Supplemental Information

Endothelial Smad4 Restrains the Transition to Hematopoietic Progenitors via Suppression of ERK Activation

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Inventory of Supplemental Information

Supplemental Data include 9 Supplemental Figures and Supplemental Materials and Methods.

Supplemental Figure 1

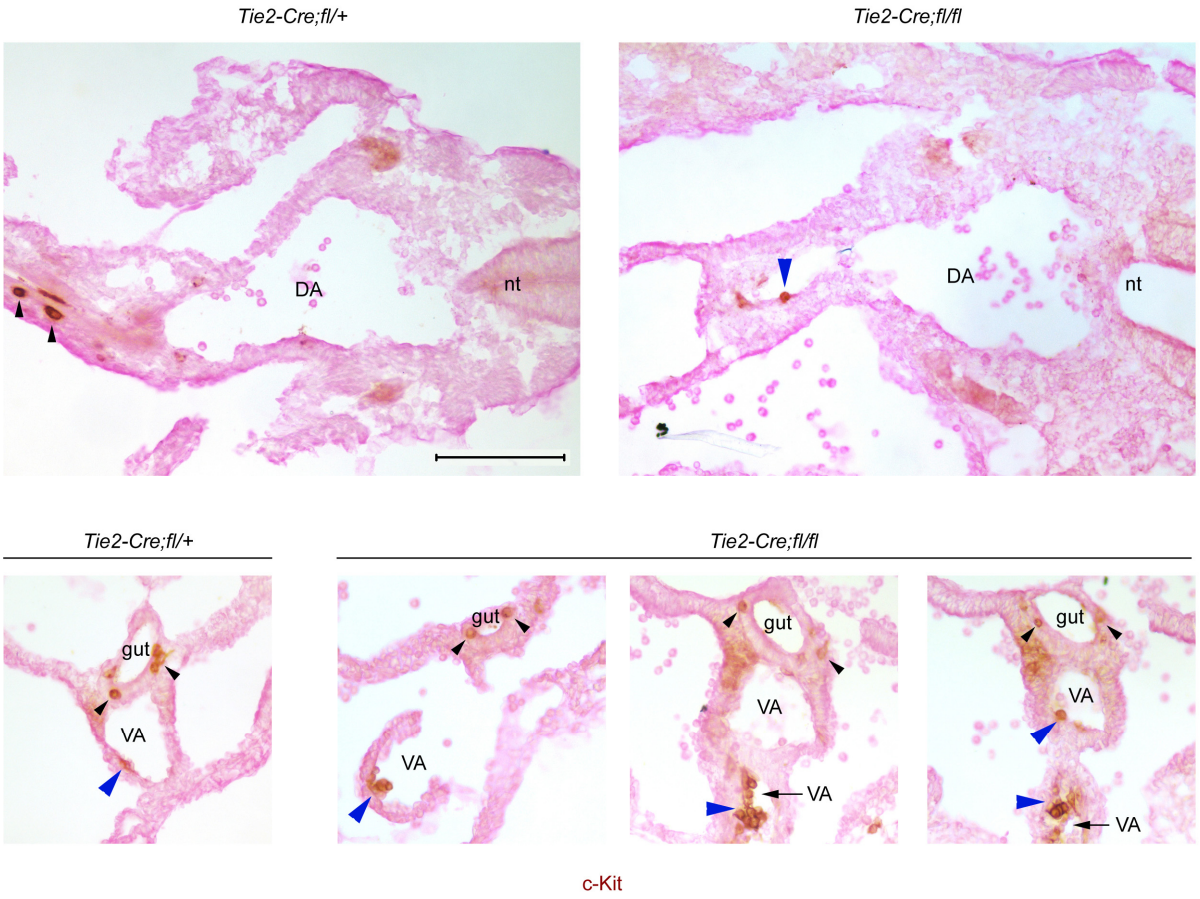

Figure S1. Increased intra-vascular hematopoietic clusters in the *Tie2-Cre;Smad4*$^{fl/fl}$ embryos. Whole mount immunohistochemistry staining of c-Kit was done at 18 sp. The embryos were then frozen sectioned at 20 μm and slightly stained with eosin. Continuous sections of the whole embryos were screened and representative images are shown. Note that the hematopoietic cluster in DA was detected exclusively in the mutants (upper). Moreover, hematopoietic clusters in the VA were much easily detected in *Tie2-Cre;Smad4*$^{fl/fl}$ embryos (lower). Blue arrowheads indicate the c-Kit^{+} intra-vascular clusters. Black arrowheads indicate the primordial germ cells (PGCs) dispersed in the wall of the gut. DA, dorsal aorta; nt, neural tube; VA, vitelline arteries. Scale bar: 100 μm.

