Thrombospondin-1 Signaling through CD47 Inhibits Self-renewal by Regulating c-Myc and Other Stem Cell Transcription Factors

Sukhbir Kaur, David R. Soto-Pantoja, Erica V. Stein, Chengyu Liu, Abdel G. Elkahloun, Michael L. Pendrak, Alina Nicolae, Satya P. Singh, Zuqin Nie, David Levens, Jeffrey S. Isenberg, and David D. Roberts

Supplemental Methods, Figure Legends, and Figures

Extended Methods

Reagents

The thrombospondin-1-derived CD47-binding peptide 7N3 (^{1102}FIRVVMYEGKK^{1112}) and the inactive control peptide 604 (FIRGGMYEGKK) were synthesized by Peptides International[1]. Human thrombospondin-1 was purified from platelets obtained from the NIH Blood Bank as described[2]. A somatic mutant of the Jurkat human T lymphoma cell line lacking CD47, JinB8, was provided by Dr. Eric Brown[3]. Jurkat T cells, JinB8, Raji human Burkitt's lymphoma cells with c-Myc under the control of an IgH enhancer, B16 F10 murine melanoma, and Rat1 fibroblasts expressing the conditional c-Myc fusion protein (MycER™ [4]) were cultured using RPMI 1640 medium containing 10% FBS, penicillin/streptomycin, and glutamine (Invitrogen).

RNA extraction and Real Time PCR: Total RNA was extracted using TRIzol (Invitrogen) 24-36 h after transfection or as indicated. Whole organs were homogenized in TRIzol. cDNA was prepared using First Maxima First Strand cDNA Synthesis kit for RT-qPCR (Fermentas). Real Time PCR was performed using the primers listed in supplemental Table 1 and SYBR Green PCR master mix (Appliedbiosystems) on an Opticon I instrument (Bio-Rad) with the following amplification program: 95 °C for 15 min, 40 cycles of 95 °C for 15 s, 58 °C for 20 s, 72 °C for 25 s, and 72 °C for 1 min. Melting curves were performed for each product from 30 to 95 °C. The fold changes in mRNA expression were calculated by normalizing to hypoxanthine phosphoribosyltransferase (HPRT1) and TATA box binding protein associated factor (TAF9) for mouse tissues and endothelial cells, or β-2 microglobulin (B2M) mRNA levels for spleen and isolated splenocytes. B2M was used for normalization of mRNA levels in human cells. Note that

the total RNA yield per cell was higher for all CD47-null and CD47-deficient cells and tissues as compared to WT. Equal amounts of total RNA from WT and CD47 null mouse correspondingly showed differences expression for many housekeeping genes, but the above noted reference genes showed minimal differences in Ct values.

Microarray processing

Samples were prepared according to Affymetrix protocols (Affymetrix, Inc). RNA quality and quantity was ensured using the Bioanalyzer (Agilent, Inc) and NanoDrop (Thermo Scientific, Inc) respectively. Per RNA labeling, 300 nanograms of total RNA was used in conjunction with the Affymetrix recommended protocol for the GeneChip 1.0 ST chips.

The hybridization cocktail containing the fragmented and labeled cDNAs were hybridized to The Affymetrix Mouse GeneChip® 1.0 ST chips. The chips were washed and stained by the Affymetrix Fluidics Station using the standard format and protocols as described by Affymetrix. The probe arrays were stained with streptavidin phycoerythrin solution (Molecular Probes, Carlsbad, CA) and enhanced by using an antibody solution containing 0.5 mg/mL of biotinylated anti-streptavidin (Vector Laboratories, Burlingame, CA). An Affymetrix Gene Chip Scanner 3000 was used to scan the probe arrays. Gene expression intensities were calculated using GeneChip® Command Console® Software (AGCC) and Expression Console™ Software. Cel files generated by the Affymetrix AGCC program were imported in the Partek Genomic Suite software and RMA (Robust Multichip Analysis) normalization, log2 transformation and probe summarization was performed. Anova pairwise comparisons and PCA (Principle Component Analysis) were performed within Partek Genomic Suite. The GEO accession numbers for the

microarray data is GSE43133.

GeneSet Enrichment Analysis (GSEA) was used to tests whether an established gene signature is significantly enriched for genes differentially expressed between WT, CD47 null, CD47 null EB-like clusters and established embryonic stem cell lines. Description of the GeneSet enrichment analysis (GSEA) and the MSigDB can be found at http://www.broadinstitute.org/gsea/. [5].

Teratoma Formation

The v6.5 mouse ES cell line was used as a positive control for testing teratoma formation. These mES cells were cultured in DMEM medium containing 15% fetal bovine serum and 1000 IU/ml LIF (Leukemia Inhibitory Factor). For teratoma formation, the mES cells or CD47-/- endothelial cells were trypsinized, washed once in PBS, and finally resuspended in PBS at 5 x 10^6/ml for mES and 1 x 10^7/ml for CD47-/- The cells suspension was chilled on ice and then mixed with 50% volume of cold Matrigel (4^{o}C). The cell-Matrigel mix was draw into a cold 1 ml syringe, and 0.15ml was quickly injected subcutaneously into NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ mice near the region where the hind thigh and the abdomen meet. Therefore, ~5x 10^5 mES cells or 1x10^6 CD47-/- cells from EB-like clusters were injected at each site. Two weeks after the injection, the mice were observed daily for tumor growth. When the tumor reached 2 cm in length, the mouse was euthanized, and the tumors were dissected out for morphological observation.

Cell culture medium for macrophage differentiation

Mouse L929 cells (a kind gift from Alan Sher, NIH) were grown in DMEM Growth medium (DMEM with high glucose, 10% FBS, 2mM L-Glutamine, Penicillin-Streptomycin; all

from Life Technologies) at 37°C under 5% CO2 until 100% confluent. Conditioned medium was harvested and stored at -80^{o}C.

CD47 deficient mouse cells were either cultured in the presence of Endothelial Basal Medium -2 (Lonza) or in the presence of 30% L929 conditioned medium in RPMI Growth Medium (RPMI 1640, 10% FBS, 2mM L-Glutamine, Penicillin-Streptomycin; all from Life Technologies). Cells were cultured for ten days at 37oC under 5% CO2. The macrophage marker was tested using Flow Cytometry.

Antibodies/Reagents for Flow Cytometry

Anti-mouse CD11c PE-Cy7, CD11b PE, and B220 PE were all purchased from BD Biosciences. Anti-mouse Ly-6C eFluor 450, Ly-6G PerCP-Cy5.5, and CD3e FITC were all purchased from ebiosciences. Anti-Mouse Sca-1 PE-Cy5 was a kind donation from Thomas B. Nutman (NIH). Anti-mouse CD14 APC-Cy7, CD31 AlexaFluor647, CD64 APC, and anti-mouse/human Mac-2 PE was purchased from Biolegend. All flow cytometry antibodies were titrated for optimal performance. Anti-Rat/Anti-Hamster Ig κ compensation particles were purchased from BD Biosciences.

All cells were dislodged by incubating with Versene (Life Technologies) and then scraping. They were collected on ice and washed with buffer (PBS with 3% BSA; Life Technologies). All following staining steps were performed on ice and incubated in the dark. After washes buffer was decanted and cells were stained with all antibodies or each florescence minus one control. Compensation beads were used for single color controls, when possible, as directed by the manufacturer. Otherwise, single color controls were made using a mixture of cell. Cells and

beads were washing thoroughly prior to acquisition. Data was acquired using a LSRII (BD Biosciences) and BD FACSDiva Software. Data was analyzed using FlowJo (Tree Star, Inc).

Cell culture medium for neural differentiation:

CD47 null mouse lung endothelial cells passaged for 6 months were seeded into 6-well tissue culture plates using basal EBM medium supplemented with FGF2 and EGF (~5-20 ng/ml), heparin and gentamycin sulfate. EB-like cluster resembling neurospheres appeared after 24-36 h. The cells were then plated onto non-tissue culture dishes in heparin-free differentiation medium. Neural precursor cells were visible after 6 days.

Neurospheres were collected and dispersed using StemPro Accutase (GIBCO). The cells were cultured using EBM medium supplemented with FGF2 and EGF (~5-20 ng/ml), Gentamycin sulfate and StemPro Neural supplement (GIBCO). The heterogenous neural phenotype cells were cultured and passaged for long term.

Cell culture medium for smooth muscle cell differentiation:

CD47 null mouse lung endothelial cells were plated in to 6-well tissue culture plates using Smooth Muscle Basal Medium (Lonza) supplemented with PDGF (10 ng/ml) and TGF-β1 (5 ng/ml). The EB-like clusters were harvested and transferred to 1% gelatin (Sigma) coated plates. The EB-like clusters differentiated into smooth muscle cells after 6 days. The differentiated smooth muscle cells were stained for smooth muscle actin.

