

The Leukemia-associated Mll-Ell Oncoprotein Induces Fibroblast Growth Factor 2 (Fgf2)-dependent Cytokine Hypersensitivity in Myeloid Progenitor Cells*

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Chirag A. Shah[‡], Ling Bei^{†§}, Hao Wang[‡], Leonidas C. Plataniias^{†§}, and Elizabeth A. Eklund^{†§1}

From [‡]The Feinberg School of Medicine and Robert H. Lurie Comprehensive Cancer Center of Northwestern University, Chicago, Illinois 60611 and the [§]Jesse Brown Veterans Administration Medical Center, Chicago, Illinois 60612

Background: *MLL* fusion proteins, including Mll-Ell, induce overexpression of HoxA9 and HoxA10 in the bone marrow.

Results: Mll-Ell induces HoxA9- and HoxA10-dependent *FGF2* transcription. Autocrine production of Fgf2 contributes to cytokine hypersensitivity in Mll-Ell expressing myeloid progenitor cells.

Conclusion: Mll-Ell induces Fgf2 production by myeloid progenitors and differentiating myeloid cells.

Significance: Proliferative effects of Fgf2 may influence the pathogenesis of leukemias with *MLL* gene translocations.

The subset of acute myeloid leukemias (AML) with chromosomal translocations involving the *MLL* gene have a poor prognosis (referred to as 11q23-AML). The *MLL* fusion proteins that are expressed in 11q23-AML facilitate transcription of a set of *HOX* genes, including *HOXA9* and *HOXA10*. Because Hox proteins are transcription factors, this suggests the possibility that Hox target genes mediate the adverse effects of *MLL* fusion proteins in leukemia. Identifying such Hox target genes might provide insights to the pathogenesis and treatment of 11q23-AML. In the current study we found that Mll-Ell (an *MLL* fusion protein) induced transcriptional activation of the *FGF2* gene in a HoxA9- and HoxA10-dependent manner. *FGF2* encodes fibroblast growth factor 2 (also referred to as basic fibroblast growth factor). Fgf2 influences proliferation and survival of hematopoietic stem cells and myeloid progenitor cells, and increased Fgf2-expression has been described in AMLs. We determined that expression of Mll-Ell in myeloid progenitor cells resulted in autocrine production of Fgf2 and Fgf2-dependent cytokine hypersensitivity. Therefore, our results implicated increased Fgf2 expression in progenitor proliferation and expansion in 11q23-AML. Because small molecule inhibitors of Fgf-receptors are in human clinical trials, this suggested a potential therapeutic approach to this treatment refractory leukemia.

HoxB3, HoxB4, HoxA9–11, and Meis1 in CD34⁺ bone marrow cells (1). In this leukemia subset, expression of these proteins was also aberrantly sustained in differentiating, CD34[−] myeloid cells. Further analysis determined that this pattern of gene expression was characteristic of AML with chromosomal translocations or partial tandem duplications involving the *MLL* gene (referred to as 11q23-AML), chromosomal translocation involving the *MYST3* and *CREBBP* genes, and a poor prognosis subset of cytogenetically normal AML (2–7).

More than 20 different fusion partners have been described for the *MLL* gene in 11q23-AML (2). The expressed *MLL* fusion proteins include common N-terminal domains from *MLL* and C-terminal domains from the various different partner genes. Studies in murine models supported a functional role for *MLL* fusion proteins in Hox expression and leukemia. Engineered expression of various *MLL* fusion proteins in murine bone marrow cells increased Hox expression in a manner that was consistent with human 11q23-AML (1, 5, 8). *MLL* fusion proteins also expanded the hematopoietic stem cell (HSC) and myeloid progenitor cell bone marrow populations *in vitro* and induced a myeloproliferative neoplasm that progressed to AML over time *in vivo* (1, 5, 8). These results were similar with all of the *MLL* fusion proteins tested.

Other murine models supported a functional role for Hox proteins in leukemogenesis. Human and murine *HOX* genes are clustered in four groups (A–D) on four chromosomes (9, 10). *HOX1–4* genes are most highly transcribed in HSC, and transcription of *HOX7–11* is most active in committed progenitor cells (10). Consistent with this expression profile, engineered overexpression of HoxB3 or HoxB4 in murine bone marrow expanded the HSC population and led to an myeloproliferative neoplasm *in vivo* (11, 12). Overexpression of either HoxA9 or HoxA10 in murine bone marrow was characterized by expansion of the bipotential myeloid progenitor population (granulocyte/monocyte progenitors (GMP)) (13–17). *In vivo* overexpression of HoxA9 or HoxA10 resulted in an myeloproliferative neoplasm that evolved to AML in a manner similar to *MLL* fusion proteins (1, 17–19).

Clinical correlative studies defined a poor prognosis subset of acute myeloid leukemia (AML)² with increased expression of

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¹ To whom correspondence should be addressed: The Feinberg School of Medicine, Northwestern University, 303 E. Superior St., Lurie 5-105, Chicago, IL 60611. Tel.: 312-503-3208; Fax: 312-503-0189; E-mail: e-eklund@northwestern.edu.

² The abbreviations used are: AML, acute myeloid leukemia; HSC, hematopoietic stem cell; Fgf2, fibroblast growth factor 2; HD, homeodomain; RA, retinoic acid; DMF, dimethylformamide; GMP, granulocyte/monocyte progenitor cell; SCF, stem cell factor.

These studies suggested the possibility that Hox proteins were involved in expansion of the leukemia stem cell population in 11q23-AML. In various forms of AML this population has generally been defined as a GMP with HSC-like characteristics (20). However, the set of Hox target genes that mediated bone marrow progenitor expansion was not defined.

We used chromatin immunoprecipitation-based screening techniques to identify HoxA10 target genes that might be relevant to leukemogenesis (21–26). In these studies we identified the gene encoding fibroblast growth factor 2 (Fgf2, or basic Fgf) as a HoxA10 target gene (26). Fgf2 is produced by bone marrow stromal cells, hematopoietic progenitor cells, and mature phagocytes (27). Increased Fgf2 production by AML cells is described, and a search of publically available data bases specifically associated this with 11q23-AML (28, 29). Fgf2 plays a key role in expanding hematopoietic progenitor cell populations and is essential for hematopoietic and embryonic stem cell maintenance (30).

In myeloid progenitor cells, Fgf2 binding to Fgf-R1/R2 activates phosphoinositol 3-kinase (31, 32). Phosphoinositol 3-kinase activates Akt kinase, which inhibits glycogen synthase kinase 3 β . Serine/threonine phosphorylation of β -catenin by glycogen synthase kinase 3 β leads to β -catenin ubiquitination and degradation. Therefore, Fgf2-induced activation of phosphoinositol 3-kinase stabilizes β -catenin and enhances β -catenin activity (26).

We previously found that autocrine production of Fgf2 contributed to cytokine hypersensitivity of HoxA10-overexpressing myeloid progenitor cells in a phosphoinositol 3-kinase and β -catenin-dependent manner (26). The possibility that Fgf2 contributed to the pathogenesis of 11q23-AML was not previously explored.

HoxA9 and HoxA10 have highly conserved DNA binding homeodomains, suggesting the possibility of common target genes (33). However, HoxA9 and HoxA10 diverge outside of the homeodomain, suggesting the possibility of differential effects on some target genes. Consistent with the latter, we found that *CYBB* was a common target gene for HoxA10 and HoxA9 (the only previously identified common target gene) (34–37). *CYBB* encodes gp91^{phox}; a rate-limiting component of the NADPH oxidase that generates H₂O₂/O₃⁻ during the innate immune response.

We found that HoxA10 interacted with and repressed a *CYBB* cis element in myeloid progenitor cells (34, 36). During myeloid differentiation, phosphorylation of conserved tyrosine residues in the DNA binding homeodomain (HD) of HoxA10 decreased binding affinity for *CYBB* (34, 36). HoxA9 activated *CYBB* transcription through the same cis element, but in this case phosphorylation of the conserved HD tyrosine residues of HoxA9 increased *CYBB* binding affinity during differentiation (37). These studies identified an antagonistic role for HoxA9 and HoxA10 during myeloid differentiation. This was consistent with previous studies indicating that HoxA10 was involved in a differentiation block in AML, but HoxA9 conferred a myeloid phenotype upon leukemia cells (13, 18).

However, in the current study we found that HoxA9 and HoxA10 activated common cis elements in the *FGF2* promoter in myeloid progenitor cells and throughout myeloid differenti-

ation. Expression of the leukemia-associated *MLL* fusion protein Mll-Ell also activated these two *FGF2* cis elements in a HoxA9- and HoxA10-dependent manner. This resulted in autocrine production of Fgf2 by Mll-Ell-expressing myeloid progenitor cells and Fgf2-dependent cytokine hypersensitivity. This is a previously un-described and therapeutically targetable mechanism for *MLL* fusion proteins to influence expansion of myeloid progenitor cells.

MATERIALS AND METHODS

Plasmid Vectors—The cDNA for human HoxA10 was obtained from C. Largman (University of California, San Francisco) (38). The HoxA9 cDNA was generated by reverse transcription and PCR from U937 cells as described (37). These cDNAs were subcloned into the mammalian expression vector pcDNAamp (Invitrogen) and the murine retroviral vector pMSCVpuro (Clontech, Mountain View, CA), as described (21, 22). A vector with the Mll-Ell fusion protein was obtained from D. E. Zhang (University of California, San Diego, CA). HoxA10- and HoxA9-specific shRNA and scrambled control sequences were designed using the Promega website (Promega, Madison, WI) and subcloned into the pLKO.1puro vector (from Dr. Kathy Rundell, Northwestern University, Chicago). Several sequences were tested, and the most efficient were combined.

The *FGF2* 5'-flank was obtained from the genomic DNA of U937 cells by PCR. The sequence was compared with the published *FGF2* 5'-flank sequence (ENSEMBL database), and promoter fragments were subcloned into the pGL3-basic reporter vector (Promega) as described (26). Other constructs were generated with mutation of identified Hox binding sites in the *FGF2* promoter. Reporter constructs were also generated using the pGL3-promoter vector (with a minimal promoter and reporter) and three copies of the proximal (–287 to –266 bp) or distal (–448 to –425 bp) Hox binding cis elements from the *FGF2* promoter as described (26).

Oligonucleotides—Oligonucleotides were custom-synthesized by MWG Biotech (Piedmont, NC). These oligonucleotides represent Hox consensus sequences from the *FGF2* promoter; –287 to –266 bp (5'-GAGAAAGTTGAGTTTAAACTTTTA-3'), –448 to –425 bp (5'-AATTTTAAAGTTTATGCCCCATT-3'), or Hox-consensus mutants of these sequences (26).

