*GATA1*s exerts developmental stage-specific effects in human hematopoiesis

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Data Supplement

Supplemental Materials and methods

Cells and cell culture

Human cell lines (K562 and HEK293T) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). Culturing and maintenance were performed according to the supplier's instructions. Human $CD34^+$ fetal liver cells (gestational week 16-18) were purchased from Novogenic Laboratories. Neonatal and adult $CD34^+$ HSPCs were obtained from cord blood and mobilized peripheral blood of healthy donors, respectively, and enriched using anti-CD34 immunomagnetic microbeads (Miltenyi Biotech). All investigations were performed in accordance to the Declaration of Helsinki and informed consent was obtained according to local laws and regulations. All HSPCs were expanded in StemSpan SFEM (StemCell Technologies) with 1% streptomycin/penicillin (Sigma-Aldrich), 100ng/mL SCF, 100ng/mL FLT3, 50ng/mL TPO, 20ng/mL IL6 (all from Peprotech) and 0.75μM Stemregenin1 (SR1) (StemCell Technologies). For megakaryocytic differentiation, StemSpan SFEM with 1% streptomycin/penicillin, 1x CD-Lipid Concentrate (Gibco, Life Technologies), 100ng/mL TPO and 10ng/mL SCF was used. For combined megakaryocytic/erythroid differentiation, the cells were cultured in StemSpan SFEM with 1% streptomycin/penicillin, 1% L-Glutamine (Millipore), 1x CD-Lipid Concentrate, 100ng/mL SCF, 50ng/mL TPO, 10ng/mL IL3 and 10ng/mL IL6 for 7 days. On day 7, the concentration of SCF was reduced to 10ng/mL and 0.2U/mL EPO was included in the culture media.

Lentiviral vector construction and assessment of CRISPR-Cas9 spacer sequences

Single guide RNAs (sgRNAs) targeting *GATA1* or *Luc* gene were cloned into the pL40C-CRISPR.EFS.mNeon vector, as previously described[1]. The protospacer sequences used in this study are shown in Supplemental Table 1. The efficacy of the different *GATA1*-targeting sgRNAs was evaluated using a fluorescence-based reporter assay, as described previously[1]. Furthermore, the efficiency of the two sgRNAs with the highest knock out rate (GATA1.1 and GATA1.2) was validated with an off-target activity screening. For this purpose, K562-Cas9-BSD cells were transduced with lentiviral particles carrying the GATA1.1 and GATA1.2 sgRNAs cloned into the SGL40C-mU6-RFP657 vector. Five days later, genomic DNA was isolated from transduced and control cells using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions.

Lentiviral transduction

Production of lentiviral particles was achieved by co-transfection of HEK293T cells with the sgRNA vectors, pMD2.G and psPAX2, using the polyethylenimine (PEI) method. The viral particles were highly concentrated by ultracentrifugation of the cellular supernatant and further using the Lenti-X™ Concentrator (TaKaRa). 3×10^6 fetal, neonatal and adult HSPCs were transduced (10^6 cells transduced per condition) using the Retronectin-based virus infection method (TaKaRa) and sorted on a FACSAria cell sorter (BD Biosciences), as previously described[2]. The percentages of sorted GFP^+ cells are shown in Supplemental Table 3. Similarly, K562 cells were transduced and subjected to single cell sorting, in order to obtain single cell clones.

Genotyping of GATA1s-K562 clones and transduced HSPCs

To identify mutations at the targeted locus, the *GATA1s*-K562 clones that appeared as full length GATA1-negative, according to the Western blot, were selected. Also, GATA1s-expressing fetal and adult HSPCs, on day 17 of combined megakaryocytic/erythroid differentiation, were harvested. Genomic DNA was isolated using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. Exon 2 of *GATA1* gene was amplified by PCR (Phusion DNA Polymerase, NEB) and PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen). The primer sequences used for this purpose are given in Supplemental Table 2. Sequences were assessed by Sanger sequencing and further analyzed for the identification of random insertions and deletions using the TIDE online tool[3].

Western blot

K562 cells obtained from single clones were lysed with Pierce™RIPA buffer (Thermo Scientific) supplemented with cOmplete™ULTRA Tablets Mini EASYpack protease inhibitors (Roche). Protein concentration was calculated according to the number of cells lysed and equal amounts per sample were analyzed by SDS-PAGE, blotted on polyvinylidene difluoride (PVDF) membrane, blocked with 5% fat-free milk and incubated with primary antibody (goat anti-GATA1 polyclonal IgG and mouse anti-β-Actin polyclonal IgG, Santa Cruz). The blots were further incubated with an HRP-conjugated secondary antibody (donkey anti-goat IgG-HRP and bovine anti-mouse IgG-HRP, Santa Cruz) and developed using Amersham™ECL Prime Western Blotting Detection Reagent (GE Healthcare).

Functional validation of GATA1s-K562 clones

7.5×10^4 cells from *GATA1s*-K562 clones #1 and #2, as well as from WT K562 cells were seeded in a 24-well and cultured in the appropriate medium. Every 3 days, the cells were harvested by pipetting and a sample was used for determining the cell counts with a CytoFLEX platform (Beckman Coulter). Furthermore, the cells were analyzed for the expression of cell surface markers by means of flow cytometry, according to standard protocols[10]. The fluorochrome-coupled antibodies used were: anti-CD41-PECy7, anti-CD61-PECy7, anti-CD42b-APC, anti-CD42b-PE, anti-CD235a-PECy7, anti-CD71-APC-AF750 and anti-CD117-APC (all antibodies from Beckman Coulter). All measurements were performed using the FACSCanto (BD Biosciences) and the data obtained were analyzed with FlowJo v10 (FlowJo, LLC).

