Supplementary Information for

The GABA Receptor GABRR1 is Expressed on and Functional in Hematopoietic Stem Cells and Megakaryocyte Progenitors

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SI Materials and Methods

Cell Isolation and Culture. Human bone marrow mononuclear cells were purchased from AllCells and cultured in StemSpan™ Serum-Free Expansion Medium II (SFEM II, StemCell Technologies), supplemented with 20ng/ml hSCF (PeproTech), hTPO, hIL-3, hIL-6 and Flt3 (R&D Systems). Mouse bone marrow mononuclear cells were isolated from mice and cultured in StemSpan™ SFEMI (StemCell Technologies), supplemented with mTPO and mSCF (PeproTech). GABA (80uM), Muscimol (80uM), TACA (300uM) or SR 95531 hydrobromide (400nM) (Tocris) are added in the presence of all the cytokines. Medium was changed every other day.

Mouse injection. Mice were randomly grouped and injected once every day using GABA agonists and antagonists. GABA 100 mg/kg, Muscimol 2 mg/kg, TACA 2 mg/kg and SR95531 4 mg/kg was injected by i.p.. The mice were treated for 7 days and then complete blood cell counting was done with the HemaTrue Hematology Analyzer (HESKE).

Transplantations and peripheral blood analyses. B6.SJL-Ptprca Pepcb/BoyJ (Jackson Laboratory) recipient mice were lethally irradiated at a single dose of 9.1 Gy. GR+ and GR- HSCs were sorted out from C57BL/6J mice by flow cytometry and transplanted into recipient mice, together with 2×10^5 supporting cells from the same strain of recipient mice, by retro-orbital venous plexus injection. Peripheral blood was analyzed at 4, 8, 12 and 16 weeks after transplantations. Each time blood was collected from the tail vein and were subsequently lysed using BD Pharm Lyse Buffer (BD Pharmingen), as per the manufacturer's protocol, for 3 min on ice. Leukocytes were stained with antibodies against CD45.1, CD45.2, CD11b, Gr-1, B220, CD3, and NK-1.1.

Virus Production and Transduction. 293T cells are cultured in DMEM supplanted with 10% FBS. Virus was prepared through transfection in 293T by calcium phosphate method with package vectors pVsVg and psPAX2 and backbone vector. Virus was harvested 48 hours later and dead cells were removed with 0.45um filter. Virus supernant was concentrated and Retronectin (Clonetech) was used to pre-coat nontreated plates following manufacturer's instruction. After BSA blocking and PBS washing, virus was coated into the plate by spinning at 2000g for 2 hours. Cells were reseeded in those well for virus transduction. Three days later, 2ug/ml puromycin was added in fresh medium for 7 days.

Colony-Forming Unit Assay. Colony forming assay was performed using the MegaCult™-C Complete Kit with Cytokines (Stemcell Tech, 04971)) following manufacturer's instruction. Colonies were counted after 12-14 days of culture and megakaryopoietic colonies were stained using antibody provided in the kit.

Flow Cytometry. Mouse bone marrow cells were harvested, washed, and resuspended in PBS containing 2% FBS, and subjected to the staining process with antibodies at their optimal concentrations. Cells were firstly blocked with Rat IgG. Then, to enrich HSCs and progenitor populations, cells were stained with APC-conjugated anti-c-Kit (2B8) and then with anti-APC magnetic beads (Miltenyi Biotec) after washing. The c-Kit+cells were eluted from LS columns (Miltenyi Biotec). Those cells then stained with the antibody cocktail directed against cell surface antigens as follows: for early progenitor analysis, Sca-1, Flk2, CD150, CD34, IL-7R, CD16/32, c-kit and the lineage markers Ter-119, B220, CD3, CD4, CD8a, Gr-1, CD11b, CD41. For late stage populations, cells were stained with antibodies specific for Sca-1, Endoglin [CD105], CD150, CD16/32, c-kit [CD117] and the lineage markers for 30min at 4°C. DNA dye was used for the live/dead cells staining.

