*Journal of Reproduction and Development*, Vol. 52, No. 3, 2006

**—Full Paper—**

# PKA Implicated in the Phosphorylation of Cx43 Induced by Stimulation with FSH in Rat Granulosa Cells

**Keiichiro YOGO[1)], Takuya OGAWA[2)], Motofusa AKIYAMA[1)], Norihiro ISHIDA-KITAGAWA[1)], Hiroshi SASADA[3)], Eimei SATO[3)] and Tatsuo TAKEYA[1)]**

*[1)]Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara 630-0101, Japan, [2)]Life Sciences Division, Lawrence Berkeley National Laboratory, 1 Cyclotron Rd, Mail Stop 84R0171, Berkeley, CA 94720-8268, USA and [3)]Laboratory of Animal Reproduction, Graduate School of Agricultural Science, Tohoku University, 1–1 Tsutsumidori-amamiyamachi, Aoba-ku, Sendai 981-8555, Japan*

**Abstract.** Connexin 43 (Cx43)-mediated gap junctional communication in granulosa cells is crucial for germ line development and postnatal folliculogenesis. We previously showed that follicle-stimulating hormone (FSH) promoted phosphorylation of Cx43 in rat primary granulosa cells. We further identified Ser365, Ser368, Ser369, and Ser373 in the carboxy-terminal tail as the major sites of phosphorylation by FSH, and found that the phosphorylation of these residues was essential for channel activity. In this study, we investigated the protein kinase(s) responsible for FSH-induced phosphorylation. H89, a cyclic AMP-dependent protein kinase (PKA) inhibitor, inhibited FSH-induced phosphorylation both *in vivo* and *in vitro*, whereas PD98059, a mitogen-activated protein kinase kinase (MEK) inhibitor, had little effect on the phosphorylation level. $Ca^{2+}$-dependent protein kinase (PKC) appeared to negatively regulate phosphorylation. Phosphopeptide mapping with or without H89 treatment indicated that PKA could be responsible for phosphorylation of the four serine residues. In addition, the purified catalytic subunit of PKA could phosphorylate the recombinant C-terminal region of Cx43, but not the variant in which all four serine residues were substituted with alanine. These results suggest that FSH positively regulates Cx43-mediated channel formation and activity through phosphorylation of specific sites by PKA.
**Key words:** Connexin 43, FSH, Gap junction, Granulosa cells, PKA

(**J. Reprod. Dev. 52:** 321–328, 2006)

Gap junctions are specialized plasma membrane associations containing intercellular channels that are formed by the association of two hemi-channels (connexons), one provided by each of the communicating cells [1–3]. Each connexon is a hexamer subunit, or connexin, which is needed to form a central pore and is the product of members of a growing multigene family. At present, 20 different genes have been identified in mammals that code for different members of the connexin family [4]. Their primary sequences in proteins are fairly conserved throughout the species, with four hydrophobic membrane spanning domains and two extracellular domains. In contrast, the carboxy-terminal tail region is unique in each connexin protein, and this diversity could be an indicator and/or cause of the potential structural and regulatory complexities of gap junction channels (GJC). Indeed, connexins are expressed in

Accepted for publication: January 13, 2006
Published online: February 13, 2006
Correspondence: K. Yogo (ke-yogo@bs.naist.jp)

a variety of cells, and each cell type has its own characteristic pattern of expression of connexin genes reflecting their functional roles. In ovarian follicles, for example, intercellular communication between oocytes and somatic cell components is well recognized as crucial for both folliculogenesis and oogenesis [5, 6], and transfer of ions, metabolites, and second messenger molecules through GJC might play a principal role in the process [7, 8]. We previously detected transcripts of five connexin (Cx) genes, including those for Cx43 and the newly identified Cx60, in porcine ovarian follicles [9, 10], and observed that follicle-stimulating hormone (FSH) could induce Cx43 mRNA expression in rat ovarian follicles. Subsequent treatment with leutenizing hormone (LH) significantly reduced the Cx43 mRNA level [10], suggesting that Cx43 is a FSH-responsive gene product and also that it might play an important role in the physiological functions of FSH.

Cx43-mediated channel formation and activity have been reported to be regulated at multiple levels by phosphorylation, including the assembly of connexons in the plasma membrane, degradation of connexin, and direct effects on channel activities [11–15]. In fact, we found that FSH promoted phosphorylation of Cx43 in rat primary granulosa cells [16]. We further identified Ser365, Ser368, Ser369, and Ser373 in the carboxy-terminal tail as major sites of phosphorylation on stimulation with FSH, and found that phosphorylation of these four residues was essential for channel activity [16]. However, it was not clear which kinase(s) phosphorylates the serine residues of Cx43. In the present study, we tried to identify the kinase(s) using a FSH-responsive rat primary granulosa cell culture system and *in vitro* phosphorylation assay. We found that cAMP-dependent protein kinase (PKA) plays a major role under the influence of FSH.

