Runx1 Is a Co-activator with FOXO3 to Mediate Transforming Growth Factor β (TGFβ)-induced Bim Transcription in Hepatic Cells*

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Transforming growth factor β (TGFβ) regulates essential cellular functions such as cellular proliferation, differentiation, and apoptosis. The Bcl-2 family of proteins has been implicated as mediators of TGFβ-induced apoptosis. We demonstrated previously that TGFβ induces the expression of Bim (Bcl-2-interacting mediator of cell death), a member of the BH3-only family of pro-apoptotic Bcl-2 proteins, to induce cell death in B-lymphocytes. Here, we investigated the mechanism of TGFβ-mediated Bim expression in two hepatocyte cell lines that undergo apoptosis with TGFβ, AML-12 and Hep3B. We show that TGFβ induces Bim protein and mRNA levels, and its expression is sufficient to induce cell death. Gene array results revealed that Runx1, a member of the Runx family of transcription factors, was induced by TGFβ, and this induction was confirmed at the mRNA and protein levels. Interestingly, TGFβ specifically induced the expression of Runx1 protein from an internal ribosome entry site (IRES)-dependent, cap-independent, mRNA transcript, and its overexpression was sufficient to induce hepatocyte apoptosis. Deletion and mutation analyses of the murine Bim promoter identified a putative forkhead binding element, at position −174 to −168 from the transcription start site, as the mediator of Runx1 induction. Co-immunoprecipitation, electrophoretic mobility shift assays, and chromatin immunoprecipitation assays demonstrated that Runx1 does not bind directly to the identified forkhead binding element but rather binds the transcriptional regulator FOXO3, which occupies this site. Finally, small interfering RNA knockdown of Runx1 or FOXO3 decreased TGFβ-induced Bim expression. Our results support a mechanism in which TGFβ stimulates Bim transcription by up-regulating Runx1 expression, which binds FOXO3, and the two cooperate in the transcriptional induction of Bim.

Transforming growth factor β (TGFβ)2 exerts its biological effects by inducing the formation of a heteromeric complex composed of type I (TβRI) and type II (TβRII) serine/threonine kinase receptors. Activation of the receptor complex leads to further propagation of TGFβ signaling to downstream signaling cascades, which include the Smads, mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (1–5). Smad signaling is activated by receptor-mediated phosphorylation of Smad2 and Smad3, which then form heteromeric complexes with Smad4 and translocate to the nucleus to regulate transcriptional responses to TGFβ.

The TGFβ-induced apoptotic response is well documented in many cell types, particularly in prostate epithelium, hepatocytes, and hepatoma cell lines, B-lymphocytes and B-cell lines (6). Multiple apoptotic mediators and signaling pathways have been implicated in TGFβ-induced apoptosis (3, 7, 8). In liver cells, these include death-associated protein kinase and the Smad pathway (9), Daxx and the c-Jun N-terminal kinase (JNK) pathway (10), GADD45b and the p38 pathway (11), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and the AP-1/Smad pathways (12, 13). The Bcl-2 family of proteins has also been implicated as mediators of TGFβ-induced apoptosis. In the FAO rat hepatoma cell line, TGFβ induces the caspase-dependent cleavage of Bad in a Smad3-dependent manner to produce a more potent, truncated Bad species (14). We first demonstrated that TGFβ induces the expression of the pro-apoptotic protein Bim (Bcl-2-interacting mediator of cell death) through a Smad3-dependent mechanism to induce cell death in B-lymphocytes (15). This induction of Bim has more recently been demonstrated in gastric epithelial cells undergoing TGFβ-induced apoptosis (16) and confirmed in AML12 hepatocytes and NMuMG mammary epithelial cells (17).

Bim is a member of the BH3-only family of pro-apoptotic proteins that also includes Bid, Bad, Bmf, Noxa, and Puma (18, 19). These proteins share only the single BH3 domain, which allows heterodimerization with other Bcl-2 family members. Following a pro-apoptotic stimulus, Bim initiates the mitochondrial cell death pathway by either directly activating Bax-like proteins (direct activation model) or by binding to prosurvival Bcl-2 family members and thereby releasing Bax-like proteins (indirect activation model) (19, 20). Bim proteins are expressed by a wide variety of tissues but are most prominently expressed by cells of hematopoietic origin and have been shown to be critical for apoptosis in B- and T-lymphocytes, macrophages, and granulocytes (18). Experiments using Bim knock-out mice indicate that Bim is required for B-cell receptor-induced apoptosis in immature and mature B-cells and for the negative selection of autoreactive B-cells (18). Although there is
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no reported hepatic phenotype in Bim knock-out mice (21, 22), there are reports of Bim being involved in hepatic apoptosis in vivo during fatal hepatitis (23) and Fas-induced liver damage (24).

The pro-apoptotic activity of Bim is highly regulated by pro-survival and pro-apoptotic cytokines through both post-translational and transcriptional mechanisms. Studies on the post-translational control of Bim have demonstrated that Bim is phosphorylated by ERK, leading to its subsequent ubiquitination and degradation (25, 26). In hepatocytes, we have recently demonstrated that TGFβ/Smad3 signaling induces the dual specificity phosphatase DUSP4 (MKP2) to inhibit ERK activity, thereby stabilizing Bim and promoting apoptosis (27). Studies on the transcriptional control of Bim have focused largely on FOXO3, a member of the forkhead family of transcriptional regulators, which directly stimulates Bim transcription (28, 29). Multiple reports have shown that the phosphatidylinositol 3-kinase (PI3K)/Akt pathway is a negative regulator of FOXO3-stimulated Bim transcription via phosphorylation of FOXO3, leading to the binding and sequestration of this transcription factor by cytosolic 14-3-3 proteins (30, 31).

The prominent role of Bim as a mediator of apoptosis is gaining in importance. Thus, an understanding of how Bim expression is regulated under various physiological and pathological conditions is of significance. There are relatively few studies that directly address the transcriptional regulation of Bim. Herein, we examined the mechanism through which TGFβ mediates the transcriptional induction of Bim using hepatocyte cell lines that undergo apoptosis in the presence of TGFβ. We demonstrate that TGFβ stimulates Bim transcription by up-regulating the expression of the transcription factor Runx1 through an IRES-dependent mechanism. TGFβ-induced Runx1, in turn, binds to FOXO3, residing at its forkhead binding site, and the two cooperate to stimulate Bim promoter transactivation.

EXPERIMENTAL PROCEDURES

Reagents—TGFβ was a generous gift from Genzyme Inc. (Cambridge, MA). Rabbit anti-Bim antibody was obtained from BD Biosciences. Rabbit anti-phospho-FOXO3 (Ser-253), rabbit anti-total FOXO3, and rabbit anti-cleaved caspase-3 (Asp-175) were purchased from Cell Signaling Technology (Beverly, MA). Mouse anti-Bcl-X, (H-5), rabbit anti-Hsp-90 (H-114), and normal mouse and rabbit IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-AML-1 (Runx1) was obtained from Active Motif (Carlsbad, CA) and mouse anti-FLAG antibody from Sigma. Sheep anti-mouse IgG-HRP and donkey anti-rabbit IgG-HRP were purchased from Amersham Biosciences (GE Healthcare). HRP-protein A was from Zymed Laboratories Inc. (San Francisco, CA). LY294002 and rapamycin were obtained from Calbiochem. The mouse anti-HA antibody (12CA5), protease inhibitor mixture tablets, and the cell death detection ELISA Plus kit were purchased from Roche Diagnostics. Tris-buffered saline-casein for Western blotting was obtained from Pierce Chemical Co. ExpressFect was ordered from Denville Scientific (Denville, NJ). The Dual-luciferase reporter system was purchased from Promega (Madison, WI) and the SuperScript III RT kit from Invitrogen. The RNeasy and Oligotex RNA isolation kits were obtained from Qiagen (Valencia, CA). All primers were ordered from IDT Technologies (Coralville, IA). ON-TARGETplus SMARTpool siRNA, non-targeting pool siRNA, and DharmaFECT 4 reagent were obtained from Dharmacon (Lafayette, CO). Reagent chemicals were obtained from Sigma.

