Oocyte-derived BMP15 but not GDF9 down-regulates connexin43 expression and decreases gap junction intercellular communication activity in immortalized human granulosa cells

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ABSTRACT: In the ovary, connexin-coupled gap junctions in granulosa cells play crucial roles in follicular and oocyte development as well as in corpus luteum formation. Our previous work has shown that theca cell-derived bone morphogenetic protein (BMP)4 and BMP7 decrease gap junction intercellular communication (GJIC) activity via the down-regulation of connexin43 (Cx43) expression in immortalized human granulosa cells. However, the effects of oocyte-derived growth factors on Cx43 expression remain to be elucidated. The present study was designed to investigate the effects of oocyte-derived growth differentiation factor (GDF)9 and BMP15 on the expression of Cx43 in a human granulosa cell line, SVOG. We also examined the effect relative to GJIC activity and investigated the potential mechanisms of action. In SVOG cells, treatment with BMP15 but not GDF9 significantly decreased Cx43 mRNA and protein levels and GJIC activity. These suppressive effects, along with the induction of Smad1/5/8 phosphorylation, were attenuated by co-treatment with a BMP type I receptor inhibitor, dorsomorphin. Furthermore, knockdown of the central component of the transforming growth factor-β (TGF-β) superfamily signaling pathway, Smad4, using small interfering RNA reversed the suppressive effects of BMP15 on Cx43 expression and GJIC activity. The suppressive effects of BMP15 on Cx43 expression were further confirmed in primary human granulosa-lutein cells obtained from infertile patients undergoing an in vitro fertilization procedure. These findings suggest that oocyte-derived BMP15 decreases GJIC activity between human granulosa cells by down-regulating Cx43 expression, most likely via a Smad-dependent signaling pathway.

Key words: BMP15 / connexin43 / GDF9 / human granulosa cell / smad

Introduction

It has been widely accepted that oocytes are not passive recipients of developmental signals from cumulus/granulosa cells while residing inside the growing follicles but are active modulators and governors of follicle growth and ovulation. Within this microenvironment, a highly coordinated interplay between oocytes and the somatic supporting cells of the ovarian follicles governs the development and maturation of these cells and the acquisition of meiotically competent oocytes (Eppig, 2001). In addition to pituitary-secreted gonadotrophins, the oocyte-derived growth factors growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) also participate in the regulation of several specific target genes related to ovulation and luteinization (Pangas and Matzuk, 2005; Diaz et al., 2007). As members of the transforming growth factor β (TGF-β) superfamily, both GDF9 and BMP15 bind to their type I and type II membrane receptors and trigger serine/threonine protein kinase activity. This ligand-receptor binding-induced activation further phosphorylates specific Smad transcription factors: Smad2/3 in response to GDF9 and Smad1/5/8 in response to BMP15 (Kretzschmar and Massague, 1998). Subsequently, phosphorylated and activated Smad2/3 or Smad1/5/8 heterodimerize with a common Smad, Smad4, and then translocate into the nucleus where they modulate the transcription of several target genes (Zhang et al., 1996).

Gap junctions mediate cell–cell communication by allowing the passage of ions, metabolites and small signaling molecules (up to...
~1 kDa) between adjacent cells (Caspar et al., 1977; Makowski et al., 1977). A single gap junction is composed of two connexons (or hemichannels) that conjoin the apposed cell membrane and span the intercellular space (Perkins et al., 1998). Each connexon consists of six symmetric subunits of various isotopes (there are 20 members in the mouse and 21 members in the human genome) of the transmembrane protein connexin (Beyer et al., 1990; Bruzzone et al., 1996; Kumar and Gilula, 1996). In many tissues, these connexin-coupled channels form clusters at membrane junctions called gap junction plaques (Caspar et al., 1977). In mammals, ovarian follicles are the functional units of female reproductive biology. The coordination between this physiological compartment, the oocyte, the cumulus/granulosa cells and the theca cells, relies on functional gap junctions (Granot and Dekel, 2002). In the developing follicles, gap junctions between cumulus/granulosa cells are composed primarily of connexin43 (Cx43), whereas connexin37 (Cx37) mainly contributes to the gap junctions that connect the oocyte to the circumjacent cumulus cells (Wiesen and Midgley, 1993; Simon et al., 1997). The importance of connexin in ovarian development and folliculogenesis has been demonstrated by studies of connexin knockout mice. Mice lacking Cx37 exhibit an abolishment of intercellular coupling between oocytes and cumulus cells, disrupted follicle development at the antrum formation stage, ovulation dysfunction and incompetent oocytes (Simon et al., 1997). Cx43 is expressed strongly in granulosa cells throughout all stages of follicle development, and Cx43-coupling gap junctions are required to sustain granulosa cell proliferation (Ackert et al., 2001; Gittens et al., 2005). The ablation of Cx43 leads to a reduced number of germ cells in fetal gonads, retarded growth of oocytes and failed fertilization (Ackert et al., 2001).

