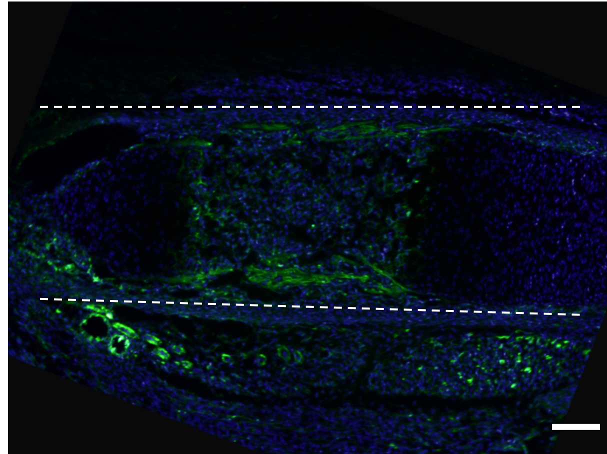
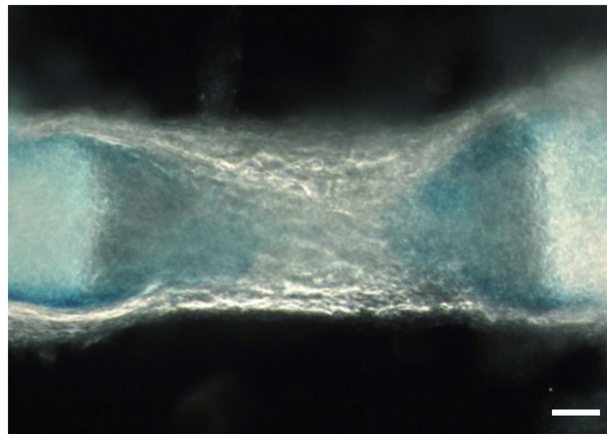
A.



Dextran FITC DAPI

B.



Alcian Blue Alizarin Red

Figure S1. A) Mouse E16 fetuses were injected with 100 µL Dextran-FITC (70 kDa) via the superficial temporal vein to reveal functional blood vessels within long bones. At E16, perfused vessels were detectable within periosteum and epiphyseal plate, but not within the middle marrow region of fetal long bone (Longitudinal femur cross section; dashed lines indicate bone edges. Green: Dextran-FITC, Blue: nuclear DAPI. Bar = 100 µm). **B**) At E16, fetal bone exhibited Alcian Blue (cartilage) staining, but no Alizarin Red (calcification) staining. (Light blue: Alcian Blue; Bar = 100 µm).

