·Basic Research·

Inhibitory effect of short-hairpin RNA expression vector-mediated osteopontin RNA interference on proliferation and invasion of prostate cancer PC-3 cells

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[Abstract] Background and Objective: Osteopontin (OPN) regulates cell migration and invasion in a variety of cancers and induces the activation of matrix metalloproteinase (MMP)-2 and MMP-9. This study was to investigate the role of OPN in the proliferation and invasion of human prostate cancer PC-3 cells and the possible functions of IκB kinase (IKK) in nuclear factor kappa B (NF-κB)-mediated signaling pathways. **Methods:** OPN short-hairpin RNA (shRNA) recombinant plasmids were transfected into PC-3 cells and different concentrations of IKK inhibitors were used to inhibit the activities of IKKα and IKKβ. The mRNA and protein expression levers of OPN, MMP-2, and MMP-9 were detected by real-time polymerase chain reaction (PCR) and Western blot. Cell cycle was detected by flow cytometry, cell proliferation by MTT assay, and cell invasion by Transwell chamber assay. **Results:** Compared with untransfected cells, the protein levers of OPN, MMP-2, and MMP-9 in OPN shRNA-transfected PC-3 cells were reduced by 55.22%, 51.71%, and 28.35%, respectively, and the abilities of cell migration and invasion were decreased by 45.48% and 51.96%, respectively ($P < 0.05$). Moreover, the inhibition of IKKβ inhibited the expressions of MMP-2 and MMP-9. **Conclusion:** OPN shRNA-mediated OPN gene silencing can inhibit the malignant biological behaviors of PC-3 cells. IKKβ may play a crucial role in the OPN-induced activation of MMP-2 and MMP-9 via NF-κB-mediated IκB/IKKβ pathways.

Key words: Prostate neoplasm, PC-3 cell, short-hairpin RNA, osteopontin, matrix metalloproteinase, RNA interference, nuclear factor-κB

The overexpression of osteopontin (OPN) is correlated with the tumorigenesis, development, invasion and metastases of many cancers.[1-4] It has been reported that OPN expression obviously increased in PC-3 cells, a human prostate cancer cell line with a high potential of metastasis.[4-7] OPN may enhance the abilities of mobility and chemical invasiveness of malignant tumor cells through regulating the activities of matrix metalloproteinase (MMP)-2 and MMP-9, which degradate extracellular matrix.[8-10] Philip *et al.*[8] reported that OPN induces nuclear factor kappaB (NF-κB)-mediated pro-MMP-2 activation through IκBα/IκB kinase (IKK) signaling pathway. Rangaswami *et al.*[10] demonstrated that OPN induces MAPK/IKK-dependent NF-κB-mediated pro-MMP-9 activation, which promotes the degradation of extracellular matrix such as type VI collagen and enhances the mobility and invasion of murine melanoma cells. It was recently reported that the OPN-induced MMP-9 expression in prostate cancer cells is closely correlated with CD44 signaling pathways, a sort of glucoprotein on the surface of cell membrane.[4,10] However, the role of OPN in the proliferation and invasion of PC-3 cells and the molecular mechanisms that OPN induces the activation of MMP-2 and MMP-9 via NF-κB signaling pathway have not completely understood. This study was to investigate the effects of OPN short hairpin RNA (shRNA) expression vector-mediated RNA interference (RNAi), which inhibited the expressions of MMP-2 and MMP-9, on the biological behaviors of PC-3 cells and the possible functions of IKK in NF-κB-mediated activation of MMP-2 and MMP-9, so as to provide potential target gene and preliminary experimental evidences for the gene therapy of human prostate cancer.

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Materials and Methods

Materials

PC-3 cells were from China Center of Type Culture Collection (CCTCC), Wuhan, China. Trizol reagents were bought from Invitrogen Co., USA; RIPA total protein extract reagents from ProMab Co.; reverse transcription kits from Toyobo Co., Japan; DNA polymerase from Fermentas Co., UK; transwell plates from Corning Inc., USA; matrigel gel from BD Co., USA; DMEM/F12 medium from Hyclone Co., USA; IKK inhibitor VII from Merck Co., USA; lipofectamine 2000 transfection kits and fetal bovine serum (FBS) from Gibco Co., USA.

Methods

Design of OPN shRNA and reconstruction of expression vector Using the GenBank sequence for human OPN mRNA (GenBank accession No. J04765.1) as a reference, we designed four candidate OPN shRNA sequences for RNAi (Table 1). The hairpin structure included 21 pairs of complementary bases, a loop composed by 9 oligonucleotides and a termination sequence. These 21-nt sequences showed no homology with other known genes in the human genome by Blast analysis. The recombinant plasmids were synthesized and purified by Shanghai GenePharma Co., Ltd. Four kinds of recombinant plasmid were respectively transfected into PC-3 cells. The most highly functional shRNA recombinant plasmid PGPU6/GFP/Neo-OPN2 was screened out by reverse transcription-polymerase chain reaction (RT-PCR) for further studies.

