



SUBJECT AREAS:  
STEM-CELL RESEARCH  
SELF-RENEWAL  
CELL SIGNALLING  
ONCOGENES

Received  
11 February 2013

Accepted  
2 April 2013

Published  
17 April 2013

Correspondence and  
requests for materials  
should be addressed to  
D.D.R. (droberts@  
helix.nih.gov)

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

Sukhbir Kaur<sup>1</sup>, David R. Soto-Pantoja<sup>1</sup>, Erica V. Stein<sup>1,2</sup>, Chengyu Liu<sup>3</sup>, Abdel G. Elkahoul<sup>4</sup>, Michael L. Pendrak<sup>1</sup>, Alina Nicolae<sup>1</sup>, Satya P. Singh<sup>5</sup>, Zuqin Nie<sup>1</sup>, David Levens<sup>1</sup>, Jeffrey S. Isenberg<sup>1,6</sup> & David D. Roberts<sup>1</sup>

<sup>1</sup>Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, <sup>2</sup>Departments of Microbiology, Immunology and Tropical Medicine, George Washington University, Washington, D.C. 20037, <sup>3</sup>iPSC and Genome Engineering Core, National Heart, Lung, and Blood Institute, <sup>4</sup>Cancer Genetics Branch, National Human Genome Research Institute, <sup>5</sup>Laboratory of Molecular Immunology, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD 20892, <sup>6</sup>Division of Pulmonary, Allergy and Critical Care Medicine and the Vascular Medicine Institute of the University of Pittsburgh, Pittsburgh, PA 15213.

**Signaling through the thrombospondin-1 receptor CD47 broadly limits cell and tissue survival of stress, but the molecular mechanisms are incompletely understood. We now show that loss of CD47 permits sustained proliferation of primary murine endothelial cells, increases asymmetric division, and enables these cells to spontaneously reprogram to form multipotent embryoid body-like clusters. c-Myc, Klf4, Oct4, and Sox2 expression is elevated in CD47-null endothelial cells, in several tissues of CD47- and thrombospondin-1-null mice, and in a human T cell line lacking CD47. CD47 knockdown acutely increases mRNA levels of c-Myc and other stem cell transcription factors in cells and in vivo, whereas CD47 ligation by thrombospondin-1 suppresses c-Myc expression. The inhibitory effects of increasing CD47 levels can be overcome by maintaining c-Myc expression and are absent in cells with dysregulated c-Myc. Thus, CD47 antagonists enable cell self-renewal and reprogramming by overcoming negative regulation of c-Myc and other stem cell transcription factors.**

CD47 is a signaling receptor for the secreted matricellular protein thrombospondin-1 and the counter-receptor for signal-regulatory protein- $\alpha$  (SIRP $\alpha$ ), which on phagocytic cells recognizes CD47 engagement as a marker of self<sup>1–3</sup>. Mice lacking CD47 or thrombospondin-1 are profoundly resistant to tissue stress associated with ischemia, ischemia/reperfusion, and high dose irradiation<sup>2,4–7</sup>. The survival advantage of ischemic CD47- and thrombospondin-1-null tissues is mediated in part by increased nitric oxide/cGMP signaling<sup>2</sup>. Radioresistance associated with CD47 blockade is cell autonomous and independent of NO signaling<sup>8</sup>, indicating that additional pro-survival signaling pathways are controlled by CD47.

Engaging CD47 in some cell types triggers programmed cell death<sup>3,9</sup>. BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) is a pro-apoptotic BH3 domain protein that interacts with the cytoplasmic tail of CD47 and is implicated in CD47-dependent cell death<sup>10</sup>. Furthermore, CD47 ligation alters localization of the dynamin-related protein Drp1, which controls mitochondria-dependent death pathways<sup>9</sup>, and some tissues in CD47-null and thrombospondin-1-null mice show increased mitochondrial numbers and function<sup>11</sup>. Mitochondrial-dependent cell death pathways involving Bcl-2 are limited by the autophagy regulator beclin-1<sup>12</sup>. We recently found that CD47 signaling limits the induction of beclin-1 and other autophagy-related proteins in irradiated cells, and blocking CD47 in vitro and in vivo thereby increases activation of a protective autophagy response<sup>13,14</sup>. This autophagy response is necessary for the radioprotective effect of CD47 blockade.

In contrast to the above noted survival advantages of decreased CD47 expression, elevated expression of CD47 confers an indirect survival advantage in vivo. CD47 engages SIRP $\alpha$  on macrophages and prevents phagocytic clearance<sup>1,15</sup>. Similarly, elevated expression of CD47 on several types of cancer cells has been shown to inhibit their killing by macrophages or NK cells<sup>16–18</sup>. Conversely, CD47 antibodies that block SIRP $\alpha$  binding enhance



macrophage-dependent clearance of tumors<sup>17,19–21</sup>, although others have shown that such clearance can occur independent of inhibitory SIRP $\alpha$  signaling<sup>22–24</sup>.

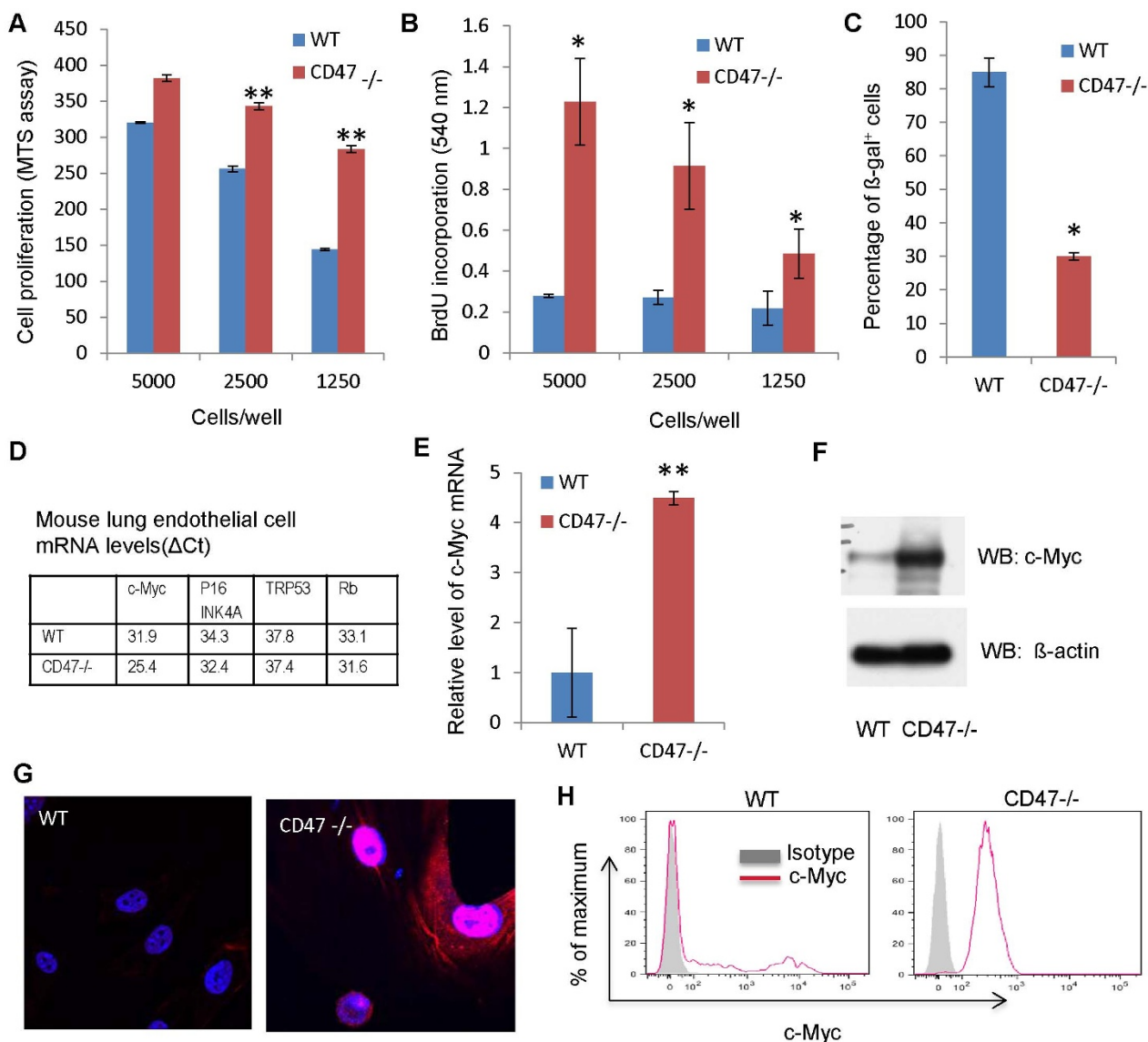
Taken together, these studies indicate two opposing roles for CD47 in cell survival. The cell autonomous advantages of decreased CD47 expression, leading to less inhibitory CD47 signaling, must be balanced against the need to maintain sufficient CD47 levels to prevent phagocytic clearance in vivo. Hematopoietic stem cells exhibit elevated CD47 expression, and high CD47 expression in the stem cell niche was proposed to be important to protect stem cells from innate immune surveillance<sup>25</sup>. In contrast to this protective function of CD47 in stem cells, we now report that loss of CD47 elevates expression of the stem cell transcription factors Sox2, Klf4, Oct4, and *c-Myc* in primary murine endothelial cells. Consequently, these cells exhibit increased asymmetric cell division and spontaneously and efficiently form clusters that resemble embryoid bodies (EBs) in serum-free media without requiring feeder cells. These EB-like clusters can