Cell Culture—Hep3B, FAO, and HepG2 cells were cultured in DMEM containing 10% fetal calf serum and antibiotics/antimycotics. AML-12 cells were grown in DMEM/F12 medium supplemented with 10% fetal calf serum, dexamethasone (40 μg/ml), insulin/transferrin/selenium mixture (Fisher Scientific), and antibiotics/antimycotics (32). COS-7 cells were cultured in DMEM containing 10% newborn calf serum and antibiotics/antimycotics, and Daudi cells were cultured in DMEM/F12 medium supplemented with 10% fetal calf serum, antibiotic/antimycotics, and 30 μM 2-β-mercaptoethanol. TGFβ was used at a final concentration of 5 ng/ml.

Western Blot Analysis—Whole cell lysates were prepared in TNMG buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 5 mM MgCl₂, 10% glycerol, and 0.5% Nonidet P-40) containing protease inhibitors. Lysates were sonicated briefly and clarified by centrifugation at 4 °C for 10 min in a Beckman tabletop microcentrifuge at maximum speed. Protein concentration of the extracts was determined using Bradford’s reagent (Pierce). Western blot analysis was performed by standard SDS-PAGE, as described previously (33).

Apoptosis Assays—Apoptosis was demonstrated by DNA ladder formation and was detected by ELISA as described previously (15). In ELISA assays, 100 × 10⁶ cells were seeded into wells of a 24-well dish. Cells were lysed in 200 μl of buffer supplied with the kit, and 20 μl of the clarified lysate was used in the ELISA. ELISA results are expressed as the ratio of the ABS₄₅₀ signal of TGFβ-treated samples normalized to the ABS₄₅₀ signal of the control sample.

Northern Blot Analysis—Northern blots were performed using mRNA as described previously (15). Total RNA (~200 μg) was isolated from a 150-mm dish of cells using an RNeasy Mini kit, and mRNA was purified from equal amounts of total RNA using an Oligotex mRNA Mini kit. Blots were hybridized in NorthernMax Prehyb/Hyb buffer (Ambion, Austin, TX) and typically washed with 0.1% SDS, 0.5 × SSC at 55 °C. Northern blots were subjected to phosphorimaging analysis to quantitate band intensity (GE Healthcare). The TGFβ probe used in Northern analyses was obtained by EcoRI/XhoI digestion of pcDNA3.1/BimEL to release the full-length insert (33). The probe used for β-actin was generated by PCR as described previously (15). Runx probes were generated by PCR as described below.

Transient Transfection and Luciferase Assays—Transient transfection of cells was accomplished using ExpressFect under serum-free conditions following the manufacturer’s instructions. After 5–6 h of transfection, the medium was replaced with complete medium with or without TGFβ as indicated. For reporter assays, about 100,000 cells/well were seeded into 12-well plates and typically transfected with 0.5 μg of the mouse 0.8 Bim luciferase promoter construct (34), 10 ng of pRL-SV40-Renilla luciferase (Promega), and up to 0.5 μg of additional plasmid DNA per well. GFP was used as a negative
control, to ensure that all cells received equal amounts of DNA. Approximately 24 h after the start of transfection, the cells were collected in 200 μl of lysis buffer, and a 20-μl aliquot was assayed for luciferase activity using Stop & Glo® reagent (Promega). For Western blot and DNA ladder experiments, 4 × 10⁶ cells were seeded into 100-mm dishes and transfected with up to 10 μg of the appropriate plasmid DNA. GFP was again used to balance the amount of DNA transfected into cells. Cells were collected 24 h later for analyses. The human pLNCX-FLAG-AML1a (Runx1A) and pLNCX-FLAG-AML1b (Runx1B) expression plasmids have been described previously (35), as have the wild-type and triple mutant pECE-HA-FKHRL1 (FOXO3) plasmids (36).

In siRNA experiments, cells were plated into a 24-well dish (50,000 cells/well) for Western blot and ELISA studies or a 6-well dish (400,000 cells/well) for RT-PCR studies. Cells were transfected with specific siRNAs or non-targeting siRNAs in serum-free medium for 5–6 h at a final concentration of 100 nM transfected with specific siRNAs or non-targeting siRNAs in 6-well dish (400,000 cells/well) for RT-PCR studies. Cells were (50,000 cells/well) for Western blot and ELISA studies or a 100-mm dish (400,000 cells/well) for RT-PCR studies. Cells were transfected with specific siRNAs or non-targeting siRNAs in serum-free medium for 5–6 h at a final concentration of 100 nM to balance the amount of DNA transfected into cells. Cells were collected 24 h later for analyses. The human pLNCX-FLAG-AML1a (Runx1A) and pLNCX-FLAG-AML1b (Runx1B) expression plasmids have been described previously (35), as have the wild-type and triple mutant pECE-HA-FKHRL1 (FOXO3) plasmids (36).

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**Gene Array**—Microarray analysis was performed using the Illumina Mouse-6 BeadChip platform as described previously (27). Briefly, total RNA was prepared as described above from control and TGFβ-treated (30 min and 1 h) AML-12 hepatocytes from two experiments. cRNA synthesis, hybridization, and washing were performed using Illumina reagents following the manufacturer’s directions. The data output from the Illumina BeadChip scanner was normalized using the rank-invariant method and analyzed using BeadStudio software.

**RT-PCR**—Total RNA was prepared as described above using an RNaseasy Mini kit. RNA was subjected to DNase digestion using DNA-free (Ambion), and cDNA was prepared using random primers supplied with the SuperScript III RT kit (Stratagene). The primers used to amplify the 3′-UTR of mouse Runx1 were 5′-AAACAAGTGGTAGCTACGCCAGC-3′ for the top strand and 5′-TGACTGATTCCTAAGACTGGGAG-3′ for the bottom strand. The PCR reaction was annealed at 55.1 °C and run for 28 cycles to generate a 524-bp product. The primers used to amplify the 3′-UTR of human Runx1 were 5′-CGCTGGAAGCAACACAGGAAG-3′ for the top strand and 5′-GGTCAAAGCAAGAAGAGCGC-3′ for the bottom strand. The PCR reaction was annealed at 55.1 °C and run for 28 cycles to generate a 524-bp product. The primers for β-actin PCR were described previously (15). The PCR reaction was annealed at 66 °C and run for 18 cycles. The IRES 5′-UTR of Runx1 was amplified from mouse primary B-cell cDNA using a top strand primer of 5′-GGCTGGCACTTCCATCCTGG-3′ and a bottom strand primer of 5′-CACAAGGCCAATTTGAGGTAAGG-3′. An approximate 1.4-kb product was generated after 35 cycles using an annealing temperature of 56.3 °C. The IRES PCR product was TA-cloned into the pGEM T-Easy vector (Promega), which was digested with EcoRI to produce a probe for Northern blots. The primary B-cells were isolated and cultured as described previously (27, 37). The cap 5′-UTR of Runx1 was amplified from cDNA prepared from a human B-cell line (Daudi) using a top strand primer of 5′-AACACAGTGGTAGCTGCTG-3′ and a bottom strand primer of 5′-AAAATGCTGTGTTAGCCATCG-3′. An approximate 171-bp product was generated after 32 cycles using an annealing temperature of 55.6 °C. The cap PCR product was purified using a PCR purification kit (Qiagen) and used for Northern blots. Daudi cells were cultured as described above. All PCR reactions used AccuPrime Taq polymerase using Buffer 1 (Invitrogen) except for the β-actin PCR reactions, which used Hot-Start Taq (Denville Scientific).

