Nkx2-8 Downregulation Promotes Angiogenesis and.Activates NF-κB in Esophageal Cancer

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Abstract
Angiogenesis is a major clinical feature of esophageal squamous cell carcinoma (ESCC), an aggressive disease of increasing incidence in developed countries. In ESCCs, the proangiogenic factor VEGF-C is an independent prognostic factor for ESCC, where understanding the mechanisms of VEGF-C upregulation may cue possible therapeutic insights. Here, we report that expression of the transcription factor Nkx2-8 is downregulated in ESCCs where it inversely correlates with progression and VEGF-C upregulation. Patients with ESCCs with lower Nkx2-8 expression exhibited reduced overall survival. Modulating expression of Nkx2-8 up or down inhibited or enhanced, respectively, proangiogenic activity in vitro and in vivo. Mechanistic investigations showed that Nkx2-8 repressed NF-κB activity by restraining nuclear localization of NF-κB p65 via downregulation of AKIP1, a NF-κB p65 binding partner, and also by directly targeting AKIP1 promoters. We confirmed evidence for the importance of the Nkx2-8/AKIP1/NF-κB axis identified in ESCC cell models through an immunohistochemical analysis of a large cohort of human ESCC specimens. Taken together, our results showed that Nkx2-8 functions as a tumor suppressor in ESCCs, the downregulation of which contributes to NF-κB activation and ESCC angiogenesis. Cancer Res; 73(12); 3638–48. © 2013 AACR.

Introduction
Esophageal cancer is one of the most aggressive and lethal gastrointestinal tract malignancies (1, 2). ESCC accounts for more than 90% of esophageal cancer cases in developing countries, and approximately 50% of all new patients with ESCCs are diagnosed in China (3–5). Despite advances in the understanding and treatment of ESCCs over the last several decades, the overall 5-year survival rate for ESCC remains low, at 5% to 40% (6, 7). Angiogenesis is a major clinical feature of aggressive ESCCs and proangiogenic factor VEGF-C has been shown to be independent prognostic factors for ESCCs (8–12). Thus, delineation of the mechanisms that regulate VEGF-C in ESCCs may allow the identification of novel targets for therapeutic intervention.
The transcription factor NF-κB, one of the major regulators of VEGF-C, is constitutively activated in various types of human cancer including ESCCs (13–16). In unstimulated cells, NF-κB p50/p65 is sequestered in the cytoplasm by its inhibitor proteins, IκBs. In response to a variety of stimuli, IκBs can be phosphorylated by IκB kinase (IKK-β), resulting in proteasomal degradation of IκBs and translocation of cytoplasmic NF-κB p50/p65 into the nucleus, thereby activating the transcription of NF-κB target genes (14–16). Recently, A-kinase–interacting protein 1 (AKIP1) was identified as a novel NF-κB p65 binding protein and found to be overexpressed in breast and prostate cancer (17–19). However, the mechanisms by which AKIP1 is upregulated in cancer remain largely unknown.

Human Nkx2-8, a novel NK2-related transcription factor, was originally identified as a developmental regulator of α-fetoprotein (20, 21). Furthermore, Nkx2-8 was reported to be downregulated in bladder, pancreatic, and non–small cell lung cancer, suggesting that Nkx2-8 downregulation might play roles in the development and progression of cancer (22–24). In this study, we show that Nkx2-8 expression by directly binding to the AKIP1 promoter, consequently resulting in an inhibition of NF-κB activity by promoting the nuclear–cytoplasmic translocation of NF-κB p65. These results provide new insights into the mechanisms regulating the activation of NF-κB in ESCCs.