readily differentiate into various lineages. *c-Myc* is a global regulator of gene expression in differentiated and stem cells<sup>26</sup> and plays a major role in this inhibitory function of CD47. Re-expression of CD47 in null cells down-regulates *c-Myc* expression and inhibits cell growth, whereas dysregulation of the *c-Myc* gene, such as commonly occurs in cancer, enables cells to tolerate high CD47 expression.

## Results

### Loss of CD47 allows self-renewal and increases *c-Myc* expression.

Primary cells isolated from CD47-null mice exhibit a remarkable advantage in adapting to the stress of tissue culture. Lung endothelial cells isolated from WT C57BL/6 mice had limited survival and proliferative capacities in primary culture as assessed by reduction of [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and bromodeoxyuridine (BrdU) incorporation (Fig. 1A, B) and rapidly became senescent upon passage (Supplemental Fig. S1). In contrast,



**Figure 1 | Enhanced proliferation and decreased senescence of CD47-null murine endothelial cells is associated with increased expression of *c-Myc*.** (A) MTS assay for cell survival and growth over 72 h expressed as % of day 0 values at the indicated plating densities of first passage WT and CD47 null cells. (B) BrdU assay for DNA synthesis. (C) Percentage of senescence-associated β-galactosidase expression at passage 3. (D) Expression of genes associated with cell immortalization in WT and CD47 null cells. (E) *c-Myc* mRNA levels in lung endothelial cells of WT and CD47 null mice. (F) CD47 limits *c-Myc* protein levels. (G) *c-Myc* expression (red) in WT and CD47<sup>-/-</sup> endothelial cells. Blue = DAPI nuclear stain. (H) Flow cytometric analysis of *c-Myc* expression in WT and CD47<sup>-/-</sup> endothelial cells. (\**p* < 0.05, \*\**p* < 0.01).

