Wip1 regulates hematopoietic stem cell development in the mouse embryo

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Materials and Methods

Mouse and Embryo Generation. Wild type C57BL/6-Ly5.2 and *Wip1*$^{+/-}$ heterozygous-Ly5.2 mice(1) were used for timed mattings and C57BL/6-Ly5.1/5.2 or C57BL/6-Ly5.1 mice (8-12 weeks) were used as transplantable recipients. *Wip1* heterozygous deficient (*Wip1*$^{+/-}$, HT) males and females were crossed to obtain *Wip1* homozygous deficient embryos (*Wip1*$^{-/-}$, KO). *Wip1*$^{+/-}$ embryos were not tested in this study since the phenotypes of heterozygotes are normal compared with wild type (*Wip1*$^{+/+}$, WT). Embryos (E10.5–E12.5) were staged by counting somite pairs(2). AGM, YS or Fetal liver were dissected and tails were used for genotyping. Mice were housed in the animal facilities and experimentation complied with the ethics committee of the affiliated hospital of Academy of Military Medical Sciences.

Hematopoietic Progenitor and Stem Cell Assays. Sing-cell suspension from fetal liver, AGM and YS was plated into assayed in 0.9% methylcellulose (Sigma) supplemented with 10 ng/ml IL-3 (Peprotech), 50 ng/ml SCF (Peprotech), 5 ng/ml IL-6 (Peprotech), and 3 U/ml Epo (sansheng pharmaceutical co.) for colony forming unit-culture (CFU-C) assay. Hematopoietic colonies were counted after 7–8 days culture. BFU-Es, CFU-GMs and CFU-Mixes were clarified in the total CFU-C counting, whereas CFU-Es and HPP-CFCs were counted on the 3rd day and 10th day, respectively. Cells from hematopoietic tissues, flow cytometric sorting or cultures were injected intravenously into irradiated recipients (9.0 Gy Cobalt-60-irradiation, split dose). Peripheral blood of recipients was taken (at 16 weeks) for Ly5.1-/Ly5.2-specific flow cytometric analysis. Recipients are considered repopulated when ≥10% of cells are donor-derived.

Explant Culture. E10.5 WT and *Wip1*$^{-/-}$ AGM were cultured for 3 days in MyeloCult™ M5300 (StemCell Technology) supplemented with 10^{-6} M hydrocortisone (Sigma) on Durapore 0.65 μm filters (Millipore) supported by stainless steel stands at the gas-liquid interface as previously described(3).

OP9/OP9-DL1 Co-culture Cells were cultured on OP9 (cytokines from Peprotech: 50 ng/ml SCF, 20 ng/ml Flt3 ligand, 20 ng/ml IL-7, 8 days) or OP9-DL1 (100 ng/ml SCF, 100 ng/ml Flt3 ligand, 100 ng/ml IL-3) for 4-6 days ± Wip1 inhibitor (25 μM,

CCT007093, Sigma)(4, 5). Cells were harvested by mechanical pipetting for further experiments.

Flow Cytometric Assay. Cells from embryonic hematopoietic tissues, cultures and adult hematopoietic tissues were stained by fluorescence conjugated/unconjugated antibodies (Online Supplementary Table S3) for 30' on ice, corresponding secondary antibodies were used if necessary. Sorted cells were collected for transplantation, co-culture or in lysis buffer for RNA extraction. Ki67 and 7-AAD staining was performed for cell cycle analysis. Embryonic cell proliferation was determined after 2-hour injection(i.p.) of 5-bromo-2'-deoxyuri-dine(BrdU, 100 mg/kg) into the pregnants(6). For apoptotic analysis, Annexin V and 7-AAD staining was performed. All antibodies were listed (Online Supplementary Table S3). Cytometry was performed on Aria II (BD Biosciences). FACS data were analyzed with FlowJo software.

Gene Expression Analysis. RNA from embryonic tissue or sorted cells was extracted according to RNeasy Micro Kit (QIAGEN) and cDNA were reversed transcribed with SuperScriptIII reverse transcriptase (QIAGEN). Real-time PCR was performed by using GoTaq® Master Mixes (Promega) and detected on Applied Biosystems 7500. Sequences of primers listed (Online Supplementary Table S2).

Statistical Analysis

All data are presented as the mean±SEM. Student's test was used for comparison of various groups. $P<0.05$ was considered statistically significant, * presents $P<0.05$, ** present $P<0.01$, *** present $P<0.001$.