Supplemental Figure 2

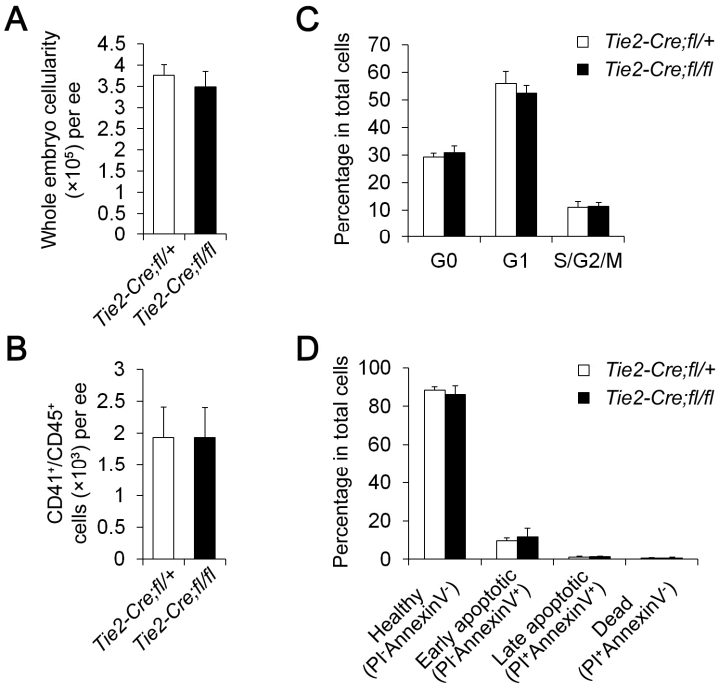

Figure S2. Cell cycle and viability of the *Smad4* conditional knockout embryos. (A) Graph showing the cellularity of the *Tie2-Cre;Smad4*$^{fl/fl}$ and control whole embryos (*n* = 3). (B) Graph showing the absolute numbers of CD41^{+}/CD45^{+} hematopoietic cells in the caudal half of *Tie2-Cre;Smad4*$^{fl/fl}$ and control embryos (*n* = 3). (C) Graph showing the cell cycle status of the caudal half total cells determined by FACS analysis of Ki67/7-AAD fluorescence (*n* = 4). (D) Graph showing the apoptotic status of the caudal half total cells determined by FACS analysis of Annexin V/PI fluorescence (*n* = 5). Data are mean ± SD. No significant difference was detected between control and mutant embryos.

