Supplemental Methods and Data

Stress erythropoiesis Cultures

Isolated murine bone marrow cells were cultured in stress erythropoiesis expansion media (SEEM) for 5 days and switched to stress erythropoiesis differentiation media (SEDM) for 3 days. SEEM contains Gibco IMDM (Invitrogen) supplemented with 10% fetal bovine serum (Gemini Bio-Products), 1% penicillin-streptomycin, 10 μg/ml insulin, 200 μg/ml transferrin, 2mM L-glutamine, 0.01g/ml bovine serum albumin, 7 μl/L 2-mercaptoethanol, 30 ng/ml GDF15 (Novoprotein), 15 ng/ml BMP4 (R&D systems), 50 ng/ml SCF (Goldbio) and 25 ng/ml SHH (GoldBio). SEDM contains Gibco IMDM, all supplements in SEEM and 3U/ml Epo. Cells were incubated at 2% O_2, 5% CO_2 when cultured in SEDM. Sorted murine spleen cells were cultured in SEEM and SEDM for indicated period of time.

Murine BMDM cultures

L929 cell line was cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. L929 supernatants were filtered and collected after 7 days. Isolated murine bone marrow cells were cultured in DMEM with 10% fetal bovine serum, 1% penicillin-streptomycin and 10% L929 supernatants for 5 days to generate BMDMs. Media were changed every 2 to 3 days.

Human BMDM culture

Human bone marrow mononuclear cells (MNCs) (ReachBio) were thawed at 37°C water according to the ReachBio instructions. MNCs were enumerated and cell concentrations were adjusted to 5×10^5 cells/mL. MNCs were cultured in Gibco RPMI-1640 (Invitrogen) supplemented with 10% fetal bovine serum (Gemini Bio-Products), 1% penicillin-streptomycin, 2 mM L-glutamine and recombinant human macrophage colony-stimulating factor (M-CSF) (GoldBio). 10 ng/mL M-CSF were supplemented for the first 24 hours and 25 ng/mL M-CSF were supplemented for the following culture period. Media were changed every 2 to 3 days. Human BMDMs were enriched after 10 to 15 days.

Tamoxifen dependent deletion of floxed mouse alleles.

Homozygous floxed mice were crossed with Rosa26-CreERT mice to generate floxed;Rosa26-CreERT mutants. To delete Stat5 in vivo, Tamoxifen was injected into mice every day for 5 days (75 mg/kg). Isolated bone marrow cells were treated with 1μM 4-hydroxytamoxifen (Sigma Aldrich) for 24 hours to induce deletion of Stat5 and β-catenin in vitro.

BMT and complete blood count assay

For BMT, $Stat5^{fx/fx}$;Rosa26-CreERT and β-$catenin^{fx/fx}$;Rosa26-CreERT bone marrow cells were isolated and cultured in SEEM with 4-OHT for 24 hours to generate $Stat5^{\Delta/\Delta}$ cells and β-$catenin^{\Delta/\Delta}$ cells. $Stat5^{fx/fx}$ or β-$catenin^{fx/fx}$ cells were also treated with 4-OHT as control. For experiments using purified SEPs as donor cells, β-$catenin^{fx/fx}$;Rosa26-CreERT, β-$catenin^{fx/fx}$ or C57BL/6-GFP bone marrow cells were expanded in SEEM for 5 days. β-$catenin^{fx/fx}$;Rosa26-CreERT, β-$catenin^{fx/fx}$ cultures were treated with 4-OHT for 24 hours to induce deletion of β-catenin as described above. CD133+Kit+Sca1+ cells from each culture were isolated by Flow sorting. Recipient mice were lethally irradiated (950 cGy) and transplanted with unfractionated donor bone marrow cells ($5x10^5$/transplant) or purified SEPs ($5x10^4$ mutant or control SEPs + $5x10^4$ GFP+ SEPs). Peripheral blood was collected retro-orbitally on indicated days and transferred to blood collection tubes coated with EDTA (BD). Hemavet 950 was used for hematological analysis.

