RNAi Screen Identifies MTA1 as an Epigenetic Modifier of Differentiation Commitment in Human HSPCs

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The molecular mechanisms regulating key fate decisions of hematopoietic stem cells (HSCs) remain incompletely understood. Here, we targeted global shRNA libraries to primary human hematopoietic stem and progenitor cells (HSPCs) to screen for modifiers of self-renewal and differentiation, and identified metastasis-associated 1 (MTA1) as a negative regulator of human HSPC propagation in vitro. Knockdown of MTA1 by independent shRNAs in primary human cord blood (CB) HSPCs led to a cell expansion during culture and a relative accumulation of immature CD34⁺CD90⁺ cells with perturbed in vitro differentiation potential. Transplantation experiments in immunodeficient mice revealed a significant reduction in human chimerism in both blood and bone marrow from HSPCs with knockdown of MTA1, possibly caused by reduced maturation of blood cells. We further found that MTA1 associates with the nucleosome remodeling deacetylase (NuRD) complex in human HSPCs, and on knockdown of MTA1, we observed an increase in H3K27Ac marks coupled with a downregulation of genes linked to differentiation toward the erythroid lineage. Together, our findings identify MTA1 as a novel regulator of human HSPCs in vitro and in vivo with critical functions for differentiation commitment. © 2022 ISEH – Society for Hematology and Stem Cells. Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/)

HIGHLIGHTS

- RNAi screen targeting 11,000 genes identified regulators of human HSPCs.
- MTA1 was identified as a negative regulator of HSPC propagation in vitro.
- Knockdown of MTA1 preserves the immature phenotype of human HSPCs in vitro and restricts their engraftment capacity in vivo.
- MTA1 is associated with the NuRD complex in HSPCs and mediates H3K27 deacetylation.

Hematopoietic stem cells (HSCs) are characterized by their capacity to self-renew and differentiate into all blood cell types. HSCs can restore the entire blood system and are used as a cornerstone therapy in life-saving transplantation procedures for leukemias and lymphomas, as well as inherited diseases of the hematopoietic system [1,2]. The human hematopoietic system is maintained by a relatively small number of HSCs where the balance between self-renewal and differentiation is tightly regulated to preserve the stem cell pool [3]. Recent studies have highlighted the importance of the epigenetic landscape and the chromatin state of HSCs in controlling cell identity, lineage priming, and fate decisions [4,5]. Yet, the more precise details of the molecular programs governing the first critical commitment steps of HSCs have remained incompletely defined. We have previously reported the feasibility of using RNAi screens to identify regulators of renewal and differentiation in primary human HSCs [6]. Using screening paradigms based on pooled lentiviral shRNA libraries targeted to cord blood (CB)-derived hematopoietic stem and progenitor cells (HSPCs), we have successfully identified genes with key roles in both normal and malignant hematopoiesis, including MAPK14 [7], JARID2 [8], and members of the cohesin complex [6]. In this study, we further built on this approach by employing next-generation short hairpin (sh)RNA libraries from The Broad Institute RNAi Consortium (TRC). Using selection assays for enhanced maintenance of the HSPC phenotype in vitro, we identified metastasis-associated 1 (MTA1) as the top-scoring candidate gene in the screen and a potent modifier of human HSPC differentiation. Knockdown (KD) of MTA1 perturbed differentiation of HSPCs, leading to an accumulation of immature cells in vitro and a profound engraftment defect in xenograft transplantation assays. Moreover, we found that MTA1 associates with the nucleosome remodeling deacetylase (NuRD) complex in human HSPCs and regulates the deacetylation of histone H3 lysine 27 (H3K27).

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KZ and JL conceived the study, designed the experiments, analyzed the results, and wrote the article. KZ performed the experiments. KZ, AS, and RG performed transplantation experiments and analyzed the microarray data. KZ and AP wrote scripts for bioinformatics analysis and analyzed the shRNA screen data. MJ performed mass spectrometry (MS) experiments and analyzed the MS data together with JH.

