Down-regulation of Smad4 enhances proliferation and invasion of colorectal carcinoma HCT116 cells and up-regulates Id2

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Abstract. The aim of this study was to determine whether the suppression of Smad4 by short hairpin RNA (shRNA) regulates the proliferation and invasion of colorectal carcinoma HCT116 cells and Id2 expression. The Smad4-shRNA expression vectors were constructed and stably transfected to HCT116 cells. The expression of mRNA and protein of Smad4 and Id2 was detected using reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting. Cellular proliferation inhibitory activity was determined by methyl thiazolyl tetrazolium (MTT) assay. Transwell assay was used to detect the effect of the inhibition of Smad4-shRNA on migration and invasion. The Smad4-shRNA vector, which inhibited Smad4 expression, was constructed and successfully transfected to HCT116 cells. The levels of mRNA and protein expression of Smad4 were markedly decreased following transfection of shRNA compared with the control groups ($P<0.05$). The abilities of proliferation, migration and invasion were increased following transfection of shRNA ($P<0.05$). The expression of Id2 was increased following transfection of shRNA ($P<0.05$). For the Smad4-down-regulated HCT116 cells, treated with or without BMP7 (25 ng/ml), no difference was found. shRNA-mediated silencing of Smad4 was able to enhance the abilities of proliferation, migration and invasion in the HCT116 cell line. Therefore, Smad4 may act as a tumor-suppressor gene in colorectal carcinoma.

Introduction

Smad4 (deleted in pancreatic carcinoma, locus 4), mapped to the 18q21 region, was discovered for its mutational inactivation in approximately 55% of pancreatic cancers (1,2). It is a key mediator of the transforming growth factor-β (TGF-β)-Smad signalling pathway, which is known to inhibit epithelial cell growth (3). When bone morphogenetic protein 7 (BMP7), which belongs to the TGF-β superfamily, is activated, a complex between BMP7 and Smad4 is formed and translocated to the nucleus to modulate transcription of BMP target genes.

Id2 is one member of the Id subfamily, which regulates cell proliferation and differentiation (4). Previous studies have suggested that Id2 is a downstream factor of BMP7, since when BMP7 is increased, Id2 is also correspondingly increased (5-7). However, since there also exists a Smad4-independent BMP7 signaling pathway (8), whether Id2 is regulated by Smad4 remains to be determined. Smad4 mutants are found in approximately 30% of colorectal cancers (9), but the function of Smad4 in colorectal carcinoma remains unclear. Here, we report that upon down-regulation of Smad4 expression by short hairpin RNA (shRNA), the human colorectal carcinoma cell line, HCT116, demonstrated enhanced cell proliferation and invasion, and the expression of Id2 also increased, which indicates that Smad4 may act as a tumor-suppressor gene in colorectal carcinoma, and that Id2 may be a downstream gene of Smad4.

Materials and methods

Cell culture. The human colorectal cancer cell line, HCT116, was obtained from the Centre for Type Culture Collection, Chinese Academy of Sciences (China). The cells were maintained in McCoy's 5A medium (Genom), supplemented with 10% fetal bovine serum (FBS) (Biowest, South America) and penicillin G-streptomycin (Genom) at 37˚C in a cell incubator with 5% CO_2/95% air. Cells were detached using trypsin-EDTA (Genom).

Reconstitution of shRNA. The oligonucleotide sequence of Smad4 (NM_005359) was selected to design the shRNA sequence, and other sequences used in breast cancer were referred to (10). The oligonucleotide sequence is outlined as follows: Smad4-shRNA: forward: 5'-CATTGGATGGGAGGC TTCATTAAGCTTTGAAGCCTCCCATCCAATGTTTTTT-3';

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Table I. The primers used in the PCR experiment.