Phenylhydrazine (PHZ) treatment and monocytes transfer

Resident splenic macrophages were depleted with chlodronate liposomes (ClodronateLiposomes. org). A single dose of chlodronate (100μl/10g body weight) is injected retro-orbitally. Donor monocytes were purified by using EasySep Monocyte Enrichment Kit (Stem Cell Technologies). 24 hours after chlodronate administration, $1x10^6$ monocytes were transferred into recipient mice by retro-orbital injection. Recipient mice were injected with a single dose of PHZ (100mg/kg body weight) intraperitoneally 24 hours after monocytes transfer.

Splenic EBI isolation

The isolation of splenic EBIs isolation was done as previously described[2-4]. In brief, spleens were minced and digested in 0.075% (*m/v*) Collagenase IV (Gibco 17104019) and 0.004% (*m/v*) DNase I (Invitrogen DN25) in RPMI1640 for 30min at 37^{o}C. The suspension was gently passed through an 18-guage needle several times and cells were washed by centrifugation. Cell pellets were resuspended with 1mL RPMI1640 containing 0.004% DNaseI and layered on top of 30% (*v/v*) FBS in Iscove's Modified Dulbecco's Medium (IMDM) followed by gravity sedimentation for 45 minutes at room temperature. The supernatant was carefully removed and the pellet was processed with Percoll (Sigma) gradient centrifugation (50%(*v/v*)/100%(*v/v*)) at 400*g* for 20min. The interface between the 50% and 100% Percoll was collected, washed with PBS, and processed for flow cytometry and imaging flow cytometry.

Immunocytochemistry

Spleen macrophages and BMDMs were fixed with 4% formaldehyde (Sigma-Aldrich) for 20 min at room temperature (RT). Cells were washed with PBST (PBS containing 0.1% Tween20) and permeabilized by 0.3% Triton X-100. Samples were blocked with 1% BSA in PBST for 30 min at RT and incubated with primary antibodies (F4/80, 123102, Biolegend and Phospho-Stat5a/b, ab32364, Abcam) at 4 °C overnight. Secondary antibodies (FITC-conjugated goat anti-rabbit IgG, sc-2012, Santa Cruz Biotechnology and Alexa 647-conjugated goat anti-rabbit IgG, A21244, Life Technologies) were applied for 1h in dark. After washing with PBST for 3 times, samples were mounted in Prolong Gold Antifade Mountant with DAPI (P-36931, Life Technologies). FV1000 confocal microscope (Olympus) was used for imaging.

Lipid extraction and liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis

Prostaglandins (PGs) were extracted from BMDMs and BMDM cell culture supernatants by using C18 Sep-pak cartridges (Waters). Briefly, culture supernatants were acidified with 1N HCl and loaded on to the Sep-Pak cartridge, bound PGs were eluted with methanol, evaporated under the stream of nitrogen gas and stored in ethyl acetate at −80° C until further analysis. For intracellular PGs, cell pellets were washed twice with DPBS, suspended in 1 mL of 80% methanol and homogenized with 0.1 mm zirconium beads using a bead beater homogenizer under standard cycle conditions.

Homogenates were centrifuged at 20000 x g for 10 mins at 4˚ C. Supernatants were transferred to a clean brown glass vial, evaporated under nitrogen, dissolved in ultrapure water, acidified and extracted as described above. After extraction, PGs were suspended in 70% methanol and analyzed by LC-MS/MS. Quantitative analysis of Δ^{12}-PGJ_2 and PGE_2 was performed using a calibration curve generated by respective standards in multiple reaction monitoring (MRM) mode. Following mass transitions were used to detect each PG: Δ^{12}-PGJ_2 (332.8/188.9; 332.8/314.9; 332.8/271.3 m/z) and PGE_2 (351/315; 351/271; 351/189). Data acquisition and analysis were performed using Analyst software (version 1.5; AB Sciex). PGs were normalized to cell number.

PKH26 cell membrane labeling

Bone marrow cells were isolated and immediately labeled with PHK26 by following the manufacture instructions (PHK26 Red Fluorescent Cell Linker Kits, Sigma-Aldrich). Cells were collected and stained with other cell surface markers before flow cytometry analysis. PKH26 were detect by using LSR-II Fortessa Flow cytometer (BD Biosciences) with 561nm laser and 575/26 filter.