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Methods

Human Material Resources

Human CB samples were obtained from maternity wards in Helsingborg General Hospital and Skåne University Hospital in Lund and Malmö, Sweden. Samples were collected following regulations set by the regional ethics committee, which includes a written consent. CB samples were processed within 24 hours by isolating mononuclear cells using the density-gradient method (Abbott, Chicago, IL USA). CD34+ cells were enriched using magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s protocol.

shRNA Screen and Bioinformatic Analysis

The shRNA screen was performed according to a previously published protocol [9], using TRC (The RNAi Consortium) shRNA lentivirus libraries TRC 1.5 and TRC 2.0 (Nos. SHPH15 and SHPH2, Merck/Sigma-Aldrich, St. Louis, MO). In short, 70 million CB-derived CD34+ cells collected from more than 100 umbilical cord units were divided through seven biological replicates. Cells were transduced with six shRNA library pools (from TRC1.5 and TRC 2.0 libraries), each consisting of around 7,000 shRNAs. With given library titers, we aimed at around 20%-30% transduction efficacy and coverage of the library between 200 and 300 CD34+ HSPCs per hairpin. As selection pressure, growth advantage under standard culture conditions was used to identify genes that affect self-renewal and differentiation in primary HSPCs. Therefore, cells were maintained in serum-free expansion medium (SFEM, Stem Cell Technologies, Vancouver, BC, Canada), supplemented with stem cell factor (SCF), thrombopoietin (TPO), and FLT3-ligand (FLT3L) (each at 100 ng/mL, Peprotech/Thermo Fisher Scientific, Waltham, MA). To evaluate the initial library distribution, cell culture samples were collected 3 days after transduction. Then, other samples were collected at week 3 followed by purification of CD34+ cells and gDNA extraction. Sample collection and processing for next-generation sequencing (NGS) analysis was done according to a previously published protocol [9].

Reads from NGS were annotated to the shRNA library. The median counts for each technical replicate for a given pool were calculated. Next, by following the edgeR guidelines, after converting the raw counts to counts per million by using the cpm function in edgeR, we retained only those hairpins that are represented as at least 1-cpm reads in at least five replicates [10]. The data then were normalized by the trimmed mean of M-values (TMM) method. After estimating the dispersion parameters, the glmFit and glmLRT functions were used to identify subsets of differentially represented hairpins between day 3 and week 3. The described analysis was performed for each pool independently, and the results were pooled. The hairpins were ordered by their respective log2 fold change median estimates. To visualize the shRNA ranking, the log2 fold change values of the raw counts were used.

For gene ranking, the log2 fold change values of the hairpins for each given gene were aggregated by taking their median value, along with a statistical test to decide if the majority of the log2 fold change values were larger than zero for that gene, and reporting the two metrics together. A one-sided Fisher’s exact test was performed on the hairpins of the same gene to decide if their log2 fold change value was greater than zero. p Values and false discovery rates (FDRs) were reported along with the median log2 fold change values.

Cell Culture

CB-Derived human CD34+ cells were cultured in tissue-treated plates. Cells were maintained in SFEM supplemented with SCF, TPO, and FLT3L at 100 ng/mL (Miltenyi Biotec, Bergisch Gladbach, Germany). Penicillin (100 U/mL) and streptomycin (100 μg/mL) were added to the cultures to prevent infections (Cytiva, Marlborough, MA USA).

Lentiviral Production of shRNA Lentiviruses and Transduction

pLKO1 vectors were used to clone a pLKO1_GFP backbone, where the puromycin-resistance gene was changed to Green Fluorescent Protein (GFP). MTA1 shRNA sequences, as well as Scrambled control (Scr), were cloned into pLKO1_GFP backbones. Scr is a nontargeting shRNA sequence, suggested by TRC. Lentiviruses were produced in the human 293T cell line according to a previously published protocol [11], where polyethylenimine (PEI, Polysciences, Warrington, PA USA) was used for transfection, and virus was concentrated using ultracentrifugation. Freshly thawed and sorted CD34+ cells were transduced at a multiplicity of infection (MOI) of 20 to obtain 30%−50% transduction efficiency. CD34+CD38−/CD90+CD45RA− sorted cells required a three times higher MOI to reach the same level of transduction efficiency as sorted CD34+ cells; thus, an MOI of 60 was used for these cells.

Cell Sorting

CB-Derived CD34+ cells were thawed at 37°C and slowly diluted by adding Dulbecco’s minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Fisher Scientific, Waltham, MA) and 100 μg/mL DNase I (Stem Cell Technologies) until diluted more than 10 times from the starting volume. The cells were incubated at room temperature for 10 min, washed, and resuspended in ice-cold phosphate-buffered saline (PBS) with 2% FBS and 2 mmol/L EDTA, then stained with antibodies and kept on ice until sorting. All cell sorting was performed on a BD Ariannu or BD Ariall (BD, Franklin Lakes, NJ USA). Cells were sorted into SFEM with cytokines used for cell cultures. Sorted cells were washed and plated to culture plates.