Gene	Forward primer	Reverse primer
Smad4	22-mer 5'CTGCTGCTGGAATTGGTGTTGA3'	20-mer 5'CTGGAGGGCCCGGTGTAAGT3'
Id2	21-mer 5'CAGCATCCCCCAGAACAAGAA3'	23-mer 5'TGGTGATGCAGGCTGACAATAGT3'
β-actin	24-mer 5'CAACTGGGACGACATGGAGAAAAT3'	23-mer 5'CCAGAGGCGTACAGGGATAGCAC3'

reverse: 5'-AATTAAAAAACATTGGATGGGAGGCTT CAAAGCTTGAATGAAGCCTCCCATCCAATGGGCC-3'; scrambled control: TTCTCCGAACGTGTCACGT.

shRNAs were then cloned into the vector pGCsi-H1/Neo/GFP [with CMV promoter, green fluorescence protein (GFP), SV40 promoter, neomycin, pUC ori, ampicillin, H1 promoter] by repeated excision and ligation successively. Briefly, a 21-nucleotide sequence of the Smad4 gene, which had no homology to genomic sequences in a BLAST search, was inserted into the pGCsi-H1/Neo/GFP cassette vector in the sense and antisense directions. The recombinant covalently closed circular DNA (cccDNA) was generated by Genechem Co. Ltd. (Shanghai, China).

Establishing a Smad4-knockdown cell line. The targeting and control vector plasmids were transfected into HCT116 cells using Lipofectamine 2000 (Invitrogen; Paisley, UK). The day before transfection, $1.5x10^5$ cells per well were seeded into 6-well tissue culture plates containing antibiotic-free McCoy's 5A medium. On the day of transfection, Lipofectamine 2000 and plasmid were diluted in serum-free OPTI-MEM medium (Invitrogen) at a ratio of 2.5:1, which had produced efficient transfection of HCT116 cells in preliminary experiments. The mixture was transfected into HCT116 cells separately. The cells were cultured in medium supplemented with geneticin (G418) (600 μg/ml, Invitrogen) for 2 weeks. Transfection efficiency was detected directly by testing the expression ratio of GFP, and the G418-resistant clones were selected as stable transfectants. Stably transfected clones were selected and maintained in a medium containing 300 μg/ml G418 for further studies. HCT116 cells stably transfected with the plasmid-expressing shRNA that targeted Smad4 were termed 'HCT116-Smad4-shRNA', and cells transfected with the empty plasmid were termed 'HCT116-HK', respectively.

Semi-quantitative RT-PCR. Total RNA extraction was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Cells grown on 6-well plates were lysed with TRIzol (1 ml/well), combined with chloroform and mixed. Supernatants were then precipitated with isopropanol, and RNA pellets were washed with 75% ethanol, air dried and then resuspended in water. A total of 2 μg of total RNA was converted into complementary DNA (cDNA) by reverse transcriptase followed by amplification of phosphatase and tensin homolog (PTEN) (SuperScript II, Invitrogen). Briefly, following inactivation at 65˚C for 10 min, 1 μl of the reaction mixture was incubated in buffer containing deoxynucleotide triphosphates (dNTPS) (0.2 mM each), oligonucleotide primers (0.2 μM each), $MgCl_2$ (3 mM), 10X buffer [200 mM Tris-HCl (pH 8.0), 500 mM KCl] and 1 unit of Taq polymerase. The primers were designed to amplify as shown in Table I, and β-actin served as a loading control. PCR was performed as follows: denaturation at 95˚C for 10 sec and 40 cycles of 95˚C for 5 sec, 60˚C for 30 sec. Gene expression was quantified by RT-PCR, and relative gene expression values were calculated by the ΔΔCt method using Sequence Detection System 2.1 software (Applied Biosystems, Foster City, CA, USA). The result of Smad4 or Id2 mRNA was normalized to the corresponding β-actin signal (ΔCt).

Western blot analysis. Cell samples were lysed in ice-cold lysis buffer (Beyotime, China) with 1% phenylmethylsulfonyl fluoride (PMSF) for half an hour, centrifuged at 10,000 x g for 20 min at 4˚C, and the protein concentration of the resulting supernatant was determined by the bicinchoninic acid (BCA) protein assay kit (Beyotime). Proteins (30 μg) were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to PVDF membranes. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS)/Tween-20 (0.05%, v/v) for 1 h at room temperature and incubated overnight at 4˚C with primary antibodies directed against Smad4 (B8; Santa Cruz Biotechnology, CA, USA), Id2 (C-20, Santa Cruz Biotechnology) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (V-18, Santa Cruz Biotechnology). The blots were washed and incubated with secondary antibody [horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG and HRP-labeled goat anti-mouse IgG (H+L) (Beyotime)], and developed with a chemiluminescent substrate, ECL Plus. Signals were detected using the Alpha Imager EP Fluorescent and Visible Light Gel Imaging System, and ImageQuant 5.0 was used for analysis. Assays were performed in triplicate for each experiment, and each experiment was repeated three times.