Table 1 Sequences of osteopontin (OPN) short-hairpin RNA (shRNA)

OPN shRNA notation	Targeted OPN mRNA sequence	Loop	Reverse complement sequence	Termination signal	Position in GenBank (J04765.1)
OPN1	CACCGCCATACCAGTTAAACAGGCT	TTCAAGAGA	AGCCTGTTTAACTGGTATGGC	TTTTTTG	154
OPN2	CACCGCAGCTTTACAACAAATACCC	TTCAAGAGA	GGGTATTTGTTGTAAAGCTGC	TTTTTTG	198
OPN3	CACCGAGCAATGAGCATTCCGATGT	TTCAAGAGA	ACATCGGAATGCTCATTGCTC	TTTTTTG	825
OPN4	CACCGCCATGAAGATATGCTGGTTG	TTCAAGAGA	CAACCAGCATATCTTCATGGC	TTTTTTG	906

The OPN shRNAs were cloned into eukaryotic expression plasmid PGPU6/GFP/Neo to evaluate the efficiency of OPN gene silencing.

Cell culture and tranfection PC-3 cells were cultured in DMEM/F12 (1:1) medium supplemented with 10% FBS in a 5% CO_2 humidified incubator at 37℃ for 48 h, then different concentrations of IKK inhibitor VII were added into the medium to inhibit the activity of IKKα and IKKβ. Recombinant plasmid PGPU6/GFP/Neo-OPN2 and mock plasmid PGPU6/GFP/Neo were stably transfected into PC-3 cells, using Lipofectamine 2000 according to the manufacturer's instructions, with a transfection efficiency of over 75%. After 48-hour culture, stable clones were selected using G418 at a final concentration of 600 μg/mL, and screened by limiting dilution assay. After fifteen passages, the cells with no loss of fluorescence had stable transfection of recombinant plasmid were named PCs; those with stable transfection of mock plasmid were named PC0 and used as control; untransfected cells were named PC3.

Detecting mRNA expressions of OPN, MMP-2 and MMP-9 by real-time PCR PC-3 cells at logarithmic growth phase were harvested and total RNAs were extracted. After removal of genomic DNA, fluorescent quantitative RT-PCR amplification was performed as follows: 50℃ for 2 min; 95℃ for 10 min; 40 cycles of 95℃ for 15 s and 60℃ for 45 s; then 70 cycles with increase of 0.5℃ per cycle from 60℃ for 10 s. The primers used in real-time PCR are showed in Table 2. According to the amplification plots and melting curves, when the parallel errors were small and unspecific amplification did not occur, we calculated the ΔCT and ΔΔCT, used RQ ($RQ=2^{-\Delta\Delta CT}$) values to assess the relative quantities of special mRNA expression in different groups.

Detecting protein expression by Western blot PC-3 cells at logarithmic growth phase were harvested and then schizolysised using RIPA Extraction Reagent Kits. The schizolysised solutions were centrifugated at 4℃ by 12 000 r/min for 5 min (Sigma 3K18 refrigerated centrifuge). The supernatant was collected and the protein concentration was detected by BCA method. Total cell lysate samples (20–40 μg protein per lane) were prepared in 1× loading buffer. The proteins were separated by 10% SDS-PAGE at 300 mA electric current and transferred onto PVDF membranes. The membranes were blocked in 5% non-fat milk for 2 h at room temperature, incubated with the primary antibody overnight at 4℃, then incubated with the secondary antibody for 1 h at room temperature. The antigen-antibody complexes were colorated and fixed using an enhanced chemiluminescence kit (BestBio Co., Shanghai, China). The images were collected using UVP software and dealed with Gelwords ID Advanced V 4.01 software. The antibodies used in Western blot assay are showed in Table 2.

Detecting cell cycle by flow cytometry PC-3 cells were collected at logarithmic growth phase, then unicell suspensions were prepared and incubated with 75% alcohol overnight. After poaching with phosphate buffered solution (PBS) for 3 times, the cell suspensions were added with RNaseA (10 mg/L), then dyed using propidium iodide (PI) in dark for 30 min. DNA quantities in cells at G_0/G_1, S and G_2/M phases were analyzed by flow cytometry. Each groups were detected in triplicate experiments.

Drawing cell growth curve by MTT colorimetric method PC-3 cells at logarithmic growth phase were harvested using 0.25% trypsase, suspended at a density of about 1×10^3 cells/μL, seeded into triplicate wells of 96-well plates at 100 μL/well, and incubated in a 5% CO_2 humidified incubator at 37℃ for 24 h, and then 200 μL of MTT (5 mg/mL) were added into each well. The cells were then incubated for 4 h and the supernatants were removed. After

Table 2 Primers used in real-time PCR and antibodies used in Western blot assay

Notation	Primer sequence	Amplification (bp)	Primary antibody titre	Corporation and batch number	Secondary antibody and titre	Target protein (kDa)
OPN	Forward, 5′-GTTAAACAGGCTGATTCTGG-3′	193	Rabbit OPN antibody	SANTA, SC-20788	Goat Anti Rabbit IgG/HRP	66
	Reverse, 5′-CATGGTCATCATCATCTTCA-3′		(1:400)		(1:40000)	
MMP-2	Forward, 5′-TACACCAAGAACTTCCGTCT-3′	153	Mouse MMP-2 antibody	ZYMED, 35-1300Z	Goat Anti Mouse IgG/HRP	72
	Reverse, 5′-GCCATCAAATACAATGTCCT-3′		(1:300)		(1:30000)	
MMP-9	Forward, 5′-GCAGAGGAATACCTGTACCGC-3′	196	Rabbit MMP-9 antibody	SANTA, SC-10737	Goat Anti Rabbit IgG/HRP	92
	Reverse, 5′-AGGTTTGGAATCTGCCCAGGT-3′		(1:500)		(1:50000)	
GAPDH	Forward, 5′-ACGACCACTTTGTCAAGCTC-3′	210	Mouse GAPDH antibody	ProMab, Mab-2005079	Goat Anti Mouse IgG/HRP	37
	Reverse, 5′-GTGAGGAGGGGAGATTCAGT-3′		(1:800)		(1:80000)	

MMP, matrix metalloproteinas.

