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A small interfering RNA targeting osteopontin as gastric cancer therapeutics

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Abstract

It has been shown that Osteopontin (OPN) protein is overexpressed in the majority of gastric cancers and associated with its pathogenesis. To better understanding of the role of OPN, RNA interference (RNAi) was used to inhibit OPN expression in the human gastric cancer cells in vitro and in vivo. BGC-823, gastric cancer cell line, was stably transfected with OPN small interfering RNA (siRNA) plasmids. OPN siRNA significantly reduced the expression of OPN in human gastric cancer cells in transient- and stable-transfection manner. In vitro down-regulation of OPN inhibited BGC-823 cell growth, anchorage-independent growth, migration and invasion. The results further showed that OPN small interfering RNA suppressed the growth, migration and invasion of gastric cancer cell through the reduction of MMP-2 and uPA expression, inhibition of NF-κB DNA binding activity, and down-regulation of Akt phosphorylation. In vivo animal studies showed that tumor growth was significantly inhibited in OPN siRNA group compared with WT. Intratumor gene therapy with polyethylenimine/OPNsi also resulted in tumor growth suppression and prolonged survival. Thus, this study demonstrated that down-regulation of OPN could suppress the growth, migration and invasion of gastric cancer cells, and OPN siRNA may offer a new potential gene therapy approach for human gastric cancer in future.

Keywords: Gastric cancer; OPN; RNA interference; Tumor therapy

1. Introduction

Gastric cancer is the second most common cause of cancer-related death in the world. Many Asian countries, including Korea, China, and Japan, have very high rates of gastric cancer. Despite the use of multimodal therapy (chemotherapy, radiation therapy, and surgery), the long-term disease-free survival rate of gastric cancer patients is still disappointingly low, particularly in the high-risk groups [1,2]. The identification of new therapeutic targets is therefore needed. Recent evidence suggests that osteopontin (OPN) plays a significant role in regulating cancer cell proliferation, invasion, migration and survival [3].

Osteopontin (OPN), a secreted phosphoprotein and also a member of the SIBLING family, functions as both an ECM component and a cytokine

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signaling through the binding to two cell adhesion molecules: integrin and CD44 [4–10]. Constitutive expression of OPN at low levels exists in several normal cell types, induced expression at high levels occurs during cellular remodeling processes and in tumor progression [5–9]. Cumulative evidences suggest that increased OPN expression has been correlated with tumor invasion, progression or metastasis in cancers of the breast [11–13], stomach [14], lung [15,16], prostate [17], liver [18,19] and colon [20,21]. In models of breast cancer and melanoma, OPN has been shown to activate molecular mechanisms that regulate tumor cell growth, migration and invasion [22,23]. Moreover, down-regulation of OPN expression by small interfering RNA approach significantly attenuates CT26 colon cancer metastasis by diminishing tumor cell motility and invasiveness [24].

At present, in the context of gastric cancer, the evidence has been less definitive. Although gene and protein profiling studies have identified a correlation between abundant expression of OPN and gastric cancer development, invasive phenotypes and survival [3,14], the lack of mechanistic or functional data has led to an incomplete understanding of how OPN mediates gastric cancer progression. In order to address this issue, we used RNA interference (RNAi) to produce specific and long-term silencing of OPN in BGC-823 gastric cancer cells. BGC-823 is an undifferentiated human gastric adenocarcinoma cell line and exhibits aggressive in vivo tumorigenic activity [25]. We have designed our experiments as a loss-of-function study using Western blot-analysis to confirm the extent and stability of OPN knockdown in our clones that stably express siRNA against OPN. We evaluate the capacity of growth, migration and invasion of OPN-silenced BGC-823 clones in vitro. Furthermore, the possible OPN-downstream mediators and OPN-dependent signaling pathways were explored. In addition, we used polyethylenimine as our gene delivery system, overcomes the limitations of transience and high cost in synthetic siRNAs, to efficiency inhibit BGC-823 tumor growth in a nude mouse model.

2. Materials and methods

2.1. siRNA sequences and constructs

Using GenBank™ sequence AK315461 for human OPN cDNA and computer analysis software developed by Ambion, Inc., we selected two candidate sequences in the OPN cDNA sequence for RNAi. The targeted OPN sequences were OPNsi-A: AATCTCCTAGCCCCACAGAAT; OPNsi-B: AAGGACAGTTATGAAACGAGT. These 21-nt sequences showed no homology with other known human genes. Synthetic, annealed, siRNA oligonucleotides were synthesized chemically and gel-electrophoresis purified (Ambion, Austin, TX) and used during transient-transfection experiments. Human mismatch or scrambled siRNA sequences (Ambion) possessing limited homology to human genes served as a negative control. For stable RNAi we designed a hairpin siRNA sequence that contains both sense and antisense siRNA sequences against OPN target B. The annealed dsDNA oligonucleotides were ligated between the BamHI and HindIII sites on the pSilencer2.1-U6 hygro vector (Ambion, Austin, TX). The control vector (si) was constructed by inserting a sequence that expresses a siRNA with limited homology to sequences in the human genomes. All inserted sequences were verified by DNA sequencing.