Methyl thiazolyl tetrazolium (MTT) assay. For the measurement of cell proliferation rate, the collected cells, which were stably transfected with 'Smad4-shRNA' or 'HK', were plated into 96-well plates in $1x10^3$ cells/100 μl medium per well. After 24 h, BMP7 (Peprotech, Asia), at the concentration of 25 ng/ml, was added to one of the 'HCT116-Smad4-shRNA'groups and one of the HCT116 groups. A total of 10 μl of MTT solution (Amresco) was added into each well every 12 h from the second to fifth day and plates were incubated for 4 h at 37˚C. A total of 150 μl dimethyl sulfoxide (DMSO) was then added to dissolve the formazan. Absorbance values (A) were measured at a wave length of 490 nm with a microplate reader. Results were expressed as the mean ± SEM. Assays were performed in four wells and each experiment was repeated three times. Growth curves were plotted according to the OD value alterations of the MTT assay.

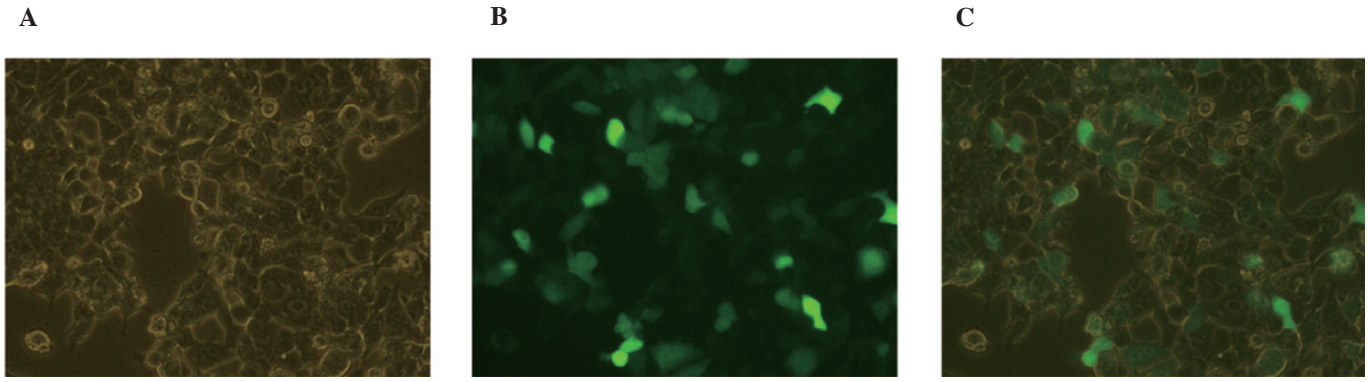

Figure 1. Expression of GFP in HCT116 cells 24 h following transfection with Smad4-shRNA as observed under fluorescence microscopy at x200 magnification. (A) Natural light. (B) Fluorescent light. (C) Both natural light and fluorescent light. Comparison between A and B was carried out, and 100 cells were counted and the number of cells expressing GPF was assessed (as observed in C). The experiment was repeated in three screen .The transfection ratio was ~30%.