Supplemental Figure 3

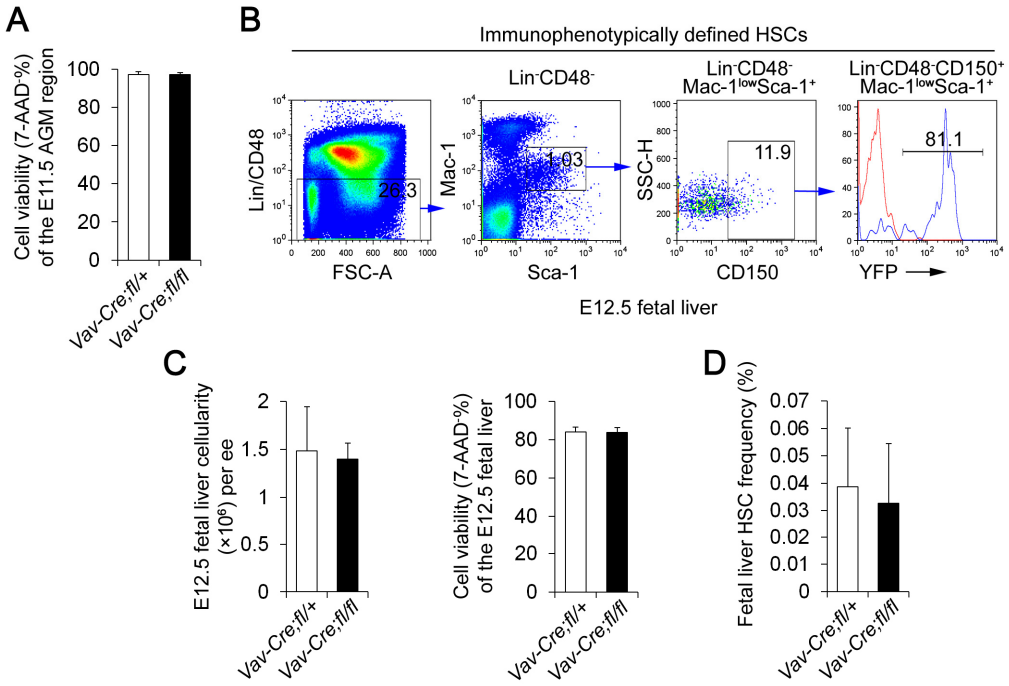

Figure S3. Deletion of *Smad4* by Vav-Cre has no influence on hematopoietic progenitor generation and homeostasis. (A) Graph showing the cell viability of the E11.5 AGM region determined by FACS analysis of 7-AAD fluorescence (*n* = 3). (B) Representative FACS analysis of EYFP fluorescence in the immunophenotypically defined HSCs ($Lin^-CD48^-CD150^+Mac1^{low}Sca\text{-}1^+$) of the E12.5 *Vav-Cre;ROSA-EYFP* fetal liver. The *ROSA-EYFP* littermate without the *Cre* transgene serves as the negative control (red line). (C) Graphs showing the cellularity (left) and the cell viability determined by FACS analysis of 7-AAD fluorescence (right) of the E12.5 fetal liver (*n* = 3). (D) Graph showing the frequencies of immunophenotypically defined HSCs in the E12.5 fetal liver (*n* = 3). Data are mean ± SD. No significant difference was detected between control and mutant embryos.

