Epithelial mesenchymal transition correlates with CD24$^+$CD44$^+$ and CD133$^+$ cells in pancreatic cancer

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Abstract. The epithelial-mesenchymal transition (EMT) has been linked to induction of a stem-cell like phenotype, characterized by altered cell surface marker expression and increased tumor formation. The aim of this study was to investigate whether EMT correlates with CD24$^+$CD44$^+$ and CD133$^+$ cells in pancreatic cancer. The morphology of untreated and gemcitabine-treated SW1990 gemcitabine-resistant cells and normal SW1990 cells were compared. NF-κB p65 expression was knocked down using siRNA. Vimentin and E-cadherin expression were analyzed using western blotting, and CD24$^+$CD44$^+$, CD133$^+$ cells were quantified by FACS. Additionally, immunohistochemistry of EMT-associated markers and stem cell-associated markers were performed in 41 cases of human pancreatic ductal adenocarcinoma. In SW1990 gemcitabine-resistant cells, gemcitabine induced a mesenchymal cell phenotype, expression of EMT-related molecular markers and increased CD24$^+$CD44$^+$ and CD133$^+$ cells compared to untreated SW1990 gemcitabine-resistant and SW1990 cells. Knockdown of NF-κB p65 inhibited the ability of gemcitabine to increase the proportion of CD24$^+$CD44$^+$ or CD133$^+$ cells and expression of EMT-related molecular markers. In human pancreatic ductal adenocarcinoma, significant correlations were observed between expression of the EMT-associated markers vimentin and E-cadherin, and stem cell-associated markers CD24, CD133 and CD44. This study demonstrated that EMT correlated with CD24$^+$CD44$^+$ and CD133$^+$ cells in pancreatic cancer. This study also suggests that EMT may induce cancer stem-like cells in pancreatic cancer, with different degrees of EMT probability inducing different proportions of CD24$^+$CD44$^+$ and CD133$^+$ cells.

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Abbreviations: EMT, epithelial-mesenchymal transition; PDAC, human pancreatic ductal adenocarcinoma; NF-κB p65, nuclear factor κB p65

Key words: epithelial-mesenchymal transition, pancreatic cancer, CD24$^+$CD44$^+$ cells, CD133$^+$ cells, nuclear factor κB p65

Introduction

Pancreatic cancer is the fourth leading cause of cancer-related deaths in Western countries, with the lowest solid cancer survival rate (1). It is characterized by rapid progression, early metastasis, and a limited response to chemotherapy and radiotherapy (2-4).

The epithelial-mesenchymal transition (EMT) is a developmental process in which polarized epithelial cells undergo multiple biochemical changes and assume a mesenchymal phenotype, with increased migratory capacity, invasiveness, resistance to apoptosis and extracellular matrix component expression (5-9). The hallmarks of EMT are a loss of epithelial cell markers, such as E-cadherin, and increased expression of mesenchymal markers, such as vimentin (9).

In addition to its role in development, tissue regeneration and fibrosis, EMT is considered a critical process in cancer progression (5-9). Induction of EMT in cancer cells results in acquisition of invasive and metastasis properties. Expression of EMT-associated molecules has been demonstrated in pancreatic cancer (10,11). A high degree of solitary cell infiltration is correlated with reduced E-cadherin and increased vimentin expression, and a poorer prognosis in pancreatic cancer (12). Furthermore, EMT can contribute to pancreatic cancer chemoresistance (13). These studies suggest that EMT may be an important mechanism in pancreatic cancer progression and contribute to the poor prognosis of this disease. Malignant stimuli such as chemotherapy, radiotherapy, and hypoxia promote EMT (14,15); therefore, EMT may play a role in adoption of pancreatic cancer cells to adverse stimuli.

Induction of EMT in human mammary epithelial cells results in a stem-cell like phenotype characterized by high CD44 and low CD24 cell surface marker expression, and the ability to form mammospheres, soft agar colonies and aggressive tumors in nude mice (16). Particular cells that are either positive for the surface antigens CD24, CD44 or CD133 have been reported to exhibit increased self renewal and tumor initiating potential, as

well as to give rise to renewed heterogeneity in pancreatic ductal adenocarcinoma (17-20). A key promoter of mobile migrating cancer stem cells, ZEB1, suppresses expression of miRNAs which inhibit a stem-like phenotype, suggesting a link between activation of EMT and maintenance of stemness (21).

Primary human pancreatic adenocarcinoma $CD44^+CD24^+ESA^+$ stem-like cells have enhanced tumorigenic potential (17). The putative pancreatic stem cell marker DCAMKL-1 also plays a key role in EMT, indicating that a relationship between EMT and stem-like cells may exist in pancreatic cancer (22).

As the relationship between EMT and $CD24^+CD44^+$ and $CD133^+$ cells in pancreatic cancer has not been fully elucidated, we investigated whether EMT correlated with a cancer stem-like phenotype in pancreatic cancer cells, and whether the phenomenon also existed in pancreatic ductal adenocarcinoma specimens.

