The transcription factor RUNX3, which mediates apoptosis and cell growth inhibition in gastric epithelial cells, is a candidate tumor suppressor that is frequently lost in gastric cancer cells. Here, we found that restoration of RUNX3 expression in the cell line not expressing RUNX3 induced apoptosis and that it physically interacted with the Forkhead transcription factor FoxO3a/FKHRL1, known to be an important regulator of apoptosis and the cell cycle. Active unphosphorylated FoxO3a/FKHRL1 was expressed in the gastric cancer cell lines. RUNX3-induced apoptosis depended on the expression of Bim, a proapoptotic BH3-only protein, and both RUNX3 and FoxO3a/FKHRL1 were required for induction of Bim expression. Furthermore, we showed that interaction of RUNX3 and FoxO3a/FKHRL1 was also indispensable for Bim expression and apoptosis in mouse embryonic fibroblasts. In the Bim promoter, RUNX3 bound to two conserved RUNX-binding elements (RBE1 and RBE2), with RBE1 being immediately downstream of a promoter, RUNX3 bound to two conserved RUNX-binding elements

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Runx transcription factors are α subunits of the polyomavirus enhancer–binding protein 2 (PEBP2) core-binding factor (CBF), which consists of α and β subunits. They have a highly conserved Runt domain responsible for DNA binding and heterodimer formation with the β subunit (PEBP2/β/CBFβ) (1). Three Runx transcription factors, Runx1, Runx2, and Runx3, have been identified in mammals. Although all these α subunits have closely related structures and biochemical properties and bind the same DNA-binding motifs (1), they possess distinct biological functions in vivo. Runx1 is essential for definitive hematopoiesis, Runx2 plays critical roles in osteoblast maturation and osteogenesis, and Runx3 is ubiquitously expressed and involved in a variety of biological activities including development of gastrointestinal tract, neurogenesis, and lineage specification of thymocytes (1). Abnormalities in human Runx (RUNX) genes have been linked with some diseases (1, 2). RUNX1/AML1 is an important translocation breakpoint in acute leukemias and heterozygous loss of RUNX2 causes cleidocranial dysplasia syndrome. RUNX3 is implicated as a tumor suppressor gene in gastric cancer.

RUNX3-deficient mice exhibit hyperplasias in gastric mucosa due to reduced apoptosis and stimulated proliferation of gastric epithelial cells. Gastric epithelial cells are less sensitive to the proapoptotic and growth inhibitory effects of transforming growth factor-β (TGF-β) (3). Furthermore, RUNX3 is inactivated in 40% of early stage and in nearly 90% of advanced stage gastric carcinomas by hemizygous deletion and hypermethylation of its promoter (3). Among the three Runx transcription factors, Runx3 is predominantly induced by TGF-β to bind and activate the germ line Ig α promoter, thereby directing Ig class switching to Igα in splenic B cells and the surface IgM+B cell line I.29 (4). RUNX3 interacts physically with Smad transcription factors (Smads), which are signal transducers of TGF-β, and induces TGF-β-dependent transcription of the germ line Ig α promoter in a cooperative manner (5, 6). Although these findings suggest that RUNX3 cooperates with its putative cofactor to exert its role as a tumor suppressor by activating proapoptotic genes, little is known about the molecular mechanisms whereby RUNX3 induces apoptosis.

Members of the FoxO subfamily of Forkhead transcription factors represent the mammalian orthologs of DAF-16, which regulates longevity in the nematode Caenorhabditis elegans. In mammals, FoxO subfamily members regulate diverse cell functions, such as apoptosis, cell cycle progression, and DNA repair (7). The serine/threonine kinase Akt/PKB (protein kinase B) regulates the activities of three FoxO proteins, FoxO1/FKHR, FoxO3a/FKHRL1, and FoxO4/AFX (8). Akt/PKB rapidly phosphorylates FoxO proteins following its activation by phosphoinositide 3-kinase (PI3K). In turn, the phosphorylated FoxO proteins associate with 14–3–3 protein, which functions as a scaffold within the cytoplasm, and are sequestered within the cytosol, rendering them unable to bind to the promoters of their target genes in the nucleus to regulate their transcription. Recently, IκB kinase has also been shown to phosphorylate and inactivate FoxO3a in breast cancer cells (9). In contrast, unphosphorylated FoxO proteins are active forms and are located in the nucleus where they bind to the promoters of their target genes.