150 μL of DMSO was added into each well, the plates were agigated for 20 min so as to dissolve the crystallizations. Finally, the absorbance values were measured using the enzyme-linked immuno assayer at a wavelength of 450 nm. The cell proliferation inhibition rate was calculated as (1−*A* value of experimental group/A value of control group) ×100%. Cell survival rates of experimental groups and control group were compared and the growth curves were drawed.

Transwell assay For migration assays, sterile polycarbonate membrane filters (Corning Inc., New York, NY) with 8-μm pores were coated with 6 μg/mL gelatin (BD Co., Franklin Lakes, NJ). The filters were hydrated with 200 μL of serum-free medium at room temperature for 60 min, then 1 mL of medium containing 5×10^4 cells were added into the top chambers of 24-well plates, and the lower chambers were filled with 0.5 mL of DMEM/F12 (1:1) medium containing 10% FBS and 0.5 mL of serum-free medium. The plates were incubated in a 5% CO_2 humidified incubator at 37℃ for 24 h. The filters were fixed with 95% alcohol for 15–20 min and stained with hematoxylin for 15 min. The cells on the upper surface were gently removed with a cotton swab and the cells on the lower surface of the filters were quantified under a microscope at 400× magnification. Each test group was assayed in triplicate.

For invasion assays, sterile polycarbonate membrane filters with 8-μm pores were coated with 50 mg/L Matrigel gelatin and the membranes were airdried at 4℃. The lower chambers of 6-well plates were filled with 1 mL of DMEM/F12 (1:1) medium containing 10 μg of fibronectin as a chemoattractant, and 0.5 mL of serum-free DMEM/F12 (1:1) containing 5×10^4 PC-3 cells was added to the upper chambers. The plates were then incubated at 37℃ in a 5% CO_2 humidified atmosphere for 48 h. Subsequently, the cells were stained with hematoxylin and the cells that had invaded the filters were recorded as migration assays.

Statistical analysis

Data are expressed as means ± standard deviation (SD). Statistical analyses were performed using SPSS11.0 statistics software. Differences between groups were compared using t-test and analysis of variance. Differences were considered to be significant when the *P* value was less than 0.05.

Results

OPN shRNA suppressed the expression of OPN, MMP-2 and MMP-9

The real-time PCR amplification plots (Figure 1A) and melting curves (Figure 1B) indicated that the differences between the parallel wells were small, thus, the amplification results were creditable. Compared with PC3 cells, the mRNA level of OPN in PCs cells was decreased significantly by 77.82% ($P < 0.05$), while PC0 cells showed no significant difference (Figure 1C).

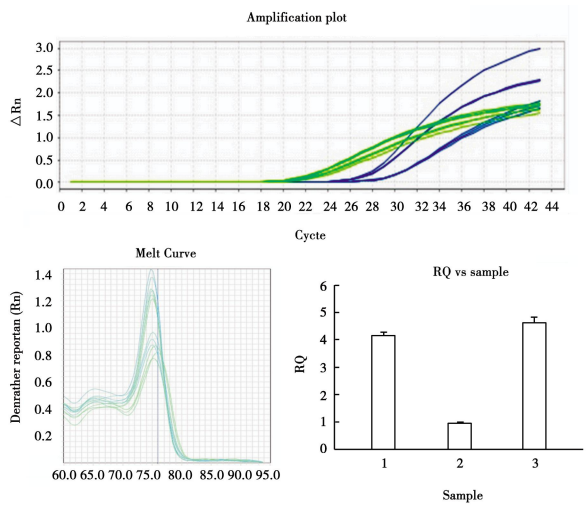

Figure 1 Osteopontin mRNA expression detected by real-time polymerase chain reaction (PCR)

A, amplification plots; B, melting curves; C, OPN mRNA relative expression in three groups.

Sample 1, PC3 cells; sample 2, PCs cells; sample 3, PC0 cells.

