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Cx31.1 acts as a tumour suppressor in non-small cell lung cancer (NSCLC) cell lines through inhibition of cell proliferation and metastasis

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Abstract

Reduced connexin expression and loss of gap junction function is a characteristic of many cancers, including lung cancer. However, there are little reports about the relation between Cx31.1 and lung cancer. This study was conducted to investigate the effect of Cx31.1 on non-small cell lung cancer (NSCLC). We found that the Cx31.1 was down-regulated in NSCLC cell lines, and the expression levels were reversely related with their metastatic potential. We ectopically expressed Cx31.1 in H1299 NSCLC cell line to examine the influence of Cx31.1 overexpression. The results showed that overexpression of Cx31.1 in H1299 cells reduced cell proliferation, induced a delay in the G_1 phase, inhibited anchorage-independent growth and suppressed cell migration and invasion. The cell cycle delay and cell migration and invasion suppressive effects of Cx31.1 were partially reversed by siRNA targeting mRNA of Cx31.1. Moreover, xenografts of Cx31.1 overexpressing H1299 cells showed reduced tumourigenicity. These results suggested that Cx31.1 has tumour-suppressive properties. Further investigation indicated that cyclin D3 may be responsible for Cx31.1-induced G_1 phase delay. Importantly, Cx31.1 increased the expression of epithelial markers, such as cytokeratin 18, and decreased expression of mesenchymal markers, such as vimentin, indicating a Cx31.1-mediated partial shift from a mesenchymal towards an epithelial phenotype. We concluded that Cx31.1 inhibit the malignant properties of NSCLC cell lines, the mechanisms under this may include regulation of EMT.

Keywords: Cx31.1 • non-small cell lung cancer • cell cycle • metastasis • cyclin D3 • EMT

Introduction

Gap junctions are channels that link the cytoplasm of adjacent cells. Gap junctional intercellular communication (GJIC) established by these channels allows for the passage of small molecules of $<$1000 Da such as ions and secondary messenger molecules. Gap junctions are made of two hemi-channels, called connexons, which are composed of six molecules of the membrane spanning connexin protein. There are more than 20 connexin (Cx) genes in the human genome [1]. They show cell-type and differentiation-dependent expression patterns. Cx31.1, also known as gap junction protein B5 (GJB5), a 273 amino acid peptide with a molecular mass of 31.088 kD, is predominantly expressed in the testes and the skin epidermis [2,3]. Cx31.1 rarely form functional gap junction channels, either with itself or with other Cx isoforms [4].

Gap junctions play important roles in various physiologic functions such as regulation of cell proliferation, cell differentiation, tissue development and cell apoptosis. In most cases, Cxs act as tumour suppressors. Greatly reduced or absence of Cx expression or GJIC have been found in various cancers, such as lung cancer, suggesting that gap junctions play an important role as tumour suppressors in maintaining cell differentiation and preventing transformation [5–9]. Conversely, when Cxs were overexpressed in carcinoma cells, tumour growth was slowed, and the cells regained the capacity to form at least partially differentiated structures [10]. In addition, mice lacking Cxs exhibited an increase in lung tissue proliferative index and lung tumour incidence [11,12], suggesting that Cxs exhibit tumour-suppressive properties.

The mechanism by which Cxs can act as tumour suppressors is not well understood. Because the primary function of Cxs has been thought to be the formation of blocks of gap junction channels that mediate the tissue homeostasis, the molecular mechanism by

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which Cxs suppress tumour formation and progression was suggested to link to the putative molecules that are exchanged *via* the Cx-modulated gap junctions [13]. However, a growing mount of reports in recent years had shown that some of the ectopic expressed Cxs did not localize to the cell–cell interface or rescue GJIC, and Cxs which did not form GJIC are sufficient to play tumour-suppressive roles, indicating a GJIC-independent mechanism of tumour suppression [14–16]. Cxs had been reported to influence cell adhesion [17], migration [18] and cell cycle [19], the key factors for tumour progression and metastasis, in a GJIC-independent manner. For example, Cx43 has been shown to modulate intercellular adhesion through interactions with the cytoskeleton [20,21], regulate the expression of several genes associated with the cell cycle, including cyclin A, cyclin D1, cyclin D2, p21 and p27 [22].

A large number of studies have been performed on members of the Cx family. However, there was little work focusing on Cx31.1. Here, we exogenously expressed Cx31.1 in NSCLC cell lines, and showed that Cx31.1 reduced tumour cell proliferation, anchorage-independent growth, migration and invasion. Moreover, development of tumours in a xenograft model was suppressed by Cx31.1. Our results suggested that Cx31.1 may act as a tumour suppressor in NSCLC cell lines.

Materials and methods

Human Cx31.1 expression constructs

The SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) was used for reverse transcription of total RNA extracted from WI-38 cell line. The primers, 5'-GATCTCGAGGCCACCATGAACTGGAGTATCTTTGAG-3' and 5'-CAGAATTCGCAAGATGGTTTTCTTCACATGGT-3', were used to amplify the entire open reading frame of Cx31.1, adding *Xho*I and *Eco*RI restriction sites to the 3' and 5' ends, respectively. The amplified fragment was digested with *Xho*I and *Eco*RI, cloned into pEGFP-N1 (Clontech, Mountain View, CA, USA), and designated Cx31.1–EGFP. The nucleotide sequences of all plasmid were confirmed by automated sequencing on an AB 3730 sequencer. Using this construct as a template, an EGFP tag was added to the C-terminus of Cx31.1. The entire open reading frame of human Cx31.1 was also cloned into pcDNA3.1(+) (Invitrogen, Burlington, Ontario, Canada), and designated Cx31.1-myc.

Cell culture and quantitative RT-PCR

All cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). The medium, foetal bovine serum (FBS), HEPES, non-essential amino acids and sodium pyruvate were purchased from Invitrogen. BEAS-2B cells were cultured in serum-free bronchial epithelial growth media (Clonetics/Lonza, Walkersville, MD, USA). WI-38 cells were cultured in DMEM medium supplemented with 10% FBS. Calu-3 cell lines were grown in Eagle's Minimum Essential Medium, supplemented with non-essential amino acids and 10% FBS. A549 cells were grown in F-12K medium supplemented with 10% FBS, 2.383 mg/ml HEPES. NCI-H1299, NCI-H460, NCI-H226 cells were grown in RPMI 1640 Medium, supplemented with 10% FBS, 2.383 mg/ml HEPES and 0.11 mg/ml sodium pyruvate. All cells were cultured in the humidified incubator at 37°C and 5% CO_2. Total RNA was prepared with RNeasy Mini Kit (Qiagen, Hilden, Germany), reverse transcription was performed according to the manual of PrimeScript RT reagent Kit (Takara Bio, Shiga, Japan). The primers for quantitative real-time PCR amplification were designed with Primer-BLAST of NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Quantitative real-time PCR was utilized to quantify the gene expression levels with 2× HotSybr PCR Reaction Mix (NuStar Laboratory, San Francisco, CA, USA). PCR reactions consisted of a hot start (10 min. at 95°C), 40 cycles of 15 sec. at 95°C and 60 sec. at 60°C. Each sample runs in triplicates for each gene. Relative expression levels of mRNA were calculated by normalizing to the level of GAPDH mRNA by using comparative threshold cycle (Ct) method, in which fold difference $= 2^{-(\Delta ct \text{ of target gene} - \Delta ct \text{ of reference})}$. Expression levels of Cx31.1, D-type cyclins (D1, D2 and D3), E-type cyclins (E1 and E2), Vimentin, Cytokeratin 18, E-cadherin and GAPDH were detected. The primers used for real-time PCR were listed in Table 1.

