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Differential *Hox* Expression in Murine Embryonic Stem Cell Models of Normal and Malignant Hematopoiesis

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The *Hox* family are master transcriptional regulators of developmental processes, including hematopoiesis. The *Hox* regulators, caudal homeobox factors (*Cdx*1-4), and *Meis1,* along with several individual *Hox* proteins, are implicated in stem cell expansion during embryonic development, with gene dosage playing a significant role in the overall function of the integrated *Hox* network. To investigate the role of this network in normal and aberrant, early hematopoiesis, we employed an in vitro embryonic stem cell differentiation system, which recapitulates mouse developmental hematopoiesis. Expression profiles of *Hox*, *Pbx1*, and *Meis1* genes were quantified at distinct stages during the hematopoietic differentiation process and compared with the effects of expressing the leukemic oncogene Tel/PDGFRβ. During normal differentiation the *Hoxa* cluster, *Pbx1* and *Meis1* predominated, with a marked reduction in the majority of *Hox* genes (27/39) and *Meis1* occurring during hematopoietic commitment. Only the posterior *Hoxa* cluster genes (*a9*, *a10*, *a11*, and *a13*) maintained or increased expression at the hematopoietic colony stage. *Cdx4*, *Meis1,* and a subset of *Hox* genes, including *a7* and *a9*, were differentially expressed after short-term oncogenic (Tel/PDGFRβ) induction. Whereas *Hoxa4-10*, *b1*, *b2*, *b4*, and *b9* were upregulated during oncogenic driven myelomonocytic differentiation. Heterodimers between *Hoxa7/Hoxa9*, *Meis1*, and *Pbx* have previously been implicated in regulating target genes involved in hematopoietic stem cell (HSC) expansion and leukemic progression. These results provide direct evidence that transcriptional flux through the *Hox* network occurs at very early stages during hematopoietic differentiation and validates embryonic stem cell models for gaining insights into the genetic regulation of normal and malignant hematopoiesis.

Introduction

Embryonic stem (ES) cells have become an established model to investigate early hematopoiesis in an in vitro setting [1,2]. Murine ES cells can be induced to differentiate by removal of the growth factor leukemia inhibitory factor (LIF) from the media. Differentiation induction leads to the production of defined colonies termed embryoid bodies (EBs) that contain developing cell populations of all 3 germ layers [1–3]. Mesoderm-derived cells within the EBs can subsequently be stimulated to produce blast-like colonies (BC) that under specific conditions can differentiate further giving rise to myeloid, erythroid, and lymphoid cells that constitute the hematopoietic lineages. Several studies have previously validated this model on the basis of gene expression and progenitor cell assays [1,2,4]. In a previous study, we carried out comparative analysis of the Wnt signaling pathway, which is a major regulator of hematopoiesis, using an ES model of differentiation and primary tissues and showed substantial overlap in expression that was both stage and organ specific [5]. This previous work emphasizes the appropriateness of the ES model as a tool to study transcriptional flux through genetic networks during hematopoietic differentiation.

The *HOX* network is comprised of the clustered class I homeobox genes, which encode master regulators of development. The mammalian 39 *Hox* genes are evolutionarily highly conserved and appear to have arisen by a process of duplication and divergence from a primordial gene (reviewed by Duboule [6]). Mammalian *Hox* genes are located as up to 13 paralogs within 4 clusters (*A*, *B*, *C*, and *D*) on separate chromosomes (7, 17, 12, and 2, in human; 6, 11, 15, and 2, in mouse). During embryonic development *Hox* genes are expressed sequentially (3′–5′) along the anterior-posterior axis, with 3′ genes such as *Hoxa1* expressed earlier and more anteriorly than 5′ genes, such as *Hoxa13* [7,8]. Deregulated

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Hox expression at the individual gene level tends to result in subtle phenotypic changes, most likely due to a high level of redundancy built into the network.

Hox expression during definitive hematopoiesis has been relatively well studied in both mouse and human with the majority of the *Hox* genes being expressed predominantly in the hematopoietic stem/progenitor cell (HSPC) compartment. *Hox* gene expression appears linked to and may specify in part hematopoietic lineage and stage of differentiation. In particular, the *A* and *B* clusters, which are preferentially expressed in the most primitive hematopoietic cells, exhibit reduced expression after lineage commitment (reviewed by Argiropoulos and Humphries [9]). Such observations led to the hypothesis that self-renewal of HSPCs is Hox dependent and that inappropriate *Hox* expression observed in myeloid leukemias underlies maintenance of the leukemia-initiating cell [10,11]. The role of *Hox* genes in hematopoiesis has also been studied using gene-targeting strategies. Both gain-of-function and loss-of-function mutants have provided insight into the role of individual *Hox* genes in hematopoiesis. Ectopic expression of *Hoxa9* results in increased HSPC self-renewal that results in acute leukemia with a long latency [12,13], whereas overexpression of *Hoxb4* results in stem cell expansion in the absence of overt leukemia [14–16]. Similarly, *Hoxa9*-deficient mice display a dramatic phenotype consistent with reduced HSPC self-renewal [17,18], whereas *Hoxb4*-deficient mice are normal [19]. The difference in phenotype between these 2 models may be related to the significantly higher expression of *Hoxa9* than that of *Hoxb4* in normal HSPCs [18].

Several studies have implicated aberrant regulation of the *Cdx* family and the *Hoxa* and *Hoxb* clusters in myeloid malignancies especially acute myeloid leukemia (AML) [20,21]. However, a role for *Hox* genes in myeloproliferative neoplasms (MPN) has not been clearly defined. The t(5:12)(q33;p13) translocation, identified in a subset of patients with the MPN chronic myelomonocytic leukemia, fuses an Ets-related transcription factor (Tel) to the PDGFRβ gene, producing the Tel/PDGFRβ (TP) tyrosine kinase-active fusion protein [22]. Previous studies using tetracycline (Tet)-regulated expression of TP recapitulated observed findings for TP^+ patient samples, in particular alterations in the hematopoietic transcriptional network that drives myelomonocytic differentiation [23,24]. In this study, we have employed a previously established hematopoietic differentiation model [5] to determine the *Hox* transcriptional profile during primitive and more definitive hematopoietic commitment. The effects of the leukemic oncogene on this gene signature were then examined to determine how perturbed myelopoiesis alters the *Hox* network. Cells were collected at key stages during hematopoietic differentiation along the mesodermal-hemangioblast-hematopoietic axis for quantitative expression profiling. Our results clearly demonstrate transcriptional flux through the *Hox* network and regulation of specific *Hox* subsets during hematopoietic differentiation of ES cells, with specific changes observed in our leukemic model. These findings are consistent with a combination of global regulation of *Hox* clusters and targeted regulation of individual *Hox* genes (or subsets) during hematopoietic commitment. This study highlights that transcriptional fluxes in the *Hox* network play a role in deregulation of myelopoiesis in MPN.