Supplemental Figure 4

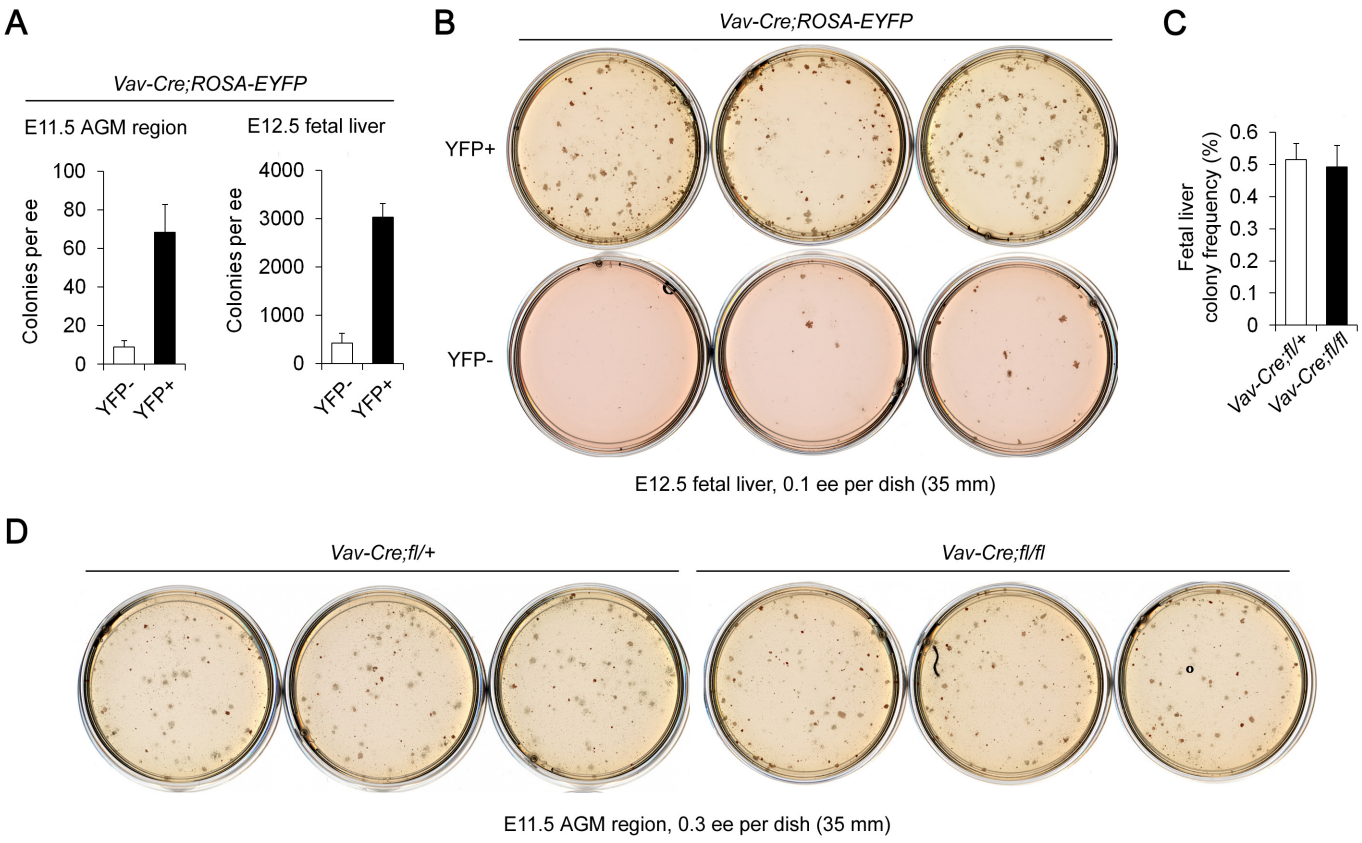

Figure S4. Deletion of *Smad4* by Vav-Cre has no influence on the hematopoietic colony forming capacity. (A) YFP^+ and YFP^- cells in the E11.5 AGM region (*n* = 4) and the E12.5 fetal liver (*n* = 4) of the *Vav-Cre;ROSA-EYFP* embryos were sorted by FACS respectively and CFU-C assay was performed. Graphs showing the colony numbers counted at day 7. (B) Representative scanning of the day 7 hematopoietic colonies from YFP^+ (upper) and YFP^- (lower) cells in E12.5 fetal liver of the *Vav-Cre;ROSA-EYFP* embryos. (C) Graph showing the frequencies of hematopoietic colonies in the E12.5 fetal liver (*n* = 3). (D) Representative scanning of the day 7 hematopoietic colonies from E11.5 AGM region. Data are mean ± SD. No significant difference was detected between control and mutant embryos.

Supplemental Figure 5

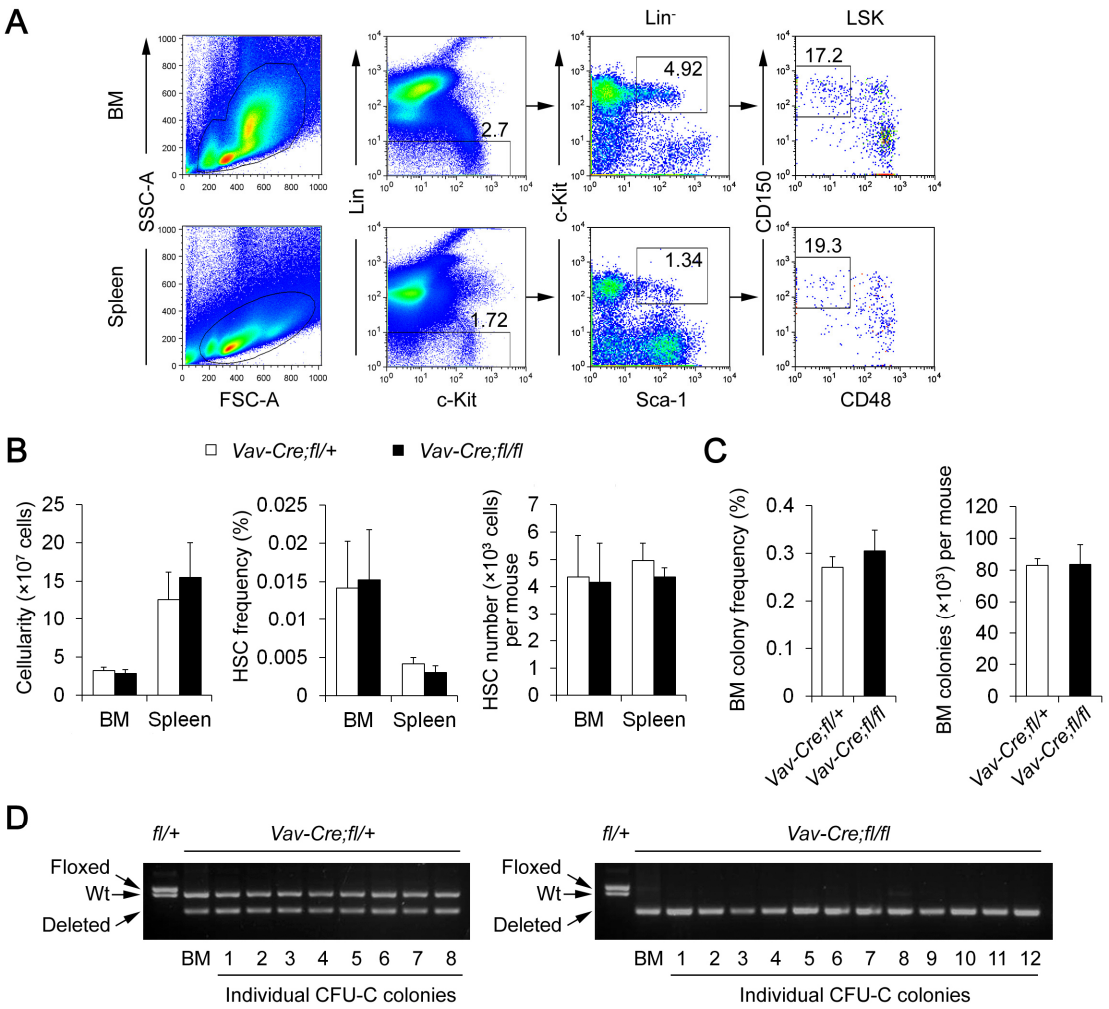

Figure S5. Deletion of *Smad4* by Vav-Cre has no influence on adult hematopoietic homeostasis. (A) Representative FACS analysis of the immunophenotypically defined HSCs ($CD150^{+}CD48^{-}Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$) in the bone marrow and spleen cells from a 8-week-old mouse. (B) Bone marrow (two femurs, *n* = 5) and spleen (*n* = 3) cellularity (left), HSC frequency (middle), and total HSC numbers (right) in the 1-2 months *Vav-Cre;Smad4*$^{fl/fl}$ mice and littermate controls. (C) Bone marrow colony frequency (left) and total colony numbers (right) in the 1-2 months *Vav-Cre;Smad4*$^{fl/fl}$ and control mice (*n* = 3). (D) PCR genotyping of bone marrow cells and individual colonies picked from CFU-C assays. The locations of *Smad4*-floxed,