Surface marker cocktail of mouse hematopoietic stem and progenitor populations:

HSC: Lin-c-kit+Sca1+CD150+Endoglin+

MPP: Lin-c-kit+Sca1+Endoglin-CD150-

GMP: Lin-c-kit+Sca1- CD41-FcgRII+

EP: Lin-c-kit+Sca1- CD41-FcgRII-Endogilin+CD150 -

MkP: Lin-c-kit+Sca1- CD150+CD41+

PreGM: Lin-c-kit+Sca1- CD41-FcgRII-Endogilin-CD150-

Pre CFU-E: Lin-c-kit+Sca1- CD41-FcgRII-Endogilin+CD150+

MEP: Lin-c-kit+Sca1- CD41-FcgRII-Endogilin-CD150+.

Human bone marrow or cord blood cells were stained in a similar way using Rat IgG for blocking and PI for viability. Human cells were stained with the antibodies cocktails as follows: CD34, CD38, CD45RA, CD123, CD71, CD105, CD41 and the lineage markers CD2, CD4, CD8, CD11b/Mac-1, CD14, CD19, CD20, CD56 and CD235a. The GABRR1 antibodies were also used for staining (Bioss Antibodies).

Surface marker cocktail of human hematopoietic stem and progenitor populations:

HSCs/MPPs: Lin-CD34+CD38-

MkP: Lin-CD34+CD38+CD123-CD45RA-CD41+

CMP: Lin-CD34+CD38+CD123lowCD45RA-
MEP: Lin-CD34+CD38+CD123-CD45RA-
GMP: Lin-CD34+CD38+CD123+CD45RA+
EP: Lin-CD34+CD38+CD123-CD45RA-CD71+CD150+.

Before analysis and sorting, cells were passed through 70um strainers. Flow cytometry and cell sorting were performed on a FACS Aria II cell sorter (BD Biosciences) and analyzed using FlowJo software (BD Biosciences).

RNA isolation and Real-Time PCR. Total RNA was extracted by RNeasy Plus Micro kit and RNeasy MinElute Cleanup Kit following manufacturer's instructions (Qiagen). Synthesis of cDNA was performed with the SuperScript™ III Reverse Transcriptase (ThermoFisher Scientific). Real time PCR was done using the Fast SYBR® Green Master Mix (ThermoFisher Scientific) on the 7900 Fast Real-Time PCR System (Applied Biosystems). PCR products were analyzed on a 2% agarose gel.

Electrophysiology. Whole-cell voltage-clamped recordings were made from the dissociated blood cells perfused in oxygenated ACSF containing the following (in mM): 119 NaCl, 26 $NaHCO_3$, 10 glucose, 1.25 NaH_2PO_4, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 2 Na-pyruvate, and 0.5 ascorbic acid, pH 7.4. The cells were visualized under infrared differential interference contrast (IR-DIC) video microscopy (Axioskop 2; Zeiss) and had a diameter of 5-10 μm. Patch pipettes (resistance of 5–7 MΩ) were pulled using borosilicate glass (WPI) on a two-stage vertical puller (Narishige). Cells were voltage clamped at various levels between −80 mV to 60mV. The pipette internal solution contains (in mM): 120 Cs-Cl, 20 tetraethylammonium-Cl, 20 HEPES, 2 EGTA, 4 MgATP, 0.4 NaGTP, 10 phosphocreatine.

Currents were evoked by puffing locally GABA of a saturating concentration at 1 mM to activate all functional GABAARs on the cell. The drug was applied through a second pipette (resistance of 3-4 MΩ) connected to Picospritzer (Cleveland, OH, USA) at a pulse duration of 100 ms and pressure of 5-10 kPa.

Gene Expression Commons. Gene expression commons (https://gexc.riken.jp/) has large-scale publicly available microarray data and perform probeset meta-analysis for a particular microarray platform. GABRR1 expression analysis in mouse hematopoietic stem and progenitor cells in gene

expression commons revealed that of 39 hematopoietic stem, progenitor and mature populations in adult mouse bone marrow, spleen, and thymus, it is uniquely expressed in megakaryocytic progenitors (MkPs), which indicates that GABRR1 may play a role in megakaryopoiesis.