## Materials and Methods

*Preparation and culture of granulosa cells*

Twenty one-day-old female SD rats (Japan SLC, Shizuoka, Japan) were injected with 2 mg of diethylstilbestrol (DES) in sesame oil once daily for 4 days, and ovaries were collected 48 h after last injection [17]. The ovaries were rinsed with Ham's F12-Dulbecco's modified Eagle's medium (F12/DMEM) supplemented with 20 $\mu$g/ml gentamycin and 0.5 $\mu$g/ml fungizone, and dissected free of fat and connective tissues. Granulosa cells were released by puncturing follicles with 26-gauge needles in F12/DMEM medium and were incubated in 0.25% trypsin and 50 $\mu$g/ml DNaseI for 1 min, followed by the addition of 50 $\mu$g/ml trypsin inhibitor. The cells were then washed three times with F12/DMEM and resuspended in culture medium (F12/DMEM, 0.1% BSA, 5 $\mu$g/ml insulin, 5 $\mu$g/ml transferrin, 20 $\mu$g/ml gentamycin and 0.5 $\mu$g/ml fungizone) supplemented with 5 $\mu$g/ml fibronectin. The cells were then plated in poly-D-lysine-coated dishes at a density of $5 \times 10^5/cm^2$ and cultured for 20–24 h in an atmosphere of 5%$CO_2$/95% air at 37 C. When necessary, granulosa cells thus prepared were stimulated with FSH (100 ng/ml; Vitro Diagnostics Inc.) for 4 h.

*Western blot analysis*

Cells were washed with phosphate-buffered saline (PBS) and lysed in lysis buffer-1 [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 2 mM PMSF, 2 mM $Na_3VO_4$, 20 mM NaF, 10 mM N-ethylmaleimide, 100 KIU/ml aprotinin and 1% Triton-X 100] for 10 min at 4 C. Cell lysates were collected in tubes and centrifuged. The supernatants were solubilized with 2X sample buffer [125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.1 mg/ml bromophenol blue, and 10% 2-mercaptoethanol]. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes (Millipore) by electrophoresis. The membranes were rinsed in PBS containing 5% BSA and 0.1% Tween-20 for 1 h, and incubated with anti-Cx43 antibody (Sigma), anti-ERK antibody (Promega), or anti-phosphorylated ERK antibody (Promega) for 1–2 h at room temperature followed by incubation with a secondary antibody (sheep anti-mouse IgG or Protein-A conjugated with horseradish peroxidase, Amersham Biosciences) in 2.5% skim milk/PBS for 1 h. After washing, signals were detected with an enhanced chemiluminescence (ECL) reagent (Amersham-Pharmacia-Biotech).

*In vitro phosphorylation reaction of recombinant Cx43-CT protein*

Rat 6x His-tagged Cx43-CT (E227-I382) or Cx43-CTS4A (S365/368/369/373A) (Fig. 1) were expressed in *E. coli.* and purified as described

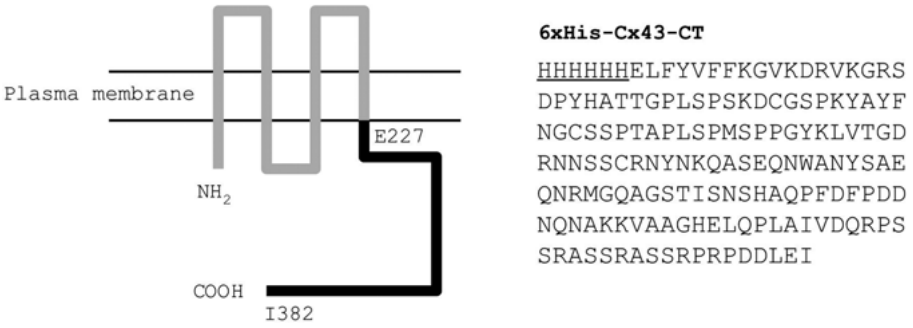


**Fig. 1.** Construction and expression of a recombinant C-terminal domain of Cx43. A cDNA encoding the cytoplasmic tail of rat Cx43 (Cx43-CT: amino acid residues from E227 to I382) was subcloned into a 6x His-tagged expression vector, pQE31 (QIAGEN).