**Nuclear Extract Preparation**—Nuclear extract preparation for EMSA, Western blotting, and immunoprecipitation experiments was performed following the method of Schreiber et al. (38). Typically, cells from one 100-mm culture dish were resuspended in 400 μl of hypotonic buffer, and the nuclear pellet was extracted in 100 μl of a high salt buffer. Protein concentration of the extracts was determined using Bradford’s reagent. Typically 5 μg of nuclear extract was analyzed in EMSA and 150 μg in Western blotting experiments.

**Rapamycin Inhibition of Protein Synthesis**—Hep3B cells were seeded into a 24-well dish at a density of 100,000 cells/well in serum-containing medium. The next day rapamycin (20 μM) was added for up to 24 h. During the last hour of each treatment [35S]methionine (PerkinElmer Life Sciences) was added to a concentration of 100 μCi/ml in serum-free medium lacking methionine. Cells were washed twice with phosphate-buffered saline and lysed in 200 μl of buffer (20 mM Tris, pH 7.4, 1% Triton X-100, 10% glycerol, 130 mM NaCl). The lysate was centrifuged, and 10 μl of the supernatant was added to 100 μl of bovine serum albumin (1 mg/ml in lysis buffer). Proteins were precipitated by adding 1 ml of ice-cold 10% trichloroacetic acid and collected by membrane filtration. Radioactivity on the membranes was determined by liquid scintillation counting.

**Co-Immunoprecipitation**—Whole cell lysates for immunoprecipitation (IP) were prepared in IP lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100) containing protease inhibitors. Cell lysates (250 μg) were incubated overnight at 4 °C with 20 μg of anti-FLAG antibody in 800 μl of IP lysis buffer containing protein G-agarose. The immune complexes were collected by centrifugation and washed four times in IP lysis buffer. The presence of forkhead protein in the immune complexes was detected by Western blotting. Nuclear extracts (1 mg) were prepared as described above, diluted 5-fold in IP lysis buffer, and precleared with protein G-agarose and rabbit IgG. After centrifugation, the supernatant was immunoprecipitated overnight at 4 °C with 15 ml of anti-Runx1 antibody and protein G. The immune complexes were collected and analyzed for forkhead protein as described above except that protein A-HRP was used as the secondary antibody.

**EMSA**—EMSA were performed following the method of Lewis and Konradi (39). Binding reactions were performed in 10 mM Hepes buffer, pH 7.9, containing 10% glycerol, 0.1 mM EDTA, 5 mM MgCl₂, 2 mM dithiothreitol, and 1 μg of poly(dI-dC) in a 20 μl volume. Nuclear extract was added to binding buffer and preincubated on ice for up to 1 h. Radiolabeled probe was added, and the binding reaction was carried out for 20
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**FIGURE 1. TGFβ induces apoptosis and increases Bim expression in hepatic cells.**

A, hepatic cells were incubated in the absence or presence of TGFβ for 48 h and then assayed for the induction of apoptosis by ELISA. The increase in apoptosis caused by TGFβ was normalized to control values for each cell type. B, Western blot showing time course of change in the expression of Bim and Bcl-Xi in hepatic cells induced by TGFβ. Hsp-90 served as a loading control. C, ELISA results showing AML-12 cell death after incubation with TGFβ for 24 h in the absence or presence of Bim siRNA. Control Western blots showed effective knockdown of Bim expression. The siRNA control (con.) used non-targeting siRNAs. D, Western blot showing the dose-dependent overexpression of FLAG-Bim (arrow) and resulting caspase-3 activation in AML-12 and Hep3B cells after 24 h. Hsp-90 served as a loading control. E, ELISA results showing dose-dependent increase in apoptosis induced by overexpression of FLAG-Bim in AML-12 and Hep3B cells after 24 h.

min at 30 °C. Where indicated, 2 µl of supershift antibody or “cold” excess oligonucleotide (200 ng) was added during the preincubation step. Samples were loaded onto 6% nondenaturing acrylamide gels and run at 250 V for 4–5 h. The gels were dried and subjected to phosphorimaging analysis. The probe used in the EMSA was a 54-bp double-stranded oligonucleotide containing a 3× concatamer of the sequence 5′-TCCGG-TAAACAGCCAGG-3′ from the human Bim promoter. The underlined sequence indicates the consensus forkhead binding site (40). This oligonucleotide was end-labeled with [γ-32P]ATP using the T4 kinase reaction to generate the EMSA probe. Three unlabeled double-stranded oligonucleotides used as cold competitors in the EMSA binding reactions were as follows: 6X-RBE, a 42-bp oligo containing a 6× concatamer of the consensus Runx binding sequence 5′-AACCA-3′ (41); FasL, a 40-bp oligo from the human FasL promoter containing four forkhead binding sites (underlined) within the sequence 5′-TCTCTATTTAAATAAATAATATAAActGGGCAA-3′ (42); and a 50-bp sequence taken from the mouse Bim promoter containing the sequence 5′-GCTGCCCAGGCAGGCTAGTTGCAGGGGCAA-3′ containing one forkhead binding site (the forkhead binding site is underlined).

**Chromatin Immunoprecipitation Assays—**ChIP and reChIP assays were performed following the method of Shang et al. (43). Briefly, Hep3B cells were seeded into 150-mm culture dishes and transiently transfected with Runx1B overnight. The next day the cells were fixed with formaldehyde, lysed in 500 µl of buffer, and sonicated. After centrifugation to clarify the lysates, 200 µl of the supernatant was precleared with IgG and then immunoprecipitated overnight at 4 °C. The immunoprecipitates were washed at 4 °C and eluted twice at room temperature with 100-µl aliquots of a freshly made sodium bicarbonate/SDS buffer. In reChIP experiments, the initial Runx ChIP was eluted using 10 mM dithiothreitol at 37 °C and then diluted 50-fold in buffer for subsequent immunoprecipitations. ChIP eluates were heated overnight at 65 °C with NaCl to reverse the protein-DNA cross-links and purified on a Qiagen PCR purification column. The column was eluted with 50 µl of buffer from which, typically, 2–5 µl of eluate was used in each 25-µl PCR reaction. PCR was performed using AccuPrime Taq with the supplied Buffer II (Invitrogen). The primers used to amplify the human Bim genomic fragment containing the FOXO3 site were 5′-GTAGGTAGCCAGGCTGAAGCTA-3′ for the top strand and 5′-AGGTCGGACAGCTAAAGG-3′ for the bottom strand. The annealing temperature of the PCR reaction was 61 °C, and
the reaction was run for 36 cycles for the FLAG-Runx1 ChIP samples and 38 cycles for the FOXO3 samples. The 155-bp product (−3135 to −2980 relative to the initiating ATG) contained the consensus forkhead binding site (−3055 relative to the initiating ATG). The sequence of this 155-bp product corresponds to the region encompassing nucleotides −254 to −96 of the mouse Bim promoter (shown in Fig. 6C). The primers used to amplify a human Bim genomic fragment 1869 bp upstream of the FOXO3 site were 5′-TGGCAGAGACAGAAAGGGACAC-3′ for the top strand and 5′-TTTTGGGCACTAAAAGGAAGC-3′ for the bottom strand. Identical PCR cycling conditions generated a 229-bp product.

