Loss of RUNX3 increases osteopontin expression and promotes cell migration in gastric cancer

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Loss of RUNX3 expression is frequently observed in gastric cancer and is highly associated with lymph node metastasis and poor prognosis. However, the underlying molecular mechanisms of gastric cancer remain unknown. In this study, we found that the protein levels of RUNX3 and osteopontin (OPN) are inversely correlated in gastric cancer clinical specimens and cell lines. Furthermore, similar inverse trends between RUNX3 and OPN messenger RNA (mRNA) expression were demonstrated in six out of seven normal-tumor-paired gastric cancer clinical specimens. In addition, low RUNX3 and high OPN expression were associated with poor prognosis in gastric cancer patients. Ectopic expression of green fluorescent protein-RUNX3 reduced OPN protein and mRNA expression in the AGS and SCM-1 gastric cancer cell lines. In contrast, knockdown of RUNX3 in GES-1, a normal gastric epithelial cell line, increased OPN expression. Although three RUNX3-binding sequences have been identified in the OPN promoter region, direct binding of RUNX3 to the specific binding site, −142 to −137bp, was demonstrated by chromatin immunoprecipitation assay. The binding of RUNX3 to the OPN promoter significantly decreased OPN promoter activity. The knockdown of OPN or overexpression of RUNX3 inhibited cell migration in AGS and SCM-1 cells; however, the coexpression of RUNX3 and OPN reversed the RUNX3-reduced migration ability in AGS and SCM-1 cells. In contrast, the knockdown of both RUNX3 and OPN inhibited RUNX3-knockdown-induced migration of GES-1 cells. Together, our data demonstrated that RUNX3 is a transcriptional repressor of OPN and that loss of RUNX3 upregulates OPN, which promotes migration in gastric cancer cells.

Introduction

Gastric cancer is one of the most common cancers in the world. Although the incidence and death rates of gastric cancers for men and women decreased from 1999 to 2008 (1), more than 700,000 gastric cancer deaths worldwide occurred in 2008 (2). Despite the recent advances in diagnosis, chemotherapy and radiation therapy, 65% of gastric cancer patients still suffer with locally advanced or metastatic disease (3). A better understanding of the molecular mechanisms and signaling pathways of metastasis will greatly improve the management of gastric cancer.

The human RUNX3 belongs to the Runx gene family (the runt-related transcription factor) and is involved in a variety of biological activities, including the development of the gastrointestinal tract (4), the neurogenesis of dorsal root ganglia (5) and T-cell differentiation (6). RUNX3 contains a highly conserved ‘runt’ domain, which directly binds to DNA by recognizing the consensus-binding sequences, TGTGGT or ACCACA, and mediates transcriptional activation or repression of target genes (7,8). The loss or reduction of the RUNX3 protein can be detected in >80% of gastric cancers. RUNX3 has been regarded as a tumor suppressor and is involved in tumor progression at different levels, such as tumor initiation (9), epithelial-to-mesenchymal transition (10) and angiogenesis (11). RUNX3 has been shown to suppress Akt transcription, leading to the downregulation of cyclin D1 and the inhibition of cell growth and tumorigenesis of gastric cancer cells (8). In addition to its growth inhibitory function, RUNX3 reduction may also contribute to gastric tumor progression. We recently demonstrated that the loss of RUNX3 expression was highly associated with increased lymph node metastasis, reduced cellular differentiation and inferior survival duration in gastric cancer patients (12). In addition, RUNX3 inhibits epithelial-to-mesenchymal transition, which promotes metastasis, through the regulation of the transforming growth factor-β (TGF-β), Wnt and Notch signaling pathways (10,13). However, the downstream target gene of RUNX3 that mediates metastasis remains unclear.