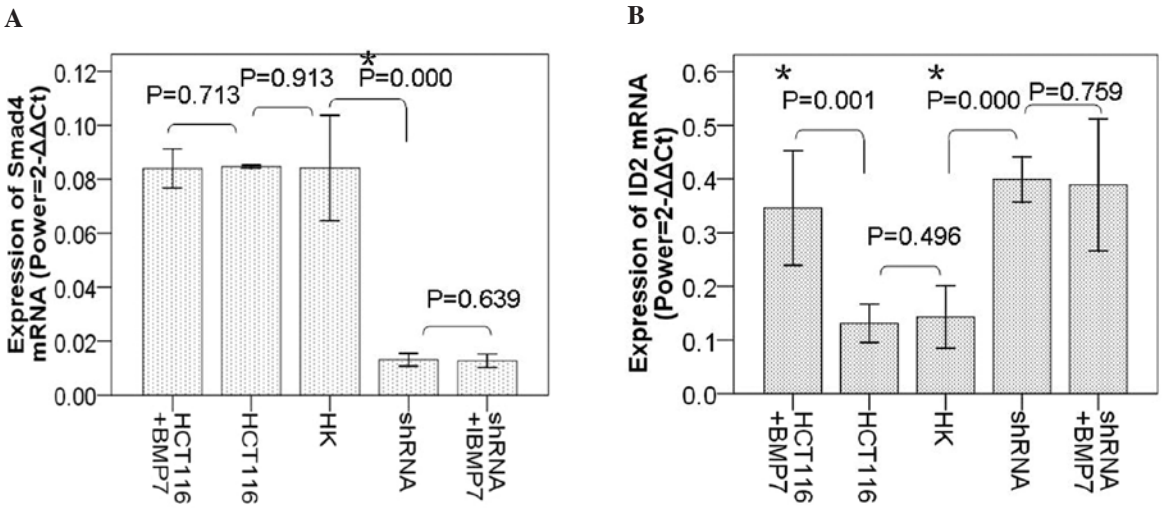

Figure 2. Expression of (A) Smad4 and (B) Id2 mRNA in human HCT116 cells. Expression of Smad4 mRNA in cells transfected with Smad4-shRNA was significantly weaker than that in HCT116 cells or in cells transfected with HK (P<0.05). For the two groups transfected with Smad4-shRNA, there was no significant difference between the cells treated with or without BMP7 (P>0.05). BMP7, bone morphogenetic protein 7.

In vitro Matrigel invasion assay. *In vitro* Matrigel invasion assay was performed using 24-well millicell inserts (Corning, NY, USA) with polycarbonate filters (pore size, 8 μm). The upper side of the polycarbonate filter was coated with Matrigel (50 μg/ml, BD Biosciences). The chambers were incubated at 37˚C with 5% CO_2 for 4 h to allow the matrix to form a continuous thin layer. The HCT116 cells and the cells stably transfected with 'Smad4-shRNA' or 'HK' were then harvested, and $2x10^5$ cells in 200 μl of 0.1% bovine serum albumin serum-free medium were placed in the upper chamber. At the same time, BMP7, at the concentration of 25 ng/ml was added to one of the 'HCT116-Smad4-shRNA' groups and one of the HCT116 groups. The lower chamber was filled with 10% serum-medium (700 μl). Cells were cultured for 24 h at 37˚C in 5% CO_2. Cells on the upper surface of the filter were removed using a cotton swab. Cells invading through the Matrigel and filter to the lower surface were fixed with 4% neutral-buffered formalin for 30 min at room temperature and stained in Giemsa stain for 15 min at room temperature. The cell number in five fields (up, down, median, left and right; x200) was counted for each chamber and the average value was calculated. Each invasion assay was performed in triplicate and repeated three times.

In vitro cell migration assay. The migration assay was used to measure cell migration through an 8-μm pore membrane in 24-well millicell inserts. The lower chamber was filled with 10% serum-medium (700 μl). Cells ($2x10^5$) in 200 μl medium supplemented with 10% FBS were placed in the upper chamber. At the same time, BMP7, at the concentration of 25 ng/ml, was added to one of the 'HCT116-Smad4-shRNA' and one of the HCT116 groups. Following 24 h of incubation, the number of migrated cells (lower side of the membrane) was counted as described above.

Statistical analysis. Statistical analyses were performed using SPSS statistical software (SPSS Inc., Chicago, IL, USA). Data were expressed as the mean ± SEM. Statistical significance of differences between groups was performed by one-way analysis of variance (ANOVA). P<0.05 was considered to