Given the pivotal role of Cx43-coupled gap junctions in sustaining normal ovarian function, the study of the regulation of gap junction activity and its forming proteins has been a subject of considerable research. Accumulating evidence has shown that the cyclic expression of Cx43 in granulosa cells of growing follicles is developmentally and hormonally regulated (Larsen et al., 1987; Mayerhofer and Garfield, 1995; Granot and Dekel, 1997; Melton et al., 2001; Wright et al., 2001). In the rat, the expression pattern of Cx43 in ovarian follicles positively correlates with changes in serum levels of gonadotrophins, indicating a role for gonadotrophins in the regulation of Cx43 expression in the female reproductive system (Larsen et al., 1987; Wiesen and Midgley, 1993). Specifically, FSH up-regulates the expression of Cx43 mRNA and protein, whereas LH or human chorionic gonadotrophin down-regulates Cx43 expression and leads to the subsequent loss of intercellular coupling within rat granulosa cell layers (Larsen et al., 1987; Wiesen and Midgley, 1993; Granot and Dekel, 2002). In addition to the endocrine effects of gonadotrophins, locally released steroid hormones such as estrogen, progesterone and androgen have been reported to regulate Cx43 and gap junction intercellular communication (GJIC) activity in several species, including humans (Andersen et al., 1993; Petrocelli and Lye, 1993; Wu et al., 2010).

Furthermore, our recent study identified exciting new roles for the theca cell-derived TGF-β superfamily members BMP4 and BMP7 in the regulation of Cx43 expression in human granulosa cells (Chang et al., 2013b). Moreover, we have clarified an important mechanism by which BMP4 and BMP7 modulate GJIC activity (Chang et al., 2013b). These data indicate that paracrine/juxtacrine signaling may be involved in the regulation of cell–cell communication, yet the roles of oocyte-derived growth factors in the regulation of granulosa cell-related connexins remain to be determined. In the present study, GDF9 was compared with BMP15 with respect to their effects on the expression of Cx43 in a human granulosa cell line and primary human granulosa-lutein (hGL) cells. We further investigated these effects relative to GJIC activity and the potential underlying cellular mechanisms of action.

### Materials and Methods

#### Preparation of primary hGL cells

Primary hGL cells were obtained with informed patient consent following approval from the University of British Columbia ethics review board. The controlled ovarian stimulation protocol for in vitro fertilization (IVF) patients consisted of either luteal-phase nafarelin acetate (Synarel, Pfizer, Kirkland, QC, Canada) or follicular phase GnRH antagonist (Ganirelix; Merck, Canada) down-regulation. Gonadotrophin stimulation began menstrual cycle day 2 with human menopausal gonadotrophin (hMG; Menopur, Ferring, Canada) and recombinant FSH (Puregon, Merck, Canada), and was followed by human chorionic gonadotrophin administration 34 h before oocyte retrieval, based on follicle size. Granulosa cells were purified by density centrifugation from follicular aspirates collected from women undergoing oocyte retrieval as previously described (Chang et al., 2013c).