Cell culture medium for hepatocyte cell differentiation:

The WT and CD47 null endothelial cells were grown in DMEM +glutamine+penicillin/streptomycin+1% insulin/transferrin/selenium supplement (Invitrogen) +hepatocyte growth factor (R&D- 20ng/ml), Oncostatin M (R&D 10 ng/ml), 10 nM dexamethasone (Waco Pure Chemical Industries Ltd, Osaka, Japan) with slight modification of [7]. The EB-like clusters were stained for the hepatocyte marker AFP after 36 h.

Cell culture medium for mesenchymal cell differentiation:

The WT and CD47 null endothelial cells were grown in BD Mosaic TM hMSC SF culture medium along with BD mosaic hMSC SF supplement (BD biosciences). CD47 null cells formed EB-like clusters after 36 h. The EB-like clusters were collected and differentiated by coating plates with BD mosaic hMSC SF surface (BD Biosciences). For direct transdifferentiation, the plates were coated with BD mosaic hMSC SF surface according to manufacturer's instructions. WT and CD47 null endothelial cells were directly plated on coated 6-well plates (BD biosciences). The transdifferentiated cells were stained using oil red after 10-days.

Oil Red O staining for mesenchymal adipocytes

Stock solution of Oil Red O (300mg of oil red powder+ 100 ml of isopropanol) was prepared the day before staining according to the manufacturer's instructions. For a working solution, 3 parts of stock solution of Oil Red O and 2 parts of deionized water were mixed. The working solution was incubated for 10 minutes at RT and filtered with Whatman filter paper several times. The differentiated cells were cultured in 12-well plates for 10-days. To assess adipogenic phenotype, cells were washed with IXDPBS and fixed with 1-2% Formalin overnight at 4^{o}C. The formalin was removed from the wells, and the cells were washed with deionized

water. Two ml of 60% of isopropanol was added to each well for 5 min. The cells were then incubated with 2 ml of Oil Red O solution for 5 min. The cells were rinsed with deionized water until clear. A 2 ml volume of hematoxylin stain was added for 1 min and then washed with water immediately. The wells were covered with water, and images were taken using phase contrast illumination.

Immunostaining of embyroid bodies and differentiated cells:

Dispersed EB-like clusters were placed on poly-D lysine coated Lab-Tek cover glass chambers and fixed with 4% paraformaldehyde for 5 min. EB-like clusters were gently washed with 1X PBS and permeabilized using 0.3% Triton X-100. The EB-like clusters were washed and blocked with 3% BSA for 1 h. Primary SOX2 (Abcam) and nestin antibodies (Covance) (1:500), KLF4, OCT4, SSEA1, SOX2 (Stemgent), c-KIT (DAKO), MYC (Epitomics), CD31 (PECAM1, BD Pharmingen) , AFP and VEGFR2 (Cell Signaling) were used for immunostaining. Alkaline phosphatase staining was performed using Alkaline Phosphatase Substrate Kit III (Vector Laboratories) according to manufacturer's instructions.

Where specified, EB-like clusters from V6.5 ES cells and CD47 null cells were cultured using ES medium containing LIF, and immunostaining was performed using Nanog, Oct4 and SOX2 antibodies (Cell Signaling) according to manufacturer's instructions.

Differentiated neural cells were cultured overnight using Lab-Tek cover glass 4-well chambers. The cells were washed twice with 1xPBS, fixed using 4% parafomaldehyde for 5 min, and washed three times. The cells were permeabilized using 0.3% Triton X-100 in PBS. The cells were washed three times 5 min each and blocked with 5% BSA for 1 h. Primary antibodies

against GFAP (DAKO), S100b (Abcam), MAP2, beta tubulin III and smooth muscle actin (Sigma) , alpha-fetoprotein (Cell Signaling) were used. Secondary antibodies (Alexa Fluor® 488 Goat Anti-Mouse IgG1 or Alexa Fluor® 488 Goat Anti-Rabbit IgG, Invitrogen) were used. Confocal images were captured using Zeiss 710 Zeiss AIM software on a Zeiss LSM 710 Confocal system with a Zeiss Axiovert 100M inverted microscope and 50 mW argon UV laser tuned to 364 nm, a 25 mW Argon visible laser tuned to 488 nm and a 1 mW HeNe laser tuned to 543 nm. A 63x Plan-Neofluar 1.4 NA oil immersion objective was used at various digital zoom settings.

Immunostaining and differentiation of EB-like clusters

CD47 null cell EB-like clusters were collected and transferred to gelatin coated T185 flask (Nunc) using RPMI complete media for 6 days. The EB-like clusters differentiated into heterogeneous colonies. The individual colonies were picked and transferred further into gelatin coated Willico dish. The colonies were cultured using appropriate differentiation media (neural smooth muscle, and hepatocyte) for 36h. The EB-like clusters were fixed with 4%PFA for 1-2 h at RT. The EB-like clusters were washed three times with 1xPBS (without Ca and Mg ions). The EB-like clusters were blocked with blocking buffer (3%BSA in PBS+0.2%TX-100) for 1-2h. The primary antibodies (1:100 in blocking buffer) for neural (ectoderm), smooth muscle actin (mesoderm) and Alpha- fetoprotein (endoderm) markers used O/N at 4C. The EB-like clusters were washed with blocking buffer three times. Secondary antibodies (1:1000 ratios of Alexa Fluor® 488 Goat Anti-Mouse IgG1 or Alexa Fluor® 488 Goat Anti-Rabbit IgG, Invitrogen) were used. The EB-like clusters were washed three times with 1X PBS. EB-like clusters were dried using Kimwipes. VECTASHIELD from Vector laboratories with DAPI used for mounting. The

confocal images were captured using Zeiss 710 Zeiss AIM software on a Zeiss LSM 710 Confocal system as above mentioned. The Z-stack images were captured and exported as an Avi File using the ZEN software.

Single cell differentiation

EB -like clusters were formed using serum free EBM media for 36 h. A single EB-like cluster was dissociated in to single cell suspension using Accutase (BD Biosciences) and was plated at limiting dilution into 96-well plates and assessed for colony formation over 7 days. A colony was picked, expanded and plated further in to 4-Well LabTek Chambers using neural, smooth muscle and hepatocytes growth media. After 7 days, the cells were stained with antibodies against TUJI (ectoderm), smooth muscle actin (mesoderm), and AFP (endoderm). WT murine lung endothelial cells were also cultured under the same conditions but were unable to differentiate and were negative for these markers (data not shown).

Embryoid body western blot

Undifferentiated EB-like clusters were cultured in either complete RPM1 or serum free media with neural growth factors for 10-15 days. Similarly, lung endothelial cells from WT and CD47-null were plated for 10-15 days with EGM2 medium at 37°C. The endothelial cells and differentiated EB-like clusters were washed with 1xPBS, and cell lysates were made using RIPA buffer. The lysates were centrifuged, and equal volumes of supernatant were boiled with 4X NuPAGE–LDS sample buffer (Invitrogen) for 5 min at 95 °C. Proteins were separated using 4-12% or or 12% Bis-Tris gels (Invitrogen). Primary SOX2 (Abcam) , nestin (Covance, 1:500), KLF4, OCT4, SOX2 (Stemgent), Tuj 1 (Neuron-specific class III beta-tubulin, Neuromics), GFAP (DAKO)

and smooth muscle actin (Sigma) , and AFP (Cell Signaling) antibodies were used at 1:1000 to perform western blots. Secondary anti-rabbit IgG or anti-mouse IgG conjugated to HRP were used at 1:5000. Super Signal West Pico chemiluminescent substrate (Thermo Scientific Fisher) was used to detect bound antibodies. For protein normalization, the blots were reprobed using a ß-actin antibody (Sigma Aldrich).

Flow cytometry

For analysis of intracellular c-Myc and Oct-4A, cells were washed twice in PBS and incubated with Accutase (BD Biosciences) in a 37^0C incubator for 10 min to dissociate the colonies into single cells. Cells were collected by centrifugation at 1500 rpm for 5 min, fixed and permeabilized using Foxp3 staining buffer kit (eBioscience) according to the manufacturer's instructions. Cells were stained with unconjugated anti c-Myc (Abcam) and anti Oct-4A rabbit monoclonal antibodies (Cell Signaling) for 30 min at 4^0C, washed twice with FACS buffer (HBSS containing 4% FBS) and incubated with goat anti-rabbit IgG-FITC (Santa Cruz Biotechnology) for 30 min at 4^0C. Cells were washed twice with FACS buffer and analyzed on a LSR II cytometer with FACSDiva software (BD Biosciences). Flow cytometry data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

BrdU staining for Asymmetric cell division

Asymmetric cell division was analyzed as described with slight modifications[8, 9]. WT and CD47 null cells (passage 1) were labeled with BrdU (1uM) for 5 days and then chased in BrdU-free medium for 24h and followed by cytochalasin B at 2 µM for 24h. The BrdU labeled cells were fixed with 70% ethanol for 30 min. The cells were denatured with 2N HCl/0.5% Triton X-100 for

60 min. The cells were washed in PBS/0.5%TX-100/0.1% BSA. The cells were stained with mouse-anti-BrdU (Calibiochem) using a dilution of 1:100 overnight at 4ºC. Secondary antibodies donkey-anti-mouse IgG-Alexa 594 or Alexa 488 (Invitrogen) were used (1:500) for 1h at RT. The cells were mounted using Vectashield (Vector Laboratories). Images were acquired at 40X using an Olympus microscope. The total cells negative for BrdU and positive for DAPI were counted manually.

