MicroRNA-124 Targets *Tip110* Expression and Regulates Hematopoiesis

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MicroRNA (miR) regulates hematopoiesis through targeting different genes post-transcriptionally. We have recently shown that *Tip110* expression is downregulated during hematopoietic stem cell differentiation. However, the underlying mechanisms are not known. In this study, we identified a conserved miR-124-binding site on the *Tip110* 3'-untranslated region (3'-UTR) and showed that *Tip110* was downregulated by miR-124 through its 3'-UTR. We then examined the relationship among miR-124 and *Tip110* expression and differentiation of human cord blood CD34⁺ cells. We found that miR-124 was expressed in a low level in human cord blood CD34⁺ cells, but it was considerably upregulated during culturing and differentiation of these cells. Moreover, we demonstrated that miR-124 expression decreased *Tip110* expression and promoted differentiation of human cord blood CD34⁺ cells, while miR-124 knockdown increased *Tip110* expression, slowed down differentiation of human cord blood CD34⁺ cells, and caused an expansion of hematopoietic progenitor cells in vitro. Finally, we used mouse embryonic fibroblasts derived from *Tip110* transgenic mice, performed the exon array analysis, and found that *Tip110* altered a number of genes in the hematopoiesis pathways. Dnmt3a as de novo methyltransferase was also significantly upregulated. That miR-124 was markedly upregulated during human cord blood CD34⁺ cell differentiation could be the result of direct loss of its promoter methylation from Dnmt3a. Taken together, our study demonstrates that miR-124 regulates *Tip110* expression and differentiation of human cord blood CD34⁺ cells and suggests important roles of miR-124/*Tip110* in hematopoiesis.

Introduction

H EMATOPOIESIS IS TIGHTLY regulated by complex multidimensional mechanisms, including those mediated by transcription factors, microRNAs (miRNAs), and epigenetic modifiers [1]. miRNAs are naturally occurring, small non-coding RNA molecules, usually 21–25 nucleotides long, present in a wide variety of organisms, highly conserved during evolution, and regulate gene expression by targeting the 3'-untranslated regions (3'-UTR) of the mRNAs [2]. miRNAs are mostly potent negative regulators of gene expression leading to gene silencing. A few hundreds of miRNAs have been identified in the human genome. In the hematopoietic system, miRNAs are required and are essential for functional hematopoiesis and regulate hematopoietic stem cells (HSCs) and lineage-committed hematopoietic progenitor cells (HPCs) [3,4]. Deregulated expression of certain miRNAs in the hematopoietic system is linked to hematologic malignancies.

miR-124 was originally identified as one of the most abundantly expressed miRNAs in the central nervous system. It is highly conserved among diverse species. miR-124

is expressed during the terminal neuronal differentiation [5]. It is not expressed in neuronal stem cells and its expression begins during the transition from neuronal stem cells to neuronal progenitors [6]. miR-124 represses various types of cancer, such as breast cancer, gastric cancer, prostate cancer, colorectal cancer, glioma, and glioblastomas, through inhibiting cellular proliferation, invasion, and inducing apoptosis. Several target genes of miR-124 have been identified to suppress tumors, such as, Ets-1, ROCK1, SMC4, TGF- α , and EAE. In addition, miR-124 promotes microglia quiescence through its target gene, CCAAT/enhancer-binding protein- α (C/EBP- α) [7–11].

Human *Tip110* was first identified as an RNA-binding nuclear protein to regulate HIV-1 gene expression and replication [12]. Our recent studies reported that *Tip110* was expressed in HSCs and *Tip110* expression levels decreased when HSCs differentiated [13]. *Tip110* also regulates proliferation of HPCs [13]. Ectopic expression of *Tip110* in quiescent CD34⁺ cord blood cells decreased apoptosis and enhanced numbers of cells entering into the cell cycle [13,14]. In the present study, we focused on the relationship among miR-124 and *Tip110* expression and CD34⁺ cell

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differentiation. Using several molecular and cell biology strategies, we demonstrated that miR-124 targeted *Tip110* expression and regulated CD34⁺ cell differentiation.