Immunohistochemistry. Skeletally mature, 8-week old, male, C57BL/6J mice (Jax 000664) were euthanized as per our Stanford approved APLAC protocol. The femurs were isolated immediately and fixed in 4% paraformaldehyde overnight at 4C. The femurs were then washed twice in PBS and decalcified in 4M EDTA at 4C for 2 weeks with EDTA change every alternate day. Following de-calcification, the femurs were then transferred into a 30% sucrose solution for 24 hours at 4C. The tissue was then embedded in OCT compound (Scigen, 4583) and stored at -20C until cryosectioning. 8um sections were taken. The sections were stored at -20C until IHC was performed. Slides for IHC were washed twice with 0.25% Triton X-100 diluted in TBS at room temperature for 5 minutes each. The slides were then blocked with 5% anti-donkey and 5% anti-goat antibodies to reduce non-specific binding of the secondary antibodies for 2 hours at room temperature. Primary antibodies (Rabbit anti-mouse GAD 65-67, Abcam GR168030 and rat anti-mouse CD31, BD Pharmingen 558736) diluted 1:250 in TBS with 1% BSA were then added to the sections and allowed to incubate at 4C overnight. After incubation, the slides were then washed twice with 0.25% Triton X-100 diluted in TBS at room temperature for 2 minutes each. The secondary antibodies (Donkey anti-rabbit AF594, Life technologies, 1256153 and goat anti-rat AF488, Invitrogen, 1156624) were then added diluted 1:500 with TBS with 1% BSA at room temperature for 1 hour. During this time, the sections were kept in the dark to prevent photo-bleaching. The slides were then rinsed twice with TBS at room temperature for 5 minutes each. Following the secondary stain, the slides were stained with DAPI (DAPI, Biolegend, B222486) diluted 1:1000 in TBS at room temperature for 10 minutes. The slides were then rinsed twice with TBS at room temperature for 5 minutes each. Aqueous mounting media (Fluoromount G, eBioscience, E099088) was used to mount the tissues. Slides were imaged using an SP8 inverted confocal microscope. Image J software (FIJITM) was used to reconstruct the confocal images.

Statistics. All the experiments were repeated at least 3 times. No blinding was done for any of these experiments. Two tailed Student's *t*-test was used for paired comparison and one way ANOVA for multiple comparisons. *p* value less than .05 was considered significant.

Data availability. The authors declare that the data supporting the findings of this study are available within the article and its Supplementary Information files.