Flow cytometry and cell sorting

Cells were collected and labeled with indicated antibodies. A list of antibodies was provided in supplemental table S1. Dead cells were determined by zombie yellow fixable viability dye (BioLegend) staining. LSR-II Fortessa Flow cytometer (BD Biosciences) was used for flow cytometry analysis. All flow cytometry data were analyzed with FlowJo software. Astrios (Becton Dickinson) cell sorter was used for cell sorting.

Epo and PGE_2 ELISA assay

Peripheral blood was collected by cardiac puncture and transferred to serum separator tubes (BD). Serum samples were separated by centrifuge, collected and stored at -80 ˚C before use. Serum Epo concentrations were measured by Mouse Erythropoietin Quantikine ELISA Kit (R&D systems). Spleens were collected and weighed on indicated days after BMT. Cells were homogenized in PBS and PGE_2 levels were measured by

ELISA immediately. Splenic and serum PGE_2 concentrations were measured by Prostaglandin E_2 Express EIA Kit (Cayman Chemical Company) and Prostaglandin E_2 Metabolite EIA Kit (Cayman Chemical Company), respectively.

Quantitative reverse transcription PCR (RT-qPCR)

Total RNA was isolated by using TriZol reagent (Invitrogen). Complementary DNA (cDNA) was generated by using the qScript cDNA SuperMix (Quanta Biosciences). qPCR was done by using StepOnePlus Real-Time PCR System (Applied Biosystems). A list of TaqMan probes is provided in supplemental table S2.

Gene Set Enrichment Analysis (GSEA)

Microarray data transcriptomics analysis was done previously[1]. The data were deposited into NCBI's Gene Expression Omnibus (GEO) for public access via GEO accession number, GSE122390. Gene expression profile was visualized by Transcriptome Analysis Console (TAC) software (Affymetrix). Normalized dataset was also processed with Gene Set Enrichment Analysis (GSEA) in order to determine statistically differentially expressed gene sets in the two groups of SEPs. Hallmark gene sets were accessed from Molecular Signatures Database (MSigDB).

Supplemental References

1. Hao, S., Xiang, J., Wu, D-C., Fraser, J., Ruan, B., Cai, J., Patterson, A., Lai, Z-C., and Paulson, R.F. (2019) Gdf15 regulates murine stress erythroid progenitor proliferation and the development of the splenic erythropoiesis niche. *Blood Advances* 3, 2205-2217.
2. Sadahira, Y., Mori, M. & Kimoto, T. Isolation and short-term culture of mouse splenic erythroblastic islands. *Cell Struct Funct* **15**, 59-65 (1990).
3. Liao, C. *et al.* Selenoproteins regulate stress erythroid progenitors and spleen microenvironment during stress erythropoiesis. *Blood*, doi:10.1182/blood-2017-08-800607 (2018).

4. Liao C, Prabhu KS and Paulson RF. Monocyte-derived macrophages expand the murine stress erythropoietic niche during the recovery from anemia. *Blood.* 2018;132(24):2580-2593.

Supplemental Tables

Table S1. Flow cytometry antibody list.

Antibodies	Fluorochrome	Source	Catalog No.
CD34	Alexa Fluor 647	BD	560230
CD133	PE	eBiosciences	12133182
CD133	PE-Cy7	BioLegend	141210
Kit (c-kit)	Brilliant Violet 421	BioLegend	1058282
Sca1 (Ly-6A/E)	APC-Cy7	BioLegend	108126
Sca1 (Ly-6A/E)	FITC	Biolegend	108106
Phospho-Stat5	Alexa Fluor 647	BD	562074
F4/80	PE-Cy7	Biolegend	123114
F4/80	APC	Biolegend	123116
Ter119	FITC	Biolegend	116206
CD45.2	PE	Biolegend	109808
CD16/32	N/A	Biolegend	101302

