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Colocalization of connexin 43 and connexin 45 but absence of connexin 40 in granulosa cell gap junctions of rat ovary

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The expression and localization of gap junction family proteins (connexins) were examined in nonstimulated and gonadotrophin-stimulated ovarian follicles of immature rats. Immunoblot and RNA blot analysis showed the presence of connexin (Cx) 43, Cx40 and Cx45 in ovarian tissue. Of these connexin proteins, Cx43 and Cx45 were identified by immunofluorescent microscopy between granulosa cells in characteristic expression patterns related to follicular developmental stages, while Cx40 was not expressed in granulosa cells but was detected in blood vessels in ovarian stroma. In some plaques of gap junction between granulosa cells, Cx45 was found to be colocalized with Cx43. In immunofluorescent microscopy, the expression of Cx43 was increased with follicular growth, but decreased after induction of ovulation by injection of human chorionic gonadotrophin. In contrast, the Cx45 protein was constantly expressed through follicular development; however, after ovulation, no staining of Cx45 was detected in the corpus luteum. Dual expression and the functional role of Cx43 and Cx45 in cell-to-cell communication in ovarian granulosa cells at various developmental stages were discussed.

Introduction

Gap junctions are aggregates of transmembrane channels, through which small molecules of less than 1 kDa can pass from the cytoplasm of one cell to its neighbours (Pitts and Simms, 1977; Bennett and Goodenough, 1978; Loewenstein, 1979). Intercellular communication through gap junctions may play an important role in the maintenance of homeostasis, embryonic differentiation and growth control in multicellular organisms (Loewenstein, 1988; Guthrie and Gilula, 1989). Each gap junction channel is formed by an interconnection of hemichannels in apposed membranes of adjacent cells. Hemichannels consist of hexamer subunit proteins, called connexins (Cx). To date, more than ten homologous connexin sequences have been cloned and characterized in the rodent genome (Willecke *et al.*, 1991).

The diversity of connexins in mammalian cells suggests that each connexin may be functionally specialized. Several mammalian tissues express more than one connexin gene. For example, rodent hepatocytes express Cx32 and Cx26 (Nicholson *et al.*, 1987; Kuraoka *et al.*, 1993), canine cardiomyocyte Cx43, Cx45 and Cx40 (Kanter *et al.*, 1992), mouse eye lens Cx43, Cx46 and Cx50 (White *et al.*, 1992), rat keratinocytes Cx26 and Cx43 (Kamibayashi *et al.*, 1993), and rat endometrium Cx26 and Cx32 (Winterhager *et al.*, 1991).

It is well known that gap junctions are well developed in avascular ovarian follicles. Intercellular communication via gap junctions may play an important role in signal transduction as well as in transport pathways of nutrition among granulosa cells and between oocyte and granulosa cells. Only Cx43 is expressed in rat ovarian granulosa cells of healthy developing follicles under the endogenous hormonal regulations (Risek *et al.*, 1990; Wiesen and Midgley, 1993, 1994). The mRNA and protein content of the Cx43 gap junction decrease markedly after the ovulatory surge of LH (Wiesen and Midgley, 1994).

Previous northern blot analyses have demonstrated the expression of Cx43 and Cx40 in rodent and canine ovary (Risek *et al.*, 1990; Beyer *et al.*, 1992), while it has been reported that mRNA encoding Cx45 is absent in the ovary (Hennemann *et al.*, 1992a). The expression and distribution of Cx45 and Cx40 protein have not been examined in detail in ovarian tissue (Beyer *et al.*, 1992; Hennemann *et al.*, 1992a; Kanter *et al.*, 1992). In this study, we raised antibodies directed against a polypeptide of carboxy-terminal amino acids from canine Cx45 and rat Cx40 protein. By using these type specific antibodies, we have examined both the expression and the localization of Cx40, Cx43 and Cx45 in rat ovary. Cx protein expression patterns were followed in the follicular developmental stage after injection of human menopausal gonadotrophin (hMG) and hCG. Expression of Cx43 was increased as follicles matured and decreased after ovulation, while Cx45 was constantly expressed through follicular development. These two Cx proteins colocalized partially in ovarian granulosa cells. These data suggest that each connexin plays a distinct role in the development of ovarian follicles and the signal transduction between granulosa cells.

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Materials and Methods

Animals

Female premature Wistar rats (21 days of age) were permitted to feed and drink *ad libitum*. They were injected i.p. with 15 iu of hMG (Nikken, Tokyo). After 48 h, some rats were killed under anaesthesia by ether; others were injected intraperitoneally with 20 iu of hCG (Teikokuzoki, Tokyo), and killed 1 h or 5 h later under ether anaesthesia. The ovaries were immediately removed and processed for immunofluorescence microscopy and immunoblotting, or frozen in liquid nitrogen for isolation of RNA.

Preparation and purification of site-directed antibodies

Site-specific antibodies for Cx40 and Cx45 were prepared against synthetic oligopeptide sequences corresponding to the carboxy-terminal amino acid residues 334–345 of rat Cx40 [GYHSDKRRLSKA] (Beyer *et al.*, 1992), or residues 385–396 of canine Cx45 [SKSGDGKNSVWI] (Kanter *et al.*, 1992). Japanese white rabbits were immunized three times at 2- or 3-week intervals with 1 mg of the synthetic peptide which was conjugated to keyhole limpet haemocyanin (KLH; Calbiochem, La Jolla, CA) through bifunctional cross-linker GMBS (N-γ-maleimidobutyryloxysuccinimide ester; Calbiochem). The peptides (1 ml) were mixed with about the same volume of Freund's complete adjuvant (Difco Laboratories, Detroit, MI) and injected s.c. at multiple sites on the back. Antisera were collected 1–2 weeks after the final injection. Specific immunoglobulins from the antisera were affinity-purified by using the antigen peptides coupled to Affigel-10 (Bio-Rad Laboratories, Richmond, CA) according to the supplier's protocol. Affinity-purified anti-Cx40 or anti-Cx45 antibodies were concentrated using Centriprep concentrators (Amicon, MA) and stored at −40°C. The specificity of the antibodies was examined by immunoblot analysis.