In vitro differentiation assays

Upon cell sorting, 8×10^4 HSPCs were seeded into a 48-well and cultured in the megakaryocytic differentiation-supporting medium, as described above. Similarly, 5×10^4 HSPCs were seeded for culturing in the megakaryocytic/erythroid differentiation medium. Every 2-3 days, half of the medium was replaced with fresh, the cells were harvested by pipetting and a sample was used for determining the cell counts with a CytoFLEX platform (Beckman Coulter). On days 7, 10 and 17 the cells were analyzed for the expression of cell surface markers by means of flow cytometry, according to standard protocols[10]. The fluorochrome-coupled antibodies used were: anti-CD41-PECy7, anti-CD61-PECy7, anti-CD42b-APC and anti-CD117-PE for the megakaryocytic differentiation and anti-CD41-PE, anti-CD235a-PECy7, anti-CD71-APC-AF750 and anti-CD117-APC for the combined megakaryocytic and erythroid differentiation (anti-CD42b-

APC from BD-Biosciences, the rest from Beckman Coulter). All measurements were performed using the CytoFLEX platform and the data obtained were analyzed with FlowJo v10 (FlowJo, LLC).

Colony-forming assays

Transduced HSPCs were plated in the MegaCult™-C medium with cytokines (StemCell Technologies) according to the manufacturer's instructions and incubated for 10 days in a 37^{o}C incubator with 5% CO_2 and ≥95% humidity. Dehydration, fixation and staining were achieved with a methanol:acetone solution and staining of the CD41-expressing megakaryocytic colonies was based on an alkaline phosphatase reaction. Both steps were performed according to the instructions. Imaging, counting and classification of the colonies were performed using the Keyence BZ-9000 microscope.

Characterization of fetal, neonatal and adult CD34^{+} HSPCs

The cellular composition of fetal, neonatal and adult CD34^{+} HSPCs was characterized by means of flow cytometry. Cells from each developmental stage were stained with the following fluorochrome-coupled antibodies: anti-CD34-APC-Cy7, anti-CD38-PECy7, anti-CD7-AF700, anti-CD10-BV605, anti-CD135-BV421, anti-CD45RA-FITC, anti-CD110-PE (clone 1.6), anti-CD90-APC, anti-CD49f-PerCP-Cy5.5 (all BD Biosciences) and 7-AAD (Biolegend). All measurements were performed on the FACSAria cell sorter (BD Biosciences) and the data obtained were analyzed with FlowJo v10 (FlowJo, LLC).

Evaluation of GATA1 expression by qRT-PCR

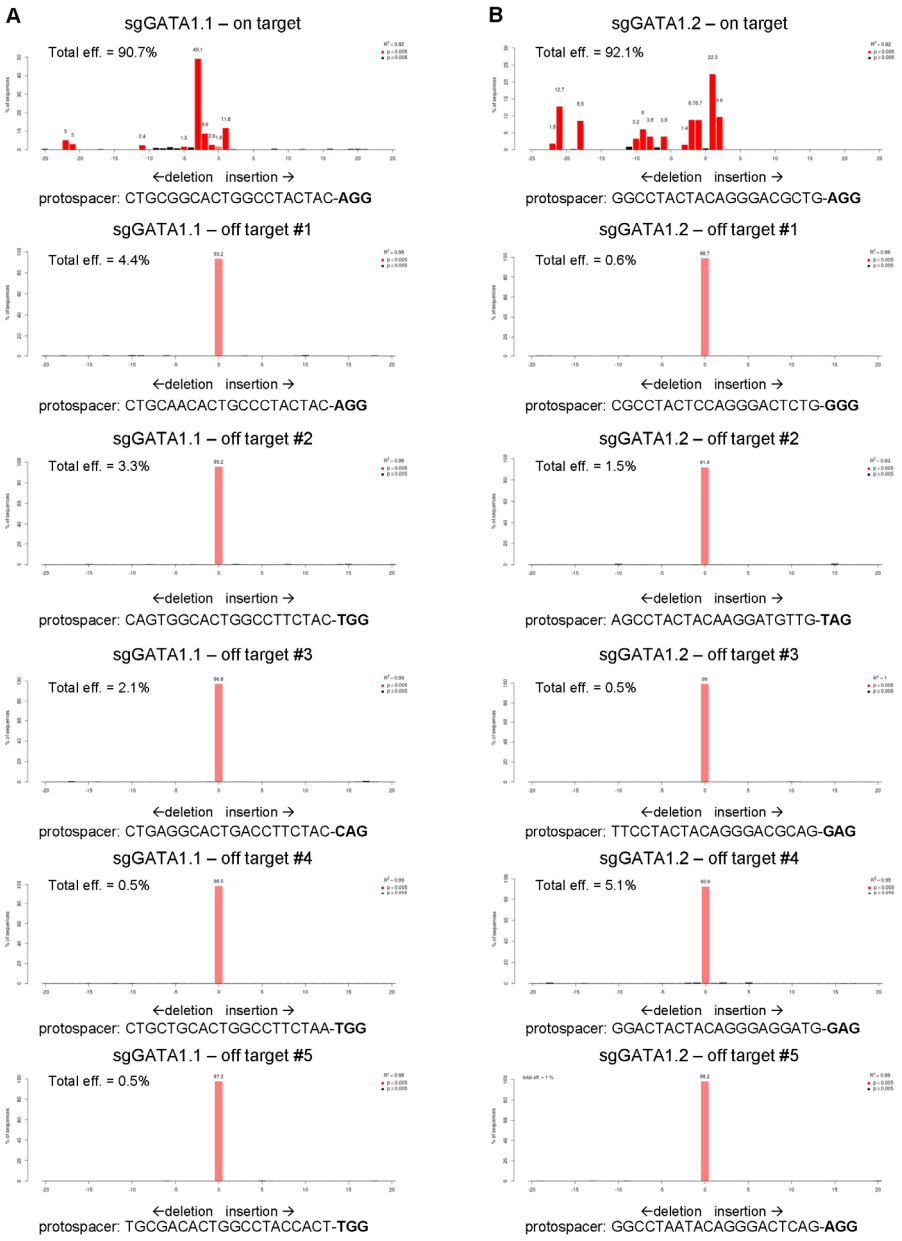
In order to assess the expression of *GATA1* in cells of different developmental stages, RNA was extracted from fetal, neonatal and adult CD34^{+} HSPCs prior to transduction,

using the RNeasy Micro Kit (Qiagen). Complementary DNA (cDNA) was synthesized from the isolated RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific) and was afterwards subjected to quantitative real-time PCR (qRT-PCR) using the SYBR® Select Master Mix (Applied Biosystems, Thermo Fisher Scientific). The primers designed for this purpose are given in Supplemental Table 2. The StepOnePlus™ Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific) was used in this assay.