RESULTS

TGFβ Induces Apoptosis and Increases Bim Expression in Hepatic Cell Lines—We had previously shown that Bim expression is increased during TGFβ-induced apoptosis in B-cells (15). In the present study we used hepatic cell lines to delineate the molecular pathway(s) regulating Bim expression, as these cells are more amenable to transfection and also undergo apoptosis in response to TGFβ treatment (9–14, 17). As shown in Fig. 1A, mouse AML-12 and human Hep3B cells undergo apoptosis in response to TGFβ, with rat FAO cells showing little response. Similarly, TGFβ was also shown to induce Bim expression in AML-12 and Hep3B cells (Fig. 1B, top panel), whereas expression in FAO cells was minimal. It was interesting that TGFβ-induced Bim expression in Hep3B cells was greater than in AML-12 cells, even though AML-12 cells demonstrated much greater apoptosis than Hep3B cells. These results are likely explained by the expression level of the major pro-survival protein in hepatocytes, Bcl-XL. TGFβ had no effect on Bcl-XL expression in Hep3B and FAO cells, but it dramatically reduced the expression level of Bcl-XL in AML-12 cells (Fig. 1B, middle panel). Western blotting for Hsp-90 showed equal protein loading of the samples (Fig. 1B, bottom panel).

The importance of Bim expression in TGFβ-induced apoptosis in AML-12 cells was demonstrated in knockdown experiments. Knockdown of Bim expression with siRNA decreased TGFβ-induced apoptosis by about 70% (Fig. 1C). Control experiments demonstrated effective knockdown of Bim by Western blotting (Fig. 1C). We next determined whether the expression of Bim was sufficient to promote apoptosis in hepatic cells. AML-12 and Hep3B cells were transiently transfected with increasing amounts of FLAG-tagged Bim for 24 h, after which the cells were collected and analyzed for Bim expression and caspase-3 activation. Overexpression of Bim protein (Fig. 1D, top panel, arrow) resulted in a dose-dependent increase in cleaved, active caspase-3 formation (Fig. 1D, middle panel). Hsp-90 showed equal protein loading (Fig. 1D, bottom panel). Overexpression of increasing concentrations of Bim was also associated with increasing cell death in AML-12 and Hep3B cells, as measured by ELISA (Fig. 1E). Collectively, these results show that TGFβ-induced Bim expression is an important mediator of cell death in hepatic cells.

TGFβ Increases Bim mRNA Levels in Hep3B Cells—To determine whether TGFβ induces Bim expression at a transcriptional level, we examined the effect of the transcriptional inhibitor actinomycin D on TGFβ-stimulated Bim expression. Hep3B cells were stimulated with TGFβ for 12 or 18 h, and actinomycin D was added during the last 6 h of another 18-h TGFβ-treated culture. In the absence of inhibitor, Bim expres-
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FIGURE 3. TGFβ Increases Bim Expression and Promoter Activity in the Presence of FOXO3. A, Western blot showing the effect of TGFβ and LY294002 (Ly) on Bim expression in Hep3B cells. Cells were treated for 8 h with TGFβ, LY294002, or both as indicated. Hsp-90 served as a loading control (Cont.). B, Western blot showing the effect of LY294002 on phospho- and total-FOXO3 expression in Hep3B cells. Both untransfected and wild-type (WT) FOXO3-overexpressing cells were studied. Cells were treated with LY294002 for 8 h. C, Hep3B cells were transfected with Bim luciferase and either wild-type FOXO3 or TM FOXO3 as indicated. After transfection, cells were treated with TGFβ for 24 h. Luciferase activity was normalized to Renilla activity for each well to control for transfection efficiency. D, Western blot showing the effect of TGFβ on phospho- and total-FOXO3 levels in Hep3B cells. Both untransfected and wild-type and TM FOXO3-overexpressing cells were studied. Cells were treated with TGFβ for 24 h after transfection. Arrow, indicates overexpressed FOXO3.

The data of Fig. 2D confirm the transcriptional induction of Bim by TGFβ, demonstrating that a 0.8-kb Bim promoter/luciferase reporter construct was transactivated by TGFβ. TGFβ treatment increased Bim luciferase activity by ~3–4-fold after 24 and 48 h (Fig. 2D). As a control, it was shown that TGFβ increased the luciferase activity of a canonical TGFβ-responsive promoter, 3TP-luciferase, ~10-fold in 24 h. Taken together, these results demonstrate that TGFβ is a transcriptional activator of Bim expression.

TGFβ Increases Bim Expression and Promoter Activity in the Presence of FOXO3—The forkhead family of transcription factors, in particular FOXO3, has previously been shown to activate Bim expression (28, 29, 44). To determine whether FOXO3 was involved in TGFβ-stimulated Bim expression, we performed initial experiments using the phosphatidylinositol 3-kinase inhibitor LY294002. LY294002 is an upstream inhibitor of Akt, blocking its phosphorylation and ability to phosphorylate and inhibit FOXO3 transcriptional activity. As shown in Fig. 3A, when LY294002 is added to Hep3B cells in the absence of TGFβ, there is a dose-dependent increase in the expression of Bim protein. Interestingly, the induction of Bim in the presence of the inhibitor is further augmented by TGFβ suggesting that, in addition to FOXO3 activity, other transcriptional modulators are required for TGFβ induction of Bim. Controls, depicted in Fig. 3B, demonstrate that the 50 µM LY294002 concentration is sufficient to inhibit Akt activity, decreasing both endogenous and overexpressed phospho-FOXO3 levels while having no effect on total FOXO3 levels. To directly determine the role of FOXO3 in TGFβ-induced Bim expression, we performed Bim luciferase assays in the absence and presence of overexpressed FOXO3 (Fig. 3C). Our results confirm that wild-type FOXO3 can stimulate Bim promoter activity and, in addition, demonstrate that TGFβ treatment augments FOXO3 Bim promoter transactivation. Furthermore, the stimulatory effect of TGFβ on Bim luciferase activity was observed even in the presence of a FOXO3 triple mutant (TM) lacking Akt phosphorylation sites. This latter result suggests that the increased Bim luciferase activity with TGFβ is not due to an effect on the phosphorylation status of FOXO3. Indeed, Western blot experiments demonstrated that TGFβ had little effect on the phosphorylation of either endogenous or wild-type overexpressed FOXO3 (Fig. 3D). These data indicate that FOXO3 transcriptional activity is not altered during TGFβ-induced Bim expression.

Runx1 Is Specifically Induced by TGFβ and Activates Bim Luciferase—The results presented indicate that in addition to FOXO3, TGFβ induction of Bim may require other transcriptional modulators (Fig. 3). Previously, it has been reported that Runx3 is a TGFβ-sensitive transcriptional activator of Bim in gastric epithelial cells (45). However, our results from a recent gene array experiment did not show any basal or TGFβ-stimulated Runx3 expression in AML12 cells (Fig. 4A and Ref. 27). We confirmed these negative results by RT-PCR (data not shown). The array did demonstrate, however, both basal and
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We next determined the ability of overexpressed Runx1 to increase Runx1 protein levels in primary B-cells (Fig. 4E). In agreement with this observation, we demonstrated by Western blotting that overexpression of Runx1B, but not Runx1A, was also able to induce a dose-dependent increase in endogenous Bim expression in Hep3B cells (Fig. 4F, lower panel) even though Runx1A was expressed at higher levels than Runx1B (Fig. 4F, upper panel). These results suggest that TGFβ induces Runx1 and that Runx1 serves as a transcriptional mediator of Bim induction.