FoxO3a has been shown to mediate apoptosis by activating proapoptotic genes in a variety of cells. Overexpression of constitutively active FoxO3a induces apoptosis by activating the Fas ligand (FasL) expression and the Fas-FasL apoptotic pathway in the human leukemia T cell line Jurkat (8). FoxO3a is responsible for Bim-mediated apoptosis in the interleukin (IL)-3-dependent pre-B cell line Ba/F3 and the IL-2–dependent T cell line CTLL-2 following the withdrawal of IL-3 and IL-2 from their cultures, respectively (10, 11). FoxO3a
also contributes to the nerve growth factor (NGF) deprivation-induced apoptosis of developing sympathetic neurons by activating Bim (12). The anti-cancer drug paclitaxel also induces Bim-dependent apoptosis by activating FoxO3a in MCF-7 breast cancer cells (13). Moreover, FoxO3a increases expression of TRAIL (tumor necrosis factor-related apoptosis inducing ligand), which is a pro-apoptotic member of the TNF family, and induces apoptosis downstream of the PTEN tumor suppressor in prostate cancer (14). Thus, FoxO3a has generally been considered an inducer of apoptosis. However, expression of the constitutively active FoxO3a protects pheochromocytoma PC12 and the colon carcinoma DLD-1 cells from apoptosis induced by oxidative stress (15, 16), as well as neutrophils from apoptosis induced by the Fas-FasL signaling in inflammation (17). The biological consequences of FoxO3a depend on cell type and stimuli surrounding cells. The transcriptional activity of FoxO3a is modulated through interaction with its cofactors.

Here, we address the mechanism through which RUNX3 exerts its tumor suppressor activity by inducing apoptosis in gastric cancer cells. RUNX3 is shown to physically interact with FoxO3a expressed in gastric cancer cell lines to activate Bim and induce apoptosis. The same interaction is also demonstrated in mouse embryonic fibroblasts, suggesting that Runx3 is involved in Bim-dependent apoptosis, which is transcriptionally regulated by FoxO3a in a variety of cell types.
EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Reagents—COS7, AGS, AGS-derived transfectant clone, and NIH-3T3 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum. MKN28, MKN45, KATO III, and SNU16 cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum. Primary mouse embryonic fibroblasts (MEFs) were prepared from embryos produced from intercrosses of Runx3+/− mice (3) at 14.5 days post-coitus and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Transfection of cDNA was performed using FuGENE 6 (Roche Applied Science) or Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s instructions. Human recombinant TGF-β1 was purchased from R&D Systems (Minneapolis, MN).

Plasmid Construction—A FLAG epitope was introduced by PCR into the N terminus of RUNX3 cDNA obtained from pEFBOS-T7-RUNX3 (18), and the resulting cDNA was fully sequenced. The cDNA was further subcloned into pcDNA3.1 (+) and pcDNA3.1(-) vectors (Invitrogen). The FoxO3a cDNA obtained from FOXO3a-Myc (9) was subcloned into pcDNA3.1 (+). Mutagenesis of the Bim promoter (19) was performed using a QuikChange multi-site-directed mutagenesis kit (Stratagene, La Jolla, CA) and the mutations confirmed by sequencing the DNA.

Ecdysone-inducible Transient Expression of FLAG-RUNX3—Cells were cotransfected with pVgRXR (Invitrogen) and recombinant pIND(SP1)/Hygro vectors (Invitrogen). The FoxO3a cDNA obtained from FOXO3a-Myc (9) was subcloned into pcDNA3.1 (+). Mutagenesis of the Bim promoter (19) was performed using a QuikChange multi-site-directed mutagenesis kit (Stratagene, La Jolla, CA) and the mutations confirmed by sequencing the DNA.