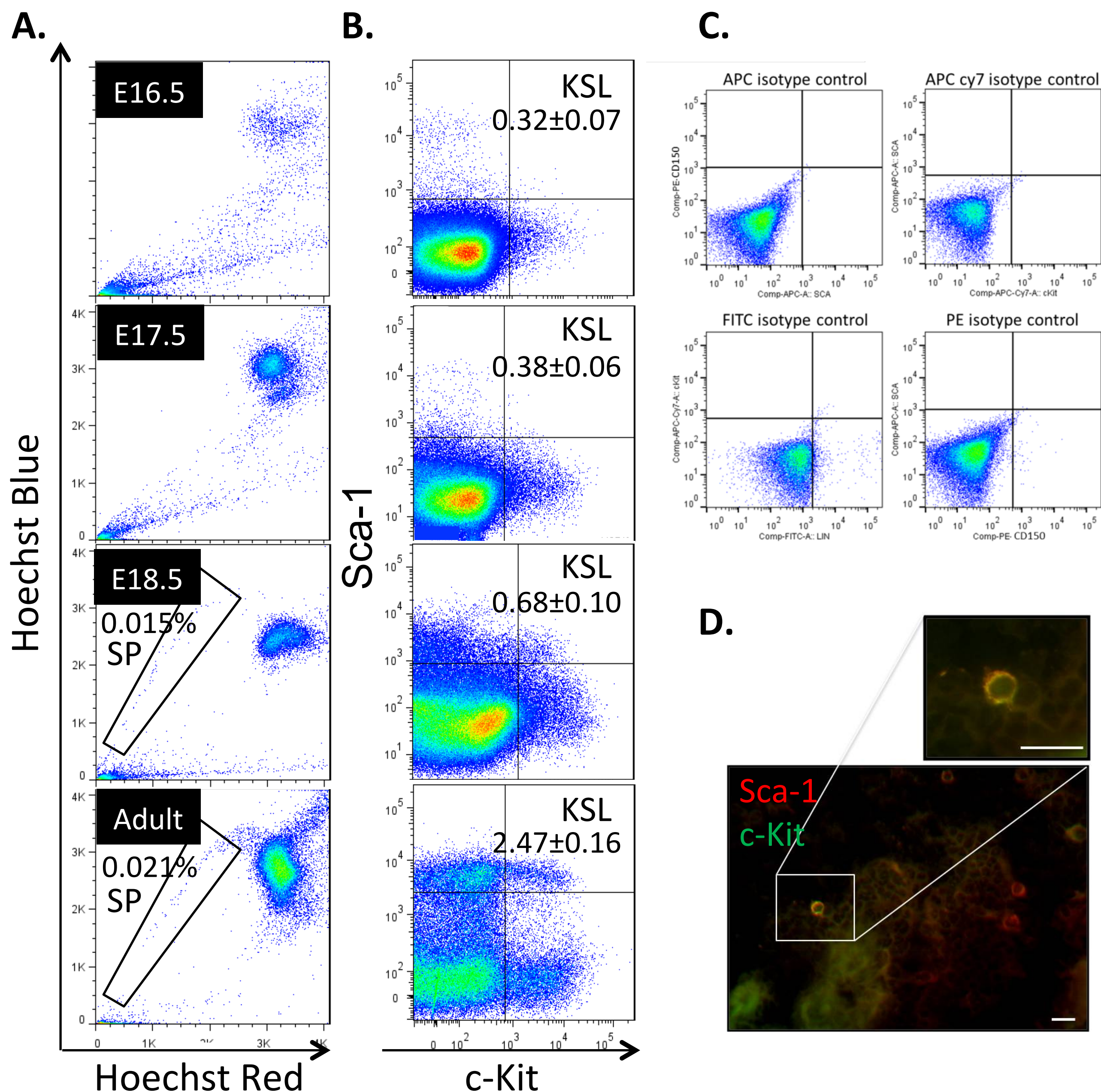