TGFβ Specifically Induces IRES-activated Runx1 mRNA Transcripts—Runx1 transcription can produce both cap-dependent mRNA transcripts and IRES-dependent mRNA transcripts, depending on which promoter region in the Runx1 gene is activated (48, 49). Therefore, it was of interest to determine the type of Runx1 mRNA transcripts up-regulated by TGFβ (shown in Fig. 4B). PCR was used to prepare probes for Northern blotting that specifically recognized the 5′-UTR region of cap- and IRES-dependent mRNA transcripts. The results of Northern analyses using these probes demonstrated that TGFβ had little effect on the expression level of cap-dependent mRNA transcripts (Fig. 5A, left panel), whereas TGFβ markedly increased the expression level of IRES-dependent mRNA transcripts (Fig. 5A, middle panel). Both cap- and IRES-specific Northern probes identified multiple Runx1 mRNA transcripts. Normalization of a representative cap- and IRES-mRNA transcript (Fig. 5A, left and middle panels, arrows) to that of the corresponding β-actin mRNA transcript (Fig. 5A, right panel) confirmed that only IRES-specific Runx1 mRNA transcripts were increased by TGFβ (Fig. 5B).

If TGFβ specifically induces only IRES-dependent Runx1 mRNA transcripts, then the observed increase in Runx1 protein following TGFβ stimulation (Fig. 4D) must be IRES transcript-dependent. To address this question, we utilized the ability of the drug rapamycin to inhibit cap-dependent translation (50, 51). In Hep3B cells, we demonstrate that 20 nM rapamycin produced a time-dependent decrease in protein synthesis, which was reduced to 37% of control levels after 24 h of treatment (Fig. 5C). We therefore treated cells with 20 nM rapamycin for 24 h prior to stimulation with TGFβ for various times and examined Runx1 protein induction by TGFβ. The results (Fig. 5D) demonstrate that even in the presence of rapamycin, TGFβ induces, in a time-dependent manner, Runx1 protein expression. Control blots demonstrated that rapamycin inhibited cytosolic phospho-p70 S6K levels (Fig. 5D, third panel), indicating that mTOR and therefore cap-dependent protein translation was inhibited by rapamycin. Blotting for nuclear GATA6 and cytosolic Hsp-90 demonstrated equal loading (Fig. 5D, second and fourth panels). These results support the idea that TGFβ stimulates Runx1 expression through an IRES-dependent pathway.

Runx1 Is a Co-activator of the Bim Promoter with FOXO3—To determine whether Runx1 was providing the additional activity required for TGFβ induction, we performed Bim luciferase assays in the presence or absence of co-transfected FOXO3 and Runx1. Overexpression of FOXO3 and Runx1

FIGURE 4. Runx1 is specifically induced by TGFβ and stimulates Bim expression. A, Illumina gene array results for AML-12 cells stimulated with TGFβ for 30 min and 1 h. Results for all Runx probes are shown. Normalized values are given. nd, not detectable above background. B, Northern blot of Runx1 mRNA levels in AML-12 cells after TGFβ incubation. The blot was hybridized with a mouse Runx1 3′-UTR-specific probe. The arrow indicates the prominent, large Runx1 transcript induced by TGFβ. β-Actin served as a loading control. C, semiquantitative PCR analysis of Runx1 induction in Hep3B cells after TGFβ incubation. β-Actin served as a loading control. DNA size markers are shown in the left lanes. D, Western blot showing the time course of Runx1 expression in Hep3B cells (top) and primary B-cells (middle) in the presence of TGFβ. Nuclear lysates of Hep3B cells and whole cell lysates of B-cells were analyzed. Hsp-90 served as a loading control (Cont.) for B-cells. E, effect of Runx1A and Runx1B overexpression on Bim luciferase activity in Hep3B cells. After transfection cells were incubated for 24 h before analysis. Luciferase activity was normalized to Renilla activity for each well to control for transfection efficiency. F, effect of Runx1A and Runx1B overexpression on Bim protein levels in Hep3B cells. After transfection, cells were incubated for 24 h before analysis by Western blotting. The level of Runx1 overexpression was demonstrated by blotting for anti-FLAG.
TGFβ Induction of Runx1 and Bim Expression

**FIGURE 5.** TGFβ specifically induces IRES-activated Runx1 mRNA transcripts. A, Northern blot of Runx1 mRNA levels in AML-12 cells after TGFβ incubation. The blot was hybridized with a human Runx1 5′-UTR cap-specific probe (left panel), a mouse Runx1 5′-UTR IRES-specific probe (middle panel), and β-actin as a loading control (right panel). Arrows indicate the Runx1 transcripts used for quantitation. The position of molecular weight standards is shown on the left. B, phosphorimaging analysis was used to quantitate the Northern blot signals in A, and the Runx1/β-actin ratio was calculated for each sample. The values for the TGFβ-treated samples were then normalized to the control value. C, effect of 20 nm rapamycin on the incorporation of [35S]methionine into protein in AML-12 cells. Cells were incubated with rapamycin for the indicated times, and during the last hour [35S]methionine was added to the tissue culture medium. Cells were collected, and the incorporation of [35S] into trichloroacetic acid-precipitable protein was determined. The values for the TGFβ-treated samples were then normalized to the control value. D, Western blot showing the effect of rapamycin on TGFβ-induced Runx1 expression in AML-12 cells. Cells were treated in the absence or presence of 20 nm rapamycin for 24 h, and then TGFβ was added for the indicated times. Rapa, rapamycin; Cyto., cytosolic.

individually increased Bim promoter transactivation, which was further augmented with TGFβ treatment (Fig. 6A). When FOXO3 and Runx1 were co-transfected, Bim promoter transactivation was increased compared with when the two were transfected individually. More importantly, Bim promoter transactivation by the combination of FOXO3 and Runx1 was not increased upon TGFβ treatment, suggesting that both FOXO3 and Runx1 mediate TGFβ transactivation of the Bim promoter. To determine the cis-acting elements in the Bim promoter that mediate TGFβ induction and to further demonstrate the role of FOXO3 and Runx1 in this response, we performed deletion and mutation analyses of the 0.8-kb Bim promoter (Fig. 6C, −699 to +96). Three truncation mutants were produced by stepwise removal of about 200 bp from the 5′-end of the 0.8-kb Bim promoter sequence to generate Bim luciferase constructs of −0.6 kb (−480 to +96), 0.4 kb (−290 to +96), and 0.2 kb (−120 to +96; see Fig. 6C). The results demonstrate that all of the constructs responded to Runx1 and FOXO3, transfected individually or in combination, except for the 0.2-kb Bim luciferase (Fig. 6B). Therefore, FOXO3 and Runx1 must act through a binding site(s) located within the first 200 bp of the 5′-end of the 0.4-kb Bim promoter sequence. Interestingly, bioinformatic analysis of this 200-bp region (MatInspector, Genomatix, Munich, Germany) identified no Runx binding site and a single FOXO binding site (position −174 to −168 relative to the transcription start site) that was also conserved in the human Bim promoter (Fig. 6C). The program also identified other FOXO binding sites as well as a single Runx binding site upstream in the full-length 0.8-kb sequence of the mouse Bim promoter. However, these binding sites were not conserved in the human sequence.

Earlier reports have demonstrated that FOXO3 and Runx3 bind to each other (52). We therefore reasoned that because there are no Runx binding sites in the 0.4-kb Bim luciferase construct, and yet this construct responds to overexpressed Runx, that Runx1 may mediate its effects through its potential interaction with FOXO3. To test this hypothesis, we mutated the FOXO3 binding site in the 0.4-kb Bim luciferase construct (see Fig. 6C) and examined its response to overexpressed Runx1 and FOXO3. The results demonstrated that the mutated 0.4-kb Bim luciferase construct (0.4 m) was much less responsive to Runx1, as well as to FOXO3, compared with the wild-type 0.4- or 0.8-kb Bim luciferase constructs (Fig. 6D). These results support the idea that Runx1 is a co-activator of Bim transcription through its interaction with FOXO3 at the forkhead binding site.