Western blot results showed that, compared with PC3 cells, the protein levels of OPN, MMP-2 and MMP-9 in PCs cells were decreased significantly by 55.22%, 51.71%, and 28.35%, respectively ($P < 0.05$), while PC0 cells showed no significant differences (Figure 2). These results demonstrated that OPN

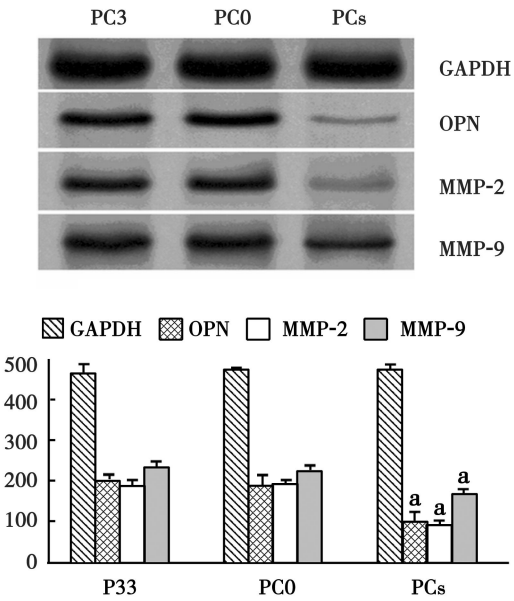

Figure 2 Expression of OPN, MMP-2, and MMP-9 proteins detected by Western blot

[a]$P<0.05$, vs. PC3 and PC0 cells, respectively.

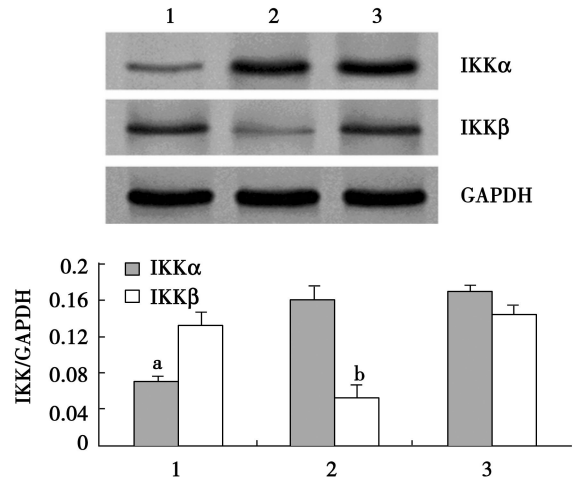

Figure 3 Expression of IKKα and IKKβ proteins detected by Western blot

Group 1, PC-3 cells pretreated with 200 nmol/L IKK inhibitor Ⅶ; group 2, PC-3 cells pretreated with 40 nmol/L IKK inhibitor Ⅶ; group 3: untreated PC-3 cells.

[a]$P<0.05$, vs. group 2 and group 3, [b]$P<0.05$, vs. group 1 and group 3.

shRNA expression vector-mediated OPN gene silencing not only downregulated OPN expression but also suppressed the expression of MMP-2 and MMP-9 in PC-3 cells.

Effects of different concentrations of IKK inhibitor VII on the activities of IKKα and IKKβ as well as the expression of OPN, MMP-2 and MMP-9

Compared with untreated PC-3 cells, the protein level of IKKα in PC3 cells treated with 200 nmol/L of IKK inhibitor VII was decreased by 57.89%, while the protein level of IKKβ in PC3 cells treated with 40 nmol/L of IKK inhibitor VII was decreased by 62.24%, with significant differences ($P < 0.05$) (Figure 3).

Compared with untreated PC3 cells, the cells treated with 40 nmol/L of IKK inhibitor VII showed no remarkable change in OPN mRNA expression, but the expression of MMP-2 and MMP-9 was inhibited significantly by 56.52% and 44.26%, respectively ($P < 0.01$); in contrast, the cells treated with 200 nmol/L of IKK inhibitor VII showed no remarkable changes in the expression of OPN, MMP-2 and MMP-9 (Figure 4). These data revealed that the specific inhibition of IKKβ downregulated the expression of MMP-2 and MMP-9. When treated with 40 or 200 nmol/L of IKK inhibitor VII, the expression of OPN, MMP-2 and MMP-9 were significantly lower in PCs cells than in PC0 and PC3 cells ($P < 0.05$) (Figure 4), indicating that OPN shRNA efficiently inhibited the expression of OPN, MMP-2 and MMP-9 mRNAs, moreover, suppressing the activity of IKKβ enhanced the inhibitory effects of

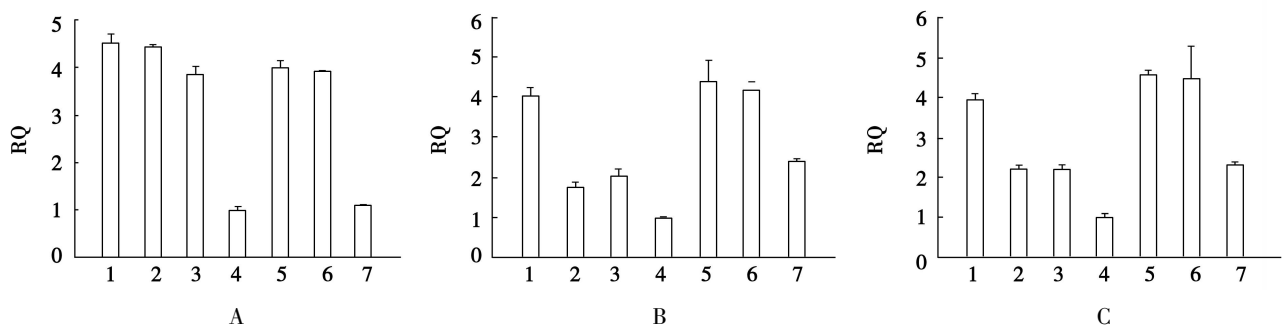

Figure 4 Relative expression of OPN, MMP-2, and MMP-9 mRNA in different groups detected by real-time PCR

