HoxA10 Regulates Transcription of the Gene Encoding Transforming Growth Factor β2 (TGFβ2) in Myeloid Cells*

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HoxA10 is a homeodomain transcription factor that is maximally expressed in myeloid progenitor cells. HoxA10 is overexpressed in a poor prognosis subset of human acute myeloid leukemia (AML) and in vivo overexpression of HoxA10 in murine bone marrow induces myeloid leukemia. HoxA10 contributes to myeloid progenitor expansion and differentiation block, but few target genes have been identified that explain the influence of HoxA10 on these processes. The current study identifies the gene encoding transforming growth factor β2 (TGFβ2) as a HoxA10 target gene. We found that HoxA10 activated TGFβ2 transcription by interacting with tandem cis elements in the promoter. We also determined that HoxA10 overexpression in myeloid progenitor cells increases TGFβ2 production by the cells. TGFβ2 stimulates proliferation of hematopoietic stem and progenitor cells. Therefore, these studies identified autocrine stimulation of myeloid progenitors by TGFβ2 as one mechanism by which HoxA10 expands this population. Because HoxA proteins had not been previously known to influence expression of pro-proliferative cytokines, this has implications for understanding molecular mechanisms involved in progenitor expansion and the pathobiology of AML.

A number of clinical correlative studies implicate Hox proteins in myeloid leukemogenesis. These studies associated increased expression of HoxB3, B4, and A9–11 in CD34+ bone marrow cells with poor prognosis in human acute myeloid leukemia (AML) (3–5). This pattern of HOX gene expression was found in AML with chromosomal translocations involving the MLL gene (11q23 leukemia) as well as with other leukemia-associated chromosomal translocations (6–9). Increased expression of this specific set of HOX genes was also described in a poor prognosis subset of cytogenetically normal AML.

Studies in murine models support a functional role for Hox proteins in leukemogenesis. Overexpression of HoxB3 or B4 in murine bone marrow expands the hematopoietic stem cell population and leads to a myeloproliferative disorder in vivo (10, 11). Overexpression of either HoxA9 or A10 in murine bone marrow induces a myeloproliferative disorder characterized by expansion of the committed myeloid progenitor population (granulocyte/monocyte progenitors or GMP) (12–16).

Although HoxA9 and A10 both expand the progenitor population, they exhibit opposing influences on differentiation; HoxA10 blocks differentiation and HoxA9 is involved in myeloid versus lymphoid lineage choice (17, 18). Perhaps consistent with this, the myeloproliferative disorder that develops in HoxA10 overexpressing mice progresses to AML over a number of months (16, 17). However, the myeloproliferative disorder in mice with HoxA9 overexpression in the bone marrow only progresses to AML in the presence of co-overexpression of the proto-oncogene Meis1 (19).

To investigate mechanisms by which overexpressed Hox proteins contribute to myeloid leukemogenesis, we have been identifying HoxA10 target genes. In myeloid progenitor cells, we found that HoxA10 represses a number of genes that encode phagocyte effector proteins (20–22). Decreased HoxA10 repression activity contributes to acquisition of phagocyte functional competence as differentiation proceeds. These studies provide a mechanism for phenotypic differentiation block by overexpressed HoxA10.

We also identified DUSP4 as a HoxA10 target gene (23). DUSP4 encodes mitogen-activated protein kinase 2 (MKP2); an inhibitor of c-Jun N-terminal kinase (JNK). Activation of DUSP4 transcription by overexpressed HoxA10 impairs JNK-induced apoptosis of progenitors and differentiating myeloid cells. This provides a mechanism for HoxA10-induced myeloid expansion in leukemia. We found that HoxA10 activates the ITGB3 gene (encoding β3 integrin) in myeloid cells (24).

Increased expression of αvβ3 integrin in HoxA10-overexp-
pressing cells might facilitate progenitor expansion via interaction with vitronectin and fibronectin in bone marrow stroma.

In the current study, we identified TGFβ2 as a HoxA10 target gene using a chromatin co-immunoprecipitation-based screening approach. This gene encodes transforming growth factor β2 (Tgfβ2), a member of the Tgfβ superfamily. Tgfβ1, -2, and -3 are homologous proteins that are encoded by separate genes (25). These proteins all bind to type I and II Tgfβ receptors (Tgfβ-R), but with differing affinities; Tgfβ1 and -3 bind most receptor isoforms with a greater affinity than Tgfβ2 (26). The functional activities of the three Tgfβ proteins also differ. Tgfβ1 stimulates cell proliferation at low concentrations, but decreases proliferation at higher concentrations (25, 27). In contrast, Tgfβ3 always represses cell proliferation and Tgfβ2 always stimulates proliferation (25, 27). The mechanism for these differences in activity are not known.

Signaling through Tgfβ-R is classically associated with activation of Smad transcription factors and consequent transcription of genes that inhibit cell proliferation. However, ligand binding to Tgfβ-R also activates mitogen-activated protein kinases (MAP kinases), in a Smad-independent manner, in some cell types (28, 29). Although activation of some MAP kinases is associated with a proliferative response, a direct connection between Tgfβ signaling, MAP kinase activation, and proliferation has not been made. Differential activation of such signaling pathways may contribute to the diverse activities of Tgfβ1 versus Tgfβ2 (30, 31).

Tgfβ2 is normally expressed in various hematopoietic cell populations (30, 31). Tgfβ2 is secreted by mature phagocytic cells and modulates phagocyte functional activities (31, 32). Of particular interest to our studies, CD34+ human bone marrow cells also secrete Tgfβ2, which stimulates proliferation in an autocrine manner (30). Therefore, regulation of TGFβ2 transcription may be a mechanism by which HoxA10 facilitates expansion of hematopoietic stem cells and myeloid progenitors. Activation of TGFβ2 transcription in leukemia by overexpressed HoxA10 might be a mechanism for expansion of the leukemia stem cell population, contributing to adverse prognosis.

MATERIALS AND METHODS

Plasmids

**Protein Expression Vectors**—The cDNA for human HoxA10 (obtained from C. Largman, University of California, San Francisco) (33) was subcloned into the mammalian expression vector pcDNA3amp (Invitrogen) and the murine retroviral vector pMSCVpuro (Clontech), as previously described (20–24). For some experiments, single base pair mutations were introduced into the HoxA10 cDNA to change tyrosine residues 326 and 343 to phenylalanine (referred to as Y-mut HoxA10), as previously described (22).

**TGFβ2 Reporter Vectors**—Various fragments of the TGFβ2 5’ flanking region were obtained by genomic PCR from U937 chromatin. The fragments were sequenced to ensure identity with the published sequence and subcloned into the pGL3-basal reporter vector (Promega). Additional constructs were generated in the pGL3-promoter vector with three copies of the proximal (−410 to −385 bp) or distal (−1506 to −1478 bp) HoxA10-binding cis elements from the TGFβ2 promoter. Such constructs were generated with the wild type proximal and distal TGFβ2 sequences, and with forms of the sequences with mutation of the Hox-binding consensus sequences (as described below).

**Oligonucleotides**

Oligonucleotides were custom synthesized by MWG Biotech (Piedmont, NC). Double-stranded oligonucleotides were synthesized for use in DNA-affinity purification assays including the −410 to −385-bp sequence (5’-CGTGGTTCAGAGAGAACGGCGAAATCTC-3’), a non-HoxA10 binding mutant sequence (5’-CGTGGTTCAGAGAACGGCGAAATCTC-3’), the −1506 to −1478-bp sequence (5’-CTTCCTCTAAATT-TATTTCTACTAATTAG-3’), or a non-HoxA10 binding mutant sequence (5’-CTTCCTCTAAATTGGCGTTCTACTT-3’).