Osteopontin (OPN) is a secreted multifunctional glyco-phospho-protein that is expressed in various tissues and plays important roles in a wide range of biological processes, such as inflammation, angiogenesis, bone remodeling, cell adhesion and migration (14). OPN binds to various receptors, including integrins and CD44, which potentially allow it to stimulate different signaling pathways and ultimately lead to tumor progression (15). Studies have indicated that OPN induces the PI3K-Akt signaling pathway through the αvβ3 integrin-mediated pathway and promotes cell migration and angiogenesis (16,17). One of the key steps involved in the process of tumorigenesis and metastasis is the degradation of the basement membrane and interstitial matrix. OPN acts as a substrate for matrix metalloproteases, and the cleaved fragments potentially lead to the degradation of the extracellular matrix, which enhances cell adhesion and migration (18). The constitutive expression of OPN has been reported to be involved in the invasion, progression and metastasis in different cancers (19,20). Elevated OPN and its receptor, CD44v9, have been correlated with the degree of lymphatic vessel invasion and lymph node metastasis in gastric cancer (21). However, the molecular mechanisms of OPN regulation in gastric cancer are not well understood.

Hormones, cytokines and growth factors can regulate OPN expression. For example, secoesteroid vitamin D3 and retinoic acid increase OPN expression in bone cells (22,23). The stimulation from platelet-derived growth factor, epidermal growth factor, TGF-β, bone morphogenic proteins (BMPs) and some inflammatory cytokines also elevate OPN expression (23). Several transcription factors have been identified to regulate OPN transcription, including Rous sarcoma oncogene (Src), estrogen receptor, vitamin D receptor, activator protein-1, T-cell factor 4 (Tcf-4), p53 and BRCA1 (24).

Because both RUNX3 reduction and OPN expression are highly associated with the metastasis of gastric cancer, we propose that RUNX3 may regulate OPN expression to influence cell migration. In this study, we found an inverse correlation between RUNX3 and OPN protein and messenger RNA (mRNA) expression in cell lines and clinical specimens of gastric cancer. The overexpression of RUNX3 in AGS gastric cancer cell line inhibited cell migration potentials...
and OPN expression at the protein and mRNA levels. In contrast, knockdown of RUNX3 increased OPN protein and mRNA expression in the normal gastric epithelial cell line,GES-1, and promoted cell migration. Reexpression of OPN reversed RUNX3-inhibited induction of cell migration. Finally, we identified a RUNX3-binding motif within the OPN promoter region. The binding of RUNX3 to the OPN promoter repressed OPN promoter activity, indicating that RUNX3 functions as a transcriptional repressor of OPN. Our study suggested that the loss of RUNX3 could promote OPN expression, which contributes to the metastasis of gastric cancer.

### Materials and methods

#### Cells line and cell culture

The human gastric cancer cell lines (AGS, NCI-N87, AZ521, SCM-1 and NUGC) were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained according to ATCC recommendations. The human gastric cancer cell lines (AGS, NCI-N87, AZ521, SCM-1 and NUGC) were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained according to ATCC recommendations. The SV40-immortalized human normal gastric epithelial cell line, GES-1, was a gift from Dr. X. H. Zhang (Laboratory for Cancer Research, VCU, Richmond, VA) and used in our experiments. The human gastric cancer cell lines (AGS, NCI-N87, AZ521, SCM-1 and NUGC) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. This cell line has a phenotype similar to normal stomach mucosal cells and forms colonies in soft agar or tumors in nude mice. The images were transformed into text files.

#### Clinical specimens

One hundred thirty-four gastric adenocarcinoma samples and corresponding non-cancerous tissues were obtained with informed consent, and the study was conducted under a protocol approved by the Institutional Review Board of National Cheng Kung University Hospital of Taiwan. For quantification PCR (QPCR) analysis, seven gastric adenocarcinoma samples and their corresponding non-cancerous tissues were obtained from the tissue bank in National Cheng Kung University Hospital under a protocol approved by the Institutional Review Board of National Cheng Kung University Hospital of Taiwan. Of 134 patients, 94 had a recorded value for overall survival and a preserved tissue section after immunohistochemistry (IHC) staining. Follow-up of the patients was carried out for up to 85 months. The survival duration was measured from the date of tissue section extraction to the date of death or the last follow-up.

#### Microarray analysis

AGS cells were transfected with green fluorescent protein (GFP) or GFP-RUNX3 for 24 h. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. During the RNA extraction, contaminating genomic DNA was removed by incubating samples with TurboTM DNase following standard procedures suggested by the supplier (Applied Biosystems, Foster City, CA). Protocols and reagents for hybridization, washing and staining followed the Affymetrix protocols. The microarray data sets were then analyzed using Gene-Spring 7.3 software (Silicon Genetics, Redwood City, CA).