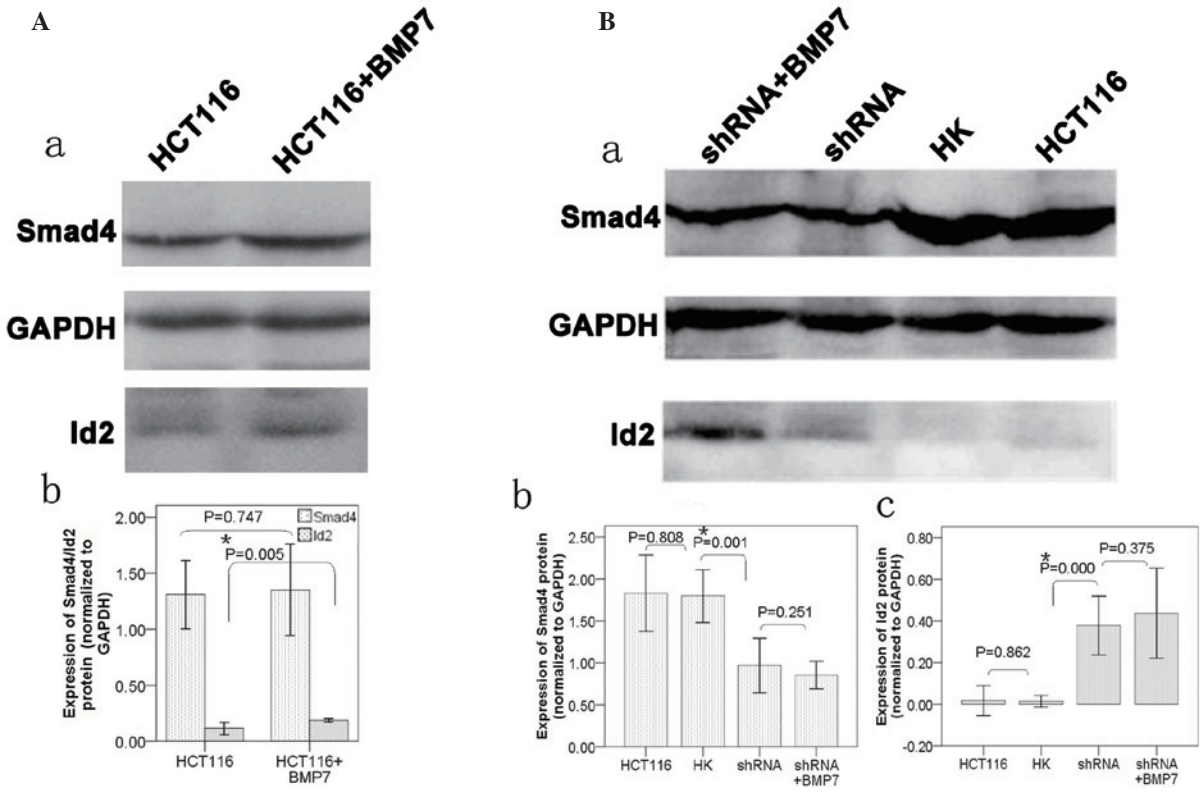

Figure 3. Protein levels in human HCT116 cells. (A) Id2 was increased in the HCT116 cells treated with BMP7 (P<0.05), but there was no significant difference in Smad4 expression between the HCT116 cells treated with or without BMP7 (P>0.05). (B-a) Expression of Smad4 protein in cells transfected with Smad4-shRNA was significantly weaker than that in the HCT116 cells or in cells transfected with HK. (B-b) Smad4 was decreased in the groups that were treated by Smad4-shRNA (P<0.05); (B-c) Id2 was increased in the groups that were treated by Smad4-shRNA (P<0.05), but there was no significant difference between the Smad4-down-regulated cells treated with or without BMP7 (P>0.05). GAPDH was used as a loading control. The graphs compare the scanning signal intensity of Smad4 expression by ImageQuant 5.0. BMP7, bone morphogenetic protein 7.

denote statistical significance. The Student-Newman-Keuls and t-test were used to analyze the differences between the two groups.

Results

Transfection of HCT116 with the adenovirus. Through sequence analysis, the Smad4-shRNA vector was identified to be constructed successfully. There were no significant differences in the transfection conditions or efficiency between the experimental and control groups. Comparison between fluorescence and natural light revealed that the transfection efficiency for Smad4-shRNA or HK groups was approximately 30% (Fig. 1). Stable shRNA transfectants were identified after selection in G418 for 14 days.

Silencing of specific genes and proteins in HCT116 cells. Following transfection of Smad4-shRNA or HK to HCT116, cells we analyzed the expression of Smad4 mRNA and protein level in HCT116 cells using real-time PCR and Western blot assay, respectively. The power ($2^{-\Delta\Delta Ct}$) values for Smad4 were significantly lower than those for HK or for the HCT116 cells (Fig. 2), which demonstrated a significantly reduced expression of Smad4 mRNA (P<0.05). However, there was no significant difference between the cells treated with or without 25 ng/ml BMP7 (P>0.05). The power ($2^{-\Delta\Delta Ct}$) values for Id2 were significantly higher than those for HK or for the HCT116 cells. There was no difference between the Smad4 down-regulated cells treated with or without 25 ng/ml BMP7 (P>0.05); however, there was a difference between the HCT116 cells treated with and without 25 ng/ml BMP7 (P<0.05) (Fig. 2).