References

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Supplemental figures and tables

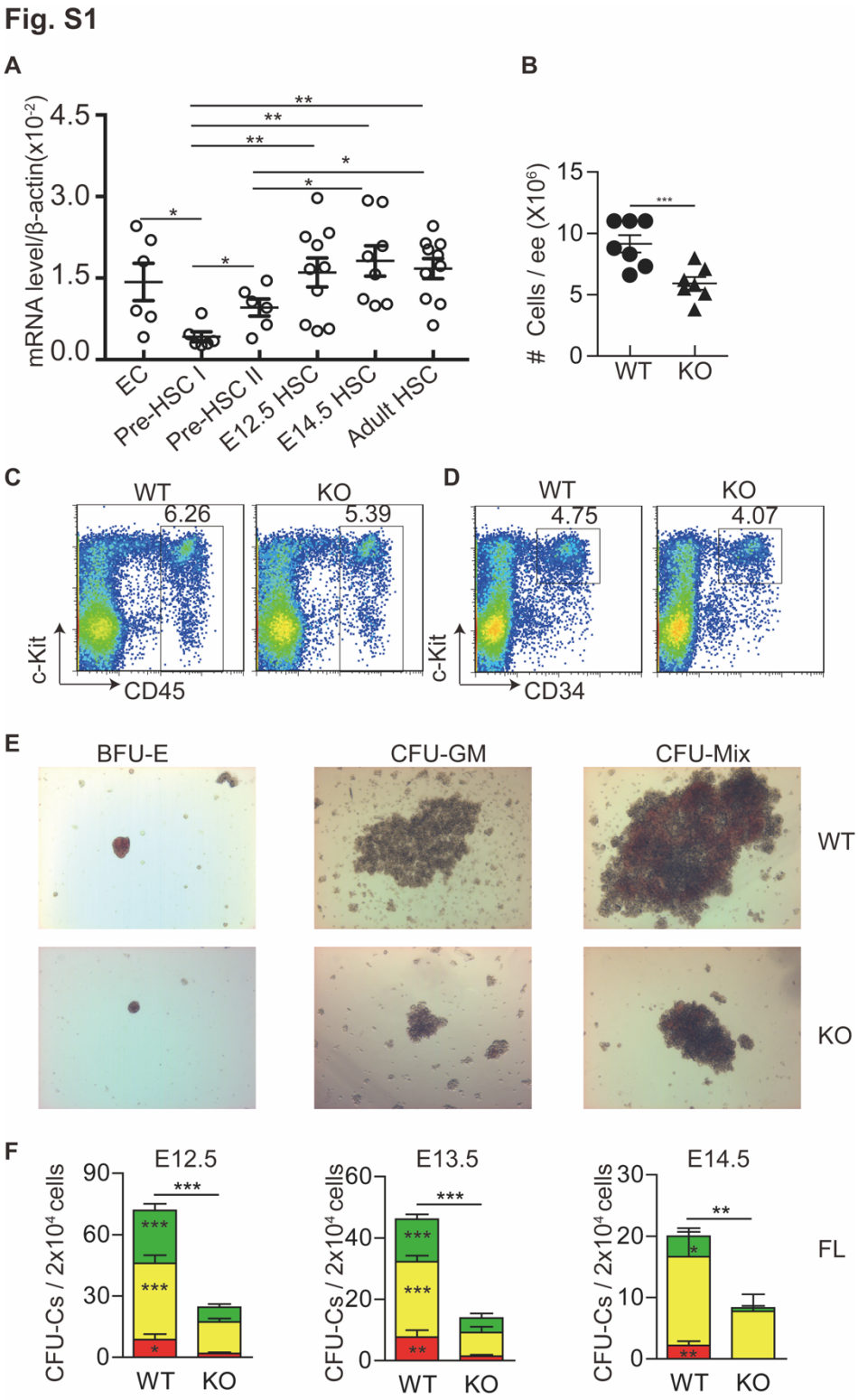

Fig. S1. *Wip1* knockout resulted in the reduction of HPCs in E12.5-E14.5 fetal liver. (A) qRT-PCR data showed the expression of *Wip1* in various indicated cell

