RUNX3 Inhibits the Expression of Vascular Endothelial Growth Factor and Reduces the Angiogenesis, Growth, and Metastasis of Human Gastric Cancer

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Abstract

Purpose: Recent studies indicated that RUNX3 exhibits potent antitumor activity. However, the underlying molecular mechanisms of this activity remain unclear. In the present study, we used a gastric cancer model to determine the effect of RUNX3 expression on tumor angiogenesis.

Experimental Design: The effects of increased RUNX3 expression on vascular endothelial growth factor (VEGF) expression in and angiogenic potential of human gastric cancer cells were determined in vitro and in animal models. RUNX3 and VEGF expression was determined in 120 human gastric cancer specimens and their relationship was analyzed.

Results: RUNX3 gene transfer suppressed VEGF expression in human gastric cancer cells. Down-regulation of VEGF expression correlated with a significantly impaired angiogenic potential of human gastric cancer cells. Furthermore, RUNX3 restoration inhibited tumor growth and metastasis in animal models, which was consistent with inhibition of angiogenesis as determined by evaluating VEGF expression and tumor microvessel formation. In gastric cancer specimens, loss or decrease in RUNX3 expression inversely associated with increased VEGF expression and elevated microvessel formation.

Conclusions: Our clinical and experimental data provide a novel molecular mechanism for the antitumor activity of RUNX3 and may help design effective therapy targeting RUNX3 pathway to control gastric cancer growth and metastasis.

Although the incidence of gastric cancer declined in the West from the 1940s to the 1980s, it remains a major public health problem throughout the world (1). In Asia and parts of South America in particular, gastric cancer is the most common epithelial malignancy and leading cause of cancer-related death. Moreover, gastric cancer remains the second most frequently diagnosed malignancy worldwide and cause of 12% of all cancer-related deaths each year (1, 2). Advances in understanding of its biology and behavior. The aggressive nature of human metastatic gastric carcinoma is related to mutations of various oncogenes and tumor suppressor genes (3–7) and abnormalities in several growth factors and their receptors (5). These abnormalities affect the downstream signal transduction pathways involved in the control of cell growth and differentiation. Specifically, they confer a tremendous survival and growth advantage to gastric cancer cells. Previous studies indicated the role of several tumor suppressor genes in gastric cancer development and progression, including the E-cadherin/CDH1 gene, TP53, and p16 (3, 6, 8–11) and, most recently, runt-related (RUNX) genes (12).

The human RUNX genes encode the α-subunit of the runt domain transcription factor PEBP2/CBF (13) and are homologues to the Drosophila genes runt (14) and lozenge (15). The mammalian and Drosophila RUNX genes share an evolutionarily conserved region of 128 amino acids, termed the runt domain, required for DNA binding and heterodimerization with the β-subunit PEBP2/CBF (13). All three runt domain family members, RUNX1, RUNX2, and RUNX3, are master regulators of gene expression in major developmental pathways and play pivotal roles in cell proliferation and differentiation in humans (16–18). Recent studies have also linked alterations of RUNX genes with carcinogenesis (12, 19–24). A drastic loss of RUNX3 expression correlates with poor prognosis of gastric cancer patients (25). Inactivation of RUNX3 seems to be caused by several mechanisms, including promoter hypermethylation (silencing) and protein mislocalization in cancer cells (25, 26). However, the molecular basis for the alteration of this novel putative tumor suppressor and its effect on cancer biology is largely unclear. Moreover, it is completely unknown
whether and, if so, how RUNX3 regulates angiogenesis and growth and metastasis of gastric cancer.

Like with other solid tumors, the growth and metastasis of gastric cancer depend on angiogenesis, which is the formation of new blood vessels from a pre-existing network of capillaries (27). Of the numerous angiogenic factors discovered thus far, vascular endothelial growth factor (VEGF; ref. 28) has been identified as a key mediator of tumor angiogenesis. Elevated expression of VEGF in human tumor biopsy specimens has been reported in cases of various cancers, including gastric cancer (2, 29, 30). In addition, increasing evidence suggests that VEGF expression is regulated by a plethora of external factors (28, 31). Moreover, loss or inactivation of tumor suppressor genes and activation of oncogenes are associated with VEGF overexpression (29–31). Thus, both genetic and epigenetic alterations involve in VEGF expression and regulation (31). It is important to determine whether RUNX3 directly regulates VEGF expression.