Transduced cells were sorted by washing and resuspending the cells in ice-cold PBS with 2% FBS and 2 mmol/L EDTA, stained with antibodies if needed, and then washing again and resuspending in ice-cold PBS with 2% FBS, 2 mmol/L EDTA, and 7-amo-actinomycin D (7AAD) for dead cell exclusion.

Flow Cytometry Analysis

Cells were washed and resuspended in ice-cold PBS with 2% FBS and 2 mmol/L EDTA with antibodies, stained at +4°C for 30 min, and then washed. Before analysis, cells were resuspended in ice-cold PBS with 2% FBS, 2 mmol/L EDTA, and 7AAD for dead cell exclusion. Flow cytometry analysis was performed on BD Fortessa or BD Fortessa x20 (BD, Franklin Lakes, NJ USA).

Quantitative Real-Time PCR

Transduced CD34+GFP+ cells were sorted 36 h after the transduction directly into RLT lysis buffer and stored on dry ice or at −80°C. RNA extraction was performed using an RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.
Superscript III (Fisher Scientific, Waltham, MA) was used for reverse transcription. TaqMan Gene expression master mix (Fisher Scientific) was used to prepare polymerase chain reactions (PCRs) according to the manufacturer’s protocol. Levels of gene expression and knockdown were evaluated with qPCR Taqman probes (Fisher Scientific) for MTA1 (Hs00950776_m1) and normalized to hypoxanthine–guanine phosphoribosyl transferase (HPRT, Hs02800695_m1) expression.

**Methylcellulose Colony Formation Assay**

Cells for colony-forming unit (CFU) assay were sorted at day 3 after transduction, unless indicated differently. CD34+GFP+ cells were sorted into methylcellulose (MethoCult H4230, Stem Cell Technology) with Iscove’s modified Dulbecco’s medium (IMDM, 4:1 Metho-Cult:IMDM), supplemented with 25 ng/mL SCF, 50 ng/mL granulocyte–macrophage colony-stimulating factor (GM-CSF), 25 ng/mL interleukin (IL-3) (Peprotech/Fisher Scientific), 5 U/mL erythropoietin (EPO, Apoteket, Stockholm, Sweden), and penicillin (100 U/mL)–streptomycin (100 μg/mL) (Cytiva). Three hundred cells per well were plated to six-well plates and incubated under standard conditions for 10–12 days, and then mature hematopoietic colonies were counted.

**Differentiation Assay**

CB-Derived CD34+ cells were transduced with lentiviruses. After 3 days the cells were washed and transferred to differentiation medium: Myelocult H5100 (Stem Cell Technologies) supplemented with 50 ng/mL SCF, 20 ng/mL GM-CSF, 10 ng/mL FLT3-L, 50 ng/mL TPO, 20 ng/mL IL-3 (Peprotech/Fisher Scientific), 5 U/mL EPO (Apopetek, Sweden), 100 U/mL penicillin, and 100 μg/mL streptomycin (Cytiva). Fluorescence-activated cell sorting (FACS) analysis was performed at days 4 and 7 after transfer to differentiation medium.

**Transplantation of Primary Human Cells and Animal Housing**

CB-Derived human CD34+ cells were sorted for CD34+ or CD34+CD90+CD45RA−CD38− cell surface markers. Six- to ten-month-old NOD Cg-Pkd<sup>emd</sup>L2rg<sup>mm1Wij</sup>/SzJ (NSG) mice were used for xenotransplantation of CD34+ cells. Transduced cells were sorted for GFP expression after 3 days and injected through the tail vein into sublethally irradiated (3 Gy) mice. Peripheral blood samples were collected from tail vein and analyzed at the indicated time points. Mice were euthanized after 16 weeks, and bone marrow and spleen samples were collected for analysis by flow cytometry. Mouse maintenance and experimental procedures followed the regional ethics committee guidelines and approval (No. 9636–18).

