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Suppression of lung cancer cell invasion and metastasis by connexin43 involves the secretion of follistatin-like 1 mediated via histone acetylation

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ABSTRACT

Although connexin has been recognized as a tumor suppressor in many types of cancer, the underlying mechanisms are poorly understood. We have previously shown that transfection of connexin43 (Cx43) cDNA retarded the growth of a highly metastatic human pulmonary giant cell carcinoma cell line, PG, both *in vitro* and *in vivo*. Here, we further demonstrate that the metastasis and invasion, but not the migration, of PG cells are also inhibited following Cx43 transfection. The diminishment of metastasis and invasion is associated with down-regulation of genes including *MMP-2*, *S100A*, *LAMA4*, and *HDAC10*, as well as up-regulation of genes such as *MTSS1* and *FSTL1* as revealed by gene chip analysis. Interestingly, the suppression effects of Cx43 are related to secreted factor(s), which are blocked by FSTL1 antibody treatment in a dose-dependent manner. Furthermore, the FSTL1 promoter was shown to be associated with acetylated histones H3 and H4 upon Cx43 transfection. These data suggest that Cx43 inhibits the invasion and metastasis of PG cells by modulating the secretion of FSTL1, which is regulated by histone acetylation. Cx43 may act as a "histone deacetylase inhibitor" to modulate gene expression and subsequent cellular functions in PG cells.

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1. Introduction

The gap junction composing proteins, connexins (Cxs), are encoded by at least 21 different genes in humans, and they play important roles in many cellular functions such as cell growth and differentiation (Meşe et al., 2007; Sáez et al., 2003). Aberrant connexin gene expression or mutation(s) is associated with many diseases such as cardiac failure, deafness, and cancer (Gerido and White, 2004; Mesnil et al., 2005; Severs et al., 2004). In fact, connexin genes have been classified among tumor suppressors (Lee et al., 1992; Yamasaki and Naus, 1996), as down-regulation of connexin and aberrant gap junctional intercellular communication (GJIC) function have been found in various human cancer cell lines and clinical cancer tissues (Cesen-Cummings et al., 1998; Grossman et al., 1994; Krutovskikh et al., 1994; Huang et al., 1999; Lin et al., 1995; Tada and Hashimoto, 1997; Tsai et al., 1996; Uchida et al., 1995). Additionally, the reintroduction of connexin genes into tumor cells results in the restoration of GJIC and the retardation of tumor growth *in vivo*, and sometimes *in vitro* (Hirschi et al., 1996; Mesnil et al., 1995; Zhang et al., 1998; Zhu et al., 1991). Furthermore, some reports suggest that connexin can suppress the invasion and metastasis of some tumor cells (Li et al., 2008; Sato et al., 2008). However, the role of connexins in tumorigenesis is perplexing given the findings that some connexin members remain expressed in some tumors and possibly correlate with tumor aggression phenotypes (Graeber and Hülser, 1998; Kanczuga-Koda et al., 2006; Saito-Katsuragi et al., 2007). It is possible that connexins and GJIC are involved in different ways, depending on the stage of the cancer (Cronier et al., 2009; Naus and Laird, 2010).

Although the well-understood function of connexins in tumor suppression is participating GJIC that allows growth regulators to be shared among cells, the tumor suppression role of Cxs may also be independent of the gap junction channel (Cronier et al., 2009; Naus and Laird, 2010). For example, retroviral delivery of Cx26 or Cx43 to the breast cancer cell line MDA-MB-231 did not increase GJIC but did result in growth suppression in vivo (Qin et al., 2002). Interestingly, the growth of C6 glioma cells was suppressed by co-culture of Cx43 transfected cells, suggesting that a diffusible factor(s) may be involved in Cx43-mediated tumor growth suppression (Zhu et al., 1992). Milk fat globule epidermal growth factor 8 (MFG-E8), monocyte chemotactic protein 1, and CCN3 (Cyr61/connective tissue growth factor/nephroblastoma-overexpressed) (NOV) have been identified

Abbreviations: CAM, chick embryo chorioallantoic membrane; ChIP, chromatin immunoprecipitation; Cx, connexin; DiI, 1,1-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; FLST1, follistatin-like 1; GJIC, gap junctional intercellular communication; RT-PCR, reverse transcription-polymerase chain reaction.

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as secreted factors involved in connexin-mediated tumor growth suppression (Fu et al., 2004; Gellhaus et al., 2004; Goldberg et al., 2000; Huang et al., 2002). However, the mechanisms responsible for non-channel-dependent activities of connexins, especially how connexin regulates these factors and how these factors affect tumor growth, are unclear and may involve multiple mechanisms that may be connexin- and cell-type-dependent.

In previous experiments, we transfected Cx43 cDNA into a highly metastatic human pulmonary giant cell carcinoma cell line, PG (Wu et al., 1987), and observed that the growth of PG cells was retarded both *in vitro* and *in vivo* (Zhang et al., 1998). In addition, extensive homologous cell coupling and heterologous communication with human lung fibroblasts were restored in the PG/C4 cell line stably expressing Cx43 (ZQ Zhang et al., 2004). To determine the underlying mechanism(s) by which Cx43 invokes tumor suppression in PG cells, we screened differentially expressed genes and proteins by two-dimensional (2D) gel proteomics and gene chip hybridization, respectively. Interestingly, a set of genes involved in tumor cell invasion and metastasis was found to be affected by Cx43 overexpression. Furthermore, we demonstrated that Cx43 was able to inhibit the *in vitro* and *in vivo* invasion and metastasis of PG cells, possibly through the secretion of follistatin-like 1 (FSTL1), which is regulated by histone acetylation.