-wild type and -deleted bands are indicated. *fl/+* indicates the tail tissue from a *Smad4*$^{fl/+}$ mouse. BM, bone marrow. Data are mean ± SD. No significant difference was detected between control and mutant mice.

Supplemental Figure 6

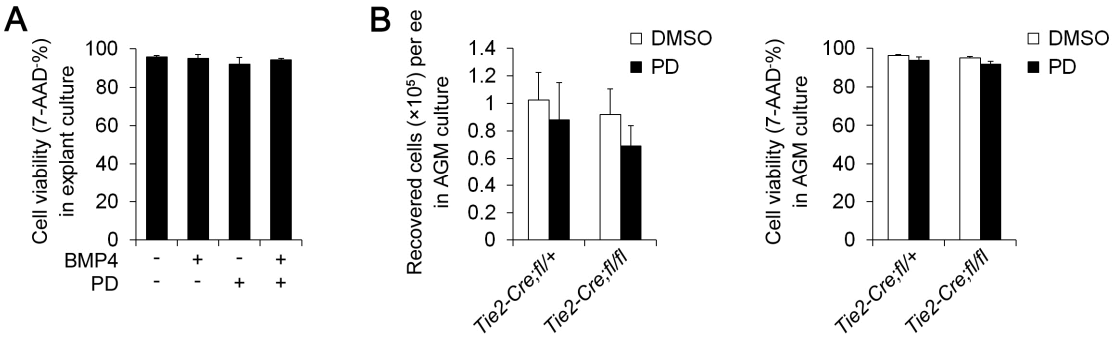

Figure S6. Viability and absolute number of the cells recovered from explant culture and AGM culture. (A) Graph showing the cell viability determined by 7-AAD staining in the explant cultures derived from E9.5 embryos (*n* = 3). BMP4 and/or PD 98059 were added as indicated. (B) Graphs showing the absolute number of recovered cells (left) and the cell viability determined by 7-AAD staining (right) in the AGM culture derived from *Tie2-Cre;Smad4*$^{fl/fl}$ and control embryos (*n* = 4). DMSO or PD 98059 was added as indicated. Data are mean ± SD. No significant difference was detected.

Supplemental Figure 7

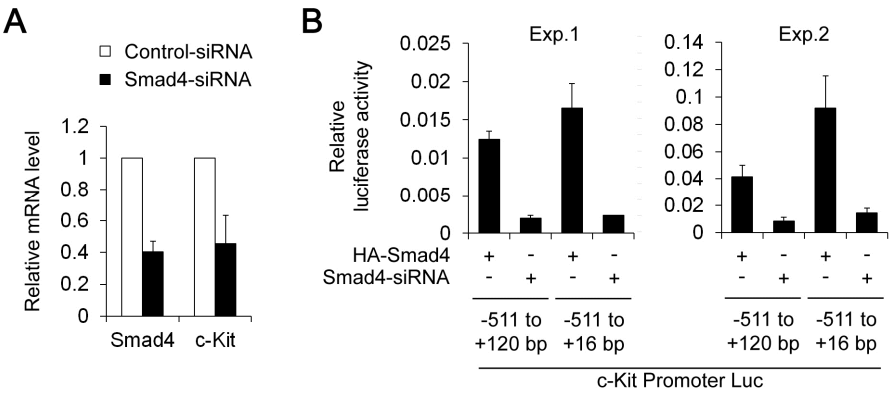

Figure S7. Up-regulation of c-Kit by endothelial Smad4 in a cell autonomous manner. (A) Realtime PCR analysis of Smad4 and c-Kit expression in control- and Smad4-siRNA immortalized embryonic endothelial cells. Relative expression fold to that of control-siRNA cells is shown (*n* = 3). (B) DNA fragments -511 bp to +120 bp (containing both the -364 bp Smad4 binding site and the +85 bp Smad binding site) or -511 bp to +16 bp (containing the -364 bp Smad4 binding site) of mouse c-Kit gene was introduced to the pGL3-basic vector. The immortalized embryonic endothelial cells were transfected with different luciferase reporter constructs co-transfected with HA-Smad4 or Smad4-siRNA plasmid for 36 hours. Graphs showing the relative promoter activities in the presence or absence of Smad4. Two independent experiments are shown. Data are mean ± SD.