Materials and Methods

Cells

293T were purchased from the American Tissue Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY) with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) at 37°C and 5% CO₂. Transfections were carried out using the standard calcium phosphate precipitation. Human cord blood was collected according to the institutional guidelines of Indiana University School of Medicine, Indianapolis, Indiana, and used to obtain CD34⁺ cells within 24 h of collection using an immunomagnetic selection (Miltenyi Biotech, San Diego, CA). CD34⁺ cells (>93% pure) were cultured in Iscove's modified Dulbecco's media (IMDM; Life Technologies) containing 10% FBS (Hyclone) and cytokines, human stem cell factor (100 ng/mL), human FLT3 ligand (100 ng/mL), and human thrombopoietin (100 ng/mL). All these three cytokines were from R&D Systems (Minneapolis, MN).

Plasmids and luciferase reporter gene assay

Human *Tip110* 3'-UTR-driven luciferase reporter plasmid pLightSwitch-*Tip110* 3'UTR was purchased from Switchgear Genomics (Carlsbad, CA), which includes the entire *Tip110* 3'-UTR (1,346 nt). Lentiviral vector expressing miR-124 (HmiR0427-MR03) and miR-124 inhibitor (HmiR-AN0074-AM03) and their paired respective controls were from GeneCopoeia, Rockville, MD. miR-124 mimic and its paired control were from Sigma, St. Louis, MO. For the luciferase reporter gene assay, 293T were transfected with indicated plasmids as well as pTK-βGal, which was used to normalize the transfection variations among all transfections. Forty-eight hours post-transfection, the cells were washed, lysed, and assayed for the luciferase activity using a luciferase assay kit (Promega, Madison, WI).

Western blot analysis

Western blot analysis of protein expression was performed as previously described [12]. Primary antibodies were mouse anti-human *Tip110* antibody [12] and mouse anti-human β-actin antibody (Sigma Chem. Co., St. Louis, MO). β-Actin was included as a loading control.

Real time polymerase chain reaction

Total RNA was isolated from cells using TRIzol (Invitrogen, Grand Island, NY). Before RNA precipitation, RNA was extracted with acid phenol:chloroform:isoalcohol (125:24:1), pH 4.5, to prevent residual genomic DNA in the RNA preparations from amplification. cDNAs were synthesized using a ScriptII RT reagent kit (Promega) and oligo d(T)(18) as the primer. *Tip110* sequences were amplified by using primers, 5'-CTT CAT CCA GGC CAC TGA TT-3' and 5'-TTG AAA CGC TCT TCC ACC TC-3'. β-Actin was used as a reference for real time PCR and amplified using primers, 5'-AAC ACC CCA GCC ATG TAC GT-3' and 5'-AGT ACT TGC GCT CAG GAG GA-3'.

Flow cytometric analysis of human cord blood CD34⁺ cells

Freshly purified and cultured human cord blood CD34⁺ cells from the different experimental groups were washed twice with PBS and stained for mouse anti-human CD34-PE/CD34-APC/CD34-FITC antibody, mouse anti-human CD38-PE antibody, mouse anti-human CD45RA-PECF594, mouse anti-human CD90-PECy7, and mouse anti-human CD49f-PerCPCy5.5 in the dark at room temperature for 30 min. All those antibodies were from BD Biosciences (San Jose, CA). Mouse IgG-FITC, mouse IgG-APC, and mouse IgG-PE were included as negative controls. The cells were subjected to flow cytometric analysis using FACS (Becton Dickinson, Franklin Lakes, NJ).

miR-124 analysis

Total RNA was isolated from human cord blood CD34⁺ cells using TRIzol (Invitrogen) according to the manufacturer's protocols and used (25 ng) for reverse transcription, followed by amplification using the TaqMan RNA-to-cDNA 1-step Kit (Applied Biosystems, Foster City, CA). Specific miR-124 primers and miRNA TaqMan probes were designed by and obtained from Applied Biosystems. Reverse transcription was performed at 48°C for 15 min. PCR was performed for 40 cycles at 95°C for 15 s and 60°C for 60 s. miR-124 levels were calculated by the comparative ΔΔCt method and normalized to endogenous small nuclear U6 RNA.

