HoxA10 Protein Regulates Transcription of Gene Encoding Fibroblast Growth Factor 2 (FGF2) in Myeloid Cells*

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Background: HoxA10 is a homeodomain transcription factor that regulates myeloid progenitor cell expansion and contributes to myeloid leukemogenesis.

Results: HoxA10 activates the FGF2 (fibroblast growth factor 2) gene in myeloid progenitor cells.

Conclusion: Increased production of Fgf2 by HoxA10-overexpressing myeloid progenitor cells stimulates proliferation through an autocrine mechanism.

Significance: Signaling pathways that are activated by Fgf2 may be rational therapeutic targets for leukemia.

HoxA10 is a member of a highly conserved family of homeodomain transcription factors that are involved in definitive hematopoiesis and implicated in the pathogenesis of acute myeloid leukemia (AML). During normal hematopoiesis, HoxA10 facilitates myeloid progenitor expansion and impedes myeloid differentiation. To better understand the molecular mechanisms that control these events, we have been identifying and characterizing HoxA10 target genes. In this study, we identified the gene encoding fibroblast growth factor 2 (Fgf2 or basic fibroblast growth factor) as a target gene that is relevant to the biological effects of HoxA10. We identified two cis elements in the proximal FGF2 promoter that are activated by HoxA10 in myeloid progenitor cells and differentiating phagocytes. We determined that Fgf2 expression and secretion are regulated in a HoxA10-dependent manner in these cells. We found that increased Fgf2 production by HoxA10-overexpressing myeloid progenitor cells induced a phosphoinositol 3-kinase-dependent increase in β-catenin protein. This resulted in autocrine stimulation of proliferation in HoxA10-overexpressing cells and hypersensitivity to other cytokines that share this pathway. Therefore, these studies identified expression of Fgf2 as a mechanism by which HoxA10 controls the size of the myeloid progenitor population. These studies also suggested that aberrant production of Fgf2 may contribute to leukemogenesis in the subset of AML with dysregulated Hox expression. Therapeutic targeting of Fgf2-stimulated signaling pathways might be a rational approach to this poor prognosis subset of AML.

HoxA10 is a highly conserved homeodomain transcription factor that plays key roles in embryogenesis and definitive hematopoiesis. HOX genes are clustered in four groups (A–D) on four chromosomes in mouse and man (1). Transcription of HOX genes is tightly regulated during hematopoiesis, progressing 5’ through 3’ through each group as differentiation proceeds. Therefore, HOX1–4 are actively transcribed in hematopoietic stem cells, and HOX7–11 (referred to as posterior or ABD HOX genes) are transcribed in committed hematopoietic progenitors (2). Activation of various HOX groups is also lineage-specific. For example, posterior HOXB genes are activated in developing lymphoid cells and HOXA genes during myelopoiesis.

Dysregulated Hox expression has been implicated in myeloid leukemogenesis, but molecular mechanisms by which Hox proteins influence this process are not well defined. Clinical correlative studies identified a subset of poor prognosis acute myeloid leukemia (AML) with increased expression of HoxB3, -B4, and -A9–11 in CD34+ bone marrow cells (3–5). In AML, expression of these Hox proteins is sustained in CD34+ cells in contrast to the usual decrease in expression during normal differentiation. This pattern of Hox gene expression is found in AML with chromosomal translocations or duplications involving the MLL gene (11q23 leukemias), in association with the MYST3/CREBBP translocation, and in a poor prognosis subset of cytogenetically normal AML (6–9).

Studies in murine models support a functional role for these 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 (common granulocyte/monocyte progenitors or GMP) (12–16). This myeloproliferative disorder evolves to AML over time in HoxA10-overexpressing mice, a process that is accelerated by constitutive activation of Shp2 protein-tyrosine phosphatase (16, 17). Overexpression of HoxA9 leads to AML in mice transplanted with bone marrow that is co-overexpressing Meis1 (18), a common Hox DNA-binding partner.

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‡ The abbreviations used are: AML, acute myeloid leukemia; TSS, transcription start site; oligo, oligonucleotide; R, receptor; GMP, granulocyte/monocyte progenitor; MSCV, murine retroviral stem cell vector; SCF, stem cell factor.
These studies suggested that specific Hox proteins are involved in expansion of various bone marrow cellular compartments. We hypothesized that Hox proteins control the balance between proliferation and death in these cell populations. However, the set of Hox target genes that explain these activities are poorly defined. In our studies, we used chromatin immunoprecipitation-based screening techniques to identify a set of HoxA10 target genes that might be involved in progenitor expansion and leukemogenesis (19–23). With the assistance of computer algorithms, such screening studies can identify potential gene networks and cognate pathways for a given transcription factor. However, descriptive studies of this nature are largely useful for hypothesis generation. Therefore, we used information from our screening studies as a starting point for functional investigations into the molecular mechanisms of Hox-induced leukemogenesis.

For example, our screen identified DUSP4 as a HoxA10 target gene (19). This gene encodes mitogen-activated protein kinase 2 (Mkp2), an inhibitor of c-Jun N-terminal kinase (Jnk). We found that activation of DUSP4 transcription by overexpressed HoxA10 impairs Jnk-mediated apoptosis in myeloid progenitor cells (19). Therefore, our studies provided a functional connection between dysregulated HoxA10 expression, increased Mkp2 protein, Jnk inhibition, and expansion of the GMP population in AML.

We also found that HoxA10 activates IJGB3 transcription in myeloid progenitor cells and differentiating phagocytes (the gene encoding β3 integrin) (20). Our studies suggested that increased αvβ3 integrin in HoxA10-overexpressing myeloid progenitor cells facilitated expansion of these cells via interaction with vitronectin and fibronectin in bone marrow stroma. Increased αvβ3 integrin expression on HoxA10-overexpressing myeloid progenitor cells leads to an increase in activation of Syk protein–tyrosine kinase, substantiating this hypothesis (20).

In a recent study, we identified the gene encoding transforming growth factor β2 (Tgfβ2) as a HoxA10 target gene (the TGFβ2 gene) (21). Tgfβ1, -2, and -3 are homologous proteins that are encoded by separate genes and bind to type I and II Tgfβ receptors with differing affinities (24). Tgfβ1 stimulates cell proliferation at a low concentration but inhibits proliferation at higher concentrations (25, 26). Tgfβ3 always represses cell proliferation, and Tgfβ2 always stimulates proliferation (25, 26). The mechanism for these differences in activity is not known. We found that Tgfβ2 expression was increased in HoxA10-overexpressing myeloid cells (21). In myeloid progenitor cells, increased Tgfβ2 secretion resulted in autocrine stimulation of proliferation in an Erk-dependent manner (21). Additionally, sustained stimulation by autocrine-produced Tgfβ2 resulted in hypersensitivity of HoxA10-overexpressing myeloid progenitor cells to other cytokines that activate Erk, such as GM-CSF and M-CSF (21).

In this study, we identified the gene encoding fibroblast growth factor 2 (Fgf2 or basic fibroblast growth factor) as a HoxA10 target gene and investigated the impact of HoxA10-induced Fgf2 expression on myeloid progenitor expansion. Fgf2 belongs to a cytokine family with 22 described members, each encoded by different genes. Fgf2 is produced by bone marrow stromal cells, hematopoietic progenitor cells, and mature phagocytes (27). Increased Fgf2 production by leukemia cells has been described in AML (28). In the bone marrow, Fgf2 plays a role in expansion of hematopoietic progenitor cell populations (29). In mature phagocytes, Fgf2 influences cell adhesion, migration, and respiratory burst activity (30). Fgf2 binds to and activates Fgf receptors (Fgf-R1–4), resulting in their dimerization and autophosphorylation. Fgf2 also binds to and activates various integrins, including αvβ3, through noncanonical interactions (31).

Stimulation of Fgf-R by Fgf2 activates a number of signaling intermediates, including phosphoinositide 3-kinase (PI 3-kinase) (32, 33). Activation of Akt by PI 3-kinase induces serine phosphorylation of glycogen synthase kinase 3 β (Gsk3β), which inhibits Gsk3β activity. Gsk3β phosphorylates β-catenin on serine/threonine residues, which leads to ubiquitination and degradation of β-catenin by the proteasome. Therefore, Gsk3β activity decreases the stability of β-catenin protein and consequently impairs expression of β-catenin target genes, including cyclin D1, c-Myc, and survivin. Therefore, Fgf2-dependent activation of PI 3-kinase would be anticipated to increase β-catenin-related cell proliferation. However, the importance of this pathway has not been previously investigated in hematopoietic progenitor cells or leukemia.

We hypothesized that increased Fgf2 expression in HoxA10-overexpressing cells results in autocrine stimulation of proliferation by increasing β-catenin. Therefore, these studies identify a previously unknown mechanism by which HoxA10 regulates proliferation and expansion of myeloid progenitor cells. This mechanism may be important in the pathogenesis of poor prognosis AML with increased Hox expression and may suggest rational targets for therapeutic approaches to this disease.