**Myeloid Cell Line Culture**

The human myelomonocytic leukemia cell line U937 (34) was obtained from Andrew Kraft (Hollings Cancer Center, Medical University of South Carolina, Charleston, SC). Cells were maintained as described (35). U937 cells were treated for 48 h with 500 units/ml of human recombinant IFNγ (Roche Applied Science) or retinoic acid + dimethylformamide for monocyte or granulocyte differentiation, respectively.

**Primary Murine Bone Marrow Studies**

Animal studies were performed according to a protocol approved by the Animal Care and Use Committees of Northwestern University and Jesse Brown Veterans Affairs Medical Center.

**Bone Marrow Harvest and Culture**—Bone marrow mononuclear cells were obtained from the femurs of WT or HoxA10−/− C57/BL6 mice (36). Sca1+ cells were separated using the Miltenyi magnetic bead system (Miltenyi Biotechnology, Auburn, CA). Bipotential myeloid progenitor cells (GMP) were cultured (at a concentration of 2 × 10^5 cells per ml) for 48 h in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1% penicillin-streptomycin, 10 ng/ml of murine GM-CSF (R & D Systems Inc., Minneapolis, MN), 10 ng/ml of murine recombinant IL-3 (R & D Systems Inc.), and 100 ng/ml of SCF (R & D Systems Inc.). Cells were maintained in GM-CSF, IL3, and SCF for 48 h, or were differentiated over 48 h in 20 ng/ml of G-CSF (granulocyte) or 20 ng/ml of murine M-CSF (monocyte) (as described in Ref. 17).

**Bone Marrow Retroviral Transduction**—Retrovirus was generated with the HoxA10/MSCV plasmid or control MSCV plasmid using the Phoenix cell packaging line according to the manufacturer’s instructions (Stratagene, La Jolla, CA). The average concentration of producer cell supernatants was 10^7 pfu/ml. Bone marrow mononuclear cells were cultured for 24 h in 10 ng/ml of IL3, 10 ng/ml of GM-CSF, and 100 ng/ml of SCF. Cells were transduced by incubation with retroviral...
supernatant in the presence of Polybrene (6 μg/ml) as previously described (17). Transduced cells were selected for 48 h in puromycin, cultured in GM-CSF, IL3, and SCF or differentiated with M-CSF or G-CSF and used for TGFβ2 expression studies and proliferation assays, as described below. Transgene expression was confirmed by real time PCR.

Quantitative Real Time PCR
RNA was isolated using TRIzol reagent (Invitrogen) and tested for integrity by denaturing gel electrophoresis. Primers were designed with Applied Biosystems software and real time PCR was performed using SYBR Green according to the “standard curve” method. Results were normalized to 18 S (for mRNA determination) or input chromatin (for chromatin immunoprecipitation studies).

Chromatin Immunoprecipitation and Gene Discovery
U937 cells were cultured with or without IFNγ or RA for 48 h. Cells were incubated briefly in medium supplemented with formaldehyde to generate DNA–protein cross-links. For CpG island array studies, cell lysates were sonicated to generate chromatin fragments with an average size of 2.0 kb (37). Lysates underwent immunoprecipitation with either HoxA10 antiserum or preimmune serum (37, 23). Antibody to HoxA10 was custom generated to a HoxA10-specific peptide, as previously described (23). Precipitated chromatin was recovered and several batches of immunoprecipitated, amplified chromatin were combined for each experiment, as described (37). Chromatin was labeled and used to screen a CpG island microarray, as described (23).

Identified genes were confirmed by independent chromatin immunoprecipitation experiments. For these studies, chromatin was co-immunoprecipitated from U937 lysates, as described above. In initial studies, lysates were sonicated to generate chromatin fragments of 500 bp. This precipitated chromatin was analyzed by PCR using primer sets to amplify various 500-bp sequences in the 5′ flank or first exon (as a negative control). PCR products were separated by agarose gel electrophoresis. In other studies, cell lysates were sonicated to generate chromatin fragments of ~200 bp. This chromatin was used in quantitative real time PCR experiments.

Myeloid Cell Line Transfections and Assays
Stable Transfectants—U937 cells were transfected by electroporation with equal amounts of a HoxA10 expression vector or empty pcDNAamp vector control plus a vector with a neomycin phosphotransferase cassette (pSRα) (30 μg each). Stable pools of cells were selected in G418 (0.5 mg/ml), and aliquots of cells were tested for HoxA10 expression by real time PCR and Western blot.

TGFβ2 Promoter Analysis—U937 cells were co-transfected with a construct with various sequences from the TGFβ2 5′ flank linked to a luciferase reporter (2.4 kb, 1.5 kb, 980 bp, 410 bp, or 360 bp TGFβ2/pGL3 or pGL3 control) (30 μg), a vector to overexpress HoxA10 (or empty vector control) (50 μg), and β-galactosidase reporter vector to control for transfection efficiency (CMVβ-gal). In other experiments, cells were co-transfected with an artificial promoter/reporter vector with three copies of the WT or Hox-binding mutant −410 to −385 bp (proximal) or −1506 to −1478 bp (distal) sequence from the TGFβ2 promoter (using the pGL3-p vector) (30 μg), a vector to express HoxA10, or a tyrosine mutant form of HoxA10 (or empty vector control) (50 μg), and CMVβ-gal. Reporter assays were performed with or without 48 h of treatment with IFNγ (400 units/ml) or retinoic acid + dimethylformamide.

Western Blots
U937 or murine bone marrow cells were lysed by boiling in 2× SDS sample buffer. Lysate proteins (50 μg) were separated by SDS-PAGE (8% acrylamide) and transferred to nitrocellulose. Western blots were serially probed with antibodies to HoxA10, phospho-ERK, total ERK, phospho-Smad2/3, total Smad2/3, and GAPDH (to control for loading). Each experiment was repeated at least three times with different batches of lysate proteins. Representative blots are shown.

In some studies, proteins were immunoprecipitated from U937 nuclear protein extracts (300 μg) under denaturing conditions using an antibody to phosphotyrosine or irrelevant control antibody (as described (22)). Proteins were separated by SDS-PAGE and Western blots were probed with antibody to HoxA10, as above. Samples of 1/10 the input protein were run in adjacent lanes to control for the abundance of HoxA10 in the various samples. Cells for these studies were U937 stable transfectants overexpressing HoxA10.

ELISA
Expression of Tgfβ2 in the medium of cultured cells was determined using the Emax ImmunoAssay System according to the manufacturer’s instructions (Promega). For these studies, cells were cultured to maintain a cell density of 0.6 × 10⁶ cells/ml. Equivalent amounts of medium were withdrawn at various time points for the assay. This assay system is specific for Tgfβ2 and does not cross-react with Tgfβ1 or -3.

Cell Proliferation Assays
Myeloid Cell Lines—U937 cells that were stably transfected with a vector to overexpress HoxA10 or empty vector control were deprived of fetal calf serum for 24 h and then treated with a dose titration of fetal calf serum (0.01 to 10%). Some cells were incubated with a blocking antibody to Tgfβ2 or irrelevant antibody control (R & D Systems, Inc.). Cell proliferation was determined by incorporation of [3H]thymidine (for the last 8 h of incubation) according to standard techniques. In other experiments, U937 cells were treated with a dose titration of human recombinant TGFβ2, with or without the ERK inhibitor PD98056, and proliferation was determined.

