.

To measure the turnover of Cx43, cells were treated with a control vehicle or FSH plus TGFβ1 for 42 h, and then CHX (5 μg/ml) was added and incubated for an additional 0–6 h to block protein synthesis. Cell lysates were then prepared and analyzed for Cx43 by immunoblotting. Quantitative analysis was conducted using the mean (±S.E.M.) density ratio of Cx43/β-actin relative to that of 0 h value from five independent experiments.

The first order decay constant (k) was determined by fitting the data to a first-order decay curve of $y = e^{-kt}$, where $y$ is signal density for Cx43 protein at time $t$, and the half-life ($t_{1/2}$) of Cx43 was determined using the formula $t_{1/2} = 0.693/k$ (Garlick et al. 1976, Beardslee et al. 1998).

Gap junction communication: scrape-loading dye transfer assay

Granulosa cells ($\sim 3.5 \times 10^6$) were cultured in matrigel-coated 35 mm dishes for 2 days and treated as described in Fig. 2. At the end of culture, the dye transfer assays were conducted as previously described (Ke et al. 2005). In brief, the confluent monolayer of cells were scraped with a sharp...
blade to create two fine linear wounds, quickly rinsed with PBS and loaded with 1 ml of Lucifer Yellow (LY, 1 mg/ml in incubation medium). The dye solution was removed 5 s later, and the culture was quickly rinsed four times with PBS. The amount of LY dye transferred from the scraped edge to the neighboring cells was examined under fluorescent microscope (×100 magnification) and photographed using SPOT image capture system (Diagnostic Instruments, Inc., Sterling Heights, MI, USA). Two representative images were taken per sample. The relative number of LY positive cells was calculated as the ratio of total number of LY-labeled cells to the cell number on the scraped edge (determined by the corresponding phase contrast image) relative to that of the control group (Yeh & Hu 2003).

**Figure 2**

Effect of selective inhibitor of PKA (PKAI), calcineurin (CNI) and TGFβRI (SB431542) on the FSH and TGFβ1-regulated gap junction communication in rat ovarian granulosa cells. Cells were treated as described Fig. 1. At the end of culture, scrape loading-dye transfer assays were conducted using a fluorescent dye Lucifer Yellow (LY), and the extent of cell coupling was determined by the number of LY positive cells (the total number of dye positive cells/the cell number on the scraped edge). Data are expressed as the mean (±SEM) number of LY positive cells relative to the control value from four independent experiments. Asterisk indicates a significant difference compared with the FSH + TGFβ1-treated group (P<0.05).

