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Growth-Suppressing Activity of the Transfected Cx26 on BICR-M1Rk Breast Cancer Cell Line

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There are accumulating evidences suggesting that connexin (Cx), a gap junction channel-forming protein, acts as a growth suppressor in various cancer cells, and this effect is attributeed to the gap junction-mediated intercellular communication (GJIC). In order to characterize the relationship between the growth-arresting activity of Cx26 and its cytoplasmic localizations after expression, we linked a nuclear export signal (NES) sequence to Cx26 cDNA before transfecting into a rat breast cancer cell line. A confocal fluorescent microscopic observation revealed that the insertion of NES minimized the nuclear expression of Cx26, and increased its cytoplasmic expression, including plasma membrane junctions. Total cell counting and BrdUrd-labeling experiments showed that the growth of the breast cancer cells was inhibited by 74% upon transfection of Cx26-NES, whereas only 9% inhibition was observed with only Cx26 cDNA.

Keywords: Cx26, gap junction channel, growth suppressor, NES, BrdUrd-labeling

Gap junctions are a collection of aqueous channels that mediate direct exchange of ions and hydrophilic small molecules (*i.e.*, metabolites, secondary messengers) between cytoplasmic compartments of contiguous cells [2, 27]. Each of these channels consists of two antisymmetric end-to-end hemichannels (also called connexon), each of which consists of six transmembrane proteins, named connexin (Cx) [3, 12, 29]. The Cx multigene family is composed of at least 20 members in mammals [34]. The gap junction-mediated intercellular communication (GJIC) makes essential contributions to cell growth regulation, metabolic corporation, and homeostasis in most mammalian tissues. Correspondingly, there is a large body of evidence that disorders of GJIC have an etiologic role in carcinogenesis [31, 35, 36]. For instance, it has been shown repeatedly that most malignant cells communicate through gap junctions to only a limited extent. Furthermore, transfection of genes coding Cx proteins into a variety of cancer cell lines often restores their communication capacity and inhibits growth *in vivo* [1, 19, 22, 23, 39].

The ability of transfected Cx subtypes in various cancer cell models to reduce cell proliferation usually correlates well with enhanced formation of functional gap junctions, being consistent with the idea that the cell growth inhibition is mediated by a passage of certain growth regulators between neighboring cells [11, 16, 18]. However, in conflict with the conventional notion, an array of studies have reported that the forced expression of Cx subtypes in certain cancer cell lines suppressed cell growth without enhancing GJIC [10, 14, 21, 38]. In addition, the expressions of Cxs in the transfected cells were shown to localize at the nucleus as well as in the cytoplasm, rather than at the junctional area of adjacent plasma membranes [10, 13, 14, 24]. In the present study, a rat breast cancer cell line, in which a dominant nuclear localization of Cx26 upon the transfection of Cx26 cDNA was previously observed, was employed in order to investigate the relationship between subcellular localization of Cx26 and its growth-inhibiting property [17]. We incorporated a nuclear external signal (NES) sequence into the Cx26 cDNA prior to transfection to manipulate the subcellular expression of Cx26.

Materials and Methods

Cell Culture

The rat BICR-M1R$_k$ cell line [25] was routinely maintained as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum, 10 units/ml penicillin, and 10 μg/ml streptomycin at 37°C in a 5% CO_2 humidified incubator.

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Plasmid Construction and Transfection

The open reading frame of the Cx26 cDNA was amplified from plasmids containing Cx26 cDNA. In order to ensure the in-frame fusion of Cx26 cDNA to the amino terminus of enhanced-GFP in a pe-GFP-N1 vector (Clontech), we used the primers containing two restriction sites for both *Eco*RI and *Xho*I. A Cx26-NES-GFP clone was constructed in the presence of a synthesized nuclear export sequence, NES, (LALKLAGLDI) [26, 33], which was designed to contain both *Eco*RI and *Xho*I restriction sites for its convenient ligation to Cx26 cDNA. Cx26-NES was digested with *Eco*RI, and then conjugated into the corresponding sites of the pe-GFP-N3 vector (Clontech). The sequences of the constructed clones (Cx26-pe-GFP and Cx26-NES-pe-GFP) were confirmed by a Perkin-Elmer 377 automatic DNA sequencer prior to further experiments. BICR-M1R_k cells (2×10^5) were transfected with 5 μg of each Cx26-pe-GFP, Cx26-NES-pe-GFP, and a mock pe-GFP vector as a control, by using the Superfect transfection reagent (Qiagen Gmbh). Each clone was selected under a 0.3 mg/ml active concentration of G418 (Geneticin, Life Technologies), and the efficiency of transfection was determined by visualizing live or fixed cells under a fluorescence microscope after 24–48 h. The individual transfected clones were prepared with a limit dilution cloning method in 96-well plates. The subcellular expression of Cx26/chimeric proteins in BICR-M1R_k cells was viewed on a Bio-Rad confocal scanning microscope (MRC 1024), under the intrinsic fluorescence of GFP.