Murine Bone Marrow—Murine bone marrow cells that had been transduced with a retroviral vector to overexpress HoxA10 or empty MSCV vector control were cultured in GM-CSF (10 ng/ml), IL3 (5 ng/ml), or SCF (100 ng/ml) and deprived of cytokines for 24 h (cultured in DMEM supplemented with 10% FCS only). Cells were stimulated for 24 h with a dose titration of GM-CSF (0.01 to 10 ng/ml in the presence of 5 ng/ml of IL3). Some cells were incubated with a
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blocking antibody to Tgfβ2 or control antibody. Cell proliferation was determined by incorporation of [3H]thymidine over the last 8 h of incubation.

In Vitro DNA Binding Assays

Isolation of Nuclear Proteins—Nuclear proteins were extracted from U937 cells by the method of Dignam (38) with protease inhibitors, as described (35).

DNA Affinity Purification Assays—Nuclear proteins (300 μg) were incubated with biotin-labeled double-stranded oligonucleotide probe representing the proximal (−410 to −385 bp) or distal (−1506 to −1478 bp) TGFβ2 promoter sequences overnight in DNA affinity purification assay buffer (25 mM HEPES (pH 7.6), 60 mM KCl, 5 mM MgCl2, 7.5% glycerol, 0.1 mM EDTA, 1 mM DTT, and 0.25% Triton X-100). The DNA-protein complexes were precipitated with 50 μl of a 50% slurry of neutravidin-coated agarose beads (Pierce). Bound proteins were eluted from the beads, separated by SDS-PAGE (10% acrylamide), and transferred to nitrocellulose. Western blots were probed with an antibody to HoxA10 obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Probes with a mutation in the Hox binding consensus sequence were negative controls in this study and total input protein was a positive control for protein loading.

Genomic Sequence Analysis

Conserved genomic sequences and consensus sequences for Hox protein DNA binding were identified using the VISTA software (Genomics Division of the Lawrence Berkeley National Laboratory (Berkley, CA) (39–41).

Statistical Analysis

Statistical significance was determined by Student’s t test and analysis of variance methods using SigmaPlot and SigmaStat software. Graphs are presented with error bars representing standard error calculations.

RESULTS

Identifying TGFβ2 as a HoxA10 Target Gene—To identify novel HoxA10 target genes, we coupled chromatin co-immunoprecipitation with a high throughput screening approach, as previously described (23). We used cells of the U937 myeloid leukemia line as a model for myelopoiesis in these studies. U937 cells can be differentiated with various agents in- cluding IFNγ, tumor necrosis factor α and phorbol myristate acetate (for monocyte differentiation), or retinoic acid and dimethylformamide (for granulocyte differentiation). U937 transfection was alleviated by phagocyte functional competence, increased sensitivity to apoptosis, and initial proliferation followed by proliferation arrest (20, 21, 34). Therefore, these cells provide a reasonable model for myelopoiesis and permitted us to identify the interaction of HoxA10 with target genes at various differentiation stages.

For the current studies, chromatin was co-precipitated from lysates of IFNγ-differentiated U937 cells with an antibody to HoxA10 or with preimmune serum. Co-precipitating chromatin was labeled and used to probe a CpG island microarray. HoxA10 co-precipitating chromatin exhibited enhanced hybridization to a CpG island from the TGFβ2 5′ flank. This CpG island was located between −595 and −886 bp relative to the ATG start codon of the gene (Fig. 1A). Because chromatin for these studies was sheared to ~2.0-kb fragments prior to co-precipitation, this identified a possible HoxA10 binding site somewhere in the proximal 2.5 kb of the 5′ flank, or within the first 1.5 kb of exon 1.

We analyzed the human TGFβ2 5′ flank for homology to the murine gene and for conserved HoxA10-DNA binding consensus sequences. Because HoxA10 frequently binds to DNA as a heterodimer with Pbx or Meis proteins, we investigated the 5′ flank for DNA-binding consensus sequences for Hox proteins, Pbx/Pbx dimers, or Hox/Meis dimers. We identified 8 conserved, potential HoxA10 binding sites within the proximal 1.5 kb of TGFβ2 5′ flank (relative to the transcription start site) (Fig. 1A).

To further define HoxA10 binding sites in the TGFβ2 5′ flank, additional chromatin co-immunoprecipitation studies were performed with U937 cells and HoxA10 antibody (or preimmune serum). To determine whether interaction between HoxA10 and TGFβ2 was differentiation stage-specific, studies were performed with or without differentiation to monocytes with IFNγ, or granulocytes with retinoic acid (RA). Co-precipitating chromatin was PCR amplified using primers to various sequences in the proximal 2.4 kb of 5′ flank and exon 1. We found that the proximal 1.5 kb, 980 bp, and 410 bp of the TGFβ2 5′ flank (relative to the transcription start site) co-immunoprecipitated with HoxA10 from untreated and differentiated U937 cells (Fig. 1B). In contrast, chromatin representing 285 bp of the proximal 5′ flank, the sequence from −1.5 to −2.4, or the first exon did not co-pre- cipitate with antibody to HoxA10. These studies identified possible HoxA10 binding between the −285 and −410-bp sequence of TGFβ2 5′ flank.

HoxA10 Activates Two TGFβ2 cis Elements—We next investigated the effect of HoxA10 overexpression on activity of the TGFβ2 promoter. For these studies, a series of reporter gene constructs were generated representing truncations of the TGFβ2 5′ flank (2.4 kb, 1.5 kb, 980 bp, 410 bp, and 360 bp from the transcription start site). These reporter constructs (or empty reporter vector) were co-transfected into U937 cells with a vector to overexpress HoxA10 (or empty expression vector). Transfectants were analyzed with or without differentiation with IFNγ or RA.

In untreated transfectants, HoxA10 overexpression significantly increased reporter expression from all except the 360-bp construct (Fig. 2A). Although differentiation did not significantly increase the activity of the TGFβ2 constructs in control transfections, HoxA10 induced a significantly greater increase in reporter expression from the 2.4-kb, 1.5-kb, 980-bp, and 410-bp constructs in differentiated transfectants in comparison to undifferentiated transfectants (Fig. 2A).

HoxA10 overexpression also induced a significantly greater increase in reporter activity from the 2.4- and 1.5-kb constructs in comparison to the increase in activity from the 980- and 410-bp constructs (approximately twice as much: 3.7 ± 0.2-fold increase versus 2.0 ± 0.1-fold increase, p < 0.001, n = 4) (Fig. 2B). These results suggested that there might be
two cis elements in the TGFβ2 promoter that were activated by HoxA10: one between 1.5 kb and 980 bp and another between 980 and 410 bp.

Neither differentiation nor HoxA10 overexpression had any effect on reporter activity from the empty, control reporter construct under any assay conditions. This minimal activity was subtracted as background.

Consistent with the possibility of two HoxA10-binding TGFβ2 cis elements, sequence analysis identified conserved Hox-binding consensus sequences between 1488 to 1481 bp and 406 to 395 bp in the TGFβ2 promoter. Therefore, reporter constructs were generated with three copies of the proximal or distal sequence linked to a minimal promoter. These constructs (or control minimal promoter/reporter vector) were co-transfected into U937 cells with a vector to overexpress HoxA10 (or empty expression vector). We found that inclusion of these mutant sequences did not significantly increase reporter activity above that of the minimal promoter/reporter control vector. This was not altered by either differentiation or HoxA10 overexpression (not shown).