Statistical analyses

Statistical data evaluation for the three groups of interest was performed using one-way or two-way ANOVA and the level of significance was set for a p value less than 0.05. All analyses were performed with GraphPad Prism 6 (GraphPad Software, Inc.).

Supplemental Figures



Supplemental Figure 1. **Evaluation of the efficacy of the GATA1s CRISPR-Cas9 system in the K562 cell line.** Decomposition plots showing the on-target (upper plots in both panels) and the off-target (5 plots for the top predicted sites) activity of GATA1.1 (A) and GATA1.2 (B) sgRNAs.

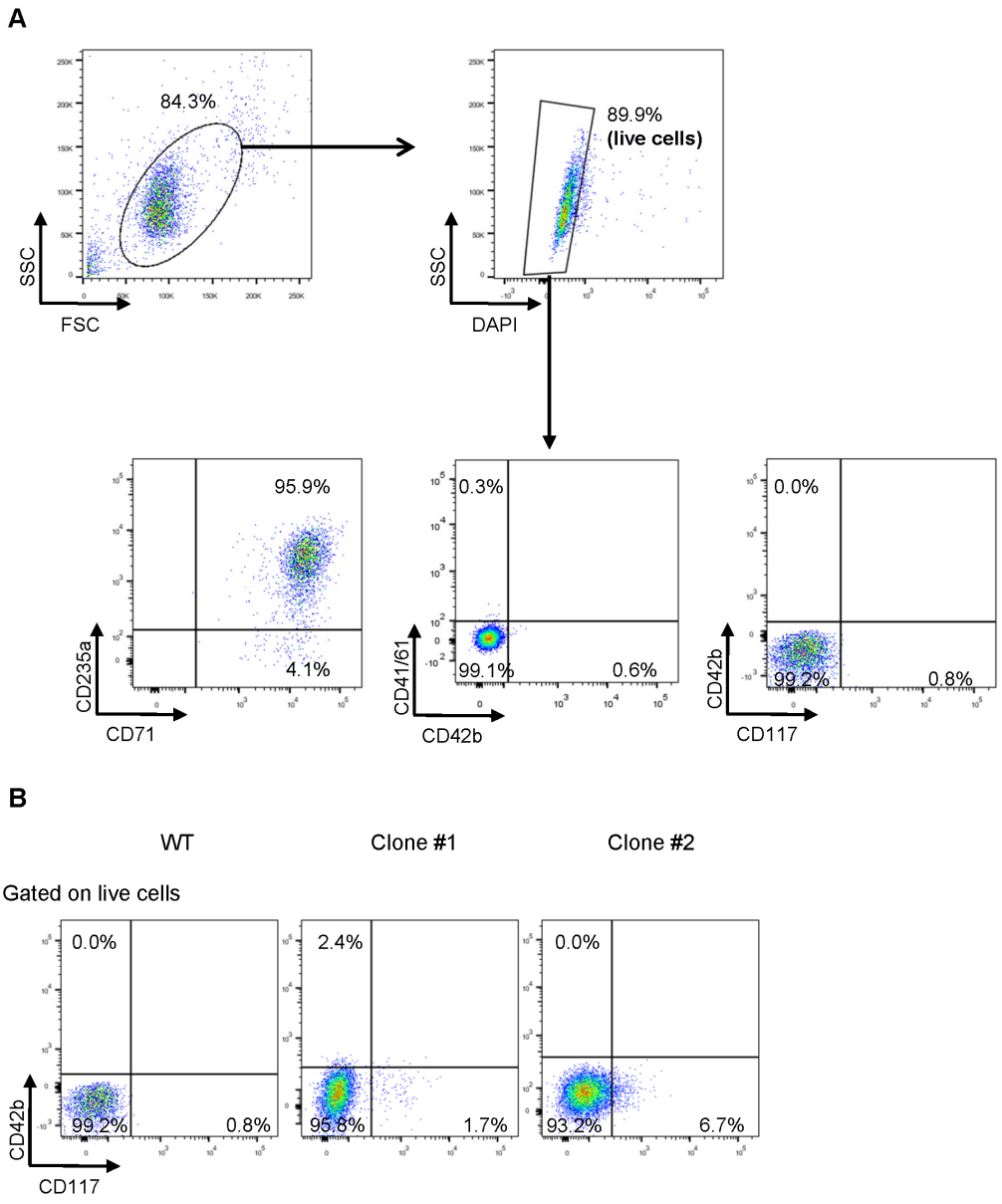
A

Clone	# 1	#2
Allele 1	-22bp	-19bp
Allele 2	-22bp	-19bp

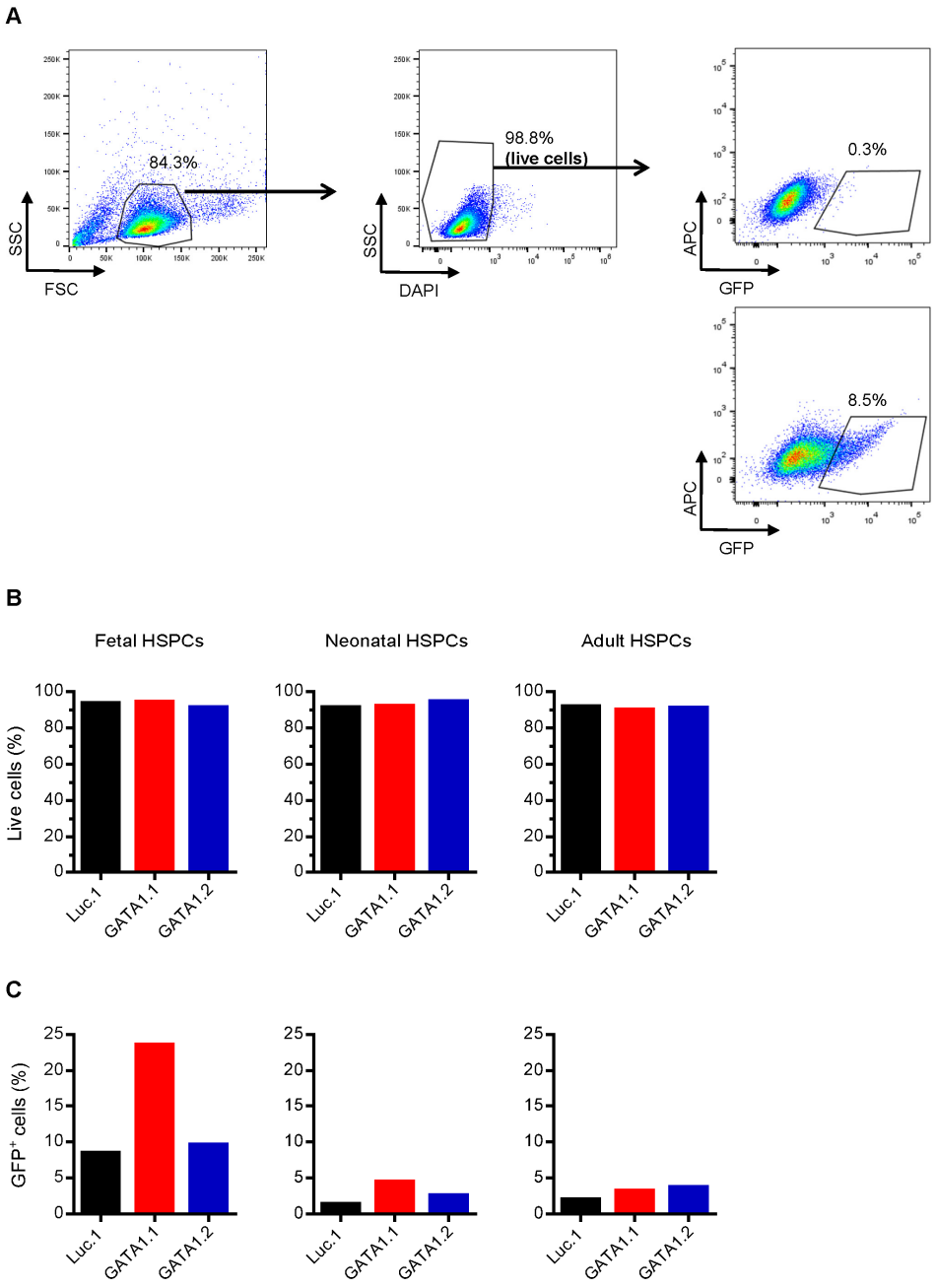
B