Lentiviral preparation and transduction

293T were transfected with lentiviral plasmids expressing miR-124, miR-124 inhibitor, or their respective controls, along with pMD-gag-pol, pRSV-Rev, and pMDL-RRE (GeneCopoeia), using the calcium phosphate transfection. The cell culture supernatants were collected 48 h post-transfection, filtered through a 0.45-μm low-protein-binding filter (Millipore, Darmstadt, Germany), and used immediately for transduction, or stored in liquid nitrogen as lentivirus stock for later transduction. Human cord blood CD34⁺ cells were transduced with lentiviruses as previously described [15]. Briefly, freshly purified human cord blood CD34⁺ cells were cultured as described above for 24 h. The cells were then incubated with lentiviruses in the presence of 8 μg/mL polybrene for 8 h. After the end of incubation, the cells were washed extensively and cultured in IMDM medium. The transduction efficiency was estimated to be 20% [15].

In vitro hematopoietic colony-forming assay

After culturing for 4 days, lentivirus-transduced human cord blood CD34⁺ cells were seeded in triplicate onto a 35-mm dish at a density of 500 cells per dish in 1 mL 1% methylcellulose culture medium containing 100 mM 2-mercaptoethanol, 2 mM L-glutamine, and 30% FBS (Hyclone). Recombinant human erythropoietin (1 U/mL), stem cell factor (SCF) (50 ng/mL), human interleukin-3 (IL-3; 10 ng/mL), and granulocyte-macrophage colony-stimulating factor (GM-CSF; 10 ng/mL) (R&D System) were included in the culture medium containing EPO (Amgen, Inc., Thousand Oaks, CA) to stimulate colonies from multipotential [CFU-granulocyte-erythrocyte-macrophage-megakaryocyte (CFU-GEMM)],

erythroid [burst-forming unit-erythroid (BFU-E)], and granulocyte-macrophage [CFU-GM] progenitor cells. Colonies were scored after 14 days of incubation at 5% CO₂ and lowered O₂ (5%) under an inverted microscope.

Preparation of mouse embryonic fibroblast and exon microarray

Pregnant *Tip110* transgenic (TG) and wild-type littermate (WT) control mice [13] were sacrificed at day 13. Each embryo was dissected from the placenta and embryonic sac. After finely mincing the tissue and treating it with trypsin, cells were washed with mouse embryonic fibroblast (MEF) culture medium (Life Technologies) and suspended in MEF medium. Total RNA was isolated using TRIzol (Invitrogen) and used for Affymetrix Exon Microarray (Affymetrix, Santa Clara, CA). Briefly, total RNA was converted into sense-strand cDNA using an Ambion WT Expression Kit (Invitrogen). The cDNA was then in vitro transcribed to create a complement RNA (cRNA) template. cRNA was combined with random primers and synthesized to produce cDNA. The cDNA was then fragmented and biotin labeled using an Affymetrix GeneChip® WT Terminal Labeling Kit (Affymetrix). The labeled cDNA was suspended in the hybridization cocktail and loaded onto GeneChip Mouse Exon 1.0 ST array (Affymetrix) for hybridization. After staining with streptavidin, signal amplification was assessed using biotinylated antistreptavidin. The images were analyzed with corresponding quality control metrics. Then, gene ex-

pression analysis was performed to identify genes of interest as follows: The first-pass analysis was performed using the Analysis Wizard of DAVID Bioinformatics Resources 6.7 from NIAID/NIH (<http://david.abcc.ncifcrf.gov/>). Normalized and filtered data were then entered into hematopoiesis pathway analysis. Genes identified in the pathway analysis were selected for analysis in KEGG pathways.

Statistical analysis

The data are mean $\pm$ SD and representative of multiple independent experiments. All experiment data were analyzed by two-tailed student's *t*-test. A *P* value <0.05 was considered to be statistically significant, and a *P* value <0.005 was considered to be highly statistically significant.