Materials and methods

Cell culture. The pancreatic SW1990 cell line was established from spleen metastasis of pancreatic ductal adenocarcinoma (PDAC). Resistant cells were obtained by culturing parental SW1990 cells in serially increasing concentrations of gemcitabine. In brief, the cells were first cultured in medium with increasing concentrations of gemcitabine, starting at the IC_{50} (3 μM), for 3 days, followed by recovery periods in drug-free medium until the cells regained exponential growth. The new IC_{50} of gemcitabine-treated cells was then evaluated by the MTT assay. The concentration of gemcitabine was then increased to the new IC_{50} (6 μM) to kill half of the cells. Then by increasing the dosage of gemcitabine in the culture medium intermittently for 24 weeks, stable cultures were obtained in which the IC_{50} was 232.2 μM. Gemcitabine was purchased from the Lily Company (Lily, France) (23).

SW1990 were previously characterized in our laboratory, and were cultured in DMEM (Hyclone, USA) supplemented with 10% newborn calf serum (NCS; Gibco, USA), 2 mM glutamine, 100 mg/ml penicillin, and 100 mg/ml streptomycin at 37˚C in a humidified chamber supplemented with 5% CO_2. SW1990 gemcitabine-resistant cells lines were cultured in DMEM, 10% NCB, 2 mM glutamine, 100 mg/ml penicillin, and 100 mg/ml streptomycin, 50 μM gemcitabine for at least 48 h before being used for protein and RNA analyses.

NF-κB p65 siRNA. A double-stranded siRNA targeted to the NF-κB p65 subunit (sense, 5'-GCCCUAUCCCUUUACGU CA-3' and antisense 5'-UGACGUAAAGGGAUAGGGC-3') with two thymidine residues introduced at the 3' end, which encoded amino acid residues 347 and 353 of the NF-κB p65 subunit was used. A non-specific control double-stranded siRNA (sense 5'-UUCUCCGAACGUGUCACGU-3' and antisense 5'-ACGU GACACGUUCGGAGAA-3') was designed. The siRNAs were produced by the GenePharma Co., Shanghai, China. SW1990 gemcitabine-resistant cells were grown to 50% confluence in 6-well or 96-well plates, transfected with siRNA in serum-free medium without antibiotic supplements using Lipofectamine™ 2000 (Invitrogen, Grand Island, NY, USA). Cells were incubated for 24 h, changed to complete media containing 50 μM gemcitabine and cultured for 72 h before western blotting.

Western blotting. Western blotting was performed using standard protocols. Briefly, $5x10^5$ cells were sonicated in RIPA buffer and 30 μg proteins was resolved on 12% polyacrylamide SDS gels and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% BSA in TBS, incubated with mouse anti-human E-cadherin 1:200 (Millipore), goat anti-human vimentin 1:200 (Millpore), mouse anti-human NF-κB p65 1:250 (Santa Cruz Biotechnology, Santa Cruz, CA) and then incubated with an alkaline phosphatase-conjugated secondary antibody. Bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Tiangen Biotech Co., Ltd., Beijing, China). A mouse anti-human GAPDH antibody 1:500 (Beyotime Institute of Biotechology) was used as a loading control.

qRT-PCR. Total RNA was extracted from cultured or transfected cells using TRIzol (Invitrogen) and NF-κB p65 mRNA expression was quantified using the NF-κB p65 primers (sense 5'-GGGGACTACGACCTGAATG-3' and antisense 5'-GGG CACGATTGTCAAAGAT-3') (25) and β-actin primers (sense: 5'-AGAAAATCTGGCACCACACC-3' and antisense 5'-TAG CACAGCCTGGATAGCAA-3') on the ABI PRISM 7000 Sequence Detection system (Applied Biosystems, Foster City, CA, USA), using SYBR Premix EX Taq (Takara, Dalian, China).

Flow cytometry analysis. Flow cytometry was performed using standard protocols. Briefly, SW1990 gemcitabine-resistant cells were transfected with NF-κB p65 siRNA for 24, 48 or 72 h, then labeled with 5 μg/ml anti-CD44 FITC and 2.5 μg/ml anti-CD24 PE or 5 μg/ml anti-CD133 PE antibodies for 10 min at 37˚C, using 2.5 μg/ml FITC rat IgG2b and 5 μg/ml PE mouse IgG1 as isotype controls. Antibodies were purchased from eBioscience, San Diego, CA, USA. Flow cytometry was performed using BD FACSCalibur flow cytometer.

Analysis of apoptosis. Apoptosis was determined using the FITC Annexin-V Apoptosis Detection kit I assay. Briefly, cells were centrifuged, washed in PBS, resuspended in 100 μl 1X binding buffer and stained with 5 ml Annexin-fluorescein isothiocyanate (FITC) and 5 ml PI at room temperature for 15 min in the dark.