Continuous growing CD47-null cells were labeled with BrdU for 10 days. One hundred percent BrdU incorporation was confirmed using confocal microscope (data not shown). The BrdU labeled cells were chased for 2 consecutive cell divisions in BrdU-free medium (72 h). The mitotic cells were obtained by gently shaking the flask. The mitotic cells were plated in glass bottom Micro Well dishes (MatTek Corporation) along with cytochalasin B for 24h. The cells formed EB like clusters and were stained with BrdU antibody and green fluorescent phalloidin conjugate. Images were captured using a Zeiss 780K confocal microscopy at 63X.

References

1. Barazi, H.O. *et al.* Regulation of integrin function by CD47 ligands. Differential effects on αvβ3 and α4β1 integrin-mediated adhesion. *J Biol Chem* **277**, 42859-42866. (2002).
2. Roberts, D.D., Cashel, J. & Guo, N. Purification of thrombospondin from human platelets. *J Tissue Cult Methods* **16**, 217-222 (1994).
3. Reinhold, M.I., Green, J.M., Lindberg, F.P., Ticchioni, M. & Brown, E.J. Cell spreading distinguishes the mechanism of augmentation of T cell activation by integrin-associated protein/CD47 and CD28. *Int Immunol* **11**, 707-718. (1999).
4. Littlewood, T.D., Hancock, D.C., Danielian, P.S., Parker, M.G. & Evan, G.I. A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. *Nucleic Acids Res* **23**, 1686-1690 (1995).
5. Subramanian, A. *et al.* Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* **102**, 15545-15550 (2005).
6. Stenberg, J. *et al.* Sustained embryoid body formation and culture in a non-laborious three dimensional culture system for human embryonic stem cells. *Cytotechnology* **63**, 227-237 (2011).
7. Ishkitiev, N. *et al.* Hydrogen sulfide increases hepatic differentiation in tooth-pulp stem cells. *J Breath Res* **6**, 017103 (2012).
8. Pine, S.R., Ryan, B.M., Varticovski, L., Robles, A.I. & Harris, C.C. Microenvironmental modulation of asymmetric cell division in human lung cancer cells. *Proc Natl Acad Sci U S A* **107**, 2195-2200 (2010).
9. Sundararaman, B. *et al.* Asymmetric chromatid segregation in cardiac progenitor cells is enhanced by Pim-1 kinase. *Circ Res* **110**, 1169-1173 (2012).
10. Bhattacharya, B. *et al.* Gene expression in human embryonic stem cell lines: unique molecular signature. *Blood* **103**, 2956-2964 (2004).

Supplementary Figure Legends

Fig S1. Continuous propagation of WT and CD47-null mouse lung endothelial cells. (**A**) Cultures were photographed 7 days after each passage. (**B**) WT cells at passage 2 showed a flattened morphology characteristic of senescent cells, but CD47-null cells maintained a typical endothelial morphology. The growth of both WT and CD47 null lung endothelial cells slowed after passages 3-5. WT cells grew very slowly and became stationary senescent cells. On the other hand, CD47 null cells initially flattened but resumed growth within 2-3 weeks. CD47 null cells restarted growth as colonies of well differentiated endothelial cells that maintained extensive cell-cell contact (cobblestone morphology) and required passage twice a week. Independent isolates of CD47 null endothelial cells reproducibly maintained their growth and morphology for at least 6 months. WT cells never resumed growth. (**C, D**) Mouse lung endothelial cells WT vs thrombospondin-1 null. Equal numbers of WT and thrombospondin-1 null murine lung endothelial cells were plated at the indicated passage numbers. After growth in EGM medium + 0.5% FBS, viable cells were quantified by trypsinization, centrifugation, and counting on a hemocytometer in the presence of Trypan blue. (**E**) CD47-null endothelial cells were stained using CD14 and CD11c antibodies and analyzed by flow cytometry. (**F**) Sca-1 expression in CD47-null endothelial cells.

Fig. S2. (**A**) Formation of embryoid bodies by continuously cultured CD47-null endothelial cells transferred into serum free neural basal medium. Sequential photographs of a representative culture are shown. (**B**) Selective formation of embryoid body-like clusters by passage 2 CD47-null endothelial cells in serum-free medium. Adherent cells (left) and nonadherent cell clusters (right) were imaged 36 h after transfer into serum-free medium. Nascent nonadherent EB-like clusters were

abundant in the CD47-null culture, but only one loose cluster of cells was observed in the WT control. The latter cells did not survive at later times.

Fig. S3. (**A**) Hierarchical cluster analysis of microarray data comparing gene expression of WT and CD47 null endothelial cells, EB-like clusters derived from CD47 null endothelial cells by culture in serum free medium for 36 h, and v6.5 ES cells. (**B**) GeneSet Enrichment Analysis (GSEA) for embryonic stem cell genes as defined by Bhattacharya et al [10] that are induced when CD47 null endothelial cells are induced to form EB-like clusters.

Fig. S4. WT (**A**) and CD47 null mouse lung endothelial cells (E) were cultured in EGM2 medium. WT (**B-D**) and CD47-null cells (**E-H**) were transferred to serum-free medium to induce embryoid bodies and stained for pluripotent stem cell markers. Top panels: Alkaline phosphate activity was observed in embryoid body cells derived from CD47-null endothelial cells (**F-G**), whereas no alkaline phosphate activity was observed in WT cells, which fail to form EB-like clusters (**B-D**). (**I,J**) Embryoid bodies derived from CD47-null cells were sectioned and stained for expression of the pluripotent stem cell markers SSEA1 and c-Kit (green). Blue = DAPI nuclear stain. Overlays are presented in each bottom right panel.

Fig. S5. Morphological, biochemical and immunofluorescence analysis of differentiated embryoid bodies derived from CD47-null cells by culturing in RPMI medium with serum for 10-15 days. Top panels show differentiated EB-like clusters under bright field and phase contrast illumination (**A&B**). Representative H&E stained section shows morphological evidence for ectodermal, mesodermal, and endodermal differentiation (**C-F**). A 5 µm formalin fixed paraffin embedded differentiated embryoid body stained with H&E (4x panel **C**) indicates the presence

of all three germ cells layers: cuboidal endodermal epithelium with slightly atypical nuclei (H&E 40x panel **D**), mesoderm or primitive mesenchyme with oval/fusiforme nuclei embedded in a myxoid matrix (H&E 40x panel **E**). Some of the cells (arrow) contain eosinophilic amorphous material. Numerous apoptotic bodies are also seen (H&E 40x panel E). Panel E shows presumptive ectoderm with pluristratified monotonous, basophilic nuclei mimicking primitive neuroectoderm (H&E 20x, panel **F**). Biochemical analysis of embryoid bodies for presence of three germ layer markers TUJI, AFP and SMA (**G**). Lower panels show representative sections of differentiated EB-like clusters stained for expression of the endothelial marker VEGFR2, which is lost upon differentiation, and the stem cell transcription factors Klf4, Oct4, c-Kit, cMyc, and the differentiation marker AFP (**H-K**).

Fig. S6. (**A**) Ectoderm differentiation marker expression by cells derived from CD47-null embryoid body-like clusters formed in serum free medium. Phase contrast image of EB-like clusters (a) and differentiation of neural precursor cells from embryoid bodies (b and high magnification in c). Neural microtubule-associated protein-2 (MAP2) expression in embryoid body cells (d) and in a differentiated adherent cell (e). Expression of glial fibrillary acidic protein (GFAP, f), neuron-specific beta III tubulin (g), and S100b astrocyte marker (h) on adherent cells grown from embryoid bodies in neural differentiation medium. (**B**). Endoderm differentiation marker expression by cells derived from CD47-null embryoid body-like clusters formed in serum free medium. Morphology of WT mouse lung endothelial cells in Hepatocyte medium (a), embryoid body formation by CD47-null lung endothelial cells in Hepatocyte medium (b), expression of endodermal marker AFP in CD47-null lung endothelial cells in Hepatocyte medium (red, c), no expression of AFP in CD47-null endothelial cells grown in EGM2 medium

(d), WT mouse lung endothelial cells in mesenchymal medium (e), and CD47 null cells in mesenchymal medium with embryoid body formation (f). Adherent cell outgrowth from differentiating embryoid bodies (g) and differentiated cells stained for adipocyte marker Oil red O staining (h-i). (**C**). Expression of the mesoderm marker smooth muscle actin by CD47 null cells grown from serum free embryoid bodies transferred into smooth muscle differentiation medium.