RT–PCR Analysis

Total RNA was separately obtained from each step of treatments, by suspending the cells in Trizol (Life Technologies) in accordance with the manufacturer's recommendation. Single-strand cDNAs were prepared from 1 μg of total RNA in the presence of avian myeloblastosis virus reverse transcriptase (Promega). The cDNAs of Cx26 and Cx26-NES chimera were amplified from the cDNA library with a polymerase chain reaction, using the following primers: 5'-ACAAGATGGATTGGGGCACA-3' as a Cx26 upstream primer, 5'-GCAATGCAT-TAGACTGGTCT-3' as a Cx26 downstream primer, and 5'-CTCGAGCTGGCCCTGAAGCT-3' as a NES downstream primer.

Western Blotting Analysis

Total protein extract (25 μg) from each clone was loaded onto a 15% gel for Cx26 protein. After electrophoresis, proteins were transferred to PVDF membranes (Bio-Rad). The blots were incubated with rabbit polyclonal anti-Cx26 (1/1,000; Zymed Laboratories) and anti-GFP antibodies. Each immunoreactive band was detected using the ECL system (Amersham). A horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham) was diluted 1:5,000 to detect the above primary antibodies.

Cell Proliferation Assay

Cells (5×10^4) were seeded on a 60-mm culture dish with DMEM at the first day of the experiment. After trypsin treatment, the collected cells were counted independently every next day of the experiment. Each counting experiment was repeated three times. The assay of cell proliferation was also carried out by using a BrdUrd labeling system. Immunostaining of BrdUrd was achieved according to a protocol described previously [32]; in each set of experiment, at least 3,000 cells were counted. The labeling index (LI) was defined as a percentage derived from the number of labeled cells divided by the total number of cells counted.

Results and Discussion

Expression of Cx26-NES in BICR-M1R_K Cancer Cells

Topological studies on the various Cx subtypes, using hydropathy analyses, limited proteolysis of membrane-embedded Cxs, and site-directed antibodies binding experiment, revealed that the Cx monomer spans the plasma membrane four times (M1–M4), forming two extracellular loops and three cytoplasmic regions (amino-, carboxyl-terminal, cytoplasmic loop) [15, 20, 37]. The NES (nuclear export signal) sequence is composed of 10 amino acids (leu-ala-leu-lys-leu-ala-gly-leu-asp-ile) (Fig. 1) and its known function is to re-target a nuclear protein to various cytoplasmic regions including the plasma membrane [26, 33].

RT–PCR experiments were performed to convince the expression of mRNAs for Cx26 and Cx26-NES after corresponding transfections. In the beginning, a mock pe-GFP vector was transfected to assess any intrinsic expression of Cx26 in BICR-M1R_K cells. Our results, as shown in Fig. 2A, indicate that neither Cx26 mRNA nor Cx26 protein is endogenously expressed in BICR-M1R_K cells. The RT–PCR bands at the positions of 709 bp and 751 bp represent the estimated sizes of Cx26 transcript and Cx26-NES transcript, respectively. In Western blotting analysis (Fig. 2B), we observed a full length of two chimeric proteins of Cx26-NES-GFP and Cx26-GFP in the corresponding clones (lanes 3 and 4). Both proteins were positioned at the predicted size of 54 kDa on SDS–polyacrylamide gel electrophoresis, with a negligible contribution of NES to the chimeric protein mass. Since the amounts of the exogenously expressed Cx26 in each cell sample might be an important factor for the cell growth, the quantities of Cx26 and GFF-conjugated Cx26 were analyzed by densitometry of the Western blots. As shown in Fig. 2C, the expression levels of Cx26 were relatively similar in all samples, although a slightly higher expression of Cx26 was obvious in the Cx26-NES-GFP clones. Unfortunately, we were not able to manipulate the expression levels of Cx26 in the current system.