Materials and Methods
Cells
Primary cultures of normal esophageal epithelial cells (NEEC) were established from fresh specimens of the adjacent noncancerous esophageal tissue, which is over 5 cm from the cancerous tissue, according to previous report (25).
esophageal cancer cell lines EC18, Eca109, HKESC1 were kindly provided by Professors S.W. Tsao and G. Srivastava (The University of Hong Kong). ESCC cell lines Kyse30, Kyse140, Kyse180, Kyse410, Kyse510, and Kyse520 were obtained from DSMZ, the German Resource Centre for Biological Material (26). The EC cell lines were grown in the Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% FBS (HyClone). All cell lines were authenticated by short tandem repeat (STR) fingerprinting at Medicine Lab of Forensic Medicine Department of Sun Yat-Sen University (Guangzhou, China).

**Patient information and tissue specimens**

This study was conducted on a total of 324 paraffin-embedded ESCC samples, which were histopathologically and clinically diagnosed at Sun Yat-Sen University Cancer Center from 2001 to 2006. For the use of these clinical materials for research purposes, prior patient consent and approval from the Institutional Research Ethics Committee were obtained. Clinical information on the samples is summarized in Supplementary Table S1. Ten ESCC specimens and the matched adjacent noncancerous esophageal tissues were frozen and stored in liquid nitrogen until further use.

**Vectors, retroviral infection, and transfection**

pMSCV/Nkx2-8–overexpressing human Nkx2-8 was generated by subcloning the PCR-amplified human Nkx2-8 coding sequence into pMSCV vector (Clontech). To silence endogenous Nkx2-8, 2 siRNA oligonucleotides (RNAi#1: GCGCA-GCCTTCTTAGATTTACCC and RNAi#2: GCTTGACCTTATTTGTATAT) were cloned into the pSuper-retro-puro vector to generate pSuper-retro-Nkx2-8-RNAi(s), respectively (24). The different regions of human AKIP1 promoter, including fragments covering nucleotides −1,276 to +119, −806 to +119, −636 to +119, and −1,276 to −783 (relative to the transcription initiation site) were cloned, respectively, into pGL3 luciferase reporter plasmid (Promega). Transfection of siRNA or plasmids was conducted using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instruction. Stable cell lines expressing Nkx2-8 or Nkx2-8 siRNA were selected for 10 days with 0.5 μg/mL puromycin 48 hours after infection.

**Western blotting assay**

Western blot was conducted as previously described (24), using anti-Nkx2-8 antibody (AvivaSysBio), anti-NF-κB p65 and anti-p84 antibodies (Cell Signaling), and anti-phospho-NF-κB p65 (Ser276) and anti-C11orf17 antibodies (Abcam). Blotting membranes were stripped and re-probed with anti-α-tubulin antibody (Sigma) as a loading control.

**Immunohistochemistry**

Immunohistochemical (IHC) analysis was conducted to study altered protein expression in 324 human paraffin-embedded ESCC samples. The procedure was carried out similarly to previously described methods (24). The degree of immunostaining of formalin-fixed, paraffin-embedded sections was reviewed and scored independently by 2 observers, based on both the proportion of positively stained tumor cells and the intensity of staining.

**Chicken chorioallantoic membrane assay**

Chicken chorioallantoic membrane (CAM) assay was conducted at the eighth day of development of fertilized chicken eggs according to a method described previously (27). A 1-cm diameter window was opened in the shell of each egg with 8-day-old chicken embryo (Yueqin Breeding Co. Ltd). The surface of the dermic sheet on the floor of the air sac was removed to expose the CAM. A 0.5-cm diameter filter paper was first placed on top the CAM, and 100 μL conditioned media harvested from ESCC cells were added onto the center of the paper. Then, the eggs were incubated at 37°C under 80% to 90% relative humidity for 48 hours. Following fixation with stationary solution (methanol: acetone = 1:1) for 15 minutes, CAMs were cut and harvested, and gross photos of CAMs were taken under a digital camera (Panasonic). The effect of conditioned media harvested from different cells was evaluated by the number of second- and third-order vessels in comparison with that treated with the media harvested from control group.