Fig. S7. Hematopoetic differentiation marker expressions by cells derived from CD47-null embryoid bodies. Morphologies of CD47-null mouse lung endothelial cells in EGM2 media (**A**) and Mouse lung endothelial cells in L929 media (**B**). Analysis by flow cytometry showed minimal expression of the macrophage marker Mac2 in EGM2 media (**C**) but expression of Mac2 in L929 media (**D**). Expression of the hematopoietic stem cell marker Sca1 was lost in CD47-null endothelial cells grown in RPM1 +L929 conditioned medium grown for 10 days (**F**). The cells were confirmed to lack CD47 expression (**E**). (**G-H**) Immunohistochemical detection of Sox2-expression (brown nuclear staining) in representative spleen sections from WT (K) and CD47 null mice (L). 40x objective.

Fig. S8. Knockdown of CD47 expression in vivo by CD47-Morphilino (**A**). Re-expression of human CD47-V5 in mouse lung endothelial cells (**B**). Relative expression of c-MYC and CD47 in transfected cells as compared to that in human umbilical vein endothelial cells (HUVEC, **C**). TSP1 reduces c-MYC expression when is CD47 re-expressed in JinB8 cells (**D**). Expression level of CD47 in transfected JinB8 cells relative to WT Jurkat cells (**E**). CD47 induced cell cytotoxicity in mouse lung endothelial cells but not in cells with dysregulated c-Myc: (**F**) Re-expression of

CD47-FLAG in the presence and absence of c-Myc-GFP in mouse endothelial cells induced cell cytoxicity. (**G**) Cytotoxicity induced by re-expression of CD47-FLAG in Raji Burkitt's lymphoma cells. (**H**). Cytotoxicity induced by re-expression of CD47-FLAG in B16 melanoma cells, Rat 1 fibroblasts and CD47 null lung endothelial cells.

Movie. S1. Z-Stack movies of differentiating embryoid bodies stained with differentiation markers. Expression of the ectoderm markers neuron-specific β III Tubulin (red) and GFAP (green) is visualized by immunofluorescent staining. Images were obtained using a 10x objective and nuclei are visualized by DAPI staining (blue).

Movie. S2. Z-Stack movies of differentiating embryoid bodies stained with the differentiation marker smooth muscle actin (red). Images were obtained using a 10x objective and nuclei are visualized by DAPI staining (blue).

Movie. S3. Z-Stack movies of differentiating embryoid bodies stained with differentiation markers for α-fetoprotein (AFP) endoderm marker. Images were obtained using a 10x objective and nuclei are visualized by DAPI staining (blue).

Figure S1

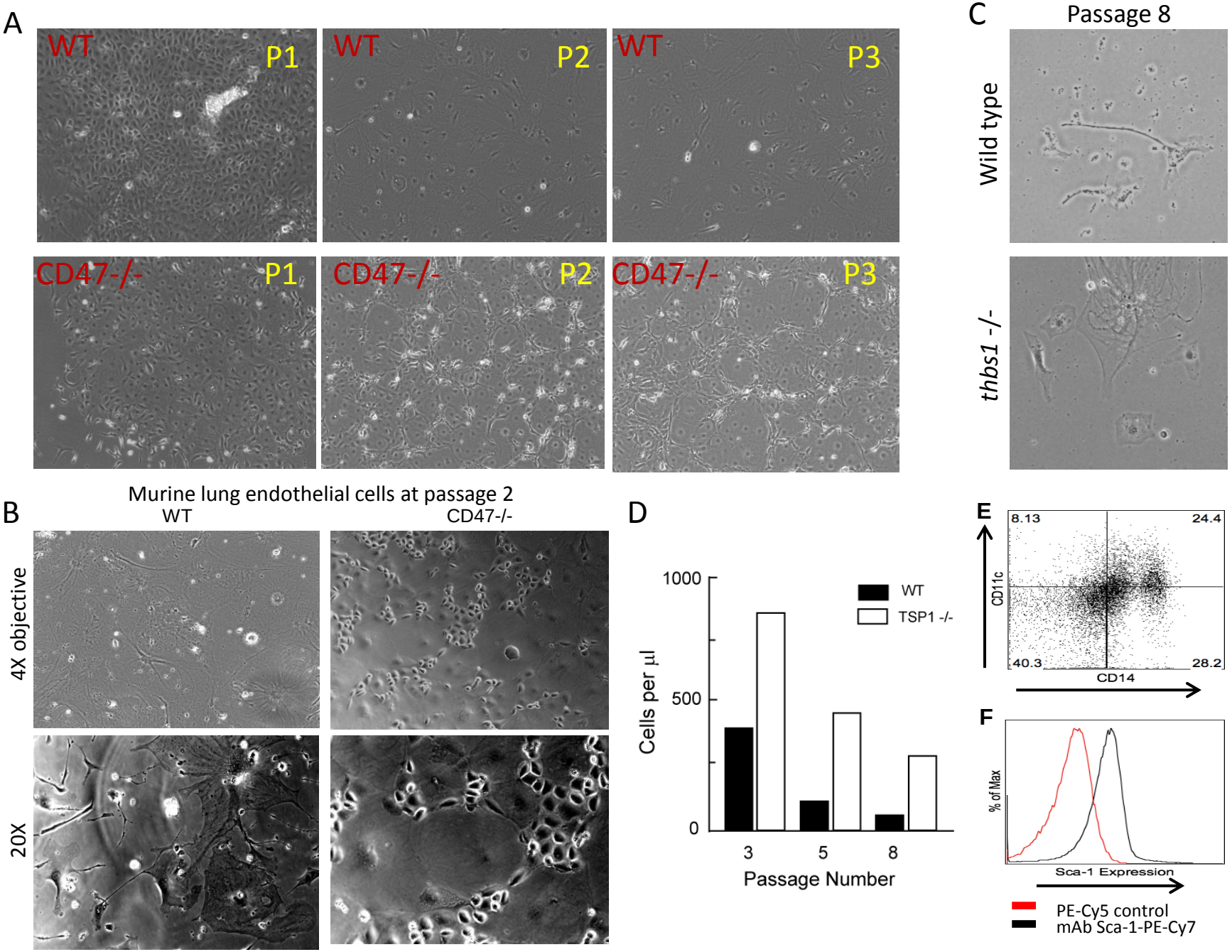
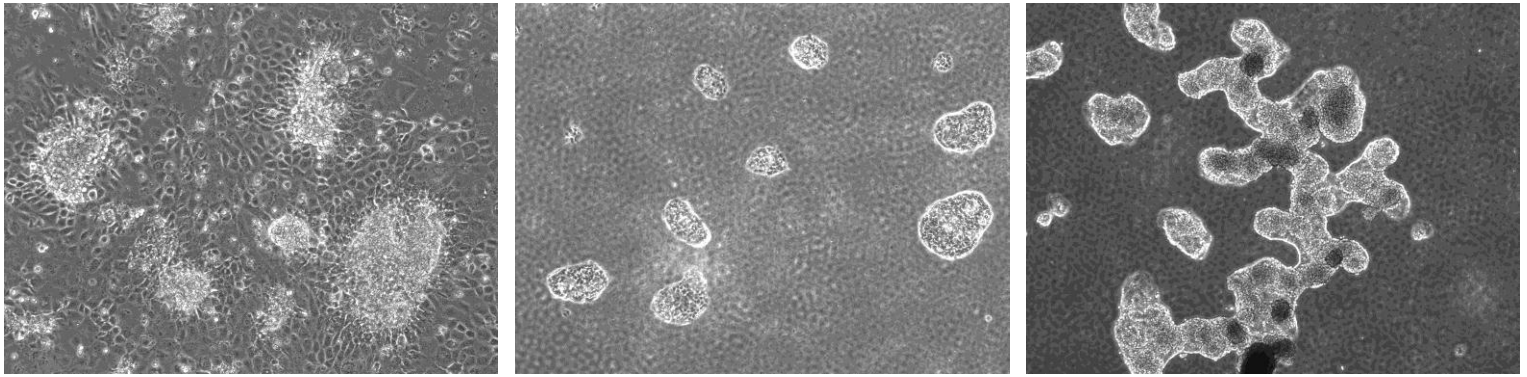