**Chromatin immunoprecipitation analysis**

Granulosa cells (~18×10⁶) were cultured in matrigel-coated 100 mm culture dishes for 2 days and treated as stated in Fig. 3 to determine the FSH and TGFβ1 regulatory effect on CRTC2, CREB and CBP binding to the Gja1 gene promoter using antibodies against CRTC2, CREB and CBP as previously described (Chen et al. 2008, Fang et al. 2012, Lai et al. 2014). In brief, cells were first fixed in 1% formaldehyde/PBS (10 min, 25 °C) to cross-link DNA and proteins, and then glycine was added (a final concentration of 125 mM; 5 min, 25°C) to stop the reaction. Cells were then scraped off, sonicated in 200 µl SDS buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0 plus protease inhibitor cocktail and 5 mM of phosphatase inhibitors...
Na$_3$Vo$_4$ and NaF) and centrifuged at 15 000 g for 15 min at 4 °C. The DNA fragment sizes were at the range of ~400–1000 bp. Small aliquots of the supernatants were kept and served as input to normalize PCR products. Supernatants were then precleared by incubation with 40 μl protein A/G plus agarose bead, 30 μl fragmented salmon sperm DNA and 0.1% BSA for 1 h at 4 °C under rotation. After centrifugation, the supernatant was incubated with CRTC2, CREB or CBP antibody (5–10 μg in buffer containing 0.01% SDS, 1.1% Triton-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0 and 167 mM NaCl plus protease inhibitors) overnight at 4 °C, followed by the addition of 50 μl protein A/G plus agarose beads and incubated for 2 h at 4 °C under rotation. Normal IgG in place of specific antibody was used as the negative control. To remove the nonspecific binding, the agarose beads were washed sequentially in the following buffers: low salt wash buffer (150 mM NaCl, 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1% Triton X-100 and 0.1% SDS), high salt wash buffer (500 mM NaCl, 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1% Triton X-100 and 0.1% SDS), LiCl wash buffer (250 mM LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl and pH 8.0) and TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The immune-precipitated products were obtained by incubating the agarose beads with 250 μl elution buffer (1% SDS, 0.1 M NaHCO$_3$) at 56 °C for 15 min followed by centrifugation. To remove RNAs and proteins, the immune complexes and input aliquots were sequentially digested with ribonuclease A (200 μg/ml in 200 mM NaCl) at 65 °C for 1 h and then with proteinase K (300 μg/ml) at 52 °C overnight. DNA was then extracted by phenol/chloroform (1:1, vol/vol), followed by isopropanol precipitation containing 0.3 M sodium acetate and 35 μg/ml glycogen, and then the precipitate was dissolved in TE buffer. PCR was performed using SensoQuest LabCycler 96 PCR system (Göttingen, Germany). For Gja1 gene, the DNA was amplified for 36 cycles (denaturation: 95 °C, 1 min; annealing: 60 °C, 35 s; elongation: 72 °C, 1 min) using antisense and sense primers (5′-CGTCTTCTCCCTCCCTCGG-3′ and 5′-GGTACCTTCTCAGCCGTTC-3′). The PCR cycle number chosen was at the linearity range of amplification. PCR products were separated on 1.5% agarose gel and visualized by ethidium bromide staining.

**Immunofluorescence analysis of Cx43**

Granulosa cells (~5×10$^5$) were cultured on matrigel-coated 12 mm cover slips for 2 days and treated as described in Fig. 4. MG132 and E64 were used according
to the earlier studies (Barrett et al. 1982, Kobayashi et al. 1992, Terret et al. 2003, Han et al. 2009). At the end of culture, cells were fixed with 4% paraformaldehyde for 15 min, washed with PBS and permeabilized with 0.05% Triton X-100 for 5 min, washed again and then blocked in 3% BSA and 3% goat serum/PBS for 1 h. Cells were then incubated with rabbit polyclonal antibody against Cx43 (Sigma) (Boswell et al. 2010) or isotypic IgG serving as a negative control for 1 h, followed by FITC-conjugated goat anti-mouse IgG for 1 h. The cover slips were then mounted on glass slides and viewed under a fluorescent microscope (Olympus BX50, Tokyo, Japan) equipped with a mercury arc lamp and photographed using SPOT image capture system (Diagnostic Instruments, Inc., Sterling Heights, MI, USA). Precaution was taken to have fluorescent images reflecting the actual emission signal for comparison among samples as previously described (Lai et al. 2013). To preclude overexposure, the photographing system was set to automatically capture the image of FSH+TGFβ1-treated cells displaying the most intense fluorescence signal. The exposure time and signal output gain value of this image was then used to capture all of the subsequent samples in a single experiment.

Co-immunoprecipitation

Granulosa cells (15–18×10⁶) were cultured in matrigel-coated 100 mm culture dishes for 2 days and treated as described in the figure legend to determine the FSH and TGFβ1 regulatory effect on Cx43 association with ubiquitin ligase Nedd4 and Cx43 ubiquitination. Briefly, cells were lysed in 0.2 ml of RIPA buffer containing a protease
inhibitor cocktail and 5 mM of phosphatase inhibitors Na orthovanadate and NaF and centrifuged at 15,000 g for 15 min. Cell lysates (control, ~800 µg; FSH+TGFβ1, ~300 µg protein) were pre-cleared with 40 µl protein-A/G plus agarose beads and 0.1% BSA, incubated for 1 h at 4 °C and then centrifuged at 15,000 g for 15 min. The supernatant were further incubated overnight at 4 °C with Cx43 antibodies (1 µg) and 40 µl protein-A/G plus agarose beads in a final volume of 500 µl. The beads were isolated by centrifugation and washed twice with cell lysis solution, and then the bound proteins were dissociated and subjected to immunoblotting analysis as described above.

