Connexin26-mediated gap junctional communication reverses the malignant phenotype of MCF-7 breast cancer cells

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A growing body of evidence indicates that the gap junction (GJ) plays a pivotal role in tumor suppression by exerting cell-cell communication. It has, however, been reported that expression of connexin26 (Cx26) protein is induced in human ductal carcinomas of the breast and that its amount increases in proportion to the grade of malignancy. We thus examined the effects of overexpressed Cx26 on growth characteristics in GJ-deficient human MCF-7 breast cancer cells that maintain the phenotype of early-stage cancers. MCF-7 cells were transfected with Cx26 cDNA, and several clones of stable transformants exhibiting a high level of cell-cell communication were established. When they were examined in terms of various growth characteristics *in vitro*, the proliferation rate and the saturation density were drastically reduced in Cx26-transfected clones compared with the mock-transfectant. The anchorage-independent growth capacity was also decreased by 50–75% after transfection of Cx26. Furthermore, the cell migration toward growth factors and cell invasion into Matrigel in a Boyden chamber were suppressed to 5–10% and 20–60%, respectively, of the control in Cx26-transfected clones. When implanted into the mammary fat pads of nude mice in the presence of an excess of 17β-estradiol, Cx26-transfected clones tended to show slower tumor growth than the mock-transfectant, although the difference was not statistically significant. Our results strongly suggest that the induction of Cx26 protein observed in human breast cancers, reported previously, may not be very relevant to the development of breast cancers, and that Cx26 can function as a tumor suppressor in breast cancer cells. (Cancer Sci 2003; 94: 501–507)

Homeostasis in cellular society is an important factor for maintenance of tissue function, and its disorder often results in dysfunction of organs and development of diseases including cancers. Among cellular apparatuses that contribute to tissue homeostasis, the gap junction (GJ) mediates gap junctional intercellular communication (GJIC) and is unique in that tiny water-soluble molecules (M_r<1000), such as inorganic ions, small metabolites and some second messengers, can travel directly between two adjacent cytoplasms through the junction.[1)] A gap junctional channel consists of two membrane-integrated hemichannels provided by each of two adjacent cells, and each hemichannel comprises a hexameric complex of connexin protein. The connexin multigene family is composed of at least 20 members in mammals.[2)]

Many studies have so far proved that down-regulation of the GJ is involved in carcinogenic pathways and that connexin proteins can function as tumor-suppressors.[3,4)] In almost all tumors, the function of the GJ is down-regulated through one or more of a variety of mechanisms, including no or reduced expression, aberrant localization, and aberrant phosphorylation or dephosphorylation of connexin protein. Moreover, enforced expression of connexin protein very often abolishes the growth capacity of transformed cells such as HeLa cells, rat BC31 bladder cancer cells and human HepG2 hepatoblastoma cells.[5–7)]

However, the function of connexin in breast cancers appears to be opposite to that confirmed in other cancers, although the *connexin26* (*Cx26*) gene was previously isolated as a candidate tumor-suppressor gene for breast cancer.[8)] In normal breast tissues, only traces of Cx26 and connexin32 (Cx32) proteins are detected in ductal and alveolar luminal cells, from which breast cancers arise.[9–11)] Expression of these two connexins is enhanced during pregnancy and further increased during lactation in the same type of cells.[12,13)] On the other hand, connexin43 (Cx43) is strongly expressed, but only in myoepithelial cells, which can not be the origin of cancers.[9–11,14)] In human breast cancers, it has been reported that expression of Cx26 is strengthened in proportion to the grade of malignancy, compared with the surrounding normal counterparts.[11)] Furthermore, while human MCF-7 breast cancer cells, representing the phenotype of early-stage cancers, express neither Cx26 or Cx32,[15)] mRNA of Cx26 has been detected in MDA-MB-231 cells manifesting the phenotype of high-grade malignancy.[16,17)] Thus, it appears that Cx26 may have the potential to elevate the grade of malignancy in breast cancers.

We therefore addressed in the present study the question of the ability of Cx26 to reinforce the malignant phenotype of the cells with low-grade malignancy. For this purpose, MCF-7 cells were transfected with human Cx26 cDNA and various growth characteristics of the resultant stable transformants were examined both *in vitro* and *in vivo*. Unexpectedly, our results indicated that overexpressed Cx26 could efficiently inhibit both the growth capacity and invasiveness of MCF-7 cells, suggesting that Cx26 is tumor-suppressive also in the breast, as well as in some other organs, and that Cx26 expressed in breast cancers might not contribute to the progression of the tumors.