**MATERIALS AND METHODS**

**Plasmids**

*Protein Expression Vectors*—The cDNA for human HoxA10 (obtained from C. Largman, University of California, San Francisco) (34) was subcloned into the mammalian expression vector pcDNA3 (Invitrogen) and the murine retroviral vector pMSCVpuro (Clontech), as described previously (19, 20, 35–37).

*FGF2 Reporter Vectors*—FGF2 5′-flank genomic fragments were obtained by PCR from the genomic DNA of U937 cells. Sequences of the fragments were compared with the published FGF2 5′-flank sequence (ENSEMBL database), and fragments were subcloned into the pGL3-basic reporter vector (Promega, Madison, WI). Other constructs were generated with mutation of identified HoxA10-binding sites in the FGF2 promoter. Reporter constructs were also generated using the pGL3 promoter vector and three copies of the proximal (−287 to −266 bp) or distal (−448 to −425 bp) HoxA10-binding cis elements from the FGF2 promoter. Constructs were generated with the wild type proximal and distal FGF2 sequences and with forms of the sequences with mutation of the Hox–binding consensus.

**Oligonucleotides**

Oligonucleotides were custom-synthesized by MWG Biotec (Piedmont, NC). Double-stranded oligonucleotides were syn-
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The human myelomonocytic leukemia cell line U937 (38) was obtained from Andrew Kraft (Hollings Cancer Center, Medical University of South Carolina, Charleston, SC). Cells were maintained as described previously (39). U937 cells were treated for 48 h with 500 units/ml human recombinant IFNγ for monocyte differentiation (Roche Applied Science) or retinoic acid + dimethylformamide for granulocyte differentiation.

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, HoxA10−/−, or HoxA10−/− C57/BL6 mice (40). Sca1+ cells were separated using the Miltenyi magnetic bead system (Miltenyi Biotechnologies, Auburn, CA). Bi-potential GMP cells were cultured (2 × 10⁵ cells/ml) for 48 h in DMEM supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, 10 ng/ml murine GM-CSF (R & Diagnostics Systems, Minneapolis, MN), 10 ng/ml murine recombinant IL-3 (R & D Systems) and 100 ng/ml SCF (R & D Systems). Cells were maintained in GM-CSF, IL3, and SCF for 48 h or were differentiated over 48 h in 20 ng/ml G-CSF (granulocyte) or 20 ng/ml of murine M-CSF (monocyte) (as in Refs. 17, 21).

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 10³ PFU/ml. Bone marrow mononuclear cells were cultured for 24 h in 10 ng/ml IL3, 10 ng/ml GM-CSF, and 100 ng/ml SCF. Cells were transduced by incubation with retroviral supernatant in the presence of Polybrene (6 μg/ml) as described previously (17, 21). Transduced cells were selected for 48 h in puromycin, cultured in GM-CSF, IL3, and SCF, or differentiated with M-CSF or G-CSF. Transgene expression was confirmed by real time PCR.

Quantitative Real Time PCR

RNA was isolated using TRIzol reagent (Invitrogen). Primers for real time PCR were designed with Applied Biosystems software. Real time PCR was performed with SYBR Green according to the “standard curve” method. Result were normalized to 18S (for mRNA determination) or input chromatin (for chromatin immunoprecipitation studies).

Chromatin Immunoprecipitation and Gene Discovery

U937 cells were cultured with or without IFNγ or retinoic acid for 48 h. Cells were incubated briefly in media supplemented with formaldehyde to generate DNA-protein crosslinks. For subcloning studies, cell lysates were sonicated to generate chromatin fragments with an average size of 2.0 kb (41). Lysates underwent two rounds of immunoprecipitation with HoxA10 antibody or irrelevant antibody (as in Refs. 19, 23, 41). The HoxA10 antibody used for these studies was a combination of two purified antibodies to different HoxA10 peptides that do not prevent DNA binding (N-20 and A-20 from Santa Cruz Biotechnology, Santa Cruz, CA). The irrelevant antibody is a purified antibody to glutathione S-transferase (anti-GST antibody, Santa Cruz Biotechnology).

Precipitated chromatin was recovered, and several batches of immunoprecipitated chromatin were combined for each experiment. Blunted ends were generated on the chromatin using Klenow enzyme. Linker oligonucleotides with BamHI sites were ligated onto the blunt-ended chromatin. The linked chromatin was recovered, digested with BamHI, and subcloned into the BamHI site of pBluescript vector. The ligation mixtures were transformed into Escherichia coli, and plasmid DNA was recovered from individual clones. This plasmid DNA was subjected to sequencing (23). Sequences were used for GenBank search to identify potential HoxA10-binding genes. Given the extensive manipulation and digestion of the DNA, the identified sequences did not always include the HoxA10-binding site that had co-precipitated with HoxA10 antibody. In previous studies, all such HoxA10-binding sites were within 1.5 kb of the recovered DNA fragment.

HoxA10 binding to genes identified in this manner was confirmed by independent chromatin immunoprecipitation experiments. For these studies, chromatin was co-immunoprecipitated from U937 lysates with an antibody to HoxA10 or irrelevant antibody, as described above. In some studies, lysates were sonicated to generate chromatin fragments of 1.5–2.0 kb. Chromatin was amplified with sets of primers representing various sequences in the gene within 3.0 kb of the immunoprecipitating chromatin. PCR products were visualized on agarose gels. In other experiments, lysates were sonicated to generate chromatin fragments of 200 bp, which were amplified by quantitative real time PCR using sets of primers flanking putative HoxA10-binding sites identified in functional (reporter gene) assays.

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 neo-mycin 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.

FGF2 Promoter Analysis—U937 cells were co-transfected with a construct with various sequences from the FGF2 5’ flank linked to a luciferase reporter (1.1 kb and 588, 467, 424, 297, 266, or 211 bp FGF2-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 −287 to −266-bp (proximal) or −448 to −425-bp (distal) sequence from the FGF2 promoter (using the pGL3-p vector) (30 μg), a vector to express HoxA10 (or empty vector control) (50 μg), and CMVβ-gal.

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 various proteins, including a loading control. Each experiment was repeated at least three times with different batches of lysate proteins. Representative blots are shown.

ELISA
Expression of Fgf2 or Tgfβ2 in the media of cultured cells was determined using the Emax Immunoassay System according to manufacturer’s instructions (Promega, Madison WI). Cells were maintained at a density of 0.6 × 10^6 cells per ml. Equivalent amounts of media were withdrawn at various time points for the assay. For other studies, murine serum was analyzed for Fgf2 or Tgfβ2 using this assay. The Fgf2 assay is Fgf2-specific and does not cross-react with other Fgfs; the Tgfβ2 assay is Tgfβ2-specific and does not cross-react with other Tgfβs.

Cell Proliferation Assays

Myeloid Cell Lines—U937 cells that were stably transfected with a vector to overexpress HoxA10 or empty vector control or co-transfected with vectors to overexpress HoxA10 (or with vector control) and express a β-catenin-specific shRNA (or scrambled shRNA control). Stable transfectants were deprived of fetal calf serum for 24 h and then treated with a dose titration of fetal calf serum (0.01−10%). Some cells were incubated with specific blocking antibodies to Fgf2, Tgfβ2, both, or control antibody (R & D Systems). Other studies were performed with a specific Fgf-R inhibitor (PD173074; Cayman Chemical Co., Ann Arbor, MI). Cell proliferation was determined by incorporation of [3H]thymidine as described previously (21).

Murine Bone Marrow—Murine bone marrow cells were transduced with a retroviral vector to overexpress HoxA10 or empty MSCV vector control and cultured in GM-CSF (10 ng/ml), IL3 (5 ng/ml), or SCF (100 ng/ml), deprived of cytokines for 24 h (cultured in DME supplemented with 10% FCS only), and stimulated for 24 h with a dose titration of GM-CSF (0.02−20 ng/ml + 5 ng/ml of IL3). Some cells were incubated with specific blocking antibodies to Fgf2, Tgfβ2, both, or control antibody (R & D Systems). Other studies were performed with a specific Fgf-R inhibitor (PD173074). Cell proliferation in response was determined as described previously (21).

In Vitro DNA Binding Assays

Isolation of Nuclear Proteins—Nuclear proteins were isolated from U937 cells by the method of Dignam with protease inhibitors, as described previously (39, 42).