previously [16]. Cell lysates were prepared as follows. Granulosa cells were stimulated with FSH (100 ng/ml) for 4 h, lysed with lysis buffer-2 (50 mM Hepes, 100 mM NaCl, 50 mM NaF, 1 mM $Na_3VO_4$, 1% Triton X-100, 2 mM PMSF, and 100 KIU/ml aprotinin), and centrifuged at 17,000 × g for 5 min. The supernatant fraction was stored at –80C prior to use. For *in vitro* protein kinase reactions, 2.5 $\mu$g of Ni-NTA resin (Qiagen)-bound Cx43-CT was mixed with 6 $\mu$l of 5X kinase buffer [100 mM Tris-HCl (pH 7.5), and 25 mM $MgCl_2$], 1.5 $\mu$l of 200 mM $MgCl_2$, 5 $\mu$l of [$\gamma$-$^{32}$P] ATP (111 TBq/mmol), and 1.5 $\mu$l of 100 $\mu$M ATP, and the volume was adjusted to 25 $\mu$l with deionized water. When necessary, 50 $\mu$M of H89 was included [18]. Five microliters of cell lysate or the catalytic subunit of PKA (Sigma) was added to the reaction mixture, which was then incubated for 30 min at 30 C. The resins were then washed and solubilized with sample buffer.

*Phosphopeptide mapping*

Two-dimensional phosphopeptide mapping and phosphoamino acid analysis were performed as described previously [19]. Briefly, labeled Cx43-CT was digested with TPCK-trypsin and the phosphopeptides were resolved on cellulose thin-layer chromatography (TLC) plates (Merck) by electrophoresis using 1% ammonium bicarbonate buffer, pH 8.9, for 30 min at 1 kV in the first dimension and ascending chromatography using phosphochromatography buffer (37.5% n-butyl alcohol, 25% pyridine, 7.5% acetic acid, and 30% deionized water) in the second dimension. The labeled peptide signals were detected by radioautography.

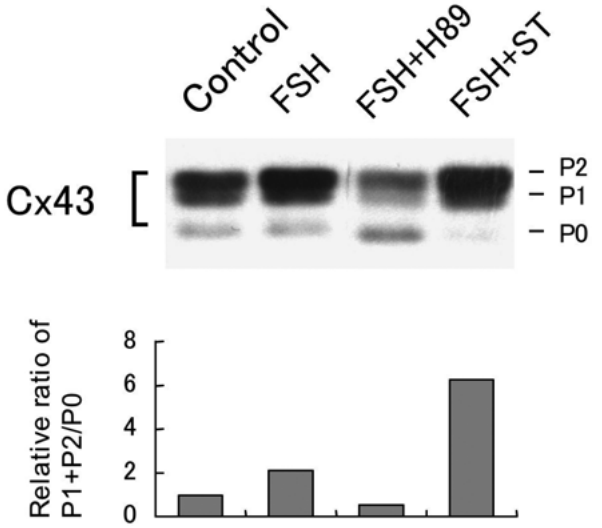


**Fig. 2.** Effects of H89 (50 $\mu$M) and staurosporin (ST) (50 ng/ml) on FSH-induced phosphorylation of Cx43. Rat primary granulosa cells were cultured with or without FSH for 4 h in the presence of the indicated inhibitor, H89 (50 $\mu$M) or ST (50 ng/ml), and subjected to Western blot analysis (*upper panel*). P1 and P2 indicate the hyperphosphorylated forms, whereas P0 corresponds to the hypophosphorylated form of Cx43. Densitometric scanning of each form of Cx43 was conducted to quantify the ratio of the P1 + P2 forms to the P0 form. The ratio was normalized to control values (*lower panel*).

## Results

*PKA enhances while PKC suppresses phosphorylation of Cx43*

To identify the possible protein kinase involved in phosphorylation of Cx43 induced by stimulation with FSH in granulosa cells, we tested inhibitors for