Materials and Methods

Hematopoietic differentiation of ES cells and ES-TP clones

Hematopoietic differentiation was performed as previously described [5]. Primary EBs were generated from single-cell suspensions of ES parental cells or ES-TP clones in the presence or absence of 500 μg/mL Tet. Cell were plated at 1×10^4/mL in low adhesion Petri dishes in the following basal media: 1% methylcellulose (Sigma-Aldrich Technologies), 1× Iscove's modified Dulbecco's medium (IMDM) (Invitrogen Life Technologies), 100 μg/mL holo-transferrin, 10 μg/mL insulin, 10^{-4} M 2-Mercaptoethanol, 50 μg/mL ascorbic acid (Sigma-Aldrich Technologies), 15% fetal calf serum (FCS; Invitrogen Life Technologies) supplemented with the following growth factors to promote good mesodermal differentiation, 10 ng/mL bone morphogenetic protein-4 (BMP-4; R&D Systems Ltd., Abingdon), 2 ng/mL Activin A, and 10 ng/mL basic fibroblast growth factor (bFGF; PeproTech™). After 3.75 days in culture EBs were harvested, washed ×3 with phosphate-buffered saline (PBS), and treated with 0.25% trypsin–ethylenediaminetetraacetic acid for 3 min. Cells were replated at 1×10^5/mL in basal media supplemented with the following growth factors to promote hemangioblast formation: 100 ng/mL stem cell factor (SCF), 10 ng/mL interleukin-6 (IL-6), and 10 ng/mL vascular endothelial growth factor (VEGF; PeproTech). After 4 days of culture, EBs and BCs were harvested as outlined and a single-cell suspension replated at 2.5×10^4/mL in basal media supplemented with 1% bovine serum albumin (Invitrogen Life Technologies) and cytokines to promote both myeloid and erythroid colony formation: 25 ng/mL GM-CSF, 25 ng/mL G-CSF, 10 ng/mL SCF, 10 ng/mL IL-3 (PeproTech), and 2 U/mL erythropoietin (EPO) (R&D Systems Ltd). After further culture for 7 days, hematopoietic colonies (HCs) were scored using a Nikon Eclipse TS100 microscope and digital camera imaging system.

Cell culture

CCE and E14tg2a (expressing the Tet-sensitive transactivator) murine ES cells [25] were routinely cultured on tissue culture plates (Nunc) coated with 0.1% (v/v) porcine gelatin (Sigma-Aldrich Technologies) as described previously [23]. The ES-TP clones were cultured as previously described [24]. For induction of expression of TP clones were washed ×3 with PBS and incubated in LIF-containing media in the presence of 500 ng/mL Tet to prevent expression of TP, or no Tet to induce the expression of TP.

Conditional expression of TP

TP clones (TP1 and TP14) were plated at 1×10^5–1×10^6 cells per gelatin-coated 100 mm tissue culture dish. Cells were cultured with and without Tet for 24, 48, or 72 h to induce the short-term expression of TP. After treatment, the cells were harvested and washed ×3 with ice-cold PBS before being lysed as described previously [24]. Protein concentrations were determined using a Bio-Rad protein assay kit. Delayed TP activation was initiated by removal of Tet at the BC stage (day 4 culture—LIF) or the HC stage (day 8—LIF) of development. BC cells with delayed TP activation cells

were collected at day 8—LIF (day 4 TP^+) and the corresponding HC cells at day 15—LIF (day 7 TP^+). Delayed TP-activated cells were examined by colony assays and total RNA isolated for polymerase chain reaction (PCR) and real time quantitative (RQ)-PCR analyses.

Immunoblotting and antibodies

For immunoblotting, 20 μg of each cell lysate was fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted onto nitrocellulose. Primary antibodies were used at the following dilutions: 0.1 μg/mL anti-phosphotyrosine 4G10 (05–32; Upstate Biotechnology); 0.5 μg/mL PDGFRβ antibody, which recognizes TP (CST 3162; New England Biolabs); and 1:2,000 anti-SHP-2 (sc-293; Santa Cruz Biotechnology Inc.). Secondary antibodies conjugated to horseradish peroxidase (Dako) were used at 1:10,000 dilution and blots developed using ECL (Amersham Pharmacia).

FDCP-Mix cells

Factor dependent cell Paterson (FDCP)-Mix cells stably expressing the *TP* oncogene or empty vector (EV) were maintained in Fishers medium with 20% (v/v) horse serum supplemented with 10 ng/mL IL-3 (R&D Systems). For differentiation induction, FDCP-Mix cells were washed 3 times in PBS and cultured in IMDM with 20% fetal bovine serum (Invitrogen Life Technologies) and cytokines to induce monocytic and neutrophilic commitment (0.1 ng/mL IL-3, 10 ng/mL GM-CSF, 5 ng/mL M-CSF, and 10 ng/mL G-CSF; PeproTech).

Flow cytometry

During hematopoietic differentiation, cells were harvested at the primary EB, and secondary EB/BC colony for ES cell, or at days 0, 4, and 7 of differentiation for the FDCP-mix cells and analyzed by fluorescence-activated cell sorting (FACS). EBs were treated with trypsin for 2 min, neutralized with FCS, washed ×3 with PBS, and resuspended at 0.5×10^5 cells/100 μL of FACS Buffer (PBS with 2% FCS and 0.02% sodium azide). Cells were blocked with 1 μg of Purified Rat Anti-Mouse CD16/CD32 Fc Block™ (Becton Dickinson, BD 553142) for 1 h at 4°C. Labeling was performed using 0.5 μg of each flurochrome-conjugated antibody or relevant isotype control for 1 h at 4°C in the dark. Secondary staining was performed for a further 30 min for any indirect stains: Flk-1 (VEGF-R2, Ly-73; BD 555308), Brachyury (Abcam 20680), Sca 1 (BD 558162), c-Kit (CD 117; BD 553356), CD 11b (BD 552850), Gr1 (BD 553129), FITC Secondary (BD 554020), Step-Avidin Secondary (BD 554064). Cells were washed in 2 mL of FACS Buffer, pelleted 1,200 rpm for 5 min at 4°C. Cells were resuspended in 300 μL of FACS Buffer and analyzed on a FACSCanto™ II flow cytometer (BD) and data analyzed using FlowJo software.

PCR analyses

Total RNA was prepared using RNAeasy Plus extraction kit (Qiagen). RNA (1 μg) was reverse-transcribed using Superscript reverse transcriptase and oligo dT primers (Invitrogen Life Technologies). PCR was performed using 2 μL of cDNA and standard conditions with gene-specific primers as previously reported [5].

Real-time quantitative PCR

RQ-PCR was performed as previously reported [26]. Briefly, total RNA was extracted from cells using RNAesay Plus extraction kit, cDNA was prepared, and RQ-PCR was achieved using TaqMan™ probe-based chemistry (Applera) and the ABI PRISM 7500 system (Perkin Elmer-Applied Biosystems). The murine *Hox*-specific oligonucleotide sets were designed using Primer Express™ (Applera) and validated by standard PCR cloning and DNA sequencing of at least 5 colonies. *Hox* target copy numbers (copies per 50 ng RNA equivalents) were obtained from plasmid-derived standard curves [27]. The 18S rRNA predeveloped assay reagent (Applera) was used as endogenous control. RQ-PCR data are reported as either cycle threshold (C_T) values corrected to endogenous control, which refers to the accumulation of sufficient PCR product to transect a user-defined threshold (lower C_T values indicate higher gene expression) or as fold change, which was calculated using the $2^{-\Delta\Delta CT}$ method and assumes doubling of the amount of product with each PCR cycle.

Statistical analysis

Data generated from biological and technical replicates were analyzed by the Paired, 2-tailed Student's *t*-test: $^+P < 0.05$ and $^*P < 0.001$.