HoxA10 Binds to Two TGFβ2 cis Elements—We next investigated whether HoxA10 influences these TGFβ2 cis elements directly (by interacting with the cis elements) or indirectly (by influencing another protein that interacts with the cis elements). In the first set of experiments, we investigated in vitro HoxA10 binding to the TGFβ2 cis elements using a DNA-affinity purification assay. For these studies, U937 nuclear proteins were incubated with biotin-labeled, double-stranded synthetic oligonucleotides representing the proximal (−410
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A

U937 Transfectants
TGFβ2 promoter activity

| 3600bp/pGL3     | HoxA10 | Control |
|-----------------|--------|---------|
|                 |        | Untreated | IFNγ | Retinoic Acid |
|                 |        | p=0.6    |      |              |

| 4100bp/pGL3     | HoxA10 | Control |
|-----------------|--------|---------|
|                 |        | Untreated | IFNγ | Retinoic Acid |
|                 |        | p=0.1    |      |              |

| 14000bp/pGL3    | HoxA10 | Control |
|-----------------|--------|---------|
|                 |        | Untreated | IFNγ | Retinoic Acid |
|                 |        | p=0.3    |      |              |

| 1.5kb/pGL3      | HoxA10 | Control |
|-----------------|--------|---------|
|                 |        | Untreated | IFNγ | Retinoic Acid |
|                 |        | p=0.4    |      |              |

| 2.4kb/pGL3      | HoxA10 | Control |
|-----------------|--------|---------|
|                 |        | Untreated | IFNγ | Retinoic Acid |
|                 |        | p=0.1    |      |              |

Luciferase Activity

B

U937 Transfections
Increase in TGFβ2 promoter activity + HoxA10

| 9800bp/pGL3     | Untreated | IFNγ | Retinoic Acid |
|-----------------|-----------|------|---------------|
|                 | p=0.5     |      |               |

| 1.5kb/pGL3      | Untreated | IFNγ | Retinoic Acid |
|-----------------|-----------|------|---------------|
|                 | p=0.2     |      |               |

Luciferase Activity

C

U937 Transfections
HoxA10 binding sites

| Distal/GL3-p    | HoxA10/pDNA | pcDNA |
|-----------------|-------------|-------|
|                 |             | p=0.5 |

| Proximal/GL3-p  | HoxA10/pDNA | pcDNA |
|-----------------|-------------|-------|
|                 |             | p=0.2 |

| pGL3-p          | HoxA10/pDNA | pcDNA |
|-----------------|-------------|-------|
|                 |             | p=0.8 |

Luciferase Activity

FIGURE 2. HoxA10 activates two cis elements in the TGFβ2 promoter. A, overexpressed HoxA10 activates the TGFβ2 promoter. U937 myeloid leukemia cells were co-transfected with a reporter vector with various truncations of the 5’ flank of the TGFβ2 gene (or empty reporter vector) and a vector to overexpress HoxA10 (or empty control expression vector). Transfectants were analyzed for reporter activity with or without differentiation with IFNγ or RA. Statistically significant differences in reporter expression with HoxA10 overexpression in undifferentiated transfected are indicated by *, ***, #, or & (p < 0.0005, n = 6). Statistically significant difference in reporter gene activity in HoxA10-overexpressing transfected versus without differentiation are indicated by **, ###, or && (p < 0.001, n = 6). B, HoxA10 induces a greater activity from the reporter construct with 1.5 kb from the TGFβ2 5’ flank in comparison to the construct with 980 bp of 5’ flank. Data from the transfections described in A were analyzed for the percent increase in reporter activity in transfected with HoxA10 overexpression in comparison to control transfected. A statistically significant difference in HoxA10-induced activity from the 1.5-kb versus 980-bp constructs is indicated by * (p < 0.0001, n = 6). C, HoxA10 activates two cis elements in the TGFβ2 promoter. U937 cells were co-transfected with a reporter construct with three copies of the −410 to −385 bp (proximal) or −1506 to −1478 bp (distal) sequence from the TGFβ2 5’ flank linked to a minimal promoter (or minimal promoter control vector) and a vector to overexpression HoxA10 (or empty expression vector control). Transfectants were analyzed for reporter activity untreated, or after differentiation with IFNγ or RA. Statistically significant increase in reporter expression with HoxA10 overexpression is indicated by * or *** (p < 0.0001, n = 8). A significant increase in reporter expression in HoxA10-overexpressing transfected with versus without differentiation is indicated by ** or # (p < 0.001, n = 8).

to −385 bp) or distal (−1506 to −1478 bp) TGFβ2 cis elements. As negative controls, proteins were incubated with double-stranded oligonucleotides with mutation of the Hox-binding consensus.

Proteins interacting with the probes were purified by affinity to neutravidin, separated by SDS-gel electrophoresis, and identified by Western blot. We found that HoxA10 co-purified with these two TGFβ2 cis elements in a manner that depended upon integrity of the putative HoxA10-binding site (Fig. 3A).

We also investigated in vivo HoxA10 binding to these cis elements using quantitative chromatin co-immunoprecipitation. For these studies, cell lysates were sonicated to generated −200-bp chromatin fragments prior to immunoprecipitation. Chromatin that co-precipitated with HoxA10 (or preimmune serum) was amplified by real time PCR using primers flanking the proximal (−410 to −385 bp) or distal (−1506 to −1478 bp) HoxA10-activated cis elements (generating products of ~100 bp). We found that the two cis elements specifically co-precipitated with HoxA10 from untreated and differentiated U937 cells (Fig. 3B). Interaction of HoxA10 with the TGFβ2 cis elements was significantly greater in differentiated U937 cells. Also, binding to the proximal cis element was significantly greater in RA-treated cells in comparison to cells treated with IFNγ.

These studies demonstrated interaction of HoxA10 with two positive cis elements in the TGFβ2 promoter. These studies indicated that HoxA10 bound to the promoter in myeloid...
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FIGURE 3. HoxA10 binds to two cis elements in the TGFβ2 promoter. A, HoxA10 binds in vitro to the proximal and distal TGFβ2 cis elements. Nuclear proteins were isolated from U937 cells without or with IFNγ or RA-induced differentiation for use in DNA-affinity purification assays. Proteins were incubated with biotin-labeled double-stranded, synthetic oligonucleotide probes representing the −410 to −385 bp (proximal) or −1506 to −1478 bp (distal) sequence from the TGFβ2, or with control oligonucleotides with mutations of the Hox binding consensus. DNA-bound proteins were recovered by affinity to neutravidin-agarose and separated by SDS-PAGE. HoxA10 was identified by Western blot. Total input proteins were a positive control for this experiments. B, HoxA10 binds in vivo to the proximal and distal TGFβ2 cis elements. Chromatin co-immunoprecipitation was performed with U937 cells and an antibody to HoxA10 (or irrelevant control antibody). Cell lysates were sonicated to generate average chromatin fragments of 200 bp prior to immunoprecipitation, and untreated cells were compared with cells differentiated with IFNγ or RA. Co-precipitating chromatin fragments were analyzed by real time PCR using primer sets that flank the −410 to −385 bp (proximal) or −1506 to −1478 bp (distal) sequence from the TGFβ2. Results were normalized to total input (not precipitated) chromatin. Statistically significant differences in HoxA10 binding in untreated versus differentiated U937 cells are indicated by *, **, or # (p < 0.002, n = 4). Statistically significant difference in binding of HoxA10 in IFNγ-treated versus RA-treated cells is indicated by *** (p = 0.03, n = 4).