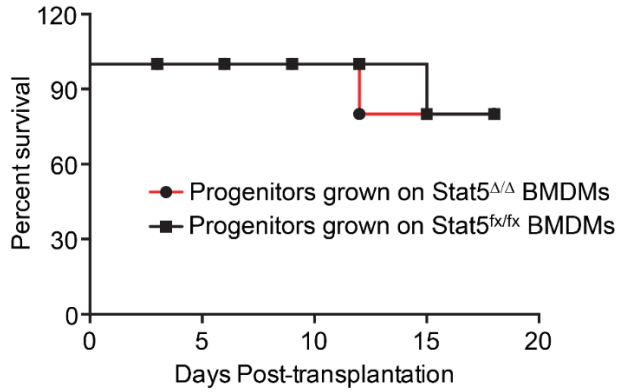
Table S2. TaqMan probe list

Gene	Catalog No.
18s	Hs99999901_s1
EpoR	Mm00833882_m1
Wnt1	Mm01300555_g1
Wnt2	Mm00470018_m1
Wnt2b	Mm00437330_m1
Wnt3	Mm03053665_s1
Wnt3a	Mm00437337_m1
Wnt4	Mm01194003_m1
Wnt5a	Mm00437347_m1
Wnt5b	Mm01183986_m1
Wnt6	Mm00437353_m1
Wnt7a	Mm00437354_m1
Wnt7b	Mm01301717_m1
Wnt8a	Mm01157914_g1
Wnt8b	Mm00442107_m1
Wnt9a	Mm00460518_m1
Wnt9b	Mm00457102_m1
Wnt10a	Mm00437325_m1
Wnt10b	Mm00442104_m1
Wnt11	Mm00437328_m1
Wnt16	Mm00446420_m1
Wnt2b(human)	Hs00921614_m1

Wnt8a(human)	Hs00230534_m1
HPGDS	Mm00479846_m1
PPARγ	Mm00440940_m1
PTGES	Mm00452105_m1

Supplemental Data

A



B

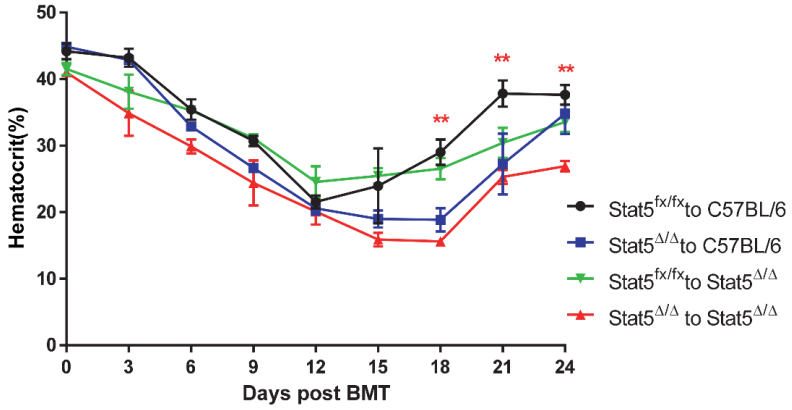


Figure S1. (A) Survival of mice transplanted with SEPs generated on Stat5$^{fx/fx}$ or Stat5$^{\Delta/\Delta}$ BMDMs. Sorted CD34+CD133+KS donor bone marrow cells were cultured on Stat5$^{fx/fx}$ or Stat5$^{\Delta/\Delta}$ BMDMs in SEEM media. Lethally irradiated C57BL/6 recipient mice were transplanted with 5,000 cultured CD34+CD133+KS donor cells. n=5 mice per group. (B). Analysis of hematocrit on indicated days after BMT. 100,000 unfractionated bone marrow cells from indicated donor mice were transplanted to the indicated recipient mice. Two-way ANOVA followed by Bonferroni's multiple comparisons. Data represent means ± SEM. ** p< 0.01. * represent p values of comparisons between Stat5$^{\Delta/\Delta}$ to C57BL/6 group and Stat5$^{\Delta/\Delta}$ to Stat5$^{\Delta/\Delta}$ group. n=5 mice per group.