Apoptosis Detection—Apoptosis was quantified using an annexin V-fluorescein isothiocyanate apoptosis detection kit II (BD Biosciences) according to the manufacturer’s instructions. Briefly, cells were trypsinized, washed, stained with fluorescein isothiocyanate-conjugated annexin V and propidium iodide, and then analyzed by flow cytometry using a FACS vantage (BD Biosciences). The data were analyzed using the Cell Quest software (BD Biosciences). Apoptosis was also examined using cell death detection ELISA PLUS assays (Roche Applied Science), which measure the presence of cytoplasmic histone-associated mono- and oligonucleosomes as a result of apoptosis, in accord with the manufacturer’s instructions.

Semi-quantitative RT-PCR Analysis and Real-time Quantitative RT-PCR Analysis—Total RNA was isolated using an RNasy mini kit (Qiagen, Hilden, Germany) and treated with DNase I using an RNase-free DNase set (Qiagen). Equal amounts of total RNA were reverse-transcribed using an Omniscript reverse transcriptase kit (Qiagen). The first-strand cDNA was used as a template. The primers used for semi-quantitative RT-PCR analyses of human Fasl (5’-CTCTGGAGATGG-GAAAGACCC-3’ and 5’-ACCAGAGAGCTCAGATACG-3’) (20), human Bim (5’-GAGAAGTGAACATTTGACG-3’ and 5’-GA-CAATGTAACGTTACAGTCG-3’) (21), human TRAIL (5’-GACC-CGCTGCCAGGATCTG-3’ and 5’-TGTCCTGATCCTGCACTG-3’) (14), and human GAPDH (5’-CACCCATGGCAATTCCAT-G-3’ and 5’-TCTAGACGCGAGGTCAAGT-3’) (21) were described previously. The primers used for real-time quantitative RT-PCR analyses of RUNX3, human Bim, and human GAPDH were Assays-on-Demand gene expression products (Applied Biosystems, Foster City, CA). The primers used for real-time quantitative RT-PCR analyses of mouse BimL (5’-GTCCTCCAGTGGGTATTTCT-3’ and 5’-TCTCCGTGCAGTGCACG-3’) and mouse GAPDH (5’-GAAGGATGAACTGCAGATACG-3’ and 5’-CCCGATGTACAACGGAGGTCTCTC-3’) were described previously (22). The primers for GAPDH served as an internal control for normalization of the results.

Small Interfering RNA—Control non-targeting siRNA and siRNA duplexes targeting human Bim were purchased from Dharmacon (Lafayette, CO). Cells were transfected with the siRNA duplexes using Lipofectamine 2000 (Invitrogen) and subsequently subjected to Western blot and apoptosis detection analyses at 72 h after transfection.

Luciferase Assay—Cells were transiently transfected with luciferase reporter constructs, mixtures of expression plasmids encoding RUNX3 (18), PEBP2β2 (23), or FoxO3a-TM (8), and an internal control pRL-TK vector (Promega, Madison, WI). Luciferase activity was measured after 20 h using the dual-luciferase reporter assay system (Promega) in an LB 960 Microplate Luminometer Centro (Berthold Technologies, Bad Wildbad, Germany). Luciferase activity was normalized for the transfection efficiency using the Renilla luciferase activity from pRL-TK.

Electrophoretic Mobility Shift Assay—COS7 cells were transfected with the indicated cDNA expression plasmids, and whole-cell lysates were prepared. Complementary oligonucleotides corresponding to FBE-RBE1 (5’-GGGAAAACATATAACACCACA-3’) and...
RBE2 (5'-GCTCAACTACGCGAGTCCTCAAGAGC-3') of the Bim promoter were biotinylated at their 3' ends, annealed, and used as probes. Binding reactions were performed and analyzed in a nondenaturing gel as described previously with minor modifications (5, 24). For supershift assays, whole-cell lysates were preincubated with an anti-Runt domain (anti-RUNX) 6B4 monoclonal antibody (25) or anti-HA monoclonal antibody (12CA5, Roche Applied Science), and the DNA-protein complexes were visualized using a LightShift chemiluminescent EMSA kit (Pierce). For competition experiments, whole-cell lysates were preincubated with a 200-fold molar excess of unlabeled complementary oligonucleotide probes.