**Enzyme-linked immunoassay for progesterone**

Granulosa cells (~5 x 10^5) were cultured on a matrigel-coated 24-well plate for 2 days and treated as described in Supplementary Figures 2 and 4, see section on supplementary data given at the end of this article. Progesterone levels in the conditioned media were determined using enzyme immunoassay as previously described (Ke et al. 2005, Chen et al. 2007, Fang et al. 2012, Lai et al. 2013). Antisera against progesterone and progesterone-conjugated horseradish peroxidase (P-HRP) were kindly provided by Dr Leang-Shin Wu (National Taiwan University, Taipei, Taiwan). In brief, 96-well plates were first coated with progesterone antibody overnight at 4 °C and washed off, and then progesterone standards and conditioned medium samples were incubated in the presence of P-HRP for 2 h at room temperature. The wells were again washed off, and peroxidase substrate 2,20-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium (Amresco Inc, Solon, OH, USA) was added and incubated for 2 h. The absorbance of reaction products was determined at 410 nm using a multimode microplate reader (TECAN sunrise, Männedorf, Switzerland). The sensitivity of the assay was 6 pg, and the intra- and inter-assay coefficients of variation were <3% and <7% respectively.

**Reverse transcription-quantitative real-time PCR**

Granulosa cells (~2 x 10^6) were cultured in matrigel-coated 35 mm culture dishes for 2 days and treated as described in Supplementary Figure 5, see section on supplementary data given at the end of this article. At the end of culture, cells were extracted for total RNA with TRizol Reagent (Invitrogen, Life Technologies) according to the manufacturer’s instruction. An RNA sample (2 µg) was then reverse-transcribed to cDNA using MMLV reverse-transcriptase (Toyobo Co., Osaka, Japan). Each 20 µl of PCR reaction mixture contained 1 X SYBR Green PCR Master Mix (Toyobo Co., Osaka, Japan), 50 ng cDNA and 300 nM of each specific primer of Cx43 (forward: 5’-CAAGGTAAGATGAGGGG-3’ and reverse primer: 5’-AGACATAAGCCAGAGTGGAG-3’). Quantitative real-time PCR (qPCR) was performed using StepOne Plus Real-Time PCR System (Applied Biosystems). Each sample was assayed in duplicate, and a mean value was used for the determination of mRNA levels by the comparative Ct (2^[-ΔΔCt]) method using L19 as the reference gene.

**Statistical analysis**

Quantitative data were analyzed by analysis of variance and Duncan’s multiple range tests at a significance level of 0.05 using the general linear model of the SAS program (SAS Institute, Inc., Cary, NC, USA). Differences between two treatment groups were analyzed using the Student’s t-test at a significance level of 0.05.

**Results**

**Potential role of CRTC2 in FSH and TGFβ1-regulated biogenesis of Cx43 gap junction**

To understand whether CRTC2 is an important mediator in the FSH and TGFβ1 regulation of Cx43 synthesis and gap junction function, we first employed selective inhibitors to determine the involvement of cAMP/PKA and calcium/calcineurin signaling and TGFβ1 in FSH and TGFβ1 regulation of Cx43 protein level and gap junction function. Pretreatment for 1 h with a specific inhibitor of PKA (PKAI, 20 µM), calcineurin (CNI, 50 µM) or TGFβ1 RI (SB431542, 1 µM) near complete inhibited FSH and TGFβ1-RI induced Cx43 synthesis and gap junction function. The sensitivity of the assay was 6 pg, and the intra- and inter-assay coefficients of variation were <3% and <7% respectively.