Cell transfection and bulk-selected H1299 cells stably expressing Cx31.1

Transfection was mediated by Lipofectamine™ 2000 (Invitrogen). H1299 cells were transfected with the Cx31.1–EGFP or control mock vector. Forty-eight hours after transfection, the cells were harvested and the green fluorescence positive cells were sorted with FACS Calibur (BD Biosciences) and then selected with 500 μg/ml G418 (Sigma-Aldrich, St. Louis, MO, USA) for 2 weeks. The EGFP fluorescence was monitored under a fluorescent microscope. Two weeks later, the bulk-selected H1299 cells stably expressing Cx31.1–EGFP or EGFP were maintained in RPMI 1640 with 250 μg/ml G418. Bulk cultures were used to avoid clone-specific effects.

RNA interference

Online software siRNA Target Finder of Applied Biosystems was used to design siRNA against Cx31.1 (http://www.ambion.com/techlib/misc/siRNA_finder.html), followed by a BLAST search to evaluate the unicity of the siRNA sequences in the human transcriptome. Three siRNAs, targeting nucleotides 4–24, 145–164 and 463–483, were selected to be chemically synthesized by GenePharma (Shanghai, China). A siRNA lacks homology to the genome was employed as a negative control. The sequences of negative control were as following: sense: 5'-UUC UCC GAA CGU GUC ACG UTT-3'; anti-sense: 5'-ACG UGA CAC GUU CGG AGA ATT-3'. Cx31.1–EGFP bulk cultures were transfected with these siRNAs at a concentration of 50 nM, mediated by Lipofectamine™ 2000 (Invitrogen). The silencing efficacies were evaluated by reduction of Cx31.1 mRNA expression assessed by quantitative real-time PCR and Western blotting with antibody against EGFP.

Cell cycle and cell proliferation analysis

Cell cycle analysis was performed with flow cytometer. For bulk-selected cultures, 1×10^6 cells were trypsinized, washed with PBS, permeabilized in 70% ethanol overnight, incubated with propidium iodide (PI, 10 μg/ml) containing RNase at 4°C for 30 min. The percentages of cells in different

Table 1 Primers used for real-time PCR amplification

Genes	Forward primer (5' to 3')	Reverse primer (5' to 3')
Cx31.1	GTCCTATTGCCGGCTGCTGGG	GCGCCCAAAGGCTGTGGAGT
Cyclin D1	TGCGGAAGATCGTCGCCACC	GCAGCTGCAGGCGGCTCTTT
Cyclin D2	GCGCTACCTTCCGCAGTGCT	CCCAGCCAAGAAACGGTCCAGG
Cyclin D3	TGCAGTGCAAGGCCTGGGTG	TCTGCTCCTGACAGGCCCGC
Cyclin E1	ACAGGACGGCGAGGGACCAG	TCCGAGGCTTGCACGTTGAGT
Cyclin E2	TGAGCCGAGCGGTAGCTGGT	TTCCGTCTGGCTGGGCTGGG
Vimentin	ACAAAGCCCGCGTCGAGGTG	GGTCAAGACGTGCCAGAGACGC
Cytokeratin 18	ACCGAGAACCGGAGGCTGGA	TCTGCAGAACGATGCGGGCA
E-cadherin	TCGTCACCACAAATCCAGTGAACAACG	GCTGTGGAGGTGGTGAGAGAGACC
GAPDH	CTTAGATTTGGTCGTATTGG	GAAGATGGTGATGGGATT

phases of the cell cycle were measured with a FACStar flow cytometer (Becton-Dickinson, San Jose, CA, USA) and analysed with ModFit software (Verity Software House, Inc., ME, USA). For transiently transfected H1299 cells, the same procedure was used, except that an analysis gate was set on green fluorescence positive cells.

Cell proliferation analysis was performed with Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manual of the manufacture. Briefly, 8000 cells were grown in each well of 96-well plate. Ten microlitres of the CCK-8 solution was added to each well of the plate (designated 0 hr). After an incubation of 1.5 hrs, the absorbance at 450 nm was measured using a Labsystems Multiskan Ascent Photometric plate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA). Each experiment was performed in triplicates.

Colony formation assay

The anchorage-independent colony formation assays were performed in soft agar in six-well plate. Five thousand cells per well were mixed thoroughly in medium containing 0.35% agarose (Cat.A9045; Sigma-Aldrich). Each performed in triplicate. The plates were incubated in a 5% CO_2, at 37°C incubator for 3 weeks. The colonies were examined under a microscope and colonies with more than 10 cells were counted.

Cell migration and invasion assay

The invasive and metastases potential of the following three groups of cells were estimated with cell invasion and migration assays: (i) the human lung bronchus epithelial cell line BEAS-2B and five NSCLC cell lines; (ii) H1299 stably expressing Cx31.1–EGFP or EGFP; (iii) H1299 cells stably expressing Cx31.1–EGFP interfered with siRNA anti-Cx31.1 or negative control. The cell invasion assay was performed in transwell plates (8-μm pore size, 6.5-mm diameter, Corning) pre-coated with MATRIGEL Basement Membrane Matrix (coating conc.: 1 mg/ml; BD Biosciences, San Jose, CA, USA). Briefly, a total of 5×10^4 cells in 0.1 ml media (with 1% FBS) were seeded into the upper chamber, with 0.6 ml of medium (10% FBS) under the upper chamber. Plates were incubated in a humidified incubator at 37°C and 5% CO_2. Forty-eight hours later, chambers were removed and the non-invading cells were scraped using a cotton swab from the upper side of the chamber. The cells under the chamber were fixed with methanol for 10 min., stained with 1% toluidine blue for 10 min. and washed twice with 95% ethanol. The cells migrated through matrigel were counted under microscope.

Migration assays were performed with the same procedure, except that the insert transwell chambers were not coated with Matrigel and medium containing 10% FBS was used for the cell suspensions.

Subcutaneous tumourigenicity assay

Male athymic BALB/c nude mice were purchased from Slac Laboratory Animal (Shanghai, China). Mice were housed in sterilized cages and fed autoclaved food and water. Mice of 6 weeks age were used in study protocol. Bulk-selected Cx31.1–EGFP overexpressing H1299 cells and EGFP expressing control cells were trypsinized, washed and resuspended in PBS, and diluted to the concentration of 10^7 cells/ml. Cell viability was confirmed by trypan blue exclusion. 1×10^6 cells suspended in 0.1 ml of PBS were injected s.c. in the flanks of nude mice ($n = 5$). Tumour sizes were monitored three times per week. Tumour volume was calculated as $\pi LW^2/6$.