types. Endothelial cells (EC, $CD31^{+}VE\text{-}Cadherin^{+}CD41^{-}CD43^{-}CD45^{-}Ter119^{-}$), pre-HSC I ($CD31^{+}CD45^{-}CD41l^{ow}c\text{-}Kit^{+}CD201^{high}$), pre-HSC II ($CD31^{+}CD45^{+}c\text{-}Kit^{+}CD201^{high}$) from E11.5 AGM region. E12.5 HSCs ($Lin^{-}Sca1^{+}Mac1^{low}CD201^{+}$) and E14.5 HSCs ($CD45^{+}CD150^{+}CD48^{-}CD201^{+}$) from fetal liver. Adult HSCs ($Lin^{-}Sca1^{+}c\text{-}Kit^{+}CD34^{-}CD135^{-}CD150^{+}CD48^{-}$) from bone marrow of 8-week old mice. (B)The cell number of fetal liver in the E13.5 embryos. (C) Representative flow cytometric analysis of phenotypic HPCs ($CD45^{+}$) (C) and ($CD34^{+}c\text{-}Kit^{+}$) (D). (E) Morphology of hematopoietic colonies derived from E12.5 fetal liver cells. (F) CFU-C assay showed the number of colonies per $2x10^{4}$ fetal liver cells from E12.5 to E14.5. Colony types were indicated by color bars. E12.5, E13.5, E14.5 n=3, 3, 2 respectively. *P <0.05, **P <0.01, ***P <0.001.

Fig.S2

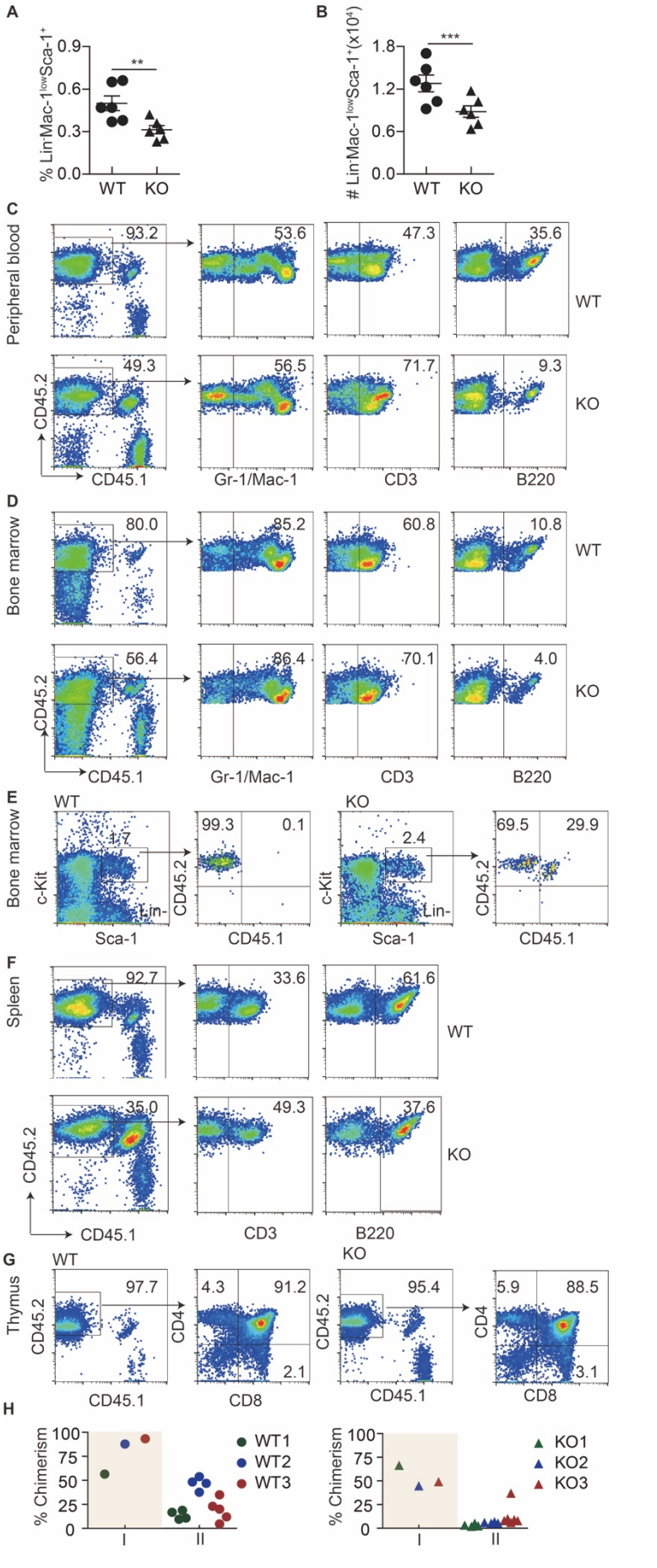
A
% Lin-Mac-1lowSca-1+
0.9
0.6
0.3
0.0
**
WT
KO
B
Lin-Mac-1lowSca-1+(x10⁴)
1.8
1.2
0.6
0.0
***
WT
KO
C
Peripheral blood
93.2
53.6
47.3
35.6
WT
49.3
56.5
71.7
9.3
KO
CD45.2
CD45.1
Gr-1/Mac-1
CD3
B220
D
Bone marrow
80.0
85.2
60.8
10.8
WT
56.4
86.4
70.1
4.0
KO
CD45.2
CD45.1
Gr-1/Mac-1
CD3
B220
E
Bone marrow
WT
1.7
c-Kit
Lin-
Sca-1
99.3
0.1
CD45.2
CD45.1
KO
2.4
c-Kit
Lin-
Sca-1
69.5
29.9
CD45.2
CD45.1
F
Spleen
92.7
33.6
61.6
WT
35.0
49.3
37.6
KO
CD45.2
CD45.1
CD3
B220
G
Thymus
WT
97.7
CD45.2
CD45.1
4.3
91.2
2.1
CD4
CD8
KO
95.4
CD45.2
CD45.1
5.9
88.5
3.1
CD4
CD8
H
% Chimerism
100
75
50
25
0
I
II
WT1
WT2
WT3
% Chimerism
100
75
50
25
0
I
II
KO1
KO2
KO3