SI Figures

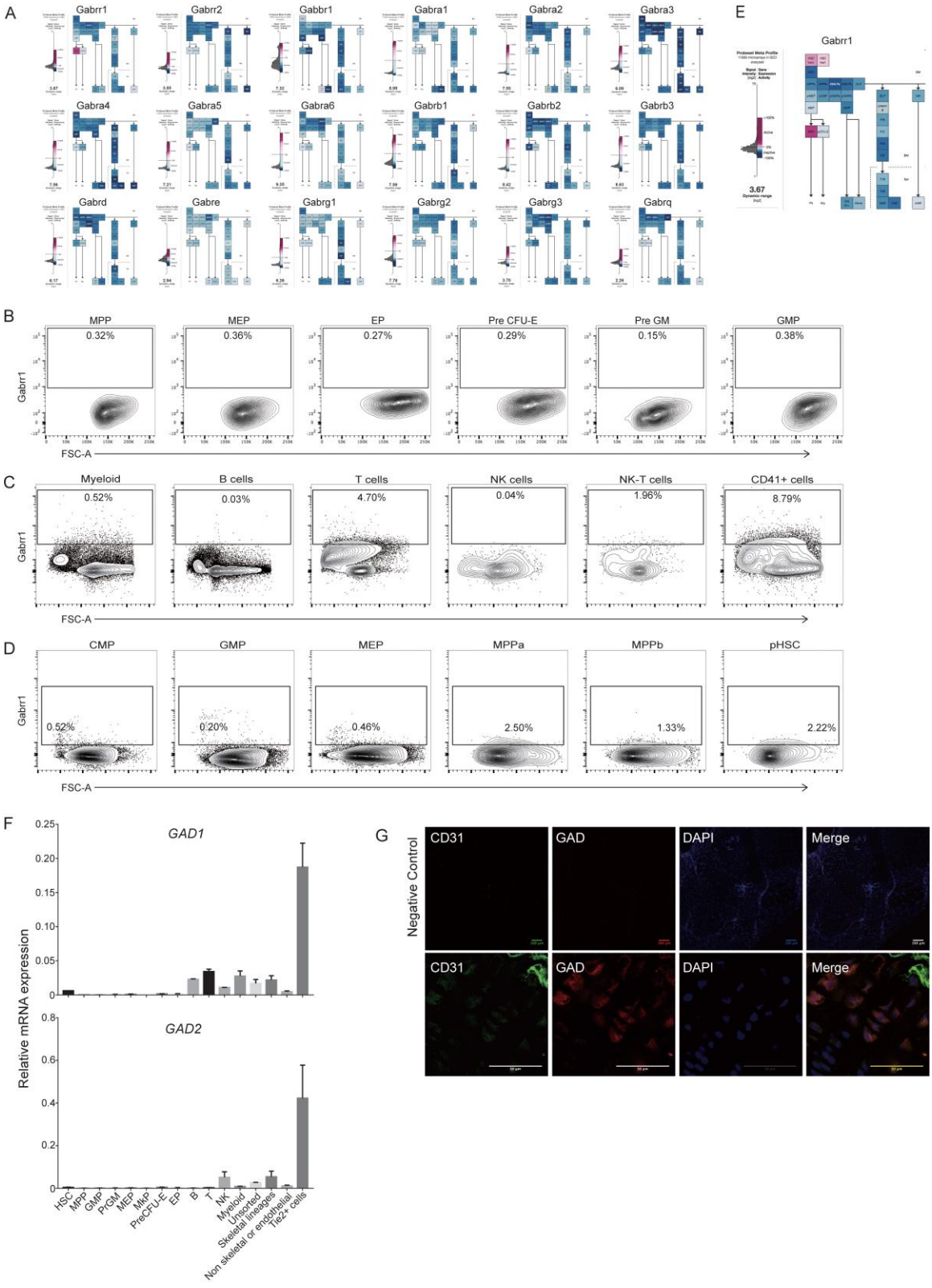
A
Gabrr1
Gabrr2
Gabbr1
Gabra1
Gabra2
Gabra3
Gabra4
Gabra5
Gabra6
Gabrb1
Gabrb2
Gabrb3
Gabrd
Gabre
Gabrg1
Gabrg2
Gabrg3
Gabrq
E
Gabrr1
B
MPP
0.32%
MEP
0.36%
EP
0.27%
Pre CFU-E
0.29%
Pre GM
0.15%
GMP
0.38%
Gabrr1
FSC-A
C
Myeloid
0.52%
B cells
0.03%
T cells
4.70%
NK cells
0.04%
NK-T cells
1.96%
CD41+ cells
8.79%
Gabrr1
FSC-A
D
CMP
0.52%
GMP
0.20%
MEP
0.46%
MPPa
2.50%
MPPb
1.33%
pHSC
2.22%
Gabrr1
FSC-A
F
GAD1
GAD2
Relative mRNA expression
0.25
0.20
0.15
0.10
0.05
0
0.8
0.6
0.4
0.2
0
HSC
MPP
GMP
PrGM
MEP
MkP
PreCFU-E
EP
B
T
NK
Myeloid
Unsorted
Skeletal lineages
Non skeletal or endothelial
Tie2+ cells
G
Negative Control
CD31
GAD
DAPI
Merge
CD31
GAD
DAPI
Merge

Figure S1. A). GEXC analysis showed the gene expression pattern of family members of GABA receptors in mouse hematopoietic cells in pooled microarray data. B). Gabrr1 expression analysis in mouse HSPCs from bone marrow mononuclear cells (related to Figure 1C). C). Gabrr1 expression analysis in different mature blood cells. D). Gabrr1 expression in immuno-phenotypically defined HSCs (pHSCs), MPPa and MPPb and other HSPC populations. Data are representative of n=6 mice. E). GEXC analysis showed the gene expression pattern of Gabrr1 in microarray data reported by Sanjuan-Pla et al., 2013. F). GAD1 and GAD2 expression in different hematopoietic and niche cell populations by real time PCR analysis. Data were presented as mean±SD from three replicates of the same group and are representative of three independent experiments G). Immunostaining for the coexpression of endothelial marker CD31 and GADs. Top panel is negative control, which showed staining with secondary antibodies only. Scale bar, 50uM.