Monoclonal mouse anti-Cx43 antibody (Zymed Laboratories, San Francisco, CA) was used for immunoblot analysis and immunocytochemistry.

Preparation of Triton-insoluble fractions

Ovaries of the premature rats were homogenized on ice by Polytron in homogenization buffer (1 mmol $NaHCO_3$ l^{-1} (pH 7.2), 1 mmol phenylmethylsulfonyl fluoride l^{-1} (PMSF), 100 mmol iodoacetamide l^{-1} (IAA), 10 mmol *N*-ethylmaleimide l^{-1} (NEM)). After centrifugation at 100 000 ***g*** for 20 min at 2°C, the pellet was dissolved in homogenization buffer supplemented with 1% (v/v) Triton-X100, and centrifuged at 100 000 ***g*** for 20 min at 2°C to separate Triton-insoluble fractions containing gap junction plaques (Musil and Goodenough, 1993). Finally, the pellet was sonicated in homogenization buffer supplemented with 20 mmol NaOH l^{-1} and then centrifuged at 100 000 ***g*** for 20 min at 2°C (Hertzberg, 1984). The pellet was eluted with 100 mmol Tris–HCl l^{-1} (pH 7.0), 0.1% (w/v) SDS. Amounts of proteins were determined by a bicinchorinic acid (BCA) protein assay reagent kit (Pierce Chemical, Rockford, IL) and stored at −40°C.

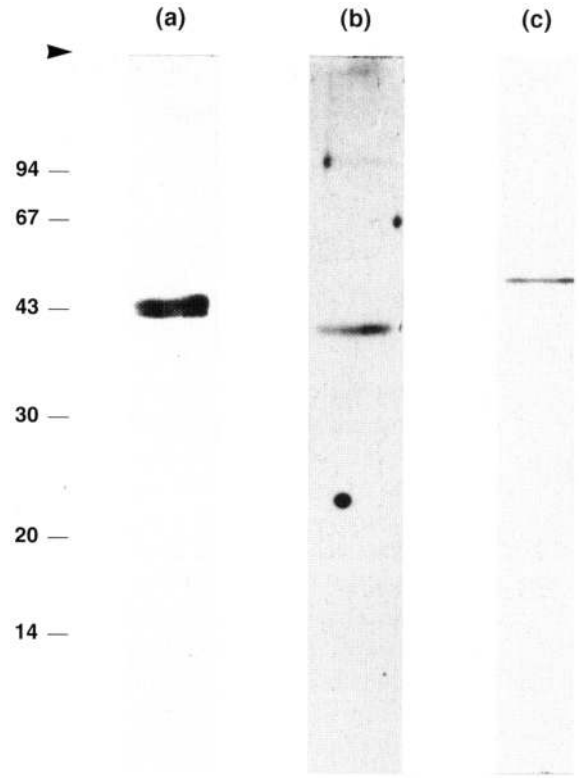


Fig. 1. Immunoblot analysis of rabbit antibodies raised against residues 334–345 of rat Cx40 and residues 385–396 of canine Cx45. Each well of electrophoresis gel was loaded with 10 μg protein of Triton-alkali extracted rat atria. Replicas were probed with (a) mouse monoclonal anti-Cx43 antibody, (b) with rabbit polyclonal anti-Cx40 antibody, (c) with rabbit polyclonal anti-Cx45 antibody. Immunoreactive bands were visualized by ECL system (Amersham) after labelling with HRP-conjugated secondary antibodies. Mobility of molecular mass standard is indicated in kilodaltons. In (a), the upper band of double bands crossreacted with anti-Cx43 antibody may represent a phosphorylated form of Cx43.

SDS-PAGE and immunoblot analysis

Samples were dissolved in Laemmli buffer (Laemmli, 1970), and incubated at 37°C for 30 min to avoid aggregation of gap junction proteins. SDS-PAGE was performed according to the method of Laemmli in 12% gel (Laemmli, 1970), and proteins were visualized with silver stain (Sil-Best stain, Nacalai tesque, Kyoto).

For immunoblot analysis, proteins separated by electrophoresis were transferred electrophoretically to nitrocellulose sheets at 100 V for 1 h. After blocking with 5% (w/v) skimmed milk (Difco) in PBS containing 0.1% (v/v) Tween-20 (T-PBS/milk) for 1 h, the blots were incubated with primary antibodies diluted at 1 μg ml^{-1} with T-PBS/milk at room temperature for 1 h. They were then washed four times in T-PBS for 15 min each, blocked again for 15 min, and incubated for 1 h at room