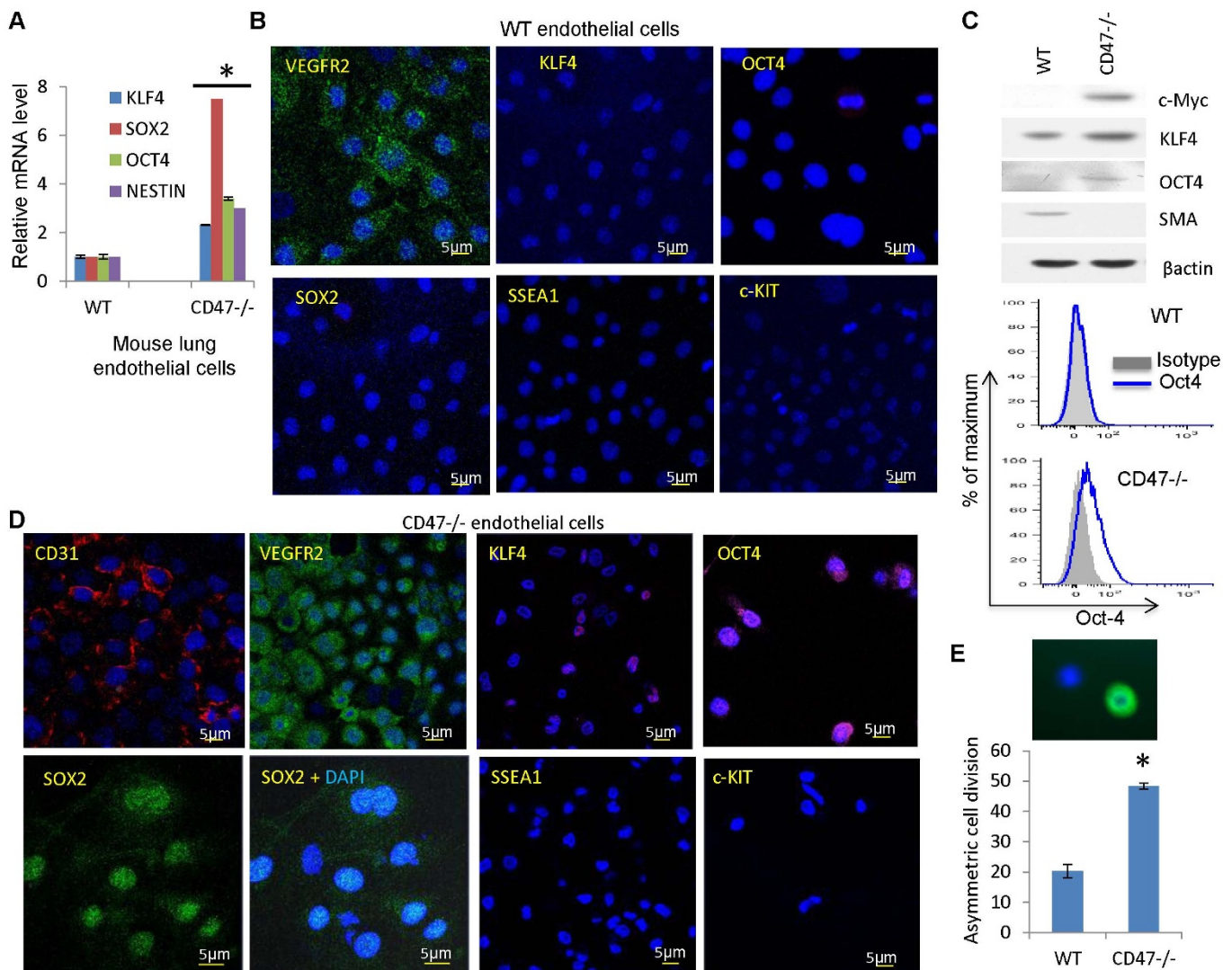


CD47-null lung endothelial cells at first passage showed enhanced plating efficiency and proliferation at several cell densities (Fig. 1A, B). Upon repeated passage, WT primary cells became flattened and vacuolated, whereas CD47-null endothelial cells consistently maintained a well differentiated cobblestone morphology for several months in continuous culture (Supplemental Fig. S1). Continuously proliferating cell lines were reproducibly obtained when primary CD47-null cells were repeatedly passaged but very rarely from WT cultures. The CD47-null cells generally lacked expression of the senescence-associated acidic  $\beta$ -galactosidase marker<sup>27</sup> that rapidly appeared in the WT cells (Fig. 1C). High frequency generation of continuously proliferating cell lines was also observed for lung endothelial cells cultured from mice lacking the CD47 ligand thrombospondin-1 (Supplemental Fig. S1).

The ability of CD47-null and thrombospondin-1-null cells to continuously proliferate could reflect either increased escape from senescence or induction of a self-renewing stem cell phenotype. Genes that enable primary cells to escape senescence and become

immortalized include *p53*, *Rb*, *p16-INK4A*, and *c-Myc*<sup>28</sup>. Of these, only *c-Myc* mRNA levels were significantly elevated relative to HPRT1 mRNA levels in the primary cell cultures and remained elevated upon repeated passage (Fig. 1D, E). Protein levels for *c-Myc* were also elevated in primary CD47-null lung endothelial cells as detected by western blotting (Fig. 1F, 2C), immunofluorescence (Fig. 1G) and flow cytometry (Fig. 1H). Most *c-Myc* was nuclear, but filamentous cytoplasmic staining was also noted in the CD47-null cells, consistent with the known *c-Myc* association with microtubules<sup>29</sup>. Characteristic of a pure endothelial cell culture<sup>30</sup>, the continuously growing CD47-null cells uniformly expressed VEGFR2 and heterogeneously expressed CD31 (Fig. 2D). Absence of vascular smooth muscle cell contamination was indicated by the lack of detectable  $\alpha$ -smooth muscle actin expression, although this was detectable at low levels in primary WT endothelial cell cultures (Fig. 2C).

**CD47 coordinately regulates stem cell transcription factors.** Because elevated *c-Myc* expression also promotes self-renewal of



**Figure 2 | Stem cell and differentiation marker expression in WT and CD47-null endothelial cells.** (A) mRNA expression levels of stem cell transcription factors in WT and CD47 null lung endothelial cells. (B, D) Stem cell and differentiation marker expression in WT (B) and CD47 null mouse endothelial cells (D). The endothelial cells were stained using the indicated antibodies and DAPI (blue). Scale bars = 5  $\mu$ m. (C) Protein expression of stem cell transcription factors and smooth muscle actin (SMA) assessed by western blotting and flow cytometry (Oct4) in cultured WT or CD47 null endothelial cells in EGM2 medium. (E) Asymmetric cell division frequencies in second passage WT and CD47<sup>-/-</sup> endothelial cells equilibrium labeled with BrdU and chased for one cell division. Asymmetric division was scored by counting BrdU<sup>+</sup> (green)/DAPI<sup>+</sup> nuclei adjacent to BrdU<sup>-</sup>/DAPI<sup>+</sup> nuclei.





stem cells<sup>31</sup> and is necessary under some conditions for embryonic stem cell self-renewal<sup>32</sup>, we examined the expression of additional transcription factors that support stem cell reprogramming<sup>33</sup> and found significant elevation of mRNA for Sox2, Klf4, and Oct4 and of the stem cell marker nestin in primary CD47-null endothelial cells (Fig. 2A). Oct4 protein expression was detected by immunofluorescence in a majority of the CD47 null cells but not in WT cells (Fig. 2B, D). Sox2 and Klf4 were expressed in a subset of CD47-null cells, whereas Klf4 and c-Myc were undetectable by immunofluorescence in WT endothelial cells, and Sox2 positive cells were rarely seen (Fig. 2B, D). Some Sox2 staining in CD47 null cells was cytoplasmic, consistent with its reported subcellular localization in early embryonic cells<sup>34</sup>, but the majority of staining was nuclear. Western blotting confirmed elevated levels of Klf4 and Oct4 in CD47-null versus WT cells (Fig. 2C). Flow cytometry also confirmed increased Oct4 expression in CD47-null cells (Fig. 2C).

These data suggested that CD47-null endothelial cell cultures contain a larger fraction of stem cells, which characteristically exhibit asymmetric cell division<sup>35,36</sup>. We examined the frequency of asymmetric division in WT and CD47-null endothelial cell cultures uniformly labeled using BrdU by chasing with unlabeled medium for 24 h in the presence of cytochalasin B to inhibit cytokinesis. Asymmetric division was indicated by adjacent DAPI<sup>+</sup> nuclei where only one cell retained BrdU staining (Fig. 2E upper panel). Such cells were significantly more abundant in the CD47-null cultures (Fig. 2E lower panel).

**Efficient formation of embryoid body-like clusters by CD47-null cells.** Ectopic expression of Klf4, Sox2, Oct4, and c-Myc or ALK2 bearing an activating mutation in human umbilical vein endothelial cells enables efficient generation of multipotent or induced pluripotent stem (iPS) cells<sup>37,38</sup>, suggesting that self-renewal of the CD47-null endothelial cells might arise from increased numbers of stem cells in these cultures. Continuously propagated CD47-null endothelial cells expressed Sca-1, and 24% of the cells were CD14<sup>+</sup>/CD11<sup>+</sup> (Fig. S1). These are characteristic markers for endothelial precursor cells<sup>39</sup>. However, CD47-null endothelial cells did not express detectable levels of pluripotency marker SSEA-1 or the stem cell marker c-Kit (Fig. 2D). Because elevated expression of c-Myc, Sox2, Oct4 and Klf4 in some types of primary cells is sufficient to induce cystic EB formation<sup>40</sup>, we examined whether loss of CD47 circumvents the need to artificially elevate these factors for inducing EBs. Indeed, transfer of early passage CD47-null endothelial cells, as well as cultures grown continuously for up to 6 months, into serum-free medium in the absence of any feeder cells within 2 days induced efficient formation of floating cell aggregates that resembled EBs and continued to proliferate in this state (Fig. 3A, Supplemental Fig. S2). These were never observed when primary WT endothelial cells were placed into the same medium, and WT cells did not survive in serum-free media. Cells in the EB-like clusters exhibited strong nuclear c-Myc staining by immunofluorescence (Fig. 3A) and flow cytometry indicated a subpopulation with stronger c-Myc expression than that observed in primary CD47-null endothelial cultures (compare Fig. 3B and Fig. 1H). Unlike CD47-null cells in endothelial cell medium, cells in the EB-like clusters expressed additional stem cell markers including alkaline phosphatase, nestin, SSEA-1 and c-Kit (Fig. 3A, S4). Consistent with their expression of stem cell markers, cells in EB-like clusters frequently exhibited asymmetric cell division (Fig. 3C).