As shown in Fig. 3, Smad4 protein expression was similar to the results of mRNA expression. The scanning signal intensity of Smad4 proteins for cells transfected with Smad4-shRNA was significantly weaker than those of HCT116 cells or cells transfected with HK (P<0.05). The efficiency of Smad4-shRNA in the transfected cells by Western blotting was 46.20% [1 - (Smad4 - shRNA group/HK group)]. We also observed in the Western blot analysis that, as the Smad4 protein decreased, the Id2 protein increased. The results for BMP7 were also similar to the results of RT-PCR (Fig. 3).

Effects of Smad4-shRNA on cell proliferation activity. The proliferative activity of tumor cells is significant. The growth curve revealed that the difference in the cell growth inhibition rate between the HCT116 cells transfected with 'Smad4-shRNA' and the other three groups was not statistically significant in the first 24 h. However, from 36 to 60 h, significant differences were observed (Fig. 4B), but no significant difference was found between the HCT116 cells and the cells transfected with HK, or between the 'Smad4-

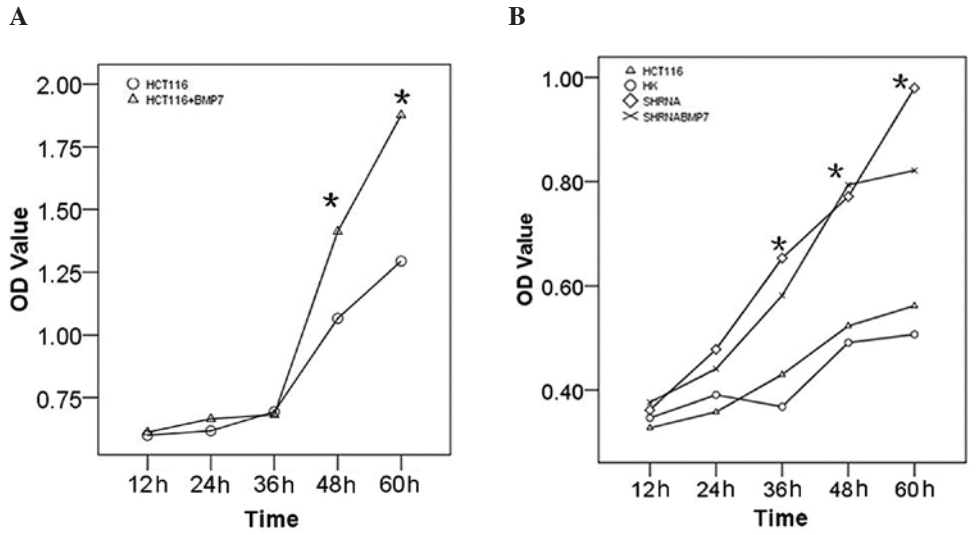

Figure 4. The growth curves showing the cell proliferation activity according to the values of 490 nm wavelength light absorption in all of the cell groups. (A) There was a difference in cell proliferation activity between the HCT116 cells treated with and without 25 ng/ml BMP7 from 48 to 60 h. (B) From 36 to 60 h, a significant difference in cell growth enhancement was noted between the cells transfected with 'Smad4-shRNA' and the cells transfected with HK ($P<0.05$). However, there was no difference between the Smad4-down-regulated cells treated with or without BMP7 ($P>0.05$).