FIGURE 3. HoxA10 binds to two cDNA elements in the TGFβ2 promoter. A, HoxA10 binds in vitro to the proximal and distal TGFβ2 cis elements. Nuclear proteins were isolated from U937 cells without or with IFNγ or RA-induced differentiation for use in DNA-affinity purification assays. Proteins were incubated with biotin-labeled double-stranded, synthetic oligonucleotide probes representing the −410 to −385 bp (proximal) or −1506 to −1478 bp (distal) sequence from the TGFβ2, or with control oligonucleotides with mutations of the Hox binding consensus. DNA-bound proteins were recovered by affinity to neutravidin-agarose and separated by SDS-PAGE. HoxA10 was identified by Western blot. Total input proteins were a positive control for this experiments. B, HoxA10 binds in vivo to the proximal and distal TGFβ2 cis elements. Chromatin co-immunoprecipitation was performed with U937 cells and an antibody to HoxA10 (or irrelevant control antibody). Cell lysates were sonicated to generate average chromatin fragments of 200 bp prior to immunoprecipitation, and untreated cells were compared with cells differentiated with IFNγ or RA. Co-precipitating chromatin fragments were analyzed by real time PCR using primer sets that flank the −410 to −385 bp (proximal) or −1506 to −1478 bp (distal) sequence from the TGFβ2. Results were normalized to total input (not precipitated) chromatin. Statistically significant differences in HoxA10 binding in untreated versus differentiated U937 cells are indicated by *, **, or # (p < 0.002, n = 4). Statistically significant difference in binding of HoxA10 in IFNγ-treated versus RA-treated cells is indicated by *** (p = 0.03, n = 4).

HoxA10 Overexpression in Myeloid Cells Increases TGFβ2 Expression—We next determined if activation of the TGFβ2 promoter by overexpressed HoxA10 was reflected in increased Tgfβ2 expression. For these studies, we compared U937 cells that were stably overexpressing HoxA10 with control vector-transfected cells. Tgfβ2 mRNA expression was determined by real time PCR. We found that differentiation of control U937 cells was not associated with increased expression of Tgfβ2 (Fig. 4A), consistent with the promoter function studies, above. Tgfβ2 expression was significantly increased by HoxA10 overexpression and was significantly greater in differentiated HoxA10-overexpressing transfectants in comparison to untreated HoxA10-overexpressing cells (Fig. 4A). This was also consistent with the TGFβ2 promoter assays.

We hypothesize that Tgfβ2 production by myeloid progenitor cells acts in an autocrine fashion to stimulate progenitor expansion under conditions of HoxA10 overexpression (such as AML). Because autocrine stimulation requires the presence of Tgfβ receptors, we investigated expression of Tgfβ-R1 and -RII in the U937 stable transfectants, discussed above. We found that both receptors were expressed in control U937 cells and there was a small, but statistically significant increase in expression in HoxA10-overexpressing cells (Fig. 4A). Expression of Tgfβ-R1 in both control and HoxA10-overexpressing U937 cells was increased by differentiation with IFNγ or RA (Fig. 4A).

We next determined if increased Tgfβ2 mRNA in HoxA10-overexpressing cells was reflected in increased Tgfβ2 protein. Tgfβ2 mRNA encodes a precursor protein of ∼40 kDa, which is cleaved to a ∼12-kDa active secreted protein. We found increased production of both pro-Tgfβ2 and the cleavage product in HoxA10-overexpressing U937 cells in comparison to control cells (Fig. 4B). Because Tgfβ-R activation is associated with activation (phosphorylation) of Smad or MAP kinase proteins, we also used these stable transfectants to investigate these pathways. We found increased phosphorylation of extracellular signal-related kinase (ERK, a MAP kinase) in HoxA10-overexpressing cells (Fig. 4B). However, there was no increase in activation of Smad2 or -3 (Smads associated with Tgfβ-R activation) in HoxA10-overexpressing cells.

We also determined whether increased expression of the Tgfβ2 protein in HoxA10-overexpressing cells resulted in increased secreted Tgfβ2. For these studies, HoxA10-overexpressing and control U937 stable transfectants were cultured for 48 h with or without IFNγ or RA. Medium was analyzed for Tgfβ2 expression by ELISA (using an antibody that does not recognize Tgfβ1 or -3). We found significantly increased Tgfβ2 protein in the medium of HoxA10-overexpressing U937 cells in comparison to control cells (Fig. 4C). Differentiation with IFNγ or RA further increased Tgfβ2 production by HoxA10-overexpressing cells, but not control cells (Fig. 4C).

However, U937 cells are a leukemia line that might be anticipated to have abnormalities in cytokine expression and signaling. Therefore, we investigated HoxA10-induced Tgfβ2 expression using primary murine bone marrow cells. For initial studies, we employed bone marrow from transgenic mice in which the HOXA10 gene was disrupted by homologous recombination (36). These mice are characterized by urogeni-
HoxA10 Activates TGFβ2 Transcription

**A** Real Time PCR
U937 Stable Transfectants

- Untreated
- + IFNγ
- + Retinoic Acid

**B** Western Blot
U937 Stable Transfectants

- Control vector
- HoxA10 vector

**C** TGFβ2 ELISA
U937 Stable Transfectants

- Untreated
- + IFNγ
- Retinoic Acid

FIGURE 4. HoxA10 overexpression in U937 myeloid cells increases Tgfβ2 mRNA expression and Tgfβ2 protein secretion. A, expression of Tgfβ2 mRNA is increased in HoxA10-overexpressing U937 cells. U937 stable transfectants were generated with a vector to overexpress HoxA10 or with empty expression vector control. Expression of HoxA10, Tgfβ2, Tgfβ3-R1, and Tgfβ3-RII was determined by real time PCR. Expression of these genes in undifferentiated transfectants was compared with transfectants treated with IFNγ or RA. A statistically significant increase in HoxA10 expression is indicated by *. A statistically significant increase in Tgfβ2 expression in HoxA10-overexpressing cells in comparison to control cells is indicated by **. Statistically significant increase in Tgfβ2 expression in differentiated HoxA10-overexpressing cells in comparison to undifferentiated cells is indicated by *** (p = 0.001, n = 4). Statistically significant increase in Tgfβ3-R1 or Tgfβ3-RII expression in HoxA10-overexpressing cells is indicated by # and ##, respectively (p < 0.001, n = 4). An increase in Tgfβ3-R1 expression with differentiation of control or HoxA10-overexpressing cells is indicated by # and & respectively (p ≤ 0.0003, n = 4). B, expression of Tgfβ2 protein is increased in HoxA10-overexpressing U937 cells. The U937 stable transfectants, described above, were also analyzed for protein expression by Western blot (WB). Total cell lysate proteins were separated by SDS-PAGE and blots were probed with antibodies for HoxA10, Tgfβ2, total or phospho-ERK, total or phospho-Smad 2/3, or GAPDH (as a loading control). C, secretion of Tgfβ2 protein is increased in HoxA10-overexpressing U937 cells. The U937 stable transfectants secreted of Tgfβ2, described above, were also determined. Cells were maintained at a constant cell concentration and medium was collected and analyzed for Tgfβ2 using an ELISA that does not cross-react with Tgfβ1 or -3. A statistically significant increase in Tgfβ2 protein in HoxA10-overexpressing transfectants in comparison to control transfectants is indicated by * (p = 0.0003, n = 3). A statistically significant increase in Tgfβ2 production in HoxA10-overexpressing transfectants with differentiation is indicated by ** (p = 0.002, n = 3).

Myeloid progenitors were isolated from the bone marrow of HoxA10−/− and control wild type (WT) mice and cultured in GM-CSF, IL3, and SCF. We previously demonstrated that cells cultured under these conditions are predominantly bipotential GMP (Sca1-CD34+CD38−) (17). Some cells were ex vivo differentiated with G-CSF or M-CSF to produce granulocytes or monocytes, respectively. Our previous studies demonstrated that more than 80% of cells are differentiated under these conditions (CD34−CD38+ and Gr1+ or Mac1+, respectively) (17).