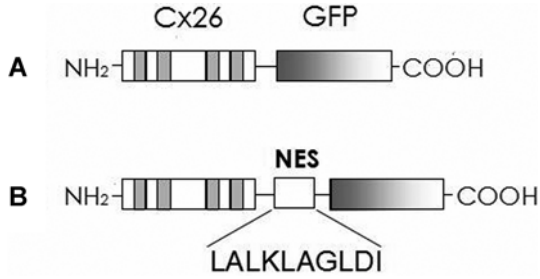

Fig. 1. Diagrammatic illustration of the chimeric DNA constructs, Cx26-GFP (**A**) and Cx26-NES-GFP (**B**).
The Cx26 cDNA was fused in-frame to the N-terminus of an enhanced green fluorescent protein (GFP) in a pe-GFP vector. The four transmembrane domains of Cx26 are depicted by grey shading.

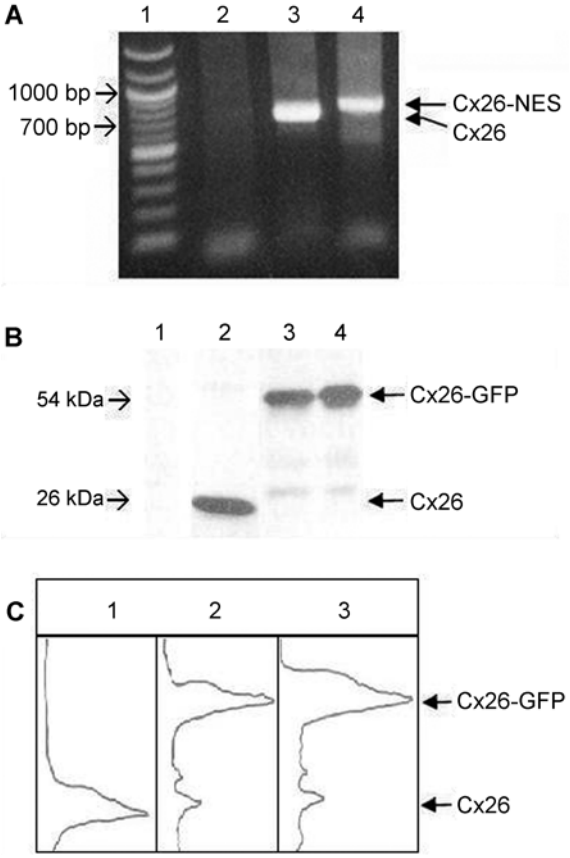

Fig. 2. RT–PCR, Western blot, and quantitative analyses.
A. RT–PCR analysis of the total RNAs isolated from the Cx26-transfected cells. Lane 1: Size standards (1,000 and 700 bps are marked); lane 2: the control cells into which only mock pe-GFP vector was transfected; lane 3: the cells transfected with Cx26 cDNA; lane 4: the cells transfected with a chimeric construct of the Cx26-NES sequence. **B**. The Western blot analysis of the cell homogenates of the transfected clones. A polyclonal rabbit anti-Cx26 antibody (Zymed Lab.) was used as a primary antibody. Each lane contained approximately 25 μg of total protein. Lane 1: The cell homogenate transfected with pe-GFP vector only; lane 2: the cell homogenate transfected with pcDNA3.1 vector containing Cx26 cDNA; lane 3: the cell homogenate transfected with pe-GFP vector containing Cx26 cDNA; lane 4: the cell homogenate transfected with pe-GFP vector containing Cx26 cDNA and NES. **C**. Quantitative analysis of the Cx26 expression in each sample of transfected cells by densitometry. Lanes 1, 2, and 3 are the densitometric analyses of lane 2, lane 3, and lane 4 of the Western blotting shown in **B**, repectively.