**Microarray Expression Profiling**

CB-derived CD34+CD38−CD90+CD45RA− cells were transduced and cultured for 60 hours. Nine thousand GFP+ cells/sample were sorted into RLT buffer (Qiagen). RNA was isolated using the RNeasy Micro Kit (Qiagen). The NuGEN Ovation FicoSL WTA System V2 Kit Protocol was used for the preparation of SPIA cDNA from RNA, followed by fragmentation and terminal biotin labeling according to the NuGEN Encore Biotin Module manual. Three micrograms of fragmented and labeled SPIA cDNA was hybridized to Affymetrix Human Genome U133 PM Array Plates. The Affymetrix GeneTitan system was used for hybridization, washing, and staining. GeneChips were scanned using an Affymetrix GeneTitan system controlled by the Affymetrix GeneChip Command Console software Version 3.3 (AGCC). Gene level-normalized expression signals were calculated from Affymetrix CEL files using the RMA algorithm[12] in the Affymetrix GeneChip Expression Console Version 1.3 software. For value definition, the log2 gene level RMA signal intensity was used.

**Western Blot**

GFP+CD34+ cells were sorted 3–5 days after transduction with shRNA lentiviruses. Cells were either collected for lysis immediately or cultured several days to obtain more cells and then collected for lysis. Collected cells were washed with ice-cold PBS twice and lysed on ice for 10 min in RIPA buffer (Fisher Scientific) supplemented with 1 × proteinase and phosphatase inhibitor cocktail (Fisher Scientific). The lysate was centrifuged for 15 min at +4°C at maximum speed, and supernatant was collected. Sample buffer (Laemmlli buffer, BioRad, Hercules, CA) supplemented with 5% 2-mercaptoethanol and 1 × proteinase and phosphatase inhibitor cocktail (Fisher Scientific) was added to the supernatant at a 1:1 ratio. Samples were boiled at 95°C for 2–5 min and then stored at −80°C or kept on ice until gel loading.

Proteins were separated using Bolt gels according to the manufacturer’s protocol (Fisher Scientific). The iBlot2 system was used to transfer the proteins to a polyvinyl difluoride (PVDF) membrane according to the manufacturer’s protocol (Fisher Scientific). The PVDF membrane was washed once in 1 × PBST buffer (Fisher Scientific) and blocked in 2% blocking solution (Fisher Scientific) for 1 h at room temperature. Membranes were incubated overnight at +4°C with primary antibodies at recommended concentrations in 1% blocking solution. Membranes were washed three times (5 min for each wash) with 1 × phosphate-buffered saline with Tween 20 (PBST) buffer, and secondary horseradish peroxidase (HRP)-conjugated antibodies in 1% blocking solution were added to the membranes at a 1:5,000 concentration for 1 h of incubation at room temperature. Membrane was washed three times with 1 × PBST, and proteins were detected by chemiluminescence according to the manufacturer’s protocol (Fisher Scientific).

**Co-Immunoprecipitation and Mass Spectrometry**

Thawed (or transduced) CD34+ cord blood cells were enriched for CD34+ by FACS sorting at day 3. Sorted cells were washed three times with PBS. Sorted cells were lysed using RIPA buffer (Thermo-Fisher) supplemented with protease and phosphatase inhibitors. Chromatin immunoprecipitation (ChIP)-grade Protein A/G Magnetic Beads (Fisher Scientific) were incubated with MTA1 antibody (Cell Signaling Technology, Danvers, MA USA) for 30 min or rabbit monoclonal antibody (Cell Signaling Technology) as an isotype control. Cell lysate was prewashed using non-antibody-treated beads (1 hour) and then incubated with antibody-treated beads overnight. Supernatant was removed, and beads were washed three times for 10 min using RIPA buffer (Fisher Scientific). Samples were eluted by heating the beads in Laemmlli buffer (BioRad) for 5 min at 100°C. Samples were loaded on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel for 30 min at 80 V. The gel was stained with fix-stain solution (0.05% Coomassie Brilliant Blue R-250, 50% methanol, 10% glacial acetic acid) for 1 hour. Gel was washed with destaining solution (30% methanol, 10% glacial acetic acid) for
4 hours. The visible bands were cut and stored at −20°C for further use. During handling, samples and buffers were kept on ice. Incubations were performed on a rotating holder at +4°C.

A detailed description of mass spectrometry and other methods is available in the Supplementary Data (online only, available at www.exphem.org).

Statistics

Statistical analysis was done using GraphPad Prism 9 (GraphPad, San Diego, CA). A two-tailed unpaired Student t-test was used to analyze groups of two. Multiple groups were analyzed with one-way analysis of variance (ANOVA). Results with a p value ≤ 0.05 were considered significant (p values represented in figures: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001, ns = not significant).

Accession Numbers

Microarray data discussed in this article have been deposited in the Gene Expression Omnibus (GEO) under GSE206083.