Materials and Methods

Vector construction. Human Cx26 cDNA[15)] has several odd ATG sequences in the 5′ untranslated region, possibly leading to a frame shift when expressed as a transgene product. To eliminate such a possibility, a cDNA fragment containing only the coding region of human Cx26 was amplified by PCR with the following set of primers: forward, 5′-AGAAGCTTCCCTGTTCTGTC-3′; reverse, 5′-ACAGGGGATCCAAATGGTTGC-3′. After digestion of the fragment with both *Hin*dIII and *Bam*HI, the cDNA fragment was inserted into the corresponding sites of pcDNA3.1(+) vector (Invitrogen Corp., Carlsbad, CA) such that Cx26 might be driven by CMV promoter. The absence of unexpected mutations in the construct was verified.

Cell culture and DNA transfection. MCF-7 cells and their transfected subclones were maintained in minimum essential me-

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dium (MEM) containing 10% fetal calf serum (FCS), 100 units/ml penicillin and 100 μg/ml streptomycin.

To determine cell growth, 5×10^4 cells were seeded into 60-mm dishes in triplicate in 4 ml of medium with 10% FCS. The cells were grown under the above-described conditions and counted every 3 days with a hemocytometer. Dead cells, as determined by trypan blue staining, were left out of the count.

A 5×10^5 aliquot of MCF-7 cells was transfected with 7 μg of Cx26/pcDNA3.1 by using Geneporter II reagent (Gene Therapy Systems, San Diego, CA). After 3 weeks of selection with 500 μg/ml G418, G418-resistant MCF-7 cells were subcloned by the limiting dilution method. The stable transformants were maintained in a medium containing 100 μg/ml G418.

Northern blotting analysis. Total RNA was extracted from each clone by TRIzol reagent (Invitrogen Corp.). Total RNA (20 μg) was electrophoresed on a 1.5% agarose-formaldehyde gel and immobilized on a Hybond-N^+ membrane (Amersham Pharmacia Biotech, Little Chalfont, England) by conventional overnight capillary transfer and UV cross-linking. After 1 h of prehybridization at 68°C in ExpressHyb Hybridization Solution (BD Biosciences Clontech, Palo Alto, CA), the membrane was hybridized with a human Cx26 cDNA probe radiolabeled with [α-^{32}P]dCTP in ExpressHyb for 1 h and then washed several times in solution I (2× SSC, 0.05% sodium dodecyl sulfate (SDS)) at room temperature for 40 min and twice in solution II (0.1× SSC, 0.1% SDS) at 50°C for 20 min each. The specific signals were detected on an autoradiogram.

The same membrane was stripped and reprobed with human glyceraldehyde-3-phosphate dehydrogenase cDNA to normalize for mRNA levels.

Immunoblotting analysis. Each clone was harvested at 70% confluence in a 60-mm dish, lysed with a sample buffer (60 m*M* Tris-HCl pH 6.8, 2% SDS, 12% glycerol, 0.1 *M* dithiothreitol, 1 m*M* phenylmethylsulfonyl fluoride) and sonicated. After measurement of total protein concentration in each homogenate with Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Total protein extract (30 μg) from each sample was loaded onto a 15% SDS-polyacrylamide gel and separated by electrophoresis, then transferred to a Hybond-P membrane (Amersham Pharmacia Biotech) at 1.9 mA/cm^2 for 1.5 h with a semi-dry transfer cell (ATTO Corp., Tokyo). The membrane was blocked with 5% non-fat skim milk in TBS-T for 1 h, and incubated with rabbit anti-Cx26 polyclonal antibody (Zymed Laboratories, South San Francisco, CA) at a dilution of 1:500 for 1 h, then with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (Amersham Pharmacia Biotech) at a dilution of 1:1750 for 1 h. Specific signals were revealed with an ECL western blotting analysis system (Amersham Pharmacia Biotech).

To estimate the expression level of estrogen receptors, mouse monoclonal anti-estrogen receptor antibody (Sigma, St. Louis, MO) was used at a dilution of 1:3000.

Indirect immunofluorescence. For immunocytochemistry for Cx26, cells were seeded on a Lab-TekII chamber slide (Nunc, Naperville, IL), washed with PBS, and fixed in pure acetone for 5 min at −20°C. Following the protocol of TSA Fluorescence Systems (Perkin Elmer Life Sciences, Boston, MA), the cells were soaked in 0.5% blocking agent (included in the kit) in PBS, treated with a mouse monoclonal anti-Cx26 antibody (Zymed Laboratories) at a dilution of 1:1000 at room temperature for 1 h, incubated with an HRP-conjugated anti-mouse IgG antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) at a dilution of 1:50 at room temperature for 30 min, and then incubated with Fluorophore Tyramid Amplification Reagent (included in the kit) for 10 min at room temperature. The fluorescence-stained cells were observed under a Microphoto-FXA microscope (Nikon, Tokyo).