Fig. S2. Multilineage output of hematopoietic stem cells was decreased in E12.5 fetal liver after *Wip1* deletion. Percentage (A) and Number (B) of $Lin^{-}Mac\text{-}1^{low}Sca\text{-}1^{+}$ cells in the E12.5 WT and *Wip1*$^{-/-}$ fetal liver. n=6, ***P* =0.0045, ****P* <0.001. Representative flow cytometric analysis of multilineage output in the peripheral blood (C), bone marrow (D), spleen (F), thymus(G) of repopulated recipients receiving E12.5 fetal liver cells. The donor derived-HSC in the bone marrow (E) of repopulated recipients by injection fetal liver cells. (H) The self-renewal capacity of reconstituted donors from E12.5 fetal liver.

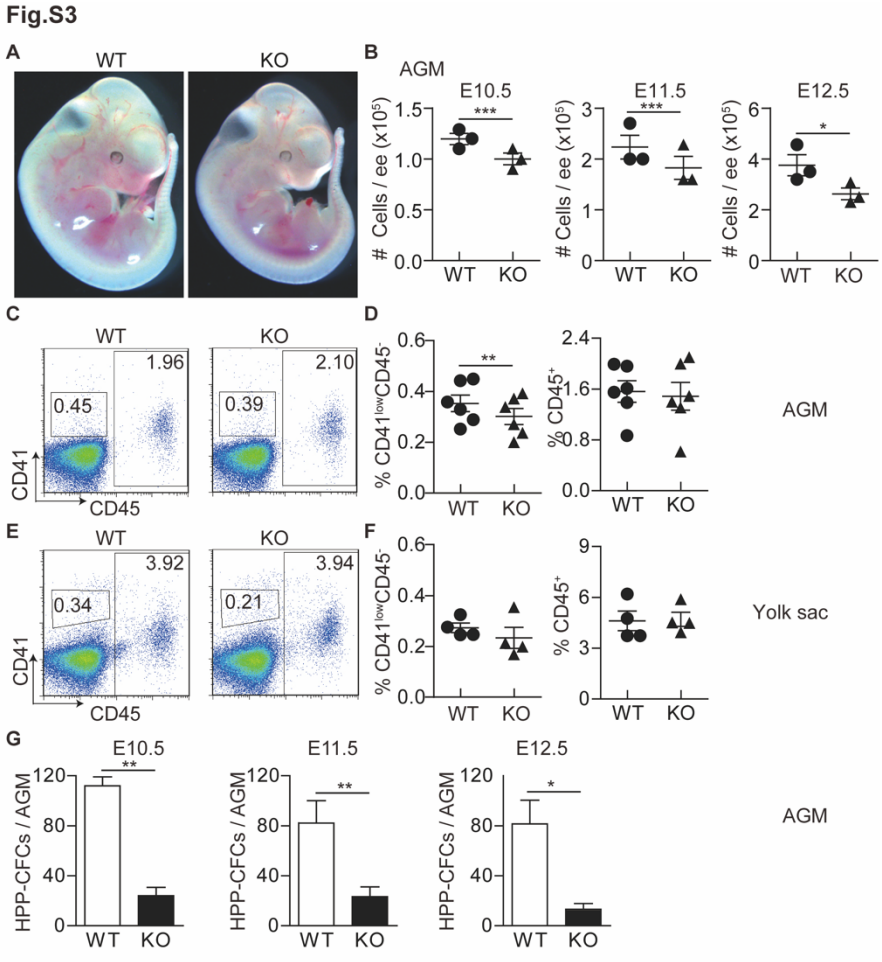

Fig. S3. Hematopoietic progenitor cells were reduced in the E10.5-E12.5 AGM and yolk sac. (A) The image of E11.5 embryos. WT 45 sp, KO 44 SP. (B) Cell numbers of E10.5-E12.5 WT and *Wip1*$^{-/-}$ AGM regions. n=3, **P* =0.0112, ****P* <0.001. Representative flow cytometric analysis of hematopoietic progenitor cells by CD41 and CD45 (C and E) and percentages of CD41^{low}CD45^{-} and CD45^{+} cells (D and F) in E11.5 WT and *Wip1*$^{-/-}$ AGM regions and YS, respectively. D, n= 6, F, n=4, ***P* =0.0037. (G) The reduction of HPP-CFCs in E10.5-12.5 *Wip1*$^{-/-}$ AGM region. n=3, 4 and 4 respectively. **P* =0.0113, ***P* <0.01.