2.2. Cell culture and transfection

The human gastric cancer cell line BGC-823 was purchased from the Institute of Cell Biology, Shanghai, China. Cell line was cultured in DMEM with 10% fetal bovine serum. Transfection was done with Superfect (Qiagen, Valencia, CA) as directed by the manufacturer and selected in hygromycin B (Invitrogen Life Technologies, Carlsbad, CA) containing medium at 400 μg/ml for BGC-823 cells. Stable transfected cell clones were tested for OPN expression by Western blotting. OPNsi-transfected BGC-823 cell clone (BGC/OPNsi) and control vector transfected BGC-823 cell clone (BGC/si) were used for the in vivo experiments.

2.3. In vitro cell growth assay

The in vitro growth rates of BGC, BGC/si and BGC/OPNsi cells were assessed at 24, 48 and 72 h. Cells were grown in monolayer culture to 60% confluence, harvested using trypsin and plated at a density of 5×10^3 cells/well into separate wells of a 96-well plate (Costar, Corning Inc., NY). DMEM-10% FBS supplemented with 100 IU/ml penicillin and 100 mg/ml streptomycin was used as culture medium. For BGC/si and BGC/OPNsi, 400 μg/ml hygromycin B was added to the culture medium. The cells were incubated with MTT (0.5 mg/ml)

after 24, 48 and 72 h. The color intensity was measured at 490 nm using an enzyme-linked immunosorbent assay reader (Dynatech). The experiments were performed in triplicate. The cell viability was expressed as A_{490nm}.

For anchorage-independent growth assays, cells were seeded in 0.3% agarose over a 0.6% agarose bottom layer at a density of 500 cells per well in 24-well plate. After 3 weeks, the numbers of colonies greater than 100 μm in diameter were counted.

2.4. In vitro migration and invasion assays

The motility and invasiveness of BGC-823 cells transfected OPN siRNA were evaluated in 24-well transwell chambers with upper and lower culture compartments separated by polycarbonate membranes with 8-mm sized pores (Costar 3422, Corning Inc., NY). Wild type (WT) BGC-823 and transfected control siRNA were used as controls. Cells were grown to 60% confluence, harvested using Cell-stripper (Cellgro, Herndon, VA), washed with 1 × PBS and resuspended in DMEM-0.1% BSA. Prior to plating cells into the transwells, DMEM-0.1% BSA was incubated in the top chamber of each transwell at 37 °C for 1 h to saturate non-specific binding sites and then subsequently removed. 5×10^4 cells suspended in 100 ml of DMEM-0.1% BSA were plated into the top chamber. DMEM-10% FBS was placed into the bottom chamber to act as a chemoattractant. After 24 h incubation at 37 °C in 5% CO2 humidified air, the cells remaining at the upper surface of the membrane were removed with a cotton swab. The cells that migrated through the 8-mm sized pores and adhered to the lower surface of the membrane were fixed with 3.7% paraformaldehyde, stained with 0.2% crystal violet and washed with 1 × PBS three times. The dye was eluted using 30% acetic acid and quantification of cell number was performed using colorimetric analysis with a microplate reader (absorbance at 590 nm). The absorbance units obtained for WT BGC-823, BGC/OPNsi clones and BGC/si clones were each divided by the absorbance units obtained for WT and expressed as a migration index. By definition, WT cells were assigned an index of 1.

In a similar fashion, the invasiveness of OPN siRNA-transfected BGC-823 cells were evaluated in Matrigel™ (Collaborative Biomedical Products, Bedford, MA) coated 24-well transwell chambers. WT BGC-823 and siRNA control clones were used as controls. Matrigel was used at a concentration of 0.4 mg/ml. Cells, media, experimental conditions and analysis performed were similar to those of migration assays. The absorbance obtained for WT, BGC/OPNsi clones and BGC/si clones were each divided by the absorbance obtained for WT BGC-823 and expressed as an invasion index. By definition, WT was assigned an index of 1. Triplicate assays were performed for each group of cells in both migration and invasion assays and results are expressed as means ± SD.

2.5. Nuclear and cytoplasmic extracts and Western blot

Subcellular Fractionation was performed by centrifugation technique as described previously. Nuclear extracts were made using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL). Total cell lysate was prepared in 1 × SDS buffer. Proteins at the same amount were separated by SDS–PAGE and transferred onto PDVF membranes. After probing with individual antibodies, antigen–antibody complex was visualized by enhanced chemiluminescence's reagents Supersignal (Pierce Biotechnology). The antibodies used were anti-OPN (R&D Systems, Minneapolis, MN), and anti-uPA (Santa Cruz Biotechnology, Santa Cruz, CA), anti-p-Akt, and anti-NF-κB p65 (Boster Biotechnology, Wuhan, PR China), anti-ERK, anti-phosphorylation ERK, anti-VEGF and anti-human MMP-2, -9 (Beyotime, Haimen, PR China). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and anti-tubulin (Ambion, Austin, TX) was used as an internal control.

2.6. Electrophoretic mobility shift assay (EMSA)

EMSA was done using LightShift chemiluminescent electrophoretic mobility shift assay kit (Pierce Biotechnology). Double-stranded gel shift probes corresponding to the human consensus NF-κB sequences 21 (5′-AGTTGAGGGGACTTTCCCA GGC-3′) were end-labeled with biotin.