#### Simian virus 40 large T antigen-immortalized human granulosa cell (SV OG) culture

In this study, we used SVOG, a non-tumorigenic immortalized human granulosa cell line, which was previously produced by transfecting human granulosa-luteal cells (from a patient undergoing IVF) with the SV40 large T antigen (Lie et al., 1996). Cells were counted with a hemocytometer, and cell viability was assessed by trypan blue (0.04%) exclusion. The cells were seeded (2–4 × 10^5 cells per ml in 6-well plates) and cultured in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C. Cells were cultured in Dulbecco’s Modified Eagle’s Medium/nutrient mixture F-12 Ham (DMEM/F-12; Sigma-Aldrich Corp., Oakville, ON) supplemented with 10% charcoal/dextran-treated fetal bovine serum (HyClone, Logan, UT, USA), 100 U/ml penicillin (Invitrogen, Life Technologies, NY, USA), 100 μg/ml streptomycin sulfate (Invitrogen, Life Technologies) and 1 × GlutaMAX (Invitrogen, Life Technologies). The culture medium was changed every other day in all experiments. Finally, cells were maintained in serum-free medium for 24 h before receiving the growth factor treatment.

#### Antibodies and reagents

Polyclonal rabbit anti-Cx43 (#3512) (1:1000) and polyclonal rabbit anti-phospho-Smad1 (Ser463/465) (1:1000); Smad5 (Ser463/465) (1:1000); Smad8 (Ser426/428) (1:1000) antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Polyclonal rabbit anti-Smad1/5/8 (N–18) (sc-6031-R) (1:1000) and monoclonal mouse anti-α-tubulin (B-5-1-2) (sc-26349) (1:3000) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Recombinant human BMP15 and dorsomorphin dihydrochloride (dorsomorphin) were obtained from R&D Systems (Minneapolis, MN, USA). Recombinant human GDF9 was obtained from BioVision Incorporated (Milpitas, CA, USA). SB-431542 was obtained from BioVision Incorporated (Milpitas, CA, USA). BMP4 and BMP7 Modulate GJIC Activity in Human Granulosa Cells from BioVision Incorporated (Milpitas, CA, USA). GDF9 was tested using SVOG cells as set out in Supplementary data.
Reverse transcription and real-time quantitative PCR

Cells were washed with cold phosphate-buffered saline (PBS), and total RNA was extracted with TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions. RNA (2 μg) was reverse-transcribed into first-strand cDNA with random primers and MMLV reverse transcriptase (Promega, Madison, WI, USA). Each 20-μl qPCR reaction contained 1 x SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 20 ng cDNA and 250 nM of each specific primer. The primers used were GJA1 (Cx43), 5′-TAC ACA ACA GCA GCG GAG TT-3′ (sense) and 5′-TGG GCA CCA CTC TTT TGC TT-3′ (antisense); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-ATG GAA ATC CCA TCA CCA TCT T-3′ (sense) and 5′-CGC CCC ACT TGA TTT TGG-3′ (antisense). qPCR was performed on an Applied Biosystems 7300 Real-Time PCR System equipped with a 96-well optical reaction plate (Applied Biosystems). The specificity of each assay was validated by dissociation curve analysis and agarose gel electrophoresis of the PCR products. Assay performance was validated by evaluating the amplification efficiencies by means of calibration curves and by ensuring that the plot of the log input amount versus Cq (also known as ΔCt) had a slope <0.1. The PCR parameters were 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Three separate experiments were performed on different cultures, and each sample was assayed in triplicate. A mean value was used for the determination of mRNA levels by the comparative Cq (2−ΔCt) method with GAPDH as the reference gene.

Western blot analysis

After treatment, cells were washed with cold PBS and lysed in lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μM aprotinin, 1 μM leupeptin and 1 mM PMSF; Cell Signaling) containing a protease inhibitor cocktail (Sigma-Aldrich).Extracts were centrifuged at 20 000g for 15 min at 4 °C to remove cellular debris, and protein concentrations were quantified using a DC Protein Assay (Bio-Rad Laboratories). Equal amounts of protein were separated by 10% SDS–PAGE and transferred to polyvinylidene fluoride membranes. The membranes were blocked for 1 h in Tris-buffered solution containing 0.05% Tween 20 and 5% non-fat dried milk and incubated overnight at 4 °C. Immunoreactive bands were detected using enhanced chemiluminescence reagents or a SuperSignal West Femto Chemiluminescence Substrate (Pierce, Rockford, IL, USA), followed by exposure to CL-Xposure film (Thermo Fisher, Waltham, MA, USA). Membranes were stripped with stripping buffer (50 mM Tris–HCl pH 7.6, 10 mM β-mercaptoethanol and 1% SDS) at 50 °C for 30 min and re-probed with a mouse anti-α-tubulin antibody used as a loading control.