CD47<sup>-/-</sup> cells were grown without feeder cells in ES medium containing LIF adopted colony morphologies similar to V6.5 mouse ES cells grown in the same medium with MEF feeder cells (Fig. 3D). Immunohistochemical analysis of these cells in ES medium demonstrated that CD47 null and V6.5 ES cells contained similar subpopulations that expressed elevated levels of nuclear Oct 4, Sox2, and Nanog (Fig. 3E).

We performed microarray analyses (GSE43133) to globally assess the stem cell character of CD47 null endothelial cells and EB-like clusters derived by culturing in serum free medium for 36 h. A global principal component analysis revealed that CD47 null endothelial cells and EB-like clusters derived from them clustered near published iPS and ES cells, whereas WT endothelial cells did not (Fig. 3F). To characterize the changes in gene expression that accompany EB-like cluster formation in the CD47 null cells, we compared global gene expression in CD47 null EB-like clusters to that of CD47 null cells before removal of serum and endothelial growth factors and found 383 genes with significant changes. Of these 255 clustered with genes that showed similar up or down regulation in the V6.5 ES cells (Supplemental Fig. S3). A GeneSet gene enrichment analysis (GSEA) identified 39 of these up-regulated genes that are included in the molecular signature for human ES cells defined by Bhattacharya<sup>41</sup> (Supplemental Fig. S3). The remaining genes included endothelial-specific genes that were significantly down-regulated (e.g. thrombomodulin (*Thbd*)) and epithelial/mesenchymal transition genes that were induced when CD47 null cells formed EB-like clusters. Notably expression of *Kdr*, which encodes VEGFR2, decreased 10-fold, consistent with loss of VEGFR2 immunoreactivity in the CD47 null EB-like clusters.

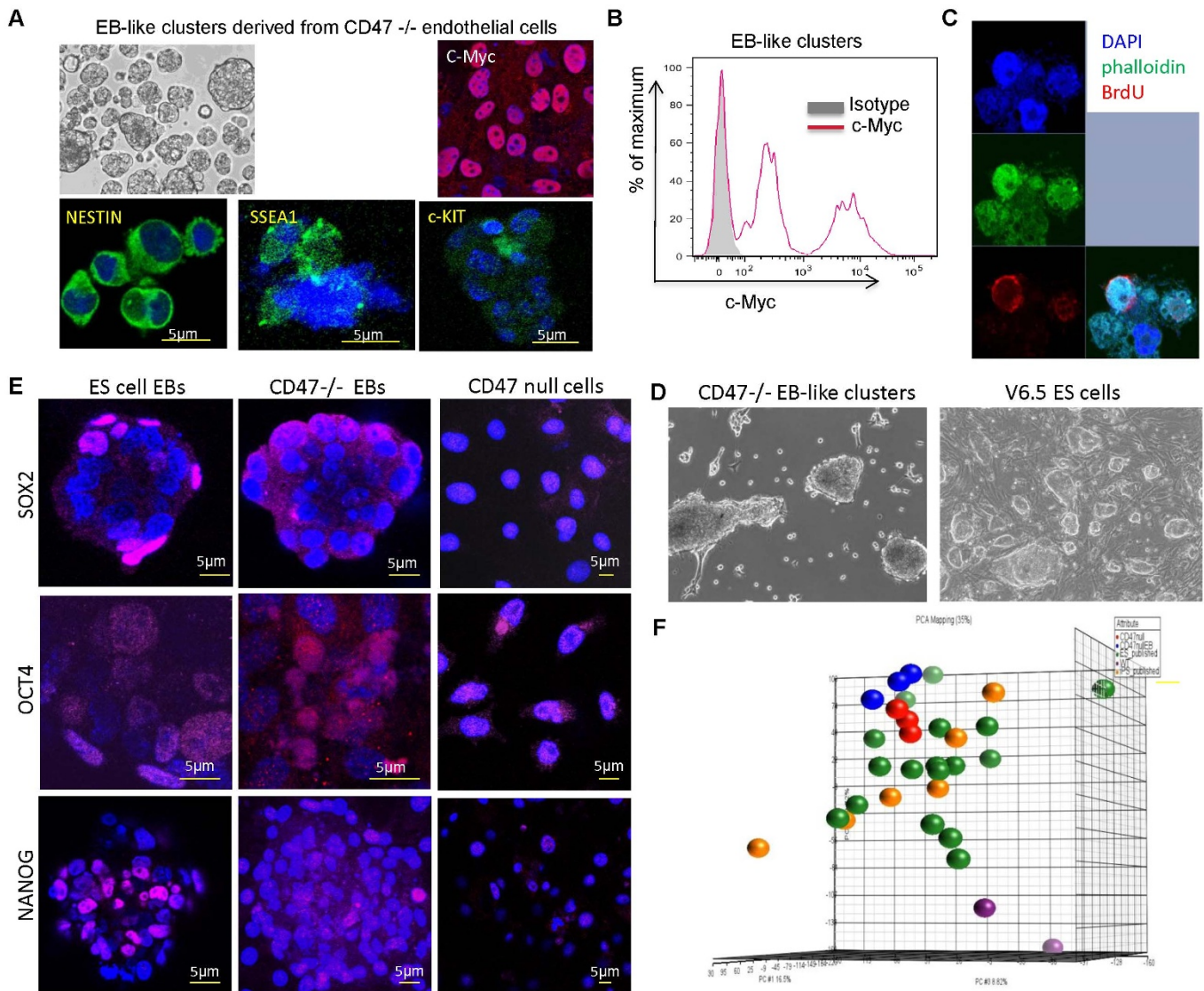
**CD47-null EB-like clusters are multipotent.** To determine whether CD47 null EB-like clusters are competent to give rise to the three germ layers, 6 day old undifferentiated EB-like clusters prepared from continuously cultured CD47-null endothelial cells were plated on gelatin-coated WillCo dishes for 36 h containing neural, smooth muscle, or hepatocyte differentiation medium (Fig. 4A–C and Movies S1–3). Appearance of cells expressing smooth muscle actin indicated mesoderm differentiation. Cells expressing neuron-specific  $\beta$  III tubulin and glial fibrillary acidic protein (GFAP) indicated ectoderm differentiation. Cells expressing  $\alpha$ -fetoprotein (AFP) indicated endoderm differentiation. Because each lineage could arise from different lineage-committed stem cells in the EB-like clusters, we expanded a single clone from CD47-null EB-like clusters and repeated the above differentiation study (Fig. 4D–F). Again, cells expressing characteristic markers of the three embryonic lineages were obtained. Therefore, the CD47-null cells are multipotent.

Morphological differentiation of EBs provides another in vitro assessment of pluripotency<sup>42</sup>. Differentiation of EB-like clusters in complete RPMI medium for 10–15 days resulted in morphological differentiation characteristic of all three embryonic germ layers (Supplemental Fig. S5). Differentiation was accompanied by loss of SSEA1 expression and decreased expression of other stem cell markers.

These results suggested that CD47 null EB-like clusters contain pluripotent cells. However, we observed limited fibrotic responses and no teratoma formation when CD47 null EB-like clusters were injected into NSG (NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/SzJ) mice under conditions where v6.5 ES cells formed teratomas. Therefore, the CD47-null EB-like clusters are probably not fully pluripotent<sup>43</sup>. Alternatively, teratoma formation may have been prevented by SIRP-dependent clearance of the CD47 null cells<sup>15</sup>.