Myeloid Cell Line Culture—The human myelomonocytic leukemia cell line U937 (39) was obtained from A. Kraft (Holings Cancer Center, Medical University of South Carolina, Charleston, SC). Cells were maintained and differentiated (with retinoic acid (RA) and dimethylformamide (DMF)) as described (39).

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 the Jesse Brown Veterans Administration Medical Center.

Bone marrow mononuclear cells were obtained from the femurs of WT or HoxA10^{-/-} C57/BL6 mice (40). Bi-potential granulocyte/monocyte progenitor cells (GMPs) were cultured (2 × 10⁵ cells/ml) for 48 h in DME media supplemented with 10% fetal calf serum, 1% pen-strep, 10 ng/ml murine GM-CSF (R & D Systems Inc., Minneapolis, MN), 10 ng/ml murine recombinant IL-3 (R & D Systems), and 100 ng/ml SCF (R & D

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Systems) followed by separation of CD34⁺ cells using the Miltenyi magnetic bead system (Miltenyi Biotechnology, Auburn, CA) (18, 23, 26). Some cells were CD34-separated after 24 h under these conditions followed by 24 h of differentiation in DME supplemented with 10% fetal calf serum, 1% pen-strep, 20 ng/ml G-CSF (R & D Systems) and 10 ng/ml IL3.

Retrovirus was generated with HoxA10/MSCV, HoxA9/MSCV or Mll-Ell/MSCV or control MSCV plasmid using the Phoenix cell packaging line according to manufacturer's instructions (Stratagene, La Jolla, CA). The average viral concentration was 10⁷ pfu/ml. Bone marrow mononuclear cells were cultured for 24 h in 10 ng/ml GM-CSF, 10 ng/ml IL3, and 100 ng/ml SCF. Cells were transduced by incubation with retroviral supernatant supplemented with Polybrene (6 μg/ml) as described (18, 23, 26). Transduced cells were selected for 48 h in puromycin followed by CD34 selection and culture in GM-CSF, IL3, and SCF (as above). Transgene expression was confirmed by real time PCR and Western blot.

Quantitative Real Time PCR—RNA was isolated using TRIzol reagent (Invitrogen). Primers were designed with Applied Biosystems software. Real time PCR was performed with SYBR Green according to the "standard curve" method. Results were normalized to 18 S and to actin (for mRNA determination) or total input chromatin (for chromatin immunoprecipitation studies).

Chromatin Immunoprecipitation—Cells were incubated briefly in media supplemented with formaldehyde to generate DNA-protein cross-links. Cell lysates were sonicated to generate chromatin fragments with an average size of 200 bp (41). Lysates underwent one round of immunoprecipitation with antibodies to HoxA9, HoxA10, or irrelevant antibody (21, 25, 26). HoxA9 and HoxA10 antibodies are not cross-reactive and 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).

Chromatin was amplified by real time PCR with sets of primers flanking the previously identified Hox binding cis elements in the *FGF2* promoter or with primers representing irrelevant regions in the proximal 2.0 kb of *FGF2* 5' flank as previously described (26).

Myeloid Cell Line Transfections and Assays—To generate stable U937 transfectant pools, cells were electroporated with equal amounts of a HoxA9, HoxA10, HoxA9 + HoxA10, or Mll-Ell expression vector (or empty control vector) plus a vector with a neomycin phosphotransferase cassette (pSRα) (30 μg each). Stable pools were selected in G418 (0.5 mg/ml), and aliquots were tested for transgene expression by real time PCR and Western blot.

For *FGF2* promoter and cis element 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, 467 bp, 467 bp with mutation of the distal cis element, 424 bp, 297 bp, 297 bp with mutation of the proximal cis element, or 266 bp of *FGF2*-pGL3 or pGL3 control) (30 μg), a vector to express Mll-Ell (50 μg), HoxA9 (50 μg), HoxA10 (50 μg), HoxA9 + HoxA10 (25 μg each), or empty vector control (50 μg), and β-galacto-

sidase 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 proximal (−287 to −266 bp) or distal (−448 to −425 bp) *FGF2* cis elements (with the pGL3-p vector) (30 μg), a vector to express Mll-Ell, HoxA9, and/or HoxA10 (as above), and CMVβ-gal. In other experiments these reporter plasmids were co-transfected with vectors to overexpress HoxA9, HoxA10, or Mll-Ell with vectors to express HoxA10- or HoxA9-specific shRNAs (or scrambled control shRNA) and CMVβ-gal. Transfectants were analyzed with or without granulocyte differentiation with RA + DMF.

Western Blots—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 in the media of cultured cells was determined using the Emax ImmunoAssay System according to manufacturer's instructions (Promega). Cells were maintained at a density of 0.6 × 10⁶ cells/ml. Equivalent amounts of media were withdrawn at various time points for the assay (26). The assay is Fgf2-specific and does not cross-react with other Fgfs.

Cell Proliferation Assays—U937 stable transfectants with HoxA9, HoxA10, Mll-Ell, or empty control vector were deprived of fetal calf serum for 24 h followed by treatment with a dose titration of fetal calf serum (0.01–10%). Some cells were incubated with a specific blocking antibody for Fgf2 or control antibody (R & D Systems). Other cells were incubated with the Fgf-R1 inhibitor, PD173074 (Cayman Chemical, Ann Arbor, MI). Cell proliferation was determined by incorporation of [³H]thymidine (23, 26).

In other studies WT or HoxA10^{−/−} murine bone marrow cells were transduced with a retroviral vector to overexpress HoxA9, HoxA10, and Mll-Ell or with empty MSCV vector, cultured in 10 ng/ml GM-CSF, 10 ng/ml IL3, 100 ng/ml SCF, deprived of cytokines for 24 h (in DME with 10% FCS), and stimulated for 24 h with a dose titration of GM-CSF (0.01–10 ng/ml + 5 ng/ml IL3). Some cells were incubated with Fgf2 blocking antibody (or control antibody) or Fgf-R1 inhibitor (or buffer control). Cell proliferation was determined as above (23, 26).

In Vitro DNA Binding Assays—Nuclear proteins were isolated by the method of Dignam *et al.* (42) as described (28). Oligonucleotide probes were prepared, and DNA binding was analyzed by electrophoretic mobility shift assay (EMSA) and DNA affinity purification techniques as described (18, 21–26). For EMSA, antibody to HoxA9 or HoxA10 (or irrelevant control antibody) was included in binding assays. For DNA affinity purification, Western blots of affinity-purified proteins were probed with antibodies to HoxA9, HoxA10, Ell, or irrelevant control antibody.

For all experiments at least three different batches of nuclear proteins were tested in two independent experiments. Nuclear protein integrity and equality of loading was determined in con-



Human sequence in black
 Mouse sequence in blue
 Hox consensus sequences in red
 Hox binding cis elements **underlined and bold**

FIGURE 1. **The proximal 500 bp of FGF2 promoter has consensus sequences for Hox-protein binding.** Sequence analysis identified homology between the human (black) and murine (blue) FGF2 promoter sequences. Hox binding consensus sequences are shown in red, and the previously identified functional cis elements are in bold and underlined.

control EMSA with a probe representing a classical CCAAT box from the α -globin gene promoter.

Statistical Analysis—Statistical significance was determined by Student’s *t* test and analysis of variance using SigmaPlot and SigmaStat software. Graphs are presented with error bars representing S.E. calculations. *p* values of ≤ 0.02 were considered statistically significant.

RESULTS

FGF2 Promoter Activity Is Increased in Mll-Ell-expressing Cells—We investigated the effect of Mll-Ell on the FGF2 promoter by co-transfecting U937 myeloid leukemia cells with a series of reporter vectors containing FGF2 promoter sequences and vectors to express Mll-Ell or empty control vector. Other cells were co-transfected with these reporter constructs and a vector to overexpress HoxA10 as a positive control. Based on the highly conserved DNA binding HD of HoxA9 and HoxA10, some cells were co-transfected with the reporter vectors and a HoxA9 expression vector.

We previously defined two FGF2 promoter cis elements that conformed to a derived Hox-DNA binding consensus sequence; that is, a proximal cis element between -297 and -266 bp and a distal cis element between -424 and -467 bp (from the transcription start site) (Fig. 1) (26). The FGF2-promoter constructs used in the current studies were designed around the cis elements, and some constructs included a mutation of the Hox-binding consensus sequences in the cis elements.

Because we previously found that HoxA9 and HoxA10 regulated CYBB transcription in a differentiation stage-specific manner, transfectants were assayed with or without differentiation to granulocytes with retinoic acid and dimethyl formamide (RA/DMF) (39).

We found that activity of reporter constructs with 467 bp (both cis elements) or 297 bp (proximal only) of FGF2 promoter was significantly increased in Mll-Ell transfectants in comparison to control ($p < 0.001$, $n = 6$) (Fig. 2A). This increase was significantly greater in transfectants with the 467-bp construct

versus the 297-bp construct ($388\% \pm 60\%$ increase with Mll-Ell versus $235\% \pm 10\%$ increase, $p = 0.01$, $n = 6$; Fig. 2A). Mutation of the single Hox binding consensus in the 297-bp construct abolished Mll-Ell-induced reporter activity (Fig. 2A). Mutation of the distal cis element in the 467-bp FGF2 promoter construct decreased Mll-Ell-induced promoter activity so that it was not significantly different from the wild type 297-bp construct ($p = 0.8$, $n = 6$).

Although there was a trend to increased activity of these FGF2 reporter constructs in RA/DMF-differentiated transfectants versus control transfectants, this did not reach statistical significance with or without Mll-Ell ($p \geq 0.06$, $n = 6$; Fig. 2A). This was consistent with the known production of Fgf2 by both myeloid progenitor cells and mature phagocytes.

We found the same pattern of increased reporter activity in U937 cells that were co-transfected with these FGF2 promoter constructs and vectors to overexpress HoxA9 or HoxA10 (Fig. 2B). There was no significant difference in the efficiency of activation of the FGF2 promoter constructs by HoxA9 versus HoxA10 ($p > 0.8$, $n = 6$). This was in contrast to the CYBB promoter, which was repressed by HoxA10 in myeloid progenitor cells but activated by HoxA9 during myeloid differentiation. Also in contrast to the CYBB promoter, HoxA9- or HoxA10-induced activity of the FGF2 promoter was only slightly increased by differentiation under each of the conditions studied ($p \geq 0.06$, $n = 6$; Fig. 2B).