#### Generation of recombinant plasmids and promoter assay

Flag-RUNX3 and GFP-RUNX3 were constructed as previously reported (8). The full-length OPN (OPNA isoform) was used in our study because OPNAS was the major isoform expressed in most of the gastric cancer cell lines analyzed (data not shown). For the OPN expression plasmid construction, a 1641 bp fragment of the human OPN gene (NCBI accession number NM_001040058) was generated by PCR amplification using the following primers: forward, AGTACAGCTGAGATGTTAGTTTT; reverse, TAAATTCAGCTGACAGATGCA. The PCR products were cloned into the pRSET vector. The images were transformed into text files containing intensity information using Affymetrix GeneChip Operating software. Microarray data sets were then analyzed using Gene-Spring 7.3.1 software (Silicon Genetics, Redwood City, CA).

#### Quantitative real-time PCR

Real-time PCR amplification of RUNX3, OPN and GAPDH was performed for 20 s at 95°C, followed by 50 cycles at 95°C for 3 s and annealing at 60°C for 30 s using a StepOnePlus Real-Time PCR System (Invitrogen). The results were normalized to those of the housekeeping gene GAPDH and are expressed as a ratio of the percentage of individual genes to the GAPDH control. The specific primer sequences for each gene are as follows: RUNX3 forward, AGGTAGTGGGATCTCCTCAGT; reverse, AGCCGAGATTGTTGGGATTGATTG; and GAPDH forward, ACTTGTACACAGGTTATGATTA; reverse, AGGCCTACTGTGTCGATCA. The PCR product was separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

#### Western blot analysis

Western blots were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. Western blot analysis was performed using anti-GFP (1:2000, sc-9996; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-OPN (1:1000 for OPN (Abcam, Cambridge, MA). The staining intensity of RUNX3 was measured using an Odyssey imaging system (Li-Cor, Inc., Lincoln, NE). The results were normalized to those of the housekeeping gene GAPDH.
anti-Flag M2 (1:5000, F1804; Sigma), anti-β-actin (1:5000, A5441; Sigma), anti-OPN (1:1000, Abcam) and anti-RUNX3 (1:1000, obtained as described previously) (12). The bands were quantified using NIH imaging software (Universal Imaging Corp., Downingtown, PA).

Cell migration assay
The migratory potential of cells was evaluated using transwell inserts with 8 mm pores (Biosciences, La Jolla, CA). After 24 h post-transfection, 1 × 10^5 cells in serum-free medium were added to each upper insert. The bottom chamber contained standard medium supplemented with 10% fetal bovine serum. After 20 h incubation, a cotton-tip swab removed non-migrated cells on the upper surface of the filters gently, and invaded cells on the lower membrane surface were fixed in methanol, stained with Giemsa, photographed and counted. Cell numbers in six randomly selected fields of each independent experiment were counted under a light microscope at ×200 magnification.

Enzyme-linked immunosorbent assay
To examine the amount of secreted OPN in the culture medium, an enzyme-linked immunosorbent assay (ELISA) was performed using a Quantikine ELISA kit for human OPN (R&D Systems, Minneapolis, MN). Briefly, the culture medium was collected from the GES-1 RUNX3-knockdown stable clone 24 h after seeding or from AGS and SCM-1 cell cultures, which were transfected with GFP and GFP-RUNX3 for 24 h. After centrifugation to remove the cell debris, the culture medium was aliquoted and stored at −80°C. The amount of OPN in the culture medium was analyzed according to the manual provided by the manufacturer.

Statistical analysis
Statistical evaluation was performed with SPSS software (version 11.05, SPSS, Chicago, IL). For QPCR, promoter activity assay, migration assay and ELISA data were collected from three independent experiments and presented as the mean ± SEM. The statistical analysis between the two groups was conducted with Student’s unpaired t-test. Correlations between RUNX3 and OPN expressions in paraffin-embedded tissues were determined by Pearson’s correlation coefficient. P < 0.05 was considered statistically significant. The mRNA expression of RUNX3 and OPN between normal and clinical tumor specimens was analyzed using a Wilcoxon signed-rank test.