Further evidence for multipotency was obtained when undifferentiated CD47-null EB-like clusters were dispersed and cultured in neural medium on a gelatin coated substrate (Supplemental Fig. S6). Ectodermal differentiation was indicated by expression of the neuronal markers MAP2, GFAP, neuron-specific  $\beta$  III tubulin, and the astrocyte marker S100b respectively (panels d–h). Some nonadherent colonies formed from these cells exhibited extensive neurite formation (panels a–c).

CD47 null endothelial cells cultured in hepatocyte growth medium developed into cystic EB-like clusters and then differentiated into cells that expressed the hepatocyte marker  $\alpha$ -fetoprotein



**Figure 3 | Stem cell marker expression in CD47 null EB-like clusters.** (A) Top left: Typical appearance of EB-like clusters photographed under phase contrast. Top right: Staining with c-Myc antibody (red) and DAPI (blue). Bottom panels: EB-like clusters or cells dissociated from them were stained using the indicated antibodies (green) and DAPI (blue). Scale bars = 5 μm. (B) Flow cytometric analysis of c-Myc expression in CD47-null cells dissociated from EB-like clusters. (C) Detection of asymmetric cell division in cells from CD47-null EB-like clusters equilibrium labeled with BrdU and then chased for two cell divisions without BrdU and stained with anti-BrdU (red) and phalloidin to visualize actin (green). Blue = DAPI. (D) Morphologies of CD47 null EB-like clusters and V6.5 ES cells growing in ES medium with LIF. The V6.5 culture also contains a MEF feeder layer. (E) CD47 null EB-like clusters and V6.5 ES cells cultured as in D and CD47 null endothelial cells in endothelial growth medium (right panels) were stained using the indicated antibodies (magenta) and DAPI (blue). Scale bars = 5 μm. (F) Principal component analysis of global gene expression data for WT (purple) and CD47 null endothelial cells (red), and CD47 null EB-like clusters (blue) compared with published expression data for iPS (gold) and ES cells (green).

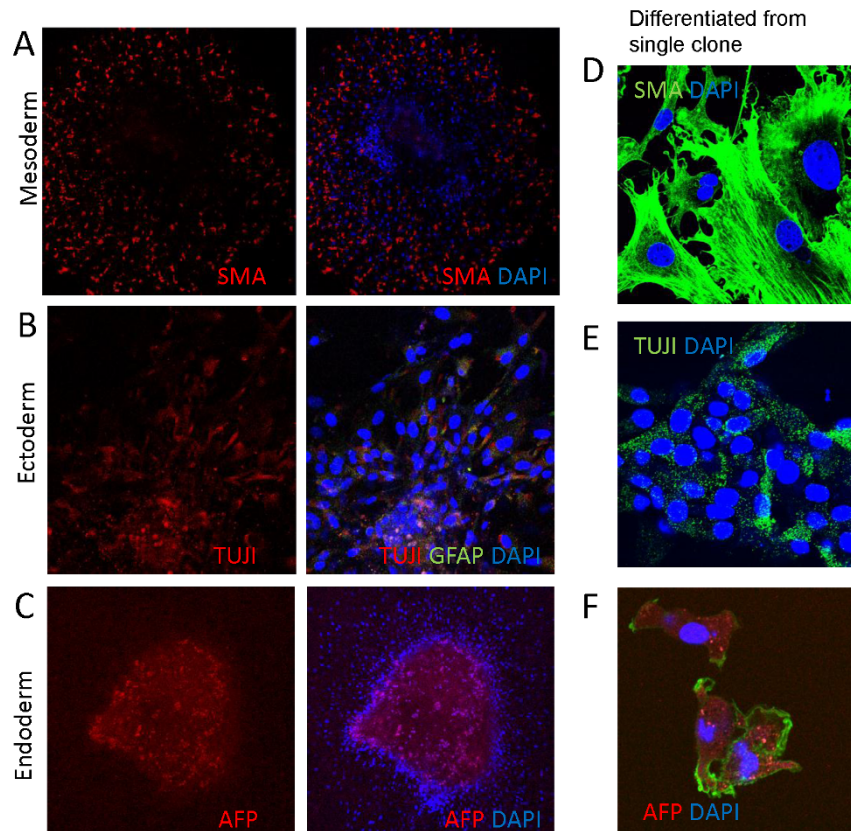
(AFP, Supplemental Fig. S6). The CD47-null endothelial cells from which these were derived did not express AFP. Although, a few WT endothelial cells survived in the hepatocyte medium, EB-like clusters never formed, and no expression of AFP was observed.

WT and CD47 null endothelial cells were cultured in mesenchymal cell medium for 10 days. Only CD47 null cells formed EB-like clusters. Some colonies of the mesenchymal differentiated cells exhibited oil red O<sup>+</sup> lipid vacuoles characteristic of adipocytes (Supplemental Fig. S6). Dispersion of CD47-null EB-like clusters into serum-free smooth muscle medium containing platelet-derived growth factor and transforming growth factor-β1 resulted in differentiation of cells with typical vascular smooth muscle morphology and expressing the lineage marker smooth muscle actin (Supplemental Fig. S6).

**Hematopoietic differentiation of CD47 null endothelial cells.** To determine their potential to differentiate into a myeloid lineage, CD47-null endothelial cells were cultured with L929 conditioned medium as a source of macrophage colony stimulating factor (M-CSF)<sup>44</sup>. After 10 days a change in cell morphology was accompanied by a marked increase in the percentage of Mac2<sup>+</sup> cells in treated CD47-null cells compared to the same cells in endothelial growth medium and loss of Sca-1 expression (compare Supplemental Figs. S1 and S7).

**In vivo regulation of c-Myc and tissue stem cell abundance by CD47.** Increased expression of c-Myc mRNA compared to that in WT mice was detected in several organs from CD47-null mice (Fig. 5A). Because the highest elevation occurred in CD47-null





**Figure 4 | Differentiation of CD47-null EB-like clusters.** (A) EB-like clusters were cultured in the presence of serum for 6 days to induce substrate adhesion and then transferred in to the indicated lineage specific media for 36 h and stained with smooth muscle actin antibody (red) to detect mesodermal cells. (B) Differentiated EB-like clusters were stained with the ectoderm neural markers glial fibrillary acidic protein (GFAP, green), neuron-specific beta III tubulin (TUJI, red). (C) Differentiated EB-like clusters were stained with anti- $\alpha$ -fetoprotein (red) to detect endodermal cells. In all panels DAPI was used to visualize nuclei (blue). (D–F) A single clone isolated from a CD47-null EB-like cluster in serum-free medium was expanded and then differentiated in the indicated lineage-specific medium for 7 days and stained for the indicated markers. Blue = DAPI, green in (F) = actin.

spleen, we examined several major cell types from this organ. B ( $CD19^+$ ) and  $CD4^+$  T cell populations showed significant up-regulation of c-Myc mRNA, whereas  $CD8^+$  T cells and monocytes did not (Fig. 5B). Nestin, Sox2, KLF4, and Oct4 mRNA levels were also markedly elevated in spleen from CD47-null mice (Fig. 5C). Consistent with the lesser elevation of c-Myc mRNA levels in lung, Oct4, Sox2, and nestin mRNA levels were moderately elevated in lung, but KLF4 was not elevated in this organ (Fig. 5D). Sox2 is normally expressed by Clara cells in conducting airways<sup>45</sup> and was similarly expressed in WT and CD47-null lung bronchiolar epithelium, but cells expressing higher levels of cytoplasmic Sox2 were selectively observed throughout the alveolar space of the CD47-null lung (Fig. 5E–F).