WT	CTGCGGCACTGGCCTACTACAGGGACGCTGAGGCCTACAGACACTCCCCAGGTAACTCCATT CTGCGGCACTGGCCTACTACAGGGACGCTGAGGCCTACAGACACTCCCCAGGTAACTCCATT
#1	CTGCGGCACTGGCCTACTACA----------CTCCCCAGGTAACTCCATT CTGCGGCACTGGCCTACTACA----------CTCCCCAGGTAACTCCATT
#2	CTGCGGCACTG----------AGGCCTACAGACACTCCCCAGGTAACTCCATT CTGCGGCACTG----------AGGCCTACAGACACTCCCCAGGTAACTCCATT

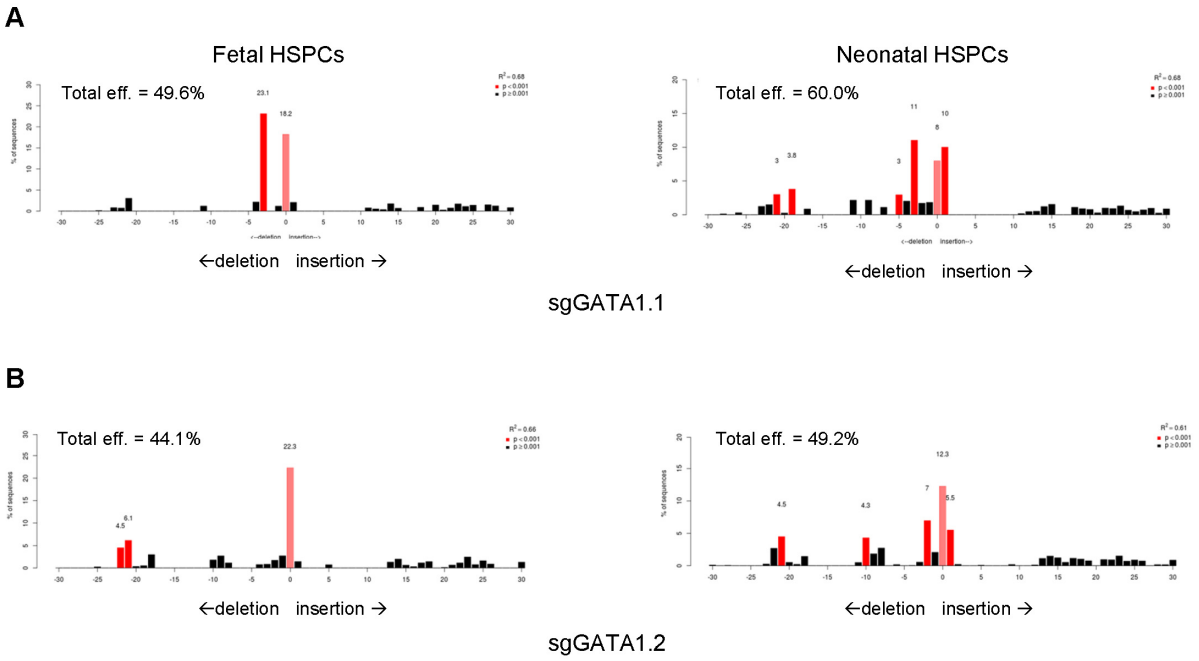
Supplemental Figure 2. Validation of the mutations introduced by the GATA1s CRISPR-Cas9 system in K562 cells. (A) Summary of the deletions identified in each allele of *GATA1* gene in the two GATA1s-K562 clones of Figure 1 B-D. (B) Alignment of sequences obtained for *GATA1* gene from WT K562 cells and the two GATA1s-K562 clones of Figure 1 B-D. The target sequences of GATA1.1 and GATA1.2 sgRNAs are highlighted with blue and orange color, respectively.