In vitro invasion assay. The Matrigel invasion assay was performed using 24-well Transwell chambers (8.0 μm pore size with polycarbonate membrane; Corning Life Sciences, Lowell, MA, USA;) following the manufacturer's instructions. The lower compartment was seeded with $5x10^4$ cells in 600 μl complete medium for 12 h, then the media was changed to serum-free medium and the upper chamber was coated with 100 μl 1 mg/ml matrigel (BD Biosciences, Bedford, MA, USA) and incubated for 3 h when 0.6 ml 10% FBS-DMEM was added to the lower chamber, and further incubated for 24 h. The non-invading cells were removed with cotton swabs. Cells migrating to the bottom of the membrane were stained with 0.1% crystal violet for 30 min at 37˚C, washed with PBS, soaked in ice-cold 33% acetic acid and oscillated for 10 min. The absorbance was measured at 570 nm using a microplate reader (Tecan, Shanghai, China).

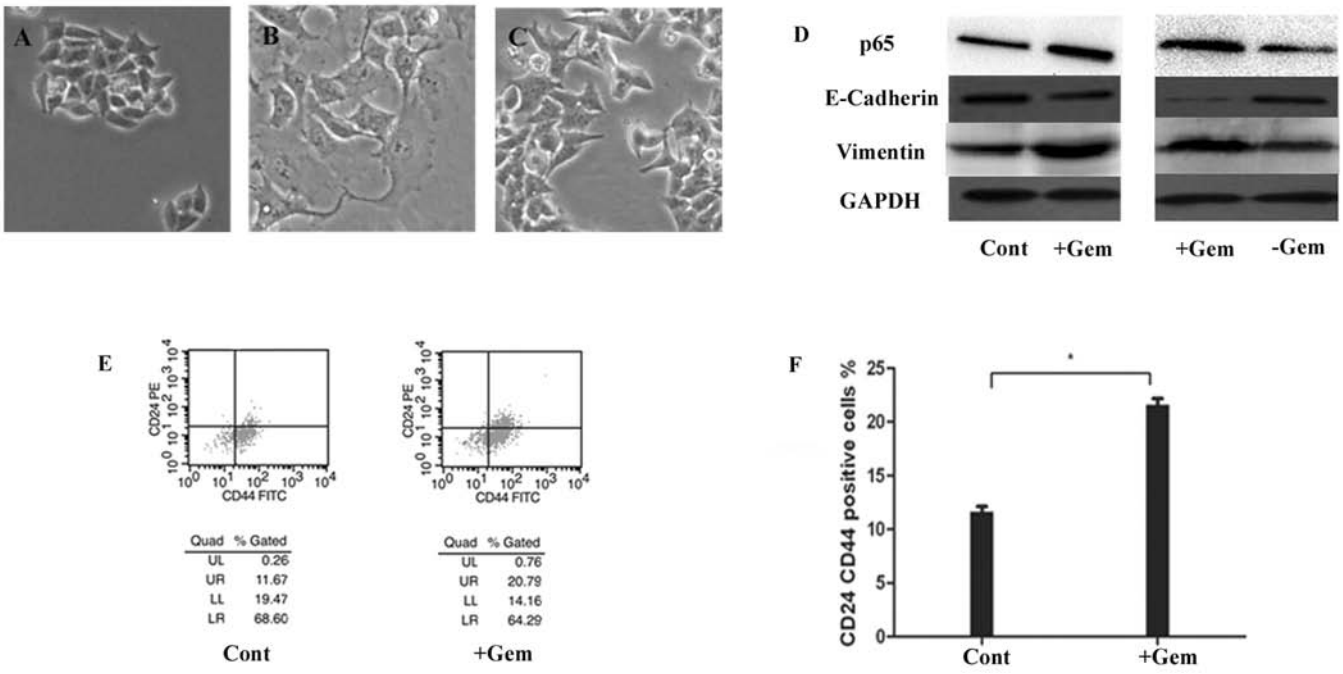

Figure 1. (A) Normal SW1990, (B) gemcitabine-resistant SW1990 cells treated with 50 μM gemcitabine for 48 h and (C) for 1 week after gemcitabine removal, indicating that the gemcitabine-induced phenotype is partly reversible (original magnification, x200). (D) Western blots of E-cadherin, vimentin and NF-κB p65 in SW1990 gemcitabine-resistant cells (Cont) untreated and (+Gem) 48 h after gemcitabine treatment and (-Gem) 24 h after gemcitabine removal. GAPDH was used as an internal loading control. (E) FACS analysis of CD44 and CD24 in SW1990 gemcitabine-resistant cells untreated (Cont) and treated with 50 μM gemcitabine (+Gem). (F) In SW1990 gemcitabine-resistant cells, gemcitabine significantly increased $CD24^+CD44^+$ cells (+Gem, n=3 per group, $^*P<0.01$) compared to untreated cells (Cont).

Immunohistochemical staining. Samples were obtained from 41 cases of pancreatic ductal adenocarcinoma confirmed by pathological analysis between 2007 to 2010 at The First Affiliated Hospital of Nanjing Medical University.