Electrophoretic Mobility Shift Assays—Oligonucleotides probes were prepared and EMSA were performed, as described previously (17, 19, 20, 21, 23). Some assays were incubated with synthetic double-stranded oligonucleotide competitors (200-fold molar excess), as indicated. Antibody to HoxA10 (or pre-immune sera) was included in other binding assays. For binding reactions with a HoxA10 antibody, disruption of the complex (not supershift) was anticipated because the HoxA10 peptide used to generate the antibody is in close proximity to the DNA-binding homeodomain (custom produced by Covance, Princeton Township, NJ), as in our previous studies (17, 20, 21). The HoxA10 antibodies used for chromatin immunoprecipitation (see above) are less efficient for these in vitro assays relative to this HoxA10 antiserum.

For all experiments, at least three different batches of nuclear proteins were tested in at least two independent experiments. Integrity of the nuclear proteins and equality of protein loading was determined in control EMSA with a probe representing a classical CCAAT box from the α-globin gene promoter. Antiserum to HoxA10 (not cross-reactive with other Hox proteins) was obtained from Covance Research Products (Richmond, CA) and from Santa Cruz Biotechnology (Santa Cruz, CA).

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 (Berkeley, CA) (43–45).

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 FGF2 as HoxA10 Target Gene—We identified FGF2 as a potential HoxA10 target gene by sequence analysis of chromatin fragments that co-precipitated with a HoxA10 antibody. We identified a set of putative HoxA10 target genes using this technique, as we have reported previously (19–23). Chromatin immunoprecipitation was performed using U937 cells, a leukemia line that is best characterized as a GMP. U937 cells undergo differentiation to monocytes when treated with interferon β (IFNβ) or phorbol myristate acetate, and granulocytes when treated with retinoic acid and dimethyl formamide. U937 differentiation is associated with phagocyte functional competence, initial proliferation followed by proliferation arrest, and increased sensitivity to apoptosis (36–38). Therefore, these cells provide a reasonable model for investigations of myeloid progenitor function and differentiation.

For this study, chromatin was co-precipitated from U937 cell lysates with an antibody to HoxA10, and co-precipitating chromatin was recovered, subcloned into a plasmid vector, and sequenced, as described previously (23, 46). Using this approach, we identified an ~150-bp sequence from the proximal 5’ flank of the FGF2 gene. Although cell lysates were soni-
cated under conditions that generated chromatin of an average size of 2.0 kb, we found that most of the chromatin fragments we recovered by this technique were less than 500 bp in size (see our previous results) (23, 46).

We performed in silico analysis of the proximal 2.0 kb of FGF25/H11032 flank to identify conserved HoxA10-DNA-binding consensus sequences. Because HoxA10 frequently binds to DNA as a heterodimer with Pbx or Meis proteins, we searched the consensus sequences for binding of Hox proteins, Hox/Pbx dimers, or Hox/Meis dimers. We identified five potential HoxA10-binding sites in the proximal 3.0 kb of the FGF25/H11032 flank (relative to the transcription start site (TSS)). All of these sites were within the proximal 1.1 kb of FGF25 flank (Fig. 1A; 5 flank shown from the TSS).

To verify HoxA10 binding to the FGF25 flank, independent chromatin immunoprecipitation experiments were performed. For these studies, lysates were prepared as described above, and chromatin was immunoprecipitated with a HoxA10 antibody or preimmune serum. Co-precipitating chromatin was amplified by PCR using primers to various regions of the FGF25 flank. PCR products were separated by agarose gel electrophoresis. Input (nonprecipitated) chromatin was a positive control for these studies.

FIGURE 1. Identification of FGF2 as a HoxA10 target gene. A, there are multiple conserved HoxA10-DNA-binding consensus sequences in the 5' flank of the FGF2 gene. The proximal 5' flank sequences of the human and murine genes were compared for homology and conserved DNA-binding consensus sequences for Hox proteins or Hox/Pbx or Hox/Meis heterodimers. The location of the sequence identified in HoxA10 co-precipitating chromatin is identified by red brackets. The HoxA10-binding functional cis elements are underlined in red. The transcription start site is indicated.

B, HoxA10 binds to the FGF25 flank in vivo. Chromatin co-immunoprecipitation (IP) was performed using U937 cells and an antibody to HoxA10 or preimmune serum. Co-precipitating chromatin was amplified by PCR using primers to various regions of the FGF25 flank. PCR products were separated by agarose gel electrophoresis. Input (nonprecipitated) chromatin was a positive control for these studies.
We found that HoxA10 overexpression significantly increases reporter activity from constructs with 1.1 kb and 588, 467, 424, and 297 bp of FGF2 5' flank (p < 0.001, n = 6) (Fig. 2A). In contrast, reporter activity of constructs with 266 and 211 bp of FGF2 5' flank is not significantly different with versus without overexpressed HoxA10 (p > 0.5, n = 6) (Fig. 2A). We found that reporter activity in non-HoxA10-overexpressing transfectants is not significantly different for any of these FGF2 promoter constructs (p = 0.2, n = 6). However, in HoxA10-overexpressing transfectants, reporter activity from various constructs fell into two groups as follows: a higher expression group and a lower expression group. Constructs with 1.1 kb and 588 and 467 bp constitute the higher expression group, and HoxA10-induced reporter expression from these constructs is not significantly different from each other (p = 0.8, n = 6) (Fig. 2A). Similarly, reporter activity is not significantly different in
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HoxA10-overexpressing transfectants with 424-bp versus 297-bp constructs (lower expression group) \((p = 0.6, n = 6)\) (Fig. 2A).

We examined HoxA10-induced reporter activity using constructs with 467-bp versus 424-bp FGF2 promoter as representatives of these two groups. We found that HoxA10 overexpression exerts an \(\sim 2\)-fold greater effect on the 467-bp construct \((187.5 \pm 8.8\% \text{ increase with HoxA10})\) in comparison with the 424-bp construct \((87.6 \pm 10.9\% \text{ increase with HoxA10})\) (Fig. 2B). This difference is statistically significant \((p = 0.0001, n = 6)\) and is consistent with one HoxA10-binding cis element between \(-424\) and \(-467\) bp and another between \(-297\) and \(-266\) bp.

Sequence analysis identified potential Hox-DNA-binding consensus sequences between \(-271\) and \(-282\) bp and between \(-441\) and \(-431\) bp in the FGF2 promoter. The function of these consensus sequences as HoxA10-binding cis elements was first investigated by introducing mutations into the FGF2 promoter. For these studies, a mutant construct was generated with the \(-467\)-bp sequence with mutation of the distal Hox-binding consensus, and another was generated with \(-297\) bp with mutation of the single Hox-binding consensus. Therefore, the mutant \(-467\)-bp construct includes one remaining potential Hox-binding cis element, and the mutant \(-297\)-bp construct does not include a potential Hox-binding cis element.

These constructs were co-transfected into U937 cells with a vector to overexpress HoxA10 (or control vector). Comparison was made with transfection experiments using WT promoter sequence constructs. We found that mutation of one of the two potential Hox-binding cis elements in the \(-467\)-bp construct significantly decreases HoxA10-induced promoter activity \((p < 0.001, n = 4)\) (Fig. 2C). HoxA10-induced activity of the mutant \(-467\)-bp construct is not different from activity of the wild type \(-297\)-bp construct \((p = 0.6, n = 4)\) (Fig. 2C). We also found that mutation of the remaining potential Hox-binding cis element in the \(-297\)-bp construct abolishes the HoxA10-induced increase in reporter activity \((p = 0.8, n = 4)\) for promoter activity from the WT and mutant \(-297\)-bp FGF2 promoter sequences with \(\textit{versus without HoxA10}\).

In control experiments, HoxA10 overexpression does not affect activity of empty control reporter construct under any assay conditions. Activity of the control pGL3-basic plasmid is less than 10\% that of constructs containing FGF2 promoter sequence and was subtracted as background.

We investigated activity of these putative cis elements in additional transfection experiments with reporter constructs containing three copies of the proximal \((-287\) to \(-266\) bp) or distal \((-448\) to \(-425\) bp) FGF2 sequence linked to a minimal promoter. U937 cells were co-transfected with these constructs (or control minimal promoter/reporter vector) and a vector to overexpress HoxA10 (or empty expression vector). Transfectants were analyzed as above.

We found that HoxA10 overexpression significantly increases reporter expression from both of the FGF2 sequence-containing constructs \((p < 0.0001, n = 6\) for both constructs) (Fig. 2D). Reporter activity of the proximal and distal cis element containing constructs is equivalent in HoxA10-overexpressing transfectants \((p = 0.8, n = 6)\). The occurrence of multiple HoxA10-binding cis elements in the FGF2 promoter is consistent with previous studies of the TGFβ2, DUSP4, CYBB, and NCF2 promoters (19, 21, 35, 36).

 Constructs were also generated with three copies of the proximal or distal FGF2 cis elements with mutation of the Hox-binding consensus. These reporter constructs were co-transfected into U937 cells with a vector to overexpress HoxA10 (or empty expression vector). We found that inclusion of these mutant sequences does not significantly increase reporter activity above that of the minimal promoter/reporter control vector. This is not altered by HoxA10 overexpression (Fig. 2D).