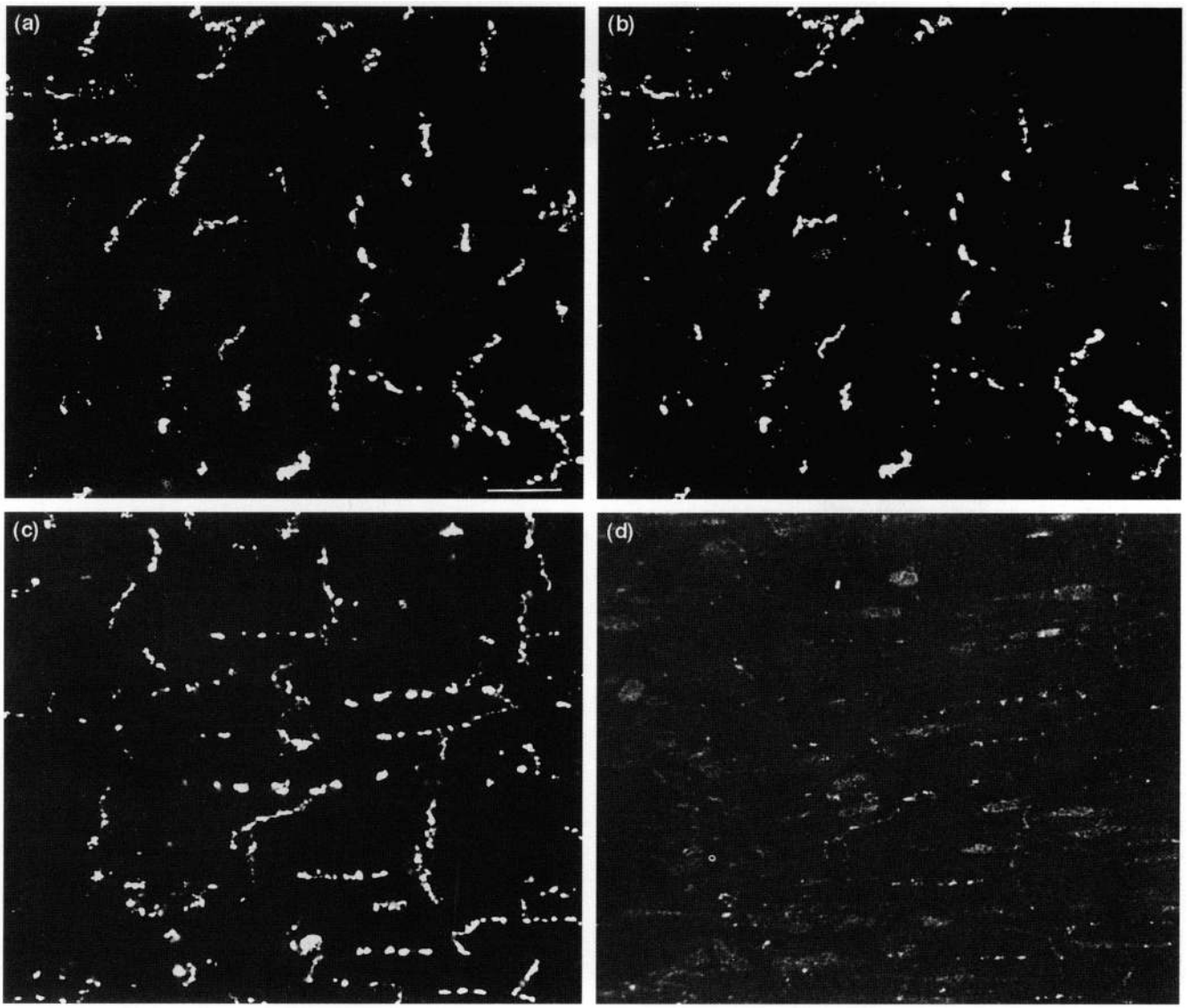


Fig. 2. Double immunolabelling of rat heart sections stained either with (a) anti-Cx43 and (b) anti-Cx40 antibodies or with (c) anti-Cx43 and (d) anti-Cx45 antibodies. Primary antibodies were visualized with fluorescein isothiocyanate-labelled anti-mouse IgG (a, c) or rhodamine isothiocyanate (RITC)-labelled anti-rabbit IgG (b, d). Cx40 and Cx45 are colocalized with Cx43 in intercalated disc of rat myocardium. Scale bar represents 20 μm.

temperature with horseradish peroxidase (HRP)-labelled goat anti-rabbit or anti-mouse IgG (Bio-Rad Laboratories) diluted 1:4000 with T-PBS/milk at room temperature. The localization of peroxidase was detected using enhanced chemiluminescence (ECL) western blotting detection reagents (Amersham), and exposed to Hyperfilm (Amersham). The following proteins were used as molecular mass standards (Pharmacia, Uppsala): phosphorylase b (mol. wt 94 000); BSA (67 000); ovalbumin (43 000); carbonic anhydrase (30 000); soybean trypsin inhibitor (20 100); and α-lactalbumin (14 400).

Immunofluorescence microscopy

For indirect immunohistochemistry, whole tissues of immature rat ovary were frozen in liquid nitrogen-cooled OCT compound (Tissue Tek; Miles, Elkhart, IN). Sections, 3–4 μm thick, were mounted on glass slides, immersed in absolute acetone at −20°C for 5 min, rinsed in PBS three times for 5 min each, and then incubated with 1% (w/v) BSA in PBS for 20 min at room temperature to reduce nonspecific binding. They were incubated for 1 h at room temperature with a mixture of affinity-purified rabbit polyclonal anti-Cx40 (10 μg ml^{-1}) or anti-Cx45 (50 μg ml^{-1}) and monoclonal anti-Cx43 (10 μg ml^{-1}). After three rinses in PBS, the sections were incubated for 1 h at room temperature with a mixture of goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) and goat anti-rabbit IgG conjugated to rhodamine (Tago, Burlingame, CA) at 1:50 dilution in BSA–PBS, and then washed three times in PBS for 5 min each. Samples were examined on a

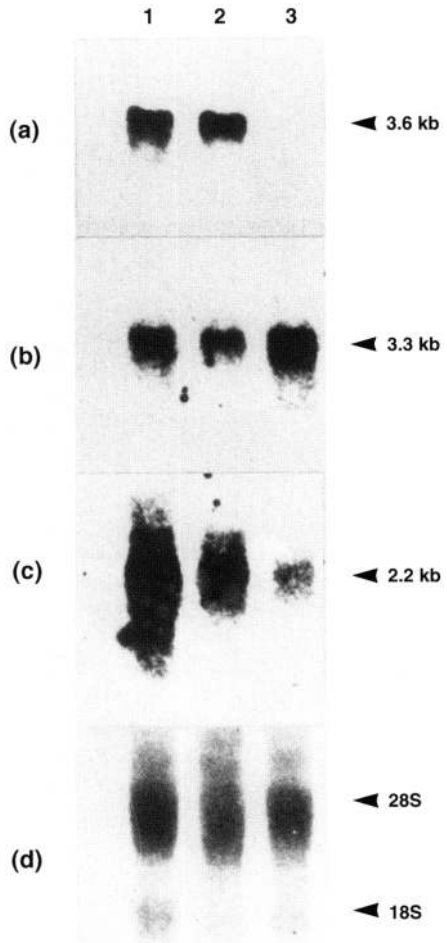


Fig. 3. Northern blot analysis in 21-day-old human menopausal gonadotrophin-stimulated rat ovary, heart and lung. Total RNAs (20 μg) were fractionated by electrophoresis in a 1.5% agarose-formaldehyde gel and hybridized with [α-^{32}P] dCTP-labelled probes against (a) Cx43, (b) Cx40 and (c) Cx45. (d) Methylene blue staining pattern of ribosomal RNAs transferred to nylon membrane. Lane 1, rat hMG-stimulated ovary; lane 2, rat heart; lane 3, rat lung.

confocal laser scanning microscope (LSM-GB200) (Olympus, Tokyo).