Results
Loss of RUNX3 is correlated with the upregulation of OPN in gastric cancer cell lines
To study the relationship between RUNX3 and OPN in gastric cancer, we first analyzed the expression of both proteins in the normal gastric epithelial cell line, GES-1, and other various gastric cancer cell lines. Compared with GES-1 cells, the reduction of the RUNX3 protein and upregulation of OPN were observed in all gastric cancer cell lines tested (Figure 1A). The results from the QPCR analysis showed an inverse correlation between RUNX3 and OPN mRNA expression in the GES-1 cells and other gastric cancer cell lines (Figure 1B). To study whether the inverse expression between RUNX3 and OPN reflects the functional regulation of biological processes in gastric cancer cells, a microarray was performed to examine the gene expression profile of the RUNX3-overexpressed AGS cells. Gene Ontology analysis of the downregulated genes (log 2 > 1) in the microarray analysis showed that OPN-modulated events were involved in RUNX3-modulated biological processes (P = 0.012; Figure 1C). This result was confirmed by western blot, which showed that the overexpression of GFP-RUNX3 inhibited OPN protein expression in AGS cells (Figure 1D) and OPN mRNA expression in both AGS and SCM-1 cells (Figure 1E). However, the knockdown of RUNX3 by a lentivirus-based shRNA induced upregulation of OPN at the protein and mRNA levels in GES-1 cells (Figure 1F).

Inverse correlation between RUNX3 and OPN expression in clinical specimens of gastric cancer
Next, we examined the protein expression of RUNX3 and OPN in clinical specimens of gastric cancer patients by IHC analysis. Consistent with its transcriptional regulatory function, RUNX3 was mainly expressed in the nucleus, whereas OPN staining was localized in the cytoplasm (Figure 2A). From the serial sections of paired normal gastric tissue (N) and tumor (T) of gastric cancer patients, RUNX3 was highly expressed in normal gastric tissues but lost in tumors. In contrast, OPN was rarely found in normal gastric tissues but highly expressed in tumors (Figure 2A). To investigate the correlation between RUNX3 and OPN, we enrolled 134 patients with gastric cancer, and the expression of RUNX3 and OPN in the clinical specimens was scored from 0 (no expression) to 3 (high expression) according to their IHC staining intensity. The result from the scatter plot of scoring showed an inverse correlation between RUNX3 and OPN protein expression in the clinical specimens (P = 0.012; Figure 2B). QPCR analysis from seven normal-tumor-paired specimens showed that RUNX3 mRNA expression was downregulated, whereas OPN was upregulated in the tumor tissues compared with the paired normal tissues (Figure 2C). Among the 134 patients, the IHC score of the specimens from 94 patients with clinical follow-up data was analyzed to investigate the prognostic significance of RUNX3 and OPN protein expression. In a Kaplan–Meier log-rank analysis, low expression of RUNX3 (score 0 and score 1) and high expression of OPN (score 2 and score 3) was significantly correlated with poor overall survival of gastric patients (P = 0.01 and 0.04 for RUNX3 and OPN, respectively; Figure 2D). These data confirmed the inverse correlation between RUNX3 and OPN expression in gastric cancer clinical specimens and cell lines.