Results

miR-124 targets *Tip110* 3'-UTR and downregulates *Tip110* expression

To determine the molecular mechanism for regulation of *Tip110* expression during HSC differentiation, we focused on the 3'-UTR of the *Tip110* gene. Using the TargetScan analysis (www.targetscan.org), we found that the 3'-UTR of human *Tip110* mRNA contained a complementary site for the seed region of miR-124 (Fig. 1A). The aligned region was indeed conserved among *Tip110* of various species, such as mouse, chimpanzee, rhesus, bushbaby, rat,

FIG. 1. Regulation of *Tip110* expression by miR-124 through its 3'-UTR. (A) Sequence alignment between human miR-224 seed sequence and 3'-UTR of *Tip110* mRNA from different species, including mouse, chimpanzee, rhesus, bushbaby, rat, and guinea pig. (B, C) 293T were transfected with miR-124 mimic (miR-124_m) or its control (Ctrl_m). Forty hours after transfection, cells were harvested for cell lysates and western blotting (B) or RNA isolation and real time polymerase chain reaction (qRT-PCR) (C). β-Actin was included as the loading control for western blotting (B) and as a reference for qRT-PCR (C). (D) Scheme of human *Tip110* 3'-UTR-containing luciferase reporter plasmid pLightSwitch-*Tip110*.3'UTR. (E, F) 293T were transfected with miR-124_m and pLightSwitch-*Tip110*.3'UTR (E) or miR-124-binding site-deleted pSwitchgear-*Tip110*.3'UTR (F). Forty-eight hours after transfection, cells were harvested for cell lysates and luciferase reporter gene assay. pTK-βGal was included for transfection efficiency normalization (E, F). The data in (D, E) are mean $\pm$ SEM and representative of three independent experiments. 3'-UTR, 3'-untranslated region.

and guinea pig. To determine whether *Tip110* mRNA expression is regulated by miR-124, 293T were transfected with miR-124 mimics, which are chemically synthesized double-stranded RNAs that mimic mature endogenous miRNAs in transfected cells. Compared with the miRNA mimic control, the miR-124 mimic expression considerably decreased *Tip110* protein expression (Fig. 1B) as well as *Tip110* mRNA expression (Fig. 1C). To determine whether *Tip110* 3'-UTR is involved in miR-124-induced *Tip110* mRNA and protein downregulation, we constructed a reporter plasmid by inserting the *Tip110* mRNA 3'-UTR immediately next to the 3' end of the luciferase gene (Fig. 1D). Similarly, coexpression of the reporter plasmid and miR-124 mimic led to significant decreases in the *Tip110* 3'-UTR-mediated luciferase activity (Fig. 1E). To further ascertain the specificity of the miR-124 interaction with *Tip110* 3'-UTR in *Tip110* mRNA and protein downregulation, another reporter plasmid containing the deletion of the miR-124-binding site from the *Tip110* 3'-UTR were transfected with miR-124 mimics. Compared with the control, coexpression of the mutated reporter plasmid and miR-124 mimic showed no significant changes in the luciferase activity (Fig. 1F). Taken together, these results indicate that *Tip110* is likely a target of miR-124 through its 3'-UTR.

miR-124 expression is inversely correlated with CD34⁺ cell differentiation

Several studies have demonstrated miRNA expression changes during hematopoiesis and hematopoietic differentiation of CD34⁺ cells [1,16,17]. However, it is not known whether miR-124 expression changes during hematopoietic