Fig.S4

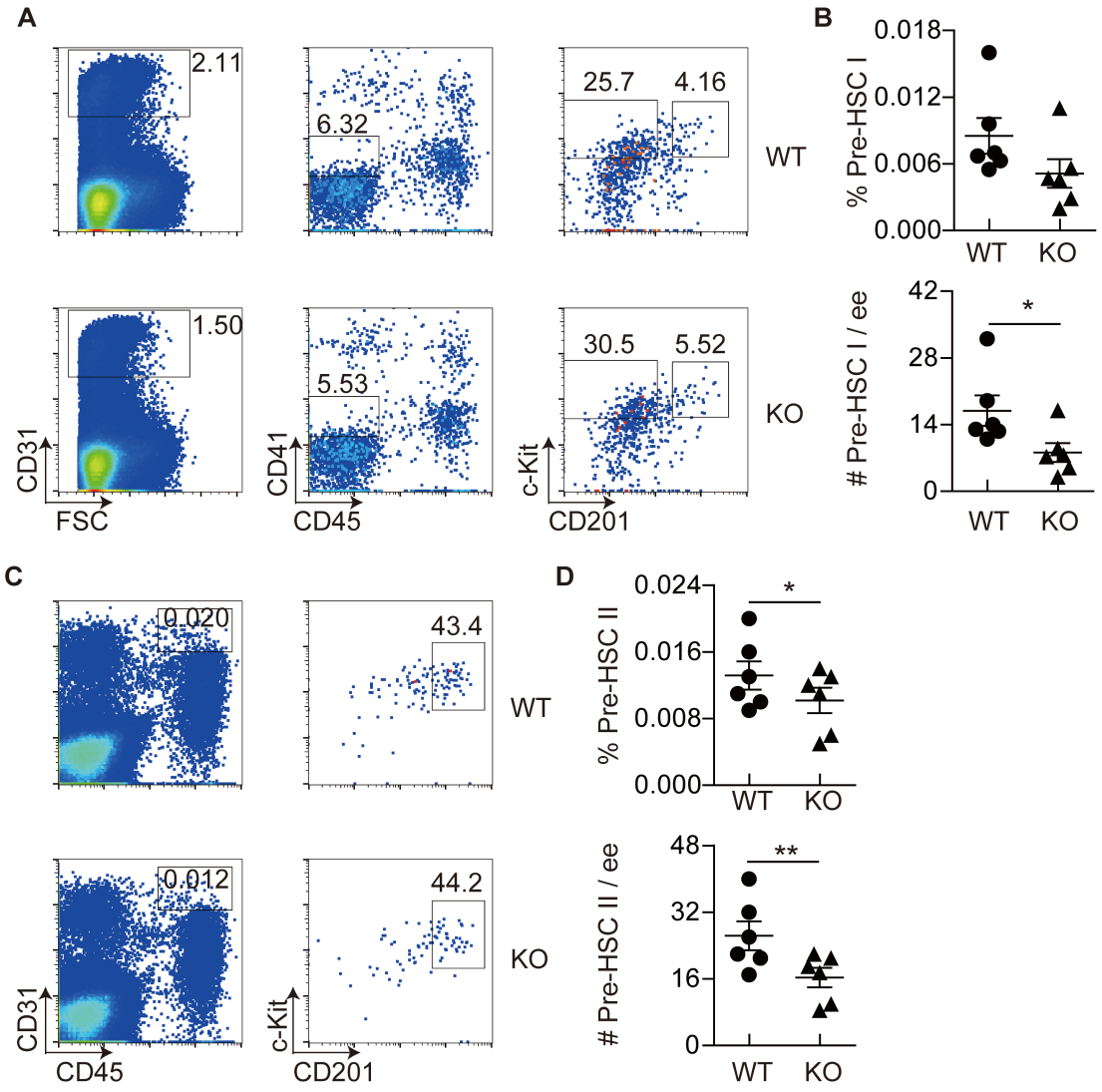

Fig. S4. Phenotypically defined pre-HSCs were decreased in the E11.5 AGM. (A) Flow cytometric analysis presented the percentage of pre-HSC I ($CD31^{+}CD41^{low}CD45^{-}c\text{-}Kit^{+}CD201^{high}$) and pre-HSC II ($CD31^{+}CD45^{+}c\text{-}Kit^{+}CD201^{high}$) in E11.5 WT and *Wip1*$^{-/-}$ AGM region. The percentages and absolute numbers of pre-HSC I (B) and pre-HSC II (D), respectively. n=6, *P <0.05, **P=0.0016.

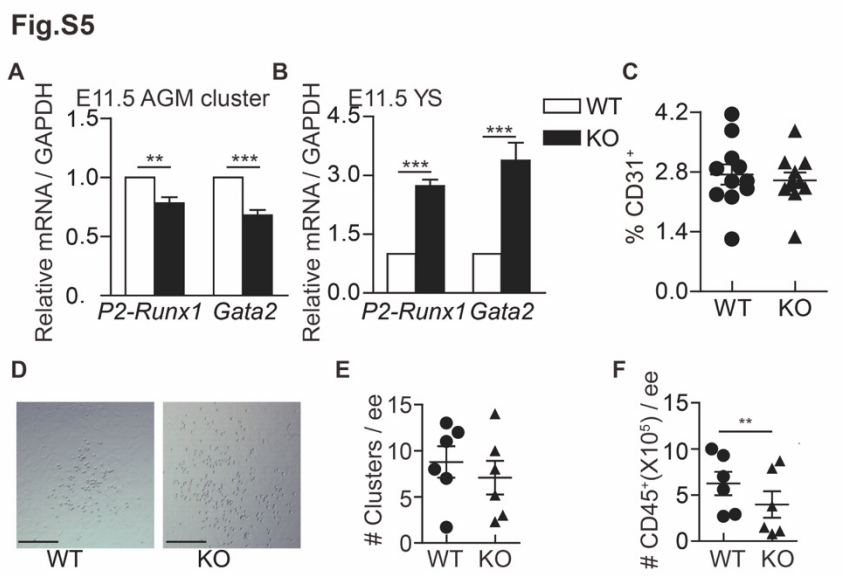

Fig.S5. *Wip1* was involved in the expansion and/or maturation of HPCs. (A) Gene expression from $CD31^{+}$c-Kit^{high} hematopoietic cluster cells of E11.5 AGM region. n=6, ***P*=0.0033, ****P*=0.0004. (B) The expression of hematopoiesis related genes was increased in the E11.5 *Wip1*$^{-/-}$ Yolk sac. n=6, ****P* <0.001. (C) The percentage of $CD31^{+}$ cells in the E11.5 AGM region. P=0.156, n=11. (D) Morphology of hematopoietic clusters generated from endothelial cells ($CD31^{+}CD41^{-}CD45^{-}Ter119^{-}$) of E10.5 AGM after 3-day OP9 co-culture. Numbers of cluster (E) and $CD45^{+}$ (F) hematopoietic cells from endothelial cells after 3-day and 8-day OP9 co-culture, respectively. n=6, ***P* = 0.0058. Scale bars: 50 μm.