Western Blot Analysis and Immunoprecipitation—After lysing cells on ice, whole-cell lysates were resolved by SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Billerica, MA). The membranes were sequentially probed with an appropriate primary antibody and a horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Buckinghamshire, UK), and immunocomplexes were detected using ECL Western blotting detection reagents (Amersham Biosciences). For immunoprecipitation, whole-cell lysates were incubated overnight with anti-FLAG M2 affinity gel Freezer-Safe (Sigma), and the immune complexes were analyzed by Western blot analysis. Anti-FLAG M2 and anti-actin (AC-15) monoclonal antibodies were purchased from Sigma. An anti-Bim monoclonal antibody (14A8) was purchased from Chemicon (Boronia, Victoria, Australia). Anti-FKHRL1, anti-phospho-FKHRL1(Thr32), and anti-phospho-FKHRL1(Ser253) monoclonal antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). An anti-AU1 monoclonal antibody was purchased from Covance (Berkeley, CA). An anti-Smad3 monoclonal antibody (FL-425) was purchased from Santa Cruz Biotechnology.

RESULTS

Restored Expression of RUNX3 Induces Apoptosis in the AGS Gastric Cancer Cell Line in the Absence of TGF-β —Runx3-deficient gastric epithelial cells are less sensitive to the proapoptotic effects of TGF-β (3). In addition, human gastric cancer cell lines including AGS, MKN28, and KATO III lose endogenous RUNX3 expression (3) and fail to respond to...
TGF-β to undergo apoptosis, while they express endogenous RUNX1 and PEBP2β2. To assess the proapoptotic role of RUNX3 in TGF-β-induced apoptosis, we generated an AGS-derived transfectant clone (AGS/RUNX3) that expressed FLAG-tagged RUNX3 in an ecdysone-inducible manner. AGS and AGS/RUNX3 cells were stimulated with an ecdysone analog, ponasterone A, in the presence or absence of TGF-β for 48 h and then analyzed for the expression of FLAG-RUNX3 and the induction of apoptosis. Real-time quantitative RT-PCR analyses showed that treatment with ponasterone A induced RUNX3 mRNA in AGS/RUNX3 cells but not AGS cells (Fig. 1A). The mRNA level of RUNX3 induced in AGS/RUNX3 cells was similar to that in the SNU16 human gastric cancer cell line that expresses endogenous RUNX3 (3) and responds to TGF-β to undergo apoptosis (26, 27). Western blot analysis confirmed ponasterone A-induced expression of FLAG-RUNX3 in AGS/RUNX3 cells but not AGS cells (Fig. 1B). TGF-β did not affect the level of RUNX3 expression. The level of apoptosis was analyzed using annexin V staining. In the absence of TGF-β, the percentage of early apoptotic cells that were positively stained with annexin V but not with propidium iodide was increased from 3.67 to 27.7% following FLAG-RUNX3 expression in AGS/RUNX3 cells but was not increased in AGS cells (Fig. 1C). Cell death detection ELISA assays also showed that apoptosis was induced by ponasterone A but not by TGF-β in AGS/RUNX3 cells (Fig. 1D). The TGF-β stimulation did not increase the percentage of the apoptotic cells. It is important to note that in AGS/RUNX3 cells the induction of apoptosis was dependent on ponasterone A but not on TGF-β.

To investigate the involvement of Smad transcription factors (Smads) that are signal transducers of TGF-β in RUNX3-induced apoptosis, we examined the transcriptional activities of Smads in AGS/RUNX3 cells. Cells were transfected with a luciferase reporter construct, (CAGA)12MLP-Luc (28), that is responsive to the binding

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**Figure 6.** Both RUNX3 and FoxO3a are required for Bim expression in AGS/RUNX3 cells stimulated with ponasterone A. A, AGS and AGS/RUNX3 cells were unstimulated or stimulated with ponasterone A. Whole-cell lysates were separated by SDS-PAGE and analyzed by Western blot with an anti-Bim or anti-β-actin monoclonal antibody. B, AGS/RUNX3 cells were transfected with a control plasmid or an expression plasmid for FLAG-RUNX3-(1–187) or AU1-FoxG1. The cells were then left unstimulated or stimulated with ponasterone A. Whole-cell lysates were analyzed by Western blot with an anti-Bim, anti-FLAG, anti-AU1, or anti-β-actin antibody.