Immunohistochemistry was performed on formalin-fixed and paraffin-embedded 4 μm sections for EMT-associated markers using anti-vimentin and anti-E-cadherin antibodies (Santa Cruz Biotechnology) and the stem cell-associated markers using anti-CD24 (Santa Cruz Biotechnology), anti-CD133 (Abcam, Cambridge, MA, USA) and CD44 (Santa Cruz Biotechnology) antibodies in an automated standard avidin-biotin complex procedure, visualized using diaminobenzidine and counterstained with hematoxylin. In each section, the number of positive cells in at least 10 randomly chosen x200 fields of view was determined. E-cadherin expression was quantified using Image-Pro Plus version 6.0 (Media Cybernetics, Inc., Bethesda, MD).

Statistical analysis. Each experiment was conducted at least three times. Values are reported as mean ± SD. Statistical analysis was performed using SPSS 13.0. Differences between groups and controls were assessed using the Student's t-test. The Pearson correlation test was used to determine the relationship between individual markers. $P<0.05$ was considered statistically significant.

Results

Phenotype changes. Phenotypic differences between gemcitabine-resistant and normal SW1990 cells, including a loss of cell-cell contracts, spindle fibroblastic morphology, and cell scattering were observed. Gemcitabine treatment in SW1990 gemcitabine-resistant cells induced acquisition of spindle cell morphology and reduced cell-cell contacts, beginning 24 h after gemcitabine treatment and reaching a maximum at 48 h (Fig. 1A and B). Changes in cellular morphology were present 72 h after gemcitabine removal, and were partly reversible after 1 week (Fig. 1C).

EMT-associated protein expression. Expressions of vimentin and E-cadherin, two EMT markers, were analyzed in gemcitabine-treated SW1990 gemcitabine-resistant cells. Vimentin was almost undetectable in normal SW1990 (24), but it was observed in SW1990 gemcitabine-resistant cells. After 48-h gemcitabine stimulation, vimentin NF-κB p65 expression increased and E-cadherin expression decreased (Fig. 1D). E-cadherin expression increased and that of vimentin, NF-κB p65 decreased 24 h after gemcitabine withdrawal in SW1990 gemcitabine-resistant cells (Fig. 1D).

$CD24^+CD44^+$ cells. Flow cytometry analysis (Fig. 1E) showed a significantly higher $CD24^+CD44^+$ ratio in gemcitabine-treated SW1990 gemcitabine-resistant cells (20.79%) than in untreated cells (11.67%, Fig. 1F, $P<0.01$). The proportion of $CD24^+CD44^+$ cells in SW1990 gemcitabine-resistant cells was higher than in normal SW1990 cells.

Effect of p65 knockdown on EMT markers. Expression of p65 was quantified using qRT-PCR, non-specific control siRNA or mock-transfected SW1990 gemcitabine-resistant cells treated with gemcitabine and western blot analysis of p65, vimentin and E-cadherin was performed at 24, 48 and 72 h.

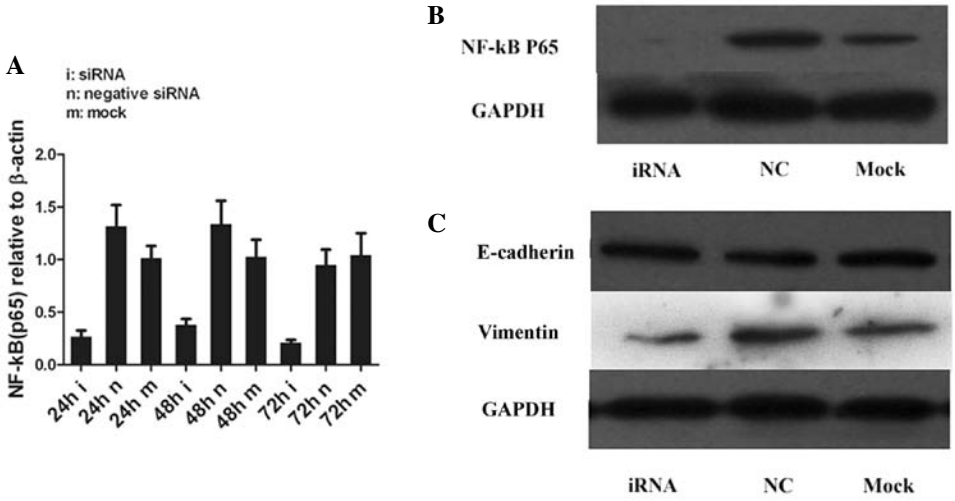

Figure 2. (A) qRT-PCR quantification of NF-κB p65 in p65 siRNA (i), control siRNA (n) or mock (m) SW1990 gemcitabine-resistant cells at 24, 48 and 72 h post-transfection relative to β-actin. (B) Western blot analysis of NF-κB p65 expression in p65 siRNA (iRNA), negative control siRNA (NC), and blank control culture medium (Mock) SW1990 gemcitabine-resistant cells 48 h post-transfection. GAPDH was used as an internal control. (C) Western blot analysis of E-cadherin and vimentin in SW1990 gemcitabine-resistant cells 48 h post-transfection with p65 siRNA (iRNA), negative control siRNA (NC), and blank control culture medium (Mock). GAPDH was used as an internal control.