In the present study, we examined the relationship between the expression of RUNX3 and VEGF expression and microvessel density (MVD) status in tumor tissue specimens obtained from patients with resected gastric cancer. We found that lost RUNX3 expression directly correlated with increased VEGF expression and tumor angiogenesis. We also found for the first time that restoration of RUNX3 expression dramatically suppressed the angiogenic potential of human gastric cancer cells, which correlated with down-regulation of VEGF expression via promoter repression in vitro, and attenuation of tumorigenicity and abrogation of metastasis in animal models. Our clinical and mechanistic data provide a novel molecular basis for the antitumor activity of RUNX3 and for targeting RUNX3 pathway to control human gastric cancer.

Materials and Methods

Human tissue specimens and patient information. We used human gastric cancer tissue specimens preserved in the Gastric Cancer Tissue Bank at Shanghai Jiaotong University Affiliated First People’s Hospital (Shanghai, China). Primary gastric cancer in these patients was diagnosed and treated at the hospital from 2000 to 2002. None of them underwent preoperative chemotherapy and/or radiation therapy. We selected 120 cases to represent all of the stages and histologic types of malignant gastric cancer. Specifically, the patients consisted of 75 (62.5%) men and 45 (37.5%) women; 64 (53.3%) ages ≤60 years and 56 (46.7%) ages >60 years with mean age of 60.5 years; 67 (55.8%) intestinal type, 39 (32.5%) diffuse type, and 14 (11.7%) mixed type; 17 (14.2%) tumor-node-metastasis stage (TNM) I, 26 (21.7%) TNM stage II, 64 (53.3%) TNM stage III, and 13 (10.8%) TNM stage IV (Table 1). All tissues were obtained with patient consent for the present study and all laboratory work involving in human specimens was approved by the Institutional Review Board of and done at Shanghai First People’s Hospital.

Cell lines and culture conditions. The human gastric cancer cell line NCI-N87 was purchased from the American Type Culture Collection (Manassas, VA), and the SK-GT5 gastric cancer cell line was obtained from Gary K. Schwartz (Memorial Sloan-Kettering Cancer Center, New York, NY). Both cell lines express RUNX3 at relatively low levels (25). The cell lines were maintained in plastic flasks as adherent monolayers in MEM supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, l-glutamine, and a vitamin solution (Flow Laboratories, Rockville, MD). Endothelial cell tube formation and proliferation assays. Tumor cells (3 × 10⁴) were cultured in 1 mL serum-free medium for 24 hours, and the medium was collected and centrifuged to remove any cell debris before its use as a conditioned medium. The tube formation assay was done as described previously (32). Briefly, 250 μL growth factor–reduced Matrigel (Becton Dickinson, Franklin Lakes, NJ) was pipetted into each well of a 24-well plate and polymerized for 30 minutes at 37°C. Human umbilical vascular endothelial cells were harvested and suspended in conditioned medium from either control cells or genetically altered cells cultured for 48 hours in modified Eagle’s medium containing 1% fetal bovine serum. Then, 2 × 10⁴ human umbilical vascular endothelial cells in 300 μL conditioned medium were added to each well and incubated at 37°C in 5% CO₂ for 20 hours. The cultures was photographed with bright-field microscopy using a Sony (Tokyo, Japan) digital camera equipped with an Optimas 6.2 program. To obtain optimal contrast and visual effect, the color scheme of the original photos was inverted so that the white color represents endothelial cells on black background. The degree of tube formation was assessed as the percentage of cell surface area versus total surface area (32). For proliferation assay, 2 × 10⁴ human umbilical vascular endothelial cells in 200 μL conditioned medium were added to 96-well plate and incubated at 37°C in 5% CO₂ for 24 hours and [³H]Tdr was added at 0.1 mCi/mL. [³H]Tdr incorporation was determined 12 hours after the addition of [³H]Tdr as described previously (33).

Restoration of RUNX3 expression. Recombinant adenoviruses harboring RUNX3 (Ad-RUNX3) and enhanced green fluorescent protein (EGFP; Ad-EGFP) were generated with the AdEasy Adenoviral Vector System (Stratagene, La Jolla, CA). The replication-defective adenoviruses were expanded in HEK293 cells and purified by double CaCl₂ gradient centrifugation to achieve a titer of ~10¹⁰ plaque-forming units/mL. For adenoviral transduction, tumor cells were seeded in culture plates. Twelve hours later, the cells were incubated for 2 hours at 37°C in serum-free medium alone or with Ad-RUNX3 or Ad-EGFP at a multiplicity of infection of 20. After washing with serum-free medium, the transduced cells were replated with DMEM and incubated for indicated times before harvesting supernatants or cells for further uses.