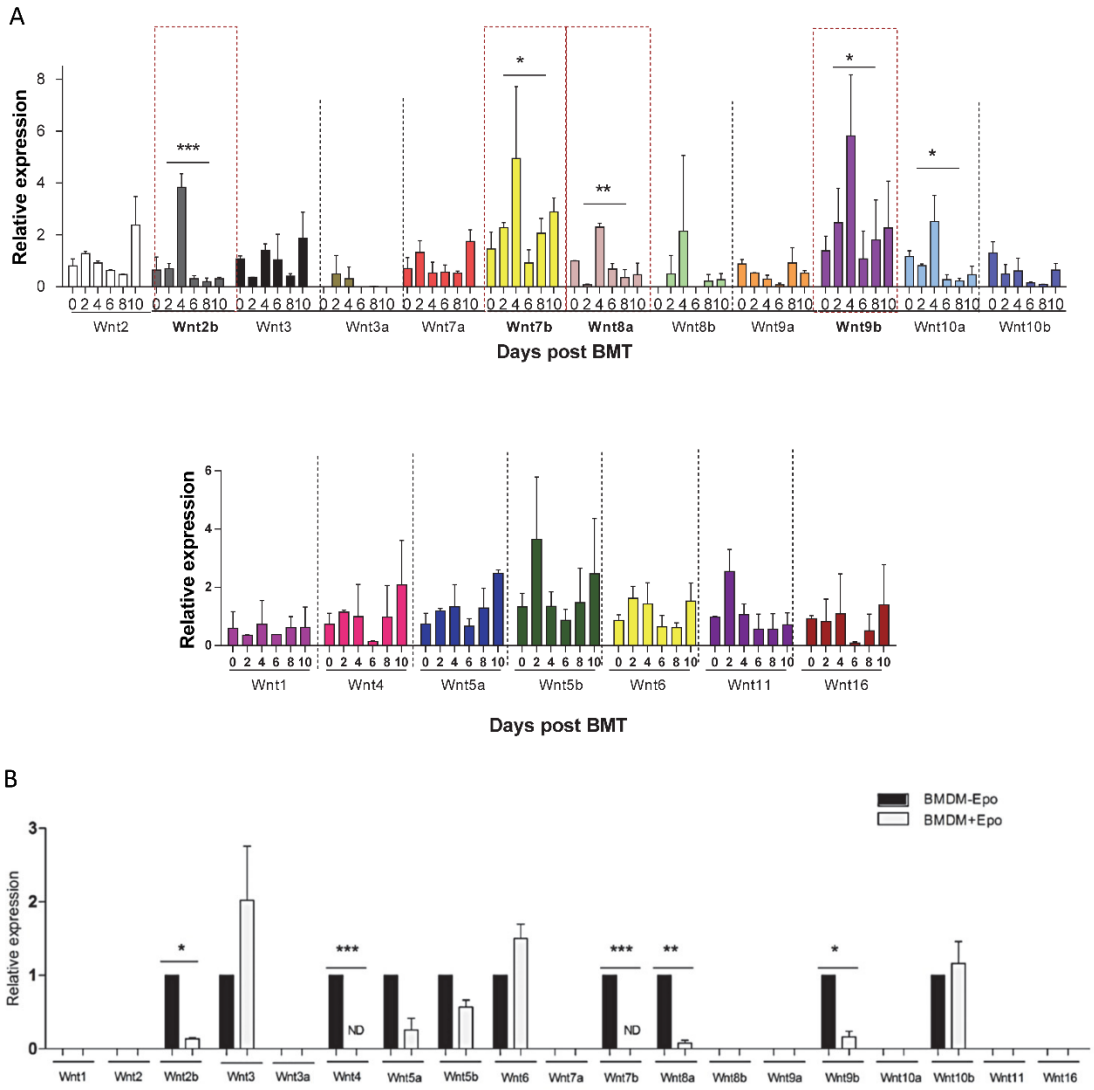


Figure S2. Analysis of the mRNA expression of Wnt family members during stress erythropoiesis. (A). mRNA expression of canonical Wnt ligands (top) and non-canonical Wnt ligands (bottom) in spleen cells from day 0 to day10 after BMT. 500,000 isolated C57/BL6 bone marrow cells were transplanted into lethally irradiated C57/BL6 recipient. Samples were collected every 2 days for analysis. n=2 mice per time point. One-way ANOVA. Data represent means ± SEM. * p< 0.05, ** p< 0.01, *** p< 0.001 (B). mRNA expression of canonical and non-canonical Wnt ligands in BMDMs treated ± Epo for 24 hours. n=3 per group. ND, not detected. Two-way ANOVA followed by Bonferroni's multiple comparisons. Data represent means ± SEM. * p< 0.05, ** p< 0.01, *** p< 0.001.