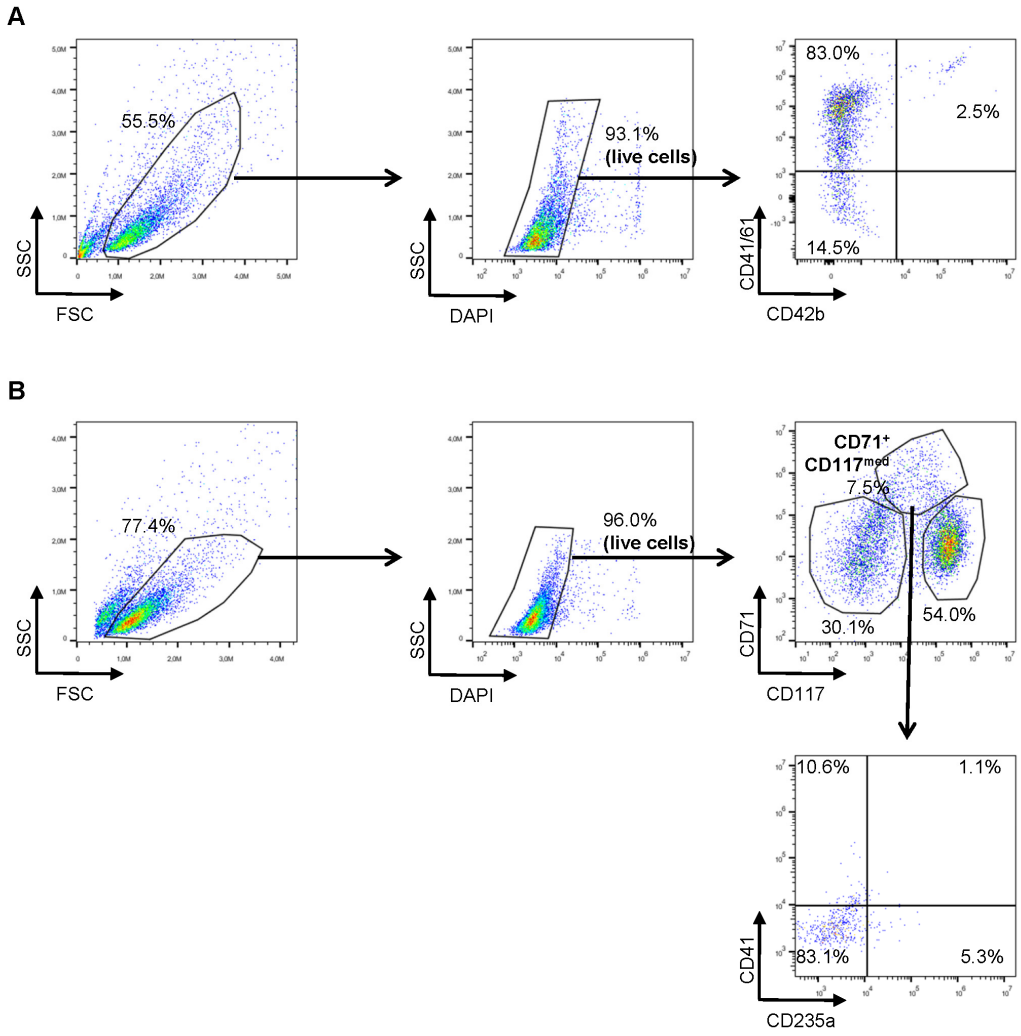
Supplemental Figure 3. Functional validation of *GATA1s*-K562 clones. (A) Gating strategy applied for flow cytometric analysis of *GATA1s*-K562 clones. Data from the WT clone are shown. K562 cells were gated on a forward scatter (FSC)/ side scatter (SSC) plot (upper left). Live cells were further gated (upper right) to detect erythroid (bottom left), megakaryocytic (bottom middle) or proliferation markers (bottom right). (B) Flow cytometric analysis of WT and *GATA1s*-K562 cells for expression of the indicated cell surface markers.