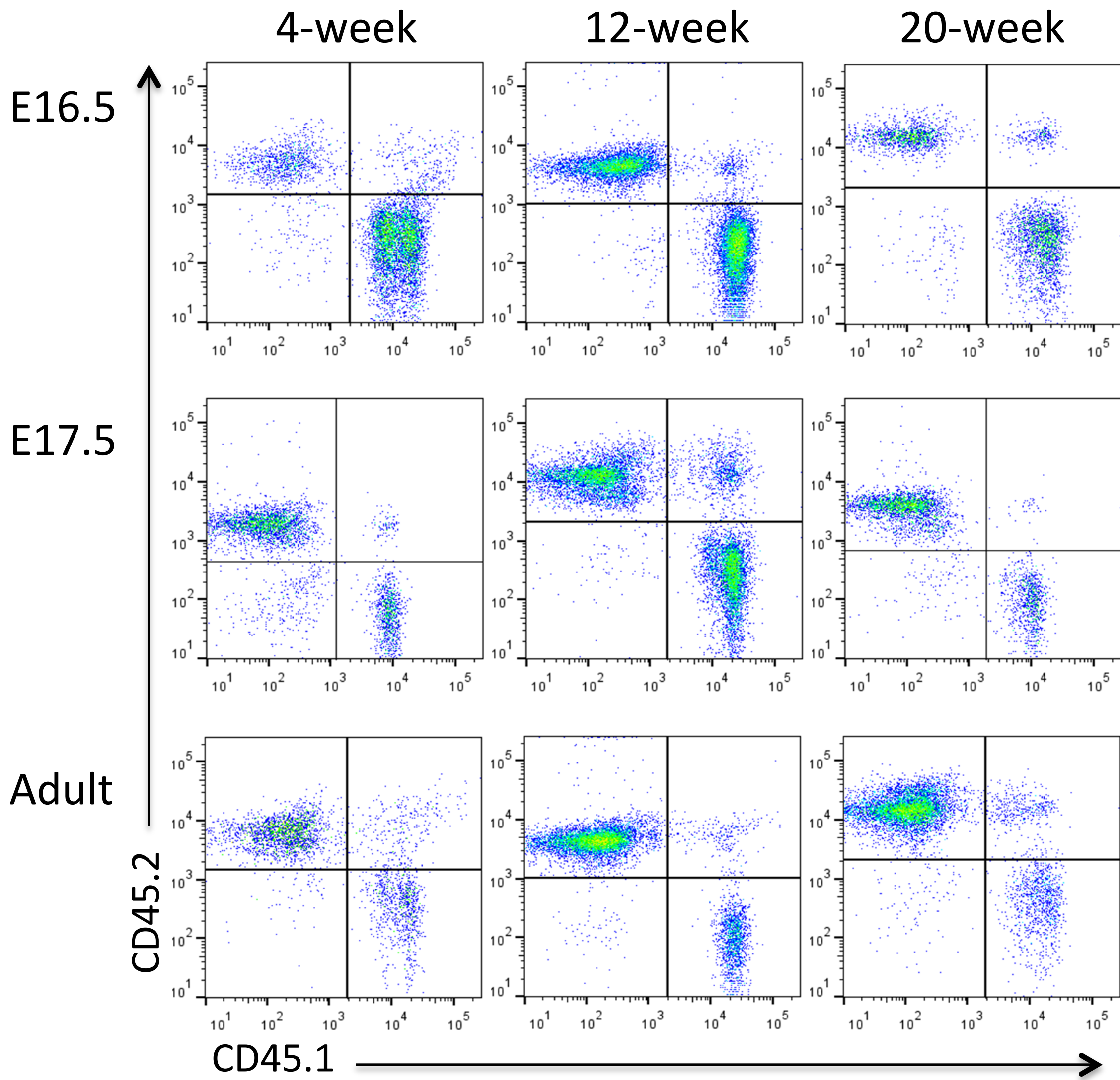