A
WT
P1
WT
P2
WT
P3
CD47-/-
P1
CD47-/-
P2
CD47-/-
P3
C
Passage 8
Wild type
thbs1 -/-
B
Murine lung endothelial cells at passage 2
WT
CD47-/-
4X objective
20X
D
1000
500
0
Cells per µl
WT
TSP1 -/-
3
5
8
Passage Number
E
8.13
24.4
40.3
28.2
CD11c
CD14
F
% of Max
Sca-1 Expression
PE-Cy5 control
mAb Sca-1-PE-Cy7

Figure S2

A Formation of embryoid body-like clusters by continuously grown CD47-null endothelial cells transferred into serum-free medium

time →



B Selective formation of embryoid body-like clusters by passage 2 CD47-null endothelial cells in serum-free medium

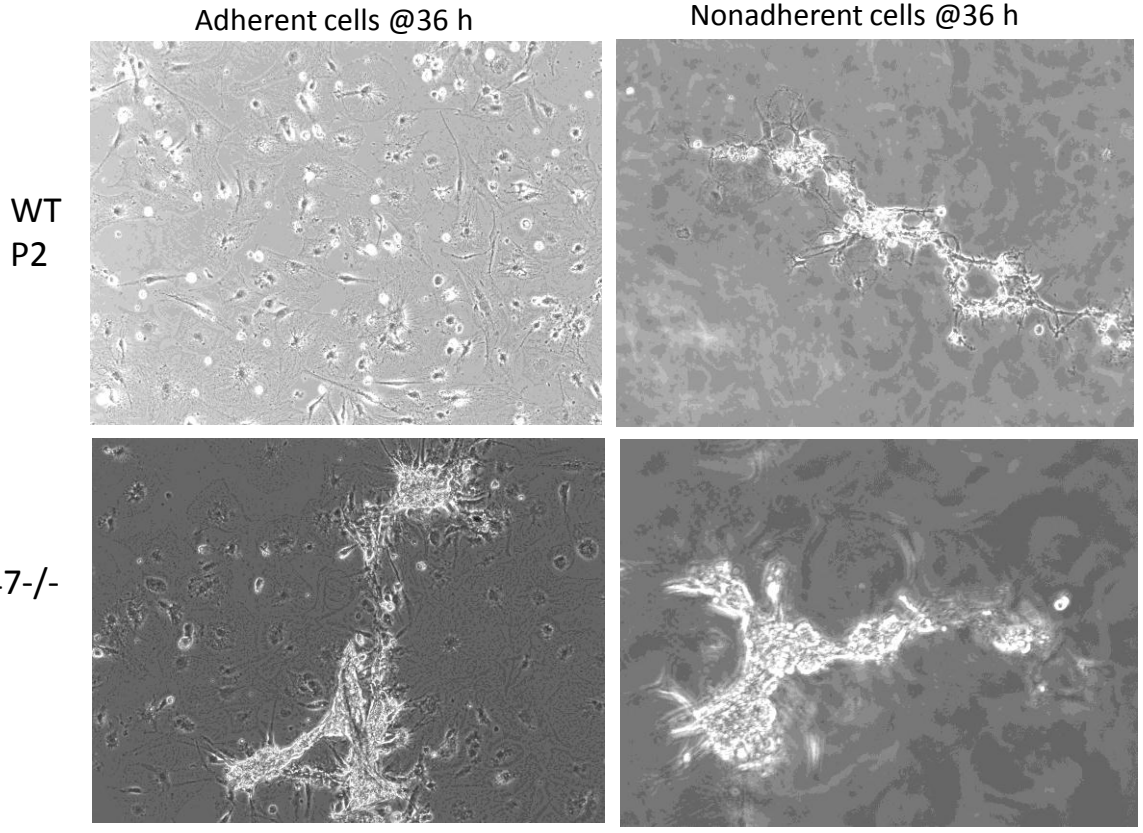
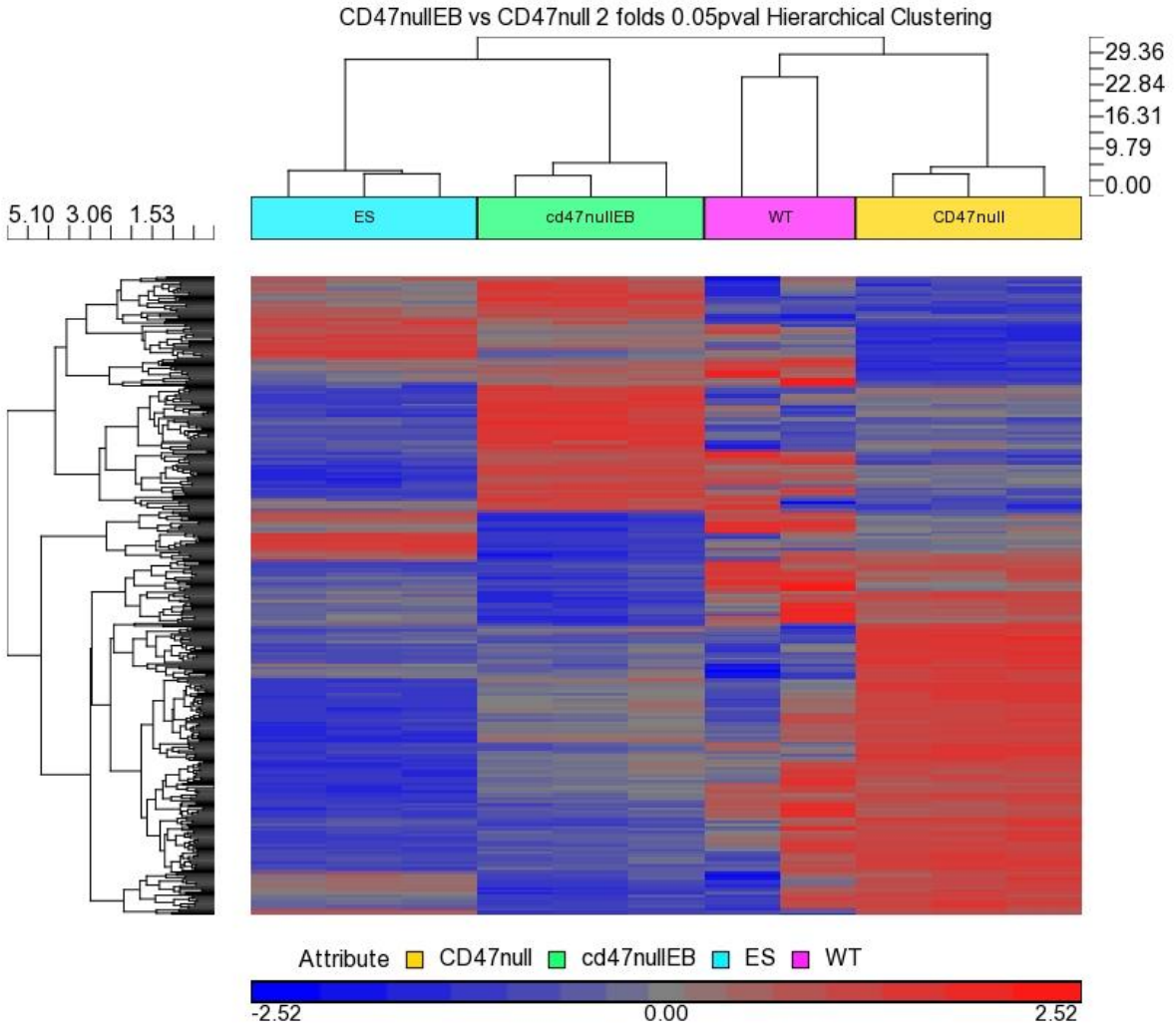


Figure S3

A


CD47nullEB vs CD47null 2 folds 0.05pval Hierarchical Clustering
29.36
22.84
16.31
9.79
0.00
5.10 3.06 1.53
ES
cd47nullEB
WT
CD47null
Attribute CD47null cd47nullEB ES WT
-2.52
0.00
2.52

B

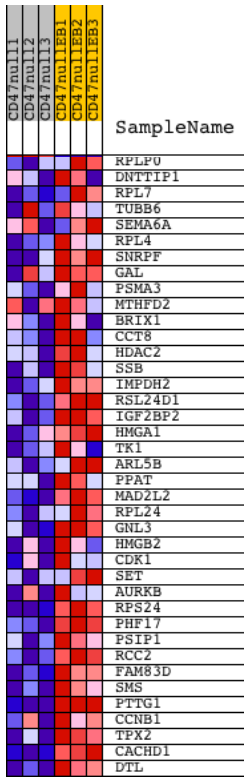

CD47null1
CD47null2
CD47null3
CD47nullEB1
CD47nullEB2
CD47nullEB3
SampleName
RPLP0
DNTTIP1
RPL7
TUBB6
SEMA6A
RPL4
SNRPF
GAL
PSMA3
MTHFD2
BRIX1
CCT8
HDAC2
SSB
IMPDH2
RSL24D1
IGF2BP2
HMGA1
TK1
ARL5B
PPAT
MAD2L2
RPL24
GNL3
HMGB2
CDK1
SET
AURKB
RPS24
PHF17
PSIP1
RCC2
FAM83D
SMS
PTTG1
CCNB1
TPX2
CACHD1
DTL