Results

Hematopoietic differentiation of ES cells

Differentiation of the E14tg2a and CCE ES cell lines was performed using a modified protocol based on the original work of Keller and colleagues [1]. The directed differentiation was validated by morphological analysis that confirmed appropriate formation of 1° EBs, BCs, and HCs at key stages of hematopoietic development. Hemoglobinized erythroid cells were observed within the BCs and distinct CFU-GM, BFU-E, and GEMM colonies were identified by day 15 of culture (Fig. 1A) consistent with these established models [1,5]. The differentiation model was validated and characterized based on expression of known ES self-renewal and differentiation genes. Expression of self-renewal markers such as *Oct-4* and *Nanog* decreased during differentiation in congruence with upregulation of maturation and lineage-specific markers such as *FGF5*, *FIK-1*, *Hbb-b1*, *PU1*, and *MafB* (Fig. 1B and as previously reported [5]). Maturation through the key stages of development was also associated with altered levels of brachyury, Flk-1, c-Kit, and Sca-1 (Supplementary Fig. S1; Supplementary Data are available online at www.liebertonline.com/scd) and differential expression of the *Hox*-regulator caudal homeobox-4 (*Cdx4*) gene and *Hoxb4* (Fig. 1C).

Complete Hox *network profiling during hematopoietic differentiation of ES cells*

Undifferentiated murine ES cells displayed a wide range of expression of *Hox* elements with corrected C_T values between 34 and 19, corresponding to 20 and 3×10^5 copies for *Hoxd9* and *Hoxa1*, respectively. All *Hox* genes were expressed to a measurable amount ($C_T < 36$) in both murine ES cell lines (Fig. 2 and data not shown). The vast majority of the

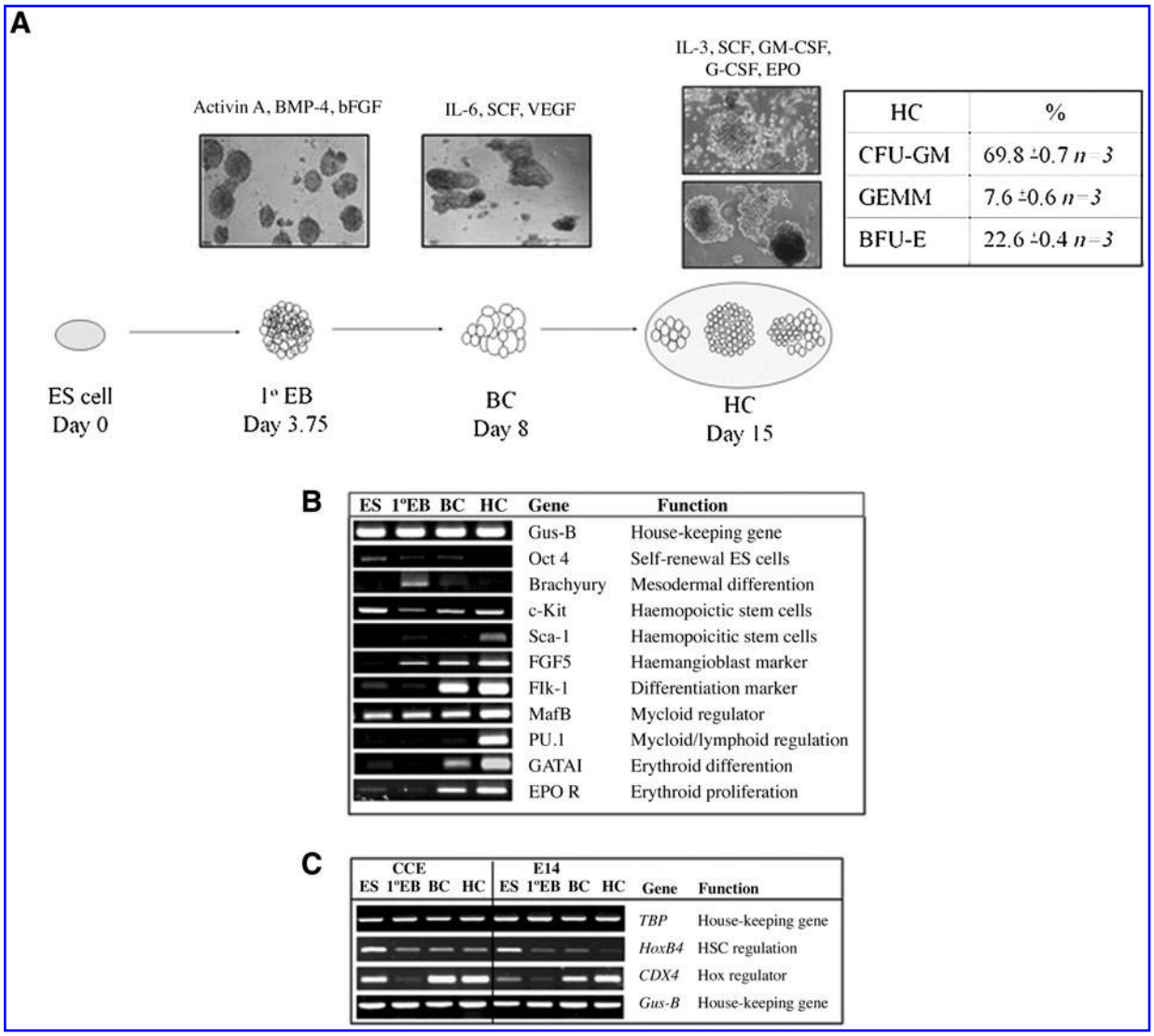

FIG. 1. In vitro hematopoietic differentiation of murine embryonic stem (ES) cells. **(A)** Schematic diagram of the 3 stages involved in hematopoietic differentiation of ES cells and the growth factor combinations used. Photographs of representative primary embryoid bodies (EB), blast colonies (BC), and hematopoietic colonies (HCs) taken at 10× magnification. Table of the percentage of morphologically identified colonies within the HC by day 15. **(B)** Reverse transcription-polymerase chain reaction (RT-PCR) analysis at key ES cell differentiation stages. Expression of known self-renewal and corresponding lineage-specific markers were examined, with the housekeeping gene *GusB* as control. **(C)** RT-PCR analysis of *Hoxb4* and *Cdx4* expression obtained from cells at the key stages of development. The low expression housekeeping genes (*TBP* and *GusB*) were included as positive controls.

Hox genes (27/39) displayed reduced expression after induction of the differentiation program, and this reduction was either transient (12/27) or sustained (15/27) for the duration of the differentiation process (Fig. 2 and Supplementary Fig. S2a–d). Of the 8 genes that showed measurable ($\Delta C_T \geq 2$) upregulation upon EB formation, only 1 was from the *Hoxa* cluster (*a13*), 2 from the *Hoxc* cluster (*c8* and *c10*), and 5 from the *Hoxd* cluster (*d1*, *d3*, *d4*, *d9*, and *d10*). Transition from the EB to the BC development stage resulted in measurable upregulation in 20/41 genes analyzed, including the 3-amino loop extension (TALE) genes *Pbx1* and *Meis1* as well as downregulation in *Hoxb1* and *c13*. Further differentiation to the HC stage resulted in global upregulation of the *Hoxc* cluster and general downregulation in the other genes with the notable exception of *Hoxa13*, *b13*, and *Meis1* (Supplementary Fig. S2a–d). Due to their preponderance in hematopoiesis, further comparative analysis of *Hoxa* expression was performed during hematopoietic differentiation of both E14tg2a and CCE cell lines. Two distinct patterns of expression emerged whereby the 3′ genes (*Hoxa1*-*Hoxa7*) were downregulated during hematopoietic differentiation and the 5′ genes (*Hoxa9*-*Hoxa13*) tended to be upregulated during this process (Fig. 3). These data suggest coordinated expression of *Hox* genes in ES cells.