The WT \(-287\) to \(-266\)-bp and \(-448\) to \(-425\)-bp cis element constructs were also used to determine the effects of increasing HoxA10-overexpression on cis element activity. For these experiments, U937 cells were co-transfected with the minimal promoter/reporter constructs with the proximal or distal HoxA10-binding cis elements (or control vector) and a vector to overexpress increasing amounts of HoxA10 (or empty expression vector). We found that activation of the FGF2 cis elements increases in a HoxA10-dose dependent manner in these transfectants (Fig. 2E). Graded expression of HoxA10 in these transfectants was verified in control experiments (Fig. 2F).

HoxA10 Binds to Two FGF2 Cis Elements—We next determined whether HoxA10 interacts with these FGF2 cis elements. We first investigated \textit{in vitro} HoxA10 binding using EMSA. For these studies, nuclear proteins were isolated from U937 cells, incubated with double-stranded synthetic oligonucleotide probes representing the \(-287\) to \(-266\)-bp or \(-448\) to \(-425\)-bp FGF2 sequences, and protein-DNA complexes were identified by native gel electrophoresis. To determine the binding site specificity of shifted complexes, some binding assays were preincubated (prior to probe addition) with an excess of unlabeled competitor oligonucleotides representing homologous probe sequence (“self”-competitor), the sequence of the other FGF2 cis element, or oligonucleotides with mutation in the putative Hox-binding sites of these two FGF2 cis elements. Other assays were preincubated with competitor oligonucleotides representing HoxA10-binding cis elements from the CYBB, DUSP4, and TGFβ2 genes. As a negative control, additional binding assays were preincubated with an oligonucleotide competitor with an irrelevant region from the CYBB gene.

We found that the \(-287\) to \(-266\) oligonucleotide probe binds two low mobility complexes that are cross-competitive with self-oligonucleotide, the \(-448\) to \(-425\)-bp FGF2 sequence, and HoxA10-binding sites from these other genes (Fig. 3A). Binding of these complexes is not competed for by mutant FGF2 competitor oligonucleotides or by an irrelevant oligonucleotide (Fig. 3A). Similar results were obtained with the \(-448\) to \(-425\)-bp FGF2 sequence probe (Fig. 3B).

We also investigated whether the protein complexes that bound to these two probes were cross-immunoreactive with HoxA10. For these experiments, nuclear proteins were preincubated with an antibody to HoxA10 or preimmune serum prior to adding the oligonucleotide probes. We found that the
low mobility complexes that bind to the two FGF2 probes are disrupted by HoxA10 antibody but not control preimmune serum (Fig. 3C). These studies used a custom-generated antibody that recognizes a unique HoxA10 peptide that is adjacent to the DNA-binding homeodomain. This Hoxa10 antiserum is different from the HoxA10 antibodies used for chromatin immunoprecipitation, which are less efficient in this assay.
We also used quantitative chromatin immunoprecipitation to determine whether HoxA10 binds in vivo to these cis elements. For these studies, cell lysates were sonicated to generated ~200-bp chromatin fragments prior to immunoprecipitation with an antibody to HoxA10 or irrelevant control antibody. Co-precipitating chromatin was amplified by real time PCR using primers flanking the proximal (-287 to -266 bp) or distal (-448 to -425 bp) FGF2 cis elements. Primers used in these experiments were designed to generate a product of ~100 bp. We found that the two cis elements specifically co-precipitate with HoxA10 from lysates of U937 cells (Fig. 3D).

**HoxA10 Increases Fgf2 Expression in Myeloid Leukemia Cells**—We next determined whether Fgf2 is expressed in a HoxA10-dependent manner. For these studies, U937 cells were stably transfected with a vector to overexpress HoxA10 or with empty control vector. To determine whether differentiation stage influenced Fgf2 expression, some transfectants were treated with IFNγ to induce monocyte differentiation or retinoic acid (RA) to induce neutrophil differentiation. Expression of Fgf2 mRNA was determined by real time PCR.

We found that Fgf2 mRNA expression in control transfectants is not significantly altered by differentiation (p = 0.6, n = 6) (Fig. 4A). We also found that HoxA10-overexpression significantly increases expression of Fgf2 mRNA, with or without differentiation (p < 0.01, n = 6) (Fig. 4A).

The hypothesis of these studies is that increased Fgf2 production by HoxA10 overexpressing myeloid progenitor cells leads to autocrine stimulation of proliferation. Therefore, we determined whether the two major Fgf2 receptors, Fgf receptor 1 and Fgf receptor 2 (Fgf-R1 and Fgf-R2), are expressed in these U937 transfectants. We found that both receptors are expressed, and the level of expression is not altered by differentiation or HoxA10 overexpression (not shown).

We also determined expression and secretion of the Fgf2 protein in the stable transfectants. We found that Fgf2 has relatively more abundant HoxA10-overexpressing transfectants in comparison with control transfectants by Western blot of cell lysate proteins (Fig. 4B). In addition, more Fgf2 is present in the media of HoxA10-overexpressing transfectants in comparison with control transfectants by ELISA (Fig. 4C; using an Fgf2-specific antibody). Differentiation of the transfectants with IFNγ or retinoic acid did not alter the amount of Fgf2 that is produced by either control or HoxA10-overexpressing U937 cells (p > 0.1, n = 4), consistent with expression of Fgf2 mRNA, above.

**FIGURE 4.** HoxA10 overexpression increases Fgf2 mRNA expression and Fgf2 protein secretion in U937 myeloid leukemia cells. A, expression of Fgf2 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. Transfectants were analyzed untreated or after differentiation to monocytes with IFNγ or neutrophils with retinoic acid (RA). Expression of HoxA10 and Fgf2 was determined by real time PCR. Statistically significant increase in HoxA10 expression is indicated by * A statistically significant differences in gene expression are indicated by * or ** (p < 0.002, n = 4). NS, nonstatistically significant. B, expression of Fgf2 protein is increased in HoxA10-overexpressing U937 cells. The U937 stable transfectants, described above, were also analyzed for Fgf2 protein expression by Western blot (WB). Total cell lysate proteins were separated by SDS-PAGE, and blots were serially probed with antibodies for HoxA10, Fgf2, or GAPDH (as a loading control). C, secretion of Fgf2 protein is increased in HoxA10-overexpressing U937 cells. Secretion of Fgf2 by the U937 stable transfectants, described above, was determined by ELISA. Cells were maintained at a constant cell concentration, and media were collected and analyzed for Fgf2 using an antibody that does not cross-react with other Fgf proteins. Statistically significant increase in Fgf2 in HoxA10-overexpressing transfectants in comparison with control transfectants is indicated by * (p < 0.0001, n = 3).
HoxA10 Increases Fgf2 Expression in Primary Myeloid Progenitor Cells—The studies in the above section were performed using a myeloid leukemia cell line with recognized abnormalities in cell proliferation and survival. Therefore, we also investigated the role of HoxA10 in Fgf2 regulation in experiments with primary murine bone marrow cells. To determine whether HoxA10 plays an essential role in Fgf2 expression, we used bone marrow from transgenic mice with targeted disruption of the HoxA10 gene (17). These mice exhibit urogenital and reproductive abnormalities (17) and mild pancytopenia that worsens with age. We also investigated the effect of HoxA10 overexpression on Fgf2 production in WT murine bone marrow.

For these experiments, myeloid progenitor cells were isolated from the bone marrow of HoxA10−/− and WT mice. Some WT cells were transduced with a retroviral vector to overexpress HoxA10 or with empty control vector (MSCV) (17, 21). Cells were cultured in GM-CSF, IL3, and SCF, a cytokine combination that produces predominantly bipotential granulocyte/macrophage progenitors (GMP; Sca1−CD34−CD38−) (17). Some cells were differentiated ex vivo with G-CSF or M-CSF to produce granulocytes or monocytes, respectively. More than 80% of cells are differentiated under these conditions (to CD34−CD38− and Gr1− or Mac1+) (17).

We first investigated Fgf2 mRNA expression in by real time PCR. We found that Fgf2 mRNA expression is equivalent in WT murine bone marrow cells under the three cytokine conditions (p = 0.8, n = 6) (Fig. 5A). We also found that Fgf2 mRNA expression is significantly less in HoxA10−/− cells in comparison with control cells under these conditions (p < 0.001, n = 6) (Fig. 5A). However, relatively less Fgf2 mRNA is expressed in HoxA10−/− cells after ex vivo differentiation to granulocytes in comparison with HoxA10−/− cells cultured under GMP conditions or after ex vivo monocyte differentiation (p = 0.02, n = 6) (Fig. 5A).