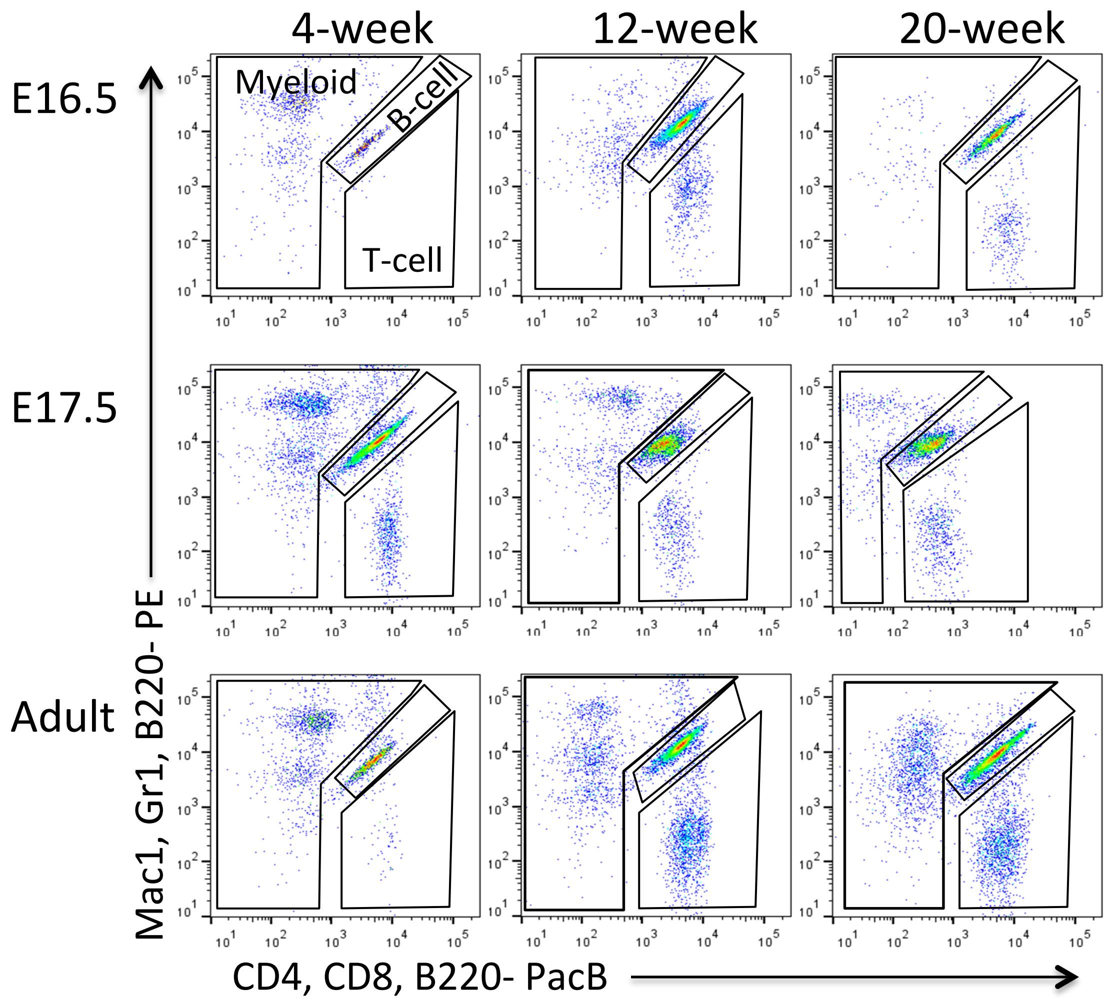