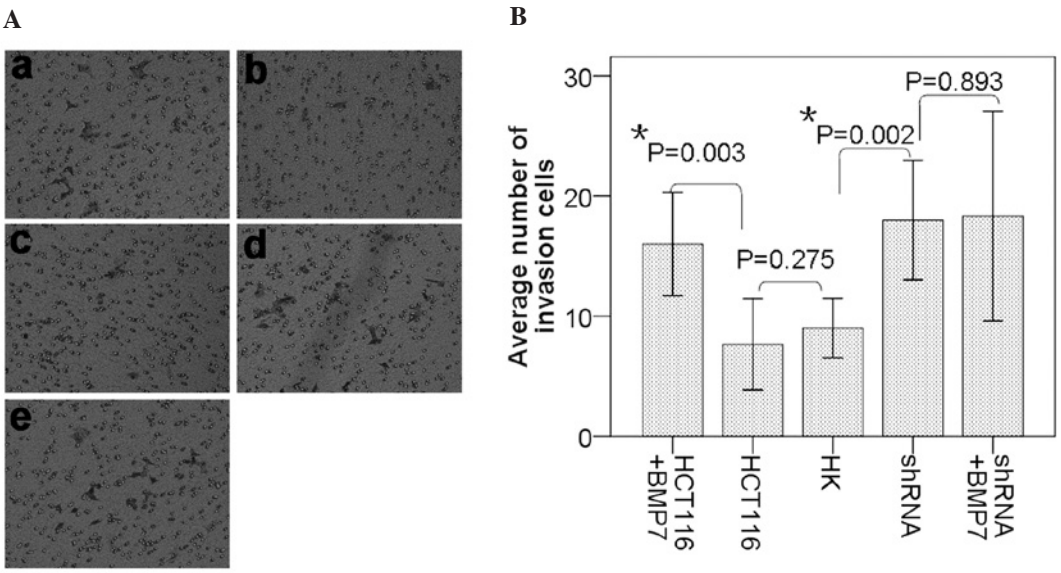

Figure 5. Silencing of Smad4 enhances the invasive ability of HCT116 cells. The number of invading cells was determined by counting the cells stained with Giemsa on the lower side of the membrane. (A-a) HCT116 cells; (A-b) HCT116-HK cells; (A-c) HCT116-Smad4-shRNA cells; (A-d) HCT116-Smad4-shRNA cells + 25 ng/ml BMP7; (A-e) HCT116 cells + 25 ng/ml BMP7. (B) Comparison of the numbers of transmembrane cells in the invasion experiments. In the invasion experiments, the invasive ability in the groups that were treated by Smad4-shRNA was different from the HCT116/HK group ($P<0.05$), but no difference was noted between the Smad4-down-regulated cells treated with or without BMP7 ($P>0.05$).

shRNA' cells treated with or without 25 ng/ml BMP7. There was, however, a difference between the HCT116 cells treated with and without 25 ng/ml BMP7 from 48 to 60 h (Fig. 4A). The results demonstrated that knockdown of Smad4 in the HCT116 cells by shRNA could alter the cell proliferation activity *in vitro*, and that the BMP7 had no effect on the Smad4 down-regulated HCT116 cells, but did have an effect on the HCT116 cells.

Invasion and migration power assay in vitro. Following 24 h of incubation, the HCT116 cells demonstrated weaker invasion/migration activities compared with the cells transfected with 'Smad4-shRNA' (Figs. 5 and 6). The differences between the HCT116 and HK groups had no statistical significance. Moreover, the HCT116 cells in 'Smad4-shRNA' group displayed a significantly stronger transmembrane invasion/migration activity compared with those in the HK group and in HCT116 cells. The transmembrane invasion/migration activity of the HCT116 cells treated with or without BMP7 was different, while the activity of the Smad4-down-regulated HCT116 cells treated with or without BMP7 was similar. These findings suggest that a decrease in Smad4 expression