Binding of RUNX3 suppresses OPN promoter activity
Because the RUNX3 expression levels affected not only protein expression but also the mRNA levels of OPN, we then hypothesized that RUNX3 may transcriptionally inhibit OPN expression. Within the promoter region (~1 to ~2267) of OPN, we found three identical RUNX3-binding site consensus sequences (TGTTGGT and ACCACA), RBS1 (~1971~1986), RBS2 (~1392~1387) and RBS4 (~142~137) (Figure 3A and B). We also found that RBS3* (~662~657) had one-base difference (ACCAGC) compared with the RUNX3-binding site consensus sequence. To investigate which binding sites were required for suppression of OPN expression, we constructed multiple luciferase reporter constructs driven by full-length or truncated OPN promoters. A luciferase reporter assay showed that cotransfection of GFP-RUNX3 and full-length OPN promoter-reporter plasmids significantly reduced luciferase activity, suggesting that RUNX3 functions as a transcriptional repressor of OPN. Among all of the truncated OPN promoter-reporter constructs containing different combinations of RUNX3-binding sites, transfection of GFP-RUNX3 only resulted in the reduction of luciferase activity when it was cotransfected with the RBS2–RBS3*–RBS4 and RBS3*–RBS4 combinations of truncated OPN promoter-reporter plasmids (Figure 3C). These results showed that RBS3* and RBS4 might play a role in RUNX3-mediated OPN repression. To demonstrate the binding of RUNX3 on the OPN promoter, AGS cells were transfected with Flag-tagged RUNX3 and ChIP was performed. The ChIP results showed that Flag-RUNX3 directly bound to RBS4 but not to other consensus RUNX3-binding sequences on the OPN promoter (Figure 3D). To reveal the importance of RBS3* and RBS4 to RUNX3-mediated OPN repression, we constructed RBS3* and RBS4 mutations (from ACCAGC to AAAACG and ACCACA to ATTACA, respectively) in the RBS3*–RBS4-truncated OPN promoter-reporter plasmids. The results from a luciferase reporter assay showed that mutation of RBS3* did not affect GFP-RUNX3-mediated repression of RBS3*–RBS4 OPN promoter activity. In contrast, a RBS4 mutation completely reversed the repressive function of RUNX3 on RBS3*–RBS4 OPN promoter activity (Figure 3E). These results showed that the direct binding of RUNX3 on RBS4 was responsible for the transcriptional repression of OPN in gastric cancer cells. Next, we investigated which domains of RUNX3 are required for OPN repression. For this purpose, AGS cells were cotransfected with a full-length OPN promoter-reporter plasmid and full-length or truncated fragments of RUNX3 plasmids, and a luciferase reporter assay was performed. The results showed that only full-length RUNX3 was capable of repressing OPN promoter activity.
RUNX3 inhibits OPN transcription and cell migration

indicating that all the functional domains of RUNX3 were required for its repressive function on OPN transcription (Figure 3F).

Expression of RUNX3 inhibits the migration of gastric cancer cells through the downregulation of OPN

Our previous studies have shown the negative role of RUNX3 and positive role of OPN on metastasis in gastric cancer (12,26). Thus, it is important to understand whether RUNX3 expression may inhibit cell migration potential through the downregulation of OPN expression. As shown in Figure 4A, the infection of two different clones of lentivirus-carrying OPN shRNAs efficiently decreased OPN protein expression in AGS and SCM-1 cells. A transwell migration assay showed that the knockdown of OPN significantly inhibited cell migration potentials of infected AGS and SCM-1 cells (Figure 4A). Similar to the effect of OPN knockdown, overexpression of RUNX3 also inhibited the migration ability of AGS and SCM-1 cells. Cotransfection of OPN with RUNX3 reversed the inhibitory effect of RUNX3 on cell migration (Figure 4B). However, the knockdown of RUNX3 by shRNA significantly facilitated cell migration of GES-1 cells. Double knockdown of RUNX3 and OPN dramatically blocked RUNX3-knockdown-induced cell migration of GES-1 cells (Figure 4C). It has been shown that secreted OPN binds to its receptor, such as CD44, and regulates the migration of cancer cells. Indeed, knockdown of RUNX3 in GES-1 cells significantly increased the amount of OPN protein in the culture medium. In contrast, the overexpression of RUNX3 in AGS and SCM-1 cells decreased OPN

Fig. 1. Inverse correlation between RUNX3 and OPN in gastric cancer cell lines. (A) RUNX3 and OPN protein levels in normal gastric epithelial cell line, GES-1, and gastric cancer cell lines were analyzed by western blot. (B) RUNX3 and OPN mRNA levels in normal gastric epithelial cell line, GES-1, and gastric cancer cell lines were analyzed by QPCR. The data were presented as fold change of mRNA levels in cancer cell lines compared with mRNA levels in GES-1 cells. (C) Gene Ontology analysis of downregulated genes in GFP-RUNX3-overexpressed AGS cells. (D) AGS cells were transiently transfected with different amounts of GFP-RUNX3 plasmids for 24 h. The expression of GFP-RUNX3 and endogenous OPN was analyzed by western blot. Quantification of band pixels is labeled below each lane and normalized with β-actin, as well as the first lane of vector control. (E) AGS (upper panel) and SCM-1 (lower panel) cells were transiently transfected with GFP-RUNX3 for 24 h. The protein expression of GFP and GFP-RUNX3 was shown by western blot (left panel). The mRNA level of OPN was analyzed by QPCR (right panel). (F) GES-1 cells were infected with lentivirus-carrying RUNX3 shRNA. The protein and mRNA levels of OPN were analyzed by western blot (left panel) and QPCR (right panel), respectively. *P < 0.05 upregulation and #P < 0.05 downregulation compared with GFP-only or scramble control groups.
protein levels in culture medium (Figure 4D). These results suggested that the loss of RUNX3 in gastric cancer cells promotes cell migration through the upregulation of OPN production and secretion.