Figure S2. Related to Figure 1. A) Fetal bone marrow cells were stained with Hoechst dye to detect an SP population within fetal bone marrow; SP cells were not present in fetal bone marrow until E18.5. **B**) KSL cells were characterized by expression of c-Kit and Sca-1 proteins and lack of blood lineage protein expression (CD4, CD8, CD45R, Ter119, Gr-1 and Mac-1). Mac-1 was excluded from lineage cocktail for fetal bone marrow analysis since fetal HSC are positive for Mac-1. KSL cells were present in fetal bone marrow starting at E16.5, the onset of HSPC activity. **C**) Isotype controls were tested for each conjugated antibody. Single compensation controls and unstained controls were also performed. **D**) Femurs isolated from P0 embryos were fixed and stained with anti-c-Kit and anti-Sca-1 antibodies and with Alexa 594 and Alexa 488 conjugated secondary antibodies, respectively. Merged images show HSPC expressed both c-Kit and Sca-1 in the central bone marrow cavity. Bar=20 μm (insert: magnified view of a double positive HSPC). All flow cytometry studies were conducted at least three times (Data represent mean ± SEM).



	4-week	12-week	20-week
E16.5	14.1 % ± 1.20	30.82 % ± 7.81	32.84 % ± 10.06
E17.5	56.46 % ± 4.40	76.89 % ± 4.60	83.71 % ± 3.97
Adult	57.72 % ± 5.04	73.55 % ± 5.73	85.8 % ± 3.30

Figure S3. Related to Figure 2. Fetal whole bone marrow cells isolated from femurs at E16.5 and onwards showed long term engraftment. Engraftment levels were determined at 4, 12 and 20 weeks after transplantation by analyzing peripheral blood donor/recipient ratio using anti-CD45.2 and CD45.1 antibodies respectively (Data represent mean ± SEM, N=3).