Supplemental Figure 8

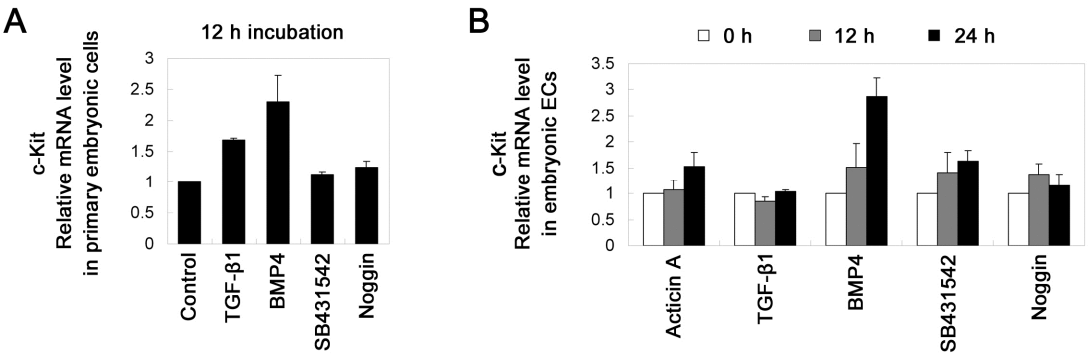

Figure S8. Up-regulation of endothelial c-Kit transcripts by BMP4. (A) Real-time PCR analysis of c-Kit expression in E9.5 primary embryonic cells treated with the indicated regents for 12 hours (*n* = 3). Relative expression fold to that of control group is shown. (B) Real-time PCR analysis of c-Kit expression in the immortalized embryonic endothelial cell line treated by the indicated regents for 12 to 24 hours (*n* = 3). Relative expression fold to that of groups without treatment is shown. Data are mean ± SD.

Supplemental Figure 9

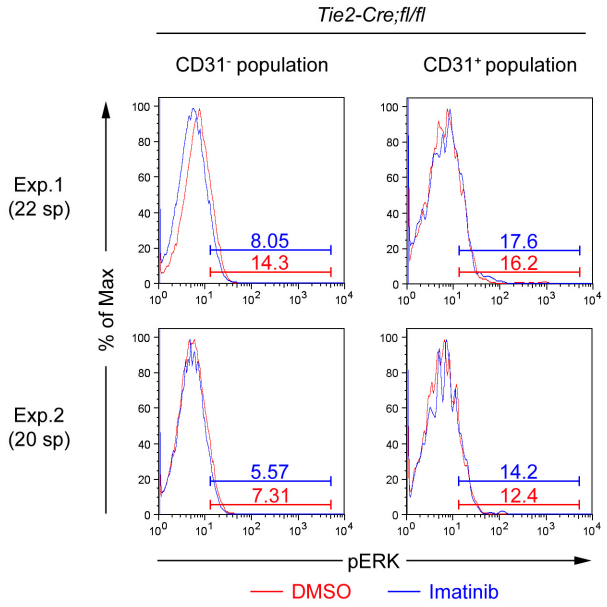

Figure S9. Effect of Imatinib on pERK expression in *Tie2-Cre;Smad4*$^{fl/fl}$ embryos.

Explant culture was performed using the caudal half of E9.5 *Tie2-Cre;Smad4*$^{fl/fl}$ embryos. DMSO or Imatinib was added in the culture system for 20 hours. Cells recovered from the culture were immediately analyzed by FACS for CD31 and pERK expression. FACS analysis of pERK expression in CD31^{-} (non-endothelial cells) and CD31^{+} (predominantly endothelial cells) populations in two independent experiments is shown. Blue lines indicate the Imatinib-treated group, and red lines indicate the DMSO-treated group. Note that Imatinib treatment slightly down-regulated pERK expression in CD31^{-} but not CD31^{+} population.

Supplemental Materials and Methods

Animal care, embryo dissection and single cell isolation

Animals were kept under specific pathogen-free conditions and handled in accordance with institutional guidelines. Littermates were used in all experiments. Embryos were staged by somite counting: E9.0, 13–20 sp; E9.5, 21–30 sp; E10.0, 31-35 sp; E10.5, 36-40 sp; and E11.5, 46-50 sp. The detection of vaginal plug was designated as 0.5. For analyzing embryos before E10.0, the caudal half was dissected just under the heart, and limbs were removed. For analyzing E10.5 embryos, the caudal half was dissected between forelimbs and hind limbs, and fetal liver was removed. The AGM region of E11.5 embryos and the fetal liver of E12.5 embryos were dissected as previously reported [1]. Primary embryonic single-cell suspension was acquired by type I collagenase digestion except for those used in the AGM culture. For some experiments, $Tie2^+$ cells from caudal half were sorted by using magnetic beads (MACS Anti-Biotin MicroBeads, Miltenyi Biotech) purification with biotin-conjugated anti-mouse Tie2 antibody (TEK4, eBioscience) according to the manufacturer's instructions. Bone marrow cells were isolated by flushing the long bones (two femurs) with HBSS containing 2% heat-inactivated bovine serum. Spleen cells were obtained by crushing the spleen between two glass slides. The cells were filtered with a 45 mm nylon mesh. Genotyping was performed as described previously [2].