2.7. Animal studies

Six- to eight-week-old female Nude mice were obtained from the Planned Parenthood Research Institute, Shanghai, People's Republic of China. All animals in this study were housed under patho-

gen-free conditions and were maintained in accordance with guidelines of the Committee on Animals of the Second Military Medical University, Shanghai, China. BGC-823, BGC/si, or BGC/OPNsi-B2 gastric cells in mid-log-growth phase were harvested by trypsinization. Single-cell suspensions (2×10^6 cells in 0.1 mL HBSS) were injected s.c. into the nude mice. The tumors were measured every 4 days with a caliper, and the diameters were recorded. Tumor volume was calculated by the formula: $a^2b/2$, where a and b are the two maximum diameters. When tumors reached 2.0×2.0 cm, the duration of survival was recorded, the mouse was euthanized.

For the OPNsi gene therapy experiments, 2×10^6 BGC-823 cells were injected s.c. into nude mice. Five days later, when the tumors were palpable, the mice were divided into three groups. Group 1 mice were used as untreated controls. Group 2 received intratumor injections (20 μg/mouse) of polyethylenimine/control vector twice weekly for various times. Group 3 received intratumor injections with 20 μg polyethylenimine/OPNsi twice weekly as described for group 2. Tumor size was measured as described above.

2.8. Statistical analysis

Data are expressed as means ± SD. Statistical analysis of data was done by Student's t test using SigmaPlot software. Difference was considered to be statistically significant at $P < 0.05$.

3. Results

3.1. RNAi decreases OPN expression

BGC-823 human gastric cancer cells are highly invasive and normally constitutively express OPN at high levels. To suppress OPN expression in BGC-823, we selected two 21-nt targets within the OPN cDNA for RNAi. Based on these targets, we designed double-stranded 21-nt siRNA constructs encoding sense and antisense siRNA, and the OPN levels were measured using Western blotting. As shown in Fig. 1A, OPN expression was significantly inhibited by OPNsR-A and OPNsR-B transient-transfection. In OPNsR-A and OPNsR-B transient-trans-

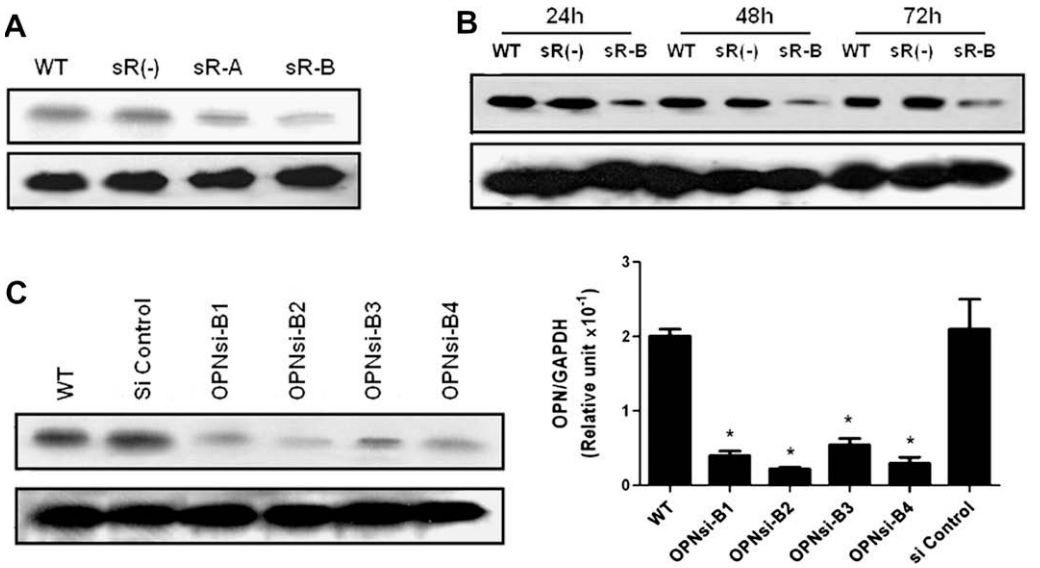

Fig. 1. Inhibition of OPN expression in BGC-823 cells by RNAi. (A) Western blot showing levels of OPN protein in cell lysates from WT (lane 1), sR(-) (si control, lane 2), sR-A (lane 3) and sR-B (lane 4). GAPDH levels served as an internal control for loading. (B) Western blot showing that RNAi-mediated silencing of OPN by transient-transfection is time-dependent. Using OPNsi-B; the extent of OPN suppression was measured at 24, 48 and 72 h (lanes 3, 6 and 9). OPN protein levels in WT (lanes 1, 4 and 7) and si control (lanes 2, 5 and 8) were used as controls. Levels of GAPDH are shown and were used as an internal control for loading. (C) Western blot and spot densitometry showing the extent of OPN protein suppression in cells stably transfected with OPNsi-B at 1 month after initial transfection. Results for WT (lane 1), OPNsi-B1, OPNsi-B2, OPNsi-B3 and OPNsi-B4 (lanes 2–5) and clonal cell lines stably transfected with si control plasmids (lane 6). Levels of GAPDH in corresponding cell lines are also shown and served as an internal control for loading. Histograms showing spot densitometry for the bands (OPN/GAPDH) are shown below the Western blot. The means ± SD of triplicate assays for each cell line is shown. $^*P < 0.05$, compared with WT control.

fected cells, OPN levels were reduced by ~50% and ~80%, respectively, compared with WT BGC-823. No significant difference was observed in OPN protein levels between BGC-823 and BGC/si cells. This RNAi-mediated effect was specific, as GAPDH levels did not differ significantly amongst the treated cells and controls. Using siRNA against target B, we further determined that transient-transfection mediated RNAi is time-dependent as OPN silencing was maintained for up to 72 h (Fig. 1B).