VEGF protein measurement. The VEGF protein level in the culture supernatants, mouse serum, and ascites were determined using the Quantikine VEGF ELISA kit (R&D Systems, Minneapolis, MN), which is a quantitative immunometric sandwich enzyme immunoassay. A curve of the absorbance of VEGF versus its concentration in the standard wells was plotted. By comparing the absorbance of the samples with the standard curve, we determined the VEGF concentration in the unknown samples (29).

Western blot analysis. Whole-cell lysates and nuclear extracts were prepared from human gastric cancer cell lines and tissues. Standard Western blotting was done using a rabbit polyclonal antibody against human RUNX3 in a 1:200 dilution (Active Motif, Carlsbad, CA) or FLAG M2 (Sigma-Aldrich, St. Louis, MO) and VEGF (clone A20, Santa Cruz Biotechnology, Santa Cruz, CA) and the anti-rabbit IgG antibody, horseradish peroxidase–linked (Fab’2), fragment obtained from a donkey (Amersham, Arlington Heights, IL). Equal protein sample loading was monitored by probing the same membrane filter with an anti-β-actin antibody (29). The probe proteins were detected using the Amersham enhanced chemiluminescence system according to the manufacturer’s instructions.

VEGF promoter constructs, site-specific mutagenesis, and analysis of VEGF promoter activity. The full-length and its deletion mutant VEGF promoter reporters in pGL3 luciferase constructs were generated as described previously (29). Site-specific mutagenesis of the full-length VEGF promoter was done by using a QuikChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The activity of VEGF promoter constructs was analyzed as described previously (29). In brief, plasmids containing firefly luciferase reporters were cotransfected into tumor cells in triplicate with an internal control pMiniTK-RL using the LipofectAMINE method (Invitrogen, Carlsbad, CA). The pMiniTK-RL contained a full-length Renilla luciferase gene under the control of a minimal TK promoter (636-757 bp from pTK-RL; Invitrogen). In some of the experiments, the reporters
were cotransfected with a RUNX3 expression vector or control vector. The activity of both firefly and Renilla luciferase reporters was determined 24 hours later using the Dual Luciferase Assay kit (Promega, Madison, WI). Specific VEGF promoter activity was calculated as described previously (29).

**Chromatin immunoprecipitation.** SK-GT5 cells were seeded to ~80% confluence in 10-cm culture dishes and transfected with Ad-RUNX3 or Ad-EGFP at a multiplicity of infection of 20. Twenty-four hours later, chromatin immunoprecipitation was done by using the chromatin immunoprecipitation assay kit (Upstate Cell Signaling Solutions, Lake Placid, NY) according to the manufacturer’s instructions. Briefly, DNA cross-linking proteins were cross-linked with DNA and lysed in SDS lysis buffer. The lysate was sonicated to shear DNA to 200 to 500 bp. After preincubation with a salmon sperm, DNA/protein A-agarose slurry for 30 minutes at 4°C, chromatin samples were immunoprecipitated overnight with no antibody, anti-FLAG, or anti-RUNX3 antibody. The VEGF promoter regions flanking putative RUNX3-binding sites were amplified by using respective forward and reverse primers. Routinely, only 10% of chromatin DNA from control samples was used as an input control. The PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light.

**Electrophoretic mobility shift assay.** SK-GT5 cells were seeded to ~80% confluence in 15-cm culture dishes and transduced with Ad-RUNX3 or Ad-EGFP at a multiplicity of infection of 20. Twenty-four hours later, the cells were collected and nuclear extracts were prepared as described previously (29). Electrophoretic mobility shift assay (EMSA) was done by using the Gel Shift Assay System (Promega) according to the manufacturer’s instructions. Briefly, the oligonucleotides as indicated in each experiment were annealed and 5’-end labeled with [α-32P]ATP by using T4 polynucleotide kinase (Amersham Life Sciences, Piscataway, NJ) according to standard procedures. The nuclear extracts (10 μg each) were preincubated in binding buffer for 10 minutes at room temperature; this was followed by the addition of the [α-32P]ATP-labeled double-strand oligonucleotide and a second incubation at room temperature for 25 minutes. For the supershift assay, specific RUNX3 and FLAG antibodies were added and incubated for 15 minutes at room temperature before the addition of a labeled probe. Samples were then loaded on a 4.5% polyacrylamide gel, and electrophoresis was done at 4°C for 4 hours at 200 V. The gel was then dried for 40 minutes at 80°C and exposed to Kodak film (Eastman Kodak, Rochester, NY) at ~70°C.