**Figure 7.** Effects of Bim silencing by siRNA on RUNX3-induced apoptosis of AGS/RUNX3 cells. A and B, AGS/RUNX3 cells were transfected with control siRNA or Bim siRNA and then stimulated with ponasterone A. The expression of Bim was analyzed by Western blot with an anti-Bim or anti-β-actin monoclonal antibody (A). Cells were stained with annexin V and propidium iodide (PI) and analyzed for the induction of apoptosis by flow cytometry (B).
of activated Smads. Luciferase assays were performed in the presence or absence of ponasterone A and/or TGF-β. The transcriptional activity of (CAGA)\textsubscript{12}MLP-Luc was not detected in the absence of TGF-β and was greatly increased in the presence of TGF-β (Fig. 1E). The ponasterone A stimulation did not increase the TGF-β-induced transcriptional activity of (CAGA)\textsubscript{12}MLP-Luc. These results indicated that Smads did not play a critical role in RUNX3-induced apoptosis in AGS/RUNX3 cells.

**RUNX3 and FoxO3a Cooperate to Mediate Apoptosis in Gastric Cancer Cell Lines**—FoxO3a is a critical inducer of apoptosis in PTEN-mediated tumor suppression of prostate cancer (14, 29) and in paclitaxel-mediated tumor suppression of breast cancer cells (13). Expression of proapoptotic Bim that mediates TGF-β-dependent apoptosis in B lymphocytes (30) is transcriptionally up-regulated by FoxO3a, resulting in induction of apoptosis in breast cancer cells (13). To assess the involvement of FoxO3a in RUNX3-induced apoptosis of gastric cancer cells, we examined the activity of FoxO3a in AGS, MKN28, KATO III, and SNU16 gastric cancer cell lines. Western blot analysis showed that active unphosphorylated FoxO3a, but not inactive phosphorylated (Thr\textsuperscript{32}, Ser\textsuperscript{253}) FoxO3a, was constitutively expressed in all these cell lines (Fig. 2).

We next investigated whether FoxO3a is indispensable for RUNX3-induced apoptosis in AGS/RUNX3 cells. FoxG1/Qin is a member of the FoxO subfamily and inhibits the transcriptional activities of FoxO1, FoxO3a, and FoxO4 (31, 32). AGS/RUNX3 cells were transfected with the AU1-tagged FoxG1 cDNA, stimulated with ponasterone A, and examined for the AU1-FoxG1 expression and the induction of apoptosis. Exogenous expression of AU1-FoxG1 was not altered by ponasterone A stimulation (Fig. 3A). Cell death detection ELISA showed that the RUNX3-induced apoptosis was significantly suppressed by AU1-FoxG1 expression (Fig. 3B), indicating that FoxO3a is required for the RUNX3-induced apoptosis in AGS/RUNX3 cells.

SNU16 cells express both endogenous RUNX3 (Fig. 1A) and active FoxO3a (Fig. 2), and respond to TGF-β to undergo apoptosis (26, 27). Although the amount of apoptosis was greatly increased after TGF-β stimulation in SNU16 cells, there was a basal level of apoptosis without TGF-β stimulation. We investigated whether endogenous RUNX3 and FoxO3a are involved in the basal level of apoptosis. SNU16 cells were transfected with a cDNA for FLAG-tagged dominant-negative truncated RUNX3, FLAG-RUNX3-(1–187) (33) or AU1-FoxG1. The cells expressed either of the cDNAs (data not shown). The transfected cells were cultured in normal culture medium. Cell death detection ELISA showed that the expression of either FLAG-RUNX3-(1–187) or AU1-FoxG1 significantly suppressed the basal level of apoptosis in SNU16 cells (Fig. 4).