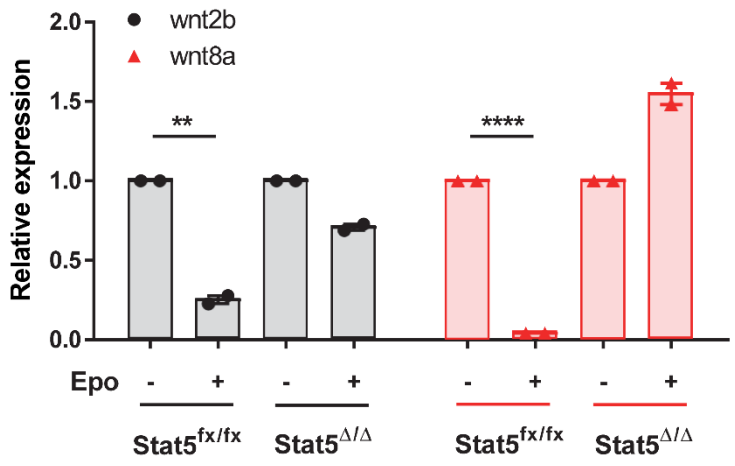


Figure S3. Epo dependent repression of Wnt2b and Wnt8a requires Stat5. mRNA expression of Wnt2b (left) and Wnt8a (right) in ex vivo cultured spleen macrophages from $Stat5^{fx/fx}$ and $Stat5^{\Delta/\Delta}$ mice treated as indicated ± Epo for 24 hours. Student t-test (2-tailed). Data represent means ± SEM. ** p< 0.01, **** p< 0.0001.

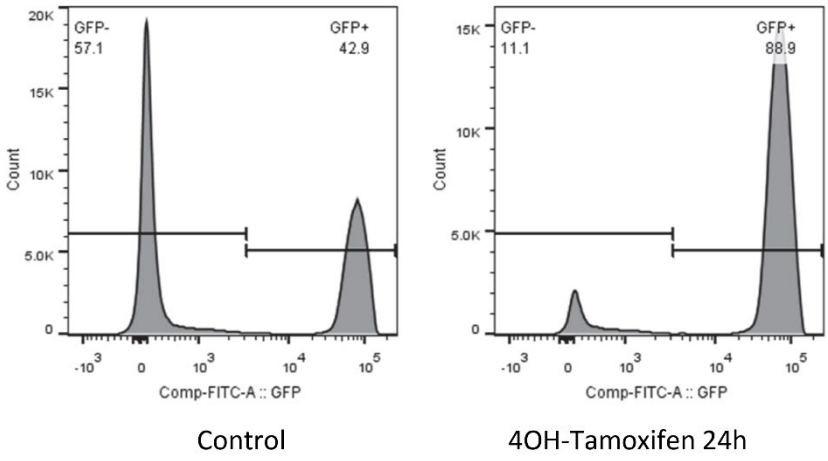


Figure S4. Mutation of β-catenin inhibits SEP expansion. Representative flow cytometry diagrams of GFP+ population (control cells) versus GFP- population (β-$catenin^{\Delta/\Delta}$ cells) isolated form co-cultures as described in Figure 3.