Northern blot analysis

Total RNA was isolated from immature rat ovary, adult rat heart and lung using a single-step thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987).

The concentration of RNA was determined by measuring absorption at 260 nm. Twenty micrograms of RNA were run on a 1.5% agarose/formaldehyde gel containing 0.2 μg ethidium bromide ml^{-1}. Gels were capillary-blotted in 20 × standard saline citrate (SSC) onto nylon membranes (Hybond N; Amersham), and fixed by baking for 2 h at 80°C in a vacuum oven. Membranes were pretreated with 50% (v/v) formamide, 5 × SSC, 0.01% (w/v) salmon sperm DNA (Sigma, St Louis, MO), 0.05% (w/v) SDS, 5 × Denhardt's reagent (0.1% (w/v) Ficoll (Type 400; Pharmacia), 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) BSA (Fraction V; Sigma)) at 42°C for 2 h. The blots were then hybridized overnight in the same buffer with [α-^{32}P] dCTP (Du Pont/NEN Research Products, Boston, MA) labelled probes. Stringent washes were repeated for 10 min at room temperature with 2 × SSC and 0.1% (w/v) SDS twice, followed by 10 min washes at 60°C with 1 × SSC and 0.1% SDS twice, and finally by 3 h washes at 60°C with 0.1 × SSC and 0.1% SDS twice. Autoradiographs were obtained by exposing the hybridized blot to imaging analyser BAS-2000 (Fujix, Tokyo). The following probes were used for hybridization: (1) Cx43 cDNA fragment (*Eco* R1, 1400 bp) (Beyer *et al.*, 1987), (2) mouse Cx45 cDNA fragment (*Eco* R1/XhoI, 2074 bp) (Hennemann *et al.*, 1992b), and (3) mouse Cx40 genomic DNA fragment (PstI, 1000 bp) (Hennemann *et al.*, 1992a).

Results

Characterization of site-directed rabbit anti-Cx40 and anti-Cx45 antibodies

Immunoblotting. The affinity-purified antibodies raised against residues 334–345 of rat Cx40 and residues 385–396 of canine Cx45 were characterized by separating the Triton-insoluble fraction of rat atrium on SDS-PAGE and transferring it to nitrocellulose sheets. Immunoblotting with anti-Cx43 antibody revealed that Cx43 protein was present as two bands running between 40 kDa and 45 kDa. The higher molecular mass band may represent the phosphorylated form of Cx43 (Fig. 1, lane a) (Musil *et al.*, 1990; Granot and Deckel, 1994). Immunoblotting with anti-Cx40 antibody revealed a single protein band with relative molecular mass (M_r) of 40 000 that migrated just below cardiac Cx43 (Fig. 1, lane b). Antibody against Cx45 labelled a single protein band with M_r of 45 000 that migrated just above cardiac Cx43 (Fig. 1, lane c). These two antibodies did not crossreact with other members of the Cx family proteins (data not shown).

Immunofluorescence microscopy. The specificity of the antibodies was further characterized by examining frozen sections of rat heart by immunofluorescence microscopy. The punctate or macular staining in the intercalated disc was recognized in the tissues stained with anti-Cx43, anti-Cx40 and anti-Cx45 antibodies (Fig. 2). Double-labelling experiments either with anti-Cx43 and anti-Cx40 antibodies (Fig. 2a, b) or with anti-Cx43 and anti-Cx45 antibodies (Fig. 2c, d), suggest that these immunoreactive sites are colocalized in intercalated disc. The expression of Cx45 was found to be lower than that of Cx43 (Fig. 2d).

Expression of Cx40, Cx43 and Cx45 in rat ovary

Northern blot analysis. Northern blot analysis was carried out to examine the expression of mRNA encoding Cx in ovary. Total RNA extracted from hMG-stimulated ovaries of immature rat, adult rat heart and lung was fractionated and hybridized with α-^{32}P-labelled cDNA probes of Cx43, Cx40 and Cx45. Under highly stringent conditions, a single