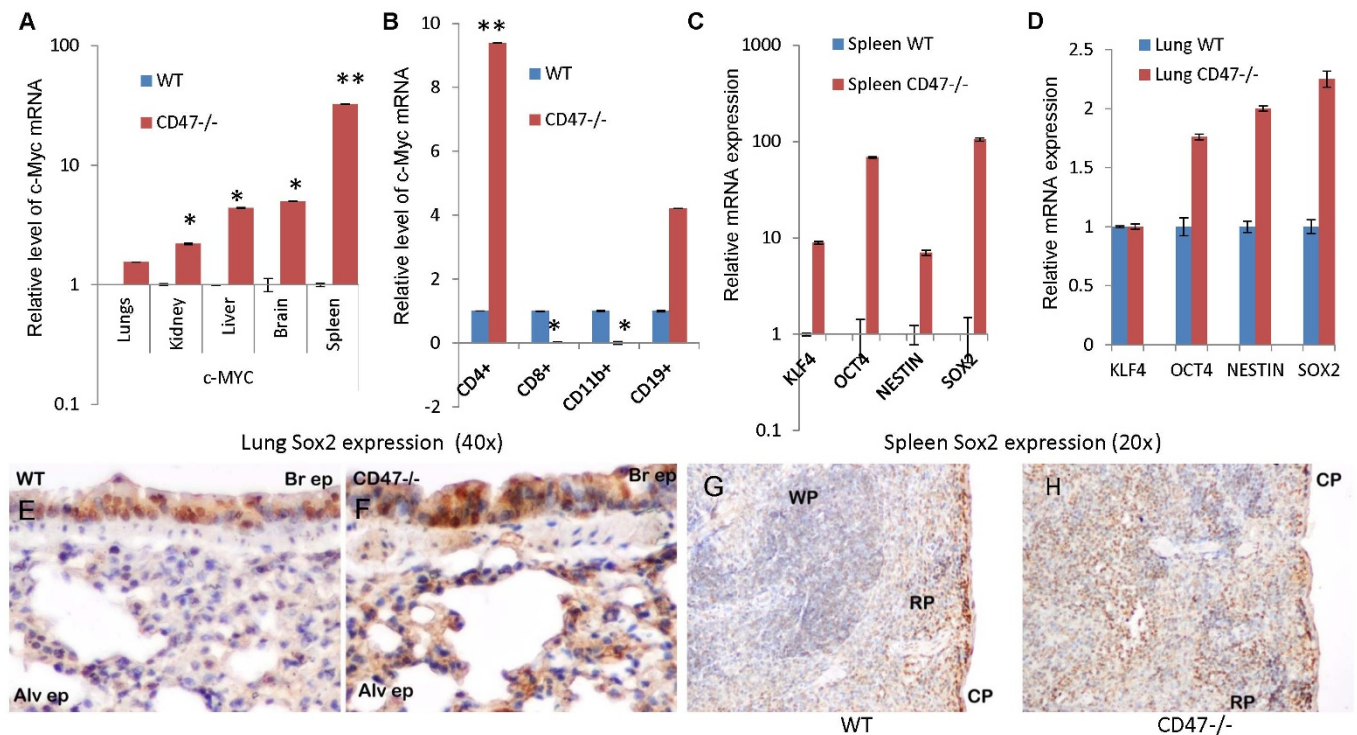
The spleen of adult mice contains a pool of multipotent  $CD45^-/Hox11^+$  stem cells that reside in the sub-capsular red pulp and are capable of differentiating into diverse lineages<sup>46,47</sup>. Consistent with these reports, we observed a limited number of cells with nuclear Sox2 protein expression in the sub-capsular region of WT mouse spleen (Fig. 5G) and sparse expression of Sox2-expressing cells in other compartments of the spleen. Similar subcapsular Sox2 immunoreactivity was seen in spleen sections from CD47 $^{-/-}$  mice, but more extensive staining was observed in the adjacent red pulp (Fig. 5H, Supplemental Fig. S7). These differences in Sox2 protein expression are consistent with the whole organ mRNA expression data and suggest that the absence of CD47 increases the number of tissue resident stem cells in vivo.

**CD47 expression acutely inhibits c-Myc expression.** The above results establish a genetic linkage between CD47, maintenance of

stem cells, and c-Myc expression but do not prove that CD47 signaling directly controls expression of c-Myc or the other stem cell transcription factors. To clarify this relationship, WT splenic T cells from c-Myc-EGFP knock-in mice<sup>26</sup> were treated with a previously validated CD47-targeting antisense morpholino<sup>48</sup> and resulted in a 7-fold increase in c-Myc mRNA level at 24 h (Fig. 6A). Intraperitoneal injection of the CD47 morpholino into WT mice significantly decreased CD47 protein expression in vivo (Supplemental Fig. S8) and resulted in induction of c-Myc as well Oct4 and Sox2 mRNA levels in spleen at 48 h (Fig. 6B). These results are consistent with our previous report that intraperitoneal injection of this CD47 morpholino confers radioprotection to mice<sup>8</sup>.

Conversely, re-expression of CD47 in CD47-null endothelial cells by transiently transfecting a human CD47 expression plasmid inhibited their proliferation and viability (Fig. 6C). c-Myc mRNA and protein levels fell when CD47 was re-expressed at a level sufficient to cause growth inhibition (Fig. 6D–E, Supplemental Fig. S8). Growth suppression by transiently expressing CD47 could be partially overcome by co-transfecting the cells with a c-Myc expression vector (Fig. 6C). Transient re-expression of CD47 in the null endothelial cells also decreased mRNA levels for KLF4, Sox2, and nestin (Fig. 6F). Notably, over-expressing c-Myc alone increased nestin and Oct4 mRNA expression, but co-expression of c-Myc with CD47 did not overcome the inhibitory effect of CD47 expression on KLF4, Sox2, or nestin, indicating that these CD47 signaling targets are Myc-independent.

**Thrombospondin-1 controls c-Myc via CD47.** The JinB8 somatic mutant of the Jurkat human T lymphoma cell line lacks CD47<sup>49</sup> and



**Figure 5 | Loss of CD47 is associated with up-regulation of stem cell transcription factors in vivo.** (A) Relative c-Myc mRNA expression in lung, kidney, liver, brain and spleen of CD47-null versus WT mice. (B) c-Myc mRNA levels in purified splenic cell populations from WT and CD47 null mice. (C) mRNA expression levels in lung from WT and CD47-null mice. (D) mRNA expression levels in spleen from WT and CD47-null mice. (\* $p < 0.05$ , \*\* $p < 0.01$ ). (E–H) Increased frequency of Sox2 expressing cells in tissues from CD47-null mice. The alveolar (Alv) regions of lung tissues from WT (E) generally lack Sox2-positive cells (brown stain), whereas CD47-null lung shows more positive cells (F). In contrast, similar uniform Sox2 staining was observed in bronchiolar epithelium (BrEp) from WT and null mice, consistent with its previously reported expression in Clara cells<sup>45</sup>. (G–H) Paraffin embedded sections of representative spleen tissues from WT (G) and CD47<sup>-/-</sup> (H) mice were stained with a specific antibody to Sox2. Sections were examined under light microscopy showing subcapsular (CP), red pulp (RP) and white pulp (WP) staining.