In order to assess the phenotype of tumor cells in which OPN expression was inhibited over long-term, we directed our attention to generating stable, OPN down-regulated clonal cell lines. Based on the higher efficiency of OPNsi-B compared with OPNsi-A, we selected target sequence B for use in designing our siRNA expression plasmids. BGC-823 cells were transfected with purified pSilencer2.1-U6 hygro vector expression vectors containing either the OPN siRNA insert (BGC/OPNsi) or the control siRNA insert (BGC/si). At 72 h after transfection, Western blot-analysis of BGC/OPNsi cells confirmed a 70% decrease in OPN expression compared with WT BGC-823 (data not shown). BGC/si cells showed similar levels of OPN protein compared with BGC-823. At 1 month, Western-blot-analysis of our clones demonstrated decreased OPN protein expression of 4.1-fold (BGC/OPNsi-B1), 3.2-fold (BGC/OPNsi-B3) and 4.4-fold (BGC/OPNsi-B4), compared with BGC-823. Our most significant clone, BGC/OPNsi-B2, demonstrated 5.1-fold decrease in OPN expression. BGC/si cells showed OPN protein levels similar to WT BGC-823. Protein levels of GAPDH, a housekeeping gene, were similar between different cell lines indicating that the RNAi-mediated knock-down of OPN was specific and did not result from a global decrease in gene expression (Fig. 1C).

3.2. Effect of OPN small interfering RNA on gastric cancer cell growth, migration and invasion in vitro

It has been reported that OPN-induced cell growth, migration and invasion in B16F10 melanoma cells [23], therefore we first checked the effect of OPN small interfering RNA on gastric BGC-823 cell growth and anchorage-independent growth in vitro. As shown in Fig. 2A and B, OPN siRNA, but not control siRNA, significantly inhib-

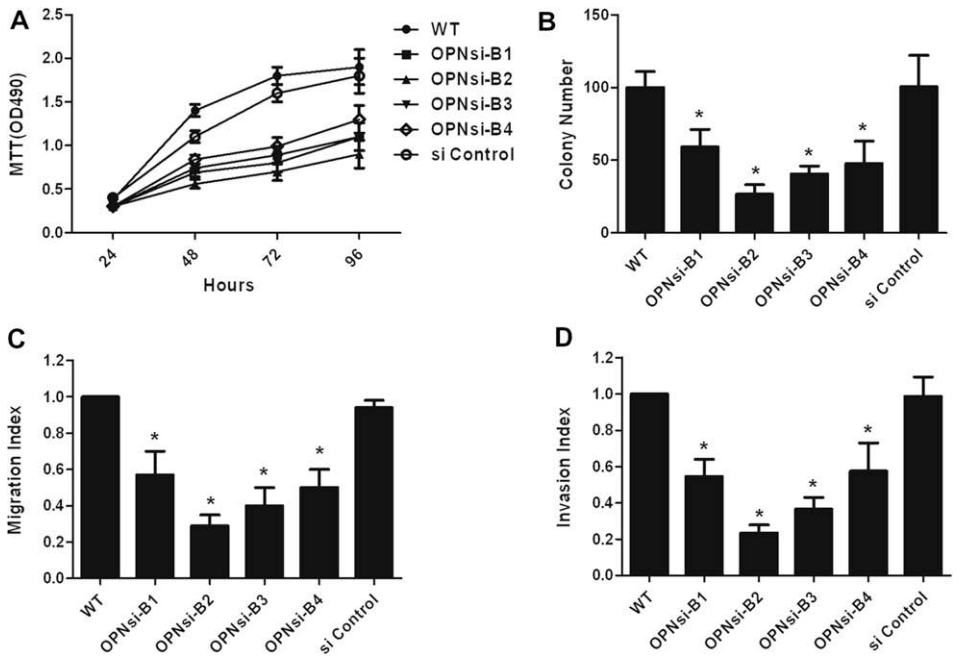

Fig. 2. Effect of OPN siRNA on BGC-823 cell growth, migration and invasiveness. (A) Effect of OPN siRNA on BGC-823 cell proliferation. At 24 h post-transfection, BGC-823 cells were seeded in 96-well plates with complete medium for MTT assay. Values were given as the means ± SD of six wells. $^{*}P < 0.05$, compared with WT control. (B) BGC-823 cells were seeded in semisolid soft agar medium to monitor anchorage-independent growth. The numbers represented the mean number of colonies of three independent experiments ± SD. ($^{*}P < 0.05$). (C) The impact of OPN silencing on in vitro BGC-823 cell migration. The migration of WT, OPNsi-B1, OPNsi-B2, OPNsi-B3 and OPNsi-B4, si control cells were assessed by incubating cells in transwell chambers for 24 h. Migrated cells were stained with 0.2% crystal violet, subjected to elution by acetic acid and quantified in a microplate reader (A590) by definition, WT cells were assigned a migration index of 1. (D) The impact of OPN silencing on in vitro BGC-823 cell invasiveness. The invasiveness of WT OPNsi-B1, OPNsi-B2, OPNsi-B3 and OPNsi-B4, si control cells were assessed by incubating cells in Matrigel-coated transwell chambers for 24 h. WT cells were assigned an invasion index of 1. The data represent the means ± SD of triplicate assays. $^{*}P < 0.05$, compared with WT control.

ited the growth and anchorage-independent growth of BGC-823 cells, especially BGC/OPNsi clone B2 cells was significantly reduced. Similar rates of growth were observed in WT BGC-823 and BGC/si cells.