Activity of the empty control reporter construct was not altered by co-transfection with vectors to express Mll-Ell, HoxA9, or HoxA10 or differentiation with RA/DMF. This minimal activity was subtracted as background.

Mll-Ell Increases FGF2 Promoter Activity in a HoxA9- and HoxA10-dependent Manner—To verify that Mll-Ell influenced FGF2 transcription through the proximal and distal cis elements, U937 cells were co-transfected with a construct containing three copies of the distal or proximal FGF2 cis element linked to a minimal promoter and reporter (or control minimal

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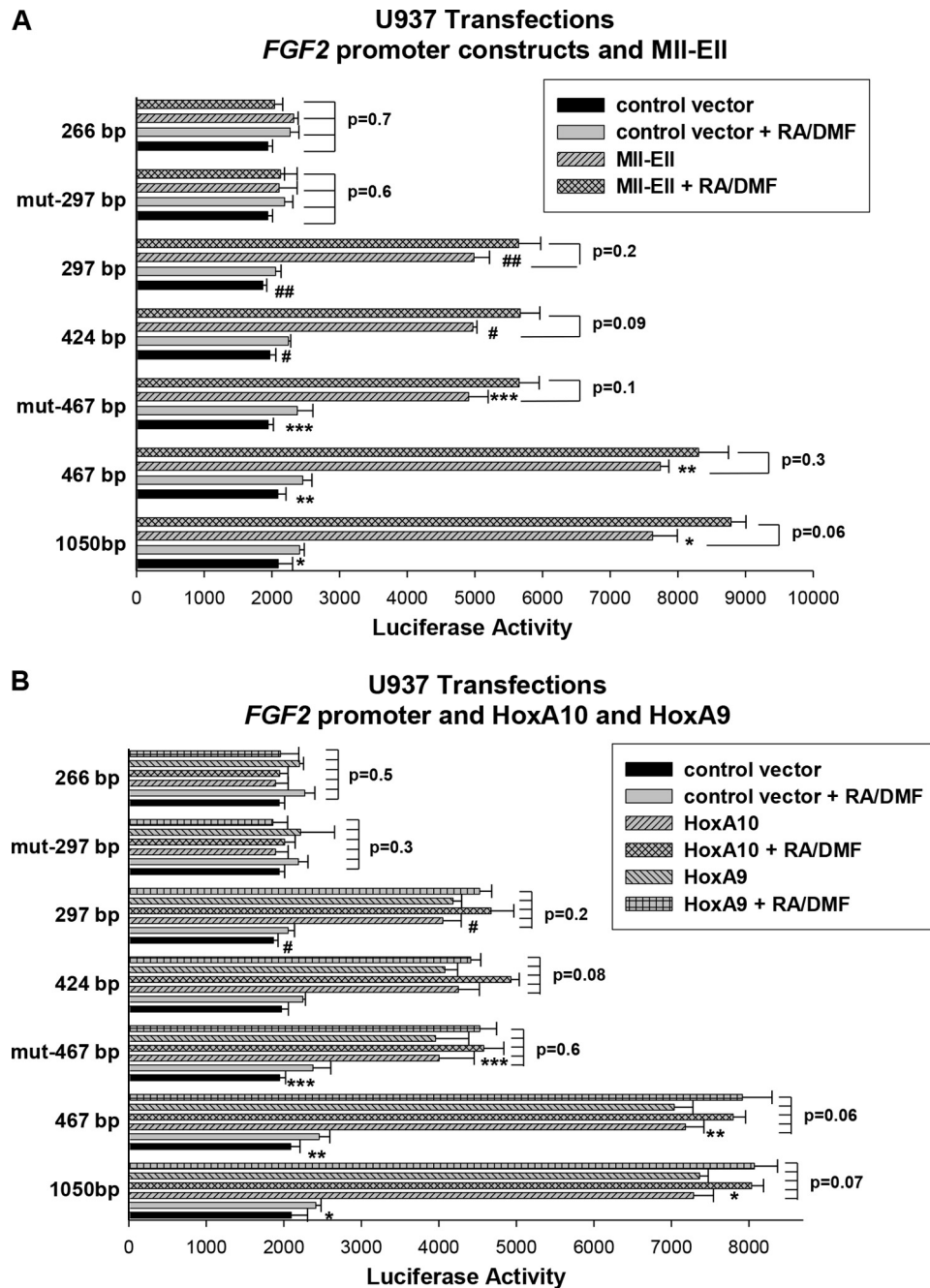


FIGURE 2. Activity of the *FGF2* promoter is increased by expression of MII-EII or overexpression of HoxA9 in myeloid leukemia cells. *A*, MII-EII expression increases activity of the *FGF2* promoter. U937 cells were co-transfected with a series of *FGF2* promoter/reporter constructs and a vector to express MII-EII or control expression vector. Some *FGF2* promoter/reporter constructs have mutations in Hox binding consensus sequences (i.e. mut-467 and mut-297). Transfectants were analyzed for reporter expression with or without granulocyte differentiation (with RA/DMF). Statistically significant differences in reporter activity are indicated by *, **, ***, #, or ##. *B*, overexpression of HoxA9 or HoxA10 increases activity of the *FGF2* promoter in a non-differentiation stage-specific manner. U937 cells were co-transfected with the *FGF2* promoter/reporter constructs (described above) and vectors to express HoxA9 or HoxA10 (or control vector). Transfectants were analyzed for reporter activity with or without differentiation. Statistically significant differences in reporter expression are indicated by *, **, ***, or #.

promoter/reporter vector) and a vector to express MII-EII or empty expression vector. Some cells were co-transfected with these reporter constructs and a vector to overexpress HoxA10 as a positive control. To determine if there were differences in regulation of the two cis elements by HoxA9 versus HoxA10, some cells were transfected with the reporter constructs and a vector to overexpress HoxA9 or equivalent amounts of HoxA9 + HoxA10 (keeping the total amount of Hox vector constant). Because phosphorylation of conserved tyrosine (Tyr) residues

in the HDs of HoxA9 or HoxA10 regulates *CYBB* cis element binding, some cells were co-transfected with the reporter constructs and vectors to express HD-Tyr mutant forms of HoxA9 or HoxA10 (35, 37). Transfectants were assayed with or without differentiation with RA/DMF.

We found that MII-EII significantly increased activity of both the proximal and distal *FGF2* cis elements ($p < 0.0001$, $n = 6$; Fig. 3A). This effect of MII-EII was not significantly altered by differentiation, although there was a trend for increase ($p \geq$

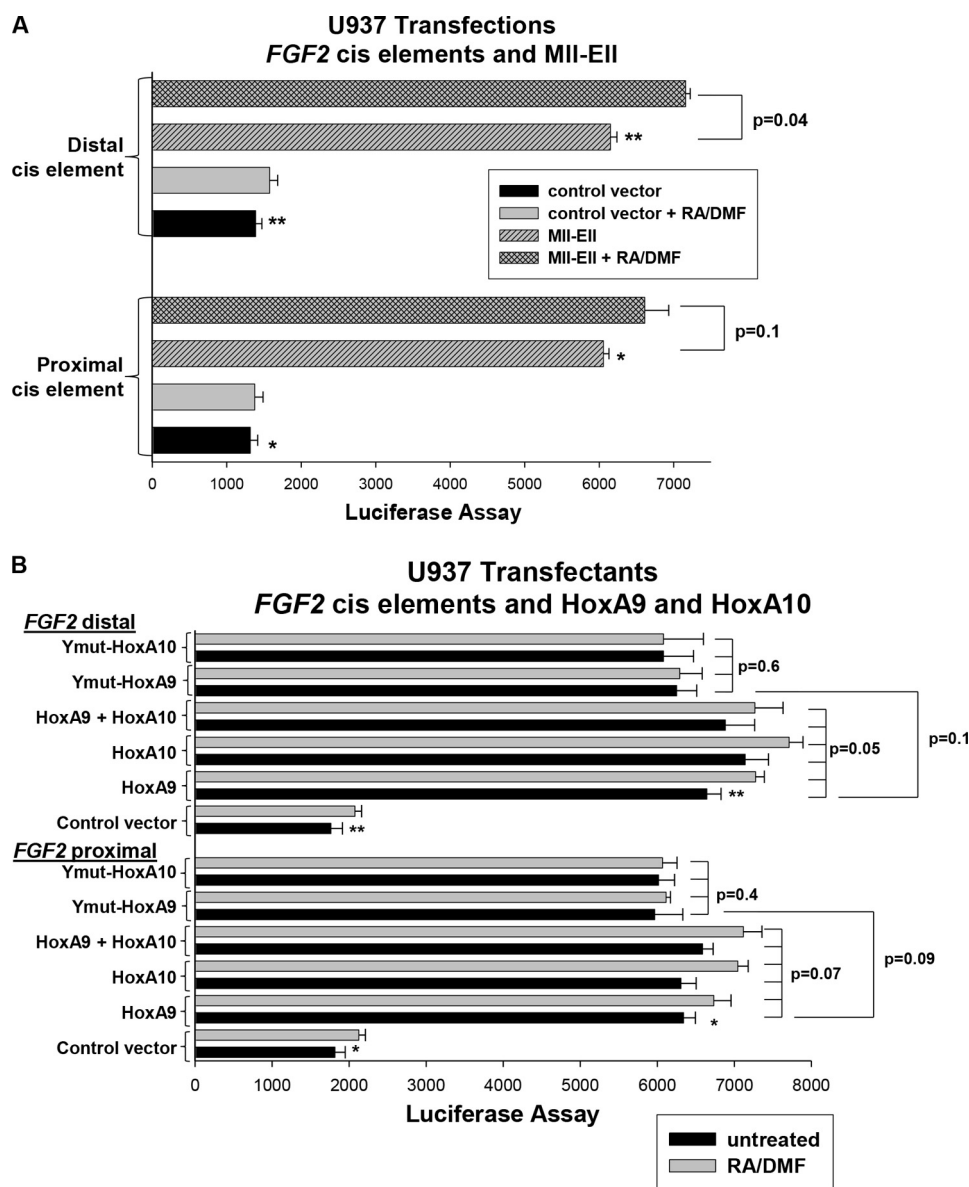


FIGURE 3. Activity of two *FGF2* cis elements is increased by expression of Mll-Ell or overexpression of HoxA9 and HoxA10 in myeloid leukemia cells. *A*, activity of the proximal and distal *FGF2* cis elements is increased by Mll-Ell expression. U937 cells were co-transfected with minimal promoter/reporter constructs with multiple copies of the proximal or distal *FGF2* cis elements (or control vector) and a vector to express Mll-Ell or empty control vector. Transfectants were analyzed for reporter activity with or without differentiation (with RA/DMF). Statistically significant differences are indicated by * or **. *B*, HoxA9 and HoxA10 activate two *FGF2* cis elements in an additive manner. U937 cells were co-transfected with the minimal promoter/reporter constructs with multiple copies of the proximal or distal Hox binding *FGF2* cis element (or control vector) and vectors to overexpress HoxA9, HoxA10, 1/2 HoxA9 + 1/2 HoxA10, HoxA9 with mutation of a conserved HD-Tyr residue (*Ymut-HoxA9*), HoxA10 with mutation of a conserved HD-Tyr residue (*Ymut-HoxA10*), or control vector. Plasmid vectors were adjusted so that the total amount of Hox expression vector was the same in Hox-overexpressing experiments. Transfectants were analyzed for reporter activity with or without differentiation. Statistically significant differences with versus without Hox-overexpression are indicated by * and **.