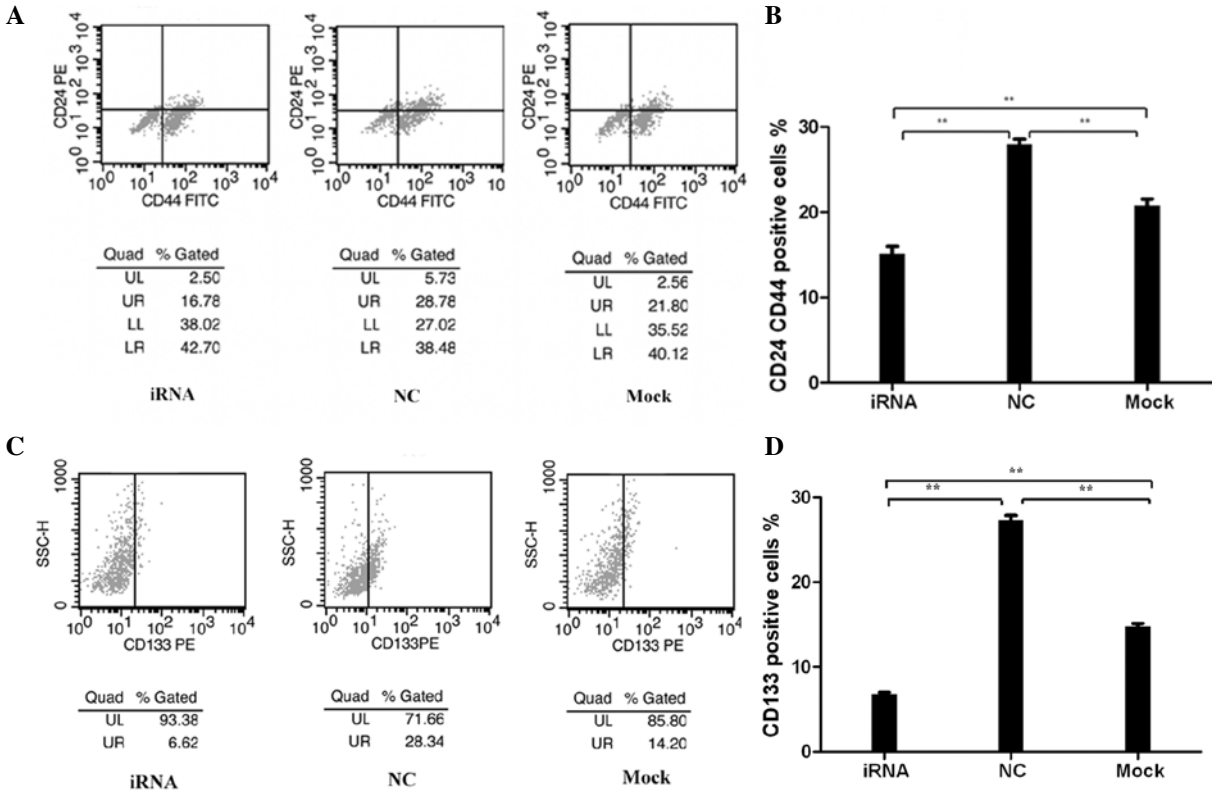

Figure 3. (A) FACS analysis plots and (B) quantification of CD44 and CD24 in SW1990 gemcitabine-resistant p65 siRNA (iRNA), control siRNA (NC), and mock-transfected cells 48 h after transfection, n=3, **P<0.01. (C) FACS analysis plots and (D) quantification of CD133 in the p65 siRNA (iRNA), control siRNA (NC) and blank control culture medium (Mock) SW1990 gemcitabine-resistant cells 48 h after transfection, n=3; **P<0.01.

siRNA reduced p65 mRNA expression compared to control siRNA and mock-transected cells at all time points (Fig. 2A), and p65 protein was undetectable in p65 siRNA-transfected cells (Fig. 2B). p65 siRNA significantly downregulated the EMT-associated protein vimentin, and control siRNA induced a slight upregulation of vimentin, compared to control mock-transected cells (Fig. 2C). No significant change in E-cadherin was observed (Fig. 2C). The phenotype of siRNA-transfected cells significantly changed.

Effect of p65 knockdown on stem-like cell associated-proteins. $CD24^+CD44^+$ and $CD133^+$ cells in SW1990 gemcitabine-resistant cells were reduced 48 h after p65 siRNA transfection, compared to control siRNA and mock-transected cells (Fig. 3,