Granulosa cells (~2 x 10^6) were cultured in matrigel-coated 35 mm culture dishes for 2 days and treated as described in Supplementary Figure 5, see section on supplementary data given at the end of this article. At the end of culture, cells were extracted for total RNA with TRizol Reagent (Invitrogen, Life Technologies) according to the manufacturer’s instruction. An RNA sample (2 µg) was then reverse-transcribed to cDNA using MMLV reverse-transcriptase (Toyobo Co., Osaka, Japan). Each 20 µl of PCR reaction mixture contained 1 X SYBR Green PCR Master Mix (Toyobo Co., Osaka, Japan), 50 ng cDNA and 300 nM of each specific primer of Cx43 (forward: 5’-CAAGGTAAGATGAGGGG-3’ and reverse primer: 5’-AGACATAAGCCAGAGTGGAG-3’). Quantitative real-time PCR (qPCR) was performed using StepOne Plus Real-Time PCR System (Applied Biosystems). Each sample was assayed in duplicate, and a mean value was used for the determination of mRNA levels by the comparative Ct (2^[-ΔΔCt]) method using L19 as the reference gene.

**Statistical analysis**

Quantitative data were analyzed by analysis of variance and Duncan’s multiple range tests at a significance level of 0.05 using the general linear model of the SAS program (SAS Institute, Inc., Cary, NC, USA). Differences between two treatment groups were analyzed using the Student’s t-test at a significance level of 0.05.
control groups; moreover, following 24 h of recovery period these cells were still responsive to FSH and TGFβ1 challenge as evidenced by progesterone production (Supplementary Figure 4). Additionally, pretreatment with PKA, CNI or SB431542 completely suppressed the FSH+TGFβ1-stimulated gap junction function as indicated by Lucifer Yellow dye transfer activity (Fig. 2). These results implicate that cAMP/PKA and calcium/calmodulin signaling crucially mediate FSH and TGFβ1 induction of Cx43 gap junction formation and function in rat ovarian granulosa cells, and TGFβ1 acts through TGFβRI to augment the FSH effect.

To further explore the potential role of CRTC2 in FSH and TGFβ1 induction of Gja1 gene expression in rat granulosa cells, chromatin immunoprecipitation (ChIP) assays were performed using CRTC2, CREB and CBP antibody. Both CRTC2 and CBP are important transcription coactivators of CREB. Our recent study demonstrated that treatment with FSH in the absence or presence of TGFβ1 (FSH±TGFβ1) similarly stimulated an early-phase dephosphorylation activation of CRTC2 within 30 min to 1 h, whereas treatment with FSH+TGFβ1 but not FSH alone induced a late-phase activation of CRTC2 at 24–48 h (Fang et al. 2012); we therefore chose two treatment time points for conducting the ChIP experiment, 45 min and 24 h. Short period (45 min) exposure to FSH±TGFβ1 similarly increased CRTC2, CBP and CREB binding to Gja1 promoter (Fig. 3). Additionally, long period (24 h) exposure to FSH+TGFβ1 but not FSH alone also increased CRTC2, CBP and CREB binding to Gja1 promoter (Fig. 3). These results suggest that FSH-initiated early-phase and FSH+TGFβ1-induced late-phase expression of Cx43 involves key recruitment of CRTC2, CREB and CBP onto Gja1 promoter. Also, FSH±TGFβ1 increased Gja1 mRNA level while TGFβ1 alone had no significant effect (Supplementary Figure 5).