**Animals.** Female athymic BALB/c nude mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were housed in laminar flow cabinets under specific pathogen-free conditions and used when they were 8 weeks old. The animals were maintained in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care in accordance with the current regulations and standards of the U.S. Department of Agriculture, Department of Health and Human Services, and NIH. All experiments involving use of animals were done at The University of Texas M. D. Anderson Cancer Center (Houston, TX) with approval from its Institutional Animal Care and Use Committee.

**Tumor growth and metastasis.** To prepare tumor cells for inoculation, cells in the exponential growth phase were harvested by brief exposure to a 0.25% trypsin/0.02% EDTA solution (w/v). Cell viability was determined using trypan blue exclusion, and only single-cell suspensions that were >95% viable were used. Tumor cells (1 × 10⁶ per mouse) were then injected into the wall of stomach of nude mice in groups of 10. The animals were killed 60 days after the tumor cell injection or when they had become moribund. Next, the primary gastric tumors were harvested and weighed. In addition, each liver of the mouse was fixed in Bouin’s solution for 24 hours to differentiate the neoplastic lesions from the organ parenchyma; metastases on the surface of the liver were counted (double blinded) with a dissecting microscope.

**Immunohistochemistry of human normal and tumor tissue specimens.** Sections (5-μm thick) of formalin-fixed, paraffin-embedded tumor specimens were prepared and processed as described previously (34). RUNX3 and VEGF protein expression was detected with RUNX3 and VEGF antibodies. A positive reaction was indicated by a reddish-brown precipitate in the nucleus and cytoplasm. Two independent investigators scored the sections without the knowledge of patient outcome (double blinded). An average value of two independent scores was used for scoring.

### Table 1. Patient characteristics and RUNX3 expression

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total, n = 120 (%)</th>
<th>Negative, n = 59 (%)</th>
<th>Weak, n = 42 (%)</th>
<th>Strong, n = 19 (%)</th>
<th>P</th>
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<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Female</td>
<td>45 (37.5)</td>
<td>19 (42.2)</td>
<td>17 (37.8)</td>
<td>9 (20.0)</td>
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<tr>
<td>Male</td>
<td>75 (62.5)</td>
<td>40 (53.3)</td>
<td>25 (33.3)</td>
<td>10 (13.3)</td>
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</tr>
<tr>
<td><strong>Age (y)</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>≥60</td>
<td>64 (53.3)</td>
<td>33 (51.6)</td>
<td>20 (31.3)</td>
<td>11 (17.2)</td>
<td>0.647</td>
</tr>
<tr>
<td>&gt;60</td>
<td>56 (46.7)</td>
<td>26 (46.4)</td>
<td>22 (39.3)</td>
<td>8 (14.3)</td>
<td></td>
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<tr>
<td><strong>Pathological type</strong></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Papillary</td>
<td>17 (14.2)</td>
<td>4 (23.5)</td>
<td>9 (52.9)</td>
<td>4 (23.5)</td>
<td>0.434</td>
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<tr>
<td>Tubular</td>
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<td>24 (50.0)</td>
<td>15 (31.3)</td>
<td>9 (18.8)</td>
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<tr>
<td>Diffuse</td>
<td>28 (23.3)</td>
<td>15 (53.6)</td>
<td>8 (28.6)</td>
<td>5 (17.9)</td>
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<td>Mucinous</td>
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<td>1 (33.3)</td>
<td>2 (66.7)</td>
<td>0 (0)</td>
<td></td>
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<td>Signet ring</td>
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<td>5 (62.5)</td>
<td>3 (37.5)</td>
<td>0 (0)</td>
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<tr>
<td>Mixed</td>
<td>16 (13.3)</td>
<td>10 (62.5)</td>
<td>5 (31.3)</td>
<td>1 (6.3)</td>
<td></td>
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<tr>
<td><strong>Stage</strong></td>
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<tr>
<td>I</td>
<td>17 (14.2)</td>
<td>6 (35.3)</td>
<td>8 (47.1)</td>
<td>3 (17.7)</td>
<td>0.135</td>
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<td>26 (21.7)</td>
<td>8 (30.8)</td>
<td>12 (46.2)</td>
<td>6 (23.1)</td>
<td></td>
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<tr>
<td>III</td>
<td>64 (53.3)</td>
<td>37 (57.8)</td>
<td>17 (26.6)</td>
<td>10 (15.6)</td>
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<tr>
<td>IV</td>
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<td>8 (61.5)</td>
<td>5 (38.5)</td>
<td>0 (0)</td>
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<td><strong>Lauren’s classification</strong></td>
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<td>Intestinal</td>
<td>67 (55.8)</td>
<td>28 (50.8)</td>
<td>26 (31.3)</td>
<td>13 (17.9)</td>
<td>0.084</td>
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<tr>
<td>Diffuse</td>
<td>39 (32.5)</td>
<td>23 (48.7)</td>
<td>11 (35.9)</td>
<td>5 (15.4)</td>
<td></td>
</tr>
<tr>
<td>Mixed</td>
<td>14 (11.7)</td>
<td>8 (42.9)</td>
<td>5 (50.0)</td>
<td>1 (7.1)</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE:** Pearson’s χ² test was done to determine the statistical significance of the relationship of RUNX3 expression with various variables.
was presented in the present study. Depending on the percentage of positive cells and staining intensity, RUNX3 and VEGF staining positivity was classified into three groups: negative, weak, positive, and strong positive (34). For CD34 staining, tissue sections were processed and stained with a 1:100 dilution of a monoclonal goat anti-CD34 antibody (Santa Cruz Biotechnology) and peroxidase-conjugated anti-goat IgG antibody and then counterstained with Mayer's hematoxylin (Biogenex Laboratories, San Ramon, CA). The slides were mounted and examined using a bright-field microscope. For quantification of tumor MVD, vessels in five high-power fields (>200 magnification (>20 objective and ×10 ocular)) were counted by two independent investigators without knowledge of the patient outcome (double blinded), based on the criteria of Weidner et al. (35). An average value of the two scores was presented in the present study. MVD was divided into three groups: low (<50 vessels per five high-power fields), moderate (50-100 vessels), and high (>100 vessels) as described previously (34, 35).