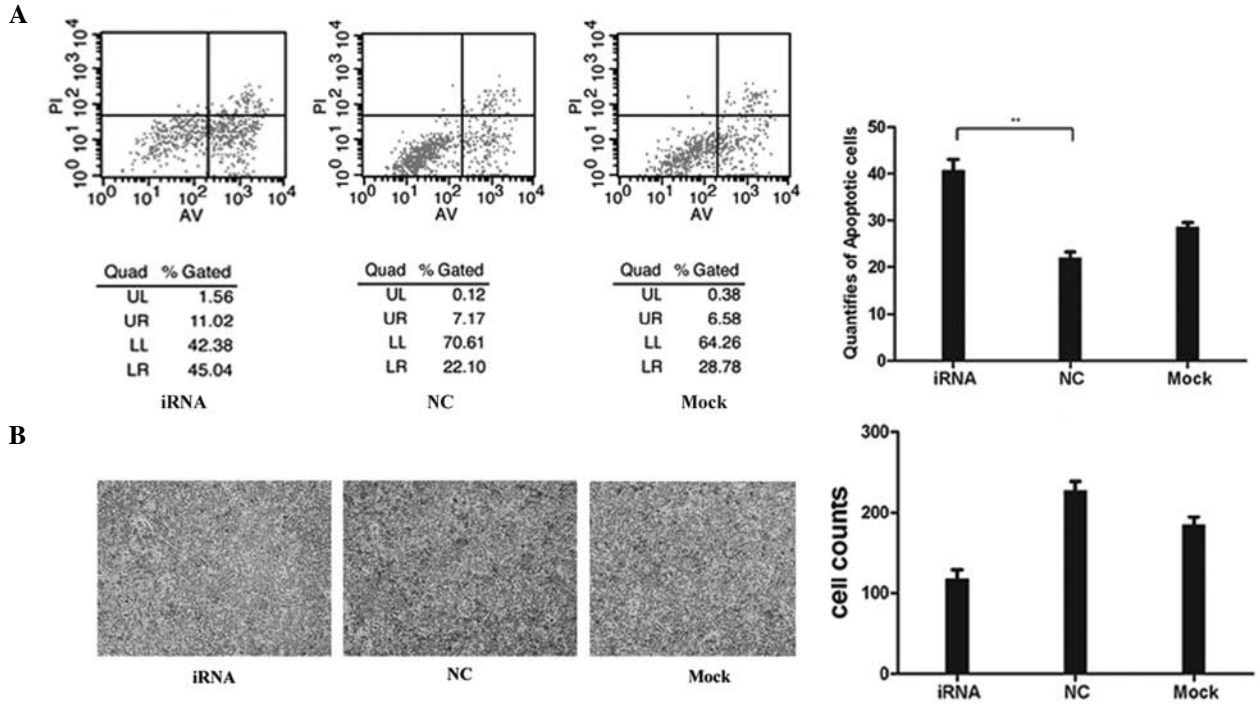

Figure 4. (A) Quantification of apoptosis in p65 siRNA (iRNA), control siRNA (NC) and blank control culture medium (Mock) SW1990 gemcitabine-resistant cells 48 h after transfection using flow cytometry. 'LR' quantifies the number of apoptotic cells. n=3, **P<0.01. (B) Representative photographs of invading cells using the Matrigel invasion assay in p65 siRNA (iRNA), control siRNA (NC) and blank control culture medium (Mock) SW1990 gemcitabine-resistant cells 48 h after transfection.

P<0.01). Notably, the proportion of both $CD24^+CD44^+$ and $CD133^+$ cells in control siRNA transfected cells was higher than in mock-transected cells (Fig. 3, P<0.01). This phenomenon disappeared in 72 h as the result of qRT-PCR quantification of NF-κB (Fig. 2A). The results suggest that cytotoxicity of Lipofectamine 2000 may stimulate the EMT and increase the proportion of stem-like cells.

The proportion of $CD24^+CD44^+$ cells increased in SW1990 gemcitabine-resistant cells treated with 50 μM gemcitabine for several generations, while p65 knockdown deceased the proportion of $CD24^+CD44^+$ cells. When gemcitabine was removed, the phenotype completely reversed; however, the proportion of $CD24^+CD44^+$ cells remained higher than in the parental cells.

Effect of p65 siRNA on apoptosis and invasion. We examined apoptosis in p65 siRNA transfected SW1990 cells stimulated with 50 μM gemcitabine by flow cytometry and a significant increase in apoptotic cells was observed (45.04%) compared to siRNA control (28.78%) or control (22.10%) transfected cells. (Fig. 4A, P<0.01)

To determine the ability of tumor invasion, we evaluated cell invasiveness. The invasive ability significantly decreased by 55% in p65 siRNA-transfected SW1990 cells stimulated with 50 μM gemcitabine (Fig. 4B), compared with control siRNA cells; however, the invasive ability of control siRNA cells was higher than mock-transected cells. These results suggest that knockdown NF-κB p65 using siRNA can suppress the invasive ability of SW1990 gemcitabine-resistant cells.

EMT marker and stem-like cell proteins in pancreatic ductal adenocarcinoma. In human pancreatic cancer tissues, CD44 was localized to cell membranes while CD24 expression was detected in the membranes and cytoplasm of the cancer cells. CD133 was expressed in the cytoplasm of the cancer cells. Vimentin was detected in the cytoplasm of mesenchymal cells and some cancer cells. E-cadherin was expressed on the membranes of normal glands and cancer cells (Fig. 5).