A, OPN mRNA relative expression; B, MMP-2 mRNA relative expression; C, MMP-9 mRNA relative expression. Group 1, PC3 cells; group 2, PC3 cells treated with 40 nmol/L IKK inhibitor VII; group 3, PC0 cells pretreated with 40 nmol/L IKK inhibitor VII; group 4, PCs cells pretreated with 40 nmol/L IKK inhibitor VII; group 5, PC3 cells treated with 200 nmol/L IKK inhibitor VII; group 6, PC0 cells pretreated with 200nmol/L IKK inhibitor VII; group 7, PCs cells pretreated with 200 nmol/L IKK inhibitor VII.

RQ, relative quantity.

OPN shRNA on the expressions of MMP-2 and MMP-9.

Western blot results of OPN, MMP-2 and MMP-9 expression (Figure 5) were similar to real-time PCR results. When treated with 200 nmol/L of IKK inhibitor VII, no significant changes in the expression of OPN, MMP-2 and MMP-9 proteins were observed; when treated with 40 nmol/L of IKK inhibitor VII, the expression of MMP-2 and MMP-9 proteins were reduced significantly by 19.72% and 25.75% ($P < 0.05$); when transfected with OPN shRNA and treated with 200 nmol/L of IKK inhibitor VII, the expression of MMP-2 and MMP-9 proteins were reduced significantly by 55.62% and 51.47% ($P < 0.01$).

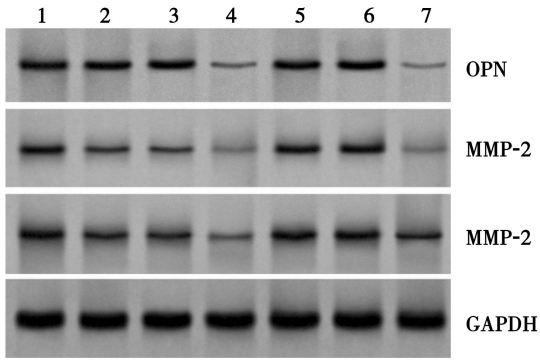

Figure 5 Expression of OPN, MMP-2, and MMP-9 proteins in PC-3 cells detected by Western blot

Lane 1, PC3 cells; lane 2, PC3 cells treated with 40 nmol/L IKK inhibitor Ⅶ; lane 3, PC0 cells pretreated with 40 nmol/L IKK inhibitor Ⅶ; lane 4, PCs cells pretreated with 40 nmol/L IKK inhibitor Ⅶ; lane 5, PC3 cells treated with 200 nmol/L IKK inhibitor Ⅶ; lane 6, PC0 cells pretreated with 200nmol/L IKK inhibitor Ⅶ; lane 7, PCs cells pretreated with 200 nmol/L IKK inhibitor Ⅶ.

Effects of OPN shRNA on cell cycle

As shown in Figure 6 and Table 3, compared with PC3 and PC0 cells, the quantity of hypodiploid DNA in PCs cells was obviously increased ($P < 0.05$), while the quantity of DNA at G_2/M phase was significantly decreased ($P < 0.05$). In contrast, the former two groups showed no significant differences ($P > 0.05$). These data suggested that OPN shRNA significantly suppressed the proliferation of PC-3 cells, arrested cell cycle in S phase, and decreased the quantity of hypodiploid DNA significantly.

Effects of OPN shRNA on proliferation, migration and invasion of PC-3 cells

As shown in Figure 7, the proliferation inhibition rate of PCs cells was higher than those of PC3 and PC0 cells after 48 h culture. The proliferation of PC0 cells was slightly inhibited. The results showed that OPN shRNA suppressed the proliferation of PC-3 cells. From the results of Transwell assay (Figure 8, Table 4), compared with PC3 cells, the migration and invasion of PCs cells were decreased by 45.48% and 51.96% ($P < 0.05$), while PC0 cells showed no significant differences ($P > 0.05$). These data suggested that OPN shRNA downregulated the expression of OPN, MMP-2 and MMP-9 in PC-3 cells, thereby resulting in suppression of the proliferation, migration and invasion of PC-3 cells in vitro.

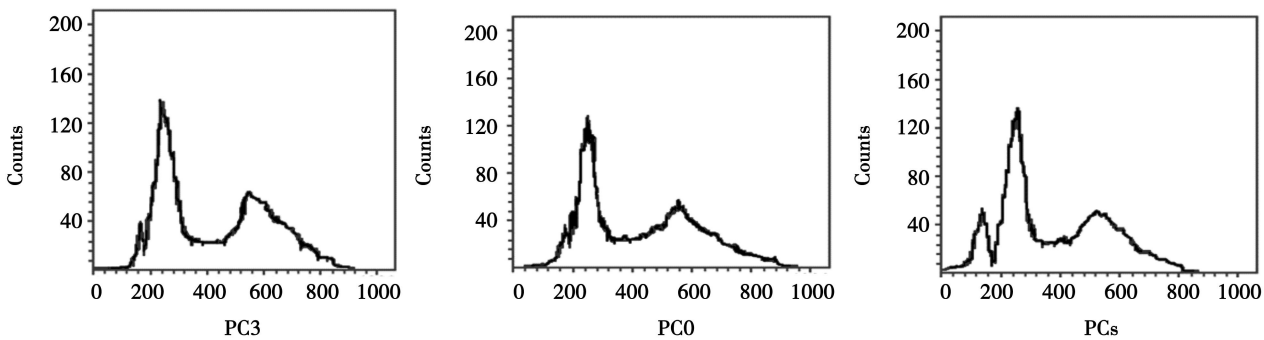

Figure 6 Cell cycle of PC-3 cells detected by flow cytometry

Table 3 Analyses of DNA quantities in different cell cycles by flow cytometry (%)