Figure S4

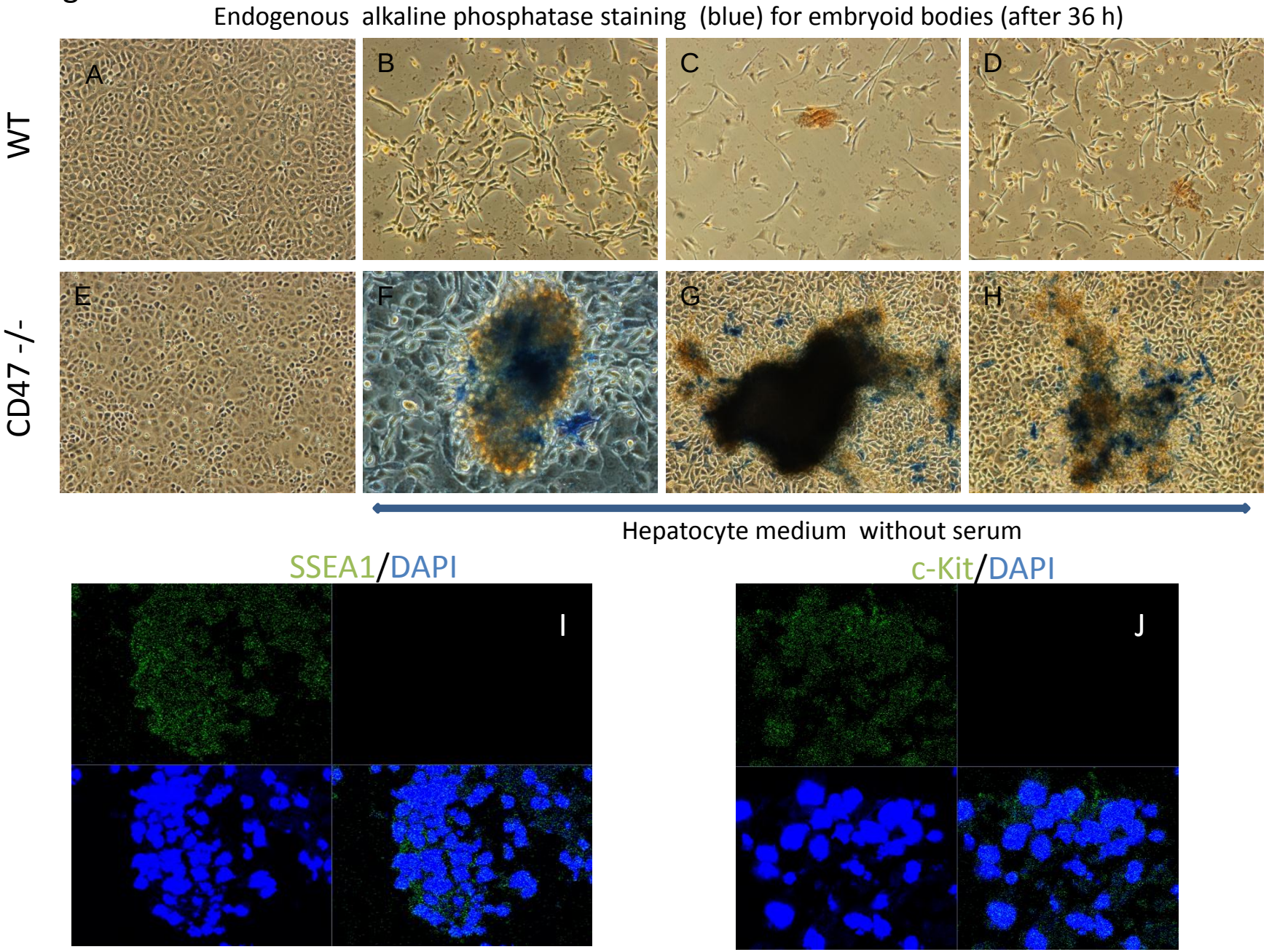
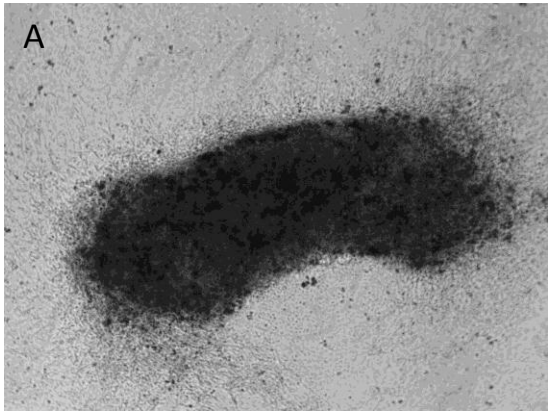
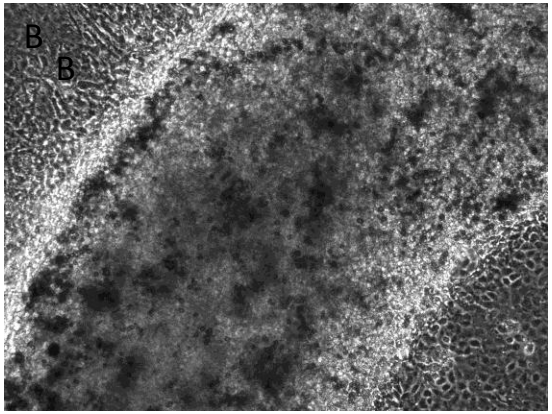

Endogenous alkaline phosphatase staining (blue) for embryoid bodies (after 36 h)
WT
CD47 -/-
A
B
C
D
E
F
G
H
Hepatocyte medium without serum
SSEA1/DAPI
c-Kit/DAPI
I
J

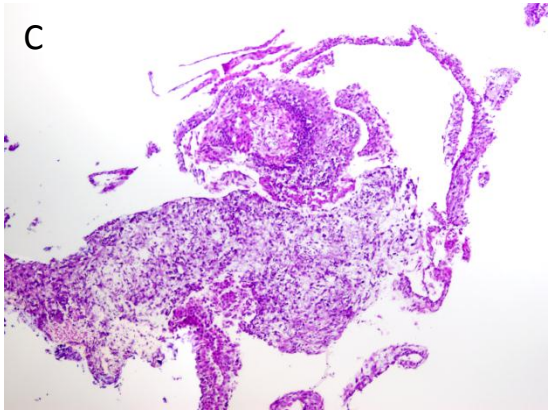
Figure S5 Differentiated embryoid body morphology and protein expression



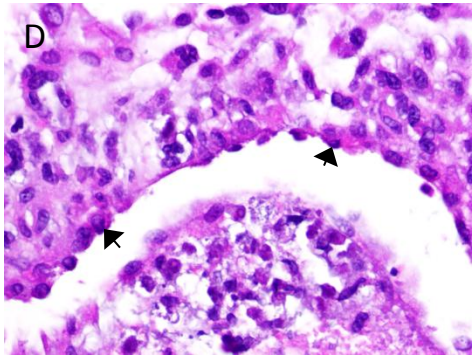
Differentiated embryoid body 15-days (4X)



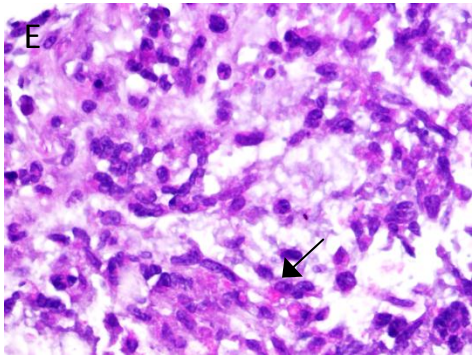
Differentiated embryoid body 15-days (20X)



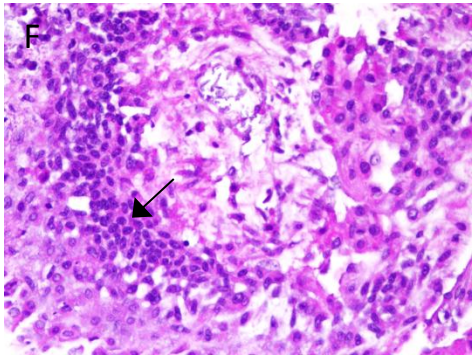
Differentiated embryoid body H&E (4X)



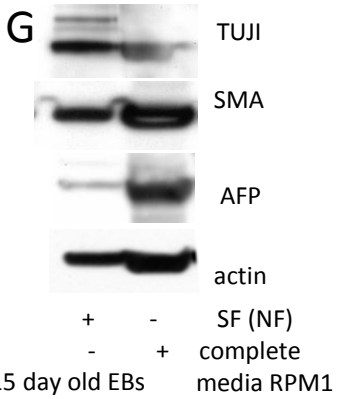
Endoderm H&E (40X)