**Electrophoretic mobility shift assay**

Electrophoretic mobility shift assay (EMSA) was conducted by using the LightShift Chemiluminescent EMSA Kit from Pierce Biotechnology according to the manufacturer’s standard protocol. EMSA DNA probes: NF-κB: sense, 5′-AGTT-GAGGGGACTTTCCCAGGC-3′, antisense, 5′-GCCTGGGAAA-GTCCCCTCAAC-3′; OCT-1: sense, 5′-TGTCGAATGGCAAAT- CACTAGAA-3′, antisense, 5′-TCTTAGTGGATTCATTCGA- CA-3′.

**Human umbilical vein endothelial cells tube formation assay**

Matrigel (200 μL) was pipetted into each well of a 24-well plate and polymerized for 30 minutes at 37°C. Human umbilical vein endothelial cells (HUVEC; 2 × 10^4) in 200 μL of conditioned medium were added to each well and incubated at 37°C in 5% CO₂ for 20 hours. Pictures were taken under a ×100 bright-field microscope and the capillary tubes were quantified by counting length.

**Chromatin immunoprecipitation**

Cells (2 × 10^6) in a 100-mm culture dish were treated with 1% formaldehyde to cross-link proteins to DNA. The cell lysates were sonicated to shear DNA to sizes of 300 to 1,000 bp. Equal aliquots of chromatin supernatants were incubated with 1 μg of anti-Nkx2-8 antibody (Abcam) or an anti-IgG antibody (Millipore) overnight at 4°C with rotation. After reverse cross-link of protein/DNA complexes to free DNA, PCR was carried out.

**Immunofluorescent staining**

Cells were treated with TNF-α (10 ng/mL) for the different time and were fixed in 4% (v/v) paraformaldehyde, permeabilized with 0.2% (v/v) Triton X-100 in PBS, and stained with a 1:100 dilution of rabbit anti-p65 polyclonal antibody.
Cell Signaling. After washing, the primary antibody was visualized with rhodamine-conjugated goat anti-rabbit IgG and nuclei were visualized with 4',6-diamidino-2-phenylindole (DAPI). Foci were observed using a laser scanning microscope (Axioskop 2 plus, Carl Zeiss Co. Ltd.).

Luciferase assay

Twenty thousand cells were seeded in triplicate in 48-well plates and allowed to settle for 24 hours. One hundred nanograms of NF-κB luciferase reporter plasmid, or luciferase reporter plasmids containing different fragments of AKIP1 promoter, or the control-luciferase plasmid, plus 1ng of pRL-TK Renilla plasmid (Promega), was transfected into cells using the Lipofectamine 2000 reagent according to the manufacturer's recommendation. Luciferase and Renilla signals were measured 24 hours after transfection using the Dual Luciferase Reporter Assay Kit (Promega) according to a protocol provided by the manufacturer.

Xenografted tumor model, IHC, and hematoxylin and eosin staining

BALB/c-nu mice (4–5 weeks of age, 18–20 g) were purchased from the Center of Experimental Animal of Guangzhou University of Chinese Medicine. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University. The BALB/c nude mice were randomly divided into 2 groups (n = 6/group). One group of mice was inoculated subcutaneously with Kyse30/vector cells (5 × 10^6) in the left dorsal flank and with Kyse30/Nkx2-8 cells (5 × 10^6) in the right dorsal flank per mouse. Another group was inoculated subcutaneously with Kyse30/scrambled vector cells (5 × 10^6) in the left dorsal flank and with Kyse30/Nkx2-8 RNA interference cells (5 × 10^6) in the right dorsal flank. Tumors were examined twice weekly; length and width measurements were obtained with calipers and tumor volumes were calculated using the equation (L × W^2)/2. On day 40, tumors were detected by an IVIS imaging system, and animals were euthanized, tumors were excised, weighed, and paraffin-embedded. Serial 6.0-μm sections were cut and subjected to IHC staining using an anti-CD31 antibody (Dako) or H&E-stained with Mayer’s hematoxylin solution. The images were captured using the AxioVision Rel.4.6 Computerized Image Analysis System (Carl Zeiss). The microvessel density (MVD) was quantified by counting the proportion of CD31-positive cells.
Statistical analysis
Statistical tests for data analysis included Fisher exact test, log-rank test, χ², and Student two-tailed t test. Multivariate statistical analysis was conducted using a Cox regression model. Statistical analyses were conducted using the SPSS 11.0 statistical software package. Data represent mean ± SD. *P < 0.05 was considered statistically significant.