Expression of Tgfβ2 in these cells was determined by real time PCR. As in the studies above, we also determined expression of Tgfβ3-R1 and -RII. In WT cells, expression of Tgfβ2 was not significantly different under any of these cytokine conditions (Fig. 5A). Expression of Tgfβ2 was significantly less in HoxA10−/− bone marrow cells in comparison to WT cells under all three cytokine conditions (Fig. 5A). Expression of HoxA10 decreased during differentiation of WT cells, consistent with previous studies (1, 2). Tgfβ3-R1 and -RII were expressed in WT and HoxA10−/− murine bone marrow cells cultured in GM-CSF, IL3, and SCF, and undergoing differentiation. We found no significant difference in Tgfβ3-R1 expression in the absence of HoxA10 (Fig. 5A). However, expression of Tgfβ3-R1 was significantly less in the HoxA10−/− cells in comparison to WT cells (Fig. 5A).

We also investigated the effect of HoxA10 overexpression on Tgfβ2 mRNA in primary murine bone marrow cells. For these studies, myeloid progenitor cells were isolated from the bone marrow of WT mice and transduced with a retroviral

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3 C. A. Shah, H. Wang, L. Bei, L. C. Platanias, and E. A. Eklund, unpublished observations.
vector to express HoxA10 (or with empty MSCV vector) (17). Cells were cultured as described above and expression of Tgfβ2, Tgfβ-R1, and -RII mRNA was determined by real time PCR. We found that HoxA10 overexpression increased Tgfβ2 mRNA expression and this effect was greatest in differentiating cells (Fig. 5B). HoxA10 overexpression slightly, but significantly, increased expression of both Tgfβ-R1 and -RII (Fig. 5B).

We next investigated whether HoxA10-dependent alteration in Tgfβ2 mRNA was reflected in secreted Tgfβ2. For these studies, myeloid progenitor cells were isolated from HoxA10−/− or WT murine bone marrow. Some WT cells were transduced with a retroviral vector to overexpress HoxA10 (or with control vector), as above. Cells were cultured as described above and medium was analyzed for Tgfβ2 production by ELISA. We found that significantly less Tgfβ2 was produced by HoxA10−/− cells in comparison to WT cells under all three cytokine conditions (Fig. 5C). Conversely, HoxA10 overexpression increased Tgfβ2 production in these cells (Fig. 5C). Tgfβ2 production in HoxA10-overexpressing cells was increased slightly, but to a statistically significant extent, by differentiation with G-CSF or M-CSF (Fig. 5C). Tgfβ2 Induces ERK-dependent Proliferation of Myeloid Progenitor Cells—Although Tgfβ-R ligand binding has been shown to activate the MAP kinase pathway, a direct func-
HoxA10 Activates TGFβ2 Transcription

A

Proliferation Assay
TGFβ2-treated U937 cells

Control
+ PD98056

Western Blot
U937 cells

B

PD98056

FIGURE 6. TGFβ2 stimulates proliferation of myeloid cells via ERK activation. A, the proliferative response of U937 cells to recombinant TGFβ2 is inhibited by the ERK inhibitor, PD98056. U937 cells were deprived of cytokines and then stimulated with a dose titration of rTGFβ2 (in the presence of 10% FCS). Some cells were treated with PD98056. Proliferation was determined by [3H]thymidine incorporation. Statistically significant decrease in proliferation in PD98056-treated cells for any given dose of rTGFβ2 is indicated by * (p = 0.0004, n = 6). B, treatment of U937 cells with rTGFβ2 results in ERK activation. U937 cells were deprived of cytokines and stimulated with rTGFβ2 (20 ng/ml), with or without treatment with PD98056, as above. Cell lysate proteins were separated by SDS-PAGE and Western blots (WB) were serially probed with antibodies to total and phospho-ERK, total and phospho-Smad2/3, or GAPDH (as a loading control).

ional connection between TGFβ2, MAP kinase activation, and cell proliferation was not previously established for myeloid cells. Therefore, we determined the impact of recombinant TGFβ2 (rTGFβ2) on U937 cell proliferation. For these studies, U937 cells were deprived of cytokines for 24 h, then treated with a dose titration of rTGFβ2 (in the presence of 10% fetal calf serum; FCS). Cell proliferation was determined by standard [3H]thymidine incorporation assays (42). We found a dose-dependent proliferative response to rTGFβ2 in U937 cells (Fig. 6A).

We next investigated the role of MAP kinase activation on TGFβ2-induced proliferation of these cells. U937 cells were again cytokine deprived and treated with a dose titration of rTGFβ2, but in the presence of PD98056. This small molecule is a specific inhibitor of ERK; a MAP kinase implicated in cytokine-induced proliferation in myeloid cells. We found that PD98056 blocked TGFβ2-induced proliferation of U937 cells.

These studies suggested that TGFβ2 stimulation of U937 cells activates ERK, resulting in proliferation. Therefore, we next determined the ERK activation status in TGFβ2-treated U937 cells. For these studies, U937 cells were treated with rTGFβ2 (20 ng/ml), with or without PD98056, and cell lysates were analyzed by Western blot. As in the proliferation assays above, cells were deprived of cytokines prior to stimulation with rTGFβ2. We found that TGFβ2 treatment of U937 cells increased ERK phosphorylation (activation) (Fig. 6B). ERK activation was blocked by PD98056 treatment of TGFβ2-stimulated cells. No increase in activation (phosphorylation) of Smad was observed in rTGFβ2-treated cells, with or without PD98056 (Fig. 6B).

HoxA10 Overexpression Increases Proliferation in a TGFβ2-dependent Manner—Based on these results, we next determined whether HoxA10 overexpression induced a TGFβ2-dependent effect on proliferation in myeloid cells. We first investigated this using HoxA10 overexpressing and control U937 stable transfectants, as described above. Cells were cultured for 24 h in serum-free medium and re-stimulated for 24 h with a dose titration of FCS. A TGFβ2-specific blocking antibody was added to some cultures and proliferation was determined by [3H]thymidine incorporation. The goal of these studies was to determine whether autocrine production of TGFβ2 modulated the proliferative response to other cytokines in HoxA10-overexpressing cells. The presumed mechanism for such an effect would be cross-stimulation of common pro-proliferative signaling pathways by TGFβ2-R and other cytokines from FCS.

We found that proliferation in HoxA10-overexpressing U937 cells was significantly greater than in control vector cells (Fig. 7A). The proliferative response in HoxA10-overexpressing cells was decreased significantly by treatment with TGFβ2-blocking antibody (Fig. 7A). Although TGFβ2-blocking antibody also decreased proliferation of control cells, the percent decrease was significantly greater in HoxA10-overexpressing cells (17.5 ± 5.5% for control cells versus 34.3 ± 3.1% for HoxA10-overexpressing cells, p = 0.03, n = 4). A possible mechanism for this was sustained ERK activation due to auto-crine TGFβ2.