HoxA10 overexpression in WT murine bone marrow cells increases Fgf2 mRNA under all three cytokine conditions (p < 0.001, n = 6), consistent with results in cell lines (Fig. 5A). Expression of Fgf2 is somewhat greater in HoxA10-overexpressing and G-CSF-differentiated cells relative to HoxA10-overexpressing cells cultured in GM-CSF, IL3, and SCF or differentiated with M-CSF. However, this difference did not reach statistical significance (p = 0.1, n = 6).

Control experiments were performed to verify HoxA10 expression in these primary murine bone marrow cells. We found that differentiation of WT cells with either G-CSF or M-CSF significantly decreases HoxA10 expression (p < 0.0001, n = 6) (Fig. 5B). This is consistent with the profile of HoxA10 expression during differentiation from CD34+ cells to CD34− cells, as described previously (2, 17). HoxA10 expression is undetectable in HoxA10−/− cells and is significantly increased in cells transduced with the HoxA10 expression vector (Fig. 5B).

Expression of Fgf-R1 and Fgf-R2 was verified in these primary cell populations by real time PCR. We found that expression of these receptors is not influenced by HoxA10 abundance or differentiation stage (data not shown).

We also determined the influence of HoxA10 expression on Fgf2 secretion by primary murine bone marrow cells. In preliminary experiments, we found that the amount of Fgf2 secreted by WT murine bone marrow cells is not significantly different under GMP conditions (GM-CSF, IL3, and SCF) versus M-CSF- or G-CSF-differentiated cells (p = 0.1, n = 4) (Fig. 5C). Significantly less Fgf2 is secreted by HoxA10−/− cells under all three cytokine conditions in comparison with WT cells (p < 0.02, n = 4), consistent with mRNA expression (Fig. 5C). Conversely, Fgf2 secretion is increased by HoxA10 overexpression under each of the three cytokine conditions (p < 0.01, n = 6). There is significantly more Fgf2 secreted into the media of G-CSF-treated, HoxA10-overexpressing cells in comparison with HoxA10-overexpressing cells treated with M-CSF or cultured under GMP conditions (p < 0.002, n = 6) (Fig. 5C).

We also investigated the role of HoxA10 in Fgf2 production in vivo. For these experiments, serum from WT, HoxA10−/−, and HoxA10−/− mice was analyzed for Fgf2 protein by ELISA. All mice in these experiments were 28 weeks of age, at which time HoxA10−/− mice exhibit mild pancytopenia. We found significantly less Fgf2 in the serum of HoxA10−/− mice in comparison with WT control mice (~30% less; p < 0.001, n = 4) (Fig. 5D). Because our previous studies determined that Tgfβ2 is also a HoxA10 target gene, we determined expression of Tgfβ2 in the serum of these mice. We found that Tgfβ2 expression is also significantly decreased in the serum of HoxA10−/− mice in comparison with WT mice (~35% less, p < 0.001, n = 4) (Fig. 5D).

HoxA10 Increases Proliferation in a Fgf2-dependent Manner—The hypothesis of these studies is that increased production of Fgf2 influences proliferation of HoxA10-overexpressing myeloid cells. We hypothesize that sustained activation of signaling pathways downstream from Fgf receptors in these cells results in hypersensitivity to other cytokines that use the same pathways. To investigate this, we performed proliferation assays using U937 stable transfectants. For these studies, HoxA10-overexpressing or control cells were deprived of serum and restimulated with a dose titration of FCS (as a source of cytokines), and proliferation was determined by [3H]thymidine incorporation.

To determine the role of Fgf2 produced by the cells, some cells were cultured in the presence of an Fgf2-specific blocking antibody (or control antibody). In previous studies, we demonstrated that production of Tgfβ2 by HoxA10-overexpressing U937 cells increases the proliferative response to FCS (21). To determine whether there is cooperation between Fgf2 and Tgfβ2, other cells were cultured in the presence of a Tgfβ2 blocking antibody, or Fgf2 blocking antibody + Tgfβ2 blocking antibody.

We found that HoxA10-overexpressing U937 transfectants are hypersensitive to the proliferative effects of FCS in comparison with control transfectants (i.e. increased proliferation at a given FCS dose in HoxA10-overexpressing cells), consistent with our previous studies (17, 21). We also found that treatment with an Fgf2-blocking antibody significantly decreases the proliferative response to FCS in HoxA10-overexpressing cells (p < 0.0001, n = 6 at all doses) (Fig. 6A). Treatment with Fgf2-blocking antibody also decreases the proliferative response in control

3 E. A. Eklund, E. Hjort, and L. Bei, unpublished observations.
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**FIGURE 5.** Fgf2 mRNA expression and protein secretion are altered in a HoxA10-dependent manner in primary murine bone marrow cells. A, Fgf2 mRNA expression in primary murine progenitor cells and differentiating cells is decreased by HoxA10 knock-out and increased by HoxA10 overexpression. Bone marrow myeloid progenitor cells were isolated from WT or HoxA10^+/−^ mice. Some myeloid progenitors from WT murine bone marrow were transduced with a retroviral vector to overexpress HoxA10 or with empty control vector (MSCV). Cells were cultured in GM-CSF, IL3, and SCF (granulocyte/monocyte progenitor conditions). Some cells were differentiated to monocytes with M-CSF or granulocytes with G-CSF. Expression of Fgf2 was determined by real time PCR. Statistically significant decrease in Fgf2 mRNA expression in HoxA10^−/−^ versus WT cells under each of the culture conditions is indicated by *, **, and *** (p < 0.0001, n = 3). Statistically significant increase in Fgf2 mRNA expression in HoxA10-overexpressing cells versus control vector transduced cells under each of the culture conditions is indicated by #, ##, and ### (p < 0.0001, n = 3). B, HoxA10 mRNA expression in primary murine progenitor cells is decreased by HoxA10 knock-out and increased by HoxA10 overexpression. Primary murine bone marrow cells, described above, were also analyzed for HoxA10 expression by real time PCR. Statistically significant decrease in HoxA10 mRNA expression in HoxA10^−/−^ versus WT cells under each of the culture conditions is indicated by *, **, and *** (p < 0.00001, n = 3). Statistically significant increase in HoxA10 mRNA expression in HoxA10-overexpressing cells versus control vector-transduced cells under each of the culture conditions is indicated by #, ##, and ### (p < 0.00001, n = 3). C, Fgf2 protein secretion in primary murine progenitor cells and differentiating cells is decreased by HoxA10 knock-out and increased by HoxA10 overexpression. Secretion of Fgf2 by primary murine bone marrow cells, described above, was also determined. Cells were maintained at a constant cell concentration, and media were collected and analyzed for Fgf2 using an ELISA that does not cross-react with other Fgf proteins. Statistically significant decrease in Fgf2 production in HoxA10^−/−^ in comparison with wild type cells under the various cytokine conditions is indicated by *, **, and *** (p = 0.0001, n = 3). Statistically significant increase in Fgf2 production from cells with HoxA10 overexpression in comparison with cells transduced with control MSCV vector under the various cytokine conditions is indicated by #, ##, and ### (p < 0.0001, n = 3). D, serum levels of Fgf2 and Tgfβ2 are decreased in HoxA10^−/−^ mice in comparison with control mice. Serum was collected from WT, HoxA10^−/−^, and HoxA10^+/−^ mice (28 weeks old) and analyzed by ELISA for Fgf2 or Tgfβ2 expression. Statistically significant differences in Fgf2 and Tgfβ2 in the serum of WT versus HoxA10^−/−^ mice are indicated by * and **, respectively (p < 0.01, n = 4).

Fgf2 interaction with Fgf-R1 results in recruitment of Fgf-R2, stimulation of tyrosine kinase activity, and receptor autophosphorylation. Therefore, we performed similar proliferation assays using HoxA10-overexpressing and control U937 cells treated with a specific Fgf receptor inhibitor (PD173074). We found that treatment with this inhibitor significantly decreases the proliferative response of HoxA10-overexpressing U937 cells to FCS (p < 0.001, n = 6 at all doses) (Fig. 6B). In contrast, there is less of an effect on control U937 cells.

Control studies were performed to determine the efficiency and specificity of the Fgf2 blocking antibody and PD173074.

In summary, we have demonstrated that Fgf2 expression is regulated by HoxA10 in primary murine bone marrow cells and that this regulation is mediated by the Fgf2 receptor. These findings have important implications for the understanding of the role of HoxA10 in hematopoiesis and for the development of new therapeutic strategies for hematological disorders.