Results
Nkx2-8 downregulation correlates with progression and poor prognosis in ESCCs
Western blotting revealed that Nkx2-8 protein was differentially reduced in all 9 tested ESCC cell lines compared with 4 primary cultured NEECs and in 10 primary human ESCC tissues compared to the adjacent non-tumor tissues, indicating that Nkx2-8 is downregulated in ESCC (Fig. 1A and B). Furthermore, IHC analysis showed that Nkx2-8 levels were inversely correlated with the clinical stage (P < 0.001), TNM classification (P < 0.001, P = 0.030, and P = 0.003, respectively), and histologic differentiation (P = 0.027) in patients with ESCCs (n = 324; Fig. 1C and Supplementary Table S2). Importantly, patients with lower Nkx2-8 expression had shorter survival (P < 0.001; Fig. 1D). Moreover, statistical analyses revealed that Nkx2-8 expression and N classification were each recognized as independent prognostic factors in ESCCs (Supplementary Table S3). Collectively, our results suggest a possible link between Nkx2-8 downregulation and ESCC progression.

Downregulation of Nkx2-8 promotes ESCC angiogenesis in vivo and in vitro
The biologic role of Nkx2-8 in ESCC progression was examined using an in vivo tumor model. As shown in Supplementary Fig. S1 and Fig. 2A and C, the Nkx2-8–overexpressing tumors were smaller, in both size and weight, than the control tumors. Conversely, the tumors formed by Nkx2-8–silenced cells were larger and had higher tumor weights than the tumors formed by control cells (Fig. 2A and C and Supplementary Fig. S2A–2C). Strikingly, the MVD in Nkx2-8–silenced tumors was drastically higher, accompanied by increased CD31-positive cells, whereas the MVD was decreased in Nkx2-8–overexpressing tumors (Supplementary Fig. S2D and Fig. 2D), suggesting that Nkx2-8 downregulation promotes angiogenesis in ESCC.

In agreement with the in vivo assay, we observed that silencing Nkx2-8 provoked, whereas overexpressing Nkx2-8 strongly inhibited, the abilities of both Kyse30 and Kyse510 ESCC cells to induce tube formation and migration of HUVECs.
and the formation of second- and third-order vessels in the CAM assay (Fig. 3A and C).

Multiple studies have reported that expression of VEGF-C, a vital proangiogenic factor, correlates strongly with ESCC progression and acts as an independent prognostic factor for ESCCs (9–11). As shown in Fig. 3D, ELISA assay showed that overexpressing Nkx2-8 significantly decreased, whereas silencing Nkx2-8 increased, the expression of VEGF-C, suggesting that Nkx2-8–mediated angiogenesis might occur via regulation of VEGF-C.

Silencing Nkx2-8 activates NF-κB signaling

As NF-κB is a key transcriptional regulator of VEGF-C (15), we then investigated whether Nkx2-8 is involved in regulation of the NF-κB pathway. As shown in Fig. 4A and C, overexpression of Nkx2-8 decreased, whereas downregulation of Nkx2-8 increased, the NF-κB luciferase activity, NF-κB DNA binding activity, and the expression of numerous well-known NF-κB downstream genes, including VEGF-C, suggesting that Nkx2-8 plays a role in the regulation of NF-κB transcriptional activity. Consistently, we found that the expression of VEGF-C mRNA and NF-κB DNA-binding ability were also dramatically reduced in Nkx2-8–overexpressing tumors and increased in Nkx2-8–silenced tumors, compared with the respective control tumors (Supplementary Figs. S2E and S3). Importantly, the expression of nuclear NF-κB p65 was dramatically decreased in Nkx2-8–transduced cells and increased in Nkx2-8–silenced cells (Fig. 4D and E), suggesting that Nkx2-8 regulates NF-κB transcriptional activity by promoting NF-κB nuclear–cytoplasmic translocation.