To determine whether TGFβ2-dependent proliferation of HoxA10-overexpressing cells correlated with ERK activation, additional studies were performed. HoxA10-overexpressing or control transfectants were deprived of cytokines followed by stimulation with FCS (10%) with or without the TGFβ2-blocking antibody. Western blots of cell lysates were serially probed with antibodies to total and phospho-ERK, and total and phospho-Smad. We found that increased ERK phosphor-
HoxA10-Overexpressing Myeloid Cells Induce Tgfβ2-Dependent Cytokine Hypersensitivity

To investigate the role of HoxA10 overexpression in Tgfβ2-dependent proliferation in non-transformed cells, similar experiments were performed with primary murine myeloid progenitors. In these studies, WT murine bone marrow cells were transduced with a HoxA10 expression vector (or empty control vector), as above. Cells were deprived of cytokines for 24 h followed by stimulation with a dose titration of GM-CSF. We found that HoxA10 overexpression resulted in hypersensitivity to GM-CSF (i.e., increased proliferation at the lower cytokine dose in comparison to control cells) (Fig. 8A). To determine whether autocrine production of Tgfβ2 contributed to hypersensitivity, some cells were treated with a Tgfβ2-blocking antibody. This antibody decreased proliferation of HoxA10-overexpressing cells (Fig. 8A).

These results suggested that autocrine production of Tgfβ2 contributed to GM-CSF hypersensitivity in HoxA10-overexpressing progenitor cells. We next determined if this involved ERK activation by autocrine Tgfβ2. For these studies, WT murine myeloid progenitor cells were transduced with a vector to overexpress HoxA10 (or with empty control vector), deprived of cytokines, and stimulated with GM-CSF (10 ng/ml) with or without Tgfβ2-blocking antibody. Cell lysates were analyzed by Western blot for activation (phosphorylation) of ERK.

We found that ERK activation was greater in GM-CSF-treated HoxA10-overexpressing cells in comparison to control cells (Fig. 8B). However, ERK activation in HoxA10-overexpressing cells was decreased by treatment with Tgfβ2-blocking antibody. These studies identified “cross-talk” between the Tgfβ2-R and GM-CSF-R at the level of ERK activation. This provided a mechanism by which autocrine activation of the Tgfβ2-receptor might increase the response to GM-CSF in HoxA10-overexpressing cells.

HoxA10 Overexpression Increases Tgfβ2 and ERK-Dependent Proliferation—In the studies above, we found that Tgfβ2 stimulated proliferation of U937 cells in a MAP kinase-dependent manner. Therefore, we investigated whether cytokine hypersensitivity in HoxA10-overexpressing cells was ERK dependent. HoxA10-overexpressing or control U937 stable transfectants were deprived of growth factors, stimulated with a dose titration of FCS, and proliferation was determined with and without treatment with PD98056. We found that ERK inhibition significantly decreased cytokine hypersensitivity in HoxA10-overexpressing cells (Fig. 9A).

To determine whether decreased proliferation in PD98056-treated cells correlated with ERK activity, Western blots were performed using cell lysates from HoxA10-overexpressing and control U937 stable transfectants. For these studies, transfectants were deprived of cytokines and re-stimulated with FCS (10%) with or without PD98056. Western blots were probed for total and phospho-ERK, total or phospho-Smad2/3, or GAPDH (as a loading control).
comparison to the effect in myeloid progenitors. We previously demonstrated that HoxA10 becomes increasingly tyrosine phosphorylated during myelopoiesis. We also previously found that tyrosine-phosphorylated HoxA10 exhibits an increased ability to activate ITGB3 transcription and decreased repression of the CYBB and NCF2 genes (20–22, 24). Using tyrosine mutant forms of HoxA10, we identified conserved tyrosine residues in the HoxA10 HD (Tyr326 and Tyr343) that are involved in these effects (20–24). Therefore, we used this HoxA10-tyrosine mutant (referred to as Y-mut HoxA10) to

FIGURE 8. HoxA10 overexpression in primary myeloid progenitor cells induces Tgfβ2-dependent cytokine hypersensitivity. A, GM-CSF hypersensitivity of HoxA10-overexpressing primary murine myeloid progenitor cells is inhibited by a Tgfβ2-blocking antibody. Murine bone marrow myeloid progenitors were isolated and transduced with a retroviral vector to express HoxA10 or with empty control expression vector. Cells were deprived of cytokines and treated with a dose titration of GM-CSF, with or without Tgfβ2-blocking antibody. Proliferation was determined by [3H]thymidine incorporation. Statistically significant differences in proliferation of HoxA10-overexpressing cells versus without Tgfβ2 antibody, at a given cytokine dose, are indicated by * (p ≤ 0.005, n = 3). B, treatment with a Tgfβ2-blocking antibody decreases ERK activation in GM-CSF-stimulated, HoxA10-overexpressing primary myeloid progenitor cells. The transduced murine bone marrow cells, discussed above, were deprived of cytokines and then treated with GM-CSF (10 ng/ml), with or without Tgfβ2-blocking antibody. Cell lysates were separated by SDS-PAGE and Western blots (WB) were serially probed with antibodies to total or phospho-ERK, or GAPDH (as a loading control).

FIGURE 9. HoxA10 overexpression in myeloid cells induces ERK-dependent cytokine hypersensitivity. A, cytokine hypersensitivity of HoxA10-overexpressing U937 cells is inhibited by a PD98056. U937 stable transfectants were generated with a vector to overexpress HoxA10 or empty expression vector control (as above). Cells were deprived of cytokines and treated with a dose titration of FCS with or without PD98056. Proliferation was determined by [3H]thymidine incorporation. Statistically significant differences in proliferation of HoxA10-overexpressing cells versus without PD98056, at a given cytokine dose, are indicated by * (p ≤ 0.0003, n = 3). B, treatment with PD98056 blocks ERK activation in HoxA10-overexpressing U937 cells. The U937 stable transfectants discussed above were deprived of cytokines and re-treated with 10% FCS, with or without PD98056. Cell lysates were separated by SDS-PAGE and Western blots (WB) were serially probed with antibodies to total or phospho-ERK, total or phospho-Smad2/3, or GAPDH (as a loading control).
investigate the role of tyrosine phosphorylation in TGFβ2 transcriptional activation.

For these studies, U937 cells were co-transfected with a reporter construct with three copies of the −410 to −385 bp (proximal) or −1506 to −1478 bp (distal) sequence from the TGFβ2 upstream linked to a minimal promoter (or minimal promoter control vector) and a vector for overexpression of HoxA10 or Y-mut HoxA10 (or empty expression vector control). Transfectants were analyzed for reporter activity untreated, or after differentiation with IFNγ or RA. Results were graphed as the fold-increase in reporter activity from the TGFβ2 cis element containing constructs with overexpression of either form of HoxA10. A statistically significantly greater effect of HoxA10 overexpression on the proximal cis element in differentiated versus untreated transfectants is indicated by * (p < 0.01, n = 3). A significantly smaller effect on activity of the proximal cis element by the Y-mutant HoxA10 in comparison to WT HoxA10 is indicated by ** (p < 0.002, n = 3). Similarly, a greater effect of HoxA10 overexpression on the distal cis element in differentiated transfectants is indicated by *** (p < 0.01, n = 3). A significantly smaller effect of Y-mutant HoxA10 in comparison to WT HoxA10 on the distal cis element is indicated by # (p < 0.001, n = 3). B, HoxA10 becomes tyrosine phosphorylated during differentiation of U937 cells. Nuclear proteins were isolated from U937 cells that were untreated, or after differentiation with IFNγ or RA. Immunoprecipitation was performed with an antibody to phosphotyrosine or irrelevant control antibody. Immunoprecipitates were separated by SDS-PAGE and Western blots (WB) were probed with an antibody to HoxA10. Nuclear proteins (1/10 the amount used for immunoprecipitation) were used to control for the abundance of HoxA10.

**DISCUSSION**

HoxA10 is a member of a specific set of Hox proteins that are overexpressed in poor prognosis human AML (3–5). This includes both differentiation stage appropriate overexpression...
in CD34+ cells, and lack of the normal decrease in expression in differentiating cells (CD34−CD38+). Overexpression of HoxA9 or A10 expands myeloid progenitors and differentiating myeloid cell populations in vivo, but the mechanism for this is not known. Our studies indicate that increased production of Tgfβ2 may be one mechanism that contributes to this effect of overexpressed HoxA10.