**Immunohistochemistry of human tumor xenograft specimens.** For VEGF and RUNX3 staining, sections (5-μm thick) of formalin-fixed, paraffin-embedded tumor specimens were deparaffinized in xylene and rehydrated in graded alcohol. For CD31 staining, frozen sections (5-μm thick) were fixed with acetone. Endogenous peroxidase was blocked using 3% hydrogen peroxide in PBS for 12 minutes. The sections were incubated for 20 minutes at room temperature with a protein-blocking solution consisting of PBS (pH 7.5) containing 5% normal horse serum and 1% normal goat serum and then incubated overnight at 4°C in anti-CD31, anti-RUNX3, or anti-VEGF antibodies. RUNX3 and VEGF expression and MVD status were assessed as described above (34, 35).

**Statistics.** The two-tailed χ² test was done to determine the significance of the relationship among the covariates. Each experiment was done independently at least twice with similar results; one representative experiment was presented. The significance of the in vitro data was determined using two-tailed Student's t test, whereas significance of the in vivo data was determined using the two-tailed Mann-Whitney U test. P < 0.05 were deemed significant.

**Results**

**Loss of RUNX3 expression correlated with increased gastric cancer angiogenesis.** We first determined the relationships among RUNX3 expression, VEGF expression, and MVD status in the primary tumor tissue of 120 patients. RUNX3 expression was classified as negative, weak, and strong in 59 (49%), 42 (35%), and 19 (16%) patients, respectively. VEGF was strongly expressed in 53 (44%) cases, weak and negative VEGF expressions were observed in 37 (31%) and 30 (25%) cases, respectively. Average vessel counts (MVD) were significantly lower in gastric cancers that had strong RUNX3 or negative VEGF expression than that in gastric cancers that had negative RUNX3 or strong VEGF expression (P < 0.01; Fig. 1A1). Furthermore, we did Pearson's χ² test to determine the significance of their relationships. The level of RUNX3 expression was inversely correlated with VEGF expression (P < 0.01; Fig. 1A2). These findings were also confirmed via analysis of consecutive sections, in which absent RUNX3 expression was consistent with increased VEGF expression and high MVD (Fig. 1B1), whereas strong RUNX3 expression was consistent with decreased VEGF expression and low MVD (Fig. 1B2). The RUNX3 staining was observed in both nuclei and cytoplasm (Fig. 1B, insets). Therefore, we provided clinical evidence that RUNX3 expression inversely associated with VEGF expression and MVD status, suggesting that RUNX3 expression negatively affected angiogenic phenotype of human gastric cancer.