**FIGURE 6.** A, proliferation of HoxA10-overexpressing U937 cells treated with a Tgfβ2 blocking antibody. As in our previous studies, we found that treatment with a Tgfβ2 blocking antibody significantly decreases the proliferative response of HoxA10-overexpressing cells to FCS. Treatment of these cells with blocking antibodies to both Fgf2 and Tgfβ2 decreases the proliferative response significantly more than either antibody alone (Fig. 6A).
For these studies, Fgf-R activation (phosphorylation) was determined by Western blots of cell lysates from these proliferation assays (at the 10% FCS dose). We found an increase in phosphorylated (but not total) Fgf-R1 in HoxA10-overexpressing transfectants in comparison with control vector transfectants, consistent with increased stimulation by Fgf2 in these cells (Fig. 6C). Treatment with either Fgf2-blocking antibody or PD173074 significantly decreases Fgf-R1 activation in HoxA10-overexpressing U937 transfectants (Fig. 6C). In other control experiments, treatment of cells with Fgf2-blocking antibody or PD173074 did not alter phosphorylation of Tgfβ-R1 or the common β chain of the GM-CSF-R (not shown).

We also investigated the role of HoxA10 in Fgf2-dependent proliferation of primary murine myeloid progenitor cells. For these studies, WT murine bone marrow cells were transduced with a HoxA10 expression vector or control vector, as described in the studies above. Cells were cultured in GM-CSF, IL3, and SCF, deprived of cytokines, and stimulated with a dose titration of GM-CSF. Some cultures were treated with Fgf2-blocking antibody or PD173034 decreases Fgf receptor activation in HoxA10-overexpressing U937 cells. Lysates from stable transfectants described in A (above; FCS dose 10%) were analyzed by Western blots probed for phospho(active) Fgf-R1, total Fgf-R1, and tubulin (as a loading control).

For these studies, Fgf-R activation (phosphorylation) was determined by Western blots of lysates from these proliferation assays (at the 10% FCS dose). We found an increase in phosphorylated (but not total) Fgf-R1 in HoxA10-overexpressing transfectants in comparison with control vector transfectants, consistent with increased stimulation by Fgf2 in these cells (Fig. 6C). Treatment with either Fgf2-blocking antibody or PD173074 significantly decreases Fgf-R1 activation in HoxA10-overexpressing U937 transfectants (Fig. 6C). In other control experiments, treatment of cells with Fgf2-blocking antibody or PD173074 did not alter phosphorylation of Tgfβ-R1 or the common β chain of the GM-CSF-R (not shown).

We also investigated the role of HoxA10 in Fgf2-dependent proliferation of primary murine myeloid progenitor cells. For these studies, WT murine bone marrow cells were transduced with a HoxA10 expression vector or control vector, as described in the studies above. Cells were cultured in GM-CSF, IL3, and SCF, deprived of cytokines, and stimulated with a dose titration of GM-CSF. Some cultures were treated with Fgf2-blocking antibody or PD173034 decreases Fgf receptor activation in HoxA10-overexpressing U937 cells. Lysates from stable transfectants described in A (above; FCS dose 10%) were analyzed by Western blots probed for phospho(active) Fgf-R1, total Fgf-R1, and tubulin (as a loading control).

For these studies, Fgf-R activation (phosphorylation) was determined by Western blots of lysates from these proliferation assays (at the 10% FCS dose). We found an increase in phosphorylated (but not total) Fgf-R1 in HoxA10-overexpressing transfectants in comparison with control vector transfectants, consistent with increased stimulation by Fgf2 in these cells (Fig. 6C). Treatment with either Fgf2-blocking antibody or PD173074 significantly decreases Fgf-R1 activation in HoxA10-overexpressing U937 transfectants (Fig. 6C). In other control experiments, treatment of cells with Fgf2-blocking antibody or PD173074 did not alter phosphorylation of Tgfβ-R1 or the common β chain of the GM-CSF-R (not shown).

We also investigated the role of HoxA10 in Fgf2-dependent proliferation of primary murine myeloid progenitor cells. For these studies, WT murine bone marrow cells were transduced with a HoxA10 expression vector or control vector, as described in the studies above. Cells were cultured in GM-CSF, IL3, and SCF, deprived of cytokines, and stimulated with a dose titration of GM-CSF. Some cultures were treated with Fgf2-blocking antibody or PD173034 decreases Fgf receptor activation in HoxA10-overexpressing U937 cells. Lysates from stable transfectants described in A (above; FCS dose 10%) were analyzed by Western blots probed for phospho(active) Fgf-R1, total Fgf-R1, and tubulin (as a loading control).
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(Fig. 7A). Treatment with Fg2-blocking antibody also significantly decreases proliferation of HoxA10-overexpressing myeloid progenitor cells in the absence of GM-CSF stimulation ($p = 0.001, n = 6$). Proliferation of control vector transduced cells is also decreased by treatment with Fg2-blocking antibody but to a significantly lesser extent.

As in our previous studies, Tgfβ2 blocking antibody significantly decreases GM-CSF-induced proliferation in HoxA10-overexpressing cells (Fig. 7A) (21). The combination of blocking antibodies to Fg2 and Tgfβ2 has a greater effect on proliferation of HoxA10-overexpressing cells relative to either antibody individually (Fig. 7A) ($p < 0.01, n = 6$). Treatment with PD173074 significantly decreases GM-CSF-induced proliferation in HoxA10-overexpressing primary murine bone marrow cells ($p < 0.001, n = 6$), but it had less of an effect on control vector-transduced cells (Fig. 7B).

Western blots of cell lysates were performed to verify the efficiency and specificity of Fg2-blocking antibody or Fg-R inhibitor, as for U937 cells. We found that treatment with either Fg2-blocking antibody or PD173074 results in a decrease in Fg-R1 phosphorylation (but not expression), but treatment with Tgfβ2-blocking antibody does not (Fig. 7C).

HoxA10 Induces an Fg2-dependent Increase in β-Catenin in Myeloid Leukemia Cells—Fg2 activates a number of signaling pathways in a variety of cell types. In endothelial cells, Fg2 increases β-catenin protein expression through activation of PI

![Graph A](image)

**Proliferation Assays**

**Transduced Murine Bone Marrow Cells**

- **Control**
- **Control + Fg2 Ab**
- **Control + Tgfβ2 Ab**
- **Control + both blocking Ab**
- **HoxA10**
- **HoxA10 + Fg2 Ab**
- **HoxA10 + Tgfβ2 Ab**
- **HoxA10 + both blocking Ab**

- [Graph B](image)

**Proliferation Assays**

**Transduced Murine Bone Marrow Cells**

- **Control**
- **Control + PD173074**
- **HoxA10**
- **HoxA10 + PD173074**

**Western Blot**

**Transduced Murine Bone Marrow**

- + - - - **Control vector**
- - + + + **HoxA10 vector**
- - - + + **Fg2 blocking Ab**
- - - + + **PD173074**
- - - - + **Tgfβ2 blocking Ab**

- **p-Fgf-R1 Ab**
- **Fgf-R1 Ab**
- **Tubulin Ab**

**FIGURE 7.** HoxA10 overexpression in primary murine bone marrow cells induces Fg2-dependent cytokine hypersensitivity. A, cytokine hypersensitivity of HoxA10-overexpressing primary murine myeloid progenitor cells is inhibited by an Fg2-blocking antibody. Primary murine myeloid progenitor cells were transduced with a vector to overexpress HoxA10 or control MSCV vector, as described above. Cells were deprived of cytokines and treated with a dose titration of GM-CSF with or without a blocking antibody to Fg2, Tgfβ2, or both. Proliferation was determined by $[^{3}H]$thymidine incorporation. Statistically significant differences in proliferation of HoxA10-overexpressing cells with Fg2-blocking antibody versus control antibody at a given cytokine dose are indicated by * ($p < 0.001, n = 3$). Significant decreases in proliferation in HoxA10-overexpressing cells treated with Fg2 blocking antibody and Tgfβ2 blocking antibody versus either antibody alone are indicated by ** ($p < 0.01, n = 3$). B, cytokine hypersensitivity of HoxA10-overexpressing primary murine myeloid progenitor cells is inhibited by an Fg-R receptor inhibitor. Primary transduced murine myeloid progenitors (described above) were also analyzed for GM-CSF-induced proliferation with or without treatment with PD173034 (a specific Fg-R inhibitor). Statistically significant differences in proliferation of HoxA10-overexpressing cells with versus without the inhibitor are indicated by * ($p < 0.01, n = 3$). C, treatment with Fg2 blocking antibody or PD173034 decreases Fg-R receptor activation in HoxA10-overexpressing primary murine myeloid progenitor cells. Lysates from stable transfectants described in A (above; 20 ng/ml GM-CSF) were analyzed by Western blots probed for phospho(active) Fg-R1, total Fg-R1, and tubulin (as a loading control).
3-kinase (32, 33). PI 3-kinase activates Akt, and Akt inactivates Gsk3β. β-Catenin is a Gsk3β substrate, and serine/threonine-phosphorylated β-catenin is ubiquitinated and degraded by the proteasome. PI 3-kinase is frequently activated in leukemia cells, and this pathway is shared with the GM-CSF-receptor. Therefore, PI 3-kinase activation was an attractive candidate for mediating proliferative effects of Fgf2 on HoxA10-overexpressing myeloid progenitor cells.