**Potential regulation of FSH and TGFβ1 on Cx43 protein stability**

To understand whether FSH and TGFβ1 regulation of Cx43 phosphorylation is related to its trafficking and degradation, BFA, E64 and MG132 were used. BFA blocks protein trafficking from the endoplasmic reticulum to the Golgi apparatus indirectly by targeting a guanine nucleotide exchange factor Sar1 and thus preventing the formation of COPII-mediated transport vesicles (Klausner et al. 1992). E64 is a lysosome inhibitor mainly targeting cysteine proteases (Bogoy et al. 2000), and MG132 is a peptide aldehydes blocking proteolytic activity of 26S proteasome (Han et al. 2009). Granulosa cells were treated with a control vehicle or FSH+TGFβ1 for 45 h, and then BFA in the absence or presence of E64 and/or MG132 were added to the culture for an additional 3 h. At the end of culture, cells were prepared for analysis of Cx43 by immunoblotting and immunofluorescence. Immunoblotting analysis demonstrates that the relative changes of Cx43-P2 and Cx43-P1 levels following administration of cell culture with BFA (± E64 and/or MG132) were largely similar in both the control and FSH+TGFβ1-treated groups (Fig. 4A). Granulosa cells given BFA reduced the Cx43-P2 level and increased the Cx43-P1 level as compared with the vehicle control, and similar results were observed in cells treated with BFA+E64 (Fig. 4A). Whereas cells given BFA+MG132 (± E64) prevented the BFA-induced decrease of Cx43-P2 level and retained the BFA-induced increase of Cx43-P1 level (Fig. 4A). Consistent with our earlier study (Ke et al. 2005), granulosa cells treated with FSH+TGFβ1 for 48 h had increased Cx43 immunostaining intensity at cell-to-cell contact sites (plaque pattern) and cytoplasm (Fig. 4B). Here, we showed that cells given BFA displayed a decrease of Cx43 immunostaining intensity at cell-to-cell contact sites, and this was prevented by the combined treatment with BFA and MG132 plus E64 (Fig. 4B). These results together indicate that initial phosphorylation modification of Cx43 (Cx43-P1) in granulosa cells begins during synthesis and trafficking in the endoplasmic reticulum (ER), while additional phosphorylation of Cx43 (Cx43-P2) occurs at the post-ER and Golgi trafficking path to the plasma membrane. Additionally, the turnover of Cx43-P2 critically involves proteasome-mediated degradation.

To explore the FSH and TGFβ1 potential regulation of Cx43 turnover, we first used a protein synthesis inhibitor cycloheximide to determine the half-life of Cx43. Granulosa cells were treated with a vehicle or FSH+TGFβ1 for 42 h, and cycloheximide was subsequently added to the cultures. At various time points (0–6 h) after the cycloheximide addition, cell cultures were terminated and cell lysates were prepared for determination of Cx43 content. Analysis of the five individual protein decay curves of Cx43 reveals that treatment with FSH+TGFβ1 increased the half-life of Cx43-P2 when compared with the vehicle control group (control, 1.75 ± 0.31 h; FSH+TGFβ1, 4.11 ± 0.61 h), while there was no significant difference regarding the half-life of Cx43-P1 (control, 1.78 ± 0.49 h; FSH+TGFβ1, 1.81 ± 0.17 h) (Fig. 5). This suggests that treatment with FSH+TGFβ1 in granulosa cells may increase the stability of Cx43-P2 but not Cx43-P1, and this involves phosphorylation modification of Cx43 at certain specific residue(s). To further substantiate that treatment with
FSH and TGFβ1 attenuates Cx43 degradation, we examined the extent of Cx43 ubiquitination and its association with Nedd4 (an E3 ubiquitin ligase). Nedd4 is suggested to be involved in the regulation of internalization and degradation of Cx43 gap junction (Leykauf et al. 2006). Granulosa cells were treated with a control vehicle or FSH (10 ng/ml) plus TGFβ1 (0.5 ng/ml) for 42 h and then given cycloheximide (5 μg/ml) for an additional 0–6 h to block protein synthesis. At the end of culture, cell lysates were prepared for immunoblotting analysis of Cx43 with β-actin serving as an internal control. To reveal clear Cx43 protein bands for quantitative analysis, a greater amount of control cell lysate was used during immunoblotting because control cells had much less Cx43 protein than the FSH+TGFβ1-treated cells. Relative density ratios were calculated using the respective 0 h cycloheximide group value as 100%. Each point represents the mean (± S.E.M.) relative density (n=5). Cx43-P2, Cx43-P1, phosphorylated forms of Cx43.