exhibited a similar over-expression of c-Myc mRNA relative to the parental Jurkat cells (Fig. 7A). Therefore, CD47 also negatively regulates c-Myc expression in human cells. Mice lacking the CD47 ligand thrombospondin-1 share most of the stress resistance phenotypes of CD47 null mice<sup>2,7</sup>, and muscle explants from thrombospondin-1-null mice exhibit increased vascular outgrowth into 3-dimensional collagen gels relative to WT explants<sup>50</sup>. Consistent with these observations and the continuous growth of thrombospondin-1-null endothelial cells, c-Myc levels in Jurkat T cells were transiently induced but then strongly inhibited by treatment with 2.2 nM thrombospondin-1 (Fig. 7B). Picomolar concentrations of thrombospondin-1 were sufficient to inhibit c-Myc expression in Jurkat cells at 24 h, but the elevated c-Myc mRNA levels in Jurkat cells lacking CD47 were not significantly inhibited by thrombospondin-1 (Fig. 7C). Re-expression of CD47 in JinB8 cells reduced c-Myc mRNA expression (Fig. 7D) and restored the ability of thrombospondin-1 to inhibit c-Myc expression (Supplemental Fig. S8). Therefore, CD47 is necessary for this activity of thrombospondin-1. The transient induction of c-Myc by thrombospondin-1 may be mediated by its other receptors expressed by Jurkat cells<sup>51</sup>.

Similar suppression of c-Myc levels was observed in the presence of a CD47-binding peptide derived from thrombospondin-1 (peptide 7N3, Fig. 7E). A control peptide with a mutated CD47 binding motif (peptide 604) was inactive. Therefore, CD47 engagement is sufficient to inhibit c-Myc expression without the participation of other thrombospondin-1 receptors.

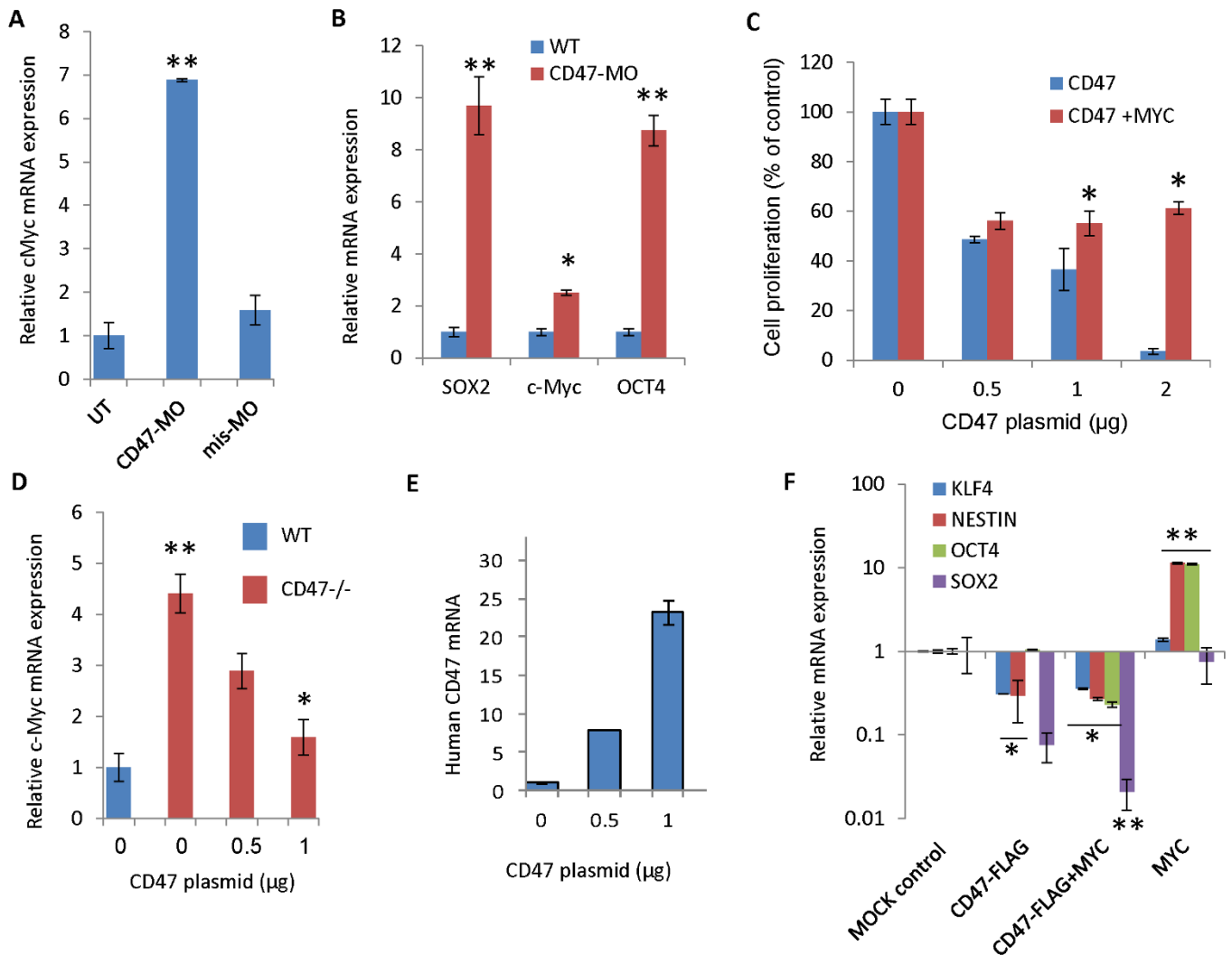
Endogenous thrombospondin-1 also controls expression of c-Myc mRNA in vivo (Fig. 7F, G). c-Myc mRNA levels were elevated approximately 3-fold in thrombospondin-1-null spleen and lung

tissues relative to the corresponding WT organs. Consistent with the data for CD47-null mice, Oct4, Sox2 and KLF4 mRNA levels were also elevated in thrombospondin-1-null spleen, but their levels were not significantly increased in lung.

**Dysregulation of c-Myc confers resistance to CD47 signaling.** One paradox that arises from the above results is that high CD47 expression appears to be a disadvantage for cells because it suppresses c-Myc expression, yet many tumor cells and some stem cells have elevated CD47 expression<sup>17,19–21</sup>. One possible explanation is that other pathways that drive cMyc expression could overcome the inhibitory effects of CD47 signaling. To examine whether c-Myc is the primary target of CD47 signaling that inhibits cell growth, we used Myc-null rat1 fibroblasts that constitutively express a tamoxifen activatable c-Myc-estrogen receptor chimeric protein<sup>52</sup>. In contrast to cells expressing only native c-Myc controlled by its endogenous promoter, transfecting the Myc-expressing Rat1 fibroblasts with CD47 did not inhibit their growth (Fig. 7H).

We previously reported that blocking CD47 conferred radioprotection to normal cells and mice, but B16 melanomas grown in these mice were not protected and instead showed enhanced radiosensitivity when CD47 was blocked<sup>8</sup>. This, combined with previous evidence that c-Myc expression is dysregulated in B16 cells<sup>53</sup>, suggested that CD47 signaling might not regulate c-Myc in these cells. Consistent with this hypothesis, transiently over-expressing CD47 in B16 melanoma cells did not inhibit their growth (Fig. 7H).