differentiation of CD34⁺ cells. To investigate the expression kinetics of miR-124 in relation to CD34⁺ differentiation, we isolated fresh human cord blood CD34⁺ cells and cultured them in the medium containing 10% FBS and cytokines, SCF (100 ng/mL), FLT3 ligand (100 ng/mL), and thrombopoietin (100 ng/mL). Freshly isolated cells (day 0) and the cells that were cultured for various days were collected for immunostaining and flow cytometry or RNA isolation for qRT-PCR. CD34⁺ cells started to differentiate, as we reported previously [13], and as the number of CD34⁺ cells decreased (Fig. 2A), so did the number of CD34⁺CD38⁻ cells (Fig. 2B), a marker for more immature cells compared with CD34⁺ cells, and the number of CD34⁺CD38⁻CD45RA⁻CD90⁺CD49f⁺ cells (Fig. 2C), a marker for purified definitive human HSCs [18]. In addition, as we demonstrated previously [13], *Tip110* mRNA expression was significantly induced at the beginning of the differentiation, followed by a gradual decrease over time (Fig. 2D). In contrast to *Tip110* mRNA expression, miR-124 was detected at a very low level in freshly isolated CD34⁺ cells (day 0) and gradually increased over CD34⁺ cell differentiation (Fig. 2E). The correlation coefficient (*r*) between the number of CD34⁺ cells and miR-124 expression, between the number of CD34⁺CD38⁻ cells and miR-124 expression, and the number of CD34⁺CD38⁻CD45RA⁻CD90⁺CD49f⁺ cells and miR-124 expression was -0.989, -0.899, and -0.915, respectively. These results suggest that miR-124 expression is inversely correlated with CD34⁺ cell differentiation.

miR-124 expression alters CD34⁺ cell differentiation

Next, we sought to determine whether manipulation of miR-124 expression would lead to changes in CD34⁺ cell

FIG. 2. Effects of CB CD34⁺ differentiation on miR-124 and *Tip110* expression. Human cord blood CD34⁺ cells were isolated and cultured in the presence of 10% fetal bovine serum and cytokines, 100 ng/mL SCF, 100 ng/mL FLT3 ligand, and 100 ng/mL thrombopoietin, for indicated days. The cells were collected. Separate aliquots were used for staining and flow cytometry analysis for CD34⁺ (A), CD34⁺CD38⁻ (B), and CD34⁺CD38⁻CD45RA⁻CD90⁺CD49f⁺ (C) or RNA isolation and qRT-PCR for *Tip110* mRNA level (D, E) and miR-124 level (F, G). β -Actin was included as a reference for *Tip110* qRT-PCR (D), and U6 was included as a reference for miR-124 qRT-PCR (E). y-Axis in (A–C) represented the number of cells of the 1 million cells counted. The data are mean $\pm$ SEM and representative of three independent experiments.

FIG. 3. Effects of miR-124 overexpression and knockdown on CD34⁺ cells. Human cord blood CD34⁺ cells were isolated, cultured for 1 day, and transduced with lentiviruses expressing miR-124 (A–D) or expressing miR-124 inhibitor (miR-124_i, E–H). Lentiviruses expressing the control miRNA (Ctrl) and control miRNA inhibitor (Ctrl_i) were included as the controls. After culturing for 4 days, the cells were collected. Separate aliquots were used for RNA isolation and qRT-PCR for the miR-124 level (A, E) and *Tip110* mRNA level (B, F) or for staining and flow cytometry analysis for CD34⁺ (C, G) or CD34⁺CD38⁻CD45RA⁻CD90⁺CD49f⁺ (D, H). U6 was included as a reference for miR-124 qRT-PCR (A, E), while β -actin was included as a reference for *Tip110* qRT-PCR (B, F). y-axis in (C–H) represented the number of cells of the 1 million cells counted. The data are mean $\pm$ SEM and representative of three independent experiments.

differentiation. First, we overexpressed miR-124 in human cord blood CD34⁺ cells through lentivirus transduction, cultured the lentivirus-transduced cells for 4 days, and determined CD34⁺ cell differentiation. MiR-124 expression in the transduced human cord blood CD34⁺ cells was confirmed using qRT-PCR (Fig. 3A). As shown before (Fig. 1), miR-124 expression decreased *Tip110* mRNA (Fig. 3B). Compared with the transduction control, miR-124 expression significantly decreased the number of CD34⁺ cells (Fig. 3C) and the number of CD34⁺CD38⁻CD45RA⁻CD90⁺CD49f⁺ cells (Fig. 3D) 4 days post-transduction. Then, we also expressed miR-124 inhibitor in human cord blood CD34⁺ cells and monitored CD34⁺ cell differentiation. As expected, miR-124 inhibitor expression decreased miR-124 in human cord blood CD34⁺ cells (Fig. 3E) and increased the *Tip110* mRNA level (Fig. 3F). In contrast to miR-124 overexpression, miR-124 inhibitor expression significantly increased the number of CD34⁺ cells (Fig. 3G) and the number of CD34⁺CD38⁻CD45RA⁻CD90⁺CD49f⁺ cells (Fig. 3H) 4 days post-transduction.