**Restoration of RUNX3 expression suppressed VEGF expression in human gastric cancer cells.** To provide casual evidence for the role of RUNX3 in tumor angiogenesis, human gastric cancer cells were transduced with adenoviral RUNX3. As shown in Fig. 2A1, exogenous RUNX3 was expressed in the tumor cells transduced with Ad-RUNX3 but not in control cells or cells transduced with control Ad-EGFP. A significant suppression of VEGF expression was evident in human gastric cancer cells transduced with Ad-RUNX3 as determined using Western blot analysis (Fig. 2A1 and A2) and ELISA (Fig. 2A3).

To determine whether RUNX3 directly regulates the expression of VEGF at transcriptional level, we transfected the deletion mutants of VEGF promoter into SK-GT5 cells with RUNX3 expression vector or control vector. As shown in Fig. 2B1, there was a progressive loss of responses of VEGF promoters to the suppressive activity by RUNX3 due to the deletion from −2,274 to −411 bp, suggesting that RUNX3 repressed VEGF transcription via its response element(s) located in between −411 and −2,274 bp of VEGF promoter.

Next, we did computer-based sequence analysis of human VEGF promoter, finding that a full-length pV2274 had at least three putative RUNX3-binding sites, whereas its truncated mutant pV411 did not. The loss of RUNX3-binding sites was consistent with the loss of responses of VEGF promoter to the suppressive activity by RUNX3 (Fig. 2B1). Furthermore, we made point mutant VEGF promoter reporters using site-directed mutagenesis. Mutation of putative RUNX3-binding site 1 (pV2274m1) led to slightly increased VEGF promoter activity but significant loss of response to RUNX3-mediated suppression of promoter activity. Similar result was obtained from mutation of RUNX3-binding site 2 (pV2274m2). More significant changes were observed in mutation of RUNX3-binding site 3 (pV2274m3) or mutations of all putative RUNX3-binding sites (pV2274m4: Fig. 2B2). The sequences and positions of three putative RUNX3-binding sites on VEGF promoter were listed in Fig. 3A. Their respective mutant sequences were identical to those of oligonucleotides used for EMSA (Fig. 3A). These data suggested that those putative RUNX3-binding sites negatively regulate VEGF promoter activity.

**Binding of RUNX3 to VEGF promoter in vitro and in vivo.** Moreover, we sought to determine whether RUNX3 binds to the regions of the VEGF promoter in vitro using a chromatin immunoprecipitation assay. Three sets of PCR forward and reverse primers flanking the putative RUNX3-binding sites were shown in Fig. 3A. To determine whether endogenous RUNX3 binds to the VEGF promoter, chromatin fragments from SK-GT5 cells were immunoprecipitated overnight with or without specific anti-RUNX3 antibody. All three sites were found to bind with RUNX3, with an apparently highest affinity in site 3 and lowest in site 1 (Fig. 3B1). To determine whether exogenous RUNX3 binds to the VEGF promoter, chromatin fragments were immunoprecipitated overnight with or without specific anti-FLAG antibody. Compared with EGFP-transduced cells, restored RUNX3 clearly bound to the target sequence of the VEGF promoter (site 3; Fig. 3B2). Similarly, both endogenous and exogenous RUNX3 were recruited to the VEGF promoter as confirmed by using both anti-FLAG and anti-RUNX3 antibodies (Fig. 3B3). Therefore, RUNX3 directly regulates VEGF transcription in a negative manner, suggesting that loss of RUNX3 expression leads to VEGF overexpression.
To further confirm the binding of RUNX3 to VEGF promoter, we did EMSA using a previously published RUNX3-binding oligonucleotide (5’-gcaagctgcatgtcccaaccacagcatcc-3’; ref. 36) as a probe (consensus probe) in the presence or absence of cold oligonucleotides corresponding to putative RUNX3-binding sites on VEGF promoter (either wild-type or mutant oligonucleotides; Fig. 3A). As shown in Fig. 3C1, all wild-type oligonucleotides effectively competed with consensus probe (Fig. 3C1, lanes 2, 4, and 6); whereas their corresponding mutant oligonucleotides did not (Fig. 3C1, lanes 3, 5, and 7). There were three major shifted bands: the lowest band (marked as “NS”) that was not affected by mutation appeared to be nonspecific, whereas the remaining two bands (marked as “X3”) that were affected by mutation appeared to contain RUNX3 protein. We then compared shift patterns using both consensus (Fig. 3C2, lanes 1-4) and VEGF site 3 (Fig. 3C2, lanes 5-7) probes and nuclear protein were extracted from Ad-RUNX3-transduced GT5 cells. Evidently, there was a highly similar shift pattern between consensus probe and VEGF site 3 probe. The putative RUNX3 bands were further shifted (super-shift) by anti-FLAG antibody (Fig. 3C2, lanes 3 and 6) but not by control IgG (Fig. 3C2, lanes 4 and 7). This observation was further confirmed using anti-RUNX3 antibody and VEGF site 3 probe (Fig. 3C3). These results strongly suggested that RUNX3 could effectively bind to putative RUNX3-binding sites of VEGF promoter in vitro.