RESULTS

Pooled shRNA Screening Identifies MTA1 as a Regulator of Human HSPC Propagation in vitro

In our previous work, we established paradigms for identifying new genetic regulators of hematopoiesis using pooled lentiviral shRNA screens in primary human HSPCs. To complement these previous screens, we here applied updated TRC libraries (Versions 1.5 and 2.0). The lentiviral libraries used in this study consisted of a total of 45,000 shRNAs targeting around 11,000 genes. This included both new shRNA sequences against genes already represented in the previous version of the library (TRC 1.0) and shRNAs targeting around 5,100 new genes that were not present in TRC 1.0. We transduced a total of 70 million CB-derived CD34⁺ cells with the shRNA libraries, and used the limited persistence of HSPCs during culture as a basis for selection of perturbations promoting maintenance or expansion of the immature phenotype. The distribution of proviral shRNA inserts was determined by NGS 3 days after transduction, as well as in purified CD34⁺ cells following 3 weeks of selection culture, as previously described [9]. After selection, the relative abundance of individual shRNAs was compared with day 3 to determine their ability to propagate CD34⁺ cells during the culture (Figure 1A).

Further analysis focused on the hairpins that were enriched in the CD34⁺ cell population at week 3, as such a selection protocol was optimized and used in our previous studies [6]. Among the enriched shRNAs after selection culture, we found new sequences targeting genes identified in our previous screens such as STAG1 and STAG2, whereas hairpins targeting housekeeping genes such as RPS12 [13] were depleted, which emphasizes the overall validity of the screen (Supplementary Figure E1, online only, available at www.exphem.org).

To identify the most prominent gene candidates, we considered only genes that were represented by at least four hairpins in the screen, and these were ranked based on the median log₂ fold changes for the shRNAs (Figure 1B; Supplementary Table E1, online only, available at www.exphem.org).

Among the candidate genes, we chose to focus on MTA1 in this study as it had the highest ranking and had not been previously studied in the context of human hematopoiesis. Four of five shRNAs for MTA1 exhibited strong enrichment in the screen (Figure 1C), and we first validated the two top-scoring shRNAs by cloning them into a GFP expressing lentiviral vector (pLKO1-GFP) to assess knockdown of MTA1 and recapitulate the screen assay. We could readily detect MTA1 expression across several subsets of cord blood HSPCs (Supplementary Figure E2A, online only, available at www.exphem.org), and found that the two shRNAs exhibited efficient knockdown at both the mRNA level (40%–60%) and protein level (75%–95%) (Figure 1D; Supplementary Figure E2B). We next transduced CB-derived CD34⁺ cells with the individual shRNA vectors and cultured them for 3 weeks while monitoring GFP levels as well as CD34 and CD90 expression. On MTA1 knockdown, we observed an increase in the frequency of the immature CD34⁺CD90⁺ population compared with the scrambled shRNA (Scr) control, as well as an increased GFP⁺ cell ratio over time in culture (Figure 1E,F). Overall, knockdown of MTA1 led to substantially increased numbers of phenotypic HSPCs over 3 weeks of culture (Figure 1G). Taken together, our findings implicate MTA1 as a novel regulator of human HSPCs.

Knockdown of MTA1 Preserves the Immature Phenotype of HSPCs

To assess the consequences of MTA1 deficiency in more detail, we transduced highly HSC enriched CD34⁺CD38⁻CD90⁻CD45RA⁻ cells with the shRNA vectors and monitored expression of EPCR, which, along with CD34 and CD90, is known to mark functional HSCs during culture [14,15]. We observed a marked increase in both frequency and numbers of CD34⁺, CD34⁺CD90⁺, and the HSC-enriched CD34⁺CD90⁻EPCR⁺ cells following the knockdown of MTA1 (Figure 2A,B). Thus, reduced levels of MTA1 preserve the most immature phenotype of cultured HSPCs, implicating a role for MTA1 in regulating the balance between renewal and differentiation in vitro. To further assess the differentiation potential of MTA1-perturbed HSPCs, we next cultured CB-derived CD34⁺ cells in the presence of differentiation-promoting cytokines (SCF, GM-CSF, FLT3-L, TPO, IL-3, EPO) (Figure 2C,D). Under these conditions, we noted an increase in CD34⁺ cells while observing significantly reduced frequencies of cells expressing the early pro-erythroblast marker CD71 for both MTA1 shRNAs, as well as reduced frequencies of CD11b⁺ positive myeloid cells for shRNA1, indicating perturbed differentiation toward both the myeloid and erythroid lineages. To functionally assess cell maturation in vitro, we further performed colony formation assays and found that MTA1⁺CD34⁺⁺ cells formed fewer colonies compared with scrambled control, irrespective of the post-knockdown culture time: plated 3 days after transduction (Figure 2E) or following 1 week of in vitro culture (Figure 2F). This indicates perturbed differentiation and a reduced capacity to form mature blood cells. In particular, shRNA1 exhibited severely reduced colony numbers and a complete lack of erythroid (BFU-E) colonies. Together, these findings suggest that MTA1 critically promotes differentiation of human HSPCs during culture.