Conditional expression of TP in an ES model of leukemia

Previous studies investigating the role of TP during hematopoietic differentiation of ES cells indicated that this

Hox	control	1° EB	BC	HC	*Hox*	control	1° EB	BC	HC
a1	**19.3 ± 0.3**	21.7 ± 0.0	**20.7 ± 0.1**	21.3 ± 0.1	*c4*	26.0 ± 0.1	30.4 ± 0.2	27.3 ± 0.4	25.1 ± 0.2
a2	21.3 ± 0.1	23.4 ± 0.0	23.9 ± 0.2	24.4 ± 0.0	*c5*	25.5 ± 0.2	28.6 ± 0.1	27.5 ± 0.1	26.7 ± 0.3
a3	**19.6 ± 0.2**	22.4 ± 0.2	23.0 ± 0.4	23.2 ± 0.2	*c6*	22.1 ± 0.2	24.6 ± 0.1	22.6 ± 0.2	**20.1 ± 0.2**
a4	**20.1 ± 0.1**	23.9 ± 0.0	23.4 ± 0.0	22.4 ± 0.2	*c8*	29.0 ± 0.1	24.5 ± 0.2	22.5 ± 0.1	21.2 ± 0.1
a5	**20.0 ± 0.1**	23.4 ± 0.2	22.4 ± 0.2	23.8 ± 0.3	*c9*	22.9 ± 0.1	26.4 ± 0.2	23.3 ± 0.0	21.4 ± 0.2
a6	**19.7 ± 0.2**	**19.3 ± 0.1**	**18.0 ± 0.1**	**19.2 ± 0.2**	*c10*	28.6 ± 0.2	24.7 ± 0.1	**20.5 ± 0.1**	**19.5 ± 0.2**
a7	**20.5 ± 0.1**	22.5 ± 0.0	21.1 ± 0.0	**20.6 ± 0.1**	*c11*	22.9 ± 0.0	26.8 ± 0.2	24.9 ± 0.2	22.3 ± 0.1
a9	22.1 ± 0.2	25.7 ± 0.3	24.4 ± 0.2	24.7 ± 0.1	*c12*	22.6 ± 0.2	26.8 ± 0.1	26.4 ± 0.1	23.9 ± 0.1
a10	22.1 ± 0.2	26.9 ± 0.1	26.7 ± 0.4	25.3 ± 0.1	*c13*	22.2 ± 0.1	25.9 ± 0.1	28.3 ± 0.2	26.9 ± 0.2
a11	21.0 ± 0.2	24.2 ± 0.3	**20.2 ± 0.2**	**18.9 ± 0.1**	*d1*	30.0 ± 0.1	26.4 ± 0.2	26.2 ± 0.2	26.8 ± 0.1
a13	27.5 ± 0.1	25.1 ± 0.4	**20.2 ± 0.2**	**19.7 ± 0.1**	*d3*	30.8 ± 0.0	27.1 ± 0.1	24.4 ± 0.2	25.2 ± 0.0
b1	21.4 ± 0.1	21.9 ± 0.1	24.1 ± 0.3	26.8 ± 0.1	*d4*	32.1 ± 0.2	28.2 ± 0.4	25.4 ± 0.0	26.6 ± 0.2
b2	22.6 ± 0.1	24.0 ± 0.1	22.9 ± 0.2	25.1 ± 0.1	*d8*	26.0 ± 0.0	27.6 ± 0.3	25.7 ± 0.0	24.8 ± 0.2
b3	21.6 ± 0.2	24.6 ± 0.2	23.4 ± 0.8	26.2 ± 0.2	*d9*	34.0 ± 0.1	29.7 ± 0.1	28.4 ± 0.1	27.9 ± 0.2
b4	22.4 ± 0.1	25.9 ± 0.5	23.2 ± 0.2	24.7 ± 0.1	*d10*	32.1 ± 0.1	26.2 ± 0.1	23.1 ± 0.2	23.9 ± 0.0
b5	24.8 ± 0.2	24.8 ± 0.0	**20.7 ± 0.1**	24.3 ± 0.0	*d11*	22.8 ± 0.2	23.4 ± 0.2	**19.7 ± 0.1**	**19.8 ± 0.2**
b6	22.3 ± 0.2	25.7 ± 0.0	24.2 ± 0.1	26.2 ± 0.2	*d12*	23.1 ± 0.2	27.4 ± 0.1	23.7 ± 0.1	26.0 ± 0.1
b7	**20.1 ± 0.1**	**23.0 ± 0.0**	**19.4 ± 0.2**	**20.4 ± 0.2**	*d13*	25.4 ± 0.3	26.1 ± 0.1	21.8 ± 0.2	22.4 ± 0.1
b8	22.3 ± 0.1	25.8 ± 0.2	23.1 ± 0.1	25.7 ± 0.0	*Pbx1*	**20.0 ± 0.1**	21.0 ± 0.1	**18.6 ± 0.1**	**19.1 ± 0.0**
b9	21.3 ± 0.1	24.8 ± 0.1	22.8 ± 0.2	25.6 ± 0.3	*Meis1*	21.7 ± 0.2	26.2 ± 0.2	21.0 ± 0.3	**19.8 ± 0.2**
b13	**20.4 ± 0.3**	24.7 ± 0.1	23.6 ± 0.2	21.1 ± 0.1					

Key: 18-21, 21-24, 24-27, 27-30, 30-35

FIG. 2. Array of *Hox-Tale* gene profiles in differentiating murine embryonic stem cells. Real-time quantitative PCR (RQ-PCR) expression analysis of *Hox* and cofactor genes at key development stages (1) undifferentiated (control), (2) primary EB (1° EB), (3) BC, and (4) HC formation. Representative mean cycle threshold (C_T) values obtained from triplicate experiments normalized to 18S rRNA ± standard error (SE) are tabulated. Lowest C_T values (18–20) reflect highest expression; highest C_T values (32–35) reflect lowest expression.

oncogene can upregulate several transcription factors involved in regulating HSC expansion, including Hoxb4 and Cdx4 [24]. Several studies have shown that the Cdx family are global regulators of *Hox* gene expression. Removal of Tet from cultured ES-TP clones induced robust expression of TP that was maintained for up to 72 h (Fig. 4A). Immunoblotting analysis showed comparable levels of TP protein in 2 independent clones that resulted in functional signal transduction from the oncogenic receptor as detected by the anti-phosphotyrosine antibody. Induced TP expression (up to 72 h) resulted in increased expression of *Hoxb4* and *Cdx4* (Fig. 4B and ref. [24]) and decreased ES cell self-renewal and proliferation as verified by reduced alkaline phosphatase staining (Fig. 4C). This resulted in altered morphology consistent with a more differentiated phenotype as previously described [24].

Complete Hox *network profiling after short-term TP expression in ES cells*

RQ-PCR profiling of the *Hox* network in ES-TP cells showed measurable expression (C_T <36) of all genes except *Hoxd3*. As was the case in the parental line a wide range of *Hox* expression was observed with C_T values ranging between 35 and 24, corresponding to between 10 and 2×10^4 copies for *Hoxb1* and *Hoxa3*, respectively. Short-term induction of TP (24 h) resulted in moderate to substantial alteration in *Hox* expression as depicted by changes in corrected C_T values (Supplementary Table S1). A subset of genes from the *Hoxa* and *b* clusters ($n = 13$) plus the cofactor genes *Pbx1* and *Meis1* were identified as candidates for further time-course study.