For immunohistochemistry for Cx26, 5-μm cryosections were immunostained by the same procedure as in the above-mentioned immunocytochemistry except that 125-fold-diluted rabbit polyclonal anti-Cx26 antibody (Zymed Laboratories) and 200-fold-diluted HRP-conjugated anti-rabbit IgG antibody (Kirkegaard & Perry Laboratories) were used as primary and secondary antibodies, respectively.

Dye-coupling assay to measure GJIC. Measurement of GJIC was done by the Lucifer yellow scrape-loading technique.[18] Culture medium was replaced with 2 ml of a dye cocktail containing 0.1% Lucifer yellow CH and 0.1% Rhodamine B dextran in PBS after confluent monolayers of cells in 35-mm dishes had been washed with PBS. Four parallel scrape lines were then made for each dish with a micropipet tip. After 2 min, the cells were washed with PBS three times and incubated in serum-free medium for a further 6 min, followed by counting of the number of cells stained with Lucifer yellow under a fluorescence microscope. Since Rhodamine B dextran is not capable of passing through GJs, cells stained with this dye are considered to be the scraped cells. Thus, the number of dye-coupled cells is obtained by excluding Rhodamine-positive cells from the cells stained with Lucifer yellow.

Anchorage-independent cell growth assay. A 5×10^4 aliquot of cells from each clone was seeded in 4 ml of MEM containing 10% FCS and 0.33% agar on 5 ml of a solidified MEM basal layer containing 10% FCS and 0.5% agar in 60-mm dishes. Fourteen days after seeding, colonies consisting of at least 10 cells in four areas (4 cm^2 each) were counted in triplicate plates. Each value was converted to that for 1 ml.

Cell migration and invasion assay *in vitro*. The abilities of cells to migrate through a vitronectin-coated filter toward FCS and to invade Matrigel were evaluated by using a Boyden chamber. For migration assay, 8-μm-pore filters of cell culture inserts (Becton Dickinson Labware, Franklin Lakes, NJ) were precoated with 30 μl of vitronectin (0.5 ng/μl) at 18 h before inoculation of cells. The monolayer cultures of each clone were trypsinized, washed with PBS twice and suspended at a density of 2×10^5 cells/ml in RPMI1640 medium containing 0.01% bovine serum albumin (BSA). A 500 μl aliquot of the cell suspension was applied to the cell culture insert on a 24-well culture plate containing 1 ml of RPMI1640 supplemented with 10% FCS. After 72 h of incubation, the top surface of the filter was cleared of cells with a cotton swab. Cells on the underside of the filter were stained with Caratz's hematoxylin, and the number of the cells that could reach the underside was counted in 5 view fields. For invasion assay, the filters were precoated with 50 μg of Matrigel (BD Biosciences Discovery Labware, Bedford, MA), dried in an incubator for 24 h, and rehydrated with 100 μl of RPMI1640 at 1 h before the cell application. The assay was conducted according to the same protocol as the migration assay.

Tumorigenicity assay in nude mice. A 1×10^6 aliquot of cells suspended in 200 μl of PBS was injected into the left second and the right fifth mammary fat pads of 5 female BALB/c-*nu*/*nu* mice of 6 weeks of age per clone under anesthesia after a 60-day-release 17β-estradiol pellet (1.7 mg) (Innovative Research of America, Sarasota, FL) was implanted s.c. into the backs of the mice. Two perpendicular diameters (d_1 and d_2) of each tumor were measured every other day and converted to tumor volume (mm^3) according to the formula: $V=(\pi/6)(d_1\times d_2)^{3/2}$.[19] Eight weeks after injection, the mice were euthanized with ether. A portion of each tumor was frozen for immunostaining, and the rest, as well as the lymph nodes, lungs, liver and brain, was fixed in 10% buffered formalin. The experiment was carried out in accordance with the Guidelines for Animal Experimentation of Akita University.

Results

Restoration of GJIC between MCF-7 cells by transfection of *Cx26* gene. Based on the results of a study by Jamieson *et al*.,[11] we initially expected that Cx26 would reinforce the malignant phenotype of human breast cancer cells, so MCF-7 cells, considered to represent the phenotype of early-stage cancers, were employed in this study. They are positive for estrogen and progesterone receptors and can not develop proliferating tumors in nude mice unless an excess of 17β-estradiol is supplemented. Further, MCF-7 cells have not yet been reported to metastasize when implanted subcutaneously into nude mice.