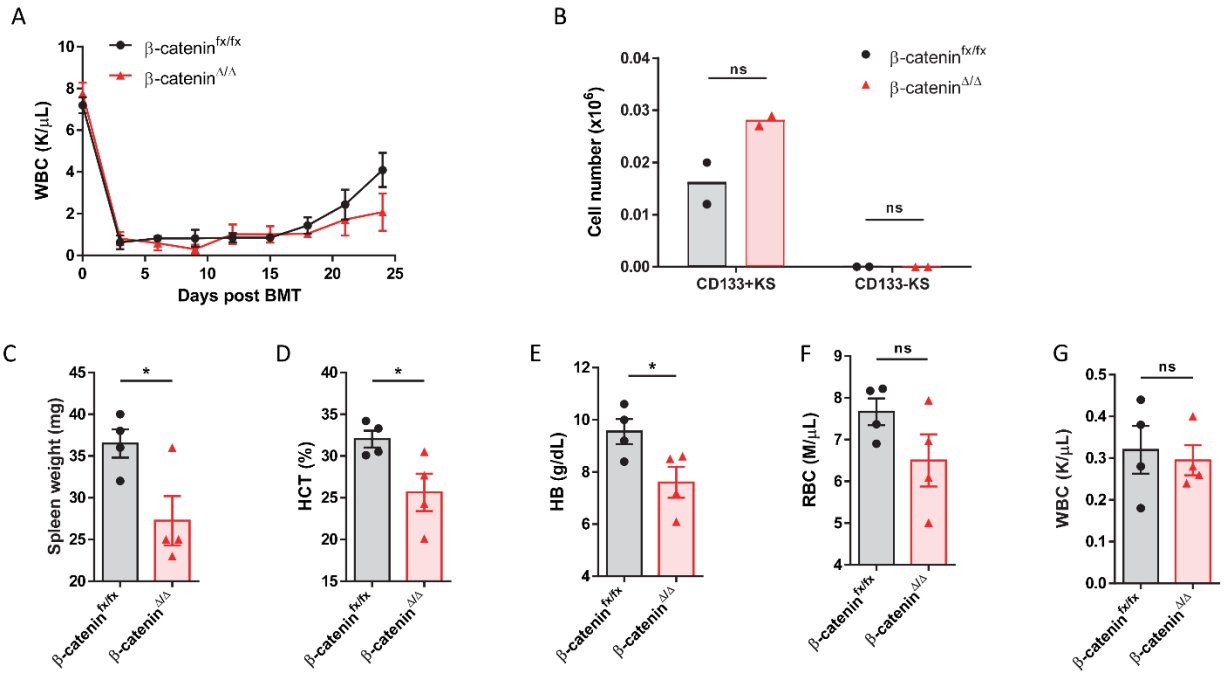


Figure S5. β-catenin$^{Δ/Δ}$ is required for SEP proliferation. (A). 500,000 unfractionated bone marrow cells isolated from β-catenin$^{fx/fx}$ or β-catenin$^{Δ/Δ}$ mice were transplanted to lethally irradiated CD45.1 C57BL/6 recipients. Analysis of WBC count after BMT. n=3 mice per group. (B-G). 50,000 GFP and 50,000 sorted CD133+KS cells isolated from β-catenin$^{fx/fx}$ or β-catenin$^{Δ/Δ}$ mice were transplanted to lethally irradiated CD45.1 C57BL/6 recipients. Student t-test (2-tailed). Data represent means ± SEM. * p< 0.05, ns, not significant. (B). Flow cytometry analysis of donor SEP homing on day 1 after BMT. CD133, Kit and Sca1 expressions are shown on cells gated on GFP-CD45.2+. (C). Analysis of spleen weight on day 8. (D). Analysis of hematocrit on day 8. (E). Analysis of hemoglobin level on day 8. (F) Analysis of RBC count on day 8. (G) Analysis of WBC count on day 8.