Immunofluorescence

For immunofluorescence, cells were washed three times in PBS, fixed in 4% paraformaldehyde for 15 min. and blocked for 30 min. in 5% normal geltin diluted in one time PBS with 0.3% Triton X-100. After a brief wash in PBS, cells were incubated with primary antibodies (c-myc, 1:100; Sigma-Aldrich; GRP94, 1:100; ProteinTech Group, Chicago, IL, USA) overnight at 4°C. The secondary antibodies conjugated with DyLight (KPL, Gaithersburg, MD, USA) were used to detect primary antibodies. Fluorescence signals were visualized with an Olympus IX71 inverted research microscope (Olympus, Tokyo, Japan).

Fluorescent visualization of lysosome

Organelle Lights Lysosomes-RFP (Invitrogen) was employed to visualize lysosome of Cx31.1 stably expressing H1299 cells according to the manual of the manufacture. Forty-eight hours after Organelle Light transfection,

cells were washed three times in PBS, fixed in 4% paraformaldehyde for 15 min. and visualized with an inverted research microscope.

Immunoblotting

Cell lysates were prepared using radio immunoprecipitation assay buffer (50 mM Tris-HCl (pH 8.0), 150 mM sodium chloride, 5 mM EDTA, 50 mM sodium fluoride, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (100 μg/ml PMSF, Sigma-Aldrich; Complete Protease Inhibitor Cocktail; Roche Diagnostics, Mannheim, Germany). Samples were centrifugated at 4°C for 15 min. to pellet insolubles. Protein concentrations were measured by a modified Lowry assay (BioRad, Hercules, CA, USA). Fifteen milligrams of protein was separated by electrophoresis on 12% SDS-PAGE gels, transferred onto polyvinylidene difluoride membranes. Membranes were blocked for 1.5 hrs with 5% non-fat milk in TBS containing 0.05% Tween-20, and incubated overnight at 4°C with primary antibodies (EGFP, 1:2000, Neomarker; Vimentin, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA; Cytokeratin 18, 1:1500; ProteinTech Group; Cyclin D3, 1:1000; ProteinTech Group; α-Tubulin, 1:3000; Sigma-Aldrich). After three washes with TBS containing 0.1% Tween-20, the blots were incubated in peroxidase-coupled secondary antibodies against rabbit or mouse IgG (1:5000, KPL) for 1.5 hrs at room temperature, washed thrice and developed using SuperSignal Substrate (Pierce, Rockford, IL, USA).

Statistical analysis

All quantitative results were presented as mean ± S.D. Experimental data analyses were conducted using unpaired, two-tailed Student's *t*-tests, and one-way ANOVA with Scheffé's *post-hoc* test where applicable. The criterion for data significance is a $P < 0.05$. The *P* values presented in the figure legends are based on the Student's *t*-test, unless otherwise stated.

Results

Cx31.1 expressions were reduced in NSCLC cell lines

As an initial test of the relation between Cx31.1 and invasiveness and metastases of NSCLC, we utilized real-time PCR to evaluate the expression levels of Cx31.1 in human lung bronchus epithelial cell line BEAS-2B and five NSCLC cell lines of different invasive potential. NSCLC cell lines showed significantly reduced expression levels of Cx31.1 compared with the human lung bronchus epithelial cell line BEAS-2B (Fig. 1). Calu-3 was reported to be a medium tumourigenicity lung cancer cell line without metastatic potential [23]. Consistently, our migration and invasion assays showed that Calu-3 had neither migration nor invasion capability. Nevertheless, its Cx31.1 expression level was significantly lower than that of BEAS-2B whereas higher than that of the cell lines with migration and invasion capability (Fig. 1). Moreover, among the cell lines with migration and invasion capability, the expression levels of Cx31.1 in the high metastatic cell lines (A549, H1299) were significantly lower than that in the low metastatic cell lines (H460, H226). These results indicated that the expression of Cx31.1 was correlated with the cell differentiation level, whereas reversely related with the metastasis potential. Thus, we further examined the potential role of Cx31.1 in tumourigenicity and metastasis.

Intracellular location of Cx31.1 ectopically expressed in H1299 cells

To investigate the intracellular location of Cx31.1, we employed two vectors, pEGFP-N1 and pcDNA3.1(+), to mediate the ectopic expression of Cx31.1 in H1299 cells through fusion to EGFP and myc in the C-terminal of Cx31.1, respectively. H1299 transfected with Cx31.1-myc were immunostained with antibody against c-myc. Both of the two constructs (Cx31.1–EGFP and Cx31.1-myc) showed similar intracellular location patterns (Fig. S1). The result suggested the fusion of EGFP in C-terminal of Cx31.1 did not alter its intracellular localization compared to Cx31.1-myc in H1299 cells.

To further investigate the localization of Cx31.1, H1299 cells overexpressing Cx31.1–EGFP were immunostained with antibody against GRP94 (HSP90B1) and transfected with Organelle Lights targeting lysosome. As shown in Figure 2, most of the Cx31.1–EGFP and GRP94 signals showed a co-localization, whereas some of the punctas of the Cx31.1–EGFP signal co-localized with lysosome.

Ectopic expression of Cx31.1 in H1299 cells reduced cell growth and induced a delay in the G_1 phase

We first examined the effect of Cx31.1 on NSCLC cell cycle. Flow cytometric analysis showed that Cx31.1–EGFP transiently transfected H1299 cell population were composed of less cells in S phase and more cells in G_1 compared with mock construct transfected H1299 cells (Fig. 3A), with a 7.9% decrease in S phase and a 8.7% in G_1 phase, respectively. Alternatively, to avoid the specific effect of EGFP tag due to its high molecular weight, we co-transfected H1299 cells with Cx31.1-myc construct and pBB14, a tail-anchored fluorescent protein expressing vector. H1299 cells co-transfected with pcDNA3.1(+) mock construct and pBB14 were used as a control. Flow cytometric analysis revealed similar cell cycle changes as that of Cx31.1–EGFP construct (Fig. 3B), confirming the delay in the G_1 phase induced by Cx31.1 overexpression.

To facilitate the visualization, detection, selection and sorting of H1299 cells expressing Cx31.1 for the following experiments, the Cx31.1–EGFP construct was used to mediate Cx31.1 ectopic expression in H1299 for further analysis.