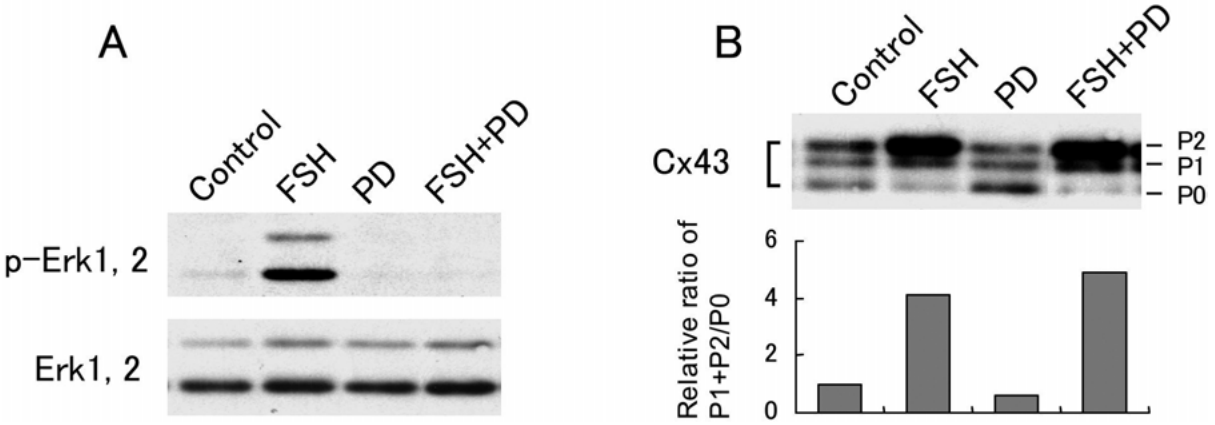


**Fig. 3.** Effect of a MEK inhibitor on Cx43 phosphorylation. (A) Granulosa cells were treated for 4 h with 50 $\mu$M of PD98059 under conditions with or without FSH, and subjected to Western blot analysis with anti-ERK antibody or anti-phosphorylated ERK antibody. (B) Granulosa cells were treated as in (A) and subjected to Western blot analysis with anti-Cx43 antibody (*upper panel*). The ratio of P1 + P2 to P0 was measured as in Fig. 1 (*lower panel*).

protein serine kinases that are known to be activated downstream of FSH signaling. As shown in Fig. 2, we confirmed that FSH increased hyperphosphorylation of Cx43 (P1 and P2) as previously reported; three bands (P0, P1, and P2) were observed by Western blotting throughout the incubation period, and a characteristic feature of the FSH-stimulated cells was a significant elevation in the levels of the P2 form after 4 h. With regard to this, when immunoprecipitated Cx43 was treated with intestine phosphatase, the slowly migrating bands (P1 and P2) disappeared and consequently, the intensity of P0 increased, implying that the appearance of the slower bands was due to hyperphosphorylation (data not shown). When H89, a PKA inhibitor, was added to the culture medium in the presence of FSH, phosphorylation appeared to be significantly suppressed, indicating a major role for a PKA-dependent pathway (Fig. 2). Since the level seen after H89 treatment appeared to be lower than that in the control cells, PKA could also play a role in FSH-unstimulated cells. In contrast, staurosporin (ST), an inhibitor of $Ca^{2+}$-dependent protein kinase (PKC), enhanced FSH-induced hyperphosphorylation. With regard to this, we found that 12-O-tetradecanoylphorbol-13-acetate, a PKC activator, decreased the level of phosphorylated Cx43 in rat primary granulosa cells (data not shown), suggesting that the PKC pathway plays a negative role in phosphorylation of Cx43.

*ERK is dispensable in phosphorylation of Cx43*

The above results showed that a PKA-dependent pathway played a major role in phosphorylation of Cx43 induced by stimulation with FSH. In this regard, extracellular signal-regulated kinase (ERK) was reported to phosphorylate Cx43 *in vivo* and *in vitro* [11, 13, 20], and FSH is also known to activate ERK in rat granulosa cells downstream from the PKA pathway [21]. We then tested the effect of PD98059, a mitogen-activated protein kinase kinase (MEK) inhibitor, on the level of phosphorylated Cx43 after FSH treatment. Although activation of ERK1/2 could be confirmed (Fig. 3A), PD98059 showed no effect on the level of phosphorylated Cx43 under the conditions used (Fig. 3B), suggesting that ERK activity was not essential for FSH-dependent phosphorylation of Cx43.

*Phosphorylation of Cx43 in vitro*

To further confirm the involvement of PKA in phosphorylation of Cx43, we carried out an *in vitro* phosphorylation reaction using a recombinant Cx43 carboxy-terminal domain (Cx43-CT). We previously found that recombinant Cx43-CT could be phosphorylated *in vitro* using rat granulosa cell lysate, and the sites of phosphorylation were identical to those observed *in vivo* [16]. We, therefore, expressed and purified 6x Histidine-tagged Cx43-CT (Fig. 1) as reported previously, and examined whether H89 treatment affected phosphorylation *in vitro*. As shown in Fig. 4, the addition of H89 to the reaction mixtures