**Bim Is Responsible for Apoptosis Induced by Cooperation of RUNX3 and FoxO3a in AGS/RUNX3 Cells**—FoxO3a induces apoptosis by transactivating the proapoptotic genes, Fasl (8), Bim (10–13), and TRAIL (14). We performed semiquantitative RT-PCR analysis to examine whether the expression levels of these genes are increased by the restored RUNX3 expression in AGS/RUNX3 cells. As shown in Fig. 5, treatment with ponasterone A increased the mRNA level of Bim, but not those of Fasl and TRAIL. Bim expression was also examined by Western blot analysis. Ponasterone A stimulation increased the expression of Bim\textsubscript{EL}, one of isofoms of Bim with the highest molecular weight in AGS/RUNX3 cells but not in AGS cells (Fig. 6A), indicating that restored RUNX3 induced Bim expression. Next, AGS/RUNX3 cells were transfected with the FLAG-RUNX3-(1–187) or AU1-FoxG1 cDNA, stimulated with ponasterone A, and examined for the expression of Bim by Western blot analysis. The expression of either FLAG-RUNX3-(1–187) or AU1-FoxG1 significantly suppressed RUNX3-induced Bim expression (Fig. 6B). These results indicated that both RUNX3 and FoxO3a were required for up-regulation of Bim.

Next, we examined the effect of siRNA-mediated Bim silencing on apoptosis induced by restored RUNX3 in AGS/RUNX3 cells. Transfection of Bim siRNA, but not the control siRNA, dramatically suppressed Bim expression (Fig. 7A) and inhibited apoptosis (Fig. 7B) in AGS/RUNX3 cells stimulated with ponasterone A. These results demonstrated that RUNX3 and FoxO3a cooperated to induce the expression of proapoptotic Bim and mediate apoptosis in AGS/RUNX3 cells.

**Overexpression of Constitutively Active FoxO3a Induces Bim Expression and Apoptosis in Runx3\textsuperscript{−/−} MEFs but Not Runx3\textsuperscript{−/−} MEFs**—To confirm the proapoptotic cooperation of RUNX3 and FoxO3a, Runx3\textsuperscript{−/−}, and Runx3\textsuperscript{−/−} MEFs were transfected with the cDNA of constitutively active FoxO3a, FoxO3a-TM (8), in which all three of the Akt phosphorylation sites Thr\textsuperscript{32}, Ser\textsuperscript{235}, and Ser\textsuperscript{315} were converted to alanines, and the induction of apoptosis was analyzed using cell death detection ELISA. Runx3\textsuperscript{−/−} MEFs underwent apoptosis following the transfecion of the FoxO3a-TM cDNA, whereas Runx3\textsuperscript{−/−} MEFs did not (Fig. 8A).

Next, we analyzed Bim mRNA levels by real-time RT-PCR in Runx3\textsuperscript{−/−} and Runx3\textsuperscript{−/−} MEFs following transfection of FoxO3a-TM cDNA. After transfection, the Bim mRNA level in Runx3\textsuperscript{−/−} MEFs increased by 3.1-fold, whereas that in Runx3\textsuperscript{−/−} MEFs only increased by
Proapoptotic Cooperation of RUNX3 with FoxO3a

**Figure 9. Activation of the Bim promoter by interaction between RUNX3 and FoxO3a.** A, two Runx-binding elements (RBE1 and RBE2) and one FBE are present in the mouse Bim promoter. Mutated versions of RBE1, RBE2, and FBE (mutRBE1, mutRBE2, and mutFBE, respectively) are shown (mutations are underlined). B, luciferase assays were performed using reporter constructs for wild-type (WT) or mutated versions (mutRBE1, mutRBE2, and mutFBE) of the Bim promoter. Runx3−/− MEFs were transfected with or without expression plasmids for RUNX3, PEBP2, and FoxO3a-TM in the indicated combinations together with the WT, mutRBE1, mutRBE2, or mutFBE promoter reporter constructs. Relative luciferase activities were measured in cell lysates after 20 h.

1.3-fold (Fig. 8B), suggesting that RUNX3 and FoxO3a are involved in Bim activation in MEFs.