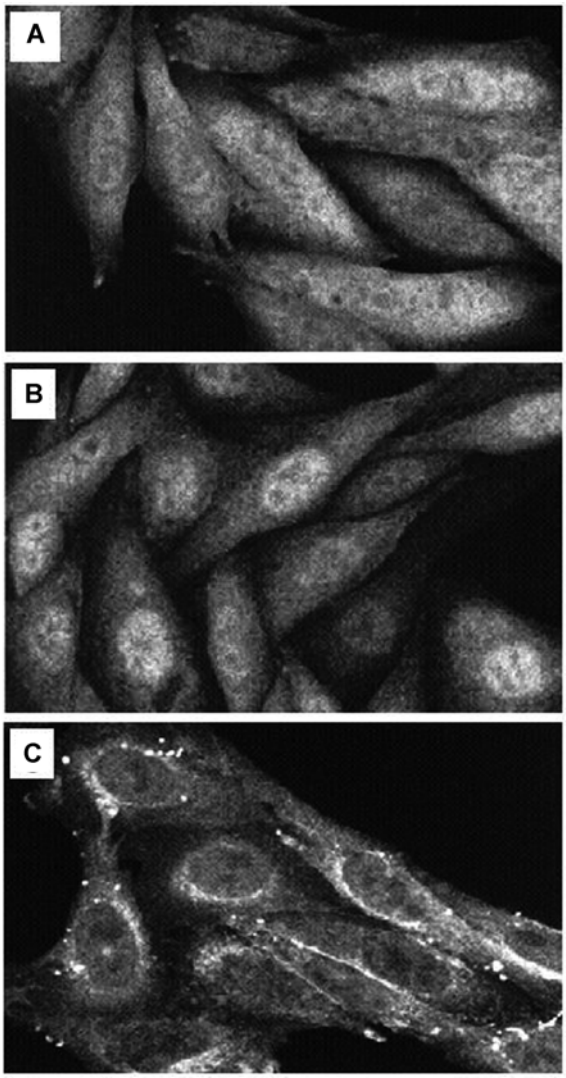

Fig. 3. The cytoplasmic localization of Cx26 in the cells transfected with Cx26 cDNA chimeras.
The expression sites were visualized by the virtue of the inherent fluorescence of GFP with a confocal scanning microscope Bio-Rad MRC 1024. (**A**) the cells transfected with a mock pe-GFP vector only; (**B**) the cells transfected with pe-GFP vector containing Cx26 cDNA, (**C**) the cells transfected with pe-GFP vector containing Cx26 cDNA-NES chimera.

Positional Shift of the Expression Site of Cx26 by NES Sequence Insertion

Subcellular locations of the chimeric proteins of Cx26-GFP and Cx26-NES-GFP were visualized by virtue of the inherent GFP fluorescence under a Bio-Rad confocal microscopy (Model: MRC1024). The fluorescent signal was observed throughout the entire cytoplasm of the cells transfected with pe-GFP vector (Fig. 3A). Interestingly, the BICR-M1R$_k$ clone, which was transfected with the chimeric cDNA construct of Cx26-GFP, contained the majority of the fluorescent signal near the nuclear envelope regions (Fig. 3B). This observation is coincident with our previous result in which the transfected Cx26 protein was shown to co-localize with the expression of lamin B1, a typical nuclear envelope protein [17]. On the other hand, the other clone of the BICR-M1R$_k$, which was transfected with the Cx26 cDNA containing the NES sequence, showed a different pattern of fluorescent signal, ranging on various regions of the cytoplasm, including the junctional area of plasma membranes, as shown in Fig. 3C. This result clearly demonstrates that the insertion of a NES sequence into the Cx26 cDNA shifted the position of Cx26 expression in the BICR-M1R$_k$ cells.