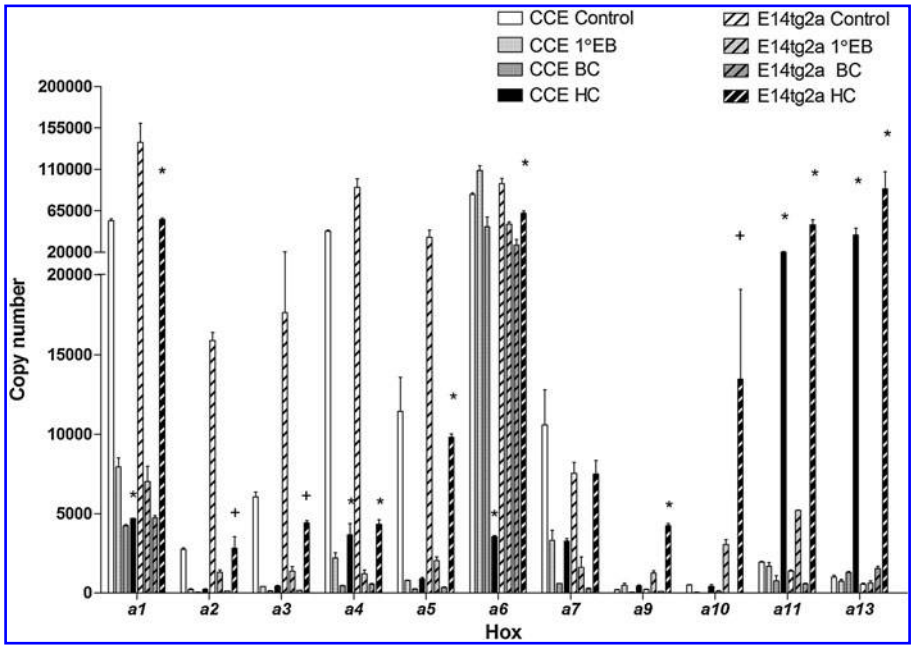

FIG. 3. Reciprocal coordinated expression of the Hoxa cluster in differentiating murine embryonic stem (ES) cells. Representative histogram plots of copy number (per 50 ng of RNA) in expression of the *Hoxa* cluster in 2 independent cell lines (CCE and E14tg2a) from primary EB (1° EB): BC and HC formation compared with undifferentiated control cells. Values were obtained from triplicate experiments and normalized to 18S rRNA ± SE. $^{\dagger}P < 0.05$ and $^{*}P < 0.001$.

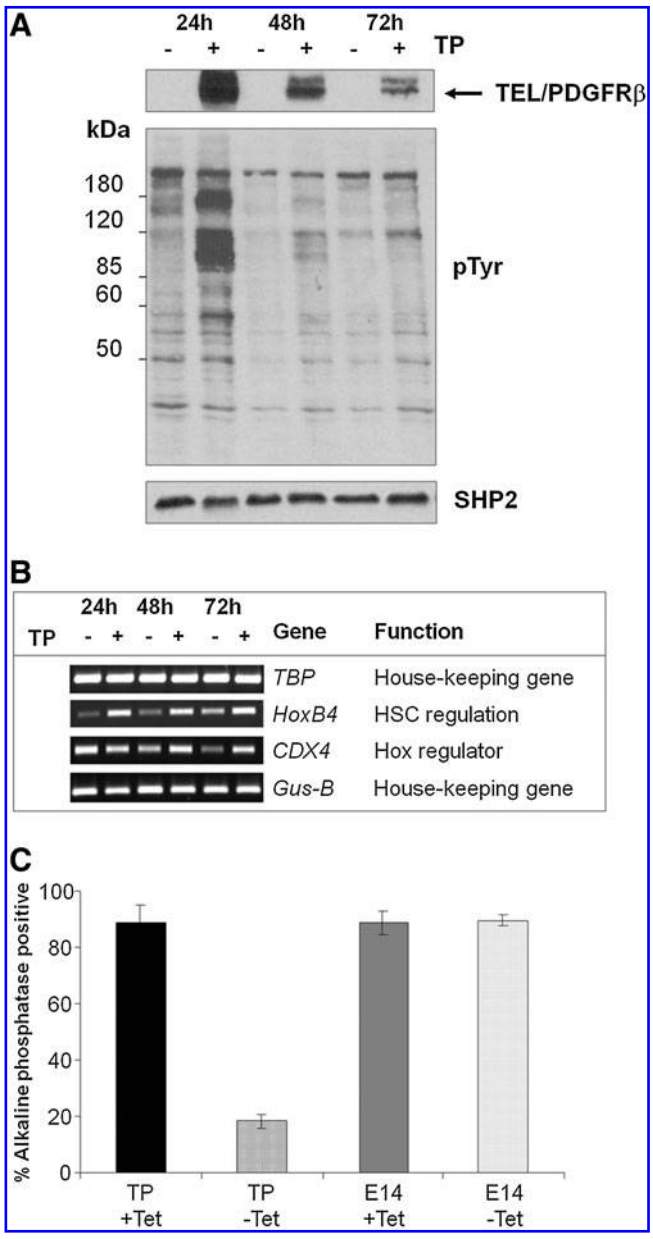

FIG. 4. Induced Tel-PDGFRβ expression in murine ES cells leads to altered gene expression and phenotype. **(A)** Representative Western blot analysis of protein extracts obtained from one of the ES cell clones stably transfected with the tetracycline (Tet)-responsive Tel-PDGFRβ receptor plasmid (TP). ES-TP clones were grown either in the presence (+) or absence (−) of Tet for 24, 48, or 72 h. Expression of Tel-PDGFRβ was confirmed using the anti-PDGFRβ receptor antibody, and activation of signaling pathways was confirmed by anti-phosphotyrosine (pTyr) antibody; SHP2 expression was used to confirm equal loading of protein. Gels representative of the level of TP expression and tyrosine phosphorylation. **(B)** RT-PCR analysis showing increased expression of *Hoxb4* and *Cdx4* after induction of Tel-PDGFRβ (+) compared with control (−) for 24, 48, and 72 h. The low expression housekeeping genes (TBP and GusB) were included as positive controls. **(C)** Histogram plots showing reduced alkaline phosphatase positivity in ES cells expressing Tel-PDGFRβ (TP −Tet) compared with noninduced (TP +Tet), nonexpressing (E14 +Tet), and treatment (E14 −Tet) controls. Values are means ± SE of 3 independent experiments.

Candidate Hox *gene expression during sustained TP activation in ES and FDCP-Mix cells*

Hox genes that demonstrated differential expression after short-term TP activation (24 h) were further analyzed in a time-course study over 3 days that correlates with substantial differentiation of TP expressing cells (Fig. 4 and [24]). The candidate genes were all expressed at measurable levels (C_T <36) throughout the time course. Significant changes in expression ($P < 0.05$) were observed throughout the time course (Fig. 5). The majority of the candidate genes demonstrated an initial reduction in expression after TP induction (9/15) that was either transient (7/9) or sustained (2/9) over the 3 days. *Hoxa6* was the most predominantly expressed of the candidate genes and was significantly upregulated at the 72 h time point. *Hoxa10* exhibited sustained repression (≥4-fold) after TP activation. Five genes, *Hoxa13*, *b2*, *b4*, *b5*, and *Meis1*, demonstrated initial upregulation in expression after TP induction that was sustained or steadily increased (up to 16-fold for *Meis1*) during the time course, as the TP expressing cells differentiate (Fig. 5). Of most interest was upregulation of *Hoxa6*, *a7*, *a9*, *b2*, *b3*, *b4*, *Pbx1*, and *Meis1* in the TP-induced differentiated cells. These genes have previously been linked to hematopoietic regulation and deregulation of myelopoiesis in leukemia.