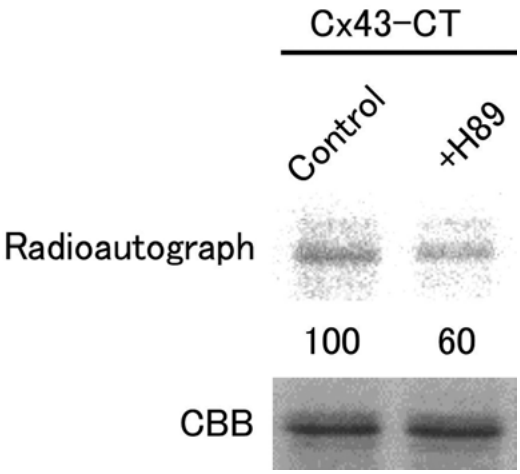


**Fig. 4.** Effect of H89 on Cx43-CT phosphorylation *in vitro*. The effects of H89 on phosphorylation reactions *in vitro* were examined using Cx43-CT as a substrate. The recombinant Cx43-CT was mixed with lysates from FSH-treated granulosa cells in the presence or absence of 50 $\mu$M of H89. Reaction products were separated by SDS-PAGE and monitored by radioautography (*upper panel*). The gel was then stained with CBB, and the amount of Cx43-CT was determined (*lower panel*). The radioactivity of each lane was normalized to the protein concentration. The relative values against lane 1 are shown under the upper panel.

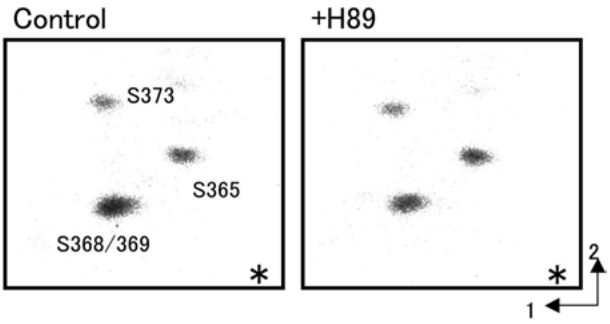


**Fig. 5.** Two-dimensional tryptic peptide mapping of Cx43-CT phosphorylated *in vitro*. Recombinant Cx43-CT was phosphorylated as in Fig. 4, then digested with TPCK-trypsin. The resulting peptides were separated as described in "Materials and Methods". Three major phosphopeptide spots corresponded to S365, S368/369, and S373 [16]. The same amount of radioactivity was applied to each sample. Representative findings of three separate experiments are shown. The origin is marked by an asterisk. Arrows 1 and 2 indicate the first dimension of electrophoresis and second dimension of the ascending chromatography, respectively.

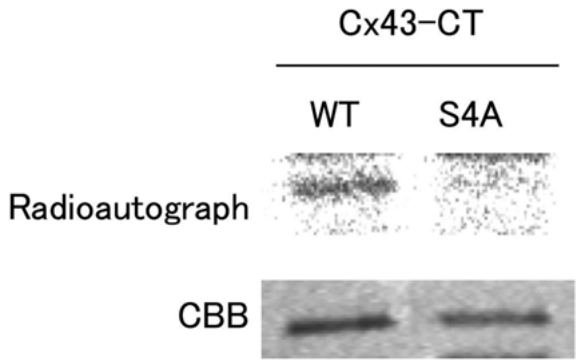


**Fig. 6.** *In vitro* phosphorylation reactions with the catalytic subunit of PKA. One unit of the catalytic subunit of PKA was incubated with either the Cx43-CT or Cx43-CTS4A in which Ser365, Ser368, Ser369, and Ser373 were substituted with alanine. Reaction products were visualized as in Fig. 4.

significantly reduced the level of phosphorylated Cx43-CT. Tryptic peptide mapping of the thus labeled Cx43-CT showed no significant changes in the mapping profiles among the H89-treated and untreated conditions (Fig. 5), indicating that PKA had no selectivity among the four serine residues, but that it could be responsible for phosphorylation of all of the residues. To further confirm the involvement of PKA, we performed *in vitro* phosphorylation reactions using the purified catalytic subunit of PKA and either Cx43-CT or Cx43-CTS4A in which Ser365, Ser368, Ser369, and Ser373 residues were substituted with alanine (Fig. 6). As a result, the potential of Cx43-CT as a substrate could be observed, and no labeling was detected with Cx43-CTS4A, indicating that these four serine residues were actually target sites for PKA.