Group	Hypodiploid DNA	G_0/G_1 phase	S phase	G_2/M phase
PC3	3.61±0.83	40.23±0.54	14.26±1.16	41.90±2.52
PC0	3.40±0.76	37.83±1.71	18.80±1.56	39.97±0.91
PCs	8.52±1.04[a]	43.40±1.07	15.76±1.28	32.33±0.83[a]

[a]$P < 0.05$, vs. PC3 group. Each group was assayed in triplicate experiments.

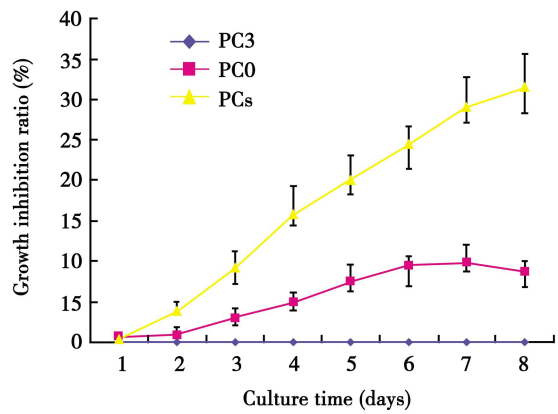

Figure 7 Growth curves of PC-3 cells in three groups

Discussion

Prostate cancer is one of the malignant tumors with a high potential of osseous metastases. Therefore, identification of the

Table 4 Comparision of cell proliferation, migration, and invasion of three groups

Group	Proliferation		Migration		Invasion	
	Culture for 5 days ($\bar{x}\pm s$)	Inhibition rate of proliferation (%)	The number of trans-membrane ($\bar{x}\pm s$)	Inhibition rate of migration (%)	The number of transmembrane ($\bar{x}\pm s$)	Inhibition rate of invasion (%)
PC3	3.31±0.02	0	151.0 ± 2.16	0	127.67±7.12	0
PC0	3.06±0.75	7.50	143.67±10.98	4.85	125.67±8.52	1.57
PCs	2.65±2.04	20.24[a]	82.33± 6.38	45.48[a]	61.33±7.52	51.96[a]

[a]$P < 0.05$, vs. PC3 cells. Each group was assayed in triple.

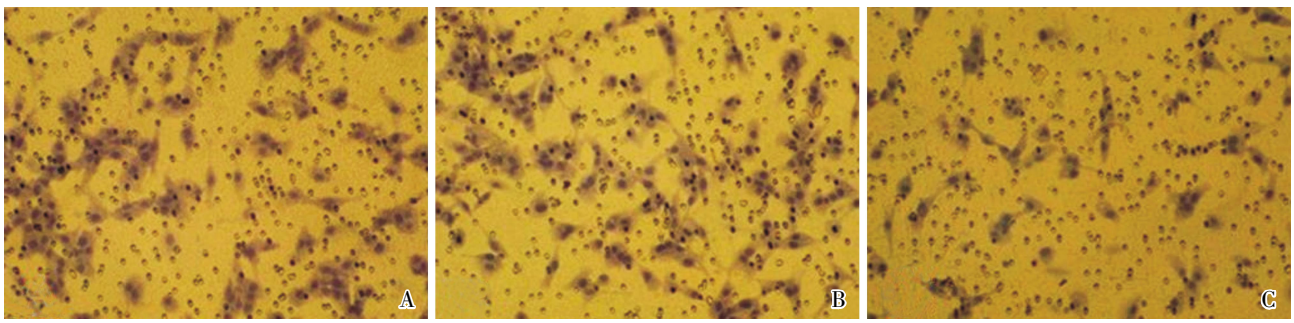

Figure 8 Invasion abilities of PC-3 cells in three groups detected by Transwell assay (×400)

After stained with hematoxylin, the PC-3 cells are showed as irregular fusiform shape. The cell numbers of pictures A, B, and C are 114, 124, and 72, respectively. A, PC3 cells; B, PC0 cells; C, PCs cells.