To avoid clone-specific effects, we used bulk selected cells for gene function analysis. Flow cytometric analysis showed that bulk cultures of Cx31.1–EGFP stably expressing H1299 cells had a 9.9%

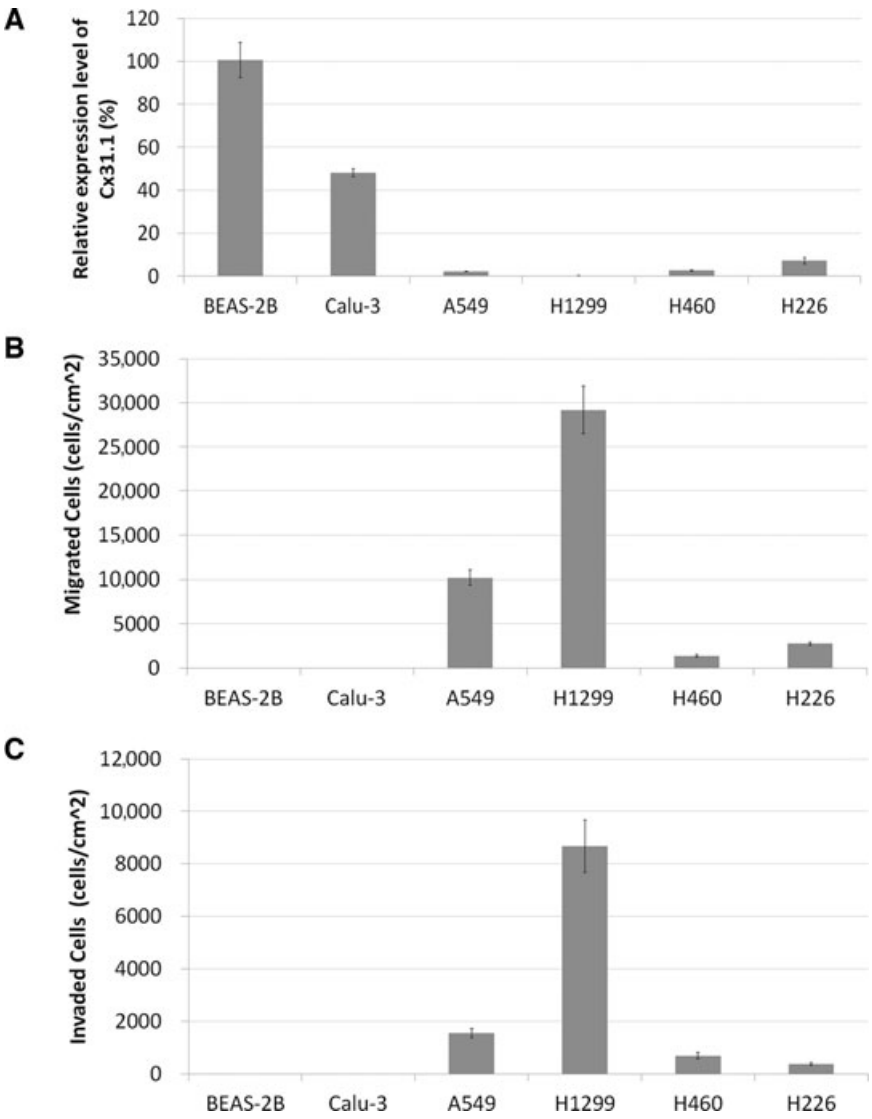

Fig. 1 Cx31.1 expressions were reduced in NSCLC cell lines and reversely related with the migration and invasion potential of NSCLC cells. (**A**) The mRNA expression levels of Cx31.1 in human lung bronchus epithelial cell line BEAS-2B and five NSCLC cell lines were determined with real-time PCR. mRNA expression level of BEAS-2B as reference. (**B**, **C**) The migration and invasion potential of NSCLC cells were determined with migration and invasion assay. 5 $\times$ 10^4 BEAS-2B or NSCLC cells in 0.1 ml media were seeded into upper chambers of Transwell uncoated (**B**, migration assay) or Matrigel matrix-coated (**C**, invasion assay). Forty-eight hours later, the cells migrated through the bottom of the Transwell were stained and counted under a reverse microscope. Each performed in triplicate. Bars represent mean $\pm$ S.D.

increase in G_1 phase, and 13.7% decrease in S phase cells compared with EGFP expressing cultures, confirming the G_1 cell cycle delay.

Cell proliferation analysis with Cell Counting Kit-8 revealed that H1299 cells stably expressing Cx31.1 had a severe reduction in cell-growth rate compared with H1299 cells stably expressing EGFP. As shown in Figure 3D, the growth rate of Cx31.1 overexpressing H1299 was about 76% and 55% of that of control cells at day 2 and day 3, respectively.

Cx31.1 overexpression suppressed anchorage-independent cell growth

We further investigated the role of Cx31.1 in anchorage-independent cell growth, a hallmark of tumour progression. After 3 weeks growth, ectopic expression of Cx31.1 in H1299 cells decreased the number of colonies in soft agar by approximately seven times (colony-formation assay; Fig. 4), suggesting that Cx31.1 overexpression suppressed anchorage-independent cell growth.

Cx31.1 overexpression in NSCLC cells reduced cell invasion and migration

To investigate the influence of Cx31.1 on invasion and metastasis, we performed migration and invasion assays. In a migration assay, we found that Cx31.1 overexpression decreased the ability of H1299 cells to migrate (Fig. 5A and B). We also examined the invasiveness of these cells, and found that Cx31.1 significantly decreased the number of cells that penetrated the Matrigel-coated

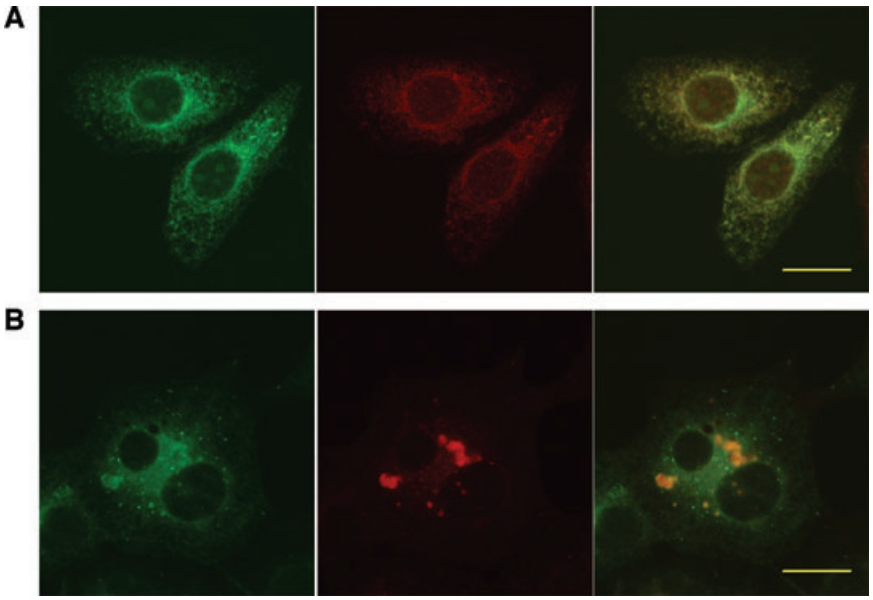

Fig. 2 The localization of Cx31.1. Most of the ectopically expressed Cx31.1–EGFP protein was restricted within endoplasmic reticulum, whereas some of the punctas of the Cx31.1–EGFP signal co-localized with lysosome. (**A**) Cx31.1–EGFP overexpressing H1299 cells (left) were immunostained with antibody against GRP94 (middle). Merged image (right) shows both Cx31.1–EGFP fusion protein and GRP94 protein signals. The Cx31.1–EGFP fusion protein signal (green) partially co-localized with GRP94 signal (red). (**B**) Cx31.1–EGFP overexpressing H1299 cells transfected with Organelle Lights targeting lysosome (middle). Merged image (right) shows some of the punctas of the Cx31.1–EGFP signal co-localized with lysosome. Scale bars, 10 μm.