To investigate this hypothesis, we first determined the effect of inhibiting PI 3-kinase on Fgf2-induced proliferation of U937 cells. For these experiments, cells were deprived of cytokines followed by stimulation with a dose titration of Fgf2 (in the presence of 10% FCS). Some cells were treated with LY294002. Proliferation was determined by [3H]thymidine incorporation. Statistically significant decrease in proliferation in LY294002-treated cells for any given dose of rFgf2 is indicated by * (p ≤ 0.001, n = 3).

**FIGURE 8.** Fgf2 stimulates PI 3-kinase-dependent proliferation of U937 myeloid leukemia cells and increases β-catenin protein and activity. A, proliferative response of U937 cells to recombinant Tgfβ2 is inhibited by the PI 3-kinase inhibitor LY294002. U937 cells were deprived of cytokines and then stimulated with a dose titration of rFgf2 (in the presence of 10% FCS). Some cells were treated with LY294002. Proliferation was determined by [3H]thymidine incorporation. Statistically significant decrease in proliferation in LY294002-treated cells for any given dose of rFgf2 is indicated by * (p ≤ 0.001, n = 3).

B, HoxA10 overexpression in U937 cells results in Fgf2-dependent increase in Akt activation, Gsk3β inactivation, and β-catenin protein expression. U937 were stably transfected with a vector to overexpress HoxA10 or with empty vector control (as described above). Cells were treated with Fgf2 blocking antibody or control antibody, as indicated. Total cell lysates were analyzed by Western blots (WB) that were serially probed with antibodies to β-catenin, phospho-Akt (activated), total Akt, serine-phosphorylated Gsk3β (inactive), total Gsk3β, or GAPDH (as a loading control). C, HoxA10 overexpression in U937 cells increases mRNA expression for β-catenin target genes. The U937 stable transfectants, discussed above, were analyzed by real time PCR for mRNA expression of β-catenin and β-catenin target genes (cyclin D1, c-Myc, and survivin). Statistically significant increase in mRNA expression with HoxA10 overexpression is indicated by *, ***, or ##. Statistically significant decrease in mRNA expression in HoxA10-overexpressing cells treated with Fgf2-blocking antibody is indicated by **, #, or ###. D, knockdown of β-catenin decreases cytokine hypersensitivity of HoxA10-overexpressing U937 cells. U937 cells were stably co-transfected with a vector to overexpress HoxA10 (or empty control vector) and a vector to express a β-catenin-specific shRNA (or scrambled shRNA control vector). Cells were analyzed for proliferation in response to a dose titration of FCS, as described in B (above). Statistically significant difference in proliferation with β-catenin knockdown is indicated by * (p < 0.001, n = 4).
Therefore, we investigated the hypothesis that HoxA10 overexpression increases β-catenin protein in an Fgf2-dependent manner. For these studies, HoxA10-overexpressing or control U937 transfectants (described above) were deprived of cytokines and restimulated with 10% FCS. Cells were treated with Fgf2-blocking antibody (or control antibody), and lysates were analyzed by Western blot.

We found that β-catenin protein is more abundant in HoxA10-overexpressing U937 cells in comparison with control cells (Fig. 8B). However, this increase in β-catenin is abrogated by treatment of the cells with Fgf2-blocking antibody (Fig. 8B). Activation (phosphorylation) of Akt is relatively greater in HoxA10-overexpressing transfectants in comparison with control transfectants, and inactivating phosphorylation of Gsk3β (phospho serine 9) is also increased in these cells (Fig. 8B). Both events were reversed by treatment of HoxA10-overexpressing transfectants with Fgf2-blocking antibody.

β-Catenin influences cell proliferation by activating transcription of target genes that include the genes encoding cyclin D1, c-Myc, and survivin. Therefore, we determined whether increased β-catenin in HoxA10-overexpressing U937 cells influences expression of these target genes in an Fgf2-dependent manner. For these studies, HoxA10-overexpressing or control U937 transfectants were deprived of cytokines and restimulated with FCS with or without Fgf2-blocking antibody, as above. Expression of β-catenin, cyclin D1, c-Myc, and survivin mRNA was determined by real time PCR.

We found that expression of each of these β-catenin target genes is increased in HoxA10-overexpressing cells, but this is reversed by Fgf2-blocking antibody (Fig. 8C). In contrast, β-catenin mRNA expression is not significantly greater in HoxA10-overexpressing cells in comparison with control cells, and it is not influenced by Fgf2-blocking antibody (Fig. 8C). This was consistent with our hypothesis that HoxA10 influences β-catenin at the level of protein stability, rather than by increasing CTNNB1 transcription.

We also investigated the role of β-catenin in cytokine hypersensitivity of HoxA10-overexpressing cells. For these experiments, U937 cells were stably co-transfected with a vector to overexpress HoxA10 (or control vector) and a vector to express a β-catenin-specific shRNA (or scrambled shRNA control). Transfectants were cytokine-deprived and re-stimulated with a dose titration of FCS, and proliferation was determined by [3H]thymidine incorporation. We found that knockdown of β-catenin significantly decreases proliferation of HoxA10-overexpressing transfectants at all FCS doses investigated (p < 0.001, n = 4) (Fig. 8D). In contrast, β-catenin knockdown has a minimal effect on proliferation of control vector transfectants in this experiment (Fig. 8D). The β-catenin-specific shRNA used in these studies decreases β-catenin protein by >70% in U937 cells (as we have previously reported) (47).

HoxA10 Induces an Fgf2-dependent Increase in β-Catenin in Primary Myeloid Progenitor Cells—Because β-catenin is likely to be dysregulated in U937 cells, we also investigated this mechanism in primary murine myeloid progenitor cells. For initial studies, WT murine myeloid progenitor cells were isolated, cultured in GM-CSF, IL3, SCF (GMP conditions), deprived of cytokines, and stimulated with recombinant Fgf2. Some cells were treated with LY294002, and proliferation was determined by incorporation of [3H]thymidine. We found that primary murine bone marrow cells exhibit a dose-dependent proliferative response to Fgf2 that is significantly decreased by PI 3-kinase inhibition (p < 0.0001, n = 4) (Fig. 9A).

We investigated the impact of HoxA10 on β-catenin expression using primary murine bone marrow cells. For these experiments, bone marrow cells were harvested from WT mice and transduced with a retroviral vector to express HoxA10 or empty retroviral control vector (as in the studies above). Cells were cultured under GMP conditions, deprived of cytokines, and restimulated with GM-CSF (20 ng/ml in the presence of FCS) with or without an Fgf2-blocking antibody. Cell lysate proteins were analyzed by Western blot. We found that β-catenin protein is increased in HoxA10-overexpressing myeloid progenitor cells in comparison with control vector-transduced cells (Fig. 9B). However, this effect of HoxA10 overexpression is significantly decreased by treatment of the cells with Fgf2-blocking antibody.

We also determined the roles of HoxA10 and Fgf2 on Akt and Gsk3β in these cells. We found that Akt activation (phosphorylation) is greater in HoxA10-overexpressing primary murine myeloid progenitor cells in comparison with control cells (Fig. 9B). We also found greater inactivating phosphorylation of Gsk3β in these cells (Fig. 9B). Phosphorylation of both of these proteins in HoxA10-overexpressing cells is decreased by treatment with Fgf2-blocking antibody (Fig. 9B).

These experiments suggest that HoxA10 overexpression increases β-catenin protein in an Fgf2-dependent manner. To determine whether this results in increased β-catenin target gene expression, additional studies were performed. In these experiments, transduced cells were treated as above, and analyzed for β-catenin, cyclin D1, c-Myc, and survivin mRNA expression by real time PCR.

We found that expression of each of these β-catenin target genes is increased in HoxA10-overexpressing primary myeloid progenitor cells in comparison with control vector-transduced cells (Fig. 9C). Treatment with Fgf2-blocking antibody significantly decreases expression of cyclin D1, c-Myc, and survivin in HoxA10-overexpressing cells (p < 0.0001, n = 6) but not control cells (Fig. 9C). β-Catenin mRNA expression is not significantly greater in HoxA10-overexpressing myeloid progenitor cells in comparison with control cells, consistent with our hypothesis.

DISCUSSION

In these studies, we identified the gene encoding Fgf2 as a HoxA10 target gene and demonstrated that HoxA10 activates FGF2 transcription in myeloid progenitor cells and differentiating phagocytes. This is a novel finding, because Hox proteins have not been previously implicated in regulation of Fgf2. Our studies demonstrate that increased Fgf2 secretion by HoxA10-overexpressing cells results in autocrine stimulation of proliferation. We determined that one mechanism for this is activation of PI 3-kinase and Akt, which results in stabilization of β-catenin protein. We also found that HoxA10-overexpressing myeloid progenitor cells are hypersensitive to cytokines that share this pathway, such as GM-CSF. Therefore, our studies
identify a mechanism for HoxA10-induced expansion of myeloid progenitor cells, a mechanism for regulation of Fgf2 activity in hematopoietic cells. This would be a possible target for molecular therapeutic approaches to HoxA10-overexpressing AML.