**RUNX3 and FoxO3a Cooperate Functionally to Activate Transcription of Bim**—We searched the proximal region of the murine Bim promoter and identified two conserved Runx-binding elements (RBE1: −660 to −655, RBE2: −29 to −24) and one conserved FoxO-binding element (FBE: −674 to −666) immediately upstream of RBE1 (Fig. 9A). The conserved Runx-binding element was also identified adjacent to one of the FoxO-binding elements in the rat Bim promoter (12) and to the FoxO-binding element in the human Bim promoter (13). PEBP2 consisting of RUNX3 and PEBP2 binds directly to the promoters of its target genes and activates their transcription through physical interactions and functional cooperation with DNA-binding cofactors such as Smads that recognize nearby sequences in the promoters (1, 5). We explored the functional interaction of RUNX3-PEBP2 and FoxO3a in the activation of the Bim promoter. Runx3−/− MEFs were transfected with a luciferase reporter construct under the control of the wild-type Bim promoter (19). As shown in Fig. 9B, the transcriptional activity of the wild-type Bim promoter was greatly increased in the presence of both RUNX3-PEBP2 and FoxO3a-TM but not in the presence of either RUNX3-PEBP2 or FoxO3a-TM alone. Furthermore, mutations in RBE1 or FBE, but not RBE2, dramatically decreased the transcriptional activity of the promoter, indicating that the transcription of Bim is activated cooperatively between RUNX3-PEBP2 and FoxO3a.

**Direct Binding of RUNX3 and FoxO3a to the Proximal Region of the Bim Promoter**—We examined whether RUNX3-PEBP2 and FoxO3a bind directly to their conserved binding elements in the Bim promoter. Electrophoretic mobility shift assays were performed using biotin end-labeled oligonucleotide probes corresponding to the sequences containing FBE-RBE1 and RBE2 and whole-cell lysates prepared from COS7 cells transfected with mixtures of cDNAs for RUNX3, PEBP2, and/or HA-tagged FoxO3a-TM as indicated. RUNX3-PEBP2 bound to both FBE-RBE1 and RBE2 probes and formed DNA-binding complexes (Fig. 10A). The binding of RUNX3-PEBP2 was inhibited by an excess of unlabeled FBE-RBE1 or RBE2 oligonucleotides. In the presence of an anti-RUNX antibody, the DNA-binding complexes were supershifted. HA-FoxO3a-TM also bound to the FBE-RBE1 probe, and this binding was blocked by an excess of unlabeled FBE-RBE1 oligonucleotides (Fig. 10B). We detected the supershift of the DNA-binding complex in the presence of an anti-HA antibody. In the presence of both RUNX3-PEBP2 and HA-FoxO3a-TM, a more slowly migrating DNA-binding complex was formed. This DNA-binding complex was supershifted by the addition of either the anti-RUNX or the anti-HA antibody, indicating that it contained both RUNX3-PEBP2 and HA-FoxO3a-TM.

These results showed that RUNX3-PEBP2 and FoxO3a physically interacted with respective cognate binding sites on the Bim promoter, thus supporting the cooperative activation of the promoter.

It has been shown that RUNX3 binds directly to Smads to form a RUNX3-Smads complex (5). Our results suggested that RUNX3-PEBP2 formed a complex with FoxO3a on the Bim promoter (Fig. 10B). To investigate the direct association between RUNX3 and FoxO3a, we performed communoprecipitation experiments using whole-cell lysates prepared from COS7 cells transfected with cDNAs for FLAG-RUNX3 and FoxO3a or FLAG-RUNX3 and Smad3. As shown in Fig. 11A, FoxO3a, as well as Smad3, was communoprecipitated with FLAG-RUNX3, demonstrating the direct association between RUNX3 and FoxO3a. Moreover, we performed communoprecipitation experiments to examine the physical interaction between RUNX3 and FoxO3a in AGS/RUNX3 cells. FoxO3a was communoprecipitated with FLAG-RUNX3 in AGS/RUNX3 cells stimulated with ponasterone A (Fig. 11B). Taken together, these results demonstrate that RUNX3 cooperates with FoxO3a to mediate apoptosis through activating Bim and that impaired expression of RUNX3 in gastric cancer
cell lines may contribute to the accelerated cell survival of gastric cancer cells.