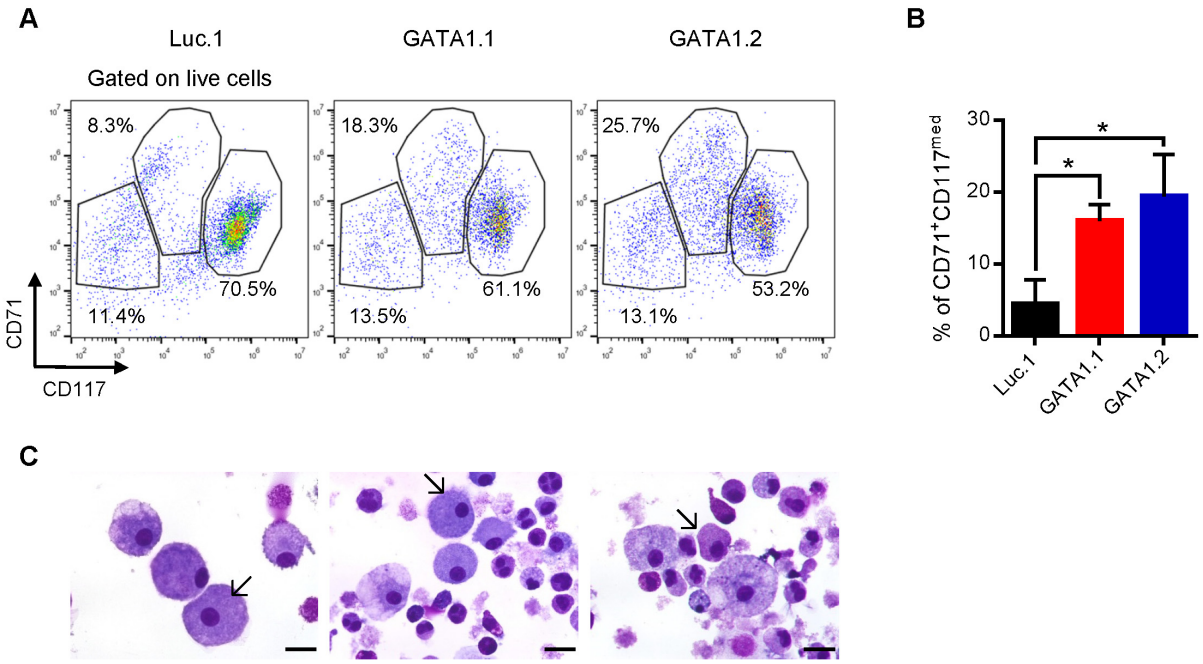
Supplemental Figure 4. Fluorescence-activated cell sorting (FACS) of transduced HSPCs. (A) Gating strategy applied in FACS of transduced fetal, neonatal and adult HSPCs. Data from the control-transduced (Luc.1) cells are shown. The cells were first gated on a forward scatter (FSC)/ side scatter (SSC) plot (left). Live cells were further gated (middle) to detect GFP-expressing cells (upper right panel shows GFP^- cells; bottom right panel shows GFP^+ cells). (B) Percentages of live-gated fetal, neonatal and adult HSPCs. (C) Percentages of GFP^+ sorted fetal, neonatal and adult HSPCs.



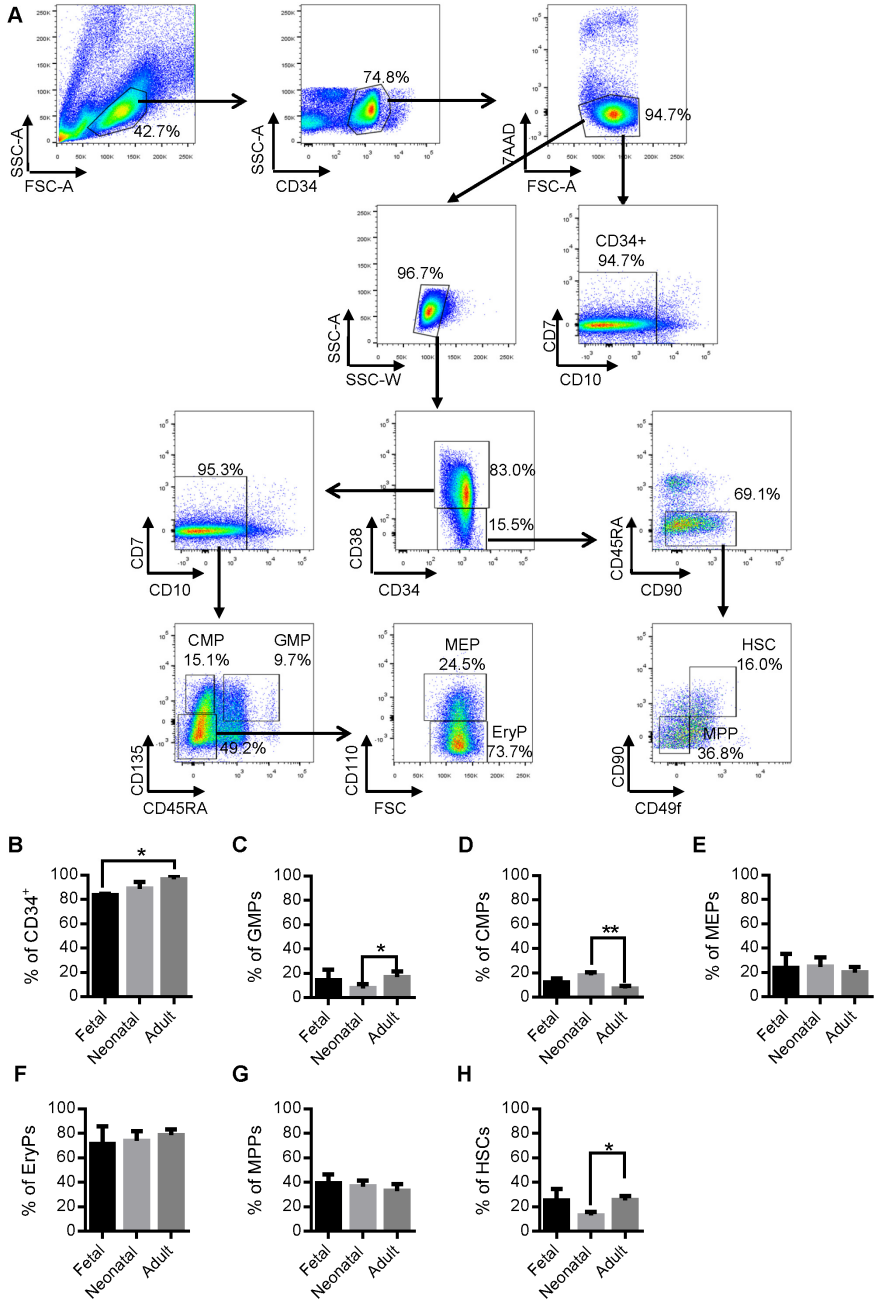
Supplemental Figure 5. Evaluation of the efficacy of the GATA1s CRISPR-Cas9 system in fetal and neonatal HSPCs. Decomposition plots showing the percentage of random insertions and deletions introduced by the CRISPR-Cas9 system, in the presence of GATA1.1 (A) and GATA1.2 (B) sgRNAs, in fetal (left panels) and neonatal (right panels) HSPCs.