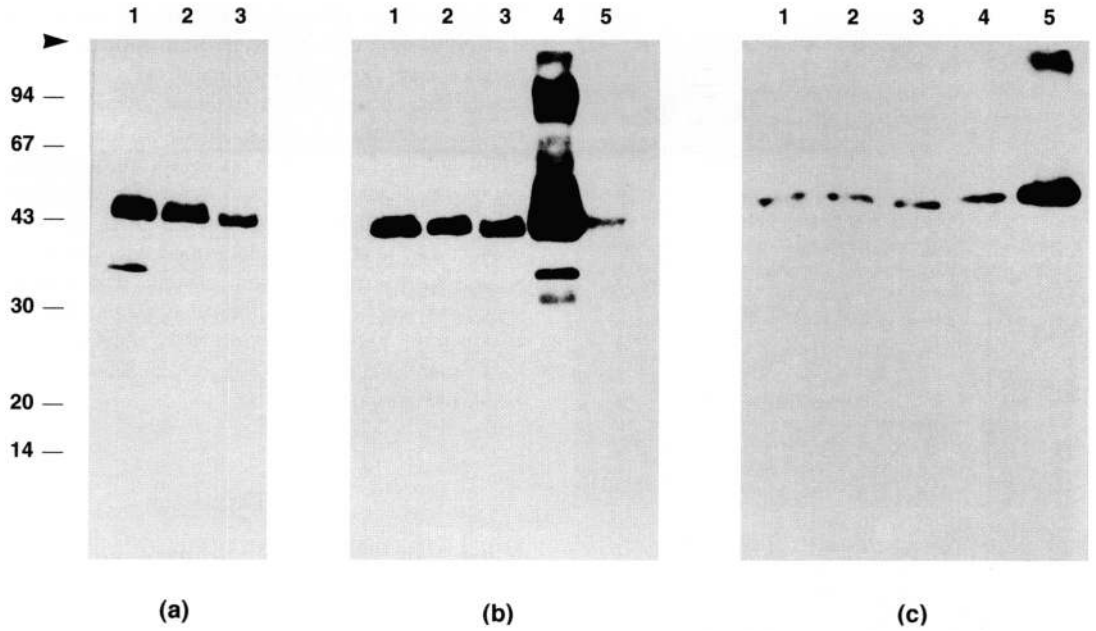


Fig. 4. Western blot analysis of nonstimulated, human menopausal gonadotrophin (hMG)-stimulated, and hMG–hCG-stimulated ovaries of 21-day-old rats. Protein extracts (10 μg per lane) were separated by SDS-PAGE and transferred to nitrocellulose. Each panel was reacted with (a) anti-Cx43, (b) anti-Cx40, or (c) anti-Cx45 antibodies. Lane 1, nonstimulated rat ovary; lane 2, hMG-stimulated rat ovary; lane 3, hMG–hCG-stimulated rat ovary; lane 4, rat lung; lane 5, rat atrium. The anti-Cx43 antibody recognizes a 43 kDa protein in rat ovary (a). We confirmed in another blot with shorter exposure that the 43 kDa broad band (a, lane 1) is caused by overlap of at least two bands that crossreacted with the anti-Cx43 antibody. The anti-Cx40 and anti-Cx45 antibodies recognized a 40 kDa band and a 45 kDa band, respectively, in rat ovary, lung, and heart (b and c). The expression of both Cx40 and Cx45 proteins in rat ovary are constant even after gonadotrophin stimulation. Mobility of molecular mass standards is indicated in kilodaltons on the left.

transcript of 3.6 kilobases (kb) of Cx43 was detected in ovary and heart (Fig. 3a), a 3.4 kb transcript of Cx40 was found in ovary, heart and lung (Fig. 3b), and a 2.2 kb transcript of Cx45 was found in ovary, heart and lung (Fig. 3c). These results demonstrate that mRNA encoding Cx43, Cx40 and Cx45 is expressed in ovaries.

Immunoblot analysis. Expression of Cx protein in rat ovaries was examined by extracting proteins from immature rat ovaries by Triton-alkali treatment and examining them by western blot analysis using anti-Cx43, anti-Cx40 and anti-Cx45 antibodies. As positive controls, protein extracts of adult rat lung and heart were used. In rat ovary, the anti-Cx43 antibody labelled a broad band which may be composed of nonphosphorylated and phosphorylated Cx43 proteins (Fig. 4a, lane 1; see also Fig. 1, lane a). Stimulation with hMG did not bring about a significant difference in Cx43 expression compared with the nonstimulated ovary (Fig. 4a, lane 2). In contrast, 5 h after hCG injection, reduced expression of the protein was detected (Fig. 4a, lane 3), which may have been due to disappearance of a slow migrating form of phosphorylated Cx43 protein. The anti-Cx40 and anti-Cx45 antibodies labelled a polypeptide band in the position of 40 kDa and 45 kDa, respectively, in all the samples examined (Fig. 4b, c). No significant changes were observed in the expression of either Cx40 or Cx45 among nonstimulated, hMG-stimulated, and hMG–hCG-stimulated ovaries (Fig. 4b, c, lanes 1, 2 and 3).

Immunolocalization of Cx40, Cx43 and Cx45 in the ovary. We defined the follicles in various developmental stages as follows. Secondary follicles were identified by the presence of an oocyte surrounded by two or more layers of follicular cells and by the absence of an antral cavity (Fig. 5a). Antral follicles were characterized by stratified granulosa cells and displacement of the oocyte to one side by the development of an eccentrically placed fluid-filled antral cavity in the mass of granulosa cells (Fig. 5b). In the 21-day-old rat ovary, numerous secondary follicles as well as a few antral follicles were observed. Human menopausal gonadotrophin prevents atresia of follicles at the pre-antral–antral boundary and promotes their continued development, which can result in a much greater number of antral follicles with various sized antra. hCG treatment brought about ovulation of mature Graafian follicles, but many follicles still remained as immature preantral follicles containing two or more layers of follicular cells.

Immunofluorescence microscopy was performed to examine the distribution patterns of Cx43, Cx40 and Cx45 in cryosection using hMG-stimulated rat ovary. The follicular developmental stage in each cryosection was examined under a light microscope after staining with methylene blue. In the secondary follicles stained with anti-Cx43 antibody, immunostaining appeared as punctate or macular fluorescence spots in discrete regions between adjacent granulosa cells (Fig. 6a). Similar fluorescence spots were detected in the granulosa cells of secondary follicles stained with anti-Cx45 antibody (Fig. 6b).