To further assess whether miR-124 affects functionally assayable HPCs, we transduced human cord blood CD34⁺ cells with lentiviruses expressing miR-124 inhibitor and performed the hematopoietic colony-forming assay. Compared with the control, miR-124 inhibitor expression in human cord blood CD34⁺ cells increased the number of granulocyte-macrophage (CFU-GM), erythroid (BFU-E), and multipotential (CFU-GEMM) progenitors (Fig. 4). Taken together, these results together suggest that miR-124 expression is a critical regulator of CD34⁺ cell differentiation and production of the different subsets of HPCs.

Link between *Tip110* and hematopoiesis

To explore the possible mechanisms that likely account for the roles of *Tip110* in hematopoiesis, we performed a genome-wide expression analysis in the context of *Tip110*

FIG. 4. miR-124 knockdown increased hematopoietic colony formation of human CB CD34⁺ cells. Human cord blood CD34⁺ cells were isolated, cultured for 1 day, and transduced with lentiviruses expressing miR-124 inhibitor (miR-124_i). Lentiviruses expressing the control miRNA inhibitor (Ctrl_i) were included as the control. After culturing for 4 days, the cells were seeded in methylcellulose medium at a density of 500 cells per mL for hematopoietic colony formation assay. The data are the ratio of the number of colonies to the number of input cells (ie, 500) and expressed as mean $\pm$ SEM and representative of three independent experiments.

FIG. 5. Regulatory pathways of hematopoiesis associated with *Tip110* expression. Mouse embryonic fibroblasts (MEFs) isolated from *Tip110* transgenic mice and congenic wild-type mice were used to isolate total RNA for the Affymetrix GeneChip® Mouse Exon 1.0 array. Genes with greater than twofold changes of expression between the *Tip110* MEF and wild-type MEF were analyzed by the KEGG pathway analysis program. A number of genes that were affected by *Tip110* expression, marked by a red star, were shown to be involved in hematopoiesis pathways.

TABLE 1. LISTED GENES AND PRIMERS USED IN THE VALIDATION STUDY

Gene	GeneBank no.	Region	Forward primer	Reverse primer	Expected size
<i>CD33</i>	NM_001111058.1	ORF:2321–2446	CTAGCAATCTGTCTCTGTCTCATC	TCTCTCTCTCTCTCTCTCTCTCT	104
<i>CD34</i>	NM_133654.3	ORF:1493–1594	GTCAAGTTGTGGTGGGAAGA	AGAGGCGAGAGAGGAGAAAT	101
<i>IL-1</i>	NM_001146088.1	ORF: 684–782	CCATAAGCAGAGGCAGAGTAG	AGTAGGGAAGGAGAGGAGTAAG	98
<i>IL-6</i>	J03783.1	ORF:55–189	CTTCCATCCAGTTGCCTTCT	CTCCGACTTGTGAAGTGGTATAG	141
<i>IL-7</i>	AC125373.4	ORP: 55–189	GCAGGAGACTAGGACCCTATAA	CAATCCTAGCCTGCCTTAGATAC	134
<i>IL-11</i>	NM_001290423.1	ORF: 819–912	GGGATCACCTGTGGCTTATT	GATCTCAGTTCCTGCTCTTC	93
<i>SCF</i>	M59915.1	ORF: 252–354	CAGCTTGACTACTCTTCTGGAC	AACACGAGGTCATCCACTATT	102
<i>Flt3L</i>	NM_013520.3	ORF:811–935	ACTCAGCCAGGTCTTATCT	ATTACAGTGTCCATCGCCATAC	124
<i>Dnmt3a</i>	NM_007872.	ORF:778–880	TCCATGAAAATGGAGGGCTC	TTGCTGATGTAGTAGGGGTC	121