Runx1 Binds the Bim Promoter through FOXO3 Binding Sites—
We next tested the ability of Runx1 to bind DNA through FOXO3 binding sites. COS cells were transfected with HA-FOXO3 in the absence and presence of increasing concentrations of FLAG–Runx1. EMSA analysis was used to demonstrate binding of FLAG–Runx1 to a 32P-labeled oligonucleotide probe containing a 3× concatamerized FOXO3 binding site from the human Bim promoter. Overexpression of FOXO3 led to the appearance of a unique band that was not apparent in control, GFP-transfected cells (Fig. 7A, left panel, arrow). Simultaneous expression of increasing amounts of Runx1 resulted in a dose-
shown (Fig. 7C, middle panel). The binding of Runx1 and endogenous FOXO3 to the forkhead site was unchanged in the presence of TGFβ (data not shown). Control experiments demonstrated that anti-IgG was not able to immunoprecipitate DNA containing the forkhead binding site (data not shown) and that the input DNA was similar for all samples (Fig. 7C, bottom panel). The association of Runx1 and FOXO3 on the forkhead site in the Bim promoter was confirmed in reChIP experiments (Fig. 7D). Taken together, these data support a model in which TGFβ stimulates Bim transcription by up-regulating Runx1 expression, which binds FOXO3 residing at its forkhead binding site and activates Bim promoter activity.

**Runx1 and FOXO3 Interact and Are Required for Endogenous Bim Expression**—To directly test whether Runx1 was serving as a Bim transcriptional co-activator by binding FOXO3, we performed co-immunoprecipitation assays. As shown in Fig. 8A (top panel), when COS cells were transfected with an equal amount of HA-FOXO3 and increasing amounts of FLAG-Runx1, there was a dose-dependent increase in co-precipitated FOXO3 in the anti-FLAG (Runx1) immunoprecipitates. Control blots demonstrated increasing concentrations of transfected FLAG-Runx1 (Fig. 8A, second panel) and equal expression of FOXO3 and equal loading using anti-Hsp-90 (Fig. 8A, bottom two panels). To determine whether endogenous Runx1 and FOXO3 bind, AML-12 cells were treated in the absence and presence of TGFβ, and Runx1 was immunoprecipitated from nuclear extracts. As shown in Fig. 8B (top panel), there was a time-dependent increase in co-precipitated FOXO3 in the anti-Runx1 immunoprecipitates. Control blots demonstrated increasing concentrations of TGFβ-induced Runx1 expression (Fig. 8B, second panel) and equal expression of FOXO3 (Fig. 8B, third panel). Equal loading was demonstrated using anti-GATA6 (Fig. 8B, bottom panel). Taken together, these results support a model in which Runx1 activation of Bim transcription requires binding to FOXO3.

If Runx1 and FOXO3 are required for endogenous Bim expression, then knockdown of these two transcription factors demonstrated that little PCR product was amplified from an upstream site in the Bim promoter that contained no FOXO3 or Runx1 sites by bioinformatic analysis. When anti-FOXO3 antibody was used for immunoprecipitations, FOXO3 was also shown to bind to the same forkhead site of the human Bim promoter and not to the upstream site (Fig. 7C, middle panel). The binding of Runx1 and endogenous FOXO3 to the forkhead site was unchanged in the presence of TGFβ (data not shown). Control experiments demonstrated that anti-IgG was not able to immunoprecipitate DNA containing the forkhead binding site (data not shown) and that the input DNA was similar for all samples (Fig. 7C, bottom panel). The association of Runx1 and FOXO3 on the forkhead site in the Bim promoter was confirmed in reChIP experiments (Fig. 7D). Taken together, these data support a model in which TGFβ stimulates Bim transcription by up-regulating Runx1 expression, which binds FOXO3 residing at its forkhead binding site and activates Bim promoter activity.

**Runx1 and FOXO3 are co-activators of the Bim promoter.** A. Hep3B cells were transfected with Bim luciferase and Runx1B, TM-FOXO3, or both as indicated. After transfection, cells were treated with TGFβ for 24 h. Luciferase activity was normalized to Renilla activity for each well to control for transfection efficiency. B, same as A except that different Bim luciferase deletion constructs were analyzed in the absence of TGFβ. C, schematic showing the 0.8-kb mouse Bim luciferase construct (−699 to +96). The numbering is based upon the transcription start site identified in the original report (40). The transcription start site is −2647 relative to the initiating ATG. Putative forkhead (FHBE), Runx (RBE), and Smad (SBE) binding sites are also identified. D, same as in B except that a new 0.4-kb Bim luciferase construct was analyzed, which contained the mutant FHBE (0.4 M) as shown in C.

dependent disappearance of this band, consistent with the idea that Runx1 binds to FOXO3 and supersedes the band. The levels of overexpressed HA-FOXO3 and FLAG-Runx1 are shown in Fig. 7B. That this band contained FOXO3 was proven true in supershift experiments (Fig. 7A, left panel) demonstrating that anti-FOXO3 and anti-HA tag antibodies were able to supershift the band, whereas anti-IgG antibodies were not. Further experiments, shown in Fig. 7A (right panel), demonstrate that the appearance of this band could be blocked by competition with three different unlabeled oligonucleotide probes, each containing forkhead binding sites (FKHD contains 3× FHBE, FasL promoter contains 4× FHBE, and the 50-bp Bim promoter region contains 1× FHBE) but not by an unlabeled oligonucleotide containing concatamerized Runx binding sites (6× Runx). These data support the idea that Runx1 binds to the Bim promoter via forkhead binding sites.

To verify that Runx1 was able to bind to forkhead sites in the endogenous Bim promoter, ChIP assays were performed following overexpression of FLAG-Runx1 in Hep3B cells. As shown (Fig. 7C, top panel), anti-FLAG antibody was able to immunoprecipitate DNA containing the same forkhead binding site in the human Bim promoter as identified previously in the mouse Bim promoter (Fig. 6C). Control experiments demonstrated that Runx1 was serving as a Bim transcriptional co-activator by binding FOXO3, we performed co-immunoprecipitation assays. As shown in Fig. 8A (top panel), when COS cells were transfected with an equal amount of HA-FOXO3 and increasing amounts of FLAG-Runx1, there was a dose-dependent increase in co-precipitated FOXO3 in the anti-FLAG (Runx1) immunoprecipitates. Control blots demonstrated increasing concentrations of transfected FLAG-Runx1 (Fig. 8A, second panel) and equal expression of FOXO3 and equal loading using anti-Hsp-90 (Fig. 8A, bottom two panels). To determine whether endogenous Runx1 and FOXO3 bind, AML-12 cells were treated in the absence and presence of TGFβ, and Runx1 was immunoprecipitated from nuclear extracts. As shown in Fig. 8B (top panel), there was a time-dependent increase in co-precipitated FOXO3 in the anti-Runx1 immunoprecipitates. Control blots demonstrated increasing concentrations of TGFβ-induced Runx1 expression (Fig. 8B, second panel) and equal expression of FOXO3 (Fig. 8B, third panel). Equal loading was demonstrated using anti-GATA6 (Fig. 8B, bottom panel). Taken together, these results support a model in which Runx1 activation of Bim transcription requires binding to FOXO3.

FIGURE 6. **Runx1 and FOXO3 are co-activators of the Bim promoter.** A, Hep3B cells were transfected with Bim luciferase and Runx1B, TM-FOXO3, or both as indicated. After transfection, cells were treated with TGFβ for 24 h. Luciferase activity was normalized to Renilla activity for each well to control for transfection efficiency. B, same as A except that different Bim luciferase deletion constructs were analyzed in the absence of TGFβ. C, schematic showing the 0.8-kb mouse Bim luciferase construct (−699 to +96). The numbering is based upon the transcription start site identified in the original report (40). The transcription start site is −2647 relative to the initiating ATG. Putative forkhead (FHBE), Runx (RBE), and Smad (SBE) binding sites are also identified. D, same as in B except that a new 0.4-kb Bim luciferase construct was analyzed, which contained the mutant FHBE (0.4 M) as shown in C.