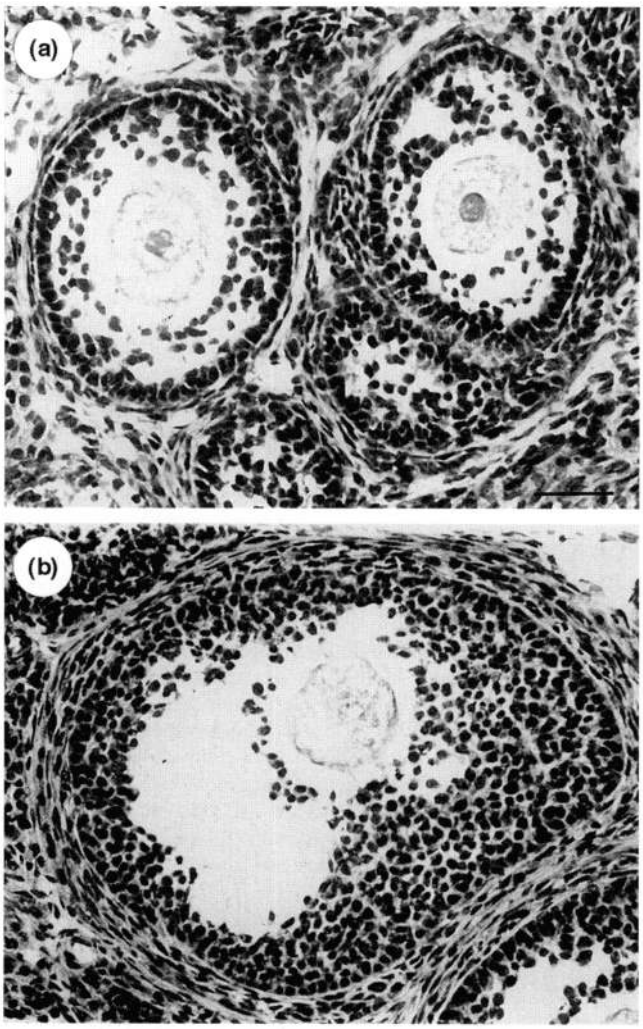


Fig. 5. Haematoxylin and eosin stain of 21-day-old immature rat ovary after injection with human menopausal gonadotrophin. In (a), secondary follicles contain an oocyte surrounded by two or more layers of follicular cells. In (b), antral follicles have the enlarged oocyte and the stratified granulosa cells around it. The oocyte is displaced by the enlargement of fluid-filled antral cavity in the mass of granulosa cells. Scale bar represents 20 μm.

The theca cells, which were identified as fusiform cells around the periphery of the follicle, contained a few punctate fluorescent spots of Cx43 and Cx45 (Fig. 6a–d).

In antral follicles, the granulosa cells displayed high expression of Cx43 (Fig. 6c) and also revealed immunofluorescence spots for Cx45 (Fig. 6d). After hCG treatment, the stromal cells of the postovulatory corpora lutea contained some punctate staining of Cx43 (Fig. 6e), but its expression was markedly decreased in comparison with that in preovulatory follicles and there were virtually no Cx45 signals (Fig. 6f). Immunofluorescence with preimmune rabbit sera showed no specific staining (data not shown). Double immunostaining using anti-Cx43 and anti-Cx45 antibody revealed that two connexins, Cx43 and Cx45, partially colocalized at some plaques in granulosa cells in antral follicles, but at other plaques only one type of Cx was identified (Fig. 7a–c).

The Cx40 gap junction protein was not detectable in granulosa cells at any follicular stage, but was detected exclusively between endothelial cells of blood vessels in ovarian stroma (Fig. 8b), which were not labelled with anti-Cx43 antibody (Fig. 8a).