0.04, $n = 6$). Two *FGF2* cis elements were equivalently activated by HoxA9, HoxA10, or HoxA9 + HoxA10 ($p > 0.2$, $n = 6$) (Fig. 3B). There was a trend to increased activity with differentiation that did not meet statistical significance for either the proximal or distal cis element with overexpression of these Hox proteins ($p \geq 0.07$, $n = 6$) (Fig. 3B). Consistent with the latter results, WT HoxA9 or HoxA10 was only slightly more efficient in activating the cis elements in comparison to HD-Tyr mutant forms of these proteins ($p \geq 0.09$, $n = 6$; Fig. 3B).

These results indicated that Mll-Ell influenced two *FGF2* cis elements that were activated by HoxA9 and/or HoxA10. To determine if activation of the cis elements by Mll-Ell required

Mll-Ell-induced expression of HoxA9 or HoxA10, cells were co-transfected with the reporter constructs, a vector to express Mll-Ell (or vector control), and vectors to express HoxA9 or HoxA10-specific shRNAs (or scrambled control shRNAs). We found that knockdown of HoxA9, HoxA10, or both significantly decreased Mll-Ell-induced *FGF2* cis element activity ($p < 0.001$, $n = 6$; Fig. 4A).

To determine if HoxA9 overexpression could compensate for loss of HoxA10 and vice versa, we co-transfected U937 cells with the proximal or distal *FGF2* cis element-containing constructs, a vector to express specific shRNAs to HoxA10 or HoxA9 (or scrambled control shRNA), and a vector to overex-

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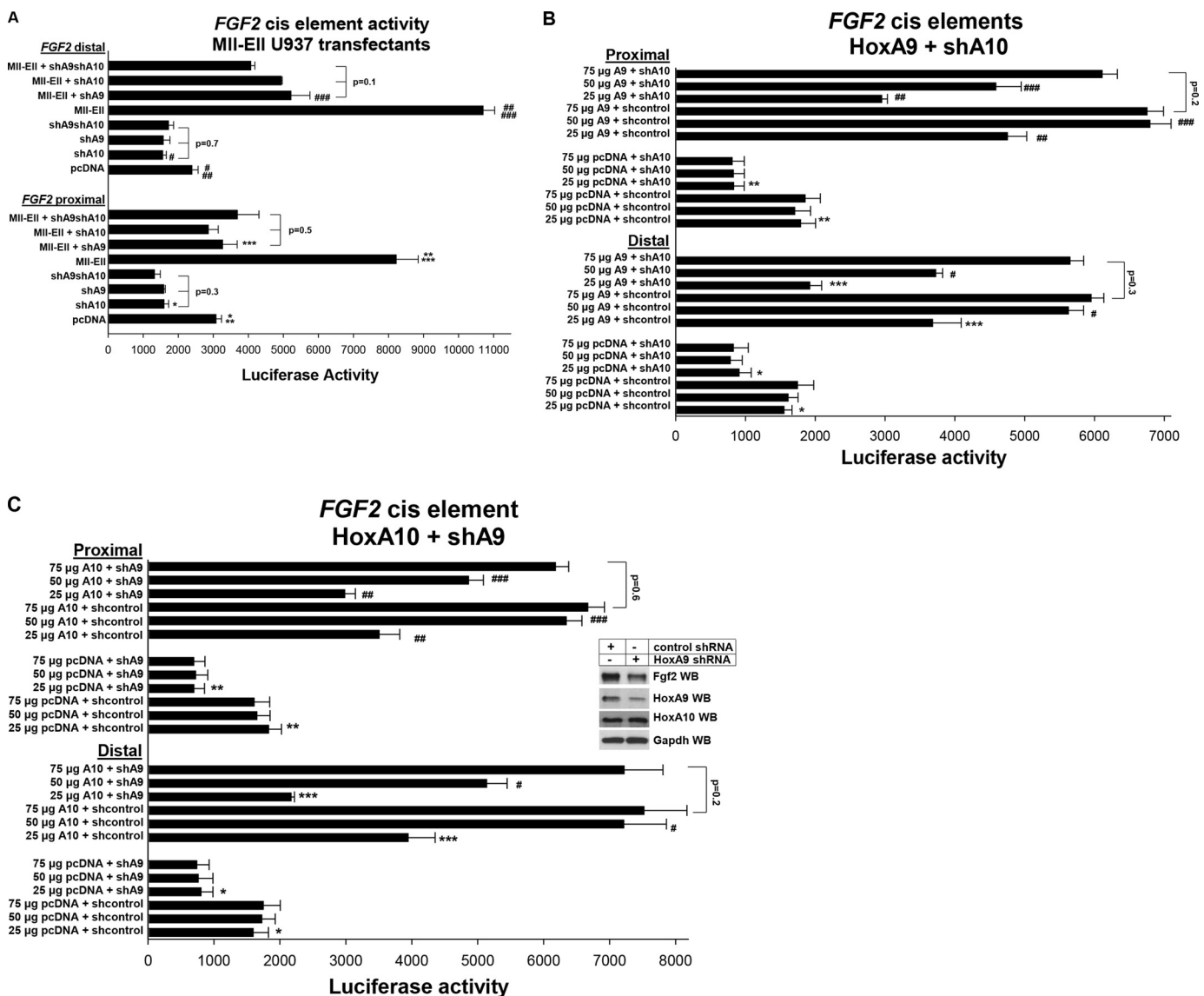


FIGURE 4. MII-EII activates the *FGF2* cis elements in a HoxA9- and HoxA10-dependent manner, and HoxA9 and HoxA10 are interchangeable for *FGF2* activation. **A**, MII-EII increases the activity of the proximal and distal *FGF2* cis elements in a HoxA9- and HoxA10-dependent manner. U937 cells were co-transfected with a minimal promoter/reporter construct with multiple copies of the proximal or distal *FGF2* cis elements (described above), a vector to express MII-EII (or empty control vector), and vectors to express HoxA9 or HoxA10 specific shRNAs (or scrambled shRNA control vectors). Cells were analyzed for reporter activity. Statistically significant differences are indicated by *, **, ***, #, ##, or ###. **B**, HoxA9 overexpression compensates for HoxA10 knockdown for activation of the two *FGF2* cis elements. U937 cells were co-transfected with the same minimal-promoter/reporter constructs as described above, a dose titration of HoxA9 expression vector (or empty control vector), and a vector to express a HoxA10-specific shRNA (or scrambled control vector). Statistically significant differences with *versus* without HoxA10-knockdown are indicated by *, **, ***, #, ##, or ###. **C**, HoxA10-overexpression compensates for HoxA9 knockdown for activation of the two *FGF2* cis elements. The converse experiment was also performed, with the same proximal or distal *FGF2* cis element containing artificial promoter-reporter constructs, a dose titration of HoxA10 expression vector, and a vector to express HoxA9-specific shRNAs (or relevant control vectors). Statistically significant differences with *versus* without HoxA9 knockdown are indicated by *, **, ***, #, ##, or ###. HoxA9 protein expression was decreased >70% using the HoxA9-specific shRNA vectors (see the *inset* Western blot (WB) of transfectant cell lysates probed with antibodies to HoxA9, HoxA10, Fgf2, or Gapdh loading control).

press HoxA9 or HoxA10 (or control vector). If HoxA9 and HoxA10 have unique effects on the cis elements, even large amounts of HoxA9 would not compensate for loss of HoxA10 and vice versa.

We found that knockdown of HoxA10 impaired the ability of overexpressed HoxA9 to activate these *FGF2* cis elements (Fig. 4B). However, we were able to overcome the effect of HoxA10 knockdown with increasing amounts of HoxA9 (Fig. 4B). We found the same effect in transfectants with knockdown of HoxA9 and HoxA10 overexpression (Fig. 4C). This suggested

that the mechanisms of *FGF2* activation by HoxA9 and HoxA10 were not unique.

Activity of the minimal promoter-reporter control vector was not altered by MII-EII, overexpression or knockdown of HoxA9 or HoxA10, or differentiation of the transfectants. This minimal activity was subtracted as background. In control experiments we identified the amount of HoxA9- or HoxA10-specific shRNA vector that decreased the endogenous message by ~70% (Fig. 4C and Ref 26). This amount was used in reporter gene assays.