Fig.S6

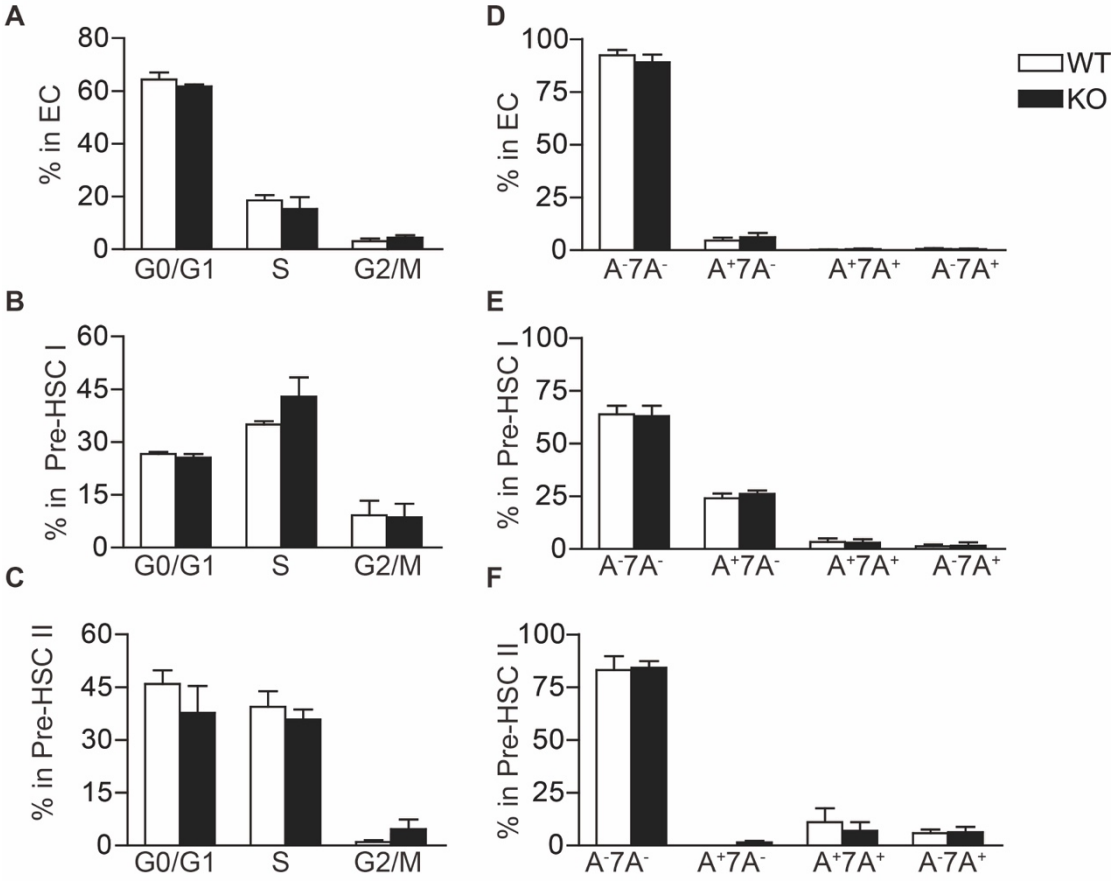

Fig. S6. *Wip1* knockout didn't disturbed the proliferation and apoptosis of pre-HSCs in the E11.5 AGM region. (A-C) Flow cytometric analysis displayed percentages of G0/G1, S, G2/M phase by BrdU/7AAD staining within ECs, pre-HSC I and pre-HSC II in the E11.5 WT and *Wip1*$^{-/-}$ AGM region. (D-F) The apoptosis status of ECs(D), pre-HSC I (E) and pre-HSC II(F) was similar between in E11.5 WT and *Wip1*$^{-/-}$ AGM region by Annexin V(A) and 7-AAD(7A) staining. EC=Endothelial cells(CD31^{+}CD41^{-}CD45^{-}), pre-HSC I (CD31^{+}CD41^{low}CD45^{-}) and pre-HSC II (CD31^{+}CD45^{+}).