MCF-7 cells were transfected either with a vector containing human Cx26 cDNA or with an empty pcDNA3.1 vector as a negative control. After 3 weeks of selection with G418 and subsequent cloning by limiting dilution, more than 20 clones of stable transformants were established, of which 9 expressed exogenous Cx26 mRNA as revealed by northern blotting analysis. Finally, three clones, expressing exogenous Cx26 to different extents, as well as the mock-transfectant, were chosen for the experiments. As shown in Fig. 1A, none of the examined clones or the parental MCF-7 cells expressed endogenous Cx26 mRNA, but Cx26-transfected clones 5, 15 and 17 expressed the exogenous Cx26 mRNA. Expression of Cx26 protein in these three transformants was also confirmed by immunoblotting analysis (Fig. 1B). The band of Cx26 protein shown by clone 15 was much less intense than those exhibited by the other two clones, enabling us to evaluate dose-dependent effects of Cx26 on various growth characteristics of the clones.

In many tumor tissues, connexin protein has been known to show aberrant subcellular localizations such as cytoplasmic or nuclear localizations.[20, 21] Similarly, in the numerous studies where cancer cells have been transfected with *connexin* genes, there is a tendency that only a minor population of overexpressed connexin proteins can be integrated into the plasma membrane,[22, 23] often resulting in insufficient restoration of GJIC. In order to examine whether significant numbers of Cx26 molecules contribute to forming GJs in the Cx26-transfected clones, an immunofluorescence study was carried out. All three Cx26-transfected clones gave numerous punctate signals indicating GJ plaques (Fig. 2). The plaques formed by clones 5 and 17 appear to be, in part, fused into strands due to excessive expression of Cx26 (Fig. 2, C and I). Furthermore, these GJ plaques were localized in a cell-cell contact area with almost no cytoplasmic signal in all of the Cx26-transfected clones, suggesting that expression of Cx26 is sufficient to induce the establishment of GJs in MCF-7 cells (Fig. 2E and data not shown).

To confirm the function of the overexpressed Cx26 proteins, GJIC of each clone was measured by scrape-loading dye-coupling assay as described in "Materials and Methods." Consistently with the expression levels of Cx26 (Fig. 1), clones 5 and 17 exhibited efficient GJIC and clone 15 showed a reticent communication capacity (Table 1). The mock-transfectant ex-

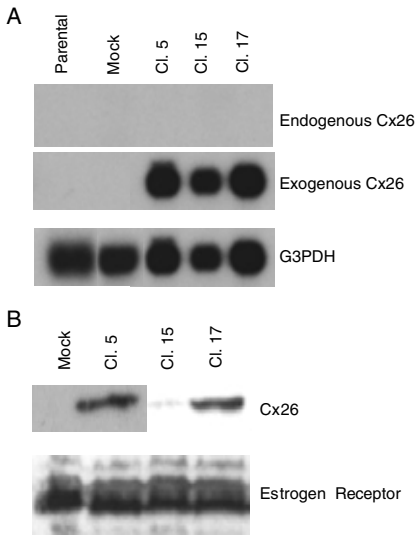

Fig. 1. Expression of Cx26 in the clones examined in this study. (A) Northern blotting analysis of Cx26. (B) Immunoblotting analysis of Cx26 and estrogen receptor.