Ectopic expression of TP in the multipotent FDCP-Mix murine cell line resulted in pTyr activation and modified differentiation as defined by increased Sca-1 and decreased CD 11b and Gr-1 expression compared with control cells (Supplementary Fig. S3). Although baseline Hox expression was an order of magnitude lower in this model compared with the ES cells, increased expression in the constitutively active TP cells (day 0) was observed for *Hoxa6*, *a7*, *a9*, *a10*, *b2*, *b3*, *b4*, *b9*, and *Meis1* (Supplementary Fig. S4). Extended differentiation of the FDCP-Mix cells, up to day 7, resulted in significantly reduced levels of Hox as expected. Meis1 expression was consistently higher than the *Hox* genes in the differentiating FDCP-Mix cell lines, and TP activation resulted in maintained increased expression of Meis1 compared with EV controls.

TP modulates key Hox *genes during hematopoietic differentiation of ES cells*

To examine transcriptional flux through the Hox network in a leukemia-differentiation model, inducible TP-ES cells were directed to undergo hematopoietic differentiation in the presence or absence of Tet. Cells were collected at time points corresponding with EB, BC, and HC formation with HCs scored to ensure that TP was driving myelopoiesis as previously reported [24]. Examination of the TP-candidate genes in this model demonstrated significant altered expression ($P < 0.05$ and <0.001) of a number of *Hox* genes at various stages of development. Moderate to substantial (up to 10-fold) changes in expression were shown between control TP-inactive and TP-active differentiating cells (Fig. 6). *Hoxa6* was the most predominant of the candidate genes in this model. Transient upregulation of the majority of genes, including *Hoxa6*, was observed at either the EB (9/15) or BC (13/15) stage of development. *Hoxa5* and *Hoxa7* expression was increased considerably (8–10-fold) with EB formation and *Hoxb1* (10-fold) with BC formation. *Hoxa6*, *a7*, *a9*, *b3*, *b4*, and *b9* were all upregulated in both the early differentiating cells

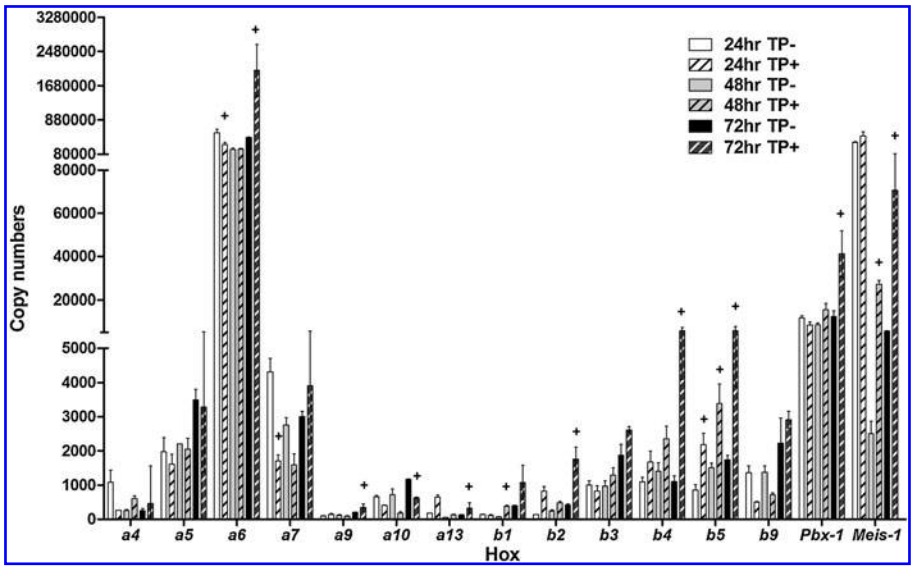

FIG. 5. Comparative expression analysis of a subset of *Hox-Tale* candidate genes after Tel-PDGFRβ induction in a leukemic murine ES cell model. Representative histogram plot of copy number (per 50 ng of RNA) from 2 independent experiments of activated Tel-PDGFRβ (TP+) or control (TP−) murine ES cells for 24, 48, and 72 h. Values obtained from triplicate experiments normalized to 18S rRNA ± SE are plotted. $^{\dagger}P < 0.05$ and $^{*}P < 0.001$.

(72 h TP expression) as well as during mesodermal and hemangioblast formation after TP expression, identifying these as potential candidates involved in TP-mediated alterations in hematopoietic transcriptional regulation. *Hoxa5* and *a10* were initially downregulated after short-term TP expression but became upregulated during mesodermal and hemangioblast commitment. All of the candidate genes apart from *Hoxb2* were downregulated in the TP expressing HCs, most notably for *Meis1* (∼4-fold).

Delayed activation of TP results in altered differentiation and gene expression

Delayed activation of TP was examined by Tet removal at the BC or HC stage of differentiation. Late activation of TP in HC cells resulted in increased myeloid colony formation at the expense of erythroid and mixed colonies (Fig. 7A). These phenotypic changes, reflected by changes in differentiation marker gene expression, were dependent on conditional TP activation and defined stage of development (Fig. 7B). Lower *Hoxb*4 and *EpoR* expression in HC cells was more dependent on development stage than TP status. In contrast, conditional activation of TP resulted in a marked upregulation of *GATA3* expression at the BC or HC stage of development. With the exception of *Hoxa10,* which showed a moderate upregulation, TP activation in BC cells had little effect on Hox-Tale expression. Delayed TP activation in HC cells, however, resulted in significant upregulation in *Hoxa4*, *a5*, *a7*, *a13*, *b2*, *b3*, *b4*, and *b5*, with a 2-fold reduction in the expression of *Hoxa6* (Fig. 7C). The candidate Hox-Tale signature increased, cumulatively, by over 75% (10,578–18,782 copies) after activation of TP in HC cells.