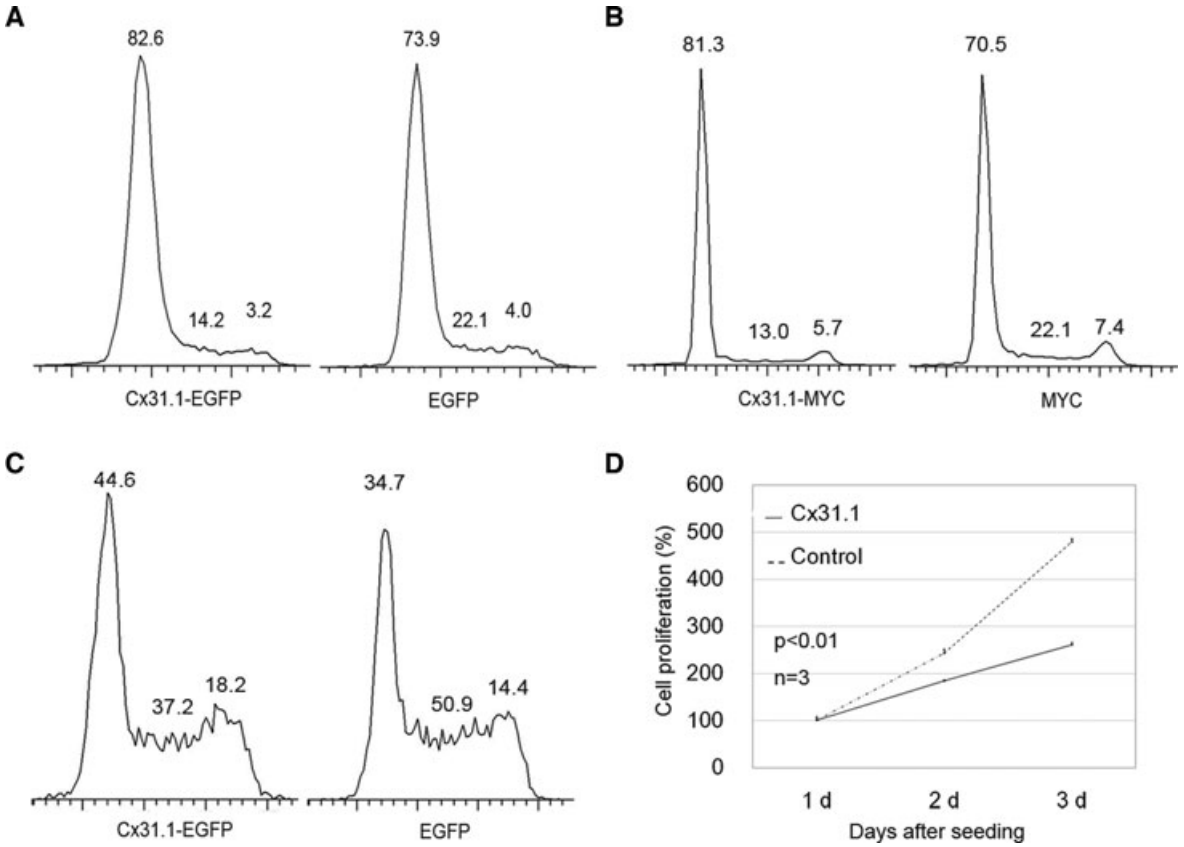

Fig. 3 Ectopic expression of Cx31.1 in NSCLC cells reduced cell growth and induced a G_1 delay. (**A**–**C**) Cx31.1 overexpression induced a G_1 delay. H1299 transiently expressing Cx31.1–EGFP fusion protein (**A**) or Cx31.1–myc fusion protein (**B**), and stably expressing Cx31.1–EGFP (**C**) all display increased numbers of cells in G_1 and decreased number of cells in S phase compared with control cells expressing EGFP. (**D**) Cell proliferation analysis with Cell Counting Kit-8 revealed that overexpression of Cx31.1–EGFP in H1299 cells reduced cell proliferation rate compared with control cells expressing EGFP.

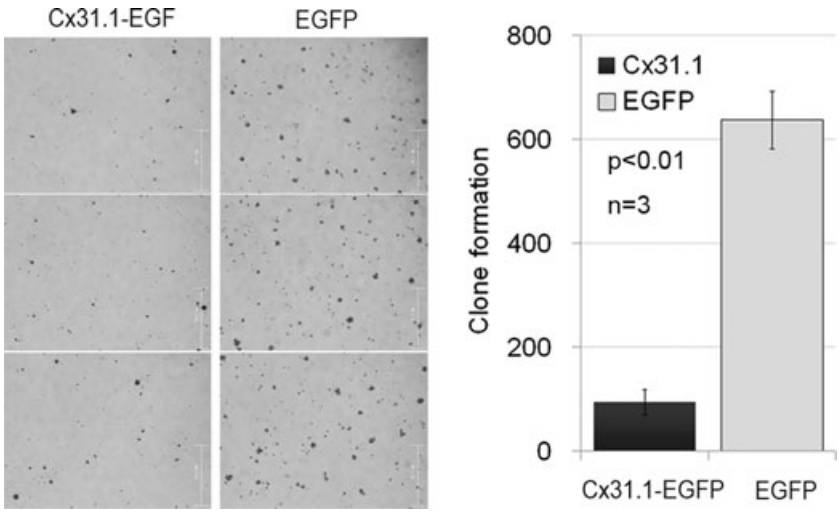

Fig. 4 Cx31.1 overexpression suppressed anchorage independent cell growth. Five thousand cells were plated in soft agar in each well of six-well plate. After incubation for three weeks, colonies were examined under a microscope and colonies with more than 10 cells were counted. Each performed in triplicate. Bars represent mean ± S.D.