Small interfering RNA transfection

Transient knockdown assays were performed using 25 nM GJA1 (Cx43)-targeting small interfering RNA (siRNA; ON-TARGETplus SMARTpool), 25 nM Smad4-targeting siRNA (ON-TARGETplus SMARTpool) or 25 nM control siRNA (ON-TARGETplus non-targeting Pool) purchased from Thermo Fisher Scientific (Lafayette, CO, USA). Cells were preincubated to 50% confluence in antibiotic-free DMEM/F12 medium containing 10% charcoal/dextran-treated fetal bovine serum, after which they were transfected with siRNA for 48 h using Lipofectamine RNAiMAX (Dharmacon, Life Technologies). The efficiency of knockdown for each target was confirmed by western blot.

Immunofluorescence staining

Cells were plated on glass cover slips, fixed with 4% paraformaldehyde in PBS for 20 min, and then permeabilized with 0.1% Triton X-100 in PBS for 5 min. After they were washed with PBS, the cover slips were mounted on microscope slides and blocked with Dako Protein Block (Dako, Mississauga, ON, Canada) for 1 h followed by incubation with a Cx43 antibody (1:150 diluted in Dako Protein Block) overnight. An Alexa Fluor 555 donkey anti-rabbit IgG (Life Technologies) was used as a secondary antibody. Finally, the cells were counterstained with chromosomal dye Hoechst 33258 (Sigma-Aldrich), rinsed with PBS, mounted in Gelvatol and imaged under a Zeiss Axioskop fluorescence microscope equipped with a digital camera (Q Imaging, Burnaby, BC, Canada).

Scrape loading and dye transfer assay

Several methods have been used to examine the intercellular communication (GJIC) between cells including fluorescence return after photobleaching, thymidine–uridine nucleotide transfer and metabolic coupling of 6-thioguanine metabolites. However, the scrape loading and dye transfer assay is the most sensitive assay to measure intercellular communication (Loch-Caruso et al., 1990).

To determine the effects of BMP15 on GJIC between human granulosa cells, we performed a scrape loading and dye transfer assay, which is based on monitoring the transfer of the fluorescent dye Lucifer yellow (MW 457.2) from one cell into the adjacent cell via functional gap junctions. The scrape-loading technique has been used to introduce macromolecular substances into cultured cells effectively by producing a transient tear in the cell membrane. Lucifer yellow cannot diffuse through intact cell membranes; however, its low molecular weight allows it to transfer to adjacent cells through intact gap junctions. After treatment, fully confluent cells were washed with PBS and scraped using a surgical blade prior to the addition of fluorescent dye (0.5% Lucifer yellow CH, potassium salt, Life Technology). After a 5-min incubation, the cells were washed thoroughly to remove background fluorescence, fixed with 4% paraformaldehyde and imaged with a Zeiss Axioskop fluorescence microscope equipped with a digital camera. GJIC was evaluated and measured as the distance from the scrape line to the furthest extent of the dye-coupled cells.

Statistical analysis

The results were analyzed by one-way analysis of variance followed by Turkey’s multiple comparison test in PRISM software (GraphPad Software, Inc., San Diego, CA, USA). The results are presented as the mean ± SEM of at least three independent experiments. Data were considered significantly different if P < 0.05.