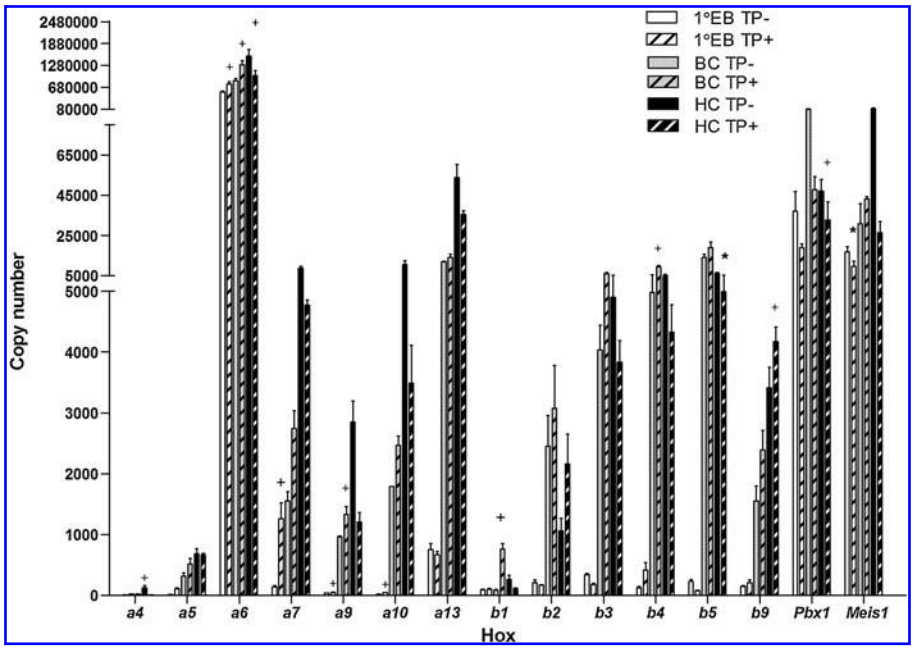

FIG. 6. RQ-PCR analysis of a subset of *Hox-Tale* genes after sustained Tel-PDGFRβ induction in a leukemic model of murine ES cell differentiation. Representative histogram plot showing copy number (per 50 ng of RNA) of the candidate genes at primary EB (1° EB): BC and HC formation after induction of Tel-PDGFRβ (TP) compared with noninduced controls. Mean values were obtained from triplicate experiments normalized to 18S rRNA ± SE. $^{\dagger}P < 0.05$ and $^{*}P < 0.001$.

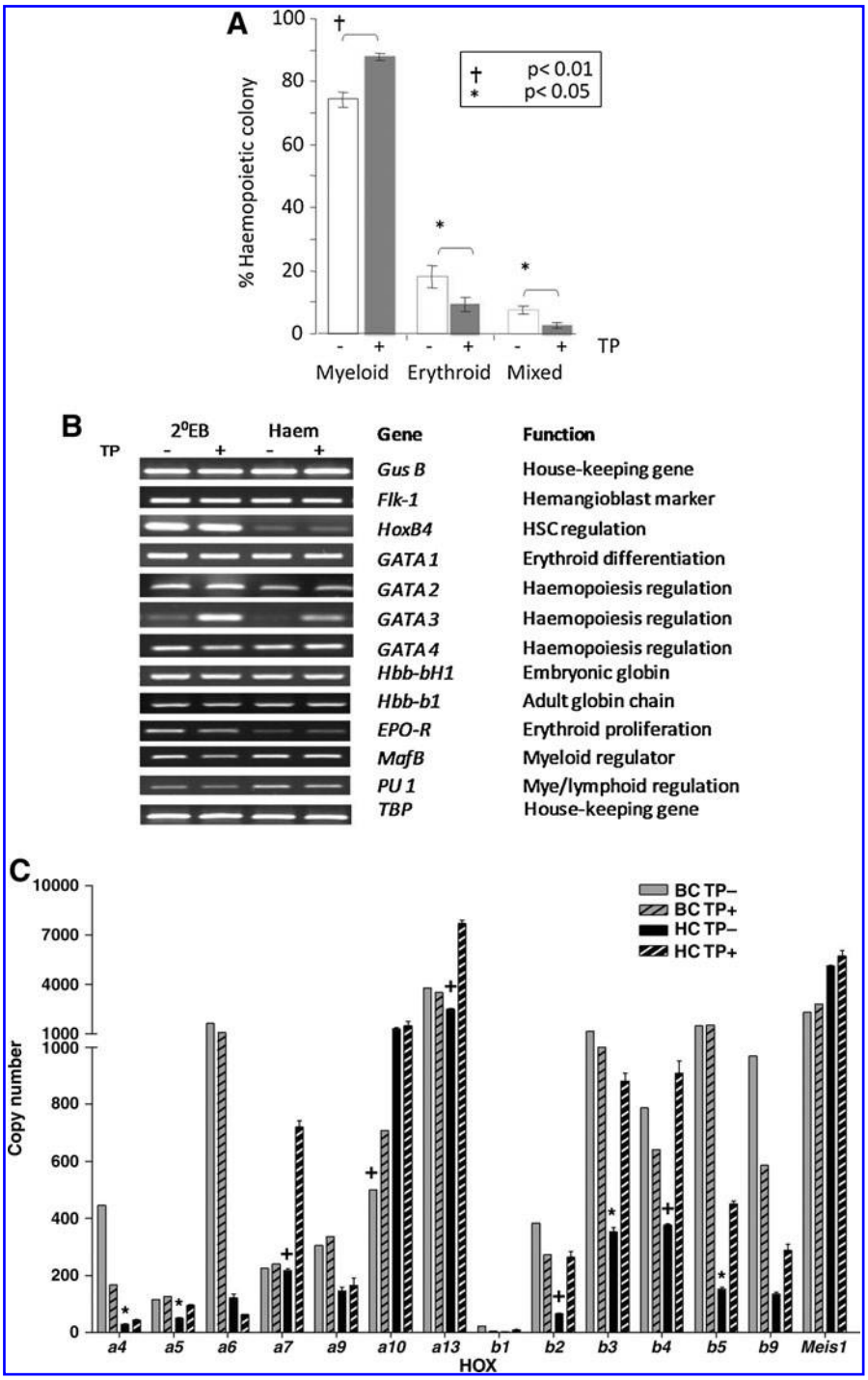

FIG. 7. Delayed TP activation results in increased myeloid differentiation and differential gene expression of ES cells. (**A**) Percentage of HC type formed by day 15 of differentiation when TP was expressed only at the HC stage (days 8–15) ($n = 3$). (**B**) Representative RT-PCR gels of differentiation markers after delayed TP activation at the BC or HC stage of development. (**C**) Representative histogram of *Hox-Tale* copy numbers (per 50 ng RNA) obtained from RQ-PCR analysis after delayed activation of TP at the BC or HC stage. Mean values were obtained from triplicate experiments normalized to 18S rRNA ± SE. $^{\dagger}P < 0.05$ and $^{*}P < 0.001$.

Discussion

The ES/EB model used to track early events involved in hematopoietic cell commitment and differentiation is now well established [1,2,28]. Several researchers have elucidated key signaling events that regulate self-renewal and lineage commitment using this system (reviewed by Park *et al.* [29]). Murine ES cells are maintained in the undifferentiated state by the presence of LIF in the culture media. LIF signals through its receptor gp130 to activate STAT3, which is sufficient for retention of ES cells in their undifferentiated pluripotent state [30]. Additional intracellular signal transduction molecules, including other STATs [31], MAPK [32], PI3K [23], TGF-Beta/Smad [33], and the members of the Wnt family [5,34,35], have been shown to play a significant role in hematopoietic development from ES cells. A modified culture system that efficiently promotes the directed differentiation of ES cells to the hematopoietic lineage [1] was used. The sequential addition of specific growth factors is critical for coordinated lineage restriction of the ES cells. BMP-4 promotes efficient formation of mesoderm; bFGF and activin A induce the differentiation of mesodermal precur-

sors to hemangioblasts; and VEGF and SCF regulates the production of fully committed hematopoietic progenitors. The stepwise production of HCs allows for molecular dissection of the key stages of hematopoietic development [36].