	Weeks	B-cells (%)	Myeloid (%)	T-cells (%)
	4	45.46	51.85	0.32
E16.5	12	72.09	6.88	19.92
	20	72.72	5.60	19.88
	4	71.18	20.19	7.27
E17.5	12	72.10	11.63	8.55
	20	68.92	7.60	14.74
	4	50.02	46.99	1.69
Adult	12	63.58	18.95	16.43
	20	53.31	32.30	12.41

Figure S4. Related to Figure 2. Fetal whole bone marrow cells isolated from femurs at E16.5 and onward showed long term engraftment, and contribution to all three blood lineages in the recipients, similar to adult bone marrow cells. Donor derived blood cells (i.e. CD45.2 positive) were evaluated for their expression of lineage markers (CD4, CD8, B220, Mac1 and Gr1). (Data represent mean ± SEM, N =3).

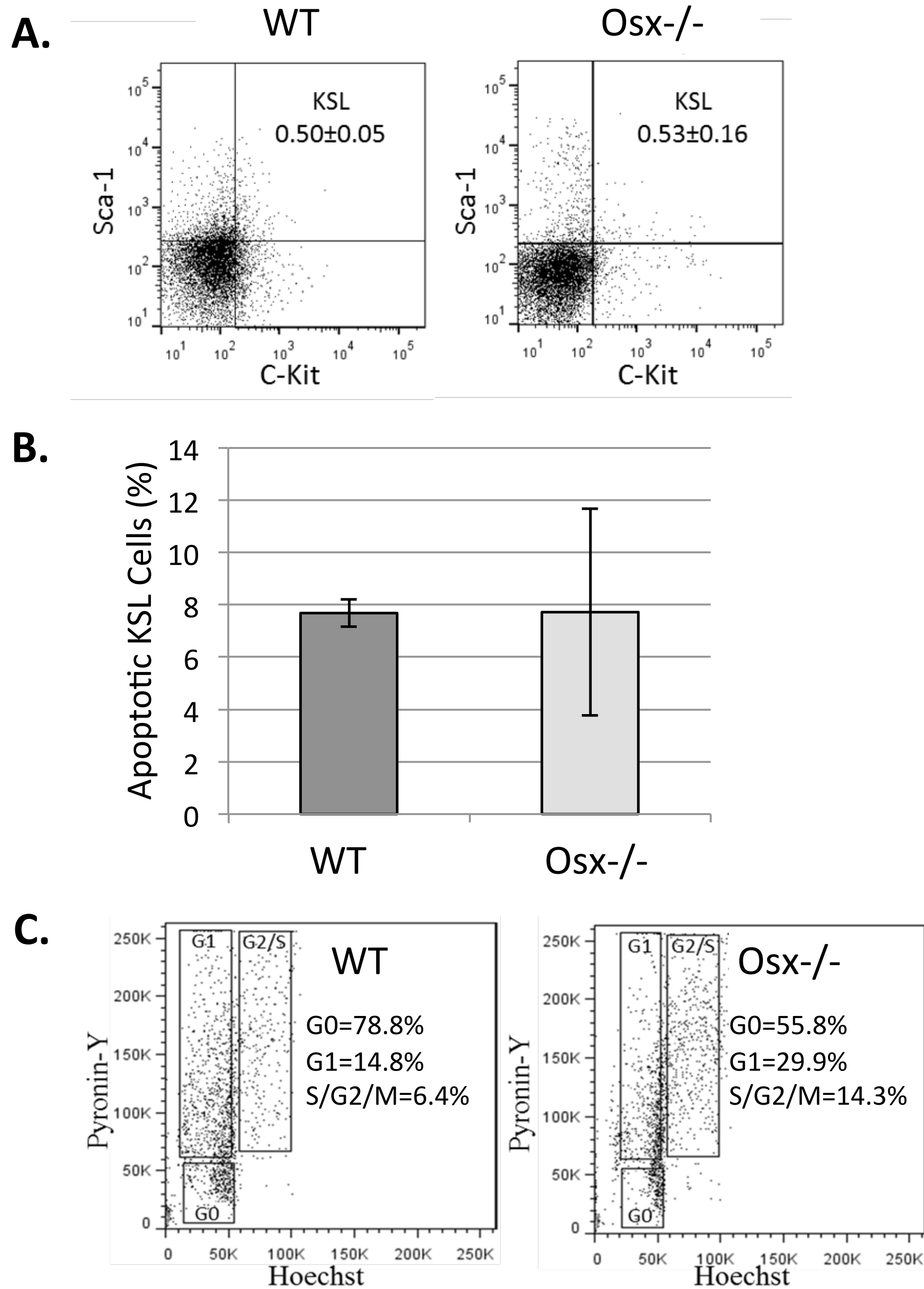


Figure S5. Related to Figure 3. Properties of KSL cells in WT and Osx-/- fetal bone marrow at E17.5. A) The proportion of KSL cells within whole bone marrow of Osx-/- mutants was comparable to that of WT littermates. Absolute KSL cell numbers, in all long bones combined, from each E17.5 WT and Osx-/- fetus were 402.70 ± 43.59 and 426.86 ± 125.64, respectively. Data (mean ± SEM, N=3; p=0.86) were normalized based on the same number of events in each group. **B**) The AnnexinV/7AAD apoptosis assay revealed no significant difference (p=0.99) in the proportion of KSL cells undergoing apoptosis in E17.5 WT (6.98, 8.71 and 7.37% KSL cells; mean ± SEM = 7.69 ± 0.52%) and Osx-/- littermates (4.95, 15.50 and 2.70% KSL cells; mean ± SEM = 7.72 ± 3.94%). **C**) Osx-/- KSL cells exhibited a decreased proportion of cells in G0, and increased proportion of cells in G1 and S/G2/M phases, relative to WT KSL cells.