Results

BMP15 but not GDF9 down-regulates Cx43 expression in SVOG cells

The concentrations of GDF9 in human follicular fluid range from 54.38 to 458.91 ng/ml (Hendarto et al., 2010), whereas there is no reported data on the concentration of BMP15 in serum or in follicular fluid. Because Cx43 is the dominant connexin in human granulosa cells and this connexin isoform is associated with a good prognosis for the corresponding oocyte (Tsai et al., 2003), we first investigated the effects of GDF9 and BMP15 on Cx43 expression in SVOG cells. Cells were treated with increasing concentrations of recombinant human GDF9 or BMP15 (1, 10 or 100 ng/ml) for 24 h, and the levels of Cx43 mRNA were examined. The results show that treatment with BMP15 significantly decreased
Figure 1  BMP15 but not GDF9 down-regulates Cx43 mRNA expression in SVOG cells. (A and C) Cells were treated with different concentrations (1, 10 or 100 ng/ml) of GDF9 or BMP15 for 24 h; Cx43 mRNA (A) and protein (C) levels were examined by reverse transcription and real-time quantitative PCR (RT-qPCR) and western blot analysis. (B and D) Cells were treated with 50 ng/ml GDF9 or BMP15 for 3, 6, 12 or 24 h; Cx43 mRNA (B) and protein (D) levels were examined by RT-qPCR. The results are expressed as the mean ± SEM of at least three independent experiments. Values marked by different letters are significantly different (P < 0.05). Ctrl, control. B15, BMP15; G9, GDF9.
Cx43 mRNA levels in a concentration-dependent manner, while treatment with GDF9 did not affect basal Cx43 mRNA levels (Fig. 1A). Moreover, treatment with 50 ng/ml BMP15, but not GDF9, significantly down-regulated Cx43 mRNA levels at any time point (3, 6, 12 or 24 h) examined (Fig. 1B). The concentration-dependent suppression of Cx43 protein levels by BMP15, but not GDF9, was confirmed by western blot analysis (Fig. 1C). Moreover, treatment with BMP15 (50 ng/ml) reduced Cx43 protein levels at different time points (3, 6, 12 or 24 h) with the maximal effect observed 12 h after treatment, whereas GDF9 had no effect at any time point examined (Fig. 1D). However, neither BMP15 nor GDF9 affected basal Cx37 mRNA levels at any concentration or time point examined (data not shown).

**Localization and distribution of Cx43 in SVOG cells**

To study the localization and distribution of Cx43 in human granulosa cells, we performed immunofluorescence staining in SVOG cells

![Image](image_url)

**Figure 2** The BMP15-induced down-regulation of Cx43 in SVOG cells is abolished by treatment with the BMP type I receptor inhibitor dorsomorphin. (A and B) To localize the distribution of Cx43 in SVOG cells, cells were transfected with 25 nM control siRNA (siCtrl) or (GJA1) Cx43 siRNA (siCx43) for 48 h. The knockdown efficiency of each siRNA was examined by western blot analysis (A). Following the transfection of the cells with 25 nM siCtrl or siCx43 for 48 h, the cells were fixed in 4% paraformaldehyde in PBS, and examined for Cx43 immunofluorescence (red) (B). (C and D) Cells were treated with 50 ng/ml BMP15 for 24 h in the presence of vehicle control (DMSO) or 5 μM dorsomorphin. The levels of Cx43 protein were examined by western blot analysis (C) or immunofluorescence microscopy (D). (E and F) Cells were treated with 50 ng/ml BMP15 for 24 h in the presence of vehicle control (DMSO) or 5 μM SB431542. The levels of Cx43 protein were examined by western blot analysis (E) or immunofluorescence microscopy (F). Cell nuclei were stained with Hoechst 33258. Scale bar represents 50 μm. The results are expressed as the mean ± SEM of at least three independent experiments. Values marked by different letters are significantly different (P < 0.05). Ctrl, control; DM, dorsomorphin.
following a transfection of control siRNA or GJA1 (Cx43)-targeting siRNA for 48 h. A western blot analysis showed that Cx43-targeting siRNA decreased Cx43 protein levels by 85–90% relative to control siRNA (Fig. 2A). Our immunostaining results showed that Cx43 was expressed clustered as red fluorescent plaques at the sites of cell–cell contacts, which is the typical appearance of aggregated Cx43 channels in gap junction plaques (Fig. 2B). Consistent with the western blot results, transfection of the cells with Cx43-targeting siRNA for 48 h dramatically decreased the amount of Cx43 immunostaining in SVOG cells (Fig. 2B).