We further evaluated whether the suppression of OPN expression would alter the ability of in vitro migration of BGC-823 cells. We assessed the motility of BGC/OPNsi clones across transwell polycarbonate membranes. As shown in Fig. 2C, cell motility in BGC/OPNsi-B1, BGC/OPNsi-B2, BGC/OPNsi-B3 and BGC/OPNsi-B4 were reduced by 55%, 29%, 40% and 50%, respectively, compared with WT. No significant differences were detected between WT BGC-823 and BGC/si.

Next, we also evaluated whether down-regulation of OPN expression would alter the ability of BGC-823 cells to invade into Matrigel™. As shown in Fig. 2D, BGC/OPNsi-B1, BGC/OPNsi-B2, BGC/OPNsi-B3 and BGC/OPNsi-B4 demonstrated 54%, 24%, 37% and 58% decreased invasiveness, respectively, compared with WT. No significant difference in invasiveness was observed between WT and BGC/si. Taken together, these data suggest that the inhibition of OPN expression in BGC/OPNsi cells resulted in decreased proliferation, cell motility and invasiveness.

3.2. OPN small interfering RNA suppresses PCNA, Ki-67 and MMP-2 expression

PCNA and Ki-67 antigen are commonly used as markers of cell proliferative activity. First, we determined that the chang of PCNA and Ki-67 expression in BGC-823 gastric cells after OPN siRNA transfection. As shown in Fig. 3, both the expression of PCNA and Ki-67 were significantly reduced in BGC/OPNsi cells. Moreover, the degree and extent of down-regulation was conserved between OPN and PCNA and Ki-67 with our most down-regulated OPN clone, BGC/OPNsi-B2.

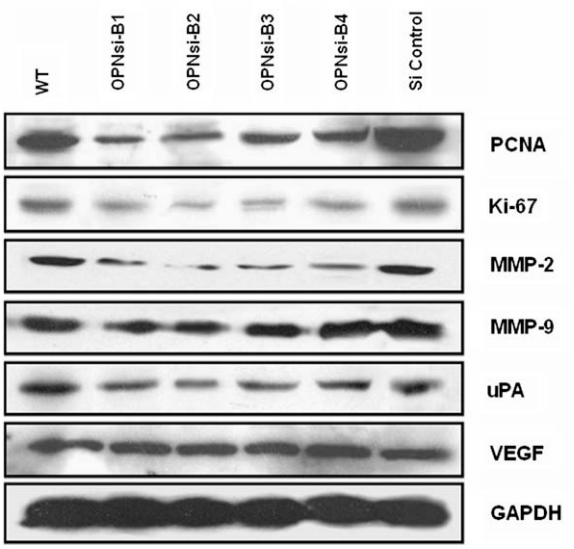

Fig. 3. OPN siRNA suppressed PCNA, Ki-67, MMP-2 and uPA expression. Protein expression of genes associated with migration, proliferation and invasioness in WT, OPN downregulated clones (OPNsi-B1 to OPNsi-B4) and control siRNA clonal cell lines. Western blotting with antibodies specific for PCNA, Ki-67, MMP-2, MMP-9, uPA and VEGF are shown. GAPDH levels are shown as an internal control for loading.

It has been shown that several proteins expression associated with OPN, such as MMP-2, MMP-9, and VEGF and uPA play important roles in breast and melanoma cell migration, growth and invasion. Therefore, we checked whether OPN siRNA affected these protein expressions in BGC-823 gastric cells. Interestingly, MMP-2 and uPA expression was decreased in each of our OPN downregulated clones (Fig. 3). No significant changes in expression were detected for MMP-9 or VEGF. The degree and extent of down-regulation was conserved between OPN and MMP-2 and uPA with our most downregulated OPN clone, BGC/OPNsi-B2, demonstrating the most significant attenuation of MMP-2 and uPA expression. These data indicated that the effect of OPN small interfering RNA on the proliferation, migration and invasion of gastric cancer cell was mainly relevant with the suppressive expression of MMP-2 and uPA.

3.3. Effect of OPN small interfering RNA on NF-κB DNA binding activity Akt and ERK activation

It has been reported earlier that OPN induces pro-MMP-2 activation through activation of NF-κB in B16F10 cells, and NF-κB DNA binding activity was involved in cell growth [26]. Therefore, we further determined whether OPN siRNA affected the NF-κB DNA binding activity. As shown in Fig. 4A, NF-κB DNA binding activity was significantly reduced in BGC/OPNsi cells, especially BGC/OPNsi cells clone B2, compared with WT BGC and BGC/si cells. Moreover, down-regulation of OPN also inhibited the amount p65 subunit of NF-κB in nuclear (Fig. 4B).

An earlier report showed that phosphatidylinositol 3-kinase/Akt and ERK signaling pathway is involved in OPN-induced tumor cell migration and invasion [22]. So we sought to determine the activity of Akt and ERK in BGC/OPNsi cells. As shown in Fig. 4B, OPNsi could not affect the ERK phosphorylation, similar to control si. In contrast, the level of Akt phosphorylation was significantly inhibited in the BGC-823 cells with OPNsi-transfected. Moreover, the degree and extent of phosphorylation down-regulation was consistent with the level of OPN expression in OPNsi-transfected BGC-823 cell clone. Taken together, these results indicate that the OPN small interfering RNA might suppress the growth, migration and invasion of gastric cancer cell through inhibition of NF-κB transcriptional activity, reduction of Akt phosphorylation.