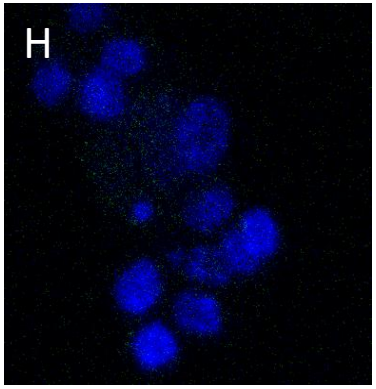


Mesoderm H&E (40X)

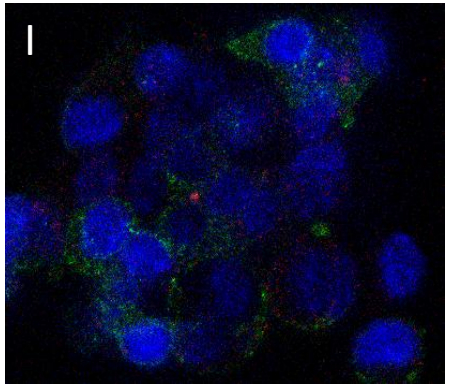


Presumptive Ectoderm H&E (20X)

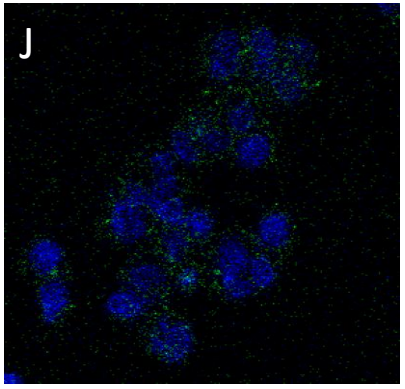




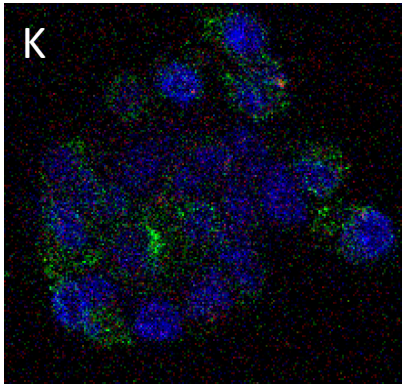
VEGFR2(G) and DAP1 (B)



KLF4 (R) and OCT4 (G)



C-kit (G) and DAP1(B)



MYC (G) and AFP (R)

No SSEA1 or CD31 were observed in differentiated EB-like clusters.

Figure S6

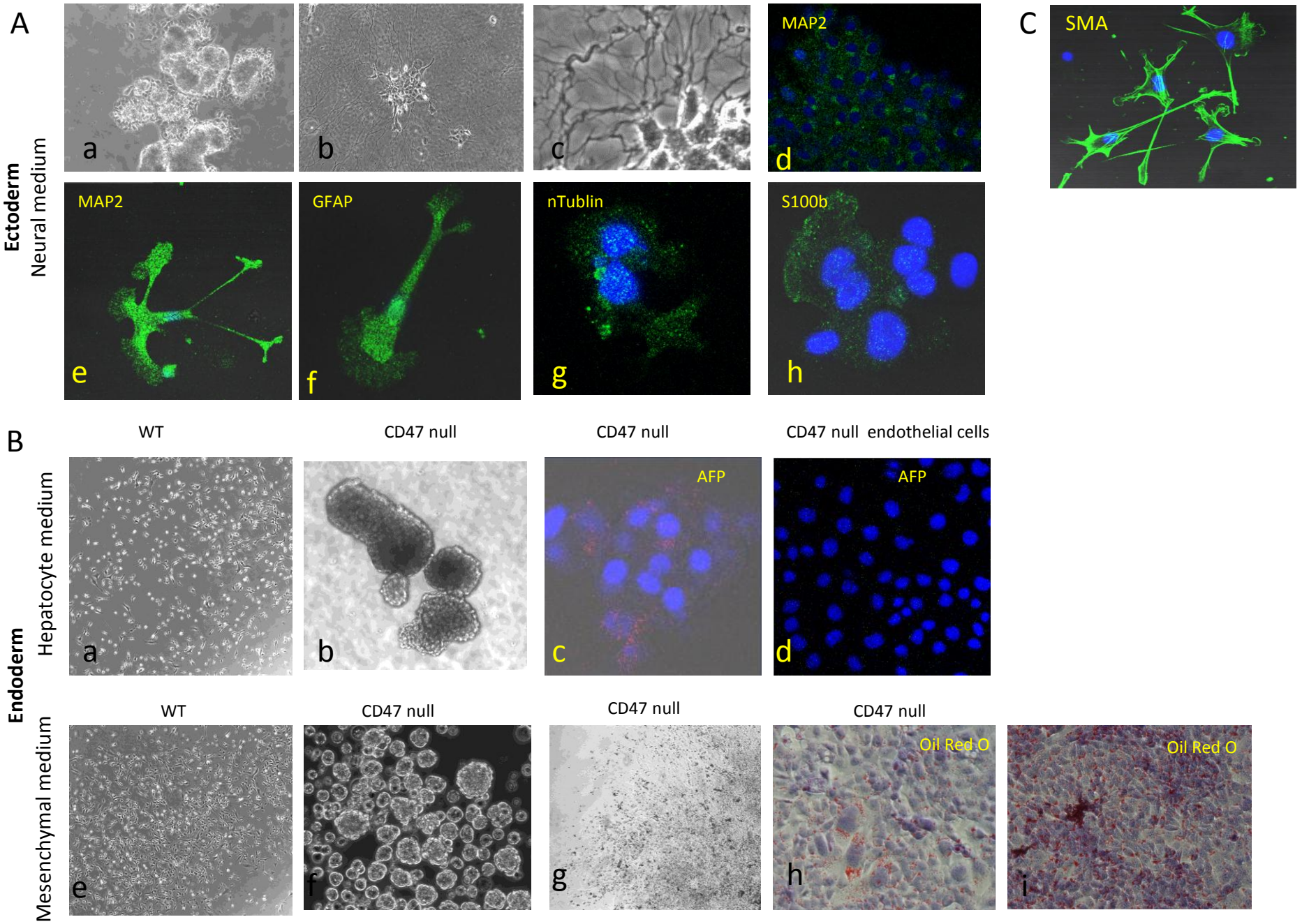
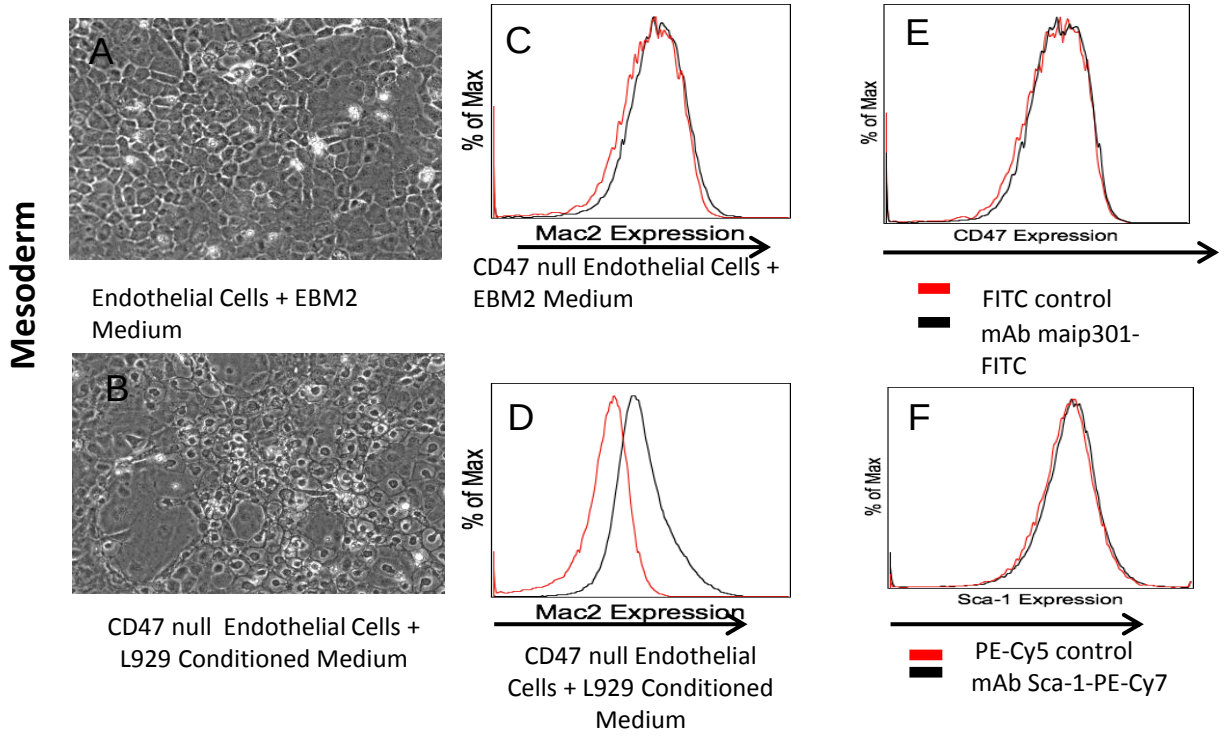
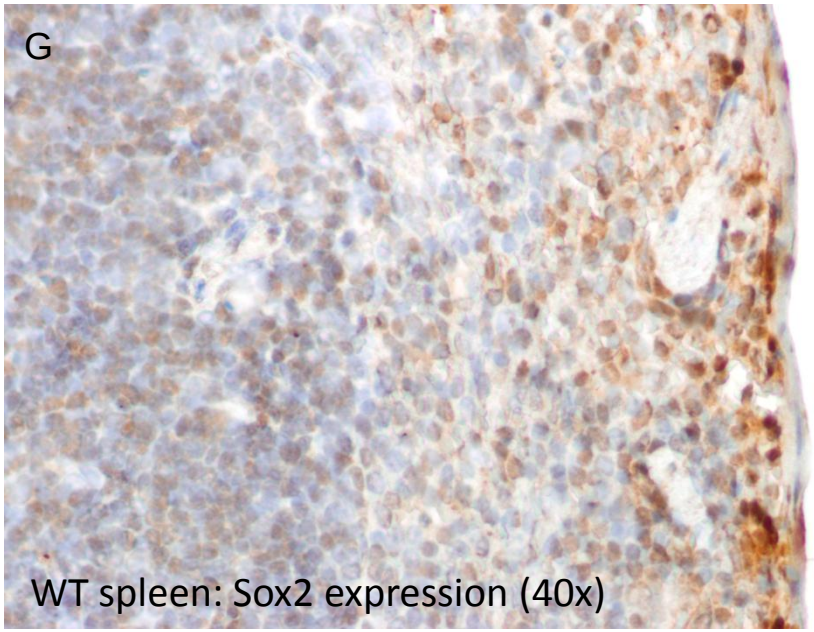