## Discussion

We and others have reported that expression of connexin 43 mRNA in granulosa cells is significantly enhanced by pregnant mare's serum gonadotropin (PMSG) and down-regulated with the additional administration of human chorionic gonadotropin (hCG) [9, 22]. FSH is the central hormone of the female reproductive system [23], and the crucial role played by FSH signaling in the female reproductive system has been illustrated by

the mouse phenotype carrying a targeted mutation in the FSH-$\beta$ subunit gene [24, 25] and by the effects of mutations in mouse and human FSH receptor molecules [26–28]. Namely, FSH-deficient female mice were infertile due to a block in folliculogenesis prior to formation of the antral follicle [24]. In a similar fashion, the indispensable role of gap junctions in the female reproductive process has been demonstrated recently by targeted disruption of certain connexin genes [29–32]. Defects in the development of oocytes and follicles have been reported in Cx37 [32, 33] and Cx43 [34, 35] knockout mice, respectively. Notably in the absence of Cx43, granulosa cells stop growing at an early preantral stage, and in Cx43 null mice, the development of follicles arrests in the preantral stage [36]. Gap junctional intercellular communication mediated by Cx43 thus seems to play indispensable roles in both germ line development and postnatal folliculogenesis, and understanding how FSH regulates Cx43-mediated GJC is important.

Signaling mechanisms downstream of the FSH receptor have been studied well. The FSH receptor is a G protein-coupled receptor, once bound with FSH, it activates adenylyl cyclase leading to production of cAMP. Then, cAMP activates the PKA-dependent pathways involving ERK and the transcription factor cyclic AMP response element binding protein (CREB), as well as PKA-independent pathways involving protein kinase B (PKB/Akt), serum and glucocorticoid-induced kinase (SGK), and p38 through cAMP-regulated guanine nucleotide exchange factor (cAMP-GEF) [37]. We demonstrated in this study that enhanced phosphorylation of Cx43 induced by stimulation with FSH in rat granulosa cells depends on the PKA pathway, while the activity of ERK was dispensable. Moreover, the catalytic subunit of PKA could phosphorylate Cx43-CT, at least *in vitro*, further supporting this notion. However, since H89 treatment could not inhibit phosphorylation of Cx43 completely *in vivo* or *in vitro*, the possibility of involvement of other kinase(s) cannot be excluded.

Various kinds of protein kinases have been shown to be involved in phosphorylation of Cx43 [38] in addition to PKA as shown in this study. Phosphorylation of connexin has been implicated in the regulation of gap junctional communication at multiple levels, including trafficking, assembly/disassembly, stability, and channel activity. For example, phosphorylation of Y265 by nonreceptor-type tyrosine kinase Src is known to lead to an inhibition of intercellular communication [39]. Further, phosphorylation of S255/279/282 by mitogen-activated protein kinase (MAPK) also disrupts gap junctional communication [11, 13]. We previously found that FSH accelerated trafficking of Cx43 and accumulation of this protein in gap junctional plaques accompanying phosphorylation in rat granulosa cells [40]. We further identified Ser365, Ser368, Ser369, and Ser373 as major sites of phosphorylation, and revealed the significance of serine phosphorylation in Cx43-mediated channel activity [16]. Namely, when all four serine residues were substituted with alanine, Cx43 appeared to lose dye transfer activity. We demonstrated in this study that these four serine residues could be the target sites of PKA. Therefore, it may be reasonable to suggest that PKA positively regulates Cx43-mediated gap junctional communication in the presence of FSH through phosphorylation of Ser365, Ser368, Ser369, and Ser373.

Finally, we showed in this study the relevance of FSH/PKA signaling to the regulation of Cx43-mediated gap junctional communication in granulosa cells. What is the significance of this finding on ovarian folliculogenesis and/or oogenesis? With regard to this, Cx37 is believed to be the only connexin protein supplied from oocytes for the gap junctional channel connecting granulosa cells and oocytes [33]. Supporting this, Gittens and Kidder recently reported using reconstituted chimeric ovaries from Cx37- or Cx43-deficient mice that expression of Cx37 in oocytes, but not granulosa cells, was prerequisite for follicular development [41]. Multiple connexins including Cx43, however, are known to be expressed in granulosa cells [9, 10], and the counterpart of Cx37 functioning in the contact region of granulosa cells and oocytes is still unknown. Clarifying the exact localization of Cx43 and its possible formation of a heterotypic gap junction with Cx37 in this region would thus be required to extend our understanding of the mechanisms of oocyte growth and maturation in ovarian follicles.