In the current studies, we identified Tgfβ2 as a HoxA10 target gene. We determined that HoxA10 activated Tgfβ2 transcription via two proximal promoter cis elements with additive activity. We also demonstrated that secretion of Tgfβ2 was increased in HoxA10-overexpressing myeloid progenitor cells in comparison to control cells. This resulted in autocrine activation of Tgfβ2-R on HoxA10-overexpressing cells, activating ERK MAP kinase, and contributing to the hypersensitivity of HoxA10-overexpressing myeloid progenitor cells to multiple cytokines. Therefore, these studies identified a previously unknown mechanism for HoxA10-induced cytokine hypersensitivity and progenitor expansion in AML.

Other genes with tandem HoxA10-binding cis elements have been previously identified (i.e. CYBB, NCF2, and DUSP4) (20, 21, 23). HoxA10 exhibits different binding affinities for the tandem cis elements in the CYBB and DUSP4 genes, which permits graded expression of these genes in differentiating cells. However, we did not find a marked difference in the affinity of HoxA10 for the two TGFβ2 cis elements in vitro or in vivo. Another function of tandem HoxA10-binding cis elements might be to facilitate assembly of large, multiprotein transcriptional activation complexes. However, we found that activities of the two TGFβ2 cis elements were additive, not cooperative. It is alternatively possible that HoxA10 interacts with these two cis elements in combination with different partners (i.e. Pbx versus Meis or other unidentified partners). In this case, different signaling events might preferentially assemble an activation complex on one or the other cis element. There was some evidence this may be occurring in the TGFβ2 promoter, because there were quantitative differences in HoxA10 binding to the two different cis elements during monocye versus granulocyte differentiation. Further investigation of this may be of interest.

We found that in vitro and in vivo binding of endogenous HoxA10 to the two cis elements increased during differentiation. However, in the absence of HoxA10 overexpression, there was no increase in TGFβ2-promoter activity or Tgfβ2 production in differentiating cells. These results suggest that HoxA10 may be the rate-limiting protein for TGFβ2 transcription. It is possible that other proteins that are differentiation stage dependent in activity are involved in activation of these cis elements, but that these proteins are inadequate to increase TGFβ2 transcription in the absence of overexpressed HoxA10. The physiologic relevance of this to normal myelopoiesis is not clear, but this mechanism might increase total Tgfβ2 production in HoxA10-overexpressing AML cells.

In previous studies, we determined that the tyrosine phosphorylation state of HoxA10 influences target gene regulation. Because HoxA10-tyrosine phosphorylation increases during myelopoiesis, this provides a possible mechanism by which overexpressed HoxA10 exhibits variable activity in differentiating cells versus progenitors. For example, we found that phosphorylation of conserved tyrosine residues in the HoxA10-HD decreased the binding affinity for negative cis elements in promoters of genes that encode phagocyte effector proteins (i.e. the CYBB and NCF2 genes) (20–22). Decreased HoxA10 binding to these negative cis elements increases CYBB and NCF2 transcription as differentiation proceeds. We also found that HoxA10-tyrosine phosphorylation decreased binding to, and activation of, DUSP4 cis elements (23). Because DUSP4 encodes the JNK-antagonist MKP2, this increases sensitivity to apoptosis during myelopoiesis. Interestingly, HD tyrosines were not involved in this effect.

In contrast, we found that phosphorylation of the HD tyrosine residues increased HoxA10 binding to the ITGB3 gene (24). The resultant increase in αvβ3 integrin expression increased binding to the extracellular matrix and motility of differentiating cells. Similarly, in the current study, we found that phosphorylation of these HD tyrosines contributed to activation of both HoxA10-binding TGFβ2 cis elements in differentiating myeloid cells.

Increased circulating Tgfβ2 in AML might influence function of mature phagocytes in addition to the effects on progenitor proliferation. Tgfβ2 is produced by neutrophils during the inflammatory response and acts as a chemoattractant for other neutrophils (31). Migration on fibronectin is especially sensitive to this mechanism, which is of interest, because of the HoxA10-induced increase in αvβ3 integrin expression, discussed above (24). The impact of HoxA10 overexpression on phagocyte function in AML will be the topic of future studies.

We found that treatment of myeloid progenitor cells with rTgfβ2 activated ERK and resulted in proliferation. We also found that HoxA10 overexpression in U937 cells induced hypersensitivity to FCS-induced proliferation, associated with ERK activation. This hypersensitivity was at least partly dependent upon autocrine production of Tgfβ2. The cytokines in FCS, which are responsible for myeloid proliferation, are not well defined. However, many hematopoietic cytokines and other growth factors induce proliferation via Ras-dependent ERK activation. Tgfβ2 overproduction in HoxA10-overexpressing cells would be a constitutive activator of ERK. Tgfβ2-dependent, constitutive ERK activation would enhance the effect of other cytokines that use this pathway by preventing negative regulatory signals from terminating the proliferative response.

HoxA10 overexpression also induced GM-CSF hypersensitivity in primary murine myeloid progenitor cells. As for HoxA10-overexpressing U937 cells, this hypersensitivity was partly abolished by blocking Tgfβ2. Ligand binding to the GM-CSF-R activates Ras, resulting in ERK-dependent proliferation. Impaired down-regulation of these events is a common mechanism for cytokine hypersensitivity in myeloid leukemia. A number of mechanisms for this have been described, including activating mutations of Ras, and inactivating mutations of Neurofibromin 1, a Ras-Gap (reviewed Ref. 43). In the current studies sustained Tgfβ2 production by HoxA10-overexpressing cells might result in...
constitutive Ras-MEK1-ERK activity, phenocopying these previously described mechanisms for Ras pathway activation in AML.

However, Tgfβ2-blocking antibody resulted in partial, but not complete, reversal of GM-CSF hypersensitivity; a result suggesting that other cytokines and signaling pathways are also influenced by HoxA10 overexpression. This might include altered cytokine receptor expression, or impairment of negative regulatory pathways in HoxA10-overexpressing cells. Possibly consistent with the first mechanism, we identified a slight increase in expression of TgfβRI and -RII in HoxA10-overexpressing myeloid cells under some conditions. Because Tgfβ1 and -3 have higher affinities for these receptors than Tgfβ2, it is difficult to predict whether increased Tgfβ-R expression alone would increase or decrease proliferation. We found that HoxA10 overexpression did not alter Tgfβ1 and -3 expression in myeloid progenitor cells (not shown). The significance of increased Tgfβ-R expression in HoxA10-overexpressing cells is the topic of ongoing studies in the laboratory.

HoxB3, HoxB4, HoxA9, and HoxA10 all influence myeloid progenitor expansion. These proteins share a highly conserved DNA-binding homeodomain, but are less conserved through the remainder of the molecule. Our results suggest the possibility that multiple Hox proteins regulate expression of Tgfβ2 and other cytokines. This will be investigated in future studies. Poor prognosis AML is characterized by overexpression of these Hox proteins and resistance of the leukemia stem cell to drug-induced cell killing. Tgfβ2 protects myeloid stem and progenitor cells from chemotherapeutic agents, such as doxorubicin, which are relevant to AML treatment (44). Increased Tgfβ2 expression in such cells provides a novel mechanism for cytokine hypersensitivity and leukemia stem cell expansion in AML with HoxA10 overexpression. This pathway could be a target for in vivo therapeutic approaches or in vitro purging of the leukemia stem cell.
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