target genes associated with the progression of prostate cancer is necessary to improve patients' survival. OPN is a secreted, sialic acid-rich phosphoglycoprotein. It acts both as chemokine and cytokine.[11] OPN has an N-terminal sequence, a highly acidic region consisting of nine consecutive aspartic acid residues, and a GRGDS cell adhesion sequence flanked by the β-sheet structure, which shows high affinities to hydroxyapatite ceramic in bone trabecula.[12] The molecular mechanisms that define the roles of OPN in tumorigenesis and metastases are complex and have not been completely understood. It has been reported that OPN induces angiogenesis through activating PI3K/AKT and ERK1/2, and enhances the proliferation and invasion of tumor cells.[13] Recently, it has been identified that OPN overexpression is associated with tumor metastases and recurrence in a variety of cancers.[1,14] Overexpression of OPN increases cell malignant tendency of human mammary epithelial cells,[15] but reduced OPN expression decreases colony formation and the potential of osseous metastases of nude mice colon cancer cells,[16] and OPN antisense oligonucleotide can inhibit OPN protein expression in murine fibroblasts and prevent tumor formation.[10] To investigate the effects of OPN on the biological behaviors of human prostate cancer PC-3 cells and its mechanisms, we preformed RNA interference (RNAi) using OPN shRNA recombinant plasmids, which were transfected into PC-3 cells and resulted in obvious downregulation of OPN expression.

RNAi refers to the transfer of double-stranded RNA (dsRNA), which has complementary sequences of the mRNA of target gene, into cells to degrade a specific mRNA, resulting in silence of the target gene and specific suppression of the expression of the functional protein. It is a important means of post-transcription gene silencing for tumor gene therapy.[17] At present, there are two sorts of RNAi applying for mammalian cells. The first is to synthesize a small interfering RNA (siRNA) by extra-organismal transcription or chemical methods, and transfect it into target cells through bangosomes or viruses. The second is to induce RNAi by short hairpin siRNA which is emerged after shRNA expression vector is transfected into target cells.[18] shRNA is not only highly specific and cheap but also not easily contaminated by RNA enzyme. After transfected into target cells, shRNA can stably express short hairpin siRNA under the function of RNA enzyme, so that it can prolong the interference time in vitro.[19] In the present study, pGPU6/GFP/Neo, a eukaryotic expression plasmid including polymerase III promotor of human U6 RNA, after transfected into PC-3 cells, emerged quantities of siRNA interference sequences under the function of U6 promoter and termination sequence. Moreover, after shRNA recombinant plasmids are transfected into target cells, the dsRNA are emerged through single strand RNA and matched-pairs of shRNA, which are behind of promoters, then RNAi is initiated. The plasmid pGPU6/GFP/Neo includes a green fluorescent protein (GFP) gene and a Neo gene. GFP gene can express green fluorescent proteins, which can be used to judge transfection efficiency. Neo gene can be used to screen positive clons through planting cells in G418 medium at a special concentration.

In this study, RNAi was successfully performed by using an OPN shRNA recombinant vector in human prostate cancer PC-3 cells in vitro. Our results showed that relative mRNA levels of OPN, MMP-2 and MMP-9 in OPN shRNA-transfected PC-3 cells were significantly decreased, compared with control groups,

however, no significant differences were detected between mock plasmid-transfected and untransfected cells. OPN shRNA-mediated gene silencing not only downregulated OPN expression but also inhibited the expression of MMP-2 and MMP-9 proteins in PC-3 cells. The results of flow cytometry, MTT and Transwell assays showed that OPN shRNA-transfected PC-3 cells were blocked at S phase, apoptosis rate increased significantly, the proliferation and invasion decreased remarkablely ($P < 0.05$). Furthermore, the decreased levels were correlated with the relative expression levels of OPN, MMP-2 and MMP-9 proteins. This study confirmed that OPN shRNA can inhibit the malignant biological behaviors of PC-3 cells and provided a preliminary experimental base for the gene therapy of human prostate cancer in the future.

The transcription factors of the NF-κB family are critical regulators of gene transcription. IKK, a large complex multiprotein that includes two catalytic subunits, IKKα and IKKβ, is responsible for IκB phosphorylation and NF-κB activation.[20] MMPs are ECM-degrading enzymes that play a critical role in embryogenesis, tissue remodeling, inflammation and angiogenesis. MMP-2 and MMP-9 play critical roles in tumor invasion, growth and matastases.[7,9,21] Philip *et al*.[7,8] reported that OPN induced NF-κB-mediated activation of MMP-2 and MMP-9 through IκBα/IKK signaling pathways in murine melanoma cells and correlated with MAPK/IKKα signaling pathways. However, previous study by Mercurio *et al*.[20] revealed that mutant versions of IKKα exert an influence on NF-κB-dependent reporter activities, consistent with a critical role for IKK in NF-κB signaling pathways in human cervix cancer cells. Our data of human prostate cancer PC-3 cells have shown that the inhibition of IKKα has no effect on the expression of OPN, MMP-2 and MMP-9, in contrast, the inhibition of IKKβ can obviously suppress MMP-2 and MMP-9 expression. Moreover, the decreased expression of MMP-2 and MMP-9 proteins in PC-3 cells, treated with 40 nmol/L IKK inhibitor VII and transfected with OPN shRNA, were higher than that of respectively applying of them, suggesting that IKKβ may play a crucial role in OPN-induced NF-κ B-mediated activation of MMP-2 and MMP-9 in PC-3 cells. Our results are not completely consistent with those reported by Philip *et al*.[7] and Rangaswami *et al*.,[8] but are consistent with those reported by Mercurio *et al*.[20] Moreover, our date showed that although knockdown of OPN can suppress the expression of MMP-2 more obvious than suppress that of MMP-9. Thus, we presumed that there are a number of signaling pathways by which OPN induces the activation of MMP-2 and MMP-9, some of them are common and others are specific. To further identify the mechanisms of these processes is one of the research projects in our laboratory.