Knockdown of MTA1 Impairs in vivo Reconstitution of Transplanted HSPCs

To further investigate the functional properties of MTA1-deficient HSPCs and the role of MTA1 in HSPC regulation in vivo, we transplanted shRNA-transduced CD34⁺ cells to sublethally irradiated NOD-SCID/IL2Rγnull (NSG) mice using two different settings. In the first setting, we did not sort transduced cells for GFP expression.
Figure 1 Pooled short hairpin (sh)RNA screen identifies MTA1 as a regulator of human hematopoietic stem and progenitor cell (HSPC) propagation in vitro. (A) Overview of the screen. Primary cord blood-derived CD34+ cells were transduced with pools of a lentiviral library containing 45,000 shRNAs. CD34+ cells were magnetically isolated after 20 days of culture to determine changes in shRNA distribution between days 3 and 20. (B) Genes ranked based on the median shRNA log2 fold change (log2 FC) between days 20 and 3. (C) Distribution of the MTA1-targeting shRNAs in the library. (D) Relative mRNA and protein levels of MTA1 in cord blood (CB)-derived CD34+ cells after knockdown by shRNAs. (E) Representative flow cytometry plots of CD34 and CD90 staining of GFP+ cells in culture 7 and 14 days following MTA1 shRNA transduction. (F) GFP+ cell ratio of MTA1 shRNA and Scr control transduced cells from days 3 (D3) to 21 (D21). (G) Relative fold increase in GFP+CD34+CD90+ cell number for MTA1 shRNA-transduced cells compared with the Scr control from days 3 to 21.
Figure 2 Knockdown of MTA1 preserves the immature phenotype of hematopoietic stem and progenitor cells (HSPCs) and perturbs differentiation into myeloid and erythroid lineages. (A) Representative flow cytometry plots of CD34, CD90, and EPCR staining of short hairpin (sh)RNA-transduced CD34⁺CD90⁺CD38⁻CD45RA⁻ cells following 1 week of culture. (B) Relative fold increase in
before transplantation, allowing competition between the transduced (GFP positive, MTA1\(^{KD}\)) and nontransduced (GFP negative, internal control) populations. We found that the overall engraftment, and in particular the contribution of GFP-positive cells, was dramatically reduced in the MTA1 shRNA1 and shRNA2 transduced groups (Figure 3A). The relative GFP ratio for MTA1\(^{KD}\) cells dropped from initially 60% to below 10% at 16 weeks posttransplantation (Supplementary Figure E3, online only, available at www.exphem.org), in contrast to the scrambled control, which showed stable engraftment. In summary, the transplantation experiments in immunodeficient mice revealed a significant reduction in human blood cell chimerism on KD of MTA1 in HSPCs. To independently corroborate this finding in a different setting, we next transplanted 2,000 shRNA-transduced GFP\(^+\)CD34\(^+\)CD90\(^+\)CD45RA\(^-\) cells to sublethally irradiated NSG mice. In contrast to the previous experiment, cells were sorted based on GFP expression before transplantation to avoid competition from nontransduced cells (Figure 3B). The analysis of peripheral blood and bone marrow after 16 weeks revealed severely reduced engraftment levels of MTA1\(^{KD}\) HSPCs, where scrambled control cells exhibited up to 10-fold higher chimerism in both parameters (Figure 3B). This suggests a functional impairment in hematopoietic reconstitution potential in MTA1\(^{KD}\) cells. Taken together, our results indicate that MTA1 is required for human HSC reconstitution and differentiation in vivo.