Pearson correlative analyses of all the individual markers (Fig. 6) indicated strong positive correlations between the expression of vimentin and CD24 (r^2=0.8601, P<0.0001), vimentin and CD44 (r^2=0.8353, P<0.0001), vimentin and CD133 (r^2=0.6807, P<0.0001). Additionally, E-cadherin and vimentin (r^2=0.2957, P=0.0002), E-cadherin and CD24 (r^2=0.2798, P=0.0004), E-cadherin and CD44 (r^2=0.3004, P=0.0002), E-cadherin and CD133 (r^2=0.1589, P=0.0098) expression showed significant negative correlations.

Discussion

Expression of vimentin and the proportion of $CD24^+CD44^+$ and $CD133^+$ cells in parental SW1990 cells is very low (24), and increased proportions of $CD24^+CD44^+$ and $CD133^+$ cells and EMT associated proteins were detected in SW1990 gemcitabine-resistant cells. During the process of obtaining gemcitabine-resistant cells, we cultured parental SW1990 cells with IC_{50} concentrations of gemcitabine and the cells appeared to have an EMT phenotype with a large number of cell death. In the recovery periods, the phenotype of EMT was partially recovered. Then we stimulated the cells with the same or lower concentrations of gemcitabine, and the cells appeared to have significant EMT phenotype with low cell death. This provides a simple and reproducible experimental model to observe changes in the proportion of stem-like cells during EMT,

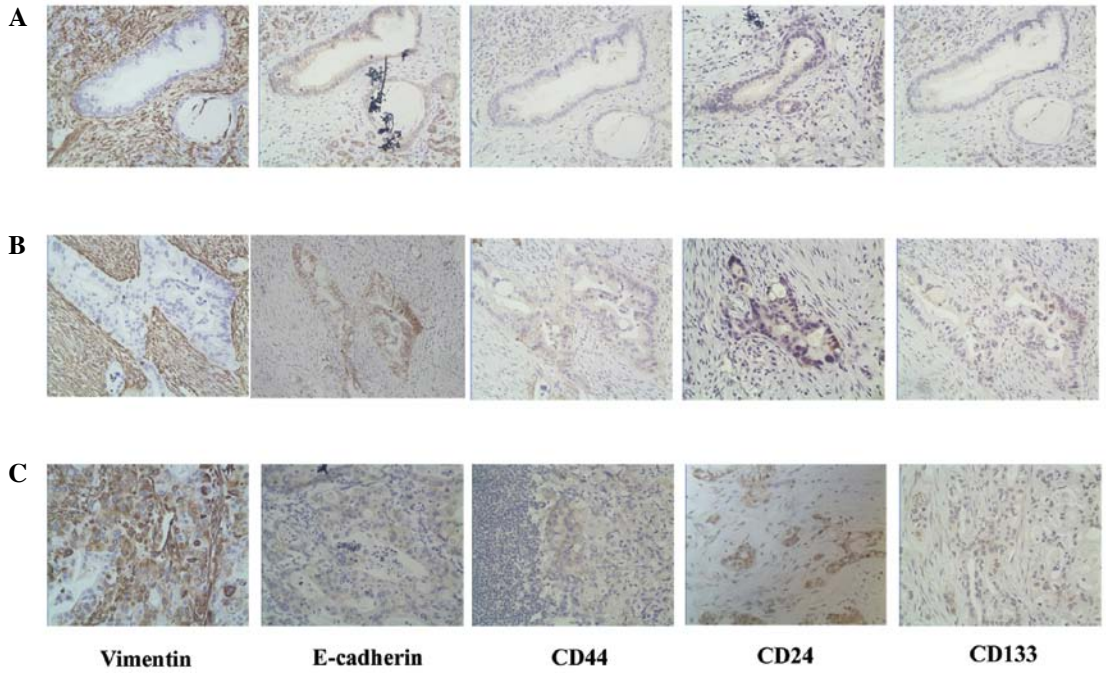

Figure 5. Immunohistochemical staining for the epithelial and mesenchymal markers vimentin, E-cadherin, and stem cell markers CD24, CD44, CD133 in three human pancreatic adenocarcinoma samples (A-C), (magnification, x200). Weak, moderate and strong expression of vimentin, CD24, CD44 and CD133 is observed in A, B and C, respectively; A, B and C illustrate weak, moderate and strong expression of E-cadherin, respectively.