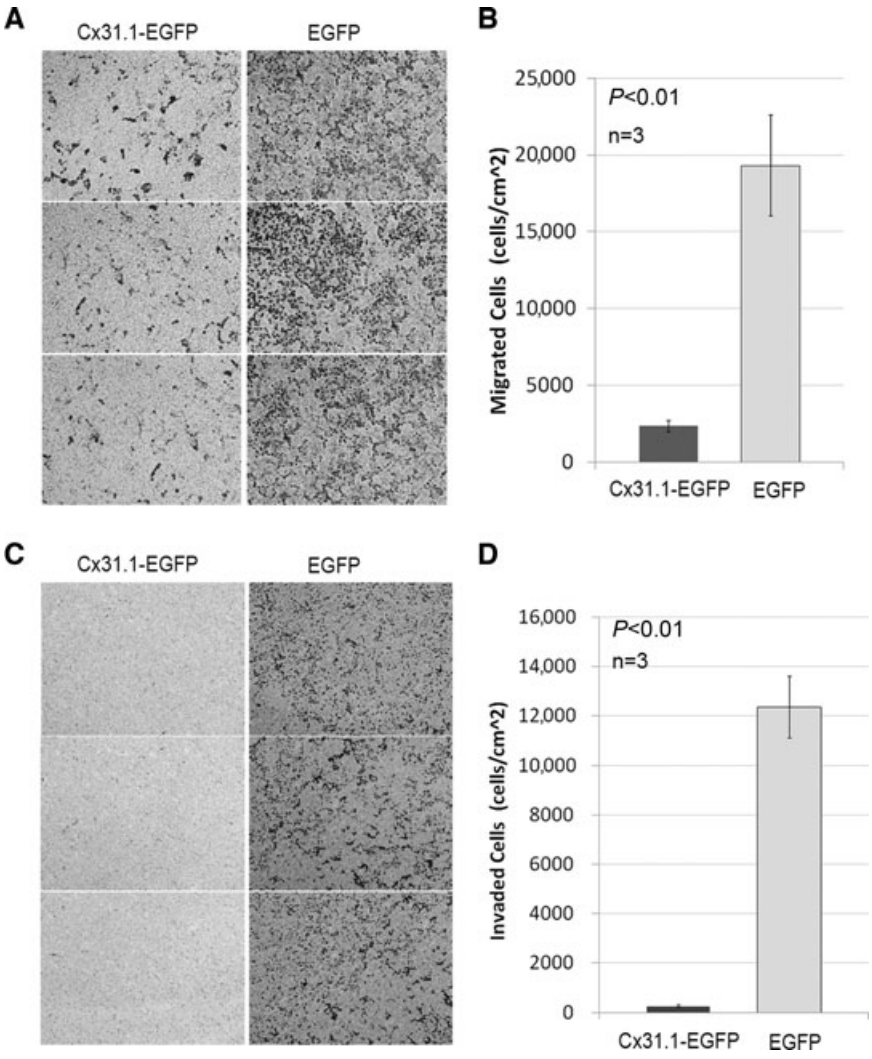

Fig. 5 Cx31.1 overexpression in NSCLC cells reduced cell migration. 5×10^4 Cx31.1 overexpressing H1299 or control cells in 0.1 ml media (1% FBS) were seeded into upper chambers of Transwell uncoated (**A** and **B**, migration assay) or Matrigel matrix-coated (**C** and **D**, invasion assay). Forty-eight hours later, the cells migrated through the bottom of the Transwell were stained and counted under a reverse microscope. Each performed in triplicate. Bars represent mean ± S.D.

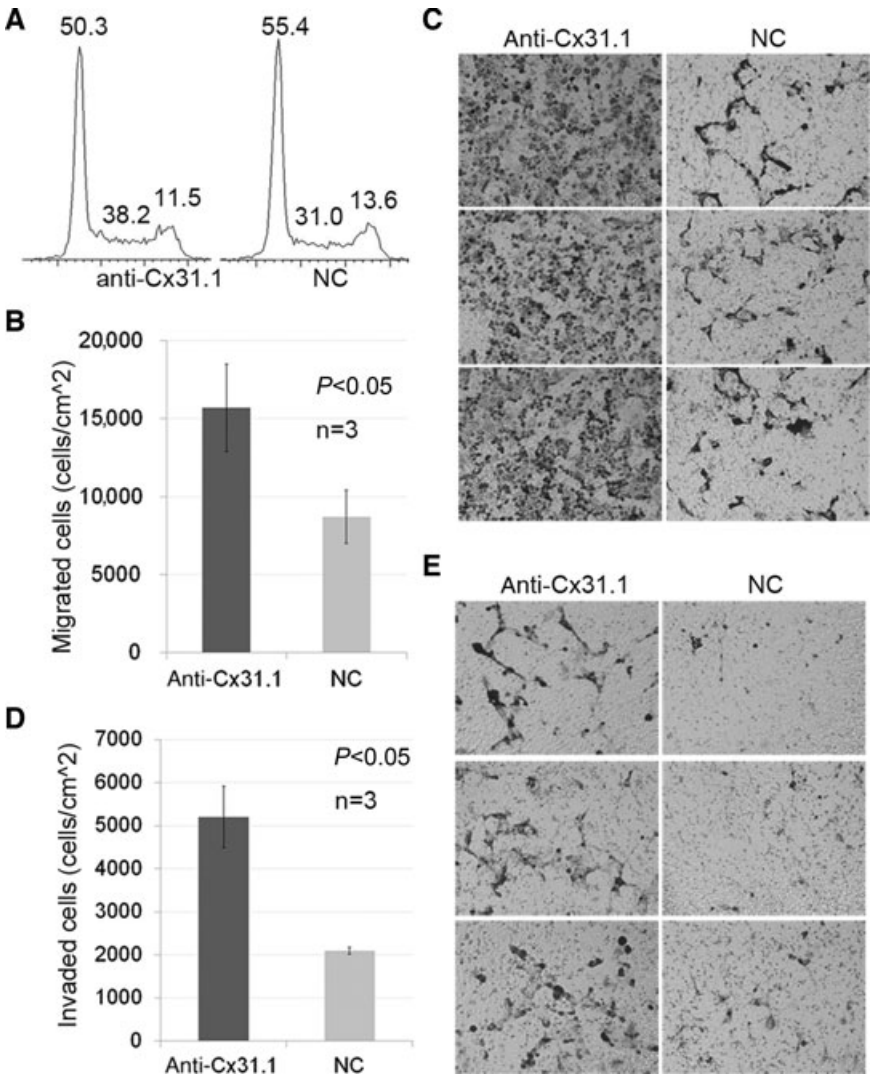

Fig. 6 SiRNA-targeting Cx31.1 reversed the Cx31.1 overexpression induced effect. (**A**) Flow cytometric analysis showed decreased number of cells in G_1 phase and increased number in S phase. (**B**–**E**) The migration (**B**, **C**) and invasion (**D**, **E**) suppressive effect of Cx31.1–EGFP were reversed by siRNA-targeting mRNA of Cx31.1 introduction. Each performed in triplicate. Bars represent mean ± S.D.

membrane (Fig. 5C and D). The results of these assays indicated that the metastatic potential of H1299 was significantly reduced by Cx31.1 overexpression.

Cx31.1 overexpression induced effect were reversed by siRNA targeting mRNA of Cx31.1

Three potential siRNAs targeting different nucleotides regions of Cx31.1 mRNA (4–24, 145–164 and 463–483) were designed by online software analysis, targeting nucleotides. We synthesized and transfected these siRNAs into Cx31.1 overexpressing H1299. According to real-time PCR analysis, the siRNA targeting nucleotides 145–164 had the best silencing efficacy (95%), and was confirmed to down-regulated Cx31.1 protein expression by 16 times (Fig. S2). This siRNA was employed for the transfection of Cx31.1 overexpressing H1299 cells. Flow cytometric analysis, migration and invasion assays revealed that the inhibition of Cx31.1 in cell proliferation, migration and invasion were partially reversed by transfection of this siRNA into Cx31.1 overexpressing H1299 cells (Fig. 6), although a full reversion could not be accomplished.