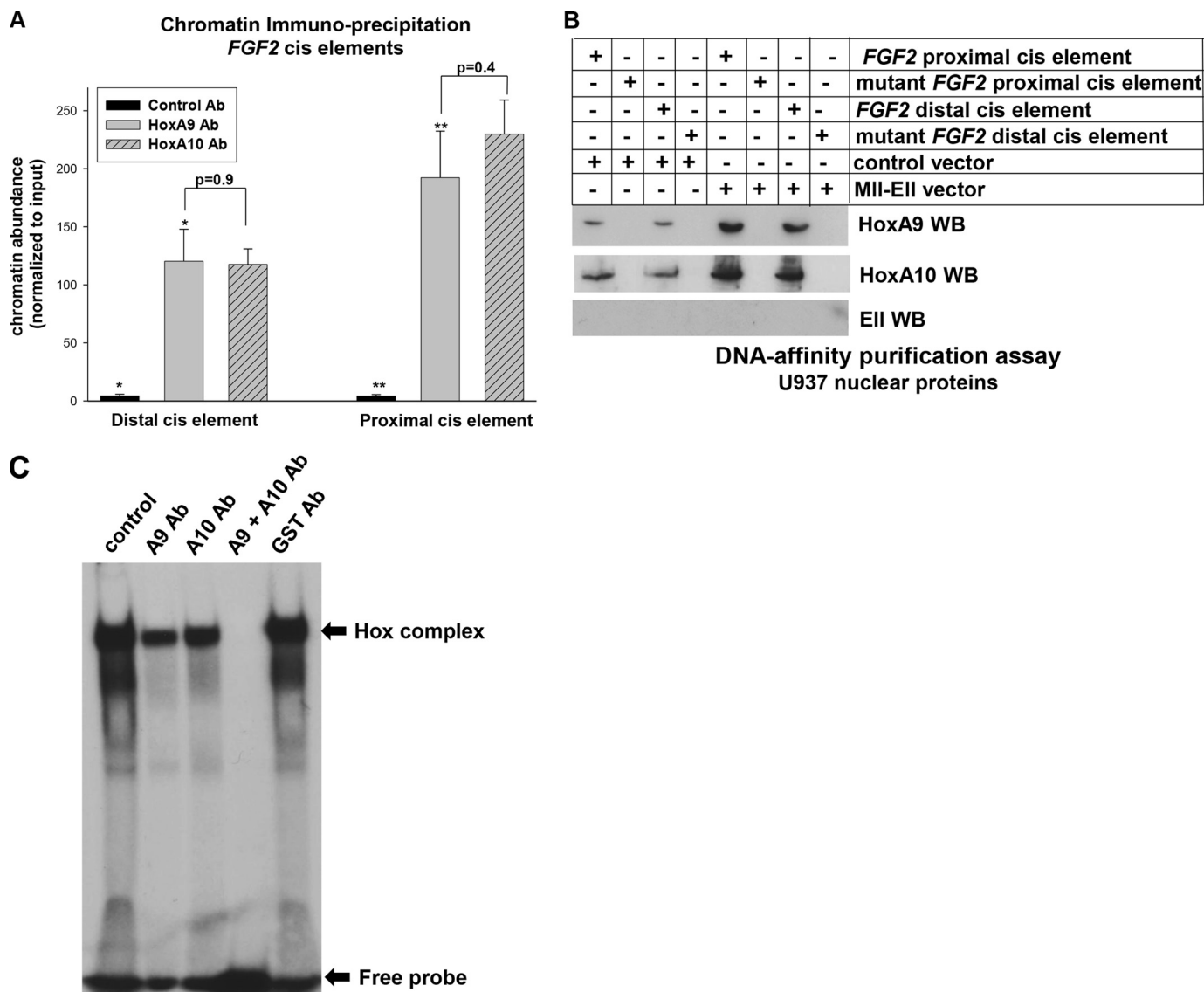


FIGURE 5. Mll-Ell increases binding of HoxA9 and HoxA10 to the *FGF2* promoter. *A*, HoxA9 binds to the previously identified HoxA10 binding *FGF2* cis elements *in vivo*. Chromatin immunoprecipitation experiments were performed using U937 myeloid cells and an antibody (Ab) to HoxA9, HoxA10 (as a positive control), or irrelevant control antibody (negative control). Co-precipitating chromatin was amplified by real time PCR with primers flanking the *FGF2* cis elements with or without mutation of the Hox binding consensus sequence. Proteins were collected by affinity of the probe to avidin-linked matrix, separated by SDS-PAGE, and identified by a Western blot (WB) with antibodies to HoxA9, HoxA10, or Ell. *B*, Mll-Ell increased binding of HoxA9 and HoxA10 to the proximal or distal *FGF2* cis elements *in vitro*. Nuclear proteins from U937 cells that were stably transfected with an Mll-Ell or empty control vector were incubated with a biotin-labeled, double-stranded oligonucleotide probe representing the proximal or distal *FGF2* cis elements with or without mutation of the Hox binding consensus sequence. Proteins were collected by affinity of the probe to avidin-linked matrix, separated by SDS-PAGE, and identified by a Western blot (WB) with antibodies to HoxA9, HoxA10, or Ell. *C*, HoxA9 and HoxA10 bind to the same *FGF2* cis elements *in vitro* by electrophoretic mobility shift assay. Nuclear proteins were isolated from U937 cells and incubated with a radio-labeled, double-stranded oligonucleotide probe representing the proximal *FGF2* cis element. Bound proteins and free probe were separated by native gel electrophoresis. Some binding assays were preincubated with antibody to HoxA9, HoxA10, both, or irrelevant control (GST) antibody. The free probe and Hox-bound DNA-protein complex are indicated by arrows.

Mll-Ell Increases Binding of HoxA9 and HoxA10 to the FGF2 Promoter—To determine if Mll-Ell increased binding of HoxA9 and/or HoxA10 to the *FGF2* promoter, we first verified binding of HoxA9 to the cis elements. For these studies, chromatin was co-precipitated from U937 lysates with an antibody to HoxA9, HoxA10 (as a positive control), or irrelevant control antibody. Lysates were sonicated under conditions that generated chromatin fragments with an average size of 200 bp (25, 26). Chromatin was amplified by real time PCR with primers flanking the proximal or distal *FGF2* cis element. We found *in vivo* HoxA9 binding to the *FGF2* cis elements (Fig. 5A).

We also performed chromatin immunoprecipitation with U937 cell lysates sonicated to generate 1.0-kb chromatin fragments. Co-precipitating chromatin was analyzed with PCR primers to amplify overlapping 500-bp sequences in the proximal 2 kb of *FGF2* 5'-flank. No additional HoxA9 binding sites were identified (not shown).

We investigated the influence of Mll-Ell on HoxA9 and HoxA10 by DNA affinity purification assays. For these studies, nuclear proteins were isolated from U937 cells that were stably transfected with an Mll-Ell expression vector (or control vector) and incubated with double-stranded oligonucleotides rep-

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representing the two *FGF2* cis elements with or without mutation in the Hox binding consensus. DNA-bound proteins were collected by affinity of the biotin-labeled probe to an avidin-linked matrix, separated by SDS-PAGE, and identified by Western blot. We found that sequence-specific HoxA9 and HoxA10 binding to both cis elements was increased by Mll-Ell (Fig. 5B). However, we did not demonstrate binding of Mll-Ell to any of these probes (Fig. 5B).

We also investigated binding to the *FGF2* cis elements by EMSA. For these studies, U937 nuclear proteins were incubated with a radiolabeled double-stranded oligonucleotide representing the proximal or distal *FGF2* cis element. Nuclear proteins were preincubated with an antibody to HoxA9, HoxA10, both antibodies, or irrelevant antibody, and bound and free probes were separated by native PAGE.

We found that both *FGF2* probes bound a low mobility protein complex that was partly disrupted by antibody to HoxA9 or HoxA10 and was completely disrupted by both antibodies together (Fig. 5C). Results are shown for the proximal cis element probe and were identical for the distal probe. We previously demonstrated that these probes generated a specific, low mobility complex with cross-competitive binding specificities with cis elements from other HoxA10 target genes (26).

Mll-Ell Induces Fgf2 Expression—We first investigated the effect of Mll-Ell on Fgf2 expression using U937 stable transfectants with an Mll-Ell *versus* control vector. We also investigated the relative effects of HoxA9 *versus* HoxA10 on Fgf2 using U937 cells stably overexpressing HoxA9, HoxA10, or HoxA9 + HoxA10 (½ each). Transfectants were analyzed with or without differentiation with RA/DMF.

We first investigated Fgf2 production by the transfectants using an Fgf2-specific ELISA to analyze cell-conditioned media. We found significantly more Fgf2 in the media of Mll-Ell-expressing U937 transfectants in comparison to control transfectants ($p < 0.0001$, $n = 3$), and this was only slightly increased by differentiation ($p = 0.08$, $n = 3$).

We also found significantly increased Fgf2 mRNA in Mll-Ell-expressing U937 cells relative to control transfectants by real time PCR ($p < 0.0001$, $n = 6$) (Fig. 6B). HoxA9 and HoxA10 mRNA was significantly increased in Mll-Ell-expressing cells ($p < 0.002$, $n = 6$; Fig. 6B). Although there was less HoxA10 mRNA *versus* HoxA9 mRNA in control cells ($p < 0.01$, $n = 6$), the increase in HoxA10 in cells expressing Mll-Ell was significantly greater than the increase in HoxA9 (568 ± 26% increase in HoxA10 with Mll-Ell *versus* 312 ± 25% increase in HoxA9, $p = 0.001$, $n = 6$; Fig. 6B). Expression of Mll-Ell was confirmed with a primer set that bridges the fusion. Western blots of lysate proteins from these cells demonstrated increased Fgf2, HoxA9, and HoxA10 protein in Mll-Ell-expressing cells in comparison to control cells (Fig. 6C).

We found significantly increased Fgf2 in the media of HoxA9-overexpressing U937 cells *versus* control transfectants ($p < 0.001$, $n = 3$), and this was not substantially altered by differentiation ($p = 0.2$, $n = 3$; Fig. 6A). Overexpression of HoxA9, HoxA10, or HoxA9 + HoxA10 also significantly increased Fgf2 mRNA ($p < 0.0001$, $n = 6$). There was no significant difference in the amount of Fgf2 mRNA expression in transfectants overexpressing HoxA9 *versus* HoxA10 *versus*

both with or without differentiation ($p = 0.2$, $n = 6$; Fig. 6D). Overexpression of HoxA9 *versus* HoxA10 was equivalent in these transfectants and approximately half as much in transfectants with both vectors (Fig. 6D). HoxA9 overexpression also increased Fgf2 protein in Western blot cells of lysates from U937 transfectants (Fig. 6E).

These studies suggested that Fgf2 was increased in myeloid progenitors and differentiating myeloid cells expressing Mll-Ell. However, U937 is a leukemia cell line that has abnormalities in proliferation and survival before introduction of oncoproteins. Therefore, we also investigated the effect of Mll-Ell on Fgf2 expression in primary murine bone marrow cells.

For these studies bone marrow cells from WT C57 Black 6 mice were transduced with an Mll-Ell retroviral expression vector (or empty control vector), cells were cultured in GM-CSF, IL3, and SCF, and CD34⁺ cells were separated. We referred to this as GMP conditions in these studies. Some cells were differentiated to granulocytes by treatment with G-CSF.

In other experiments bone marrow cells were transduced with retroviral vectors to express HoxA9, HoxA10, or HoxA9 + HoxA10 (or control vector). To determine if HoxA9 could substitute for HoxA10 for Fgf2 expression, bone marrow cells from HoxA10^{-/-} mice were also transduced with these vectors. Serum Fgf2 is decreased in HoxA10^{-/-} mice (26).