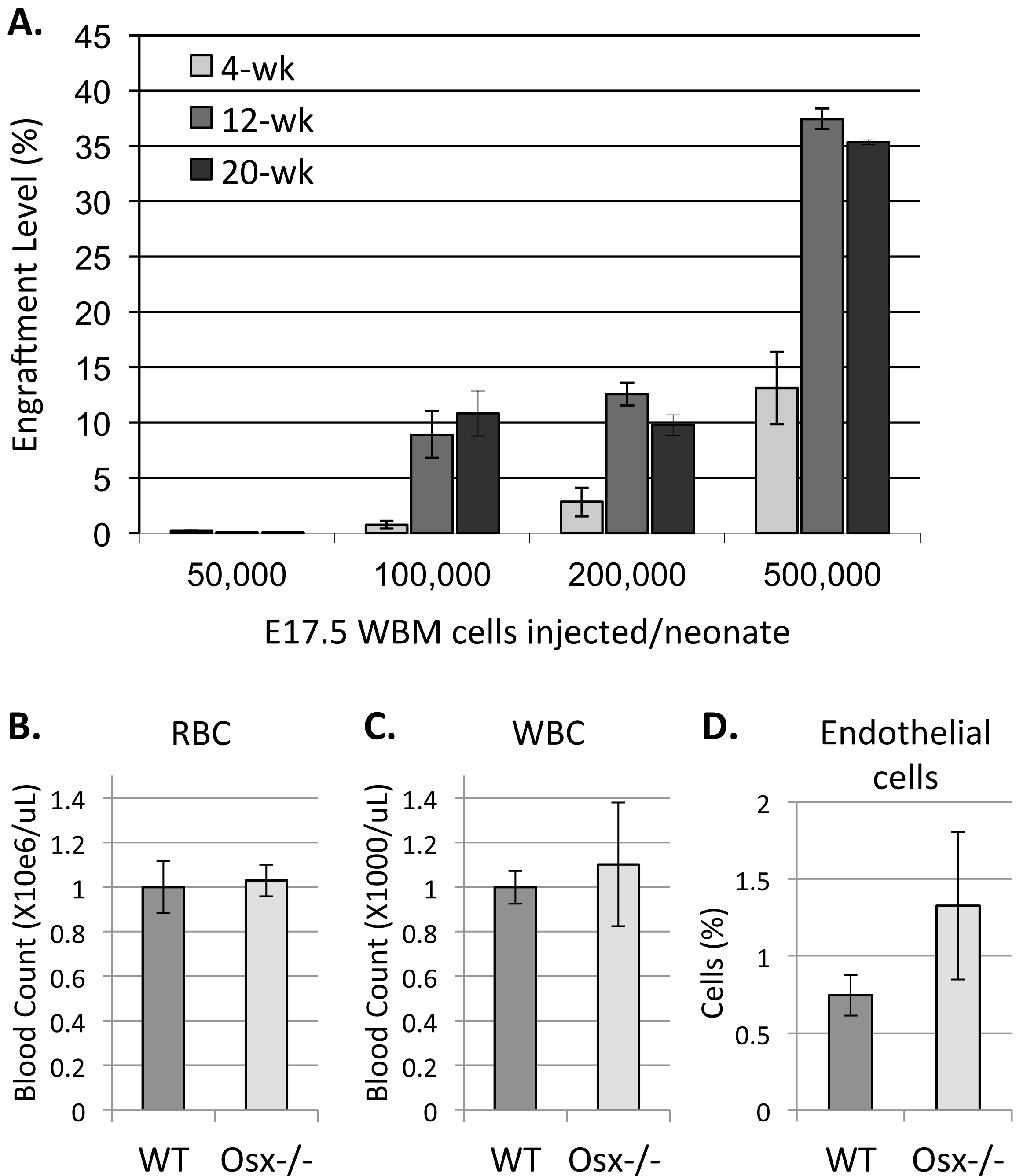


Figure S6. Related to Figure 3. A) Engraftment curve for fetal bone marrow cells. 50,000-500,000 WBM cells from E17.5 WT femurs were transplanted into sublethally irradiated neonate recipients. 100,000 WBM cells from E17.5 bone marrow were sufficient to detect the engraftment levels above 5% at 12 weeks post transplantation. (Data represent mean ± SEM, N =4). **B-D**) **Cellular composition of bone marrow from E17.5 Osx-/- and WT littermates**. There were no differences in total red blood cell (RBC; **B**) or total white blood cell (WBC; **C**) counts within bone marrow of E17.5 Osx-/- and WT littermates (Data represent normalized Mean ± SEM, N=4). Endothelial (CD31+CD45-; **D**) cell number was measured by flow cytometry and absolute percentage within WBM cells was compared (Data represent Mean ± SE, N=4). Total endothelial cell number tended to be higher in Osx-/- mutants compared to WT littermates.

Table S1. **Related to Figure 5**. Primers used for qPCR analysis. Gene accession number for each gene was obtained from NCBI database (http://www.ncbi.nlm.nih.gov/nuccore). Forward (F) and Reverse (R) primer sequences were obtained from PrimerBank database (http://pga.mgh.harvard.edu/primerbank).