Figure 3. Silencing Nkx2-8 enhances the ability of ESCC cells to induce angiogenesis in vitro. A, representative images (left) and quantification (right) of HUVECs cultured on Matrigel-coated plates with conditioned media from the indicated ESCC cells. B, cell migration assay was conducted by culturing HUVECs with conditioned media derived from the indicated ESCC cells. C, representative images of the blood vessels formed in the CAM assay after stimulation with conditioned media from the indicated cells. D, ELISA of VEGF-C expression in Nkx2-8–transduced and –silenced ESCC cells. Each bar represents the mean ± SD of 3 independent experiments. *P < 0.05.
Nkx2-8 directly downregulates AKIP1

It has been reported that AKIP1, a newly identified binding partner of NF-κB p65, enhances NF-κB–dependent gene expression via retention of nuclear NF-κB and induction of phosphorylated-p65 (p-p65) (S276; refs. 18, 19). Interestingly, analysis of the AKIP1 promoter region predicted the presence of 2 Nkx2-8–specific binding sites (NBS), suggesting that Nkx2-8 might regulate AKIP1. Indeed, the expression of AKIP1, at both protein and mRNA levels, and p-p65 (S276) were increased in Nkx2-8–silenced cells but decreased in Nkx2-8–transduced cells (Fig. 5A and B). Furthermore, we found that overexpressing Nkx2-8 decreased, but silencing Nkx2-8 increased, the luciferase activity driven by the first NBS of the AKIP1 promoter. However, neither overexpression nor knockdown of Nkx2-8 had any effect on the luciferase activities of the AKIP1 promoters that contained deleted or mutated first NBS (Fig. 5C). Moreover, ChIP assay revealed that endogenous Nkx2-8 protein bound to the first NBS in the AKIP1 promoter (Fig. 5D), indicating that Nkx2-8 regulates AKIP1 by directly targeting the AKIP1 promoter.

AKIP1 abrogates the inhibitory effects of Nkx2-8 on NF-κB activity and angiogenesis

Overexpression of AKIP1 significantly enhanced NF-κB luciferase activity in Nkx2-8–transduced cells, whereas downregulation of AKIP1 decreased the NF-κB luciferase activity in Nkx2-8–silenced cells (Fig. 6A). However, the Nkx2-8-induced nuclear–cytoplasmic translocation of NF-κB p65 and the inhibitory effect of Nkx2-8 on HUVEC tube formation were dramatically abrogated by AKIP1 overexpression (Fig. 6B and D). Importantly, the MVD of tumors formed by Kyse30/Nkx2-8/AKIP1 cells was significantly higher than that of tumors formed by Kyse30/Nkx2-8/vector cells (Fig. 6E). Taken together, these results indicate that AKIP1 abrogates the inhibitory effect of Nkx2-8 on ESCC angiogenesis.
Clinical relevance of Nkx2-8–induced NF-κB activation in human ESCC