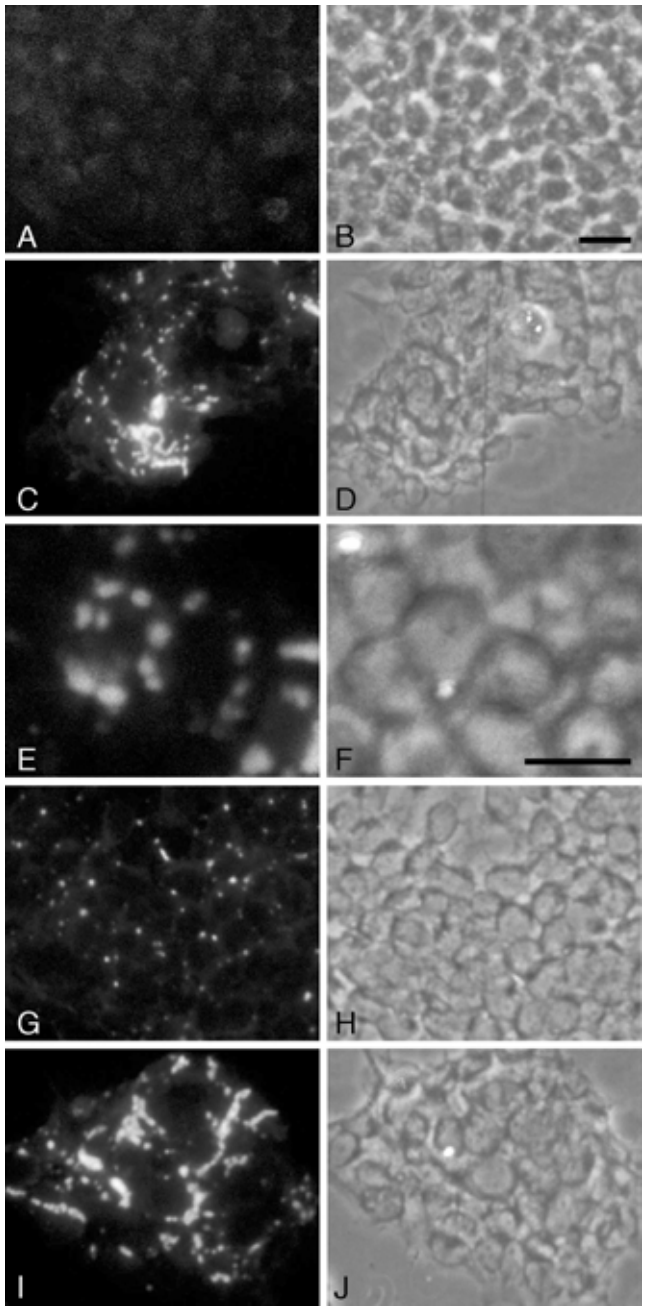

Fig. 2. Indirect immunofluorescence of Cx26 in Cx26-transfected MCF-7 cells. The immunofluorescence images (A, C, E, G and I) of Cx26 revealed by FITC were captured in the same fields as the phase contrast micrographs (B, D, F, H and J, respectively). A and B, mock-transfectant; C, D, E and F, clone 5; G and H, clone 15; I and J, clone 17. All the photographs except for E and F are at the same magnification. Scale bars, 20 μm.

hibited only a negligible GJIC capacity, indicating that, although we did not check the expression of types of connexin other than Cx26, the parental MCF-7 cells were much more likely to be deficient in GJIC even though expressing some other connexins, which may not be functional.

Growth characteristics *in vitro*. As shown by a growth curve (Fig. 3A), the proliferation rate of MCF-7 cells was drastically reduced after transfection of the *Cx26* gene compared with the mock-transfectant. It is known that GJ-mediated suppression of cell growth is often most apparent as a reduction of saturation density. This was also the case with our experiments, i.e., the saturation density of Cx26-transfected clones was 30–50% of that of the mock-transfectant (Fig. 3A).

To examine whether overexpression of Cx26 affects the anchorage-independent growth capacity of MCF-7 cells, a colony formation assay in soft agar was performed. Cx26-transfected clones could form only a 50–75%-decreased number of colonies compared with those formed by the mock-transfectant (Fig. 3B). The extent of reduction of colony-forming capacity also correlated with expression level of Cx26 protein in each clone.

Table 1. GJIC capacity of Cx26-transfected MCF-7 cells

Clones	Number of Lucifer yellow dye-coupled cells Per scraped cell±SD
Mock	0.13±0.09
Clone 5	2.04±0.69*
Clone 15	1.15±0.24*
Clone 17	2.43±0.87*

* Significantly different from the mock-transfectant at $P<0.005$.

Since MCF-7 cells express estrogen receptors and still remain responsive to 17β-estradiol, the effects of the overexpressed Cx26 on the *in vitro* cell growth were analyzed in the presence of 17β-estradiol. Prior to this experiment, we had confirmed by immunoblotting that the clones to be examined expressed similar amounts of estrogen receptor independently of the expression levels of Cx26 (Fig. 1B) and also that the expression level of Cx26 in each clone was not affected by 10 n*M* 17β-estradiol (data not shown). The growth curve indicated that overexpressed Cx26 could suppress the *in vitro* cell proliferation even in the presence of 10 n*M* 17β-estradiol, although the hormone reduced the efficiency of the growth suppression (Fig. 4A). As shown in Fig. 4B, although the anchorage-independent growth capacity of all the clones examined was significantly enhanced by 17β-estradiol, Cx26 appeared to retain a growth-suppressive activity, i.e., the colonies formed by the Cx26-transfected clones were decreased in number by 25–35% compared with those formed by the mock-transfectant.

Cell migration and invasion *in vitro*. Firstly, the ability of each clone to migrate chemotactically toward the ingredients contained in FCS was estimated by using vitronectin-coated filters in Boyden chambers. As shown in Fig. 5A, the number of the cells that reached the underside of the filter was much smaller in the case of the Cx26-transfected clones than the mock-transfectant. When the transfectants were further examined in terms of their invasion capacity into Matrigel by using Boyden chambers, the number of invasive cells was found to be significantly decreased in the Cx26-transfected clones compared with the mock-transfectant (Fig. 5B).

Overall, the results indicate that overexpression of Cx26 protein efficiently mitigated the malignant phenotype of MCF-7 in various respects *in vitro*.