Flow cytometric analysis

Antibodies used were: CD45 (30-F11), CD31 (MEC13.3), c-Kit (2B8), CD34 (RAM34), Mac-1 (M1/70), B220 (RA3-6B2), Gr-1 (RB6-8C5), CD3 (145-2C11), CD41 (MWReg30), Ter119 (TER-119), CD150 (TC15-12F12.2), CD48 (HM48-1), Sca-1 (E13-161.7), pERK (20A), and streptavidin APC-eFluor780. The biotinylated antibodies for lineage markers included: Gr-1 (RB6-8C5), B220 (RA3-6B2), CD4 (RM4-5), CD8 (53-6.7), Ter-119 (TER-119), IL-7R (A7R34). All monoclonal antibodies were purchased from eBioscience, except for CD41, CD34, CD31 and pERK from BD Pharmingen, and CD150 from BioLegend. For intracellular pERK staining, cells were firstly stained with APC-CD31, then fixed (BD Cytofix Fixation Buffer), permeabilized (BD Phosflow Perm Buffer III), and further stained with PE-pERK (ERK1/2, pT202/pY204) according to the manufacturer's instructions. For cell cycle analysis, BrdU (100 μg/g body weight, Sigma) was intraperitoneally injected into the pregnant mouse 2 hours before dissection. Cells were stained with PE-CD31 and APC-c-Kit, followed by FITC-BrdU (BrdU Flow Kit, BD Pharmingen) according to the manufacturer's instructions. FITC-Ki67 (FITC Mouse Anti-Human Ki-67 Set, BD Pharmingen) combined with 7-AAD staining was also performed. Total DNA was labelled by 7-AAD and cell cycle status (G0/G1 or S/G2/M phase) was determined by 7-AAD intensities. Ki67 signal further discriminated cells in G0 from cells in G1 phase. For apoptotic analysis, cells were stained with FITC-Annexin V and PI (FITC Annexin V Apoptosis Detection Kit I, BD Pharmingen) according to the manufacturer's instructions.

Hematopoietic assays

For AGM culture prepared from E9.0-E9.5 embryos, the caudal half was excised and digested by trypsinase. The cells were cultured in the presence of 100 ng/ml SCF (PeproTech), 10 ng/ml murine oncostatin M (R&D) and 1 ng/ml bFGF (R&D). For Explant culture with the caudal half of E9.5 embryos, tissues were deposited on 0.65-μm filters (Millipore) at the liquid–air interface in long-term medium (M5300; Stem Cell Tech) supplemented with 1 μM hydrocortisone (Sigma). For all the in vitro hematopoietic culture systems, 25 ng/ml BMP4 (R&D), 50 μM PD 98059 (Sigma), or 1 μM Imatinib (Novartis) was added as needed. For Imatinib and PD 98059, same volume of DMSO was used as the control. Methylcellulose colony-forming assay was performed using the following cytokines: 50 ng/ml SCF, 10 ng/ml IL-3, 5 ng/ml IL-6 (PeproTech), and 3 U/ml erythropoietin (Kirin). CFU-Cs were scored with an inverted microscope at day 7-10. The whole dishes were scanned using an UMAX PowerLook scanner with 1200 dpi.

LacZ staining and immunostaining

For LacZ staining, dissected E9.5 embryos or cultured cells were washed with PBS, fixed immediately in 0.2% glutaraldehyde/PBS on ice for 30 minutes, rinsed three times with PBS, and stained overnight at 37°C in X-Gal buffer (PBS containing 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM MgCl2, 0.01% Na-deoxycholate, 0.02% NP-40, and 1 mg/ml X-gal). Stained tissues were post-fixed in 4% paraformaldehyde at room temperature for 2 h, embedded in paraffin, sectioned

at 5 µm, and counterstained with 0.1% nuclear fast red. Immunohistochemistry and immunofluorescent staining were performed using standard procedures. Primary antibodies used were as follows: anti-Runx1 (EPR3099, EPITOMICS), anti-pERK (20G11, Cell Signaling), anti-BMP4 (N-16, Santa Cruz), anti-Endomucin (eBioV.7C7, eBioscience), anti-pSmad1/5/8 (Cell Signaling), PE-conjugated anti-CD31 (MEC13.3, BD Biosciences), and FITC-conjugated anti-CD45 (30-F11, eBioscience). A Nikon E600 microscope with a digital camera (RT color; SPOT; Diagnostic Instruments) was used for immunohistochemistry documentation. A ZEISS microscope with a digital camera (Axiocam; MRm) was used for immunofluorescent documentation. Images were processed using Photoshop 7 (Adobe).