2. Materials and methods

2.1. Cell culture

The culture of a highly metastatic human pulmonary giant cell carcinoma cell line, PG, and transfection with Cx43 cDNA were described previously (Zhang et al., 1998). The empty vector and Cx43 transfected clones were cultivated in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 150 μg/ml G418 (Invitrogen, Grand Island, NY).

2.2. 2D electrophoresis and mass spectrometry

Sub-confluent Cx43 transfected PG/C4 cells and vector-only control PG/A3 cells were lysed with 7 M urea lysis buffer (urea 7 M, thiourea 2 M, Tris–HCl 40 mM pH 7.4, CHAPS 4%, DTT 20 mM, PMSF 0.1%, and proteinase inhibitor cocktail). Cell lysates were separated in the 1st dimension by isoelectric focusing using immobilized pH gradient strips (pH range 4–7; Bio-Rad, Hercules, CA) and then separated in the 2nd dimension by SDS-PAGE (12%). The gels were stained with Coomassie blue R-250 and analyzed for differentially expressed proteins using PDQuest 2-D software (Bio-Rad). The spots identified as being differentially regulated were excised manually and digested in gel with sequencing-grade modified trypsin (Promega, Madison, WI). The resulting peptides were analyzed on a Qstar Pulsar I Quadrupole Time-of-Flight Mass Spectrometer (Applied Biosystem/MDS Sciex, Toronto, Canada).

2.3. cDNA microarrays

Equal amounts of cDNA from Cx43 transfectant PG/C4 cells and control PG/A3 cells were labeled with Cy5 and Cy3, respectively, and were then hybridized to a custom cDNA microarray consisting of 14,592 genes/ESTs (cat#: SBC-R-HC-100-20; Shanghai BioChip Co, Ltd., Shanghai, China). Genes were considered to be up- or down-regulated when the fluorescent intensity ratio between PG/C4 and PG/A3 cells was greater than 2 or less than 0.5. The experiment was repeated once using reversed dye labeling. Hierarchical clustering of regulated genes in this study was measured by Genespring software.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted using TRIzol® reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). For RT-PCR analysis, 3 μg of total RNA was first reverse-transcribed in a total volume of 20 μl containing: 1× RT buffer (50 mM Tris–HCl pH 8.3, 75 mM KCl and 3 mM $MgCl_2$), 20 U RNase inhibitor (Promega, Madison, WI, USA), 10 mM dithiothreitol, 50 mM deoxyribonucleoside triphosphates, 0.5 μg oligo-$(dT)_{15}$ (Promega), and 200 U Moloney murine leukemia virus reverse transcriptase (M-MLV-RT; Invitrogen). The cDNA products (1 μl of each gene) were then used as the template in PCR amplification with Taq polymerase (Takara Dalian, Dalian, China). The primers and size of each gene are listed in Supplemental Table 1.

2.5. Western blotting

Western blotting was performed using standard methods. Briefly, cells were lysed with RIPA buffer, and equal amounts of proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After blocking with 5% non-fat dried milk in Tris-buffered saline (TBS 50 mM Tris–HCl pH 7.5, 150 mM NaCl), the membrane was incubated for 1 h at room temperature with primary antibody (details of antibodies used are listed in Supplemental Table 2). Next, the membrane was washed three times with 0.05% Tween-20 in TBS and reacted with horseradish peroxidase-conjugated goat anti-rabbit (or mouse) secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). Signals were detected by enhanced chemiluminescence (Amersham Biosciences, Upsala, Sweden).

2.6. Gelatin zymography

Sub-confluent cells were incubated in serum-free medium for 24 h, and 30 μl of conditioned medium were mixed with an equal volume of non-reducing SDS gel sample buffer (5% SDS, 2% sucrose, 0.2% bromophenol blue) and applied to a 10% polyacrylamide gel containing 1 mg/ml gelatin. After electrophoresis, the gel was washed with 2.5% Triton X-100 three times for 10 min each at room temperature, then incubated in 50 mM Tris–HCl (pH 7.5) containing 200 mM NaCl, 10 mM $CaC1_2$, 5 μM $ZnCl_2$ at 37 °C for 20 h, and finally stained by Coomassie Brilliant Blue R-250 solution.

2.7. Boyden chamber assay

Boyden chambers were prepared such that the lower chamber contained RPMI 1640 medium supplemented with 10% FBS. The porous membrane (pore size, 8 μm; BD Biosciences, Bedford, MA) was coated with or without 2 mg/ml Matrigel and placed above the lower chamber. Next, 5×10^4 cells in serum-free CHO culture medium (Invitrogen, Grand Island, NY) were added to the upper chamber. After 24 h of incubation in a 37 °C CO_2 incubator, the membranes were removed, and the cells that did not migrate through the pores were scraped off using a cotton swab. The membranes were then fixed with methanol and stained with 1% toluidine blue in 1% borax. The number of migrated cells was determined by counting five random fields using an inverted Leica microscope (Leica, Wetzlar, Germany).

To ascertain whether Cx43-mediated cell invasion suppression involved a secretion factor(s), we first performed a co-culture assay in Boyden chambers. PG cells were pre-labeled with 1,1-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbo-cyanine perchlorate (DiI) (Molecular Probes), as described previously (Goldberg et al., 1995), and then mixed with PG/A3 or PG/C4 cells and plated as above in the upper chamber. Migrated cells were monitored under a fluorescein microscope. We also performed Boyden chamber assays

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