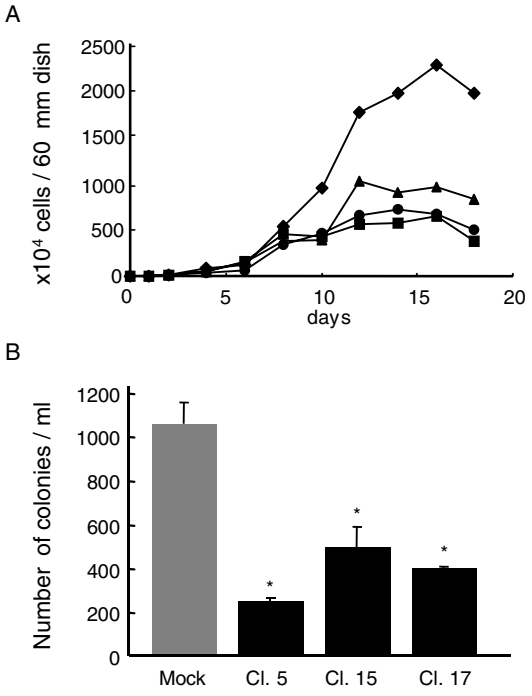

Fig. 3. Effect of overexpressed Cx26 on *in vitro* growth capacities in MCF-7 cells. (A) The proliferation curve of each clone in culture. The cells were counted every 3 days in triplicate dishes, with dead cells excluded. The SD at each point is too small to show as an error bar. ◆ mock, ■ Cl.5, ▲ Cl.15, ● Cl.17. (B) Anchorage-independent growth capacity in soft agar. Fourteen days after seeding, colonies consisting of at least 10 cells in four areas (4 cm^2 each) were counted in triplicate plates. Each value was converted to that for 1 ml. * Significantly different from the mock-transfectant at $P<0.01$.

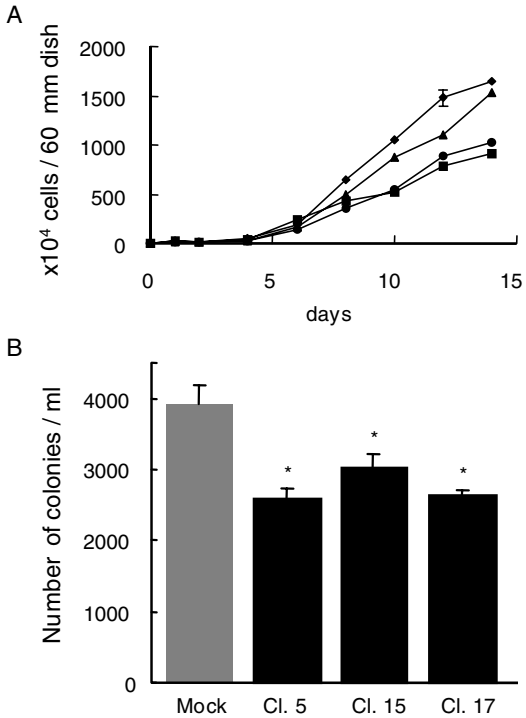

Fig. 4. Effect of overexpressed Cx26 on *in vitro* growth capacities in MCF-7 cells in the presence of 17β-estradiol. The same experiments as Fig. 3 were performed in the medium supplemented with 10 n*M* 17β-estradiol. (A) The proliferation curve of each clone in culture. No error bar is indicated when the SD is not significant. ◆ mock, ■ Cl.5, ▲ Cl.15, ● Cl.17. (B) Anchorage-independent growth capacity in soft agar. * Significantly different from the mock-transfectant at $P<0.01$.

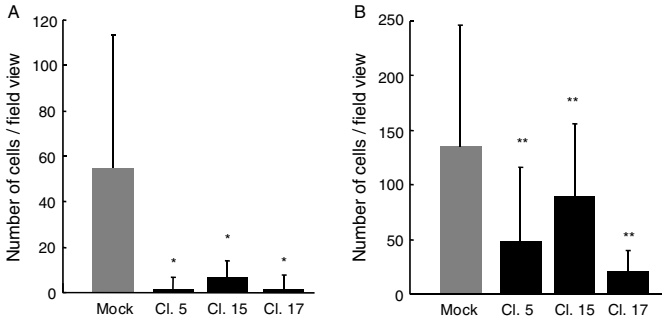

Fig. 5. Chemotactic migration to FCS and invasiveness into Matrigel. (A) Migration capacity of Cx26-transfected MCF-7 cells. The cells were seeded onto vitronectin-precoated filters of cell culture inserts and incubated for 72 h. The cells trapped by vitronectin during migration toward FCS were counted. (B) Invasion capacity into Matrigel. The cells were seeded onto the Marigel, that had been poured into cell culture inserts in advance. The cells that penetrated the Matrigel layer were counted. *, ** Significantly different from the mock-transfectant at $P<0.001$ and at $P<0.01$, respectively.