Restoration of RUNX3 expression reduced angiogenesis in vitro. To further determine the effect of restored RUNX3 expression on angiogenic potential of human gastric cancer cells, the angiogenic potentials of the supernatant of N87 cells, N87 cells transduced with Ad-RUNX3, or with control Ad-EGFP were determined by an endothelial cell tube formation assay. Representative pictures were taken in situ for tube formation in the supernatant of N87 cells, N87 cells transduced with Ad-RUNX3, or with control Ad-EGFP, and N87 cells transduced with Ad-RUNX3 (Fig. 4A). The degree of tube formation was assessed as the percentage of cell surface area versus total surface area (Fig. 4B). Furthermore, endothelial cell proliferation was also determined using [3H]TdR incorporation assay (Fig. 4C). The treatment of Ad-RUNX3 reduced the capacity of N87 cell supernatant to stimulate tube formation and proliferation of endothelial cells compared with those of control cells, suggesting that restoration of RUNX3 expression significantly impaired angiogenic potential of gastric cancer cells in vitro.

![Fig. 1. Inverse association of RUNX3 expression and MVD status.](image-url)
Inhibition of human gastric cancer growth and abrogation of metastasis by RUNX3. To determine the effect of RUNX3 on tumor growth kinetics, N87 and GT5 cells were injected s.c. into nude mice. Control tumor cells and tumor cells transduced with Ad-EGFP grew progressively, whereas RUNX3-transduced tumor cells only produced slow-growing tumors. To increase the biological relevance, N87 and GT5 cells were injected into the stomach wall of mice in groups of 10 (an orthotopic gastric cancer animal model). Control tumor cells and Ad-EGFP-transduced tumor cells produced larger tumors that metastasized to regional lymph nodes and the liver, whereas RUNX3-transduced tumor cells only produced localized small tumors (data not shown). Those findings were consistent with our previous report (25). Furthermore, the reduced tumor growth and metastasis was directly correlated with prolonged animal survival (Fig. 5A). Therefore, enforced RUNX3 expression suppressed human gastric cancer growth and metastasis.

Inhibition of VEGF expression and angiogenesis by RUNX3 in human gastric cancer growing in nude mice. To further confirm the mechanisms by which RUNX3 reduced primary tumor growth and metastasis, we examined the effect of RUNX3 on tumor angiogenesis in vivo. Microvessel formation was identified by immunostaining with anti-CD31 antibody; the number of vessels per high-power field was scored (Fig. 5B). Representative VEGF expression levels and tumor microvessel densities in both RUNX3-transduced and control tumors were shown in Fig. 5C. RUNX3 significantly inhibited VEGF expression and reduced microvessel formation in the primary tumor relative to that in the controls. The results suggest that
the altered tumor growth and metastasis by restored RUNX3 expression was directly correlated with altered VEGF expression and angiogenesis.

Discussion

Although RUNX3 has been implicated as a tumor suppressor in several tumors, including gastric cancer (12, 25, 37, 38), the effect of its alterations on tumor angiogenesis has not been examined. In the present study, we offered both clinical and experimental evidence for the first time that altered expression of RUNX3 directly influenced gastric cancer angiogenesis. Specifically, we found that dramatic loss of RUNX3 protein was associated with VEGF overexpression and increased MVD in human gastric cancer. Restoration of RUNX3 expression significantly inhibited gastric cancer cell growth in vitro and tumorigenicity and metastasis in animal models. This anti-tumor activity directly correlated with transcriptional repression of VEGF expression, a critical angiogenic molecule in human gastric cancer. Therefore, mechanistically, RUNX3 may exert its tumor suppressor activity through two general pathways: antiproliferative and antiangiogenic.