## References


1. **Goodenough DA, Goliger JA, Paul DL**. Connexins, connexons, and intercellular communication. *Annu Rev Biochem* 1996; 65: 475–502.
2. **Kumar NM, Gilula NB**. The gap junction communication channel. *Cell* 1996; 84: 381–388.
3. **Martin PEM, Evans WH**. Incorporation of connexins into plasma membranes and gap junctions. *Cardiovasc Res* 2004; 62: 378–387.
4. **Willecke K, Eiberer J, Degen J, Eckardt D, Romualsi A, Guldenagel M, Deutsch U, Sohl G**. Structural and functional diversity of connexin genes in the mouse and human genome. *Biol Chem* 2002; 383: 725–737.
5. **Eppig JJ**. Oocyte-somatic cell communication in the ovarian follicles of mammals. *Semin Dev Biol* 1994; 5: 51–59.
6. **Kidder GM, Mhawi AA**. Gap junctions and ovarian folliculogenesis. *Reproduction* 2002; 123: 613–620.
7. **Heller DT, Cahill DM, Schultz RM**. Biochemical studies of mammalian oogenesis: metabolic cooperativity between granulosa cells and growing mouse oocytes. *Dev Biol* 1981; 84: 455–464.
8. **Brower PT, Schultz RM**. Intercellular communication between granulosa cells and mouse oocytes: existence and possible nutritional role during oocyte growth. *Dev Biol* 1982; 90: 144–153.
9. **Itahana K, Morikazu Y, Takeya T**. Differential expression of four connexin genes, Cx-26, Cx30.3, Cx32, and Cx43, in the porcine ovarian follicle. *Endocrinology* 1996; 137: 5036–5044.
10. **Itahana K, Tanaka T, Morikazu Y, Komatu S, Ishida N, Takeya T**. Isolation and characterization of a novel connexin gene, Cx-60, in porcine ovarian follicles. *Endocrinology* 1998; 139: 320–329.
11. **Warn-Cramer BJ, Lampe PD, Kurata WE, Kanemitsu MY, Loo LW, Eckhart W, Lau AF**. Characterization of the mitogen-activated protein kinase phosphorylation sites on the connexin-43 gap junction protein. *J Biol Chem* 1996; 271: 3779–3786.
12. **Laing JG, Tadros PN, Westphale EM, Beyer EC**. Degradation of connexin 43 gap junctions involves both the proteasome and the lysosome. *Exp Cell Res* 1997; 236: 482–492.
13. **Warn-Cramer BJ, Cottrell GT, Burt JM, Lau AF**. Regulation of connexin-43 gap junctional intercellular communication by mitogen-activated protein kinase. *J Biol Chem* 1998; 273: 9188–9196.
14. **Zhou L, Kasperek EM, Nicholson BJ**. Dissection of the molecular basis of $pp60^{v\text{-}src}$ induced gating of connexin 43 gap junciton channels. *J Cell Biol* 1999; 144: 1033–1045.
15. **Akiyama M, Ishida N, Ogawa T, Yogo K, Takeya T**. Molecular cloning and functional analysis of a novel Cx43 partner protein CIP150. *Biochem Biophys Res Commun* 2005; 335: 1264–1271.
16. **Yogo K, Ogawa T, Akiyama M, Ishida N, Takeya T**. Identification and functional analysis of novel phosphorylation sites in Cx43 in rat primary granulosa cells. *FEBS Lett* 2002; 531: 132–136.
17. **Ogawa T, Yogo K, Ishida N, Takeya T**. Synergistic effects of activin and FSH on hyperphosphorylation of Rb and G1/S transition in rat primary granulosa cells. *Mol Cell Endocrinol* 2003; 210: 31–38.
18. **Chijiwa T, Mishima A, Hagiwara M, Sano M, Hayashi K, Inoue T, Naito K, Toshioka T, Hidaka H**. Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(*p* bromocinnamylamino)-ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. *J Biol Chem* 1990; 265: 5267–5272.
19. **Sato M, Kato JY, Takeya T**. Characterization of partly activated $p60^{c\text{-}src}$ in chicken embryo fibroblasts. *J Virol* 1989; 63: 683–688.
20. **Kanemitsu MY, Lau AF**. 1993. Epidermal growth factor stimulates the disruption of gap junctional communication and connexin 43 phosphorylation independent of 12-O-tetradeca-noylphorbol 13-acetate-sensitive protein kinase C: the possible involvement of mitogen-activated protein kinase. *Mol Biol Cell* 1993; 4: 837–848.
21. **Das S, Maizels ET, DeManno D, St Clair E, Adam SA, Hunzicker-Dunn M**. A stimulatory role of cyclic adenosine 3′, 5′-monophosphate in follicle-stimulating hormone-activated mitogen-activated protein kinase signaling pathway in rat ovarian granulosa cells. *Endocrinology* 1996; 137: 967–974.
22. **Granot I, Dekel N**. Phosphorylation and expression of connexin-43 ovarian gap junction protein are regulated by luteinizing hormone. *J Biol Chem* 1994; 269: 30502–30509.
23. **Bousfield GR, Perry WM, Ward DN**. Gonadotropins: chemistry and biosynthesis. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction. Raven Press: New York: 1994; 1749–1792.
24. **Kumar TR, Wang Y, Lu N, Matzuk MM**. Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nat Genet* 1997; 15: 201–204.
25. **Burns KH, Yan C, Kumar TR, Matzuk MM**. Analysis of ovarian gene expression in follicle-stimulating hormone $\beta$ knockout mice. *Endocrinology* 2001; 142: 2742–2751.
26. **Aittomaki K, Lucena JLD, Pakarien P, Sistonen P, Tapanainen J, Gromoll J, Kaskikari R, Sankila EM, Lehvaslaiho H, Engel AR, Nieschlag E, Huhtaniemi I, de la Chapelle A**. Mutation in the follicle-stimulating hormone receptor gene causes hereditary hypergonadotropic ovarian failure. *Cell*