**DISCUSSION**

Since gastric epithelial cells of Runx3−/− mice are less sensitive to the proapoptotic effects of TGF-β compared with those of Runx3+/+ mice, RUNX3 is implicated in the proapoptotic effect of TGF-β in gastric epithelial cells (3). We investigated whether expression of exogenous RUNX3 restored sensitivity of gastric cancer cell lines that lost RUNX3 expression and failed to respond to TGF-β stimulation. The restored expression of RUNX3 induced apoptosis in the absence of TGF-β, and TGF-β stimulation did not enhance RUNX3-induced apoptosis. These results suggested that RUNX3 possessed proapoptotic activity without involving cooperation of Smads, consistent with a recent report that showed enforced restoration of RUNX3 expression leads to cell growth inhibition and apoptosis in gastric cancer cell lines (34). Although this report indicates that expressions of caspase-3, -7, and -8 among 11 caspases are up-regulated by restored RUNX3 using RT-PCR analysis, the molecular mechanisms whereby RUNX3 induces apoptosis are still obscure. Here, we have shown that RUNX3 physically interacts with FoxO3a to activate proapoptotic Bim and mediates apoptosis in gastric cancer cells. Furthermore, the same proapoptotic interaction was found in primary cultures of mouse embryonic fibroblasts, suggesting that this mechanism operates in different tissues as well. While the proapoptotic role of Runx3 is suggested only in gastric epithelial cells (3), Runx3 is ubiquitously expressed in vivo (35) and highly expressed in spleen and thymus.

Recently, Runx3 has been shown to be involved in lineage specification of CD8 T cells by silencing CD4 and reactivating CD8 during T lymphocyte development (36, 37). The proapoptotic cooperation of Runx3 and FoxO3a might contribute to programmed cell death during T lymphocyte development.

Bim is required for normal programmed cell death induced by the NGF withdrawal in developing sympathetic neurons (38). Following NGF withdrawal, FoxO3a binds directly to two conserved FoxO-binding elements in the Bim promoter to activate its transcription and promotes apoptosis in sympathetic neurons (12). However, inhibition of FoxO3a transcriptional activity does not block NGF withdrawal-induced apoptosis indefinitely. The eventual death of sympathetic neurons mediated by Bim might require proapoptotic Runx3 with FoxO3a. The anti-cancer drug paclitaxel is used to treat breast cancers; paclitaxel treatment dramatically increases expression of FoxO3a that activates Bim and leads to induction of apoptosis in the breast cancer cell line MCF-7 but not MDA-MB-231 (13). The impaired expression of FoxO3a explains the paclitaxel-insensitive property of the MDA-MB-231 cell line. RUNX2 is ectopically expressed in human breast cancer cell lines (39), while the RUNX3 expression is lost in breast and gastric cancer cell lines. Quantitative real-time PCR analysis showed the expression of RUNX2, but not RUNX3, in MCF-7 and MDA-MB-231 cells. Since all three RUNX transcription factors recognize the same DNA-binding motifs, FoxO3a may cooperate with RUNX2 to activate Bim and induce apoptosis in breast cancer cells.

Our present results suggest that FoxO3a plays an essential role in

4 Q. C. Lau, S. Sukumar, and Y. Ito, unpublished data.
RUNX3-mediated tumor suppression by activating Bim in gastric cancer. Similar involvement of FoxO3a in tumor suppression has been shown for PTEN-mediated tumor suppression via activation of the TRAIL gene in prostate cancer (14). FoxO3a directly binds to and activates the TRAIL promoter to induce TRAIL-dependent apoptosis. The loss of PTEN, which negatively regulates the PI3K-Akt pathway, decreases the activity of FoxO3a thereby leading to impaired expression of the FoxO-regulated TRAIL that induces apoptosis in tumor cells but not in normal cells. The loss of the tumor suppressor gene PTEN frequently occurs in prostate cancer, glioblastoma, and breast cancer but not in gastric cancer. Indeed, Akt was not phosphorylated in 71.1% of gastric cancer cell lines, whereas it was in 96.3% of nonneoplastic gastric tissues (15). The loss of the tumor suppressor gene RUNX3 in gastric cancer cell lines is closely related to gastric cancer cell apoptosis (16). Akt may inhibit the RUNX3-regulated proapoptotic effect of Bim by down-regulating the activities of two DNA-binding cofactors of RUNX3, namely FoxO3a and Smads. Such cross-talk between the RUNX3 and PI3K/Akt signaling pathways may be important for homeostasis of gastric cells and could be exploited for future therapeutics against gastric cancer.

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