In vivo tumourigenicity and tumour growth was suppressed by Cx31.1 overexpression in NSCLC cell line

The ability of Cx31.1 to inhibit tumourigenicity and tumour growth was further analysed in xenograft models. Cx31.1 overexpressing H1299 cells or control cells were injected s.c. into nude mice ($n = 5$). In the control group, the mice produced visible tumours

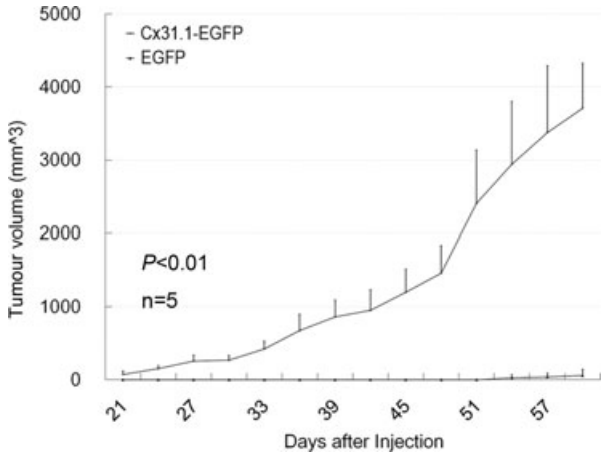

Fig. 7 Cx31.1 overexpression suppressed *in vivo* tumourigenicity and tumour growth. H1299 overexpressing Cx31.1–EGFP or control cells overexpressing EGFP were injected subcutaneously into male athymic BALB/c nude mice ($n = 5$), and the formation of experimental tumours was monitored 60 days. Mean volume of all five tumours of each group was shown. Cx31.1–EGFP overexpressing H1299 cells injected mice produced tumours six weeks later and produced smaller tumours than control group. Tumour volume was calculated as $\pi LW^2/6$. Bars represent mean ± S.D.

22 days after injection, and all the five mice produced tumours, whereas in the Cx31.1 overexpressing H1299 injected group, only three mice produced tumours 54 days after injection, and produced smaller tumours than control group (Fig. 7, Table S1). The other two mice did not produce tumours in our observation window (60 days). This demonstrated that overexpression of Cx31.1 led to suppression of tumour formation *in vivo*.

Cyclin D3 expression is down-regulated by Cx31.1 overexpression

To investigate the mechanisms through which Cx31.1 induces a delay in the G_1 phase, we examined the expression levels of cyclin D (D1, D2 and D3) and cyclin E (E1, E2). Real-time PCR indicated that Cx31.1 overexpression did not significantly affect the abundance of mRNA of cyclin D1, cyclin D2, cyclin E1 and cyclin E2 (data not shown), whereas the mRNA level of cyclin D3 was significantly down-regulated. Western blotting with antibody against cyclin D3 confirmed the down-regulation of cyclin D3 (Fig. 8).

Cx31.1 overexpression promotes a phenotypic mesenchymal to epithelial shift

The epithelial to mesenchymal transition (EMT) provides a potential mechanism by which epithelial cancer cells detach from the neighbouring cells of the primary tumour, invade through the underlying basement membrane and migrate into the surrounding stroma. Cultures of Cx31.1 overexpressing H1299 were tightly adhered to their neighbour cells, whereas the control cells did not stably contact with each other. Moreover, as shown earlier, Cx31.1 overexpressing H1299 displayed less motile and invasive character. Thus, we speculated that Cx31.1 overexpression may play a role in reversing the process of EMT.

The ratio of the intermediate filament proteins cytokeratin 18 to vimentin is often used to assess the phenotypic properties of cells that have undergone EMT [24]. Real-time PCR and Western blotting revealed that Cx31.1 overexpressing H1299 cells showed reduced vimentin levels and elevated cytokeratin 18 levels compared with control cells (Fig. 8). In addition, the mRNA expression level of E-cadherin was found to be very low in control H1299 cells but re-expressed in Cx31.1 overexpressing cells at a relative high level. It should be noticed that in real-time PCR amplification reaction, the E-cadherin expression could not be detected except for high concentration of cDNA template usage (30 times of that used in general real-time PCR), indicating that E-cadherin expressed at low level in NSCLC cells, even if it is up-regulated by Cx31.1. As expected, E-cadherin protein signal was detected in neither Cx31.1 overexpressing H1299 cells nor control cells. These results raise the possibility that Cx31.1 expression partially, yet not completely, reverts the transformed phenotype of NSCLC cells by regulating EMT.

Discussion

In this study, we have shown that Cx31.1 may act as a tumour suppressor in NSCLC cell lines. We showed that Cx31.1 was down-regulated in NSCLC cell lines, and ectopic expression of Cx31.1 in H1299 NSCLC cell line induced slowed cell growth, reduced anchorage independent cell growth, inhibited cell invasion and migration. The cell proliferation, migration and invasion inhibition effect of Cx31.1 ectopic expression was partially reversed by siRNA targeting mRNA of Cx31.1 introduction. Overexpressing of Cx31.1 partially, although not completely, promotes a phenotypic mesenchymal to epithelial shift. The cell tumourigenicity and tumour growth *in vivo* were significantly suppressed by Cx31.1 overexpression.

Since Lowenstein and Kanno first reported in 1966 that gap junctions might have a role in tumourigenesis [5], significant effort has been devoted to find the role played by gap junction and its component, Cx, in tumour progression. Although progress has been made toward associating the dysfunction of gap junction and down-regulation of Cx expression to cancers, little work has focused on the association between Cx31.1 and cancer. Al Moustafa *et al.* reported that Cx31.1 was down-regulated in head and neck squamous cell carcinoma [25]. Budunova *et al.* found that all the epidermal cell lines from SENCAR mouse skin tumours they studied were Cx31.1 negative [26]. To our knowledge, there is no report about the association between Cx31.1 and lung cancer carcinogenesis and metastasis as yet.