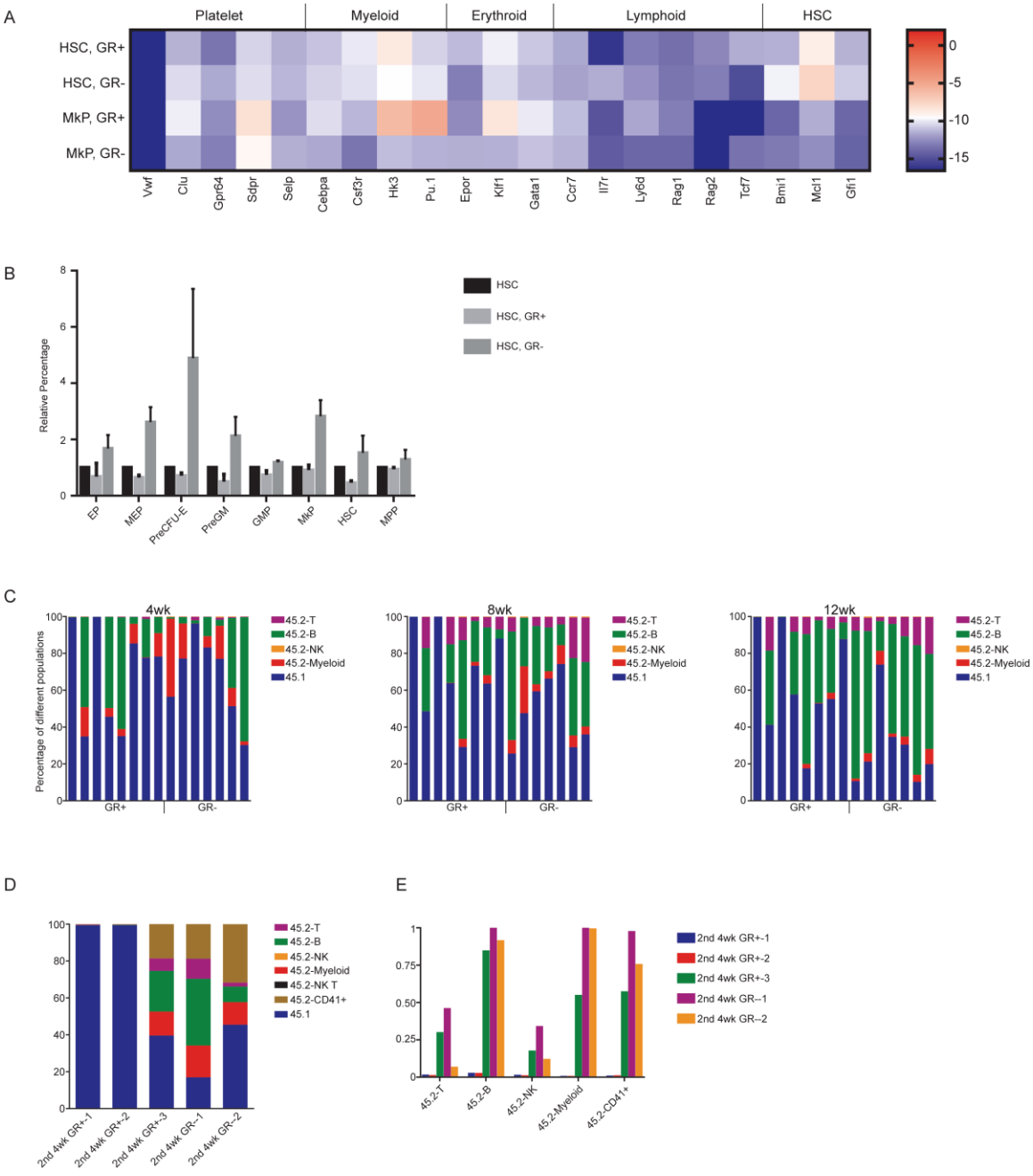
A
Platelet
Myeloid
Erythroid
Lymphoid
HSC
HSC, GR+
HSC, GR-
MkP, GR+
MkP, GR-
Vwf
Clu
Gpr64
Sdpr
Selp
Cebpa
Csf3r
Hk3
Pu.1
Epor
Klf1
Gata1
Ccr7
Il7r
Ly6d
Rag1
Rag2
Tcf7
Bmi1
Mcl1
Gfi1
0
-5
-10
-15
B
Relative Percentage
HSC
HSC, GR+
HSC, GR-
EP
MEP
PreCFU-E
PreGM
GMP
MkP
HSC
MPP
C
4wk
8wk
12wk
Percentage of different populations
45.2-T
45.2-B
45.2-NK
45.2-Myeloid
45.1
GR+
GR-
D
45.2-NK T
45.2-CD41+
2nd 4wk GR+-1
2nd 4wk GR+-2
2nd 4wk GR+-3
2nd 4wk GR--1
2nd 4wk GR--2
E