1995; 82: 959–968.
27. **Dierich A, Sairam MR, Monaco L, Fimia GM, Gansmuller A, LeMeur M, Sassone-Corsi P**. Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. *Proc Nat Acad Sci USA* 1998; 95: 13612–13617.
28. **Layman LC, McDonough PG**. Mutations of follicle stimulating hormone-$\beta$ and its receptor in human and mouse: genotype/phenotype. *Mol Cell Endocrinol* 2000; 161: 9–17.
29. **Elvin JA, Matzuk MM**. Mouse models of ovarian failure. *Rev Repro* 1998; 3: 183–195.
30. **Simon AM, Goodenough DA**. Diverse functions of vertebrate gap junctions. *Trends Cell Biol* 1998; 8: 477–482.
31. **White T, Paul DL**. Genetic diseases and gene knockouts reveal diverse connexin functions. *Annu Rev Physiol* 1999; 61: 283–310.
32. **Carabatos MJ, Sellitto C, Goodenough DA, Albertini DF**. Oocyte-granulosa cell heterologous gap junctions are required for the coordination of nuclear and cytoplasmic meiotic competence. *Dev Biol* 2000; 226: 167–179.
33. **Simon AM, Goodenough DA, Li E, Paul DL**. Female infertility in mice lacking connexin37. *Nature* 1997; 385: 525–529.
34. **Ackert CL, Gittens JEI, O'Brien MJ, Eppig, JJ, Kidder GM**. Intercellular communication via connexin43 gap junctions is required for ovarian folliculogenesis in the mouse. *Dev Biol* 2001; 233: 258–270.
35. **Juneja SC, Barr KJ, Enders GC, Kidder GM**. Defects in the germ line and gonads of mice lacking connexin43. *Biol Reprod* 1999; 60: 1263–1270.
36. **Gittens J, Mhawi AA, Lidington D, Oullette Y, Kidder GM**. Functional analysis of gap junctions in ovarian granulosa cells: distinct role for connexin 43 in early stages of folliculogenesis. *Am J Physiol Cell Physiol* 2003; 284: C880–C887.
37. **Richards JS**. New Signaling pathways for hormones and cyclic Adenosine 3′,5′-monophosphate action in endocrine cells. *Mol Endocrinol* 2001; 15: 209–218.
38. **Lampe PD, Lau AF**. The effects of connexin phosphorylation on gap junctional communication. *Int J Biochem Cell Biol* 2004; 36: 1171–1186.
39. **Lin R, Warn-Cramer BJ, Kurata WE, Lau AF**. v-Src phosphorylation of connexin 43 on Tyr247 and Tyr265 disrupts gap junctional communication. *J Cell Biol* 2001; 154: 815–827.
40. **Yogo K, Ogawa T, Akiyama M, Ishida N, Takeya T**. Gap junctions in ovarian folliculogenesis. In: Sato E, Miyamoto H, Manabe N (eds.), Animal Frontier Sciences: Life Science Update in Animal Science. Kyoto; Hokuto Shobo; 2003: 97–102.
41. **Gittens JE, Kidder GM**. Differential contributions of connexin37 and connexin43 to oogenesis revealed in chimeric reaggregated mouse ovaries. *J Cell Sci* 2005; 118: 5071–5078.