overexpression using the Affymetrix exon array. We took advantage of the *Tip110* transgenic mice [13,19]. Due to the scarcity of mouse HPCs, we used MEFs from *Tip110* transgenic mice and cognate wild-type littermates and isolated total RNA for the genome-wide expression analysis. The difference of gene expression between *Tip110* MEF and wild-type MEF was calculated as folds of changes using the GeneSpring software platform. The subsequent KEGG pathway analysis revealed a number of genes in the cellular pathways that were affected, including the hematopoiesis pathway (Fig. 5) that exhibited at least 2.0-fold changes by increased *Tip110* expression (Table 2). We next selected eight genes of hematopoietic importance and performed qRT-PCR validation assay (Table 1). Among those were five up-regulated genes, *CD34*, *IL-6*, *IL-11*, *SCF*, and *Flt3L*, and three downregulated genes, *CD33*, *IL-1*, and *IL-7*, in the *Tip110* MEF (Fig. 6). All genes, except *IL-11*, showed consistent up- or downregulation in the *Tip110* MEF between the array and the qRT-PCR, giving rise to about 87.5% validation rate.

To investigate how miR-124 is induced in HSCs during the differentiation process, we searched the potential factors regulating miR-124 expression from *Tip110*-regulated genes in the splicing array. miR-124 was reported to be methylated on CpG islands on its promoter region by *Dnmt3a* [20]. We identified *Dnmt3a* to be upregulated by *Tip110* expression

through the array, which was further confirmed by qRT-PCR (Fig. 6). Loss of *Dnmt3a* expression might be caused by downregulation by *Tip110* during HSC differentiation so as to result in less methylation on miR-124. This result suggested that miR-124 induction during HSC differentiation could be caused by alleviation of miR-124 methylation through the loss of *Dnmt3a* expression from *Tip110* down-regulation.

Discussion

miRNAs are essential for regulating hematopoiesis, including controlling self-renewal and differentiation [21]. Therefore, studies on the miRNA expression as well as their integrative analysis of affected genes are extremely important. The current study provides new insights into the role of miRNA in the function of normal human cord blood HSC self-renewal and differentiation. In this study, we have demonstrated that miR-124 directly suppresses expression of *Tip110* (Fig. 1), which is an important factor for cord blood HSC self-renewal. miR-124 expression level increases during CB HSC differentiation (Fig. 2). Manipulation of the miR-124 expression level altered cord blood HSC self-renewal and differentiation (Fig. 3) and production of HPCs (Fig. 4). Furthermore, *Tip110* expression leads to expression changes of a number of genes involved in hematopoiesis. Taken together, these results show for the first time that miR-124 is an important regulator of hematopoiesis and support further insights about the roles of *Tip110* in hematopoiesis.

miR-124 has been viewed as a potential master regulator of differentiation of various types of cells in the central nervous system [22]. Overexpression of miRNA-124 in mouse embryonic stem cells induced differentiation of these cells [23]. miRNA-124 expression is repressed in various cancer cells, such as glioblastoma, gastric cancer, ovarian cancer, and bladder cancer [7–11]. In this study, we showed for the first time that miR-124 was markedly upregulated during human cord blood CD34⁺ cell differentiation. The similarity between normal stem cells and tumorigenic cancer cells is their proliferation capability [24]. The common function of miRNA-124 on stem cell differentiation, CNS development and adult neurogenesis, and cancer cell growth inhibition is linked to its ability to suppress cell proliferation toward the quiescent stage and induce cell differentiation.