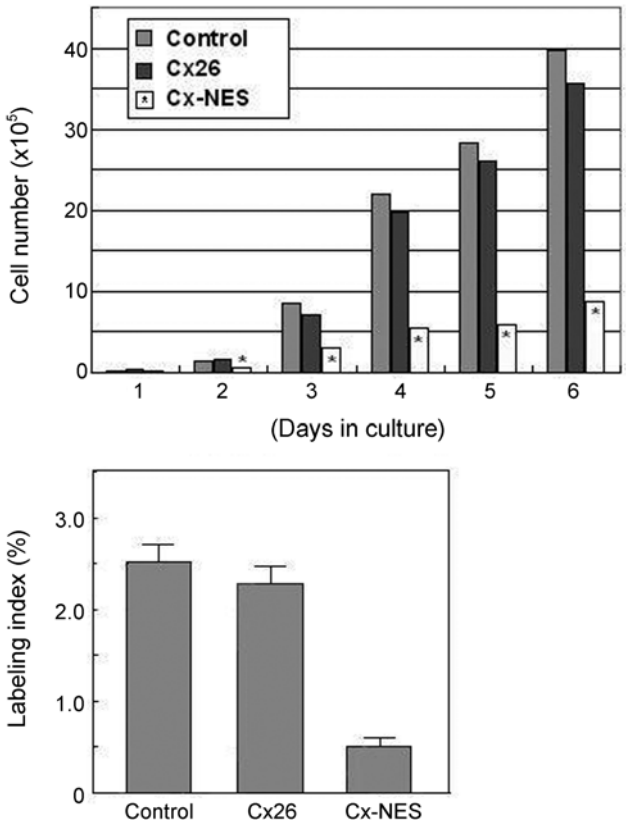

Fig. 4. Analyses on the proliferation of the transfected cells.
Upper panel: The total number of cells was counted at various growth periods (day 1 through 6). Each clones of cells that were transfected with either Cx26 cDNA or Cx26 cDNA-NES chimera were seeded at low density and then collected at various durations. Low panel: The cell proliferations of the various transfectants were analyzed by using BrdUrd labeling method. The labeling index (LI) was defined as a percentage derived from the number of labeled cells divided by the total number of cells counted. All values are expressed as means from three determinations. Three different clones that were transfected by either Cx26 cDNA or Cx26 cDNA-NES were employed for these independent experiments. The data used to construct these graphs are the means of ± S.D. of three replicates.

Cell Growth Inhibition of Cx26-NES

In order to analyze the positional shift of Cx26 expression regarding its growth-inhibitory property on the cancer cells, the total cell numbers of clones transfected with Cx26 cDNA was compared with corresponding clones with Cx26-NES transfection. As shown in Fig. 4 (upper panel), within the same time periods, the cells with only Cx26 cDNA transfection grew up to 90% of control cell growth. However, the cells transfected with the Cx26-NES construct grew up to only one-quarter of the growth observed with control cells. According to the BrdUrd incorporation assay [32], the labeling index of BrdUrd measured in the cells of Cx26-NES construct reached 24% of the index value observed in the control cells (low panel). Although conducting both the total cell-counting experiment and BrdUrd-incorporation analysis would be superfluous, we wanted to make sure that the inhibitory activity of this exogenous Cx26 upon transfection with a NES sequence was dramatically elevated.

On the other hand, we need to mention our previous study [17] that showed a significant retardation on the growth of BICR-M1R_k cells when the cells were transfected with the Cx26 cDNA alone. Although the present study was carried out with minor experimental modulations from the earlier one, including the use of the pe-GFP-N3 vector rather than the plain expression vector pcDNA3.1+, we could not determine a clear explanation for the discrepancy.

The cell growth-suppressing property of various Cx subtypes, whose expressions induce GJIC in normal cellular condition, was postulated from an accumulated fact that their unique function of cell-to-cell coupling is impaired in most cancer cells. Furthermore, the transfection of various isotypic Cx genes into many different cancer cell lines often restores their communication capacity and inhibits growth *in vivo* to a significant degree [1, 4, 9, 11, 19, 28]. On the other hand, an array of studies also showed that Cxs (or part of the Cx molecule) inhibited cell growth in the absence of major changes in cell-cell coupling [5, 10, 14, 21, 24]. Several plausible studies on the mechanism have suggested that Cx proteins may suppress cell growth through regulating various signaling molecules and/or cytoskeletal proteins, including p27, Skp2, CCN3, c-Src, and tubulin [6, 7, 8, 30, 38]. In this regard, we wanted to observe whether there is any differential expression of cyclin B, a pivotal protein involved in cell growth regulation, upon the NES-conjugated Cx26 transfection. Neither RT–PCR nor Western blotting experiment showed any changes on the expression of cyclin B (data not shown). In conclusion, the present study demonstrates that the cytoplasmic trafficking of the exogenously expressed Cx26 is intimately related with its growth-suppressing activity on BICR-M1R_k breast cancer cells.

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