Figure S2. A). Expression analysis of the hematopoietic lineages associated genes in purified mouse GR+ HSCs, GR- HSCs, GR+ MkPs and GR- MkPs by real-time PCR. Numbers indicate ΔCt values to the housekeeping gene beta-actin. B). Flow cytometry analysis of the HSPC subpopulations after differentiation in vitro from mouse GR+ HSCs and GR- HSCs. Data shown are mean ± SD from three individual samples of the same group and are representative of at least three independent experiments. C). Percentage of chimerism at 4, 8 and 12 weeks after transplanting GR+ HSCs (n = 8 mice), or GR- HSCs (n = 7 mice) into primary recipients. D-E). Multilineage reconstitution after secondary transplantation confirms the HSC identity of the purified cells. Each column represents an individual mouse.

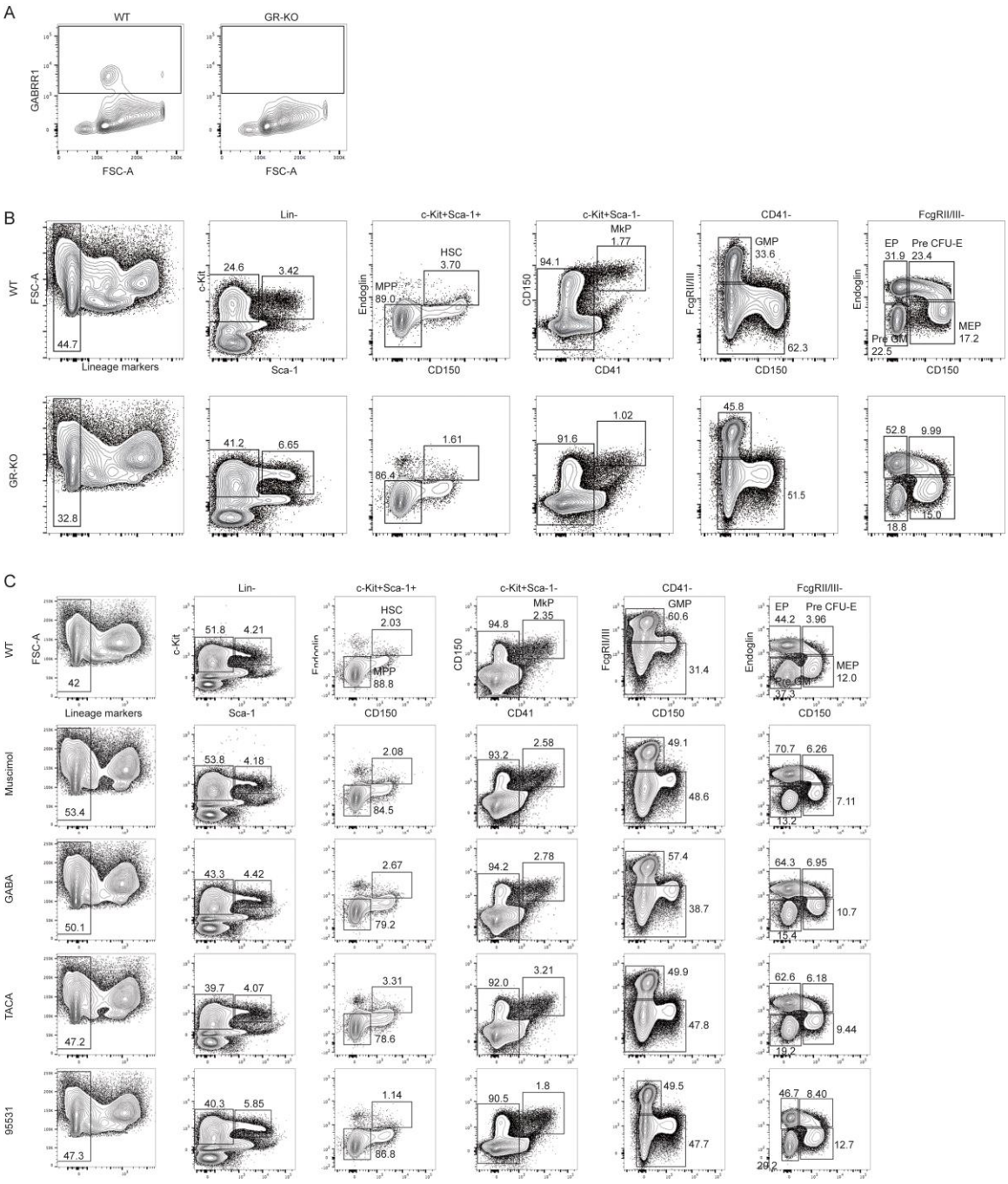
A
WT
GR-KO
GABRR1
FSC-A
B
Lin-
c-Kit+Sca-1+
c-Kit+Sca-1-
CD41-
FcgRII/III-
WT
GR-KO
FSC-A
Lineage markers
c-Kit
Sca-1
Endoglin
CD150
CD41
FcgRII/III
HSC
MPP
MkP
GMP
EP
Pre CFU-E
MEP
Pre GM
C
Muscimol
GABA
TACA
95531