FIGURE 6. **Runx1 and FOXO3 are co-activators of the Bim promoter.** A, Hep3B cells were transfected with Bim luciferase and Runx1B, TM-FOXO3, or both as indicated. After transfection, cells were treated with TGFβ for 24 h. Luciferase activity was normalized to Renilla activity for each well to control for transfection efficiency. B, same as A except that different Bim luciferase deletion constructs were analyzed in the absence of TGFβ. C, schematic showing the 0.8-kb mouse Bim luciferase construct (−699 to +96). The numbering is based upon the transcription start site identified in the original report (40). The transcription start site is −2647 relative to the initiating ATG. Putative forkhead (FHBE), Runx (RBE), and Smad (SBE) binding sites are also identified. D, same as in B except that a new 0.4-kb Bim luciferase construct was analyzed, which contained the mutant FHBE (0.4 M) as shown in C.
Bim expression in response to TGFβ demonstrate that siRNA knockdown of Runx1 or FOXO3 decreases Western blotting and RT-PCR, respectively. The results demonstrated that siRNA knockdown of Runx1 or FOXO3 decreases Bim expression in response to TGFβ (Fig. 8C, top left panel). Knockdown of Bim served as a positive control. Western blotting and RT-PCR analysis of Runx1 mRNA transcript levels (Fig. 8C, right panel) demonstrated effective knockdown of target proteins and no off-target effects. Hsp-90 served as a loading control for whole cell lysates (Fig. 8C, bottom left panel). These results support a role for Runx1 and FOXO3 in TGFβ-induced Bim expression.

FIGURE 7. Runx1 binds the Bim promoter through FOXO3 binding sites. A, EMSAs of Runx1 binding to forkhead binding sites. COS cells were transfected with a constant amount of HA-TM-FOXO3 and increasing amounts of FLAG-RUNX1B. Nuclear extracts were prepared and preincubated with supershifting antibodies (left panel) or excess, unlabeled oligonucleotides (right panel) as indicated, before the addition of the 32P-labeled 3x-forkhead probe. The probe lane contains no nuclear extract but all other components of the EMSA buffer. Arrows indicate bands specifically labeled in the presence of overexpressed FOXO3. B, control Western blots showing the expression of HA-TM-FOXO3 and FLAG-RUNX1B in the nuclear extracts used in the EMSAs described in A. C, Hep3B cells were transfected with a low (L, 10 μg) or high (H, 30 μg) dose of FLAG-RUNX1B. Cross-linked lysates were prepared, and equal amounts were immunoprecipitated with either anti-FLAG or anti-FOXO3 antibody. PCR analyses were performed on the purified ChIP extracts to amplify a band containing the FHBE in the human Bim promoter (Fox site) or an upstream site lacking a forkhead/Runx site (Upstream site). PCR was also performed on the input DNA samples obtained before ChIP analyses. D, reChIP experiments were also performed on anti-FLAG ChIP samples. FLAG-ChIP samples were immunoprecipitated with either anti-IgG or anti-FOXO3, and the FOX site was amplified. Cont, control.

In the present study, we have demonstrated a direct transcriptional activation of the murine Bim promoter by TGFβ, providing evidence that it is due to the induction of Runx1. Runx proteins are a family of transcription factors that share a Runt homology domain (RHD) and play important roles in development and cancer. Knock-out studies in mice have shown that Runx1 is vital for hematopoiesis and neural development, Runx2 for bone formation, and Runx3 for gastric epithelial cell proliferation (54). Previous studies have demonstrated that TGFβ induces the expression of Runx2 in C2C12 mesenchymal stem cells (55) and Runx3 (AML2) in I.29m B-cells (56). This is the initial observation that TGFβ induces Runx1. Runx proteins have previously been implicated in mediating growth arrest and apoptosis of TGFβ family members. For example, in the Saos2 osteosarcoma cell line, bone morphogenetic protein induces Runx2 expression, which is a direct transcriptional activator of Bax, increasing its expression and sensitizing the cells to apoptosis (57). Overexpression of Runx3 restored TGFβ-induced p21 expression and growth arrest, but not apoptosis, in a Runx3-null biliary tract cancer cell line (58) and in stomach epithelial cells (59). Gastric cancer cells expressing a dominant-negative form of Runx3 or antisense Runx3 were resis-
We were unable to detect mRNA transcripts for Runx2 or Runx3 by PCR in either Hep3B or AML-12 cells even after TGFβ/H9252 treatment (data not shown). These negative results confirmed our gene array analysis in AML-12 cells (Fig. 4A). However, we readily detected basal levels of Runx1 mRNA in both AML-12 and Hep3B hepatocytes, and its levels were induced by TGFβ (Fig. 4B and C). These results imply that both Runx1 and Runx3 isoforms are cell-specific mediators of TGFβ-induced Bim expression to elicit cell death. This idea is supported by the finding that both Runx1 and Runx3, but not Runx2, have been shown to be frequently down-regulated, along with their co-activator core-binding factor B, in gastric and hepatocellular cancers (60, 61).

Our finding that Runx1 activates the Bim promoter in cooperation with FOXO3 is not surprising given that Runx proteins have been shown previously to serve as transcriptional co-activators with other proteins, including Smads (41, 54). The forkhead site at −174 to −168, which we identified as being important for Runx1/FOXO3 activation, has also been shown to be important for activation of the rat Bim promoter (28). Other reports showing direct activation of the Bim promoter by FOXO3 have not identified the exact site(s) (29, 44). Runx3 has also been shown to directly activate the Bim promoter, although at widely divergent sites. In mouse embryonic fibroblasts, the RBE located from −609 to −603 of the mouse promoter (see Fig. 6C) was shown to be activated by Runx3 (52), whereas in gastric cancer cells, two RBEs were identified in the human Bim promoter but were located far upstream of the mouse RBE (45). Interestingly, we always detected a decreased stimulatory response to Runx1 and FOXO3 in our 0.4-kb Bim luciferase construct when compared with the 0.8-kb construct (see Fig. 6, B and D). Thus, the Runx and forkhead binding elements at the 5′-end of the mouse Bim promoter may be active in our hepatic cells as well, albeit to a lesser extent than in mouse embryonic fibroblasts.

All Runx proteins are similar in that transcription can be initiated from two different promoters, designated the distal (P1) and proximal (P2) promoters (48). The Runx1 protein produced by the P1 promoter is translated from a cap-dependent mRNA transcript and encodes a longer N-terminal amino acid sequence (19–32 amino acids) relative to that produced from the P2 promoter (5 amino acids). More importantly, the Runx1 protein produced from the P2 promoter is translated from an IRES-dependent mRNA transcript (62). The diversity of Runx1 proteins expressed by cells is further complicated because of alternative splicing of the primary mRNA transcripts and post-translational modification of the proteins themselves by phosphorylation, acetylation, and ubiquitination (48, 49). Our results indicate that TGFβ specifically induces only IRES-activated Runx1 mRNA transcripts and protein. Although many Runx1 mRNA transcripts could be detected by
Northern blotting using a probe against the common 3′-UTR of Runx1, TGFβ induction was most apparent for the largest transcript (Fig. 4B). The size of this TGFβ-induced Runx1 mRNA was similar to the largest and most abundant transcript identified with the IRES-specific probe and much greater than any transcript identified with the cap-specific probe (Fig. 5A). As further proof of their identity, the time course of TGFβ induction of the largest Runx1 mRNA transcript was also similar using either the common 3′-UTR or the IRES-specific probes. At present, it is unclear whether the presence of multiple TGFβ-induced Runx1 mRNA transcripts explains the multiple TGFβ-induced Runx1 protein bands identified on Western blots (Figs. 4D and 5D) or if this is due to post-translational modification of the Runx1 protein. Although Runx1 protein modification could be either TGFβ-dependent or -independent, there are reports that TGFβ stimulates Runx3 acetylation, which prevents its ubiquitination and degradation (63).