Discussion

RUNX3 has been considered a tumor suppressor that increases apoptosis and inhibits cell proliferation in gastric cancer. However, the role of RUNX3 in metastasis is largely unknown. In this study, we found that RUNX3 expression was inversely correlated with OPN expression at the mRNA and protein levels in gastric cancer cell lines and clinical specimens. In addition, low RUNX3 expression and high OPN expression were correlated with poor survival of gastric cancer patients. The binding of RUNX3 on the OPN promoter suppressed OPN transcription, suggesting that RUNX3 functions as a transcriptional repressor of OPN.

The transcriptional regulation of OPN is complicated, and multiple pathways are involved in its regulation. The human OPN promoter was cloned in 1994 (27). Several transcription factors that regulate OPN expression have been identified, including activator protein-1, Myc, Oct-1, upstream stimulating factor, v-Src, TGF-β/BMPs/Smad/Hox, Wnt/β-catenin/APC/GSK3β/Tcf-4, Ras/RRF and p53 (28). In our study, we demonstrated that RUNX3 functions as a transcriptional repressor of OPN, binds to the OPN promoter at the specific binding site (−142 to −137 bp) and suppresses OPN transcription. It is worth noting that all three RUNX transcription factors, RUNX1, RUNX2 and RUNX3, recognize the same consensus-binding element. RUNX2 is a vital regulator for osteoblast differentiation (29) and regulates numerous target genes that are essential for bone formation, cell growth, vascular invasion, matrix synthesis and the turnover of bone tissue (30). A previous study showed that RUNX2 binds to the OPN promoter at the binding site, which is identical to the RUNX3-binding site that we identified in the OPN promoter, and activates OPN transcription in mammary epithelial cells (31). These findings suggest the possibility that RUNX2 and RUNX3 may compete for the same binding site on the OPN promoter and induce the opposite regulation of OPN expression. RUNX3 is predominantly expressed in normal gastric mucosa but lost in gastric cancer; however, the expression of RUNX2 protein is very weak in both normal gastric mucosa and gastric cancer (32). Thus, the loss of RUNX3 may play a more critical role in the upregulation of OPN and promotion of metastasis in gastric cancer.

In addition to transcriptional regulation, OPN expression can also be regulated through different signaling pathways, such as TGF-β and Wnt/β-catenin. Recent studies have shown that pharmacological inhibition of the PI3K/Akt signaling pathway induced OPN expression by enhancing surface roughness of osteoblasts (33–35). Our previous study indicated that RUNX3 repressed Akt transcription, leading to the downregulation of β-catenin and cyclin D1 and inhibition of cell proliferation and tumorigenesis of gastric cancer (8). The results from these studies suggest that the upregulation of OPN in gastric cancer may also be mediated through RUNX3-induced Akt inhibition. However, it has been shown that OPN also activates the PI3K/Akt pathway and upregulates HIF-1α via binding to v3 integrins in gastric cancer cells (18). Because OPN is an important molecule that involves the cell growth,
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Fig. 3. RUNX3-mediated transcriptional repression of OPN. (A) Illustration of four predicted RUNX3-binding sites (RBS1–RBS4) in the OPN promoter. RBS3* had one-base difference (ACCACG) compared with consensus RUNX3-binding site (ACCACA). (B) The predicted RUNX3-binding sites presented in the DNA sequence of the OPN promoter (from −2267 to −1). (C) OPN promoter-reporter constructs, which were composed of different combinations of predicted RUNX3-binding sites, were cotransfected with GFP or GFP-RUNX3 into AGS cells. Twenty-four hours after transfection, the luciferase activity was measured. *P < 0.05 compared with individual GFP controls for each experimental group. (D) AGS cells were transfected with Flag-RUNX3. ChIP assay was performed to examine the direct binding of Flag-RUNX3 with individual predicted RUNX3-binding sites. (E) RBS3* and RBS4 were individually mutated in RBS3*–RBS4 OPN promoter-reporter constructs and cotransfected with GFP- or GFP-RUNX3 plasmid in AGS cells. Luciferase promoter assay was performed, and the luciferase activity was measured. *P < 0.05 compared with individual GFP control for each experimental group. (F) Different fragments of GFP-RUNX3 constructs were cotransfected with the full-length OPN reporter construct in AGS cells. Luciferase promoter assay was performed, and the luciferase activity was measured. *P < 0.05 compared with individual GFP control for each experimental group.
differentiation and metastasis of cancer cells, a possible feedback loop of the OPN and Akt signaling pathway may play a fundamental role to fine-tune the levels of OPN in physiological and pathological conditions.