MTA1 Associates with Members of the NuRD Complex in HSPCs and Influences H3K27 Deacetylation

The molecular function of MTA1 has been attributed to its association with the NuRD complex as an epigenetic modifier [16,17]. The complex has both chromatin remodeling (CHD3/4/5) and histone deacetylase (HDAC1/2) activity, and further consists of several non-enzymatic components that include MBD2/3 (methyl-CpG-binding domain), RBBP4/7 (retinoblastoma-binding proteins), GATAD2A/B (GATA zinc finger domain-containing proteins) and MTA1/2/3 (metastasis-associated proteins) [18,19] (Figure 4A). The NuRD complex proteins can assemble in various combinations to form a series of different complexes with specific properties and diverse roles across different tissues and cell types [20]. Next, we investigated the MTA1 interactome to validate the interaction between MTA1 and other NuRD complex members in human HSPCs. MTA1 was co-immunoprecipitated from CB-derived CD34\(^+\) lysates followed by mass spectrometry (CoIP-MS). In total, we identified 155 significantly co-immunoprecipitated proteins (Supplementary Table E2, online only, available at www.exphem.org). Several NuRD complex members (MTA1, MTA2, CHD4, HDAC1, GATAD2A, RBBP7, GATAD2B, RBBP4) were identified in the assay and clustered among the most significantly associated proteins (Figure 4B). Our interactome data describe the composition of the MTA1/NuRD complex in GFP\(^+\)CD34\(^+\), GFP\(^+\)CD34\(^+\)CD90\(^-\), or GFP\(^+\)CD34\(^+\)CD90\(^+\)EPCR\(^+\) cell number from shRNA-transduced CD34\(^+\)CD90\(^+\)CD38\(^+\)CD45RA\(^-\) cells following 2 weeks of culture. (C) Representative histograms for CD34, CD71, and CD11b expression in GFP\(^+\)CD34\(^+\)CD34\(^+\)CD90\(^-\)CD38\(^+\)EPCR\(^-\) cells cultured for 7 days under differentiation-promoting conditions. (D) Summary of CD34, CD71, and CD11b frequencies in GFP\(^+\)CD34\(^+\)CD34\(^+\)CD90\(^+\)EPCR\(^+\) cells measured at days 4 and 7. (E) Colony-forming potential of GFP\(^+\)CD34\(^+\)CD34\(^+\)CD34\(^+\)CD90\(^-\) cells transduced with MTA1 or Scr shRNAs. (F) Colony-forming potential of GFP\(^+\)CD34\(^+\)CD34\(^+\)CD34\(^+\)CD34\(^+\)EPCR\(^+\) cells that were cultured for 1 week under standard culture conditions prior to sorting and plating of the CD34\(^+\) GFP\(^+\) cells.

In summary, the transplantation experiments in immunodeficient mice revealed a significant reduction in human blood cell chimerism on KD of MTA1 in HSPCs. To independently corroborate this finding in a different setting, we next transplanted 2,000 shRNA-transduced GFP\(^+\)CD34\(^+\)CD90\(^+\)CD45RA\(^-\) cells to sublethally irradiated NSG mice. In contrast to the previous experiment, cells were sorted based on GFP expression before transplantation to avoid competition from nontransduced cells (Figure 3B). The analysis of peripheral blood and bone marrow after 16 weeks revealed severely reduced engraftment levels of MTA1\(^{KD}\) HSPCs, where scrambled control cells exhibited up to 10-fold higher chimerism in both parameters (Figure 3B). This suggests a functional impairment in hematopoietic reconstitution potential in MTA1\(^{KD}\) cells. Taken together, our results indicate that MTA1 is required for human HSC reconstitution and differentiation in vivo.

DISCUSSION

Here, we report on an extended RNAi screen building on our previously established paradigms for selection of modifiers of renewal and differentiation in primary human HSPCs. Although the screen identified both enriched and depleted (dropped out) shRNAs from the selection assay, we focused on candidate genes from the enriched fraction of shRNAs. Generally, depletion phenotypes are more prone to off-target effects from nonspecific toxicity. Moreover, robust drop-out screens require very high library coverage, which is difficult to achieve with primary cells. A common challenge when performing shRNA screens is that the level of knockdown varies between shRNAs and that the screening libraries may not contain sufficient numbers of efficient shRNAs against all genes to allow scoring in the assay. Additionally, when performing screens in heterogeneous populations of primary cells such as CD34\(^+\) cells is to achieve sufficient representation of all shRNAs in the libraries in the relevant target cells. Although we used very large numbers of primary CD34\(^+\) cells for the screen, we cannot be sure that all shRNAs had infected sufficient numbers of fully functional HSPCs in all replicates to allow scoring in the assays. Thus, although our screens serve as powerful tools for discovery of novel
regulators in primary human HSPCs and clearly can filter out strong hits, it should be emphasized that they cannot be considered as comprehensive screens in terms of assessing all genes in the libraries.