Discussion

Gap junctions containing predominantly Cx43 are well developed in granulosa cells and play critical role in ovarian functions including folliculogenesis and oocyte meiotic maturation (Mayerhofer & Garfield 1995, Granot & Dekel 1997Grazul-Bilska et al. 1997, Kidder & Mhawi 2002, Gittens et al. 2003). Our earlier work implicates that Cx43 gap junction formation/function is crucial to FSH and TGFβ1-induced steroidogenesis (progesterone production) in primary culture of granulosa cells of rat ovarian antral follicles (Ke et al. 2005). This study further reveals that FSH and TGFβ1 upregulation of Cx43 gap junctions in ovarian granulosa cells critically involves enhancing CRTC2/CREB/CBP-mediated Cx43 expression and attenuating ubiquitin ligase Nedd4-mediated proteosomal degradation of Cx43 protein.

FSH and TGFβ1 upregulation of Cx43 synthesis and gap junction formation/function is supported by the

![Figure 5](image-url)

**Figure 5**
FSH and TGFβ1 regulation of Cx43 turnover rate in rat ovarian granulosa cells. Cells were treated with control vehicle or FSH (10 ng/ml) plus TGFβ1 (0.5 ng/ml) for 42 h and then given cycloheximide (5 μg/ml) for an additional 0–6 h to block protein synthesis. At the end of culture, cell lysates were prepared for immunoblotting analysis of Cx43 with β-actin serving as an internal control. To reveal clear Cx43 protein bands for quantitative analysis, a greater amount of control cell lysate was used during immunoblotting because control cells had much less Cx43 protein than the FSH+TGFβ1-treated cells. Relative density ratios were calculated using the respective 0 h cycloheximide group value as 100%. Each point represents the mean (± S.E.M.) relative density (n=5). Cx43-P2, Cx43-P1, phosphorylated forms of Cx43.

![Figure 6](image-url)

**Figure 6**
FSH and TGFβ1 regulation of Cx43 association with ubiquitin ligase Nedd4 and Cx43 ubiquitination in rat ovarian granulosa cells. Cells were treated with control vehicle or FSH (10 ng/ml) plus TGFβ1 (0.5 ng/ml) for 48 h. At the end of culture, cell lysates were prepared for co-immunoprecipitation analysis using Cx43 with normal IgG serving as a negative control. Due to control, cells had much less Cx43 protein than the FSH+TGFβ1-treated cells; similar total content of Cx43 for both groups was loaded during immunoblotting analysis. (A) To reveal Cx43 association with ubiquitination, and (B) Cx43 association with Nedd4. Relative density ratios were calculated using the control value as one. Each bar represents the mean (± S.E.M.) relative density (n=3). Asterisk indicates a significant difference compared with the control group (P<0.05). IP, immunoprecipitation; C, control; F+T, FSH+TGFβ1.
following lines of evidence. First, FSH increased the Cx43 mRNA level in primary culture of rat ovarian granulosa cells and a rat granulosa cell line GFSHR-17 (Sommersberg et al. 2000, Chen et al. 2013). Additionally, we previously demonstrated in primary culture of rat ovarian granulosa cells from antral follicles that treatment with FSH and TGFβ1 increased Cx43 protein levels and gap junction-mediated cell-to-cell communication, and treatment with TGFβ1 alone had no significant effect (Ke et al. 2005). This study further discloses that TGFβ1 acts through TGFβRI to enhance the FSH effect in rat granulosa cells (Figs 1 and 2). However, in human granulosa cells, a recent study demonstrated that TGFβ1 upregulates Cx43 expression, and this effect occurs via TGFβRI-mediated Smad 2/3- and Smad4-dependent pathway (Chen et al. 2015). The different response toward TGFβ1 treatment alone on Cx43 in rat and human granulosa cells awaits future study. The present study further demonstrates that pretreatment with selective inhibitors of PKA and calcineurin, PKAI and CNI, each suppressed the FSH+TGFβ1-increased Cx43 protein level and cell-to-cell communication (Figs 1 and 2). While the CREB coactivator CRTC2 was reported to serve as a coincident sensor of cAMP/PKA and calcium/calcineurin (Screaton et al. 2004), we recently disclosed that PKAI suppresses the FSH-induced early phase activation of CRTC2, and CNI inhibits both the FSH-induced early-phase and the FSH+TGFβ1-induced late-phase activation of CRTC2 (Fang et al. 2012). Moreover, treatment with FSH±TGFβ1 rapidly (45 min) and similarly increased CRTC2, CREB and CBP binding to Gja1 promoter, and FSH+TGFβ1 elicited a longer (24 h) enhancement effect (Fig. 3). These lines of evidence together implicate the crucial involvement of cAMP/PKA and calcium/calcineurin signaling in the FSH and TGFβ1 regulation of Gja1 gene expression and Cx43 gap junction formation/function in rat ovarian granulosa cells.