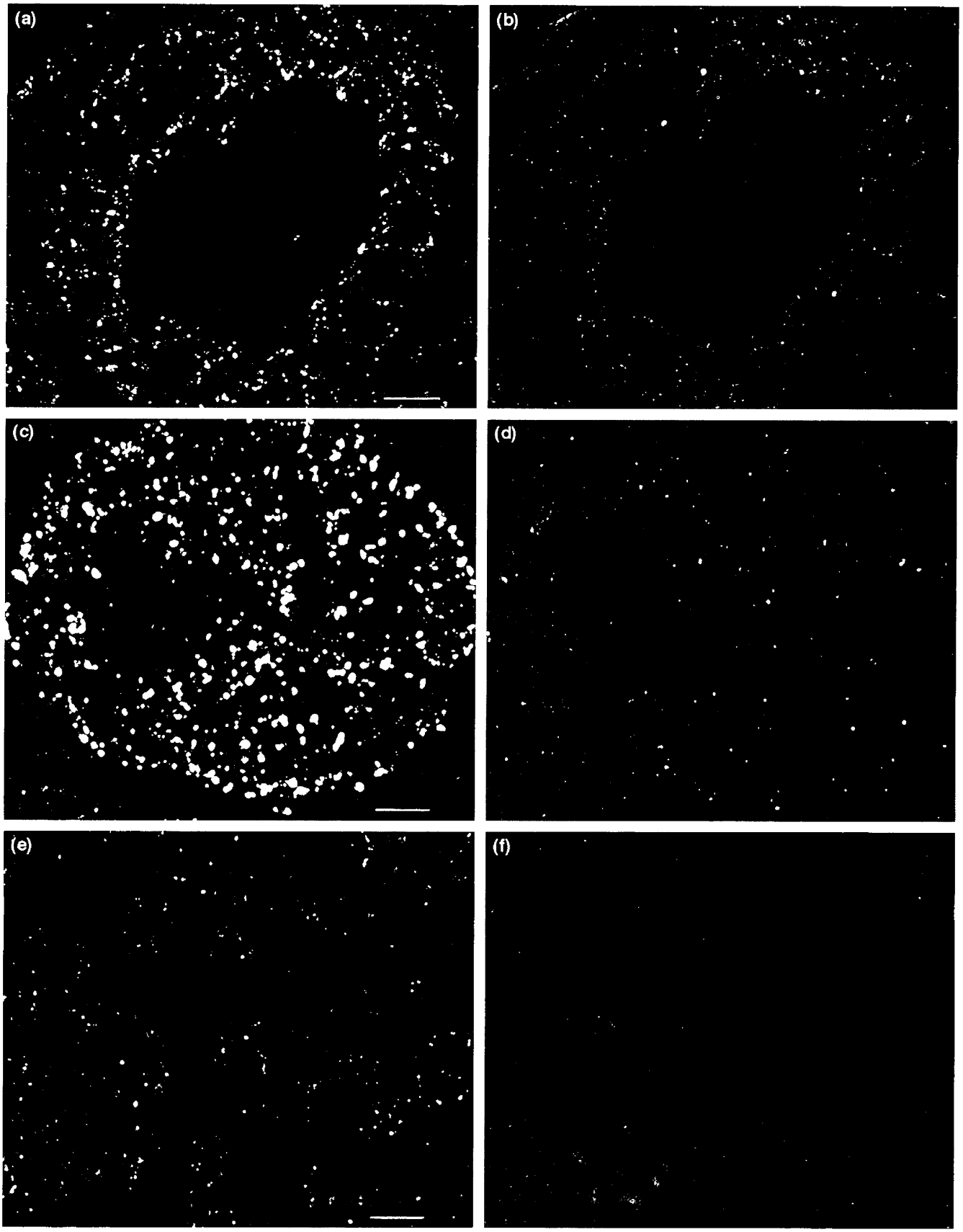
Discussion

In this study, we have detected the expression of Cx43, Cx40 and Cx45 by both western and northern blotting in whole ovarian tissues. In immunofluorescence analysis, however, Cx43 and Cx45 were found to be expressed in granulosa cells, while expression of Cx40 was too low to detect in granulosa cells and only detectable in vascular endothelium.

Previous reports have demonstrated that Cx45 is expressed in heart (Kanter *et al.*, 1992, 1993, 1994), mouse embryonic skin (Butterweck *et al.*, 1994a) and mouse kidney (Butterweck *et al.*, 1994b). The present study is the first report that Cx45, as well as Cx43, is expressed in ovarian granulosa cells. Immunofluorescence analysis indicated the expression of Cx45 in both immature secondary follicles and mature antral follicles. Although Beyer (1990) and Butterweck *et al.* (1994a) have reported that Cx45 is expressed more abundantly in embryonic immature tissues than in adult tissues; we failed to detect a marked change in Cx45 expression through the various developmental stages of ovarian follicles by immunocytochemistry. In addition, in western blot analysis, Cx45 expression remained constant in nonstimulated, hMG-stimulated, and hMG–hCG-stimulated ovarian tissues.

Cx43 and Cx45 were expressed in both immature and mature preovulatory follicles in the ovary. In addition, double immunostaining and confocal laser scanning microscopy showed that Cx43 and Cx45 were partially colocalized, within a single plaque, in the granulosa cells. Risek *et al.* (1994, 1995) have also provided supporting evidence that gap junction channels are composed of two different types of connexin within the same junctional plaque in rat uterine epithelium and embryonic epidermis. It has been reported that Cx45 gap junction channels are less permeable to ions and metabolites than are Cx43 channels in osteoblastic cells; that is, Cx43

Fig. 6. Double immunolabelling of frozen sections of 21-day-old human menopausal gonadotrophin-stimulated rat ovary stained with the monoclonal anti-Cx43 antibody (a, c, e), or the site-specific affinity-purified antibody against Cx45 antibody (b, d, f). Localization of these Cx proteins was visualized with fluorescein isothiocyanate-labelled anti-mouse IgG (a, c, e) or rhodamine isothiocyanate-labelled anti-rabbit IgG (b, d, f). Abundant punctate or macular immunofluorescent spots among granulosa cells are present in both secondary (a) and antral follicles (c) stained by anti-Cx43 antibody, while a few punctate immunofluorescent spots consistently appeared in both secondary (b) and antral (d) follicles stained with anti-Cx45 antibody. The stromal cells of postovulatory corpora lutea show decreased signals of Cx43 (e) and no Cx45 (f) protein. Scale bars represent 20 μm.



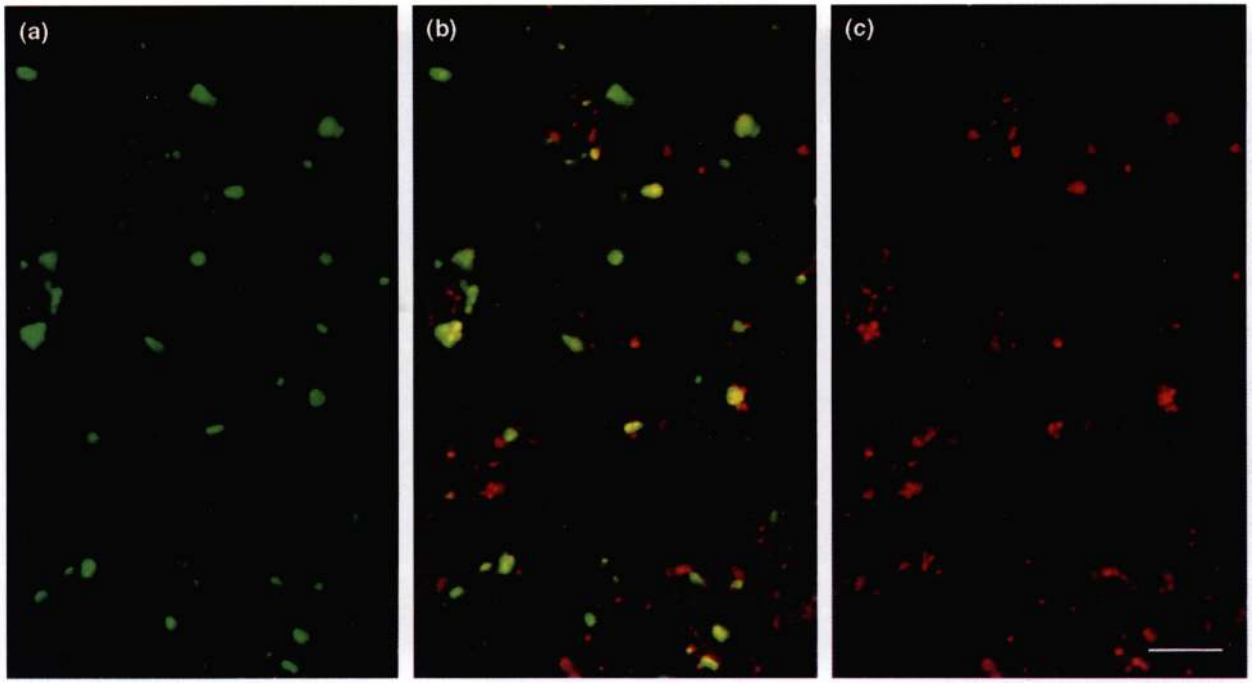


Fig. 7. Double immunolabelling of a frozen section of 21-day-old human menopausal gonadotrophin-stimulated rat ovary stained with the monoclonal anti-Cx43 antibody (a) and anti-Cx45 antibody (c). Localization of Cx proteins are visualized green with fluorescein isothiocyanate-labelled anti-mouse (a) or red with rhodamine isothiocyanate-labelled anti-rabbit (c) IgG. An overlapping image of the two fluorescence probes is shown by yellow in (b), indicating the partial colocalization of Cx43 and Cx45. Scale bar represents 5 μm.