In summary, we successfully constructed a human prostate cancer cell line, PCs, stably transfected with PGPU6/GFP/Neo-OPN2 recombinant plasmid. Our results indicated that OPN shRNA-mediated OPN gene silence can not only downregulate the expression of OPN, MMP-2 and MMP-9 in PC-3 cells but also obviously suppress the proliferation, migration and invasion of PC-3 cells in vitro. Our study also suggested that IKKβ may play a critical role in OPN-induced NF-κB-mediated activation of MMP-2 and MMP-9.

References

[1] Rittling SR, Chambers AF. Role of osteopontin in tumour progression [J]. Br J Cancer, 2004,90(10):1877–1881.

[2] Rudland PS, Platt-Higgins A, El-Tanani M, et al. Prognostic significance of the metastasis-associated protein osteopontin in human breast cancer [J]. Cancer Res, 2002,62(12):3417–3427.

[3] Shimada Y, Watanabe G, Kawamura J, et al. Clinical significance of osteopontin in esophageal squamous cell carcinoma: comparison with common tumor markers [J]. Oncology, 2005,68(2–3):285–292.

[4] Desai B, Rogers MJ, Chellaiah MA. Mechanisms of osteopontin and CD44 as metastatic principles in prostate cancer cells [J]. Mol Cancer, 2007,6: 18.

[5] Jain A, McKnight DA, Fisher LW, et al. Small integrin-binding proteins as serum markers for prostate cancer detection [J]. Clin Cancer Res, 2009,15(16):5199–5207.

[6] Castellano G, Malaponte G, Mazzarino MC, et al. Activation of the osteopontin/matrix metalloproteinase-9 pathway correlates with prostate cancer progression [J]. Clin Cancer Res, 2008,14(22):7470–7480.

[7] Thalmann GN, Sikes RA, Devoll RE, et al. Osteopontin: possible role in prostate cancer progression [J]. Clin Cancer Res, 1999,5(8):2271–2277.

[8] Philip S, Kundu GC. Osteopontin induces nuclear factor kappa B-mediated promatrix metalloproteinase-2 activation through I kappa B alpha /IKK signaling pathways, and curcumin (diferulolylmethane) down-regulates these pathways [J]. J Biol Chem, 2003,278(16):14487–14497.

[9] Rangaswami H, Bulbule A, Kundu GC. Nuclear factor-inducing kinase plays a crucial role in osteopontin-induced MAPK/I kappa B alpha kinase-dependent nuclear factor kappa B-mediated promatrix metalloproteinase–9 activation [J]. J Biol Chem, 2004,279(37)38921–38935.

[10] Desai B, Ma T, Zhu J, et al. Characterization of the expression of variant and standard CD44 in prostate cancer cells: identification of the possible molecular mechanism of CD44/MMP9 complex formation on the cell surface [J]. J Cell Biochem, 2009,108(1):272–284.

[11] Denhardt DT, Guo X. Osteopontin: a protein with diverse functions [J]. FASEB J, 1993,7(15):1475–1482.

[12] Prince CW. Secondary structure predictions for rat osteopontin [J]. Connect Tissue Res, 1989,21(1–4):15–20.

[13] Dai J, Peng L, Fan K, et al. Osteopontin induces angiogenesis through activation of PI3K/AKT and ERK1/2 in endothelial cells [J]. Oncogene, 2009,28(38):3412–3422.

[14] Cui BK, Zhang CQ, Zhang Y, et al. Osteopontin as a potential biomarker of metastasis and recurrence for hepatocellular carcinoma [J]. Chin J Cancer, 2006,25:876–978. [in Chinese]

[15] Coppola D, Szabo M, Boulware D, et al. Correlation of osteopontin protein expression and pathological stage across a wide variety of tumor histology [J]. Clin Cancer Res, 2004,10(1 Pt 1):184–190.

[16] Gardner HA, Berse B, Senger DR. Specific reduction in osteopontin synthesis by antisense RNA inhibits the tumorigenicity of transformed Rat 1 fibroblasts [J]. Oncogene, 1994,9(8):2321–2326.

[17] Paul CP, Good PD, Winer I, et al. Effective expression of small interfering RNA in human cells [J]. Nat Biotechnol, 2002,20(5):505–508.

[18] Brummelkamp TR, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells [J]. Science, 2002,296 (5567):550–553.

[19] Wadhwa R, Kaul SC, Miyagishi M, et al. Know-how of RNA interference and its applications in research and therapy [J]. Mutat Res, 2004,567(1): 71–84.

[20] Mercurio F, Zhu H, Murray BW, et al. IKK-1 and IKK-2: cytokine-activated I kappa B kinases essential for NF-kappa B activation [J]. Science, 1997,278(5339):860–866.

[21] Scorilas A, Karameris A, Arnogiannaki N, et al. Overexpression of matrix-metalloproteinase-9 in human breast cancer: a potential favourable indicator in node-negative patients [J]. Br J Cancer, 2001,84(11):1488–1496.