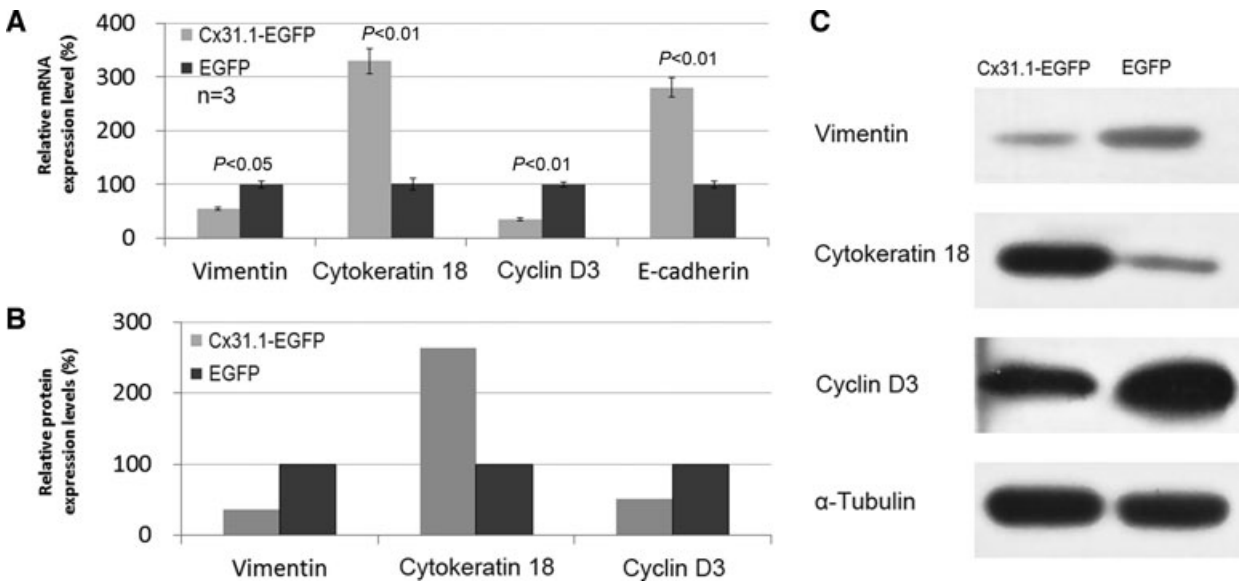

Fig. 8 Analysis of Cx31.1-regulated components related to cell cycle and EMT pathways. Real-time PCR (**A**) and western blotting (**B**, **C**) revealed that Cx31.1–EGFP overexpressing H1299 cells showed reduced vimentin and CCND3 levels and elevated cytokeratin 18 and E-cadherin levels compared with control cells overexpression EGFP.

We found that Cx31.1 was down-regulated in non-small cell lung cancer cell lines compared to normal cells. Furthermore, the Cx31.1 expression level of NSCLC cell line without metastases potential was rather higher than that of cell lines with metastases potential. Among the cell lines with migration and invasion capability, the expression levels of Cx31.1 in the high metastatic cell lines were significantly lower than that in the low metastatic cell lines, indicating that the metastatic potential of NSCLC cell lines were reversely related with their Cx31.1 expression levels.

It remains controversial whether the mechanism of Cxs act as tumour suppressors is due to the intercellular molecule exchange *via* the Cx-modulated gap junctions or due to an alternate GJIC-independent mechanism. A growing amount of evidence has accumulated suggesting that Cxs exert the tumour suppressive effect through a GJIC-independent mechanism [27–32]. It was reported that Cx31.1 do not form functional gap junction channels [4]. Consistently, our results showed that the ectopically expressed Cx31.1 co-localized with an endoplasmic reticulum protein GRP94. We speculated that the suppressive effect of Cx31.1 on NSCLC progress was GIJC independent.

The mechanism by which Cxs may act to regulate cell growth, differentiation and tumour progress in a GJIC-independent manner is through directly or indirectly regulating partners that signal and regulate cell growth and carcinogenesis. For example, it has been reported that Cx43 regulated CCN3 [33], p21 [22], whereas Cx32 interact with Src [34], and Cx26 has been shown to bind to E3 ubiquitin ligase [35] and caveolin-1 [36]. Little has known about the molecules interact with Cx31.1.

As a beginning to assess the molecules regulated by Cx31.1 expression in NSCLC cells which may be related to the Cx31.1-based mechanisms of cell growth inhibition and G1 cycle delay, we examined the expression levels of two types of cyclins: cyclin D and cyclin E, and found cyclin D3 was down-regulated in Cx31.1 overexpressing H1299 cells. As components of the cell-cycle engine, D-type cyclins govern the passage throughout G_1 phase in response to extracellular signals. Although the three D-type cyclins (D1, D2 and D3) are highly homologous and conserved in evolution, they are differentially regulated in various cancers because of distinct structural motifs, different regulation by oncogenic signalling events and tissue-specific expression patterns [37]. Several groups had reported that cyclin D3 was overexpressed in human cancers [38–40], and others found that cyclin D3 protein levels remain constant in normal epidermis and skin tumours [41], or even that overexpression of cyclin D3 inhibited mouse skin tumour development [42]. A potential tumour promotion effect of cyclin D3 on NSCLC is supported in our study where Cx31.1 overexpression reduced cell proliferation and induced a G_1 stage delay, whereas the expression level of cyclin D3 was down-regulated by Cx31.1 overexpression.

We performed immunoprecipitation to examine the possible interaction between Cx31.1 protein and cyclin D3. The lysate of Cx31.1–EGFP expressing H1299 cells were immunoprecipitated with EGFP antibody and then probed for cyclin D3. No cyclin D3 signal was detected (data not shown), indicating no direct interaction between Cx31.1 and cyclin D3. Further studies are required to investigate the mechanism by which Cx31.1 repress cyclin D3 expression.

Another important mechanism in carcinogenesis is EMT. An oncogenic EMT is thought to be one of the earliest stages of tumour metastasis. It has been reported that the pathways controlling EMT, including Wnt/β-catenin and TGF-β signalling are inappropriately activated in cancers [43]. McLachlan *et al*. reported that Cx43 and Cx26 promoted a phenotypic mesenchymal to epithelial shift [44]. To assess the molecules regulated by Cx31.1 expression that may responsible to Cx31.1 mediated metastasis suppression, we examined the markers of EMT. The results showed that expression of Cx31.1 reduced mesenchymal marker vimentin level and increased epithelial marker cytokeratin 18 level, indicating that Cx31.1 may regulate molecular pathways leading to a reverse of the EMT process.

The most important signalling pathways that regulate or mediate the EMT are Wnts and TGF-β3 pathways. They regulate several downstream effectors, including the EMT-related genes, such as Twist, Snail, Slug and Vimentin [45–49]. In oncogenic EMT, Snail, Slug and Twist induce EMT through direct binding of the transcription factors to the E boxes in the promoter of E-cadherin and inhibiting its expression [50–52]. Therefore, E-cadherin down-regulation is a key feature of EMT [53]. Earlier reports suggested that the Cx proteins and E-cadherin concurrently reduced in human lung cancers [54–56], and Cxs interact with cell adhesion associated proteins, including E-cadherin [57–59]. It was suggested that cadherins might be a prerequisite for gap junction assembly [60]. Our real-time PCR results showed that Cx31.1 overexpression slightly up-regulated E-cadherin expression, indicating that Cx31.1 overexpression mediate only a partial reversion to an epithelial phenotype of NSCLC cells. EMT is a multi-step process and regulated by many signal pathways [61]. Therefore, even though Cx31.1 may be a component in the reversion of EMT, it is not surprising that Cx31.1 expression alone does not promote the complete reversion of NSCLC cells to an epithelial phenotype.

In summary, we have shown that Cx31.1 may act in human NSCLC cells to inhibit tumourigenesis and metastasis. The mechanism may involve the downstream regulation of factors governing EMT and cell cycle.

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Conflicts of interest

The authors confirm that there are no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Fig. S1 The intracellular location of Cx31.1 proteins ectopically expressed in H1299 cells.

Fig. S2 The efficiency of siRNA targeting Cx31.1 was determined with real-time PCR (**A**) and Western blot (**B**, **C**).

Table S1 Tumour sizes of Cx31.1–EGFP stably expressing H1299 injected mice and control group

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