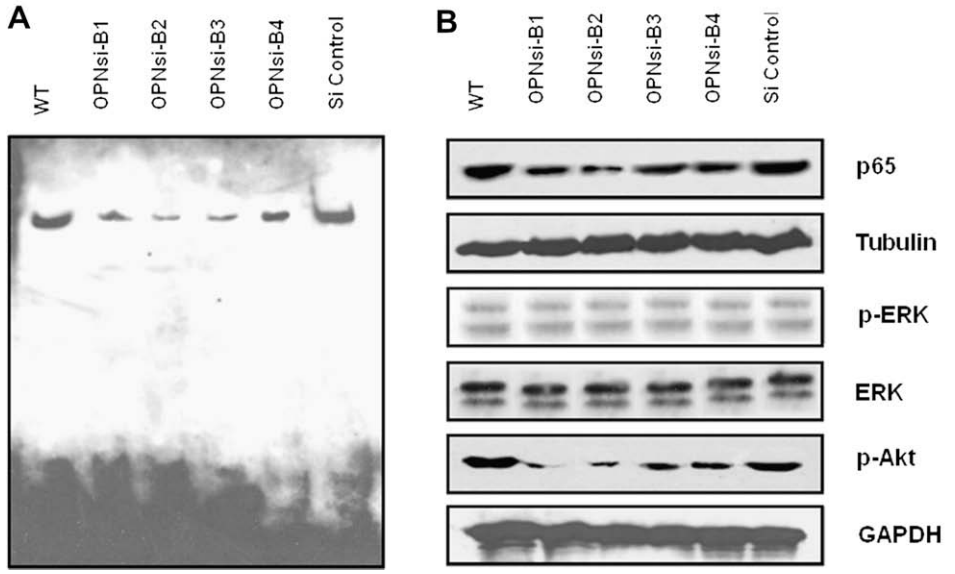

Fig. 4. Effect of OPN small interfering RNA on NF-κB DNA binding activity Akt activity and ERK activation. (A) Effect of OPN small interfering RNA on activity of NF-κB DNA binding. BGC, BGC/si and BGC/OPNsi cells were cultured in DMEM-10% FBS medium. Nuclear protein extracts were harvested 48 h latter and subjected to EMSA. B, Effect of OPN small interfering RNA on p65 level, Akt activity and ERK activation. Cytoplasmic and Nuclear protein extracts were harvested 48 h latter and subjected to Western blotting with anti-p-Akt, anti-NF-κB p65 and anti-ERK, and anti-p- ERK. anti-GAPDH and anti-tubulin were used as internal controls.

3.4. Effect of OPN small interfering RNA on gastric tumor growth in vivo

To determine whether inhibition of OPN by siRNA had an effect on tumor growth in vivo, BGC-823, BGC/si, or BGC/OPNsi-B2 cells were inoculated s.c. into nude mice. BGC-823 and BGC/si cells grew rapidly, resulting in palpable tumors 4–5 days following injection (Fig. 5A). By contrast, tumor formation was significantly slower after inoculation of BGC/OPNsi-B2. The BGC/OPNsi-B2 tumors were significantly smaller than those in both control groups. Survival time was also significantly longer for mice inoculated with BGC/OPNsi -B2 cells (Fig. 5B). No significant difference in either tumor growth or survival time was detected between the tumors induced by the BGC/si control cells and the parental BGC-823 cells.

To determine the OPN status in these tumors, RNA was extracted from tumor tissue and real-time PCR for OPN was done. OPN were normally detected in BGC-823 and BGC/si tumors, whereas the expression of OPN was significantly reduced in BGC/OPNsi-B2 tumors (data not shown), indicating that inhibition of OPN by OPNsi transfection was stable in vivo.

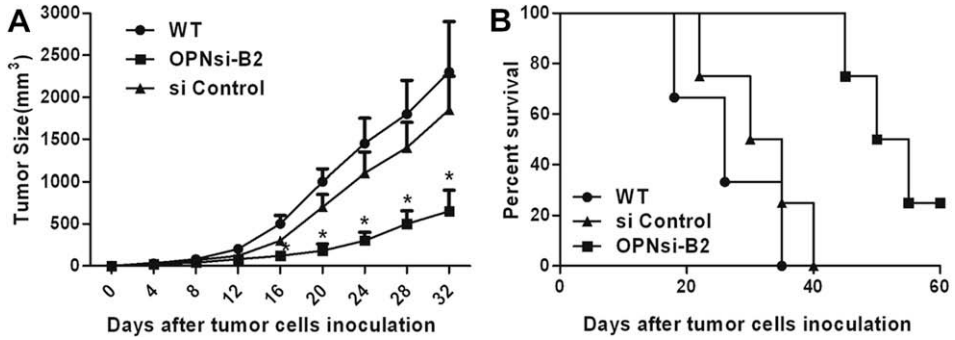

Fig. 5. Effect of OPN siRNA on BGC-823 gastric tumor growth in mice. (A) BGC-823, BGC-823/si, or BGC-823/OPNsi-B2 cells were inoculated s.c. into nude mice. Tumor growth was monitored and tumor volume was calculated. $^{*}P < 0.05$, compared with BGC-823, or BGC-823/si tumors. (B) Mice were euthanized when the tumor reached 2×2 cm in diameter. Survival curve was calculated as the percentage of surviving mice on the indicated days.