We found significantly increased Fgf2 in the media of Mll-Ell-expressing bone marrow cells in comparison to control vector-transduced cells ($p < 0.0001$, $n = 3$), and this was not altered by granulocyte differentiation ($p = 0.6$, $n = 3$) (Fig. 7A). We also found a significant increase in Fgf2, HoxA9, and HoxA10 mRNA in Mll-Ell-expressing bone marrow cells in comparison to control vector-transduced cells ($p < 0.001$, $n = 6$) (Fig. 7B). Expression of Fgf2 mRNA in Mll-Ell-expressing cells was not significantly altered by G-CSF-induced differentiation ($p = 0.2$, $n = 6$).

As noted for U937 transfectants, Mll-Ell resulted in a significantly greater increase in expression of HoxA10 *versus* HoxA9 (773 ± 49% increase in HoxA10 with Mll-Ell *versus* 372 ± 12% increase in HoxA9, $p = 0.001$, $n = 6$). Western blots demonstrated increased Fgf2, HoxA9, and HoxA10 protein in Mll-Ell-transduced murine bone marrow cells in comparison to control cells (Fig. 7C).

We found that overexpression of HoxA9 in murine bone marrow cells significantly increased Fgf2 in the media ($p < 0.001$, $n = 3$; Fig. 7A). HoxA9-induced increase in Fgf2 was not altered by G-CSF differentiation ($p = 0.5$, $n = 3$) and was not significantly different from the effect of expressing Mll-Ell or overexpressing HoxA10 or HoxA9 + HoxA10 ($p = 0.2$, $n = 3$; Fig. 7A). HoxA9 overexpression in HoxA10^{-/-} myeloid progenitor cells also significantly increased Fgf2 production ($p < 0.0001$, $n = 3$; Fig. 7A). Fgf2 secretion by HoxA10^{-/-} cells was equivalent with Mll-Ell expression or overexpression of HoxA9, HoxA10, or both ($p = 0.4$, $n = 3$; Fig. 7A).

Fgf2 mRNA was also significantly increased in HoxA9-overexpressing bone marrow cells relative to control cells ($p < 0.0001$, $n = 6$; Fig. 7D). This increase was equivalent in cells expressing Mll-Ell or overexpressing HoxA9 *versus* HoxA10 *versus* both with or without G-CSF differentiation ($p = 0.3$, $n = 6$). In these studies HoxA9 and HoxA10 were equivalently over-

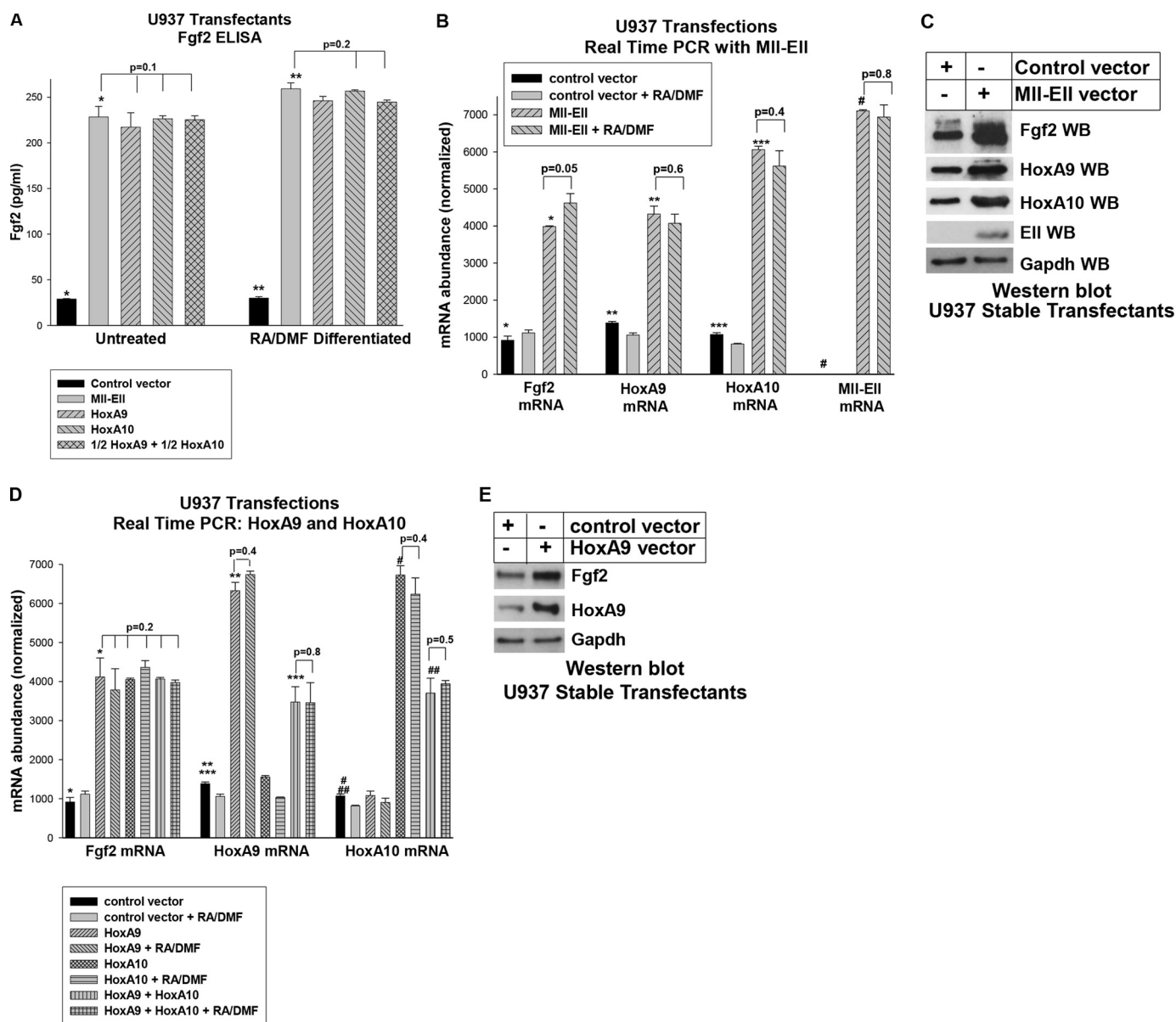


FIGURE 6. Fgf2 expression is increased in U937 cells expressing Mll-Ell or overexpressing HoxA9 or HoxA10 with or without differentiation. A, Fgf2 production by U937 cells is equivalently increased by expression of Mll-Ell or overexpression of HoxA9, HoxA10, or HoxA9 + HoxA10. U937 cells were stably transfected with vectors to express Mll-Ell or overexpress HoxA9, HoxA10, or HoxA9 + HoxA10 (1/2 of each) or with empty control vector. Media were harvested from the transfectants with or without RA/DMF differentiation and analyzed for Fgf2 by ELISA. Statistically significant differences in Fgf2 are indicated by * or **. B, Mll-Ell increases expression of Fgf2, HoxA9, and HoxA10 mRNA in U937 transfectants. U937 stable transfectants with Mll-Ell or empty control vector were analyzed for expression of HoxA9, HoxA10, and Fgf2 mRNA by real time PCR. Cells were analyzed with or without differentiation with RA/DMF. Statistically significant differences in mRNA expression are indicated by *, **, ***, or #. C, Mll-Ell increases expression of Fgf2, HoxA9, and HoxA10 proteins in U937 transfectants. Cell lysates from these stable transfectants were analyzed by Western blot (WB) with antibodies to HoxA9, HoxA10, Fgf2, EII, and Gapdh (a loading control). D, overexpression of HoxA9, HoxA10, or HoxA9 + HoxA10 in U937 cells equivalently increases Fgf2 mRNA abundance. U937 cells were stably transfected with vectors to overexpress HoxA9, HoxA10, HoxA9 + HoxA10, or empty control vector and analyzed by real time PCR for expression of Fgf2, HoxA9, or HoxA10 mRNA. Cells were analyzed with or without RA/DMF-differentiation. Statistically significant differences in expression are indicated by *, **, ***, #, or ##. E, overexpression of HoxA9 in U937 cells increases Fgf2 protein expression. Cell lysates from these transfectants were analyzed by Western blots with antibodies to HoxA9, Fgf2, or Gapdh.

expressed, and approximately half as much was expressed in cells with both vectors (Fig. 7D). Western blots of cell lysate proteins demonstrated increased Fgf2 protein with HoxA9 overexpression in comparison to control cells (Fig. 7E).

We found decreased Fgf2 mRNA expression in HoxA10^{-/-} bone marrow cells in comparison to control cells (Fig. 7F) (26). Fgf2 expression in HoxA10^{-/-} cells was equivalently increased by expression of Mll-Ell or overexpression of HoxA10, HoxA9, or HoxA9 + HoxA10 ($p = 0.8, n = 6$) (Fig. 7F). HoxA10 knock-

out did not influence HoxA9 mRNA expression (Fig. 7F). We also demonstrated similar overexpression of HoxA9 and HoxA10 in these cells and approximately half the overexpression with both vectors (Fig. 7F).

Mll-Ell Induces Fgf2-dependent Cytokine Hypersensitivity—To investigate the role of autocrine Fgf2-production in cytokine-induced proliferation of Mll-Ell-expressing cells, U937 transfectants with Mll-Ell or control vector were treated with a dose titration of FCS. Some cells were also treated with an Fgf2