Connexins including Cx43 display a rapid turnover rate, and phosphorylation has been implicated to be involved in the regulation of connexin trafficking to plasma membrane and degradation (Laird et al. 1991). This study discloses that FSH and TGFβ1 upregulation of Cx43 gap junction formation and function in ovarian granulosa cells also involves the modulation of Cx43 trafficking and degradation as indicated by the following evidence. Treatment with FSH±TGFβ1 increased the level of phosphorylated Cx43, particularly the hyper-phosphorylated forms Cx43-P2 and Cx43 gap junction plaque at plasma membrane (Ke et al. 2005). Here, we demonstrate that granulosa cells administered with FSH+TGFβ1 had an increased half-life of Cx43-P2 as compared with the control (Fig. 5). Further, cells given BFA to block protein trafficking from endoplasmic reticulum to Golgi had a reduced Cx43-P2 level and Cx43 gap junction plaque at plasma membrane together with an increased Cx43-P1 level (Fig. 4A and B), suggesting hyper-phosphorylation of Cx43 occurs during transporting to/through the Golgi apparatus and to the plasma membrane, and the level of Cx43-P2 is positively associated with Cx43 membrane plaque formation. Both proteasome and lysosome have been implicated in the degradation of Cx43 (Laing & Beyer 1995, Laing et al. 1997). In the present study, we found that MG132 (a proteasome inhibitor) prevented the BFA-induced decrease of the Cx43-P2 level and Cx43 membrane plaque, and E64 (a lysosome inhibitor) had little effect (Fig. 4A and B), suggesting that, in ovarian granulosa cells, proteasome is a key degradation pathway for Cx43. The involvement of lysosome in the degradation of Cx43 in granulosa cells cannot be totally excluded because E64 mainly inhibits lysosomal cysteine proteases (Bogyo et al. 2000). It has been proposed that the proteasomal modulation of Cx43 stability at the plasma membrane is likely through degrading connexin-interacting proteins such as E-cadherin, caveolin-1, tubulin and ZO-1, which are involved in regulating the stability, gap junction assembly and internalization of Cx43 (Fujimoto et al. 1997, Giepmans et al. 2001, Toyofuku et al. 2001, Schubert et al. 2002). The understanding of how proteasome-mediated turnover of Cx43 is controlled remains limited. Recent studies reported that Cx43 could directly interact with ubiquitin ligase Nedd4 (Leykauf et al. 2006). Here, we show that treatment with FSH plus TGFβ1 attenuated Cx43 association with ubiquitin ligase Nedd4 and Cx43 ubiquitination in rat granulosa cells (Fig. 6). These lines of evidence together implicate that FSH and TGFβ1 upregulation of Cx43 gap junction formation and function in ovarian granulosa cells also involves the modulation of Cx43 trafficking and degradation.

In all, the present study discloses that FSH and TGFβ1 modulate the synthesis, trafficking and degradation of Cx43 gap junctions in ovarian granulosa cells with crucial involvement of CRTC2/CREB/CBP in Cx43 expression and Nedd4/proteasome in Cx43 turnover. Additionally, hyper-phosphorylation of Cx43 is closely associated with its trafficking and gap junction plaque formation and protein stability.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-15-0076.
Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was supported by grants from National Science Council of Taiwan NSC100-2320-B-010-017-MY3 and NSC102-2628-B-010-002-MY3 (to J-J H) and NSC98-2311-B-002-005-MY3 (to F-C K). We are also thankful for the support from the Ministry of Education, Aim for the Top University Plan.

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