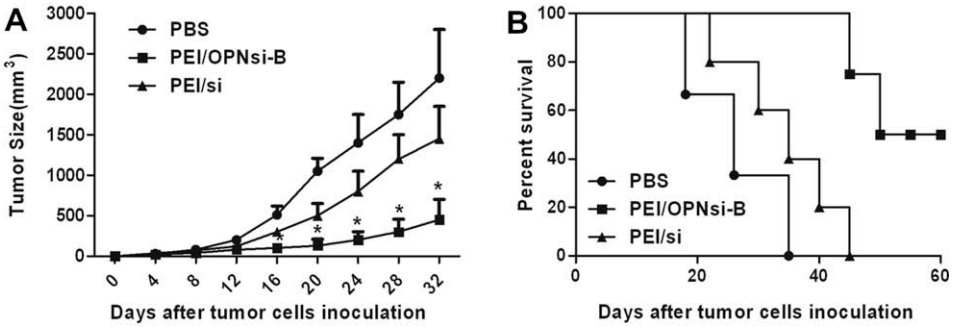

Fig. 6. Effect of OPNsi gene therapy on gastric tumor growth in mice. (A) Mice received a s.c. injection of BGC-823 cells. Three days later, mice were divided into three groups and given intratumoral injections of polyethylenimine/si, polyethylenimine/OPNsi-B2, or no treatment. Tumor size was measured at various time points. $^{*}P < 0.05$, compared with the untreated or polyethylenimine/si group. (B) Mice were euthanized when the tumor reached 2 × 2 cm in diameter. Survival curve was calculated as the percentage of surviving mice on the indicated days.

3.5. Effect of OPNsi gene therapy on gastric tumor growth

We next investigated whether OPN siRNA can be used as a gene therapy. We elected to use polyethylenimine (PEI) as our gene delivery system because of previous successful report with this nonviral vector [27]. BGC-823 cells were injected s.c. into nude mice. Five days later, the palpable tumors were injected with polyethylenimine/OPNsi-B2 or PEI/si control. As shown in Fig. 6A, PEI/OPNsi-B2 gene therapy significantly inhibited tumor growth in mice compared with the PEI/si control. Survival time for the PEI/OPNsi treated mice was also significantly longer (Fig. 6B). PEI/OPNsi-B2-treated tumors grew slowly, with ulceration appearing when the tumor reached ~8 mm in diameter. These results indicate that using polyethylenimine as OPN RNAi delivery system, could efficiency inhibit BGC-823 tumor growth in a nude mouse model.

4. Discussion

Previous studies has been demonstrated that OPN mRNA and protein expression were significantly higher in gastric cancer tissues compared with surrounding non-tumour tissues [3,28]. Here, we showed that gene therapy targeting OPN may have therapeutic benefit in the growth of gastric tumor. This was done by selectively inhibiting OPN expression and protein production using RNA-mediated interference by siRNA. Delivery of siRNA can be achieved through exogenous application of synthetic siRNA or through endogenous expression using plasmid or vector delivery to the target cell. Chemically or enzymatically synthesized siRNA is costly and has been shown to have a relatively short half-life with only transient inhibition of the target gene [29]. To overcome these shortcomings, we constructed several vector-based expression systems in which sense and antisense strands of short OPN sequences were transcribed into hairpin structures under the control of a U6 promoter and then processed into functional siRNAs by double strand-specific RNase called Dicer inside the cells [30]. We designed two different OPNsi plasmids targeting four different OPN sequences in different regions of the OPN mRNA. OPN protein expression was inhibited in the transfected BGC-823 cells as detected by Western-blot.

The role of OPN in tumourigenesis can be explained by the multiple functions of OPN in cells [31]. Several mechanisms have been proposed through studies using cultured cells. First, it is recognised that OPN has adhesive activity because its receptors all mediate cell adhesion. Second, the ability of cells to migrate may be directly tied to their tumourigenicity and OPN promotes the migration of diverse cells, including monocytes, macrophages and tumor cells, along OPN gradients [32]. In addition, OPN-deficient cells are reported to be hypomotile [33]. Third, some experiments suggest that OPN inhibits apoptosis and stimulates survival and growth of cells with inducible OPN [34], or with the addition of OPN to cell culture medium [35], through an interaction with its receptor CD44 [36]. Fourth, several studies have suggested that OPN increases tumor invasiveness by inducing proteinase [13,37]. In the present study, our data in vitro showed that down-regulation of osteopontin efficiently inhibited BGC-823 cell growth, anchorage-independent growth, migration and invasion.