Previous studies have demonstrated that the Runx1 5′-UTR, used in our study as an IRES-specific probe, does in fact direct cap-independent, IRES-initiated mRNA translation (62). In addition, a gene array study demonstrated that the Runx1 mRNA translation state, a measure of ribosome-bound mRNA, was actually increased in the presence of rapamycin over time in U87 and LAPC-4 cell lines (50). This result is opposite to that expected for a cap-dependent mRNA transcript. Thus, these two independent studies support our claim that the Runx1 mRNA transcript(s) induced by TGFβ in hepatic cells is indeed IRES-dependent. This is consistent with the hypothesis that during apoptosis there must be a switch from cap-dependent to cap-independent translation because of caspase cleavage of the cap-dependent translation machinery (64). Indeed, many IRES-dependent eukaryotic mRNAs have been identified that encode proteins associated with cell proliferation and cell death, such as c-Myc and Apaf-1, respectively (64, 65).

Taken together, our data support a model in which TGFβ stimulates Bim transcription by up-regulating Runx1 expression, which binds FOXO3, residing at its forkhead binding site, and activates Bim promoter activity and expression. Of particular interest is our finding that TGFβ specifically induces IRES-dependent expression of Runx1. Future studies are needed to determine whether the target of TGFβ-induced Runx1 activation, Bim, is also translated from IRES-dependent mRNA transcripts. Moreover, it will also be of interest to determine whether IRES-dependent mRNA translation is a common feature of TGFβ-induced apoptosis.

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REFERENCES

1. Schmierer, B., and Hill, C. S. (2007) Nat. Rev. Mol. Cell Biol. 8, 970–982
2. Rahimi, R. A., and Leof, E. B. (2007) J. Cell. Biochem. 102, 593–608
3. Siegel, P. M., and Massagué, J. (2003) Nat. Rev. Cancer 3, 807–817
4. Moustakas, A., and Heldin, C. H. (2005) J. Cell Biol. 168, 3573–3584
5. Javelaud, D., and Mauviel, A. (2005) Oncogene 24, 5742–5750
6. Schuster, N., and Kriegstein, K. (2002) Cell Tissue Res. 307, 1–14
7. Sánchez-Capel, A. (2005) Cytokine Growth Factor Rev. 16, 15–34
8. Yu, J., Zhang, L., Chen, A., Xiang, G., Wang, Y., Wu, J., Mitchelson, K., Cheng, J., and Zhou, Y. (2008) J. Cell. Physiol. 215, 422–433
9. Jang, C. W., Chen, C. H., Chen, C. C., Chen, J. Y., Su, Y. H., and Chen, R. H. (2002) Nat. Cell Biol. 4, 51–58
10. Perlman, R., Schiemann, W. P., Brooks, M. W., Lodish, H. F., and Weinberg, R. A. (2001) Nat. Cell Biol. 3, 708–714
11. Yoo, J., Ghiassi, M., Firmanova, L., Balliet, A. G., Hoffman, B., Fornace, A. J., Jr., Liebermann, D. A., Bottinger, E. P., and Roberts, A. B. (2003) J. Biol. Chem. 278, 43001–43007
12. Yamamura, Y., Hua, X., Bergelson, S., and Lodish, H. F. (2000) J. Biol. Chem. 275, 36295–36302
13. Herzer, K., Grosse-Wilde, A., Krammer, P. H., Galle, P. R., and Kanzler, S. (2008) Mol. Cancer Res. 6, 1169–1177
14. Kim, B. C., Mamura, M., Choi, K. S., Calabretta, B., and Kim, S. J. (2002) Mol. Cell. Biol. 22, 1369–1378
15. Wildey, G. M., Patil, S., and Howe, P. H. (2003) J. Biol. Chem. 278, 18069–18077
16. Ogushi, M., Kuroki, S., Fukamachi, H., O’Reilly, L. A., Kuida, K., Strasser, A., and Yonehara, S. (2005) Mol. Cell. Biol. 25, 10017–10028
17. Ramjaun, A. R., Tomlinson, S., Eddaooudi, A., and Downward, J. (2007) Oncogene 26, 970–981
18. Strasser, A. (2005) Nat. Rev. Immunol. 5, 189–200
19. Adams, J. M., and Cory, S. (2007) Oncogene 26, 1324–1337
20. Galonek, H. L., and Hardwick, J. M. (2006) Nat. Cell Biol. 8, 1317–1319
21. Bouillet, P., Metcalf, D., Huang, D. C., Tarlinton, D. M., Kay, T. W., Köntgen, F., Adams, J. M., and Strasser, A. (1999) Science 286, 1735–1738
22. Roset, R., Orlet, F., and Gil-Gomez, G. (2007) Front. Biosci. 12, 4722–4730
23. Kaufmann, T., Jost, P. J., Pellegrini, M., Puthalakath, H., Gugasyan, R., Gerondakis, S., Cretney, E., Smyth, M. J., Silke, J., Hakem, R., Bouillet, P., Mak, T. W., Dixit, V. M., and Strasser, A. (2009) Immunity 30, 56–66
24. Corazza, N., Jakob, S., Schaer, C., Frese, S., Keogh, A., stroka, D., Kassahn, D., Torgler, R., Mueller, C., Schneider, P., and Brunner, T. (2006) J. Clin. Invest. 116, 2493–2499
25. Ewings, K. E., Wiggins, C. M., and Cook, S. J. (2007) Cell Cycle 6, 2236–2240
26. Hübner, A., Barrett, T., Flavell, R. A., and Davis, R. J. (2008) Mol. Cell 30, 415–425
27. Ramesh, S., Qi, X. J., Wildey, G. M., Robinson, J., Molkentin, J., Letterio, J., and Howe, P. H. (2008) EMBO Rep. 9, 990–997
28. Gilley, J., Coffer, P. J., and Ham, J. (2003) J. Cell Biol. 162, 613–622
29. Cai, B., and Xia, Z. (2008) Apoptosis 13, 803–810
30. Burgering, B. M., and Medema, R. H. (2003) J. Leukocyte Biol. 73, 689–701
31. Fu, Z., and Tindall, D. J. (2008) Oncogene 27, 2312–2319
32. Wu, J. C., Merlino, G., and Fausto, N. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 674–678
33. Qi, X. J., Wildey, G. M., and Howe, P. H. (2006) J. Biol. Chem. 281, 813–823
34. Bouillet, P., Zhang, L. C., Huang, D. C., Webb, G. C., Bottema, C. D., Shore, P., Eyer, H. J., Sutherland, G. R., and Adams, J. M. (2001) Mammm. Genome 12, 163–168
35. Kitabayashi, I., Aikawa, Y., Nguyen, L. A., Yokoyama, A., and Ohki, M. (2001) EMBO J. 20, 7184–7196
36. Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Joo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999) Cell 96, 857–868
37. Hardy, R. R., Carmack, C. E., Shinton, S. A., Kemp, J. D., and Hayakawa, K. (1991) J. Exp. Med. 173, 1213–1225
38. Schreiber, E., Matthias, P., Müller, M. M., and Schaffner, W. (1989) Nucleic Acids Res. 17, 6419
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