**Figure 3** BMP15 but not GDF9 activates the Smad1/5/8 signaling pathway in SVOG cells. (A) Cells were treated with 50 ng/ml BMP15 or GDF9 for 30, 45 or 60 min; the phosphorylation levels of Smad1/5/8 were examined by western blot analysis. (B) Cells were treated with 50 ng/ml BMP15 for 60 min in the presence of vehicle control (DMSO) or 5 μM dorsomorphin; the phosphorylation levels of Smad1/5/8 were examined by western blot analysis. (C) Cells were treated with 50 ng/ml BMP15 for 60 min in the presence of vehicle control (DMSO) or 5 μM SB431542; the phosphorylation levels of Smad1/5/8 were examined by western blot analysis. The results are expressed as the mean ± SEM of at least three independent experiments. Values marked by different letters are significantly different (P < 0.05). Ctrl, control; DM, dorsomorphin; B15, BMP15; G9, GDF9.

**Dorsomorphin abolishes the BMP15-induced down-regulation of Cx43 in SVOG cells**

To date, three TGF-β type I receptors, ALK2, ALK3 and ALK6, have been demonstrated to be involved in BMP-mediated cellular functions (Goumans and Mummery, 2000). It has been shown that dorsomorphin, a selective inhibitor of BMP type I receptor, specifically inhibits the function of ALK2, ALK3 and ALK6 (Yu et al., 2008; Cross et al., 2011). To investigate whether ALK2, ALK3 and ALK6 are involved in the BMP15-induced down-regulation of the Cx43 expression, cells were treated with 50 ng/ml BMP15 in the presence or absence of 5 μM
dorsomorphin. As shown in Fig. 2C, BMP15-induced down-regulation of Cx43 protein was abolished by co-treatment with dorsomorphin. Consistent with the western blot results, an immunostaining assay showed that treatment with BMP15 decreased the expression of Cx43 and that this decrease was abolished by co-treatment with dorsomorphin (Fig. 2D).

To further confirm that other BMP type I receptors are not involved in the BMP15-induced down-regulation of Cx43, cells were treated with 50 ng/ml BMP15 in the presence or absence of 5 μM SB-431542, an inhibitor of ALK4, ALK5 or ALK7 (Inman et al., 2002). As shown in Fig. 2E, co-treatment of SB-431542 did not affect BMP15-induced down-regulation of the Cx43 protein. Consistent with the western blot results, an immunostaining assay showed that treatment with BMP15 decreased the expression of Cx43 and that this decrease was not altered by co-treatment with SB-431542 (Fig. 2F).

**BMP15 activates Smad1/5/8 signaling in SVOG cells**

To examine whether treatment with BMP15 activates Smad1/5/8, SVOG cells were treated with BMP15 or GDF9 (50 ng/ml) for 30, 45 or 60 min. The results showed that treatment with BMP15 increased the phosphorylation levels of Smad1/5/8 after 30-, 45- and 60-min stimulations, whereas treatment with GDF9 did not alter the phosphorylation level of Smad1/5/8 at any time point (Fig. 3A). To further investigate whether ALK2, ALK3 and ALK6 are involved in BMP15-induced Smad1/5/8 activation, cells were treated with BMP15 in the presence or absence of 5 μM dorsomorphin. As shown in Fig. 3B, treatment of the cells with dorsomorphin abolished the BMP15-induced Smad1/5/8 phosphorylation, whereas treatment of the cells with SB-431542 did not alter BMP15-induced Smad1/5/8 phosphorylation (Fig. 3C).

**The Smad signaling pathway is required in SVOG cells for the BMP15-induced down-regulation of Cx43**

Before regulating the transcription of target genes, the common mediator Smad4 forms heteromeric complexes with R-Smad, Smad2/3 or Smad1/5/8 (Shi and Massague, 2003). To investigate whether Smad signaling is required for the BMP15-induced down-regulation of Cx43, Smad4 siRNA was used to knockdown the endogenous expression of Smad4. Western blot analysis was used to examine the knockdown efficiency. As shown in Fig. 4A, after transfection for 48 h, Smad4 siRNA down-regulated Smad4 protein levels by 80–90% relative to control siRNA. In addition, transfection with Smad4 siRNA for 48 h abolished the suppressive effect of BMP15 on Cx43 protein (Fig. 4B).