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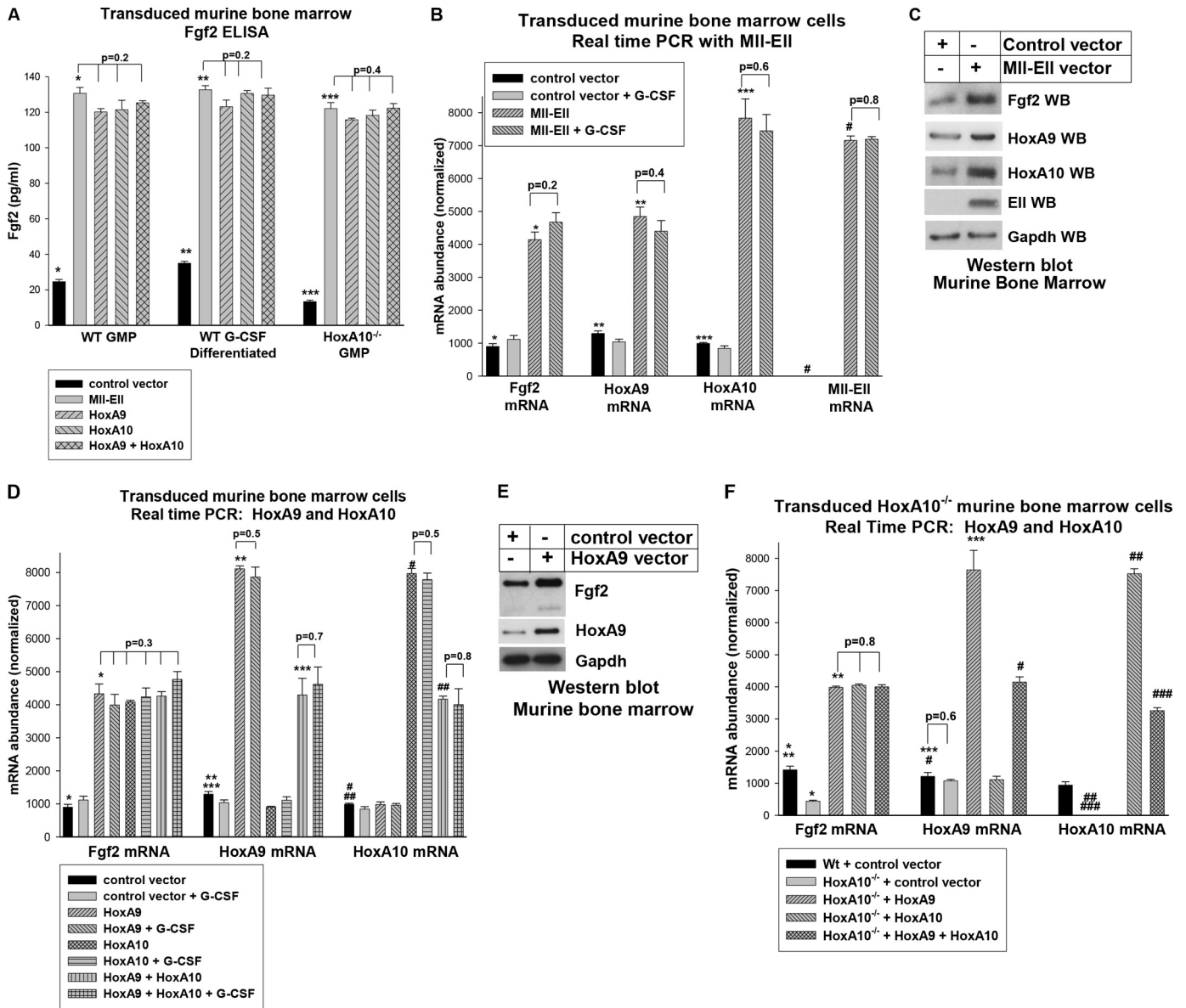


FIGURE 7. Fgf2 expression is increased in primary murine myeloid progenitor cells expressing Mll-Ell or overexpressing HoxA9 or HoxA10 with or without granulocyte differentiation. *A*, expression of Mll-Ell or overexpression of HoxA9 or HoxA10 increased Fgf2 production by murine myeloid progenitor cells. Bone marrow was harvested from WT or HoxA10^{-/-} mice and transduced with retroviral vectors to express Mll-Ell or overexpress HoxA9, HoxA10, HoxA9 + HoxA10, or empty control vector. Cells were cultured in GM-CSF, IL3, and SCF, and CD34⁺ cells were isolated (GMP conditions) or differentiated with G-CSF. Media were analyzed for Fgf2 by ELISA. Statistically significant differences in Fgf2 are indicated by *, **, or ***. *B*, Mll-Ell increases expression of Fgf2, HoxA9, and HoxA10 mRNA in primary murine bone marrow cells. The Mll-Ell-transduced cells (or control vector-transduced cells) described above were analyzed by real time PCR for Fgf2, HoxA9, HoxA10, or Mll-Ell fusion mRNA. Statistically significant differences in expression with Mll-Ell are indicated by *, **, ***, or #. *C*, Mll-Ell increases expression of Fgf2, HoxA9, and HoxA10 protein in primary murine bone marrow cells. These cells were also analyzed by Western blot (WB) with antibodies to Fgf2, HoxA9, HoxA10, EII, or Gapdh. *D*, overexpression of HoxA9 increases Fgf2 mRNA expression in primary murine bone marrow cells. Primary murine bone marrow cells were transduced with vectors to express HoxA9, HoxA10, or HoxA9 + HoxA10 or with control vector and cultured under GMP or G-CSF differentiation conditions described above. Cells were analyzed by real time PCR for Fgf2, HoxA9, and HoxA10 mRNA expression. Statistically significant differences in expression are indicated by *, **, ***, #, ##, or ###. *E*, HoxA9 overexpression increases Fgf2 protein expression in primary murine bone marrow cells. Cell lysate proteins from the transduced GMP cells, described above, were analyzed by Western blot with antibodies to Fgf2, HoxA9, or Gapdh. *F*, overexpression of HoxA9 rescues expression of Fgf2 in HoxA10^{-/-} murine bone marrow cells. Primary HoxA10^{-/-} murine bone marrow cells were transduced with vectors to express HoxA9, HoxA10, or HoxA9 + HoxA10 or with control vector and cultured under GMP or G-CSF differentiation conditions as described above. Cells were analyzed by real time PCR for Fgf2, HoxA9, and HoxA10 mRNA. Statistically significant differences in expression are indicated by *, **, ***, #, ##, or ###.

blocking antibody (or control antibody) or a specific Fgf-R1 inhibitor (PD173074). We found that FCS-induced proliferation was significantly greater in Mll-Ell-expressing U937 cells in comparison to control cells at all doses tested ($p < 0.001$, $n = 3$; Fig. 8A). Cytokine hypersensitivity of Mll-Ell transfectants was reversed by treatment with Fgf2 blocking antibody or Fgf-R1

inhibitor (Fig. 8A). The specificities of this antibody and inhibitor were previously validated (26).

We also investigated the functional significance of autocrine Fgf2 production in Mll-Ell-expressing murine bone marrow cells. For these studies cells were transduced with a retroviral vector to express Mll-Ell or control vector, cultured under

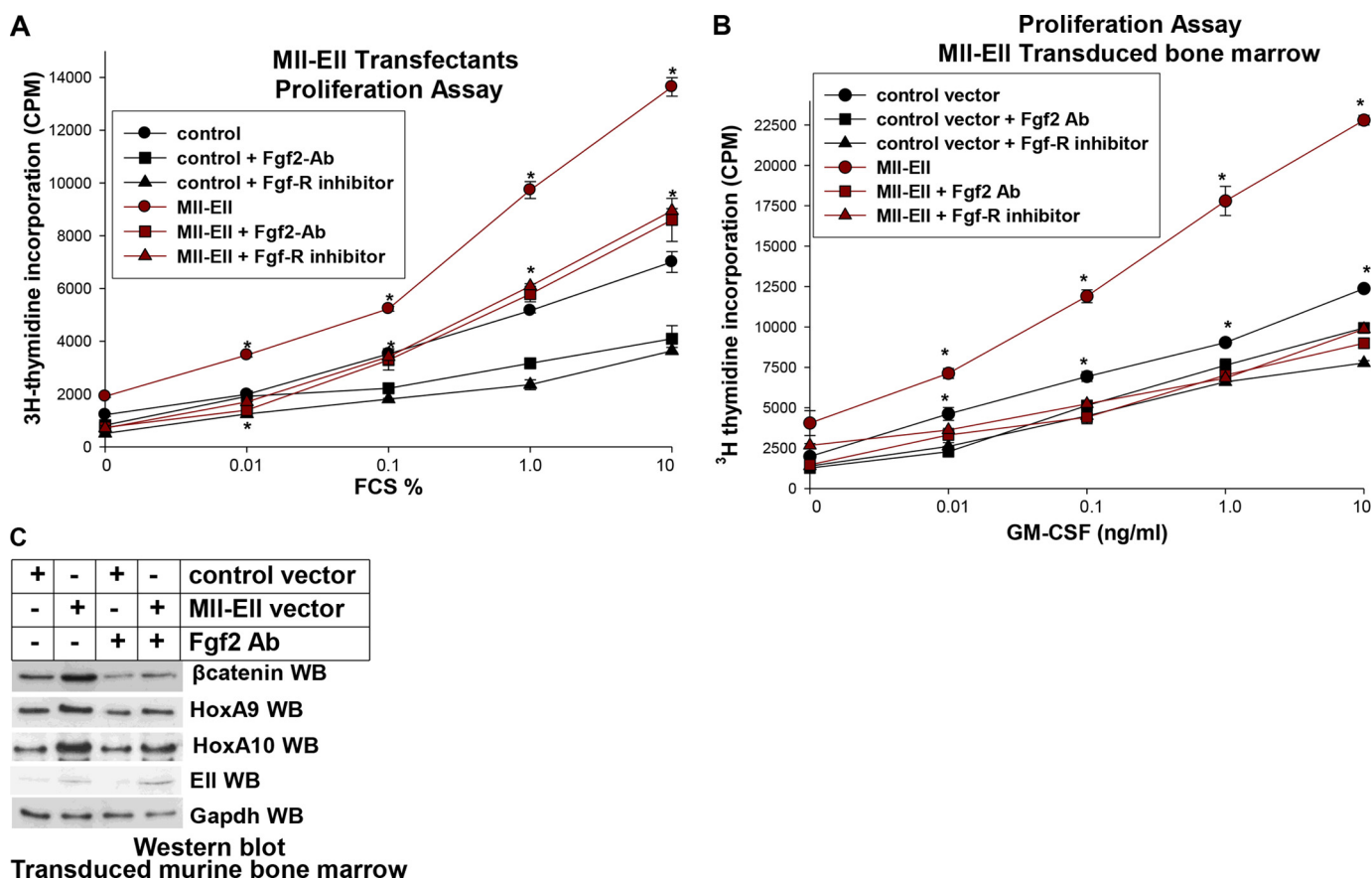


FIGURE 8. MII-EII induces Fgf2-dependent cytokine hypersensitivity in myeloid progenitor cells. A, MII-EII induces Fgf2-dependent cytokine hypersensitivity in U937 myeloid cells. U937 cells were stably transfected with a vector to express MII-EII or control vector and analyzed for proliferation in response to a dose titration of FCS (by incorporation of [³H]thymidine). Some cells were treated with a blocking antibody to Fgf2 or an Fgf-R1-inhibitor. Statistically significant differences in [³H]thymidine uptake at the same dose of FCS are indicated by an asterisk. B, MII-EII increases Fgf2-dependent GM-CSF hypersensitivity in primary murine myeloid progenitor cells. WT murine bone marrow cells were transduced with a vector to express MII-EII or empty control vector and analyzed under GMP conditions for proliferation in response to a dose titration of GM-CSF (by incorporation of [³H]thymidine). Some cells were treated with an Fgf2 blocking antibody or Fgf-R1 inhibitor. Statistically significant differences in [³H]thymidine uptake in MII-EII-expressing cells with versus without Fgf2 blocking antibody or Fgf-R1 inhibition are indicated by an asterisk. C, MII-EII increases β-catenin protein in an Fgf2-dependent manner in primary murine myeloid progenitor cells. Some transduced cells were analyzed by Western blots (WB) with antibodies to β-catenin, HoxA9, HoxA10, or Gapdh.