Although the mechanism of RUNX3 in cancer cell migration is largely unknown, it has been shown that the loss of RUNX3 in RUNX3-knockout mice increased the expression of the chemokine receptor CCR7 and promoted migration of dendritic cells to

Fig. 4. RUNX3-mediated repression of OPN inhibits the migration of gastric cancer cells. (A) Stable clones (#1 and #2) after OPN knockdown in AGS (left panel) and SCM-1 (right panel) cells were subjected into a transwell migration assay. Western blot showed the protein expression of OPN for each experimental group (inset). The cell number of migrated cells was quantified and shown as a histogram chart. *P < 0.05 compared with scramble control (S).

(B) AGS (left panel) and SCM-1 (right panel) cells were transiently transfected with Flag, Flag-RUNX3 or Flag-RUNX3 + OPN. Western blot showed the protein expression of RUNX3 and OPN for each experimental group (inset: V, vector; R, Flag-RUNX3-transfected group; O, OPN-transfected group). Representative images were taken at ×200 magnification. The cell number of migrated cells was quantified and shown as a histogram chart. *P < 0.05 downregulation compared with vector-only control. #P < 0.05 upregulation compared with Flag-RUNX3-transfected group.

(C) GES-1 cells were infected with the lentivirus-carrying RUNX3 shRNA, OPN shRNA or both shRNAs. Western blot showed the protein expression of RUNX3 and OPN for each experimental group (inset). Quantification of migrated cells was shown as a histogram chart. #P < 0.05 downregulation compared with RUNX3 shRNA-infected group. Representative images for migration assay were taken at ×200 magnification.

(D) The amount of OPN protein in the culture medium, which was collected from scramble or RUNX3-knockdown GES-1 cell cultures and GFP or GFP-RUNX3-overexpressed AGS and SCM-1 cell cultures, was analyzed by ELISA. *P < 0.05 upregulation compared with scramble control in GES-1 cell cultures. #P < 0.05 downregulation compared with GFP-only control groups in AGS and SCM-1 cell cultures.
lungs draining lymph nodes (36). The reexpression of RUNX3 inhibited migration of glioma cells through the downregulation of MMP2 (37). In our study, overexpression of RUNX3 significantly inhibited OPN production, secretion and cell migration in gastric cancer cells. However, the overexpression of OPN did not completely restore RUNX3-reduced cell migration, suggesting that the inhibitory function of RUNX3 on cell migration may be through multiple molecular mechanisms.

In conclusion, we demonstrated an inverse correlation between RUNX3 and OPN in cell lines, clinical specimens and the prognosis of gastric cancer. We showed that RUNX3 was a transcriptional repressor of OPN through consensus sequence binding from –142 to –137 in the OPN promoter. The loss of RUNX3 in gastric cancer cells promoted OPN-induced cell migration. Although more mechanistic studies are necessary to define the cofactor of RUNX3 in OPN transcriptional regulation, our study provides important evidence that expands the current knowledge of gastric cancer metastasis and suggests that the combination of RUNX3 and OPN may be valuable markers for the prognosis of gastric cancer.

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References

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