Previous studies have shown that the gastric epithelium of RUNX3+/− knockout mice exhibits a reduced rate of apoptosis and reduced sensitivity to transforming growth factor (TGF)-β1, suggesting that the tumor suppressor activity of RUNX3 operates downstream of the TGF-β signaling pathway (12, 37, 38). Given the potential role of RUNX3 in TGF-β signaling, apparently, the tumor suppressor activity of RUNX3 is realized by inducing cell cycle arrest and/or apoptosis. Indeed, the functions of RUNX proteins seem to be similar to those of TGF-β superfamily of cytokines (12). Presumably, TGF-β and RUNX proteins may cooperatively regulate cell growth and differentiation through multiple mechanisms. The foundation of their functional cooperation has been suggested by several recent studies, which have shown the physical interaction of RUNX proteins with TGF-β receptors (39) and the transcriptional repression of TGF-β target genes by RUNX proteins (40).

Finally, the clinical relevance of RUNX3 in gastric cancer staging and prognosis warrants further exploration. This is particularly important for cancers with altered RUNX3 expression. Given the potential role of RUNX3 in gastric cancer angiogenesis, the development of novel therapeutic strategies targeting RUNX3 and VEGF may provide an opportunity for improving the treatment of gastric cancer.
protein with Smads and p300 (39–42). Because TGF-β generally induces cell cycle arrest at G1–G0 by increasing the expression or activity of specific cyclin-dependent kinase inhibitors (43, 44) and many other potential targets (45–48) and that cyclin D1 is an important positive cell cycle regulator, whereas p27 is an important cell cycle inhibitor (49), whether they were actually involved in the regulation of cell cycle arrest and/or apoptosis by RUNX3 is currently under investigation in our laboratory.

On the other hand, the roles of oncogenes and tumor suppressor genes in tumor angiogenesis have been well investigated, although their molecular bases are largely unclear (27–31). Among several potential mechanisms, influences on the expression of various angiogenic molecules have been shown (27–31). For example, VEGF expression is negatively regulated by tumor suppressor genes p53, p75, and von Hippel-Lindau, which most likely occurs through their formation of complexes with Sp1 and inhibition of its binding to and transcriptional activation of the VEGF promoter (31, 50). Given the prominent role of VEGF in tumor angiogenesis, growth, and metastasis, targeting VEGF function mostly through interfering with VEGF and VEGF receptor interaction and signaling has yielded significant therapeutic benefits in both animal models and cancer patients. Thus, the functional status of those tumor suppressors may directly affect the angiogenic phenotype (31). In the present study, our in vitro and animal experiments have clearly shown that restoration of RUNX3 impaired the angiogenic potential of human gastric cancer cells, which was at least one of the potential mechanisms by which RUNX3 suppresses the growth and metastasis of human gastric cancer. The angiogenic impairment was associated with the inhibition of VEGF expression, although altered expression of other proangiogenic and/or antiangiogenic molecules are also possible. Importantly, we were the first to show that RUNX3 directly suppressed VEGF expression via transcriptional repression. Whether Sp1 signaling also is involved in the suppression of VEGF expression by RUNX3 is highly probable but remains to be defined, considering the facts that Sp1 activity is critical to VEGF expression and most oncogenes and tumor suppressor genes regulate VEGF expression via interaction with Sp1 (31). Therefore, our results not only provide a novel mechanism for the antitumor activity of RUNX3 but also suggest that combining targeting RUNX3 pathway with existing anti-VEGF/VEGF receptor pathway agents may produce more drastic antiangiogenic and antitumor activity.

In summary, the present study was the first to indicate that RUNX3 directly suppresses VEGF gene transcription, which is at least partially responsible for RUNX3-mediated inhibition of human gastric cancer angiogenesis, growth, and metastasis. Therefore, our study provides a novel molecular mechanism for the antitumor activity of RUNX3 and further underscores the importance of RUNX3 in gastric cancer development and progression and better understanding the molecular basis for aberrant RUNX3 signaling pathway may help design effective therapeutic modality to control gastric cancer growth and metastasis.

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RUNX3 Inhibits the Expression of Vascular Endothelial Growth Factor and Reduces the Angiogenesis, Growth, and Metastasis of Human Gastric Cancer

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