We analyzed these proliferation, migration and invasion makers derived from our OPN downregulated cell line BGC-823 using Western-blot for PCNA, Ki-67, uPA, MMP-2, MMP-9 and VEGF. PCNA plays an essential role in nucleic acid metabolism during DNA replication and repair [38]. It functions as the accessory protein for DNA polymerase d and is required for chromosomal DNA synthesis in S phase [39,40]. Ki-67 antigen is expressed by cells in the G1, G2, S and M phases of the cell cycle and is commonly used as a marker of proliferative activity [41]. Both antigens serve as markers for tumor proliferation and decreased survival in several carcinomas [39,42–45]. Our data indicated that the levels of PCNA and Ki-67 in OPNsi-transfected gastric cancer cells were reduced and consistent with our in vitro proliferation assays. Recent reports suggest that MMP-mediated remodel of the ECM is one of several initiating events allowing cancer cells to invade the surrounding stroma [46,47]. OPN also increases cell invasiveness in human mammary carcinoma through stimulation of uPA [48]. Our data found that MMP-2 and uPA expression were suppressed, and the expression of VEGF and MMP-9 were unchanged between BGC/OPNsi and BGC-823 cells. These data was incompletely consistent with Paul C. Kuo's report in colon adenocarcinoma [24]. Interestingly, the suppressed extent of these protein markers expression including OPN, were closely relevant with the ability of gastric cancer cell migration, invasion and growth in vitro. We postulate that the low growth, migration, and invasion derived from BGC/OPNsi cells resulted from the minority of BGC/OPNsi that retained residual OPN expression either through incomplete penetrance of the siRNA effect or BGC/OPNsi cells that acquired molecular escape mechanisms to counter the expression of OPN-specific siRNA. Our in vitro data support this hypothesis as the degree of silencing shown by Western blotting confirmed that OPN was not completely silenced. The hypothesis of residual expression is also consistent with the results of our functional assays, which show that suppression of OPN expression neither resulted in complete inhibition of cellular migration and invasion nor complete prevention of the gastric tumor growth in vivo. Importantly, Residual OPN expression in BGC/OPNsi cells also accounts for the similar levels of MMP-2 and uPA expression between WT and BGC/OPNsi cells. Together, these data suggest that the majority of BGC/OPNsi-B2 cells have suppressed expression of OPN, MMP-2 and uPA, are motility- and invasion-impaired in vitro and tumor formation was slower in vivo.

Except that we screened possible OPN-downstream targets and mediators, such as uPA, MMP-2, MMP-9 and VEGF, OPN-dependent signaling pathways were further determined in OPNsi-transfected BGC-823 cells. Cumulative evidence in models of breast cancer and melanoma suggest that there are a number of OPN-dependent signaling pathway [22,23]. Philip and Kundu have recently shown that OPN upregulates pro-MMP-2 expression in a NF-κB-dependent fashion during ECM invasion by melanoma cells [26]. Das et al. have confirmed that OPN induction of uPA secretion is NF-κB-IκBa-IKK-mediated and dependent on PI 3′-kinase/Akt activity [22,23]. However, other report demonstrated that OPN-induced AP-1-mediated secretion of uPA through activating c-Src/EGFR/ERK signaling pathways in breast cancer cells [49]. In the present study, our data showed that NF-κB DNA binding activity and PI 3′-kinase/Akt phosphorylation, not ERK activation, were significantly reduced by OPN siRNA in BGC-823 cells. Of course, earlier reports have shown that OPN also induced epidermal growth factor receptor (EGFR) mRNA expression, EGFR tyrosine kinase activity, hepatocyte growth factor receptor (Met) mRNA and protein expression and increases Met kinase activity during OPN-mediated migration in human mammary cancer cells [50,51]. OPN-induced cell migration may also be mediated by signaling through the CD44 receptor [52]. These data clearly suggest that there are a number of OPN-downstream targets and OPN-dependent signaling pathway. Thus, further identifying relevant molecular targets and signaling pathway in OPNsi-transfected BGC-823 cells is currently undergoing in our laboratory.

Our in vitro and in vivo data represent the first report describing mechanistic evidence for OPN as a mediator in growth, migration and invasion of gastric caner using BGC-823 cells, and suggest that OPN is a potential therapeutic target for gastric tumor. Thus, we elected to use polyethylenimine (PEI) as the siRNA vector delivery system to exert gene therapy because the success with polyethylenimine has been reported [27]. PEI is a cationic polymer, which is nontoxic when delivered in vivo. This polymer retains its cationic state at physiologic pH levels, prevents endosomal buffering, and does not elicit a significant immune response. The injection

of PEI/OPNsi into palpable BGC-823 murine tumors resulted in the inhibition of tumor growth, increased animal survival and decreased tumor OPN expression compared with tumor injected with polyethylenimine/si control vector. Moreover, we observed no adverse effects in the mice treated with PEI/OPNsi intratumor injection. The ultimate goal, however, is the therapeutic use of RNAi through systemic application of a specific RNAi-inducing agent in vivo. Although it has been reported that systemic application of PEI-complexed siRNAs efficiency resulted in a remarkable reduction of tumor growth in some models [53], in the present study, systemic i.v. administration of PEI/OPNsi at the same dose, only slightly suppressed gastric tumor growth (data not shown). Cumulative evidence has indicated that the majority of the injected dose of systemic administration was taken up by the reticular endothelial system in the liver and spleen, only 2–5% of the injected dose/g tissue for the tumor [54,55]. Furthermore, since PEIs with higher concentration tend to show toxicity in the lung [53], it is limited to increase the injected dose of PEI/siRNA complex. Therefore, development of a more efficient and tissue-targeted siRNA delivery system for systemic application is undergoing in our lab.

In summary, we have shown that siRNA technology can be used to specifically inhibit OPN expression. Both cell transfection and delivery by polyethylenimine resulted in selective inhibition of OPN expression, leading to decreased tumor growth in vivo. Therefore, targeting OPN with specific small molecule inhibitors may have therapeutic benefit for patients with gastric tumor.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

Acknowledgments

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