Tumorigenicity in nude mice. To assess tumorigenicity *in vivo*, Cx26-transfected MCF-7 clones as well as the mock-transfectant were injected into the bilateral mammary fat pads in female athymic nude mice, and the first appearance and growth of tumors were recorded. Since MCF-7 cells are tumorigenic in nude mice but can not develop proliferating tumors without supplementation of 17β-estradiol, a 17β-estradiol pellet was implanted at the time of the injection of each clone. As shown in Fig. 6A, all the clones examined formed proliferating tumors with similar latency in the presence of 17β-estradiol. During observation for 28 days after the injection, there was a tendency for the tumors derived from clone 15 and the mock-transfectant to be larger than those from clones 5 and 17 at many time points and for the former to manifest a higher growth rate than the latter, although the effects did not reach statistical significance. After the 30th day, increasing numbers of mice died for unspecified reasons whatever the injected clone, preventing us from obtaining accurate data. When the mice died or when the surviving mice were euthanized on day 56, all the subcutaneous tumors and tissues of the liver, lungs and brain were examined histologically. In the tumors developed by the mock-transfectant, the tumor cells grew in cord-like patterns with a stroma comprising thick collagenous bundles (Fig. 6B). This histological finding is similar to that of the tumors derived from the Cx26-transfected clones, suggesting that overexpression of Cx26 does not affect the morphology of MCF-7 cells *in vivo*. A histological examination of other organs revealed a metastasized tumor in the lung of a mouse given clone 15, but this was the only metastasis found throughout the experiment.

To verify whether the examined clones lost expression of the transgene during tumor formation, the tumors from clone 15 as well as from the mock-transfectant were examined immunohistochemically for expression of Cx26. As shown in Fig. 7, fluorescence signals corresponding to GJ plaques composed of Cx26 molecules were detected in a cell-cell contact area, suggesting that loss of expression of Cx26 was not involved in tumor formation by the Cx26-transfected clones examined.

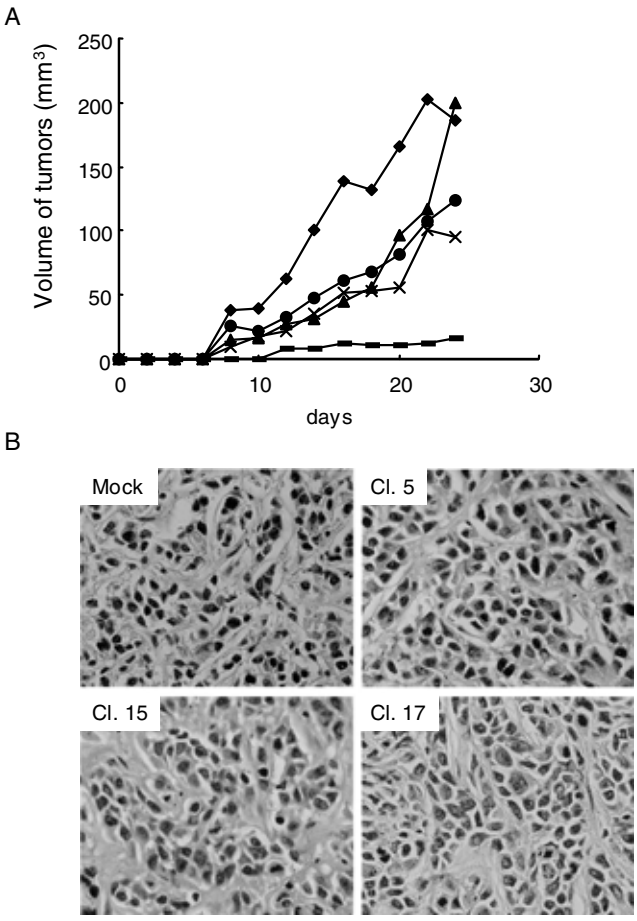

Fig. 6. *In vivo* tumorigenicity assay of Cx26-transfected MCF-7 cells. (A) Growth of tumors. 1×10^6 cells were injected into the left second and the right fifth mammary fat pads of each female nude mouse in the presence or absence of 17β-estradiol pellet. The size of each tumor was measured every other day. ▬ mock, ◆ mock+estradiol, ▲ Cl.5+estradiol, ● Cl.15+estradiol, × Cl.17+estradiol. (B) Histological findings of the tumors formed by Cx26-transfected MCF-7 cells implanted in the mammary fat pads of female nude mice.

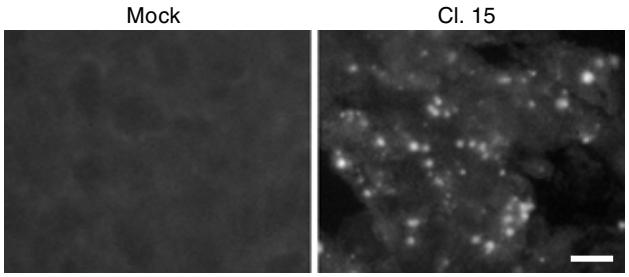

Fig. 7. Indirect immunofluorescence of Cx26 in developed tumors. Cryosections of the developed tumors were stained for Cx26. Specific signals were revealed by FITC. Scale bar, 20 μm.