BMP-4 signaling appears to play 2 distinct and sequential roles during ES-derived hematopoiesis at least in part by activation of the *Cdx-Hox* pathway [37]. FGF regulation of the *Cdx-Hox* axis has been established in other developmental models [38] and a *Cdx-Hox* code may actually control the effectiveness of FGF signaling [39]. Similarly VEGF signaling is associated with differential *Hoxb* expression [40]. Members of the TGFβ superfamily, including Activin A, have previously been identified as regulators of the *Hox* axis [41] and more recently direct interaction between specific Hox proteins and TGFβ/BMP downstream regulators have been identified [42,43]. Differential expression of *Cdx4* and *Hoxb4* was initially confirmed in the ES model and quantitative profiling of the *Hox* network extended these findings to the complete *Hox* network. *Cdx4* expression was initially downregulated during formation of the EBs in the presence of BMP-4 but became dramatically upregulated during production of BCs and hematopoietic progenitors, perhaps in part due to the biphasic activity of BMP-4.

Induction of differentiation in the normal ES model resulted in a robust downregulation of the majority of *Hox* genes, including *Hoxb4*, reflected by an increased C_T value and reduced copy number. Global downregulation of *Hox* genes is a hallmark of differentiation and may reflect global epigenetic regulation of the clusters [44,45]. The increased expression of *Hoxa13*, *c8,* and *c10* throughout the differentiation process points to specific *Hox* regulation associated with the early stages of hematopoiesis. The marked upregulation of the *Hoxd* cluster, with the exception of *Hoxd12*, also suggests privileged global regulation of this cluster during ES differentiation as previously reported [46]. This global activation may be controlled in part by an underlying mechanism of nuclear reorganization that results in looping out of the *Hoxd* cluster from its chromosomal territory [47]. Whether this nuclear reorganization is maintained in tissue-specific developmental processes such as hematopoiesis requires further study. The *Hoxa* cluster is generally well expressed in hematopoietic tissue and its deregulation is often associated with leukemia [9]. Comparative analysis of 2 independent ES cell lines undergoing hematopoietic differentiation demonstrated reciprocal coordinated expression of the *Hoxa* cluster. The more anterior 3′ subset (*Hoxa1-Hoxa7*) showed consistent reduced expression during all 3 key stages of differentiation. Conversely, the more posterior 5′ subset (*Hoxa9-Hoxa13*) showed differentiation associated downregulation followed by a moderate to substantial increase in expression upon formation of the hematopoietic progenitor cells. Both the degree and trend of expression appeared coordinated around cluster position, with Hoxa6/7 acting as the fulcrum. The trend in expression was similar for both cell lines with the E14tg2a cell line showing greater degree of expression change throughout the network.

Oncogenic activation of TP results in an MPN with accumulation of myelomonocytic cells. In the leukemic model, the transforming TP oncoprotein self-associates and activates kinase-dependent signaling pathways [48]. Previous studies demonstrate that TP inhibits ES self-renewal and promotes myeloid differentiation [24,49]. This phenotype was validated by reduction in alkaline phosphatase expression and upregulation of *Hoxb4* and *Cdx4*. Increased *Hoxb4* and *Cdx4* expression levels are known to enhance the clonogenic potential of EB-derived cells during hematopoietic differentiation [49,50]. Recent evidence suggests that Cdx4 is upregulated in 23% of AML patients, with preferential expression shown in primitive stem and progenitor cells. In addition, Cdx4-transduced bone marrow demonstrated serial re-plating ability of primitive myelomonocytic-like cells [20]. Further, the impaired hematopoietic phenotype of Cdx mutant mice can be rescued by multiple *Hox* genes [51]. Therefore, Cdx4 may play an important role in modulating the Hox network to bias myelomonocytic differentiation in the TP model.

Short-term TP activation in ES cells resulted in increased expression of *Hoxa13* and *Hoxb2* (up to 6-fold). *Hoxa13* is recognized as playing a role in normal limb development, but overexpression has been observed in models of leukemia and in small cell lung carcinoma [52]. *Hoxb2* is part of a common *Hox* repertoire associated with self-renewal [53] and expansion potential of another ES cell line (D3) and bone marrow-derived mesenchymal stem cells [54]. Extended analysis identified *Hoxa6*, *b1*, *b2*, *b4,* and *Meis1* as displaying measurable to substantial upregulation (up to 16-fold for *Meis1*) after TP activation. *Hoxb4* is well established as a self-renewal factor for ES and LT-HSCs [16] and we have previously shown *Hoxa6* to affect hematopoietic cell proliferation and self-renewal [55]. The striking and sustained upregulation of *Meis1* suggests that it plays a key role in the oncogenic pathway initiated by TP activation. Meis1 is an established Hox collaborator and its expression has been shown to track with *Hox* genes during ES differentiation [56]. *Meis1* cooperates with *Cdx4* to accelerate AML development in mice through upregulation of *Hoxa6*, *a7*, *a9*, *b8*, *b4*, and *c6* [20] and has been associated with regulating other *Hox* genes such as *Hoxb1* and *b2* [57].

Hematopoietic differentiation of TP expressing ES cells resulted in upregulation of *Hoxa6*, *a7*, *a9*, *b1*, *b2*, *b3*, *b4*, and *b9* between days 3 and 8 of differentiation when cells undergo mesodermal and hemangioblast/HSC formation. *Meis1* was also upregulated in the BC when the first hemangioblasts and HSC form. To test if the TP-induced transcriptional effects were ES cell specific, conditional TP expression (-Tet) was delayed until the BC (days 4–8) or HC (days 8–15) stage of development. Quantitative PCR analysis of the Hox candidates identified 9 genes (*Hoxa4*, *a5*, *a7*, *a13*, *b2*, *b3*, *b4*, *b5*, and *Meis1*) that were upregulated after TP expression at the HC stage rather than BC stage. To further examine the TP-induced Hox candidates in a hematopoietic progenitor cell line, an FDCP-Mix-TP$^+$ model was used [57,58]. Constitutive expression of TP in the FDCP-Mix cell line (day 0) resulted in differentiation with an associated upregulation of *Hoxa6*, *a7*, *a9*, *a10*, *b2*, *b3*, *b4,* and *Meis1* compared with EV controls. As expected the Hox expression was reduced to barely detectable levels after differentiation of the FDCP-Mix cells, validating this model. Together, these findings demonstrate that oncogene-specific disruption of genetic networks may govern the leukemic cell phenotype. Expression of a core subset of *Hox* genes (*a7*, *a9*, *b2*, *b3*, and *b4*) is enhanced, along with *Meis1* and *Cdx4*, in the presence of TP throughout hematopoietic

differentiation and may represent part of the leukemia stem cell signature along with *Nanog*, *Oct4*, *c-kit,* and *Flk-1* [24].

The temporal and spatial expression of *Hox* genes is tightly regulated at both the genetic and epigenetic level. This study demonstrates for the first time transcriptional flux through the *Hox* network during normal hematopoietic and TP-induced leukemic differentiation of ES cells. Quantitative complete *Hox* network profiling confirms and extends previous published reports and identifies a novel subset of genes with potential roles in establishing early hematopoietic commitment. Due to the high degree of redundancy built into the network, loss-of-function studies involving individual *Hox* genes and clusters have provided limited information on the importance of this family in hematopoiesis. This study highlights that this is probably due to changes in several *Hox* genes influencing hematopoietic regulation. Overall, this study has identified how the *Hox* gene expression pattern alters during key stages of hematopoietic differentiation and establishes *Hox* signatures that change in response to an active tyrosine kinase involved in an MPN. Upregulation of *Cdx4*, *Meis1*, *Hoxa7*, *a9 b2*, *b3,* and *b4* are an emerging theme in myeloid malignancies and warrant further investigation as potential targets for future therapies or intervention.

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Author Disclosure Statement

The authors have no conflicts of interest to declare.

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