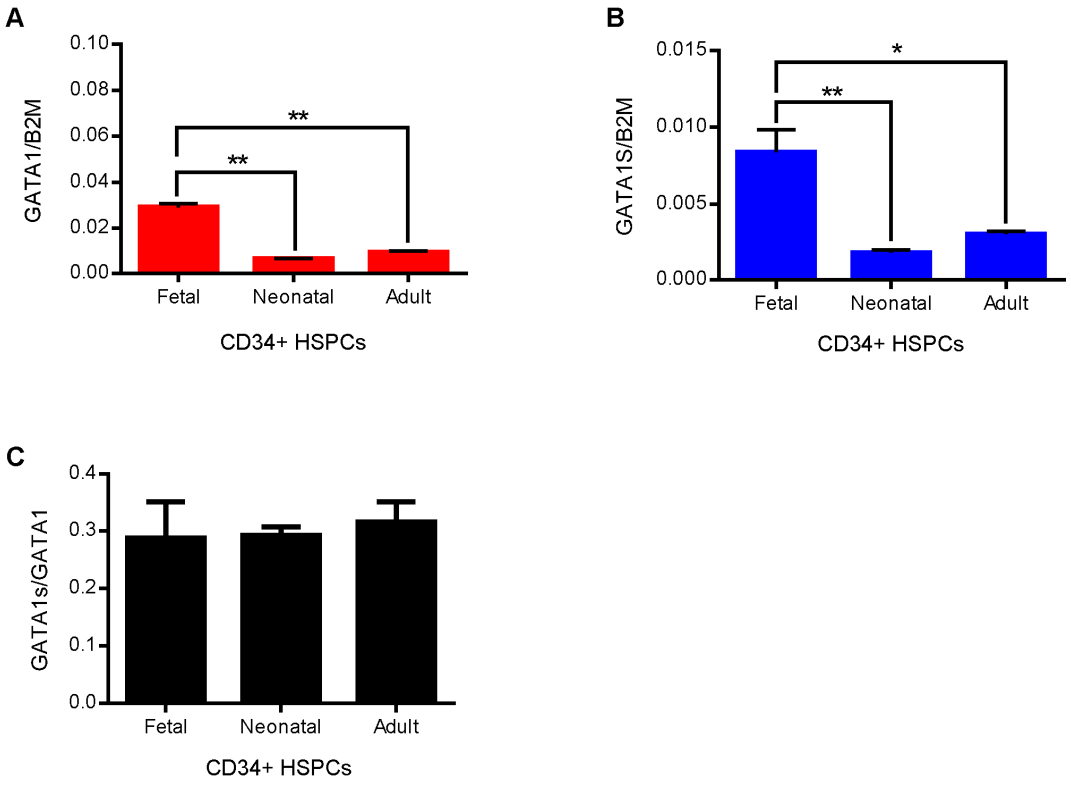
Supplemental Figure 6. Flow cytometric assessment of *in vitro* megakaryocytic and combined megakaryocytic/erythroid differentiation assays. (A) Gating strategy applied in flow cytometric analysis of transduced HSPCs grown in liquid cultures supporting megakaryocytic differentiation. Data from the control-transduced (Luc.1) cells are shown. The cells were first gated on a forward scatter (FSC)/ side scatter (SSC) plot (left). Live cells were further gated (middle) to detect megakaryocytic markers. (B) Gating strategy applied in flow cytometric analysis of transduced HSPCs grown in liquid cultures supporting combined megakaryocytic/erythroid differentiation. Data from the control-transduced (Luc.1) cells are shown. The cells were first gated on a forward scatter (FSC)/ side scatter (SSC) plot (left). Live cells were further gated (middle) to determine the $CD71^{-}CD117^{-}$, $CD71^{+}CD117^{med}$ and $CD71^{+}CD117^{+}$ populations. The $CD71^{+}CD117^{med}$ population was subsequently gated to determine the expression of the indicated cell surface markers.



Supplemental Figure 7. The accumulation of CD71^{+}CD117^{med} in GATA1s-expressing neonatal HSPCs is transient. Neonatal HSPCs were transduced with GATA1-targeting (GATA1.1 and GATA1.2) or control (Luc.1) sgRNAs.(A) Representative flow cytometry plots (from 3 independent experiments), showing CD71^{+}CD117^{-}, CD71^{+}CD117^{med} and CD71^{+}CD117^{+} cells on day 17 of combined megakaryocytic/erythroid differentiation. Percentages of each population are indicated. (B) Percentages of neonatal CD71^{+}CD117^{med} population on day 17 of combined megakaryocytic/erythroid differentiation. Data from 3 independent experiments are shown as mean±s.d. *P_{ANOVA}<0.05 (C) Microscopic images (MGG staining; x 1000 original magnification; scale bar 20μm) of neonatal CD34^{+} HSPCs on day 17 of combined megakaryocytic/erythroid differentiation. The arrows show representative mast cells.