Analysis of 324 ESCC tissue specimens using IHC analysis showed that Nkx2-8 expression was inversely correlated with the expression levels of AKIP1, p-p65 (Ser276) and CD31 (P < 0.001, P < 0.001, and P = 0.002, respectively; Fig. 7A and B). Consistently, Nkx2-8 expression in 10 freshly collected clinical ESCC samples also negatively correlated with the mRNA levels of AKIP1 and VEGF-C (P = 0.004; P = 0.013), as well as the NF-κB DNA–binding activity (r = –0.753, P = 0.007; Fig. 7C). These data further supported the notion that Nkx2-8 downregulation in ESCCs induces AKIP1 expression and activates NF-κB signaling, ultimately leading to angiogenesis and poor clinical outcomes for human ESCCs (Fig. 7D).
Figure 6. AKIP1 abrogates the inhibitory effects of Nkx2-8 on NF-κB activity and angiogenesis. A, top, NF-κB luciferase reporter activities were analyzed in Nkx2-8-transduced ESCC cells transfected with vector or AKIP1 and in Nkx2-8-silenced ESCC cells transfected with scramble or AKIP1 siRNAs. Bottom, Western blotting of AKIP1 expression in the indicated cells. α-Tubulin was used as a loading control. B, Western blotting of nuclear NF-κB p65 and total NF-κB p65 expression in the indicated cells. p84 was used as nuclear loading control. C, immunofluorescent staining of NF-κB p65 in the indicated cells treated with TNF-α (10 ng/mL) for different times. D, quantification of HUVEC tube formation cultured on Matrigel-coated plates with conditioned media from the indicated cells. E, overexpressing AKIP1 abrogated the inhibitory effect of Nkx2-8 on angiogenesis as shown by a xenograft model. Left, representative images of the tumor-bearing mice. Middle, tumor volumes were measured on the indicated days. Left, the MVD was evaluated by CD31-positive cells. Error bars represent mean ± SD of 3 independent experiments. *, P < 0.05.
Compelling evidence has indicated that activated NF-κB is associated with the initiation and progression of ESCCs (28–35). Activation of NF-κB has been reported to be associated with acid-induced esophageal epithelial cell transformation (28). Multiple downstream targets of NF-κB, such as COX-2 and interleukin-8, and NF-κB nuclear expression/NF-κB activity, were shown to be significantly increased in ESCC tissues, compared with normal esophageal tissues (29–31). Importantly, ESCCs with high activated NF-κB display aggressive pathologic features and have poor treatment outcomes (32). However, blockade of the NF-κB pathway not only inhibits ESCC progression but also reduces tumor growth and metastasis, indicating its potential as a therapeutic target for ESCC treatment.

Figure 7. Clinical relevance of the Nkx2-8/AKIP1/NF-κB axis in human ESCCs. A, Nkx2-8 levels were negatively associated with AKIP1, p-p65 (Ser276), or CD31 expression in 324 primary human ESCC specimens. Two representative cases are shown. Original magnification, ×200. B, percentage of ESCC specimens showing low or high Nkx2-8 expression relative to the level of AKIP1, p-p65 (Ser276), or CD31. C, expression analysis (left) and correlation (right) between Nkx2-8 expression and AKIP1 or VEGF-C mRNA expression and NF-κB DNA-binding activity in 10 freshly collected human ESCC samples. Each bar represents the mean ± SD of 3 independent experiments. *, P < 0.05. D, model: Nkx2-8 downregulation induces AKIP1 expression and activates the NF-κB signaling pathway, ultimately leading to angiogenesis and poorer clinical outcomes for human ESCCs.
methylation of the CpG islands in the promoter region of regulation of Nkx2-8 in pancreatic cancer is caused by hypermethylation of the Nkx2-8 promoter restores the expression of Nkx2-8, indicating that downregulation in cancer. Harris and colleagues reported that the histone methyltransferase SMYD3 could upregulate Nkx2-8 by binding to its promoter region to induce K4-dimethylation of histone H3 (39). We also observed that 4 of 10 cases of ESCC tissues expressed higher levels of Nkx2-8 mRNA but lower levels of Nkx2-8 protein, compared with the matched adjacent nontumor tissues (Supplementary Fig. S4). These results suggest that downregulation of Nkx2-8 protein in some ESCC tumors may be mediated via other unknown posttranscriptional mechanisms such as translational repression or ubiquitin-dependent protein degradation. In this respect, further investigation of the mechanisms by which Nkx2-8 protein is reduced in ESCCs will provide valuable data toward a better understanding of the development and progression of ESCCs, which might eventually lead to the development of a new therapeutic strategy for the treatment of ESCCs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: L. Song, J. Li Development of methodology: H. Gong, J. Wu Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Lin, A. Liu Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): C. Lin, H. Gong, A. Liu, X. Lin, J. Wu, M. Li Writing, review, and/or revision of the manuscript: L. Song, J. Li Study supervision: L. Song, M. Li, J. Li

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