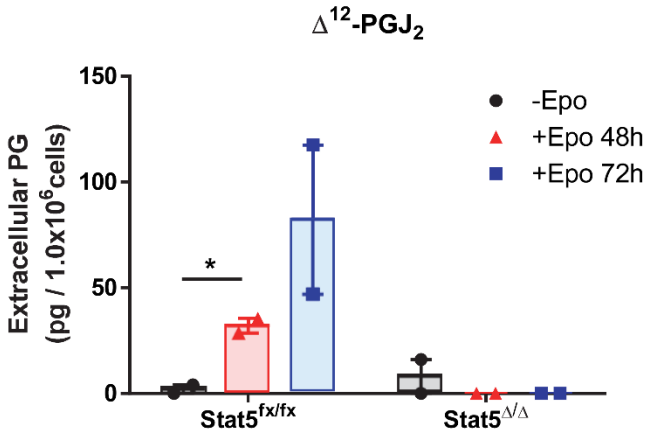
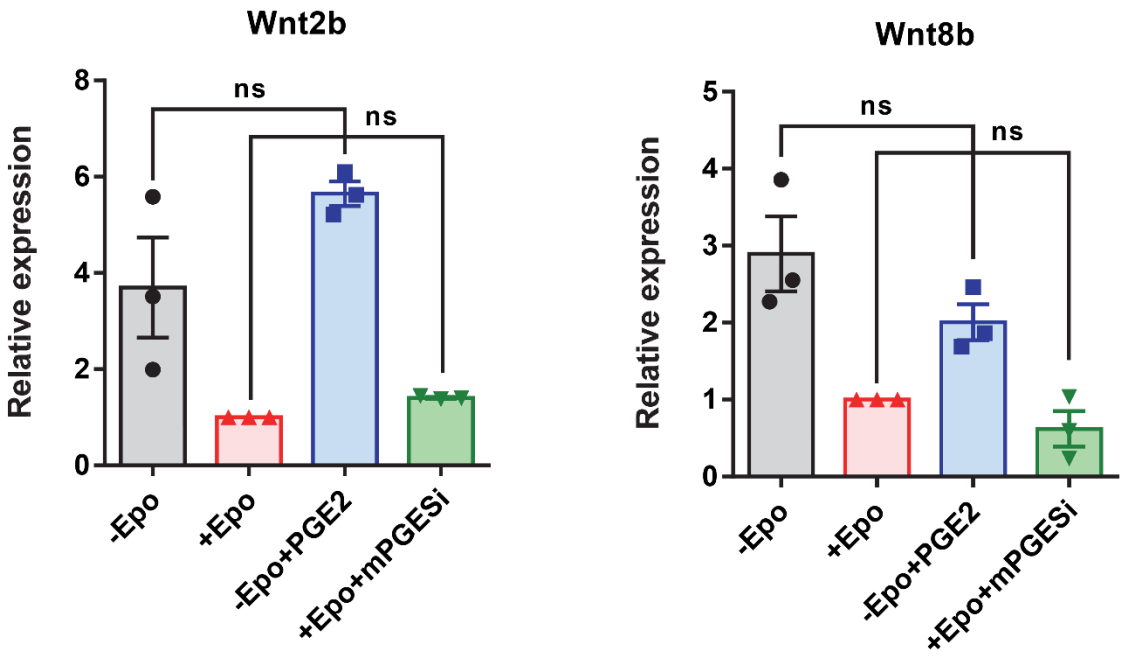


Figure S6. Production of Δ^{12}-PGJ_2 requires Epo dependent Stat5 activation. LC-MS/MS analysis of extracellular Δ^{12}-PGJ_2 in $Stat5^{fx/fx}$ and $Stat5^{\Delta/\Delta}$ BMDMs at the indicated times after Epo treatment. Two-way ANOVA followed by Bonferroni's multiple comparisons. Data represent means ± SEM. * $p < 0.05$.

A.



B.

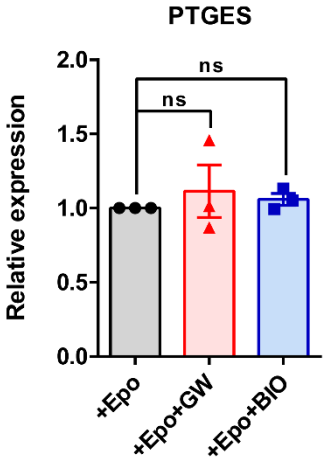


Figure S7. PGE_2 does not promote repression of Wnt2b and Wnt8a expression.

(A) mRNA expression of Wnt2b (left) and Wnt8a (right) in BMDMs treated with Epo, PGE_2 or mPGESi as indicated for 24 hours. Student t-test (2-tailed). Data represent means ± SEM. ns, not significant. (B) mRNA expression of PTGES in BMDMs treated with

Epo, GW or BIO as indicated. Student t-test (2-tailed). Data represent means ± SEM. ns, not significant.