Most of the top-ranked genes have previously not been implicated in HSPC biology, and it will certainly be of interest to follow up on these other candidate genes as well. As expected, some of the genes, such as SPOPL, SLK, KLF13, and CA4, have previously been implicated as inhibitors of cell growth and tumor suppression [23–26].

Similar to those of our previous study, the findings from this screen can serve as a resource of potential HSPC regulating genes, and we are currently establishing an open database for access to the raw data with user-friendly navigation tools (demonstration version is attached in the Supplementary Data, online only, available at www.exphem.org [shrna_app.html]).

In this study, we decided to follow up on the highest-scoring gene, MTA1, and we found that it associates with the NuRD complex in human HSPCs. While MTA1 has not been directly linked to a role in hematopoiesis, the NuRD complex has been reported to have a regulatory role in both embryonic stem cells (ESCs) and mouse HSCs by influencing stem cell maintenance and differentiation [27,28]. Similar to those of our previous study, the findings from this screen can serve as a resource of potential HSPC regulating genes, and we are currently establishing an open database for access to the raw data with user-friendly navigation tools (demonstration version is attached in the Supplementary Data, online only, available at www.exphem.org [shrna_app.html]).

Molecularly, we observed a global accumulation of acetylated H3K27 marks in MTA1-depleted cells. This suggests that depletion of MTA1 in HSCs may prevent silencing of active transcription sites of HSC-specific regulators indicated by acetylated H3K27 marks, possibly keeping cells in an immature state and compromising maturation. Indeed, when HSCs were placed in culture, H3K27Ac marks were reported to be downregulated as stem cell potential becomes compromised and the cells start differentiating [29,30]. Global inhibition of histone deacetylation using HDAC inhibitors has successfully been used to promote maintenance and expansion of human HSPCs in culture [31]. Further, it has been previously reported that NuRD-mediated deacetylation of H3K27 facilitates recruitment of PRC2 complex to direct gene repression, which also has an important role in hematopoiesis and differentiation [21]. Although the NuRD complex is usually defined as a transcriptional repressor, recent studies have indicated that it can also stimulate gene expression [32].

Strikingly, however, we observed only very mild gene expression changes from MTA1 knockdown in highly purified HSPCs, despite the profound cellular effects. Apart from a set of erythroid lineage-specific genes that were downregulated, we did not detect any alterations of distinct hallmark or gene categories. It is possible that the preserved H3K27Ac marks broadly conserve established HSPC signatures with only mild influence on individual genes that therefore escape detection. Overall, the molecular mechanisms underlying MTA1/NuRD activity in HSPCs warrant further investigation.

It will also be interesting to study MTA1 in the context of malignant hematopoiesis. Although there are no reports on recurrent mutations in MTA1 associated with leukemia, it would seem plausible that MTA1 could have a tumor suppressor role in hematological malignancies.

Figure 3 Knockdown of MTA1 impairs in vivo reconstitution of transplanted hematopoietic stem and progenitor cells (HSPCs). (A) Engraftment of human CD34+ cells transduced with MTA1 or Scr short hairpin (sh)RNAs and transplanted without prior sorting to sublethally irradiated NSG (NOD Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice (five mice for each condition). The graph indicates the frequency of total hCD45+ and hCD45+GFP+ cells in peripheral blood. (B) Engraftment of human GFP+CD34+CD38−CD90+CD45RA− cells transduced with MTA1 or Scr shRNAs sorted for Green Fluorescent Protein (GFP) expression before transplantation to sublethally irradiated NSG mice. The graphs indicate the percentages of hCD45+GFP+ cells in peripheral blood and bone marrow after 16 weeks (n = 12).
Based on our findings. On the other hand, it has been reported that MTA1 is upregulated in several cancer forms and it has been suggested to execute oncogenic functions [33,34]. The functions may be highly context dependent as the NuRD complex has been linked to a variety of oncogenic processes such as metastasis, epithelial-to-mesenchymal transition (EMT), and cell cycle progression [35]. Collectively, our work indicates that the MTA1−NuRD complex is a key genetic regulator of human HSPC fate, with important implications for the understanding of both normal and malignant hematopoiesis.

**Conflict of interest disclosure**

The authors declare no competing interests.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.exphem.2022.08.004.

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