Figure S3. A). GABRR1 could be detected in bone marrow cells of B6129SF2/J wildtype mice (left), but not in those of B6;129S4-Gabrr1^{tm1Llu}/J mice (right). B). Multicolor flow cytometry analysis of HSPC population changes in B6;129S4-Gabrr1^{tm1Llu}/J mice (bottom panel) and its approximate control B6129SF2/J (top panel). C). Flow cytometry of bone marrow HSPC subpopulation changes by multicolor flow cytometry in mice treated with agonists or antagonists of Gabrr1.

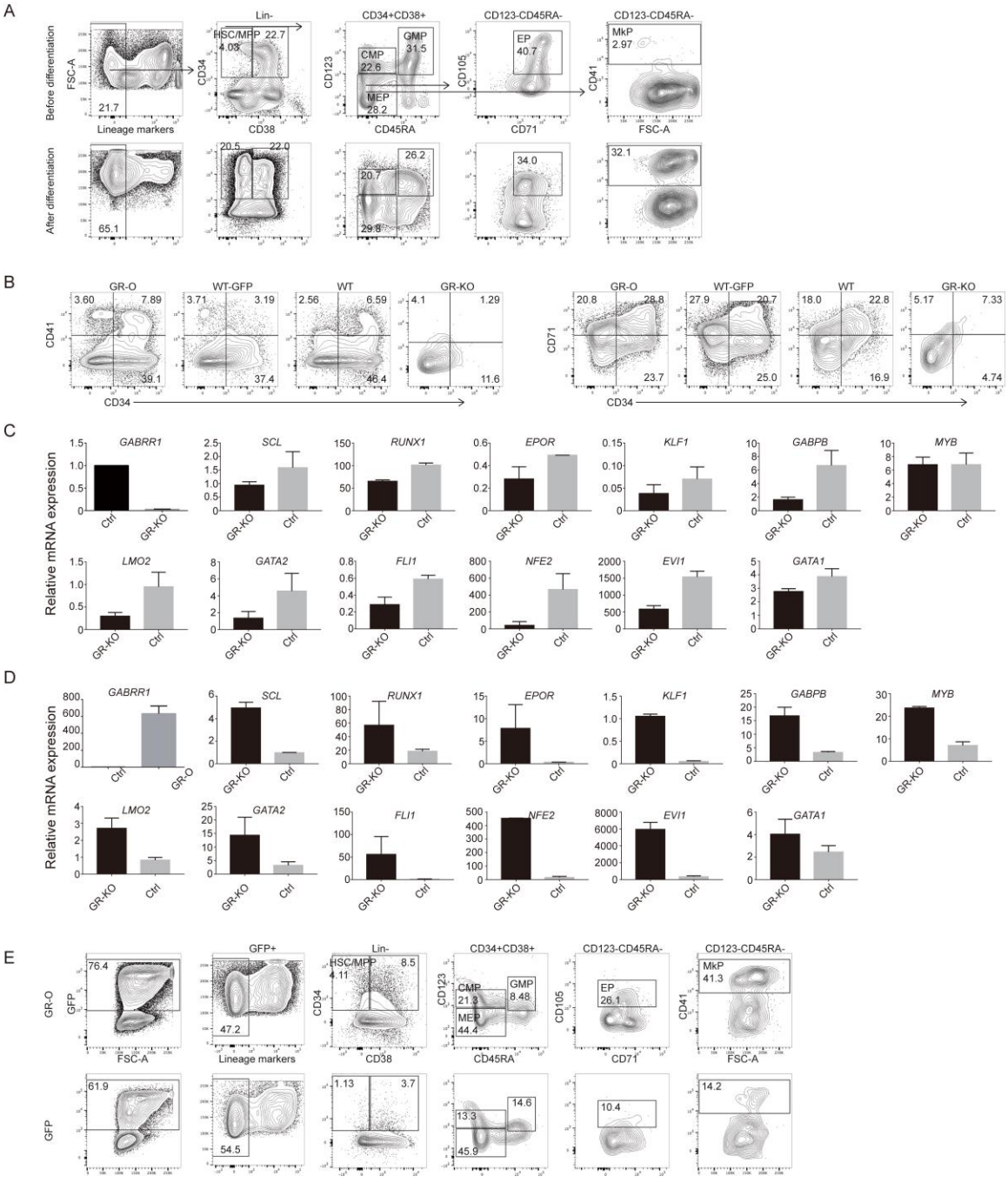
A
Before differentiation
After differentiation
FSC-A
Lineage markers
21.7
65.1
Lin-
HSC/MPP 22.7
4.03
CD34
CD38
20.5
22.0
CD34+CD38+
CMP 22.6
GMP 31.5
MEP 28.2
CD123
CD45RA
20.7
26.2
29.8
CD123-CD45RA-
EP 40.7
CD105
CD71
34.0
CD123-CD45RA-
MkP 2.97
CD41
FSC-A
32.1
B
GR-O
WT-GFP
WT
GR-KO
CD41
CD34
3.60
7.89
39.1
3.71
3.19
37.4
2.56
6.59
46.4
4.1
1.29
11.6
CD71
20.8
28.8
23.7
27.9
20.7
25.0
18.0
22.8
16.9
5.17
7.33
4.74
C
Relative mRNA expression
GABRR1
SCL
RUNX1
EPOR
KLF1
GABPB
MYB
LMO2
GATA2
FLI1
NFE2
EVI1
GATA1
Ctrl
GR-KO
D
Relative mRNA expression
GABRR1
SCL
RUNX1
EPOR
KLF1
GABPB
MYB
LMO2
GATA2
FLI1
NFE2
EVI1
GATA1
Ctrl
GR-O
GR-KO
E
GR-O
GFP
FSC-A
76.4
47.2
Lineage markers
GFP+
Lin-
HSC/MPP 8.5
4.11
CD34
CD38