GMP conditions, and assayed for proliferation in response to a dose titration of GM-CSF. We found that GM-CSF-induced proliferation was significantly greater in MII-EII-expressing versus control cells at all cytokine doses ($p < 0.001$, $n = 3$; i.e. GM-CSF hypersensitivity). GM-CSF-induced proliferation of MII-EII-expressing cells was significantly decreased by treatment with Fgf2 blocking antibody or Fgf-R1 inhibition with PD173074 ($p < 0.01$, $n = 3$) (Fig. 8B).

Fgf2 binding to Fgf-R1/R2 results in stabilization of β-catenin protein (26). We tested the hypothesis that autocrine production of Fgf2 in MII-EII-expressing cells also stabilized β-catenin protein using the transduced murine bone marrow cells described above. Cells were treated with an Fgf2 blocking antibody (or control antibody), and Western blots of cell lysates were probed for β-catenin, HoxA9, HoxA10, EII (to identify the fusion protein), or Gapdh (a loading control). We found an increase in β-catenin protein in MII-EII-expressing bone marrow cells that was abrogated by Fgf2 blocking antibody (Fig. 8C). Expression of β-catenin mRNA was not increased in MII-EII-expressing cells (not shown).

We also investigated the impact of increased production of Fgf2 in HoxA9-overexpressing U937 stable transfectants by

proliferation assays similar to studies above. We found that HoxA9 overexpression induced cytokine hypersensitivity in these cells (Fig. 9A) similar to overexpressed HoxA10 (18, 26). HoxA9-induced cytokine hypersensitivity was significantly decreased by treatment with Fgf2 blocking antibody or Fgf-R1 inhibitor ($p < 0.01$, $n = 3$; Fig. 9A).

We analyzed U937 stable transfectants overexpressing HoxA9, HoxA10 (positive control), or HoxA9 + HoxA10 for β-catenin protein expression by Western blot. We found that β-catenin protein was increased in cells overexpressing HoxA9, HoxA10, or HoxA9 + HoxA10 in comparison to control transfectants, but this was decreased by treatment with Fgf2 blocking antibody (Fig. 9B). Expression of β-catenin mRNA was not increased in HoxA9- or HoxA10-overexpressing cells (not shown), consistent with Fgf2-induced stabilization of β-catenin protein.

We also investigated the roles of HoxA9 and Fgf2 in cytokine hypersensitivity using transduced murine bone marrow cells as described above. We found that HoxA9-overexpressing murine myeloid progenitor cells exhibited significantly enhanced GM-CSF-induced proliferation at a low cytokine dose relative to control cells ($p < 0.01$, $n = 3$; Fig. 9C). We also found that

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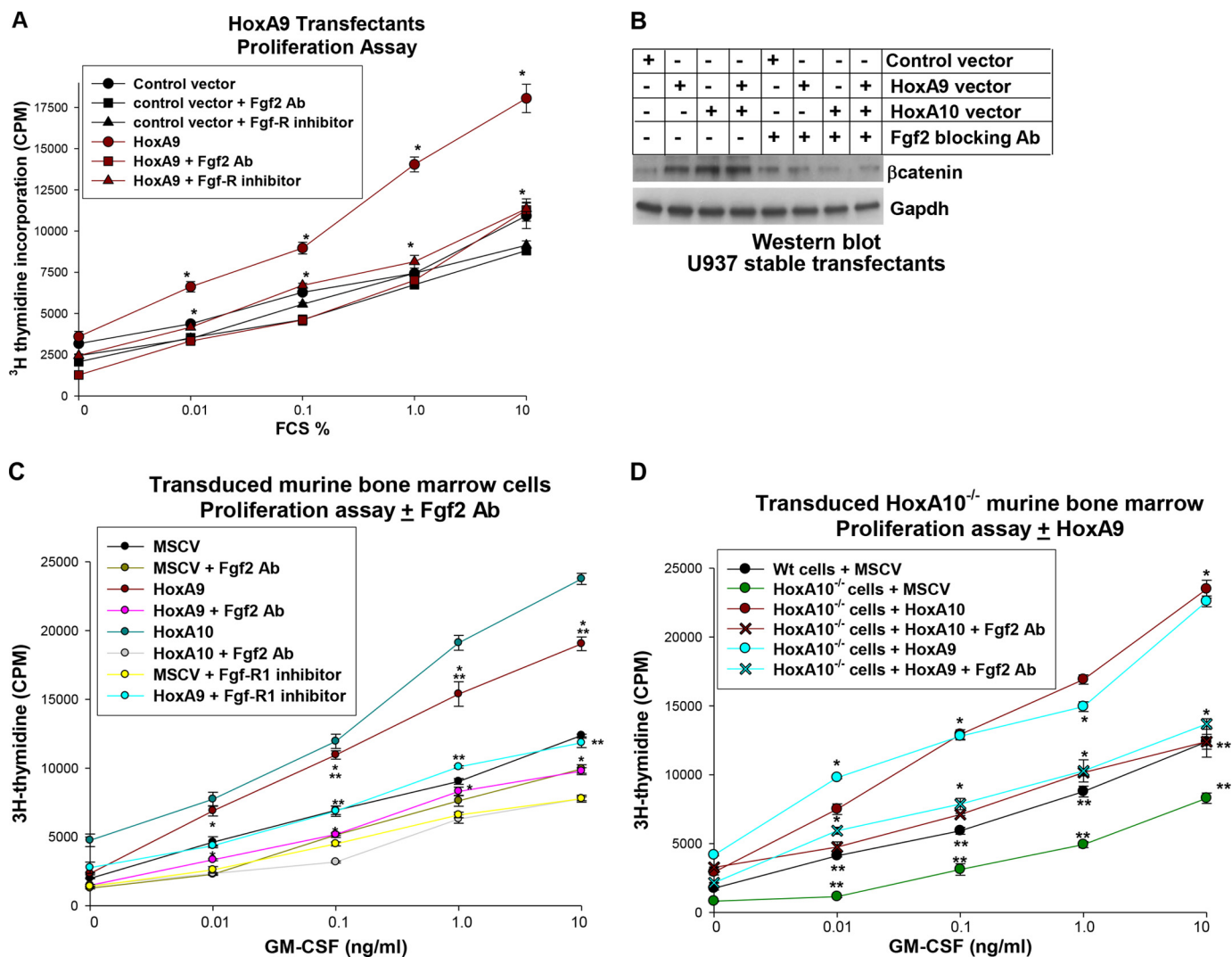


FIGURE 9. HoxA9 overexpression induces Fgf2-dependent cytokine hypersensitivity in myeloid progenitor cells. *A*, overexpression of HoxA9 induces Fgf2-dependent cytokine hypersensitivity in U937 myeloid leukemia cells. Cells stably overexpressing HoxA9 or transfected with an empty control vector were analyzed for proliferation in response to a dose titration of FCS (by incorporation of [³H]thymidine). Some cells were treated with a blocking antibody (Ab) to Fgf2 or Fgf-R1 inhibitor. Statistically significant differences in [³H]thymidine uptake at the same dose of FCS are indicated by an asterisk. *B*, HoxA9 increases β -catenin protein expression in an Fgf2-dependent manner. U937 cells that stably overexpressed HoxA9, HoxA10, or HoxA9 + HoxA10 were analyzed by Western blot with antibody to β -catenin or Gapdh with or without Fgf2 blocking antibody. *C*, overexpression of HoxA9 in murine bone marrow myeloid progenitor cells induces Fgf2-dependent GM-CSF hypersensitivity. WT murine bone marrow cells were transduced with a vector to overexpress HoxA9 or HoxA10 or with empty control vector. Cells were analyzed for proliferation in response to a dose titration of GM-CSF with or without an Fgf2 blocking antibody or Fgf-R1 inhibitor. Statistically significant differences in [³H]thymidine incorporation at the same dose of GM-CSF are indicated by asterisks (* or **). *D*, HoxA9 rescues GM-CSF-induced proliferation in HoxA10^{-/-} myeloid progenitor cells. HoxA10^{-/-} murine bone marrow cells were transduced with a vector to overexpress HoxA9 or HoxA10 or with empty control vector. Cells were analyzed for proliferation in response to a dose titration of GM-CSF with or without an Fgf2 blocking antibody. Empty vector-transduced WT cells were a control in this study. Statistically significant differences in proliferation at comparable GM-CSF doses are indicated by asterisks (* or **).

treatment of HoxA9-overexpressing myeloid progenitor cells with Fgf2 blocking antibody or Fgf-R1 inhibitor significantly decreased GM-CSF induced proliferation ($p < 0.01$, $n = 3$) (Fig. 9C). HoxA10 vector and control vector-transduced murine myeloid progenitor cells were positive and negative controls for these studies.

To determine if HoxA9 could rescue proliferation defects in HoxA10^{-/-} cells, HoxA10^{-/-} murine myeloid progenitor cells were transduced with vectors to express HoxA9 or HoxA10 (as a positive control) or with empty retroviral vector (as a negative control) and treated with a dose titration of GM-CSF. We found that GM-CSF-induced proliferation of HoxA10^{-/-} cells was impaired in comparison to WT cells at all GM-CSF doses ($p <$

0.01, $n = 3$; Fig. 9D). This was reversed, and cytokine hypersensitivity resulted, with overexpression of HoxA9 (Fig. 9D). HoxA9-induced cytokine hypersensitivity was decreased by treatment with Fgf2 blocking antibody.

DISCUSSION

Increased serum levels of Fgf2 are observed in some leukemia subjects, and increased Fgf2 mRNA expression is specifically found with 11q23-AML (28, 29). Consistent with this, we determined that expression of an 11q23-AML-associated MLL fusion protein (Mll-Ell) increased *FGF2* transcription in a HoxA9- and HoxA10-dependent manner. This resulted in autocrine production of active Fgf2 by Mll-Ell-expressing cells

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genesis of 11q23-AML. This is a topic of translational relevance and ongoing investigations.

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