channels allow transfer of molecules about 1 kDa such as intracellular second messengers, whereas Cx45 channels can pass only the smaller ions in electronic coupling (Veenstra *et al.*, 1992; Steinberg *et al.*, 1994). Therefore, Cx43 and Cx45 may well function distinctively in cell-to-cell communication in ovarian granulosa cells, even though they are colocalized in the same plaque.

We have also examined the expression of Cx43 in various follicular stages of hMG-stimulated immature rats. Before hCG injection, immunoblot analysis revealed the presence of at least two closely migrated bands positive for Cx43 antibody. Protein with slower mobility may represent the phosphorylated state of Cx43 (Musil and Goodenough, 1991; Granot and Deckel, 1994). After hCG treatment, Cx43 immunofluorescence decreased in postovulatory follicles, and Cx43 bands, especially Cx43 protein with slower mobility became decreased in immunoblotting intensity. In this condition, since many mature follicles had reached the postovulatory stage and there remained few antral follicles, the decrease of phosphorylated forms of Cx43 proteins with slow mobility may reflect the sparsity of mature antral and Graafian follicles after hCG injection. These results support previous reports that Cx43 protein is multiple phosphorylated in preovulatory mature follicles and becomes dephosphorylated in postovulatory follicles after the LH surge or after hCG treatment (Larsen *et al.*, 1981, 1987; Granot and Deckel, 1994).

The presence of mRNA encoding Cx40 in rat ovarian tissues reported by Beyer *et al.* (1992) was confirmed in this study. Immunocytochemistry, however, showed that Cx40 protein was undetectable in rat granulosa cells, but localized exclusively in endothelial cells of blood vessels in the ovarian stroma. Cx40 was also localized in the endothelial cells of blood vessels in adult lung and heart (data not shown). Beyer *et al.* (1992) reported that Cx40 is expressed in rat vascular smooth muscle cell. However, Bastide *et al.* (1993) reported that Cx40 is expressed in vascular endothelium and conductive bundles of rat heart but not in the smooth muscle layer of the artery. Our study supports the latter report.

In summary, we have shown that Cx43 and Cx45 partially colocalize at the same gap junction plaques connecting granulosa cells of rat ovary. Cx45 was constantly expressed in premature and preovulatory mature follicles but was not detected in postovulatory corpora lutea, and Cx43 was expressed in premature and increased in preovulatory antral follicles but decreased in postovulatory corpora lutea.

These stage- and cell-type specific expression patterns of different connexins may reflect the functional importance of gap junction channels for ovarian follicle development.

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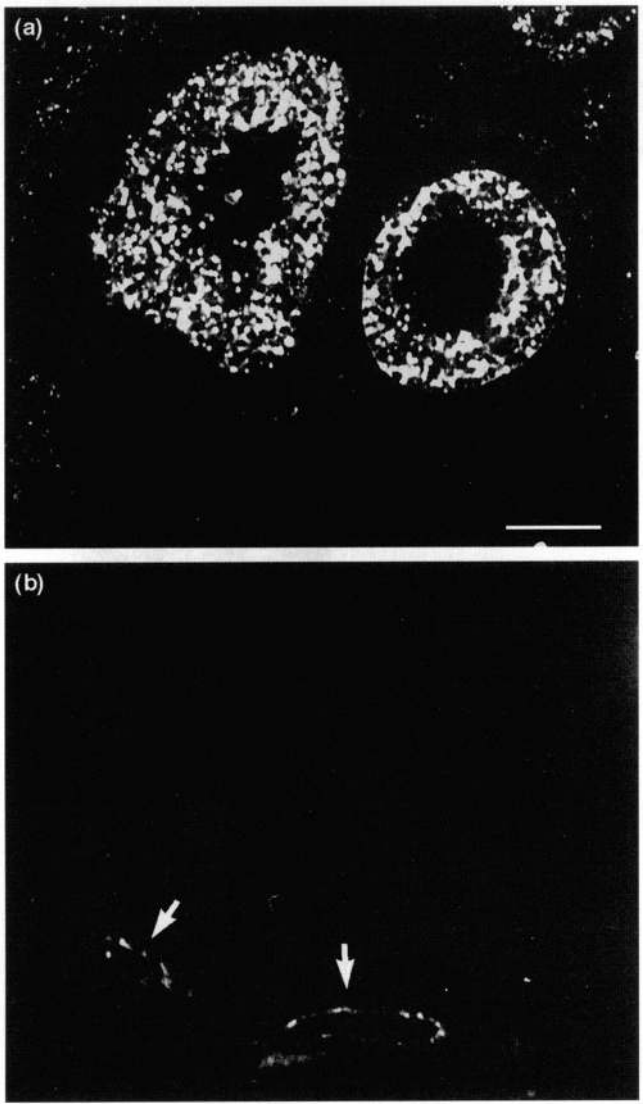

Fig. 8. Double immunolabelling of a frozen section of 21-day-old human menopausal gonadotrophin-stimulated rat ovary stained with (a) the monoclonal anti-Cx43 antibody and (b) a site-specific affinity-purified antibody against Cx40 antibody. Localization of these Cx proteins was visualized with (a) fluorescein isothiocyanate-labelled anti-mouse or (b) rhodamine isothiocyanate-labelled anti-rabbit IgG. (a) Expression of Cx43 in various follicular stages; (b) expression of Cx40 only in blood vessels (arrows) in the ovarian stroma. Scale bar represents 50 μm.

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