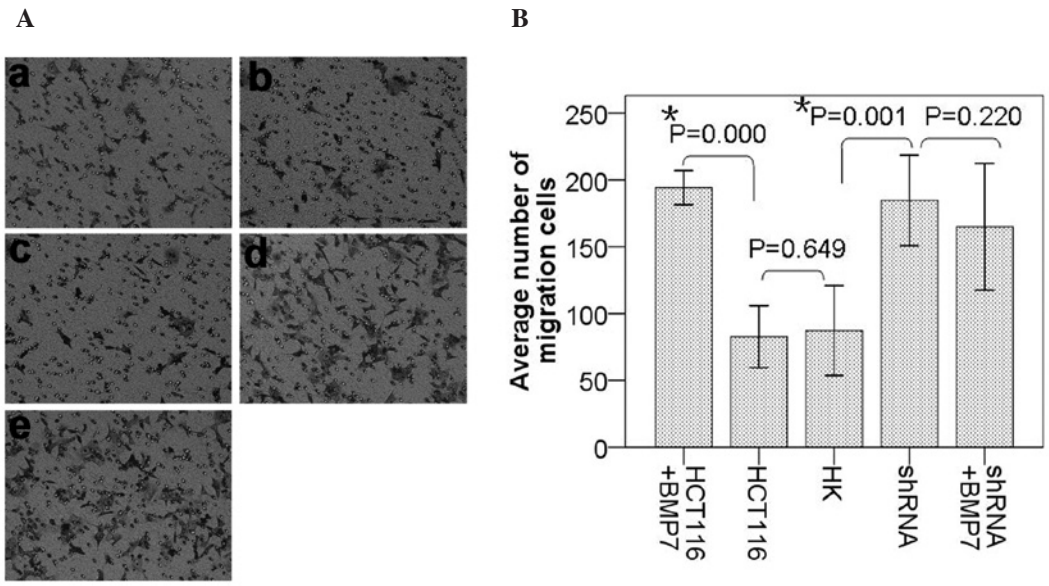

Figure 6. Silencing of Smad4 enhances the migration of HCT116 cells. The number of migrating cells was determined by counting the cells stained with Giemsa on the lower side of the membrane. (A-a) HCT116 cells; (A-b) HCT116-HK cells; (A-c) HCT116-Smad4-shRNA cells; (A-d) HCT116-Smad4-shRNA cells + 25 ng/ml BMP7; (A-e) HCT116 cells + 25 ng/ml BMP7. (B) Comparison of the numbers of transmembrane cells in the migration experiments. In the migration experiments, the migratory ability in in the groups that were treated by Smad4-shRNA was different from the HCT116/HK group ($P<0.05$), but no difference was noted between the Smad4-down-regulated cells treated with or without BMP7 ($P>0.05$).

level appears to be closely associated with the enhanced invasion and migration of the HCT116 cell line and that no effect for BMP7 was noted in the Smad4-down-regulated HCT116 cells.

Discussion

Colorectal cancer is the third most common malignancy worldwide and causes considerable morbidity and mortality (11). In 1990, Fearon and Vogelstein proposed that multistage genetic alterations were involved in colorectal carcinogenesis, typically in the adenoma-carcinoma sequence (12). Here, we demonstrated that Smad4 plays an essential role in colorectal cancer cell proliferation and invasion. When treated with Smad4-shRNA, the colorectal cancer cell line HCT116 displayed enhanced cell proliferation and invasion. These results indicate that Smad4 may be used as a colorectal cancer suppressor for gene therapy. Zhang *et al* studied the function of the gene (or protein) Smad4 using another method and obtained the same results (13). In addition, in our experiments the expression of the BMP7 target gene, *Id2,* also increased, while the reasons for this require further study.

BMPs are part of the transforming growth factor superfamily (14,15). They cooperatively bind type I and type II BMP receptors, phosphorylate the BMP-specific Smads1, 5 and 8 in turn, and then complex with Smad4. This complex translocates to the nucleus and modulates gene transcription of BMP target genes (16). Id2 is one of the BMP7 target genes and plays an essential role in cell proliferation. BMP7 signaling is also divided into Smad4-dependent and Smad4-independent pathways (8). Through Western blotting experiments, we found that when Smad4 expression was down-regulated by Smad4-shRNA, Id2 expression increased. Without Smad4 or down-regulated Smad4, Id2 did not increase any more than when BMP7 was added. Smad4 inactivation selectively removes only the tumor suppressive axis of the pathway, allowing TGF-β to preferentially overactivate the Smad-independent pathways in cooperation with other pro-oncogenic pathways. Based on our results, it may be supposed that Id2 was regulated by BMP7 through the Smad4-dependent pathways. In addition, the cell proliferation and invasion did not change when treated with BMP7 in Smad4 down-regulated HCT116 cells. These results indicate the presence of a Smad4-dependent BMP7 pathway in HCT116 cells. The enhanced proliferation and invasion abilities of HCT116 treated with Smad4-shRNA may be due to up-regulated Id2. This study may help to deepen the understanding of the Smad4 pathway in the pathogenesis of colorectal cancer, as well as gene diagnosis and treatment for colorectal cancer therapy. However, greater details of these mechanisms remain to be elucidated through further research.

Acknowledgements

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