Gene Name	GeneBank Access. No.	Primer Sequence (5'→3')
Osx (Sp7)	NM_130458	**F**-ATGGCGTCCTCTCTGCTTG **R**-TGAAAGGTCAGCGTATGGCTT
Opn (Spp1)	NM_009263	**F**-AGCAAGAAACTCTTCCAAGCAA **R**-GTGAGATTCGTCAGATTCATCCG
N-cad (cdh2)	NM_007664	**F**-AGCGCAGTCTTACCGAAGG **R**-TCGCTGCTTTCATACTGAACTTT
VE-cad (cdh5)	NM_009868	**F**-CACTGCTTTGGGAGCCTTC **R**-GGGGCAGCGATTCATTTTTCT
Vegfr2 (kdr)	NM_010612	**F**-TTTGGCAAATACAACCCTTCAGA **R**-GCAGAAGATACTGTCACCACC
Integrin β1 (Itgb1)	NM_010578.2	**F**-ATGCCAAATCTTGCGGAGAAT **R**-TTTGCTGCGATTGGTGACATT
Integrin α5 (Itga5)	NM_010577	**F**-CTTCTCCGTGGAGTTTTACCG **R**-GCTGTCAAATTGAATGGTGGTG
KitL (SCF)	NM_013598	**F**-GAATCTCCGAAGAGGCCAGAA **R**-GCTGCAACAGGGGGTAACAT
Cxcl12	NM_001012477	**F**-TGCATCAGTGACGGTAAACCA **R**-CACAGTTTGGAGTGTTGAGGAT
Nestin	NM_016701	**F**-CCCTGAAGTCGAGGAGCTG **R**-CTGCTGCACCTCTAAGCGA
Notch1	NM_008714	**F**-GATGGCCTCAATGGGTACAAG **R**-TCGTTGTTGTTGATGTCACAGT
Tie2	NM_013690	F- CGGCCAGGTACATAGGAGGAA R- TCACATCTCCGAACAATCAGC
CD44	X66084	**F-** TGCAGGTATGGGTTCATAGAAGG **R-** GTGTTGGACGTGACGAGGA
Hif1 α	NM_010431.2	**F-** ACCTTCATCGGAAACTCCAAAG **R-** CTGTTAGGCTGGGAAAAGTTAGG
VegfA	NM_009505.4	**F-** GCACATAGAGAGAATGAGCTTCC **R-** CTCCGCTCTGAACAAGGCT
P-selectin ligand (Selplg)	NM_009151	**F-** GAAAGGGCTGATTGTGACCCC **R-** AGTAGTTCCGCACTGGGTACA
Integrin α4 (Itga4)	NM_010576.3	**F-** GATGCTGTTGTTGTACTTCGGG **R-** ACCACTGAGGCATTAGAGAGC
p21	NM_001111099	**F-** CCTGGTGATGTCCGACCTG **R-** CCATGAGCGCATCGCAATC
p53	AJ297973	**F-** GTCACAGCACATGACGGAGG **R-** TCTTCCAGATGCTCGGGATAC
CD38	NM_007646	**F-** TCCCTCCGTGAGCCATTTTAC **R-** CGATGTCGTGCATCACCCA
β-Actin	NM_007393	**F**-GGCTGTATTCCCCTCCATCG **R**-CCAGTTGGTAACAATGCCATGT

Supplemental Experimental Procedures

Apoptosis Assay to analyze KSL fraction of fetal bone marrow from E17.5 Osx-/- and WT littermates

Fetal long bones from E17.5 Osx-/- and WT littermates were dissected and single cell suspensions were obtained, as described in methods section. Apoptosis assay was performed using Annexin V: PE Apoptosis kit (BD Biosciences), as described in the manufacturer's protocol. For KSL isolation, anti-c-Kit-APC-Cy7, anti-Sca-1-APC, and FITC conjugated lineage marker antibodies (CD4, CD8, B220, TER119, and Gr-1) (eBiosciences) were also added during the antibody staining step. Samples were immediately analyzed by flow cytometry (BD FACSAria).

Peripheral blood counts from E17.5 Osx-/- and WT littermates

Embryos were anesthetized with isoflurane (Baxter) followed by intra-cardiac puncture with an insulin syringe to obtain ~50 µL of blood, which was collected into ethylenediaminetetraacetic acid (EDTA)–treated glass capillary tubes. EDTA (Sigma-Aldrich) (0.5 M, 5 µL) was added, and the blood cell counts were performed using a Hemavet 950FS (Drew Scientific, Oxford, CT), according to the manufacturer's protocol.