Table S1: HSC Activity in Embryonic Tissues

Time post transplantation				> 16 Weeks	
Stage	**Genotype**	**ee[a]**	**EXP(n)**	**Re[b]/Total**	**Chimerism(%)**
E12.5 Fetal Liver	WT	0.05	3	15/17	65.0±7.8
	KO	0.05	3	6/19	12.7±4.6
E14.5 Fetal Liver	WT	0.01	2	10/10	89.9±1.0
	KO	0.01	2	15/15	71.9±3.1
E11.5 AGM (42-50 sp)	WT	1	4	4/12	21.8±9.6
	KO	1	4	0/9	0.1±0.0
E12.5 AGM	WT	1	3	6/6	92.2±1.0
	KO	1	3	2/7	10.7±8.0
E11.5 Yolk sac(41-45 sp)	WT	1	3	2/6	15.4±10.5
	KO	1	3	3/9	9.1±4.1
E12.5 Yolk sac	WT	1	3	9/9	87.3±1.4
	KO	1	3	8/11	43.2±9.7
E10.5 AGM (35-39 sp[c])	WT	1	2	11/12	56.4±8.2
	KO	1	2	4/9	27.5±11.5
E11.5 AGM T1 (41-45 sp)	WT	1	2	3/4	19.6±8.3
	KO	1	2	2/6	15.2±9.7
E11.5 AGM T2 (41-45 sp)	WT	0.5-1	3	6/12	27.6±8.7
	KO	0.5-1	3	5/16	14.3±5.6
E11.5 AGM T1 CCT	DMSO	1	2	6/7	33.3±9.0
(41-45 sp)	CCT	1	2	0/7	0.6±0.2
E11.5 AGM T2 CCT	DMSO	1	3	7/9	46.2±8.8
(41-45 sp)	CCT	1	3	5/10	13.5±5.2

[a]ee, embryo equivalent.

[b]Re, repopulated.

[c]sp, somite pairs.

Table S2. Primers for Real-Time PCR

Genes	Primer sequences
GAPDH	5'-AGGTCGGTGTGAACGGATTTG-3'
	5'-GGGGTCGTTGATGGCAACA-3'
P2-Runx1	5'-AAGATCCGAGCCCCTGTC-3'
	5'-TCACAACAAGCCGATTGAGT-3'
Gata2	5'-GCG AAA ACC AAA CTG CAT AAG C-3'
	5'-CTG TCT CCC AGA AAC CAA GAG C-3'
β-Actin	5'-GCAAGTGCTTCTAGGCGGAC-3’
	5'-AAGAAAGGGTGTAAAACGCAGC-3
Wip1	5'- CTGACT GAT AGCCCT ACT TACAAC A-3'
	5'- GAG AAG GCA TTA CTG CGA ACA-3'

Table S3. Antibody list

Antibodies	Clone	Company
APC anti-mouse CD45.1	A20	eBioscience
PE anti-mouse CD45.2	104	eBioscience
APC-efluor780 anti-mouse CD3e	145-2C12	eBioscience
APC anti-mouse CD201	1560	eBioscience
APC anti-mouse CD19	eBio1D3	eBioscience
PE-Cy7 anti-mouse Mac-1	M1/70	eBioscience
PE-Cy7 anti-mouse Gr-1	RB6-8C5	eBioscience
FITC anti-mouse B220	RA3-6B2	eBioscience
PE anti-mouse CD31	MEC13.3	BD
APC anti-mouse CD31	MEC13.3	BD
PE-Cy7 anti-mouse CD45	30-F11	eBioscience
PE anti-mouse CD45	30-F11	eBioscience
FITC anti-mouse CD41	MWReg30	BD
APC anti-mouse c-Kit	2B8	eBioscience
PE-Cy7 anti-mouse c-Kit	2B8	eBioscience
FITC anti-mouse CD48	HM48-1	eBioscience
Biotin anti-mouse CD11b	M1/70	eBioscience
Biotin anti-mouse CD8a	53-6.7	eBioscience
Biotin anti-mouse CD4	GK1.5	eBioscience
Biotin anti-mouse CD127	A7R34	eBioscience
Biotin anti-Human/mouse CD45R (B220)	RA3-6B2	eBioscience
Biotin anti-mouse Ly-6G (Gr-1)	RB6-8C5	eBioscience
Biotin anti-mouse Ter-119	TER119	eBioscience
PE anti-mouse CD150	TC15-12F12.2	Biolegend
PE-Cy7 anti-mouse Ter119	TER 119	eBioscience
FITC anti-mouse CD34	RAM34	eBioscience
APC anti-mouse Sca-1	D7	eBioscience
PerCP-Cy5.5 anti-mouse Mac-1	M1/70	eBioscience
PerCP-Cy5.5 7-AAD		eBioscience
Streptavidin-APC-Cy7	Streptavidin	BD
FITC Mouse Anti-human/mouse Ki-67 Set		BD
Cytofix/Cytoper™ Fixation/Permeabilization Kit		BD
Annexin V Apoptosis Detection kit		eBioscience