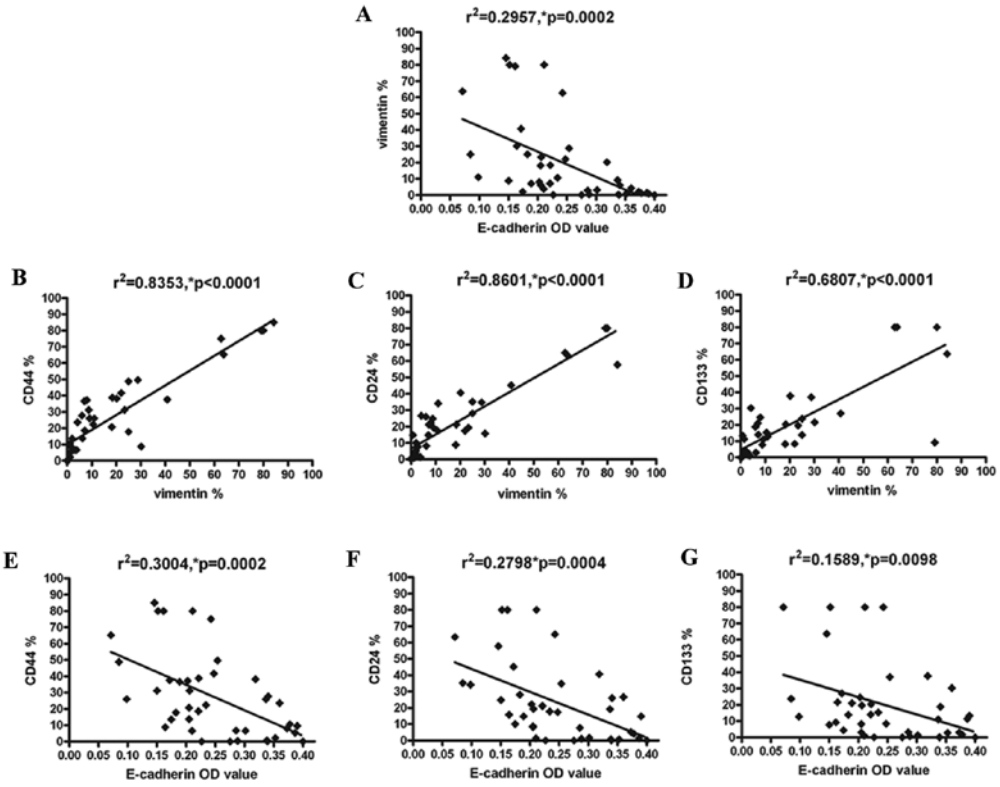

Figure 6. Correlation between cell surface marker expression in pancreatic adenocarcinoma. (A) Vimentin and E-cadherin, (B) vimentin and CD44, (C) vimentin and CD24, (D) vimentin and CD133, (E) E-cadherin and CD44, (F) E-cadherin and CD24, (G) E-cadherin and CD133.

as 50 μM gemcitabine has no significant effect on SW1990 gemcitabine-resistant cell (232.2 μM) survival.

Gemcitabine is commonly used in the treatment of pancreatic cancer (1). There are studies that evaluated treatment with gemcitabine in the context of pancreatic cancer, showing that one can develop gemcitabine-resistant cell lines and that they are associated with EMT (26). Several studies have demonstrated low (<5%) proportions of stem-like cells

in normal SW1990 cells (25). In this study, we observed that the mesenchymal cell phenotype, expression of EMT-related molecular markers and the proportion of $CD24^+CD44^+$ cells was greatest in gemcitabine-stimulated SW1990 gemcitabine-resistant cells, reduced in parental gemcitabine-resistant cells and lowest in normal SW1990 cells. These data indicate that the process of EMT and the associated proportion of stem-like cells was gradually enhanced by gemcitabine in SW1990 cells. NF-κB p65 participated in this process.

NF-κB is considered a central signaling pathway of EMT in pancreatic cancer. Inhibiting the NF-κB signaling pathway can block EMT of pancreatic cancer cell lines (27). Our results showed there was high NF-κB p65 expression in gemcitabine-stimulated SW1990 gemcitabine-resistant cells. Knockdown of NF-κB p65 could inhibit the effect of gemcitabine stimulation to increase the proportion of $CD24^+CD44^+$ and $CD133^+$ cells in 48 h; however, the proportion of $CD24^+CD44^+$ and $CD133^+$ cells in p65 knockdown gemcitabine-resistant SW1990 cells was still higher than normal SW1990 cells.

Though only one cell line (SW1990) was used in these experiments, we observed significant correlations between expression of the EMT-associated markers vimentin, E-cadherin and the stem cell-associated markers CD24, CD44 and CD133 in human pancreatic ductal adenocarcinoma specimens, which verified the reliability of the *in vitro* experiments, suggesting that EMT not only maintained pancreatic cancer stem-like cells, but also may induce cancer stem-like cells in pancreatic cancer.

Interestingly, it has been reported that CD24 is downregulated during EMT in pancreatic cancer cells (25). However, we observed increased CD24 in response to EMT. Additionally, the proportion of $CD24^+CD44^+$ and $CD133^+$ cells, vimentin expression and invasive ability in control siRNA cells was higher than mock-transected cells, indicating that transfection stimulates a degree of EMT. These data indicated that EMT in pancreatic cancer may be not a complete switch, rather a reversible effect which can occur in different degrees.

In conclusion, EMT correlated with $CD24^+CD44^+$ and $CD133^+$ cells in pancreatic cancer. EMT may induce pancreatic cancer stem-like cells, and different degrees of EMT may induce different amounts of $CD24^+CD44^+$ and $CD133^+$ cells. EMT may be one mechanism by which pancreatic cancer cells adapt to malignant stimulation. Elucidation of the molecular mechanisms underlying the progression of pancreatic cancer is crucial for developing therapeutic strategies.

Acknowledgements

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