Dysregulated Hox expression has been implicated as a prognosis-driving abnormality in AML. Specifically, increased expression of a set of HOX genes (HoxB3, -B4, and -A7–11) confers poor prognosis in this disease (6–9). In AML, these Hox proteins exhibit both increased differentiation-stage appropriate expression and inappropriately sustained expression during differentiation. Consistent with these clinical observations, forced overexpression of Hox proteins expands specific bone marrow cell populations. For example, overexpression of either HoxA9 or HoxA10 expands the GMP population (48). HoxA10 overexpression tends to block differentiation, whereas HoxA9 overexpression facilitates acquisition of granulocyte-like characteristics (17, 49). However, relatively few Hox target genes have been identified that explain these effects.

We identified a number of HoxA10 target genes that are candidates for mediating progenitor expansion. More importantly, we demonstrated a mechanistic role for dysregulated expression of several of these target genes in proliferation or apoptosis in HoxA10-overexpressing cells. For example, we
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found that apoptosis resistance in HoxA10-overexpressing myeloid progenitor cells is mediated by Mkp2-dependent inhibition of Jnk (19). Additionally, we demonstrated that cytokine hypersensitivity of HoxA10-overexpressing myeloid progenitor cells is influenced by Tgfβ2-dependent Erk activation (21). Tgfβ2 is also involved in facilitating immune effector functions of mature phagocytes, including chemotaxis and respiratory burst oxidase activity (50, 51).

We determined that activation of the gene encoding β3 integrin increased αβ3 expression on HoxA10-overexpressing myeloid cells (20). We found that increased αβ3 in HoxA10-overexpressing myeloid progenitor cells results in increased activation of Syk; a tyrosine kinase involved in proliferation. αβ3 also plays a role in adhesion and rolling of mature phagocytes. Interestingly, αβ3 integrin is activated via a noncanonical interaction with Fgf2 and Fgf-R1 (31).

In this study, we investigate the role of HoxA10 in regulating Fgf2, a cytokine with importance for ES pluripotency, hematopoietic stem cell, GMP expansion, and hematopoietic differentiation. Fgf2 is also secreted by mature phagocytes and participates in regulating migration, adhesion, and activation of the respiratory burst oxidase (30). Fgf2 plays an important role in angiogenesis, a function that is not addressed in this study, but which may contribute to leukemogenesis. Therefore, Fgf2 is similar to several other HoxA10 target genes that regulate both myeloid progenitor expansion and phagocyte functional activities. This is consistent with a role for Hox proteins in mediating cellular functions at multiple points during hematopoiesis.

We identified FGF2 as a HoxA10 target gene by chromatin immunoprecipitation. The technique that was used for these studies involves stripping proteins from co-precipitated chromatin, digestion with Klenow to generate blunt ends, adding linkers, digesting with a restriction enzyme, and subcloning. Although sonication conditions were set to generate chromatin fragments of ~2.0 kb, we found that the majority of recovered fragments were between 100 and 500 bp. This is likely to be due to the harsh conditions to which the chromatin was subjected subsequent to co-precipitation, and the use of restriction digestion prior to subcloning. In this study, the FGF2 sequence that was recovered did not include the HoxA10-binding cis elements identified by functional assays (although they are closely adjacent). This is consistent with our previous experience with this technique (23, 46).

We identified two FGF2 cis elements that are activated by HoxA10. This result is consistent with the multiple HoxA10-binding sites found in other target genes, including CYBB, NCF2, DLISP4, and TGFβ-2 (19, 21, 22, 35–37). We found that HoxA10-binding affinity for the two FGF2 cis elements is approximately equivalent. In contrast, the cis elements in some other HoxA10 target genes are of variable affinity (i.e. CYBB, NCF2, and DLISP4). The functional significance of multiple HoxA10-activated cis elements is unknown but may relate to graded expression of these target genes in a differentiation stage-specific manner or during phagocyte activation.

It is possible that genomic scanning studies might identify additional, more distal HoxA10-binding sites in FGF2. However, the intent of these studies was to determine whether FGF2 transcription is functionally regulated by HoxA10. The major goal of our work was to identify the role of Fgf2 in proliferation of HoxA10-overexpressing myeloid progenitor cells. These studies therefore determine that signaling pathways that are activated by Fgf2 may be potential therapeutic targets for AML with dysregulated Hox expression. Large scale screening studies can identify theoretical pathways of influence for transcription factors. However, it is necessary to take such studies to a functional level to identify potential therapeutic approaches.

Using EMSA, we identified low mobility shifted complexes that bind to both FGF2 cis elements. These complexes have cross-competitive binding specificities with each other and with HoxA10-binding cis elements from other genes. The shifted complexes that bind to these probes migrate as a doublet, and both bands are specifically disrupted by the HoxA10 antibody. This is consistent with our studies of previous HoxA10 target genes. In those studies, we found that HoxA10 binds DNA in partnership with Pbx1 or Pbx2. This complex recruits either the Creb-binding protein (CBP) or histone deacetylase 2 (HDAC2), depending upon whether the gene is activated or repressed by HoxA10. Therefore, the multiple bands identified by EMSA represent HoxA10 binding as a monomer, a heterodimer, or a higher order complex with a histone-modifying enzyme. Studies characterizing the various complexes that bind HoxA10 target genes and the roles of these complexes in regulated gene expression are ongoing in the laboratory.

We found that Fgf2 expression is decreased in HoxA10-deficient cells, in vitro and in vivo. We hypothesize that decreased circulating Fgf2 may be responsible for the mild cytopenias observed in HoxA10 knock-out mice. The roles of decreased Fgf2 and Tgfβ2 expression in hematopoiesis in HoxA10−/− mice are currently under investigation in the laboratory. Of significance to AML, we found that HoxA10 overexpression increased Fgf2 expression and secretion in granulocyte/monocyte progenitor cells. In studies with primary murine bone marrow cells, HoxA10 overexpression had an even greater effect on cells undergoing granulocyte differentiation. This may reflect the role of Fgf2 in the inflammatory response.

We found that increased Fgf2 secretion by HoxA10-overexpressing myeloid progenitor cells induces PI 3-kinase-dependent proliferation of these cells. This study also identifies a mechanism for hypersensitivity of HoxA10-overexpressing myeloid progenitor cells to other cytokines. We hypothesize that sustained Fgf2 expression in HoxA10-overexpressing myeloid progenitor cells induces sustained activation of PI3-kinase. This would increase the effect of other cytokines that also activate PI 3-kinase by impairing normal mechanisms that down-regulate this pathway. Specifically, we found that hypersensitivity of HoxA10-overexpressing myeloid progenitors to GM-CSF is partly reversed by treatment with an Fgf2-blocking antibody. The GM-CSF-R also activates PI 3-kinase, and hypersensitivity to GM-CSF is frequently found in myeloid leukemias.

We previously found that GM-CSF hypersensitivity of HoxA10-overexpressing myeloid progenitor cells was also partly abrogated by a blocking antibody to Tgfβ2 (21). Those studies determined that hypersensitivity was related to activation of Erk by Tgfβ2 signaling (21). It is therefore of interest that we found cooperation between Fgf2 and Tgfβ2 for cytokine
hypersensitivity in HoxA10-overexpressing cells. It is possible that these two cytokines function cooperatively by activating different signaling pathways or that there are common pathways activated by both cytokines in myeloid progenitor cells. This is a topic of ongoing investigations.

We found that HoxA10 overexpression increased β-catenin protein in an Fgf2-dependent manner. Increased β-catenin activity is associated with poor prognosis and drug resistance in AML, but the mechanism for this increase is not clear. We found increased expression of β-catenin target genes in HoxA10-overexpressing myeloid progenitor cells. We also found that β-catenin knockdown decreases cytokine hypersensitivity in HoxA10-overexpressing cells. However, the effect of Fgf2-blocking antibody was greater than the effect of β-catenin knockdown. One possibility is that Fgf2 activates additional pathways that impact proliferation. Candidate intermediates that are activated by the Fgf-R in neural or epithelial cells include Shp2-protein-tyrosine phosphatase, MAPKs, or protein kinase C. Additionally, activation of αvβ3 by Fgf2 may contribute to proliferation via Syk. These pathways will be investigated in subsequent studies.

Fgf proteins have been implicated in a number of hematologic and nonhematologic malignancies. Several Fgf-R inhibitors are being developed as therapeutic agents. Our studies suggest the possibility that such agents might be productively targeted to the poor prognosis subset of AML subjects with increased Hox expression.

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