A
Ectoderm
Neural medium
a
b
c
MAP2
d
MAP2
e
GFAP
f
nTublin
g
S100b
h
C
SMA
B
Endoderm
Hepatocyte medium
WT
CD47 null
CD47 null
CD47 null endothelial cells
a
b
AFP
c
AFP
d
Mesenchymal medium
WT
CD47 null
CD47 null
CD47 null
e
f
g
Oil Red O
h
Oil Red O
i

Figure S7


Mesoderm
A
Endothelial Cells + EBM2 Medium
B
CD47 null Endothelial Cells + L929 Conditioned Medium
C
% of Max
Mac2 Expression
CD47 null Endothelial Cells + EBM2 Medium
D
% of Max
Mac2 Expression
CD47 null Endothelial Cells + L929 Conditioned Medium
E
% of Max
CD47 Expression
FITC control
mAb maip301-FITC
F
% of Max
Sca-1 Expression
PE-Cy5 control
mAb Sca-1-PE-Cy7


G
WT spleen: Sox2 expression (40x)

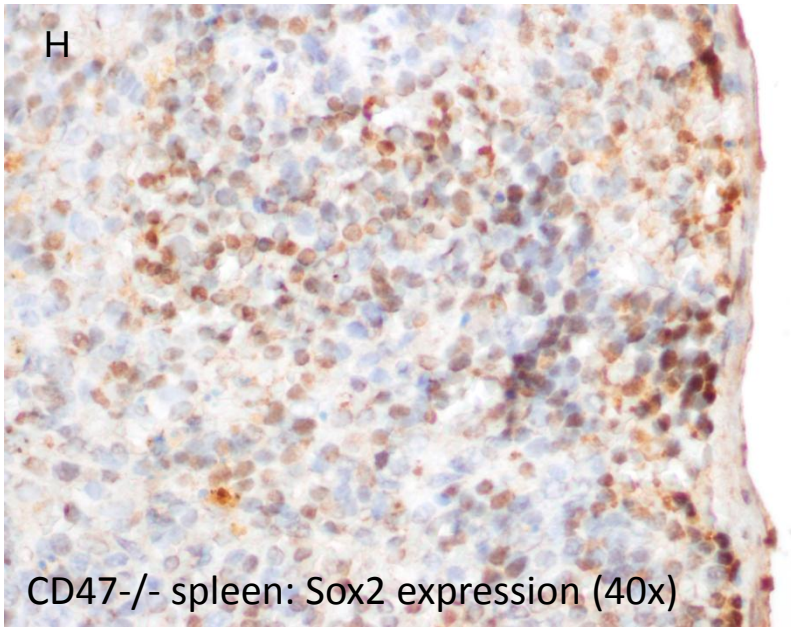
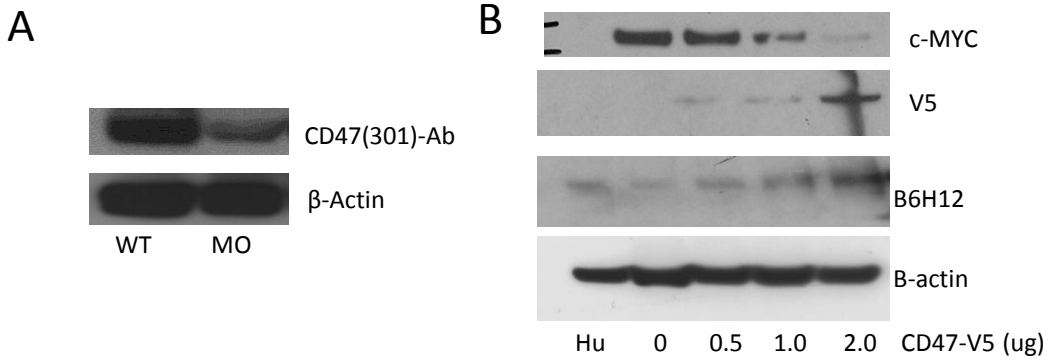
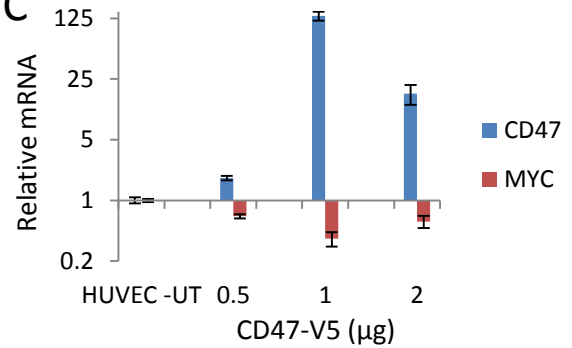

H
CD47-/- spleen: Sox2 expression (40x)

Figure S8

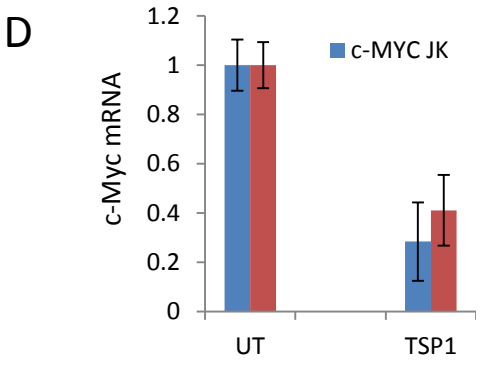
supplementary data for Figure 6 panel B

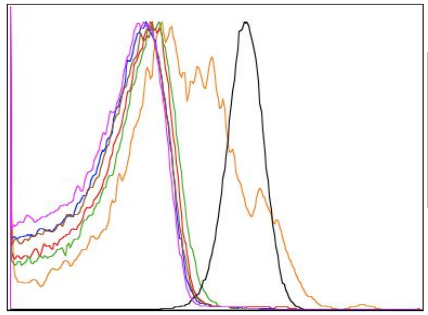


supplementary for Figure 6 panel D-E



Supplementary data for Figure 7 panel C





Sample	Live (%)	MFI (PE)
wildtype Jurkat Cells (primary Ab control)	88.1	182
wildtype Jurkat Cells (secondary Ab control)	85.9	214
wildtype Jurkat Cells	79.8	3579
CD47 deficient Jurkat Cells (primary Ab control)	72.1	212
CD47 deficient Jurkat Cells (secondary Ab control)	67.9	297
CD47 deficient Jurkat Cells	68.8	281
CD47 deficient Jurkat Cells + CD47-v5	14.9	1877