In agreement with these findings, we showed in this study that miRNA-124 promoted human cord blood CD34⁺ cell

TABLE 2. INVOLVEMENT OF *TIP110*-REGULATED GENES IN THE CELLULAR PATHWAY IDENTIFIED BY DAVID (THE DATABASE FOR ANNOTATION, VISUALIZATION, AND INTEGRATED DISCOVERY, VERSION 6.7) IN KEGG ANALYSIS (THE KYOTO ENCYCLOPEDIA OF GENES AND GENOMES)

Term	Gene count	P value
Chemokine signaling pathway	25	8.1E-6
Hematopoietic cell lineage	29	2.1E-5
Cytokine–cytokine receptor interaction	15	5.1E-5
Complement and coagulation cascades	13	2.6E-4
Systemic lupus erythematosus	15	4.8E-4
Natural killer cell-mediated cytotoxicity	16	8.9E-4
Toll-like receptor signaling pathway	14	1.1E-3
Cytosolic DNA-sensing pathway	9	5.1E-3
Cell adhesion molecules	16	8.6E-3
Axon guidance	14	1.2E-2
Glutathione metabolism	8	1.3E-2
Leukocyte transendothelial migration	13	1.4E-2

FIG. 6. qRT-PCR validation of selected genes that were altered by *Tip110* expression. Total RNA was prepared from *Tip110* transgenic and wild-type MEFs. qRT-PCR was performed using the gene-specific primers. The fold changes between *Tip110* transgenic and wild-type MEFs compared with those obtained by the above-mentioned exon array. β -Actin was included as a reference for qRT-PCR. The data are mean $\pm$ SEM and representative of three independent experiments.

differentiation. miR-124 effects on CD34⁺ cell differentiation may likely also involve the targets of miR-124, such as CDK4, CDK6, and cyclin D2 [25–28]. For example, miR-124 suppresses CDK6 expression and as a result, causes cell cycle arrest at the G1 phase and suppression of cell proliferation [29]. CDK6 itself has also been shown to regulate HSCs [30] and play a central role in hematopoietic malignancies [31]. Our previous findings that *Tip110* is an important regulator of human cord blood hematopoiesis [13], along with our current findings that *Tip110* is a novel target of miRNA-124, suggest that downregulation of *Tip110* expression during human cord blood CD34⁺ cell differentiation is likely a result of the interaction between increased miR-124 expression during human cord blood CD34⁺ cell differentiation and *Tip110* 3'-UTR. In addition, miR-124 expression could be transcriptionally inactivated by CpG island hypermethylation in human tumors [25,28]. Therefore, it would be interesting to determine whether it is possible to manipulate human cord blood stem cell hematopoiesis through miR-124 expression in vivo.

Microarrays are used to determine genome-wide gene expressions and to define gene expression regulatory networks. To identify how *Tip110* affects the hematopoiesis, we compared gene expression between the *Tip110* transgenic MEF and its cognate wild-type MEF and identified the possible gene targets of *Tip110* in hematopoiesis pathways. The results showed that *Tip110* regulates a number of genes in the hematopoiesis pathway. Among them are CD34, SCF, and Flt3L upregulated by *Tip110*. SCF and Flt3L are potent costimulating cytokines for HSCs and HPCs [32]. On the other hand, CD33, downregulated by *Tip110*, is a myeloid differentiation antigen [33,34].

Dnmt3a acts as de novo methyltransferase for modification of unmethylated DNA [35]. During hematopoiesis, Dnmt3a expression was reported to be much higher in primitive long-term HSCs than differentiated cells [36]. Loss of Dnmt3a enables HSC differentiation. Microarray data between the *Tip110* transgenic MEF and its cognate wild-type MEF identified Dnmt3a to be significantly upregulated. Oncoprotein EVI1 forms a complex with Dnmt3a for a specific target on the miR-124 regulatory region on its promoter [20]. That miR-124 was markedly upregulated during human cord blood CD34⁺ cell differentiation could be the result of direct loss of its promoter methylation from Dnmt3a.

In conclusion, the data provide additional evidence to support the notion that *Tip110* participates in the regulation of hematopoiesis and demonstrate for the first time a dynamic requirement of miR-124 for hematopoietic differentiation through downregulation of *Tip110* expression.

Author Disclosure Statement

No competing financial interests exist.

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