Discussion

Although several connexin proteins are detected in normal breast tissues, the expressed molecular species of connexin differ among cell types. While Cx43 is expressed in myoepithelial cells,[9–11, 14] Cx26 is expressed rather weakly in luminal cells,[9–11] from which most breast cancers arise. Jamieson *et al.*[11] have reported that the expression of Cx26 is enhanced in human ductal carcinomas of the breast and that the expression level is correlated with the grade of malignancy. However, when we transfected *Cx26* gene into human MCF-7 breast cancer cells in this study, overexpression of Cx26 could suppress various growth parameters of MCF-7 cells, including the growth rate, saturation density, growth in soft agar, migration and invasion (Figs. 3–5). How can this apparent discrepancy be explained? The

Cx26 molecules expressed strongly in high-grade breast cancers are localized almost exclusively in cytoplasm, but not in cell-cell contact areas,[11] suggesting that these Cx26 molecules can not function as a gap junction component and that high-grade breast cancers are likely to be deficient in intracellular trafficking of Cx26. On the other hand, Cx26 molecules overexpressed in MCF-7 cells were sorted properly into the plasma membrane (Fig. 2), probably because MCF-7 cells maintain the phenotype of early-stage breast cancers. The expression of Cx26 seen in high-grade breast cancers may be a mechanism of the fail-safe system to normalize aberrant cell growth, but may not be able to exert its tumor-suppressive activity due to disruption of the system supportive of gap junction formation.

It has been debated whether or not connexins can control cell growth in a GJIC-independent manner. Qin *et al*.[24] recently reported that either Cx43 or Cx26 inhibits, without exerting GJIC, *in vivo* tumor growth but not *in vitro* cell proliferation in human MDA-MB-231 breast cancer cells, considered to represent the phenotype of high-grade malignancy. In our study, a great majority of Cx26 molecules, giving only a faint signal in cytoplasm, were integrated into the plasma membrane of MCF-7 cells to form functional GJs (Fig. 2 and Table 1) and efficiently suppressed *in vitro* cell growth, but did not have a clear effect on *in vivo* tumor growth (Figs. 3–6). These two studies thus suggest that GJIC and cytoplasmic connexins may be capable of controlling cell growth through different mechanisms. We speculate that, while homologous GJIC between the same type of cells preferentially inhibits *in vitro* cell growth, cytoplasmic connexins are involved in suppression of *in vivo* tumor growth.

One reason why a Cx26-mediated tumor-suppressive effect was not very obvious *in vivo* in this study may be because cell-cell interaction between Cx26-transfected MCF-7 cells and other cells of the mammary fat pad weakens the Cx26-mediated growth suppression observed *in vitro*. When implanted into nude mice, Cx26-transfected MCF-7 cells have, for the first time, an opportunity to establish heterologous gap junctions with other types of cells, probably by forming heterotypic GJ channels composed of Cx26 and other connexin proteins. Accumulating evidence has indicated that heterotypic GJs have a character that is distinct from homotypic ones.[25] Consequently, the state of GJIC established *in vitro* in MCF-7 cells can be modulated by newly-formed heterotypic channels, leading to diminished tumor-suppressive activity *in vivo*. However, the possibility that the tumor-suppressive activity of Cx26 was masked by the enormous tumorigenic effect of 17β-estradiol can still not be excluded.

Although there has so far been no report describing metastases of MCF-7 cells injected into mammary fat pads, one of our Cx26-transfected clones developed a metastatic focus in the right lung, despite the lack of metastasis of the mock-transfectant. The significance of this metastasis is unclear, because only one focus was found throughout our experiments. Nevertheless, since overexpression of Cx26 has been reported to induce metastases of s.c.-injected mouse BL6 melanoma cells,[26] the fact that Cx26-transfected MCF-7 cells developed a metastatic focus after implantation into mammary fat pads should be noted for future studies.

It has been established that GJs mediate the bystander effect to reinforce suicide gene therapy, especially using *HSV thymidine kinase* gene and ganciclovir.[27] A number of trials have already been planned and executed. A crucial issue of this strategy is how to induce or up-regulate GJIC in tumor tissues. Co-introduction of *connexin* genes into tumors is the most straightforward idea and has actually been proposed. It is thus critical that the *connexin* gene should not have any oncogenic effect. Our results indicate that Cx26 does not display oncogenic effects, but rather growth-suppressive effects, and therefore support the potential usefulness of Cx26 as a mediator of the bystander effect in the therapy of breast cancers.

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