Cytospin and May-Grunwald/Giemsa staining

Different populations based on the expression profiles of CD45 and c-Kit were sorted by FACS Aria 2 flow cytometer (BD Biosciences) and transferred to slides in a cytospin centrifuge. The slides were fixed immediately in cold methanol for 5 min and stained with May-Grunwald/Giemsa following standard protocols. Images were captured using a Nikon E600 microscope with a digital camera (RT color; SPOT; Diagnostic Instruments).

Real-time PCR

The freshly isolated primary embryonic cells or immortalized embryonic endothelium established previously [3] were resuspended in the presence of 50 ng/ml BMP4, 200

ng/ml Noggin (R&D), 50 μM PD 98059, 5 ng/ml TGF-β1 (R&D), 5 ng/ml Activin A (R&D), or 50 μM ALK4/5/7 inhibitor SB-431542 (Sigma) for 12-48 hours. The Smad4-siRNA sequences used were as follows: GCAGCAGAAUGGAUUUACUTT and AGUAAAUCCAUUCUGCUGCTT. jetPRIME (Polyplus) -mediated Smad4-siRNA Oligo transfection was used to knockdown Smad4 in the immortalized embryonic endothelium. Total RNA from the caudal half, sorted populations, or cultured cells with different treatments was extracted by TRIZOL (Invitrogen) and reversed transcribed with an mRNA selective PCR kit (TaKaRa). Real-time PCR was performed with a Roche LightCycler 2.0 system using a SYBR green program. Sequences of specific primers were self-designed except for P2-Runx1 primers [4] and are listed as follows.

Gene	Primer sequence	Length (bp)	Product Tm (℃)
Hprt	5'-GCTGGTGAAAAGGACCTCT-3' 5'-CACAGGACTAGAACACCTGC-3'	249	84.2
Scl	5'-GGCAAGAAACAGGAGTGAGAAG-3' 5'-GCAAGGAAGACACAGAGGAAGA-3'	162	85.3
Runx1	5'-ACTCACTGGCGCTGCAACAA-3' 5'-AAGCTCTTGCCTCTACCGCT-3'	209	90.8
Gata2	5'-GCGAAAACCAAACTGCATAAGC-3' 5'-CTGTCTCCCAGAAACCAAGAGC-3'	235	86.8
Gata1	5'-AGCCTATTCTTCCCCCAAGTTT-3' 5'-GGTTCCTCGTCTGGATTCCATC-3'	371	90.7
Tie2	5'-CCAACAGTGATGTCTGGTCCTA-3' 5'-TCTGGGCAAATGATGGTCTC-3'	213	88.9
vWF	5'-GTGTACCACGAGGTCATCAACG-3' 5'-GAGGGCACAAAGGTCAGGAG-3'	164	89.4
P2-Runx1	5'-AAGATCCGAGCCCCTGTC-3' 5'-TCACAACAAGCCGATTGAGT-3'	70	83.8
Smad4	5'-CCTCCCATTTCCAATCATCC-3' 5'-TAGCCATCCACAGTCACAACA-3'	125	85.4
Tgfb1	5'-TGGTGGACCGCAACAAC-3' 5'-AGCCACTCAGGCGTATCAG-3'	250	87.3
Bmp4	5'-AGGCGACACTTCTACAGATG-3' 5'-GATGCTGCTGAGGTTGAAG-3'	272	92.1

Id1	5'-TCTGGGAAAGACACTACCGC-3' 5'-TCCTCAGAAATCCGAGAAGC-3'	210	87.4
Ihh	5'-TCAAGGACGAGGAGAACACG-3' 5'-CGGTCACCCGCAGTTTCA-3'	120	90.0
Shh	5'-GGCTGTGGAAGCAGGTTT-3' 5'-GTCCAGGAAGGTGAGGAAGT-3'	238	92.4
Ptch1	5'-GTCAGTGTCATCCGAGTGG-3' 5'-GAGCAAGAAACGGCAAAA-3'	214	90.5
Smo	5'-TCAATGCGTGTTTCTTCGT-3' 5'-CAGGGTCTCACTGGAGGTG-3'	128	90.1
c-Kit	5'-CGCCTGCCGAAATGTATGAC-3' 5'-AAATGTGCTTGGTGCTGTCC-3'	123	85.4

Reporter assay

DNA fragments -511 bp to +120 bp or -511 bp to +16 bp of mouse c-Kit gene was introduced to the pGL3-basic vector (Promega), Embryonic endothelial cells in 24-well plates were transfected with different reporter constructs co-transfected with HA-Smad4 or Smad4-siRNA plasmid for 36 hours. Luciferase activities were detected with dual luciferase reporter assay reagents (Promega).

References

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