Supplemental Figure 8. HSPCs of different developmental stages present only minor discrepancies in the progenitor composition. (A) Gating strategy applied in flow cytometric analysis of fetal, neonatal and adult CD34⁺ HSPCs. Data from neonatal HSPCs are shown. The cells were initially gated on a forward scatter (FSC)/side scatter (SSC) plot. Live cells were sequentially gated to detect cell surface markers characteristic of common myeloid progenitors (CMP), granulocyte monocyte progenitors (GMP), megakaryocytic erythroid progenitors (MEP), primitive erythroid progenitors (EryP), multipotent progenitors (MPP) and hematopoietic stem cells (HSC). (B-H) Percentages of CD34⁺ cells (B), GMPs (C), CMPs (D), MEPs (E), EryPs (F), MPPs (G) and HSCs (H) identified in fetal, neonatal and adult HSPCs. Data from 3 independent samples are shown as mean±s.d. *P_{ANOVA}<0.05, **P_{ANOVA}<0.01



Supplemental Figure 9. Fetal HSPCs have higher endogenous expression of both GATA1 and GATA1s. (A-B) Expression of total *GATA1* (A) and *GATA1s* (B) normalized to the expression of β2-microglobulin (*B2M*) in WT fetal, neonatal and adult HSPCs. (C) Ratio of *GATA1s* to total *GATA1* expression. Data from two replicates are shown as mean±s.d. *P_{ANOVA}<0.05, **P_{ANOVA}<0.01

Supplemental Tables

Supplemental Table 1. Target sites of sgRNAs

sgRNA	Targeted sequence
Luc.1	CACCGAGTTCACCGGCGTCATCGTC
	AAACGACGATGACGCCGGTGAACTC
GATA1.1	CACCCTGCGGCACTGGCCTACTAC
	AAACTAGTAGGCCAGTGCCGCAGC
GATA1.2	CACCGCCTACTACAGGGACGCTG
	AAACCAGCGTCCCTGTAGTAGGCC
GATA1.3	CACCGTACCCATTGCTCAACTGTA
	AAACTACAGTTGAGCAATGGGTAC
GATA1.4	CACCGGTACACCTGAAAGACTGTT
	AAACAACAGTCTTTCAGGTGTACC

Supplemental Table 2. Primer sequences

Primer name	Sequence
GATA1 exon2 forward	GAGAAATATGGAGACTGAGGTG
GATA1 exon2 reverse	CCATTTGAGAAGCTTCCAGCC
qPCR GATA1 (exon 4) forward	CATCCGGCCCAAGAAGCGCC
qPCR GATA1 (exon 6) reverse	CGCATGGTCAGTGGCCGGTT
qPCR GATA1s (exon 1-3 spanning) forward	CACCAGCCCAGTCTTTCAG
qPCR GATA1s (exon 1-3 spanning) reverse	ACAGTTGAGGCAGGGTAGA

Supplemental Table 3. Absolute number of transduced HSPCs and percentages of sorted GFP$^+$ cells

	Transduced cells	% GFP+ cells		
		Luc.1	GATA1.1	GATA1.2
Fetal HSPCs	3×10^6	8.5	23.6	9.6
Neonatal HSPCs	3×10^6	1.4	4.5	2.6
Adults HSPCs	3×10^6	2.0	3.2	3.7

Supplemental Table 4. Absolute cell numbers counted in each time point of a representative *in vitro* megakaryocytic differentiation assay

		Days								
		0	3	5	7	10	13	17	22	24
Fetal HSPCs	**Luc.1**	80000	50352	45936	48120	53040	54432	72216	103248	90720
	GATA1.1	80000	57624	71976	103620	166440	149040	138096	206784	199296
	GATA1.2	80000	54312	64272	89400	100140	93096	113040	128592	91728
Neonatal HSPCs	**Luc.1**	80000	113381	202943	255245	433651	549792	570106	564365	584458
	GATA1.1	80000	141229	236918	349885	562930	582912	563923	604992	538310
	GATA1.2	80000	106702	194911	269266	519101	508061	510048	505190	453523
Adult HSPCs	**Luc.1**	80000	54179	48852	44132	84539	97097	138994	129168	141312
	GATA1.1	80000	58181	55421	61244	104162	135626	156050	138442	151027
	GATA1.2	80000	47858	47969	55476	108744	112829	146059	120998	114595

Supplemental Table 5. Absolute cell numbers counted in each time point of a representative *in vitro* combined megakaryocytic/erythroid differentiation assay

		Days						
		0	3	5	7	10	13	15
Fetal HSPCs	**Luc.1**	50000	110688	371400	731040	1473120	2165760	1589760
	GATA1.1	50000	155016	523980	950880	1716000	2654400	1984320
	GATA1.2	50000	111768	343800	669120	1604640	2294400	1368960
Neonatal HSPCs	**Luc.1**	50000	236280	631104	812832	1114368	1705344	1699968
	GATA1.1	50000	315312	890016	1233792	2078976	3867648	4460544
	GATA1.2	50000	230400	768528	1119168	2449344	5157504	4167552
Adult HSPCs	**Luc.1**	50000	72096	158928	171504	310080	642432	847104
	GATA1.1	50000	67200	147096	168240	424512	1254336	1698624
	GATA1.2	50000	59568	133080	143328	407328	968064	1348608

Supplemental References

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2. Klusmann JH, Godinho FJ, Heitmann K, et al. Developmental stage-specific interplay of GATA1 and IGF signaling in fetal megakaryopoiesis and leukemogenesis. Genes Dev. 2010;24(15):1659-1672.

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