**BMP15 decreases GJIC in SVOG cells**

To investigate whether the down-regulation of Cx43 contributes to decreased GJIC activity, we evaluated cellular GJIC using a scrape-loading experiment.
BMP15 decreases GJIC activity in SVOG cells. (A) Fully confluent cells were treated with 50 ng/ml BMP15 for 24 h in the presence or absence of 5 μM dorsomorphin (DM). (B) Cells were transfected for 48 h with 25 nM control siRNA (siCtrl) or Smad4 siRNA (siSmad4) and then treated for 24 h with 50 ng/ml BMP15. The GJIC activity was measured by monitoring the transfer of Lucifer yellow fluorescent dye between cells, and the images were captured utilizing a fluorescence microscope (top panel). The corresponding phase contrast micrographs are shown in the bottom panel. The results are expressed as the mean ± SEM of at least three independent experiments. Values marked by different letters are significantly different (P < 0.05). Ctrl, control; DM, dorsomorphin.
dye transfer technique. As shown in Fig. 5A, treatment with 50 ng/ml BMP15 for 24 h reduced the number of Lucifer yellow dye-coupled cell layers on either side of the scrape. In addition, a 30-min pretreatment with 5 μM dorsomorphin abolished the BMP15-mediated decrease in GJIC activity (Fig. 5A). Similarly, the transfection of 25 nM Smad4 siRNA for 48 h reversed the suppressive effects of BMP15 (50 ng/ml) on GJIC activity (Fig. 5B). These results indicate that BMP15 decreases GJIC activity in SVOG cells, most likely via a BMP type I receptor-driven Smad-dependent signaling pathway.

**BMP15 but not GDF9 down-regulate Cx43 in primary hGL cells**

To closely mimic the in vivo situation and accomplish more physiologically relevant data, we also used primary culture cells to confirm the function of GDF9 and BMP15 in human granulosa cells. Primary hGL cells obtained from infertile patients undergoing an IVF procedure were cultured in vehicle control with or without GDF9 and BMP15 (1, 10 and 100 ng/ml), and the results showed that BMP15 decreased Cx43 mRNA and protein levels in a concentration-dependent manner (Fig. 6A and B). Consistent with the results in SVOG cells, treatment of hGL cells with GDF9 did not affect basal Cx43 mRNA and protein levels (Fig. 6A and B).

**Discussion**

The bi-directional communication between oocytes and their surrounding follicle cells is essential for coordinating and fulfilling the differentiation and maturation of these follicle compartments from one developmental stage to the next. In mural granulosa cells, luteinization is the final differentiation stage that is provoked by the ovulatory stimulus. Premature luteinization that is associated with high serum progesterone levels may adversely affect oocyte quality and the implantation environment (Feldberg et al., 1989). A pioneer study has shown that the removal of the oocyte–cumulus complex led to the precocious luteinization of rabbit follicles in vivo (el-Fouly et al., 1970), indicating that oocytes may secrete an anti-luteinization factor. Subsequent studies have supported the long-standing hypothesis that oocytes can regulate the production of progesterone, a hallmark of luteinized cells, by granulosa cells from the time of follicle development throughout ovulation (Elvin et al., 1999; Otsuka et al., 2000). Aside from progesterone, the underlying mechanisms that modulate the luteinization process include intracellular signaling pathways, cell adhesion factors, intracellular cholesterol and oxysterols (Murphy, 2000). The trafficking and exchange of these molecules between cumulus granulosa cells relies on functional Cx43-channeled gap junctions. Therefore, the expression pattern of Cx43 can be regarded as another parameter of luteinization. Previous
clinical data have also demonstrated that a reduction in Cx43 expression within cumulus cells at the time of oocyte collection yields good embryo competence (Hasegawa et al., 2007). Our results showing that oocyte-derived BMP15 decreases GJIC activity by down-regulating Cx43 expression suggest that oocytes may play an important role in the prevention of premature luteinization.