1.13
3.7
CD34+CD38+
CMP 21.3
GMP 8.48
MEP 44.4
CD123
CD45RA
13.3
14.6
45.9
CD123-CD45RA-
EP 26.1
CD105
CD71
10.4
CD123-CD45RA-
MkP 41.3
CD41
FSC-A
14.2
GFP
61.9
54.5

Figure S4. A). Gating strategy of human HSPCs subpopulations in multicolor flow cytometry and the HSPC population changes before and after 7 days of differentiation. Data are representative of at least three independent experiments. B). Flow cytometry analysis of CD34+CD41+ (MkP) and CD34+CD71+ (EP) percentage in 7-day differentiated CD34+ cells with GABRR1 knockout or overexpression with the non-treated or GFP-transduced cells as the control, respectively. C-D). Gene expression analysis of GABRR1, megakaryocytic and/or erythroid cells-associated genes by real time PCR from bone marrow CD34+ after knockout (C) or overexpression (D) of GABRR1 and then 7 days of differentiation. Data shown are mean±SD from three replicates of the same group and are representative of at least three independent experiments. E). Multicolor flow cytometry of human HSPCs after 7 days of differentiation of bone marrow CD34+ cells with GABRR1 overexpression. Data are representative of at least three independent experiments. GR-O, GABRR1 overexpression; GR-KO, GABRR1 knockout.