A comprehensive understanding of the molecular mechanism of the cellular response to BMP15 is crucial for developing new pharmacological strategies for clinical treatment. In the present study, the knockdown of Smad4, the central component of the TGF-β superfamily signaling pathway, reversed the inhibitory effects of BMP15 on Cx43 expression and GJIC activity in human granulosa cells. Consistent with our previous results, Smad4 associates with Smad1/5/8, which constitutes a well-characterized signaling pathway downstream of BMPs, and may mediate the suppressive effect of BMP15 on Cx43 expression. Moreover, the same effects did not occur when the responses to GDF9, which exerts its action through Smad2/3 signaling. This has been shown that unlike mouse GDF9, human GDF9 is initially produced in an inactive form because the human GDF9 prodomain is designed to limit mature growth factor (Simpson et al., 2012). The GDF9 used in the present study was shown to be biologically functional (Supplementary data). A study using combined northern blot and in situ hybridization analysis showed that BMP15 is coincidently expressed with GDF9 in the oocyte beginning at the primary follicle stage and continuing through ovulation in mice (Dube et al., 1998; Laitinen et al., 1998). Therefore, our results indicate that although GDF9 and BMP15 are close relatives and display a similar oocyte-specific expression pattern (Dube et al., 1998), their effects vary with respect to the origin of these growth factors. Previous in vitro studies have shown that in many species including human, GDF9 and BMP15 may exhibit distinct effects on reproductive functions (Paulini and Melo, 2011). In particular, GDF9 suppresses FSH-induced progesterone and estradiol production (Vitt et al., 2000); whereas BMP15 suppresses only FSH-induced progesterone production (Otsuka et al., 2001) in rodent granulosa cells. In addition, BMP15 has been shown to suppress bovine cumulus granulosa cell apoptosis; however GDF9 has no effect on apoptosis in bovine cumulus granulosa cells (Hussein et al., 2005). Furthermore, our recent studies also showed that treating SVOG cells with GDF9 or BMP15 elicits differential effects on the mRNA and protein levels of StAR, a critical regulatory protein that regulates the rate-limiting step in steroid biosynthesis (Chang et al., 2013a).

In a conditional knockout study, ovarian-specific Smad4 knockout mice are subfertile with multiple defects in folliculogenesis (Pangas et al., 2006). In these mice, the depletion of Smad4 resulted in the disrupted regulation of steriodogenesis, leading to the premature luteinization of granulosa cells and subsequently to premature ovarian failure (Pangas et al., 2006). These results highlight the critical role of a Smad4-driven signaling pathway in the timing of granulosa cell differentiation. Although it is increasingly apparent that BMP superfamily members activate both Smad and other signaling pathways (Derynick and Zhang, 2003), our results suggest that BMP15 modulates Cx43-based gap junction activity most likely through a Smad-dependent signaling pathway. However, the present data are unable to provide information as to whether Smad1/5/8 transcription factors interact with Cx43 (GJA1) promoter sequences in the nucleus following exposure to BMP15. Future studies aimed at addressing this question by generating Cx43 (GJA1) promoter constructs and transfecting them into granulosa cells would be of great interest.

In conclusion, the current study demonstrates that BMP15 down-regulates Cx43 in primary hGL cells and a transformed human granulosa cell line. In addition, the reduction of Cx43 contributes to a decrease in the activity of GJIC. Moreover, the present results indicate that the Smad1/5/8 signaling pathway is required for the BMP15-induced down-regulation of Cx43. Taken together, these in vitro studies suggest that the oocyte plays a critical role in the local regulation of cell–cell communication.

**Supplementary data**

Supplementary data are available at http://molehr.oxfordjournals.org/.

**Authors’ roles**

H.-M.C. contributed to the study design, execution, analysis and interpretation of data, manuscript drafting and critical discussion. J.-C.C., P.C.K.L. contributed to the study design, analysis and interpretation of data, manuscript drafting and critical discussion. E.T. contributed to the provision of clinical samples, manuscript drafting and critical discussion.

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**Conflict of interest**

None declared.

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