Over-expression of CD47 also failed to inhibit growth or survival of Raji Burkitt's lymphoma cells where c-Myc expression is driven by enhancer regions donated by the translocated immunoglobulin heavy chain<sup>54</sup> (Fig. 7I). In agreement with these growth data, cell



**Figure 6 | CD47 expression regulates c-Myc and stem cell transcription factor expression.** (A) Morpholino knockdown of CD47 in lung endothelial cells increases c-Myc mRNA expression. (B) In vivo morpholino knockdown of CD47 elevates c-Myc, Oct4, and Sox2 mRNA at 48 h in mouse spleen. (C) CD47 re-expression in CD47-null murine endothelial cells suppresses cell growth unless c-Myc expression is sustained. (D) CD47 re-expression in CD47 null endothelial cells alters expression c-Myc compared with WT (E) Expression level of transfected human CD47. (F) Re-expression of CD47 and c-Myc alters mRNA expression of stem cell transcription factors. (\* $p < 0.05$ , \*\* $p < 0.01$ ).

cytotoxicity (LDH release) was increased by re-expressing or over expressing CD47-FLAG in normal mouse lung endothelial cells but not in B16 melanoma, Raji Burkitt's lymphoma, and Myc null Rat 1 fibroblasts (Supplemental Fig. S8). Together, these results establish that c-Myc is the dominant target of CD47 signaling for limiting cell growth and suggest that this regulation requires 5'-regions of the *c-Myc* gene, which are absent in Raji cells.

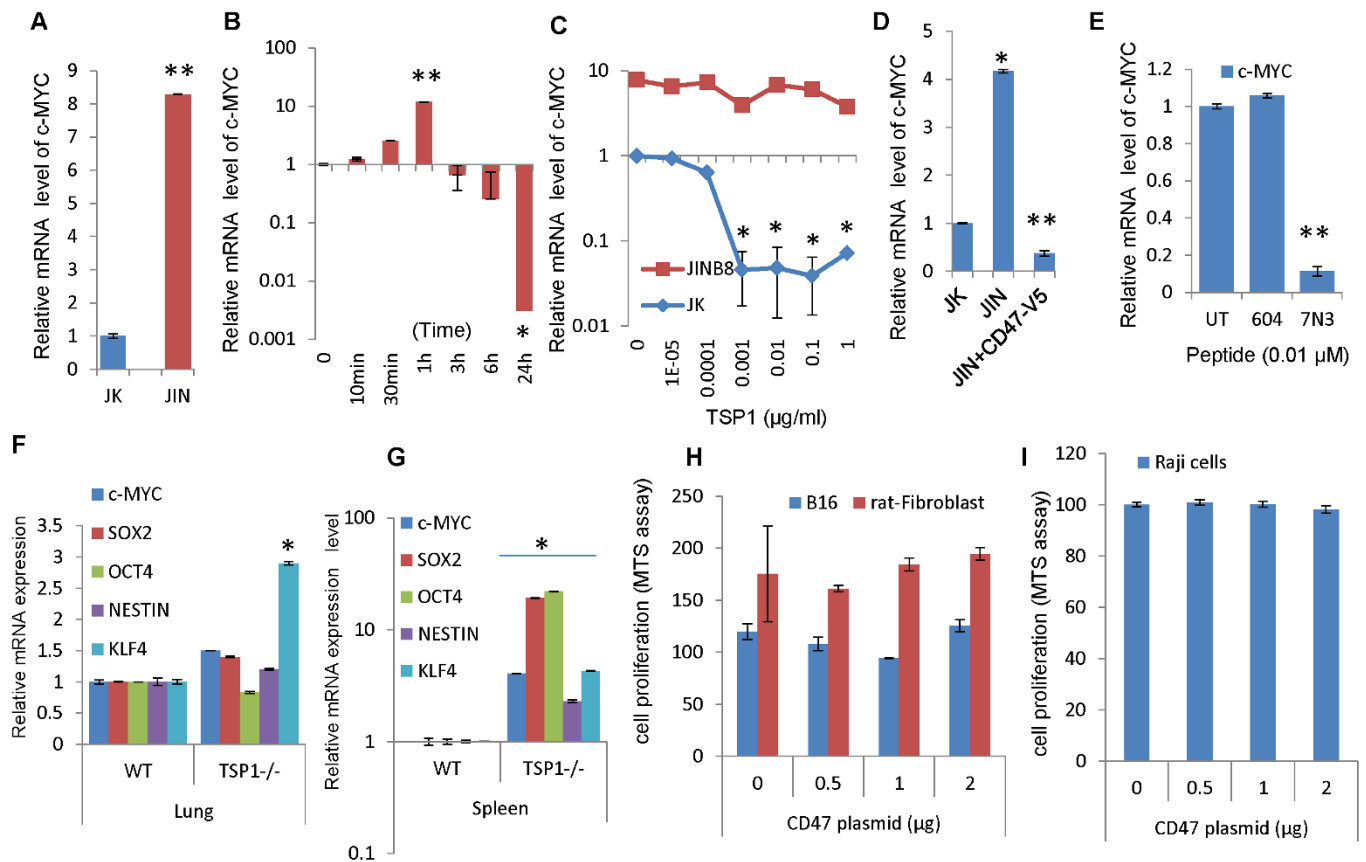
## Discussion

These results demonstrate that a population of multipotent stem cells is selectively maintained at high frequency in primary and continuously cultured CD47-null endothelial cells. These cells support long term maintenance of viable endothelial cells in medium containing endothelial growth factors, but when deprived of serum CD47-null cells spontaneously generate EB-like clusters that express pluripotency markers including alkaline phosphatase, Nanog, and SSEA-1. These in turn can be induced to differentiate into cell types representative of all three embryonic germ layers when appropriate growth factors and cytokines are provided. In contrast to exhibiting these characteristics of iPS cells, the CD47 null EB-like clusters did not form teratomas. Consistent with their lack of teratoma formation, we

found no loss of expression of the tumor suppressor gene *PTEN* or activation of oncogenes including *Ras* in CD47-null EB-like clusters. Loss of *PTEN* was reported to increase teratoma formation by pluripotent stem cells<sup>55</sup>. Others have shown that stem cells can remain pluripotent when teratoma formation is suppressed<sup>56</sup>. Thus, the CD47-null EB-like clusters may not be fully pluripotent, but their lack of tumorigenicity would be an advantage for potential therapeutic applications<sup>57</sup>.

Our data reveal that suppression of c-Myc expression is an important mechanism by which thrombospondin-1 signaling via CD47 controls cell growth and differentiation. c-Myc is now recognized to be a universal amplifier of the expression of actively transcribed genes in somatic and embryonic stem cells<sup>26</sup>, so the ability of CD47 to control c-Myc expression identifies CD47 as cell surface receptor that globally regulates gene expression. Combined with its specific regulation of the stem cell transcription factors Oct4, Sox2 and Klf4, CD47 limits the growth, self-renewal, and reprogramming capacity of primary murine cells in tissue culture. Suppression of these major stem cell transcription factors by CD47 also occurs in vivo and can be modulated by targeting CD47. A corresponding increase in the abundance of tissue stem cells, suggested by the increased numbers of





**Figure 7 | Regulation of c-Myc and stem cell transcription factors by CD47 ligation.** (A) c-Myc mRNA in Jurkat (JK) and CD47-deficient JinB8 Jurkat T cells (JIN). (B) Time-dependence for regulation of c-Myc mRNA expression by the CD47 ligand thrombospondin-1. Jurkat cells were treated with 2.2 nM thrombospondin-1 for the indicated times before isolating RNA and assessing c-Myc mRNA by real time PCR normalized to  $\beta$ 2m mRNA and expressed as a ratio to normalized c-Myc levels in control cells at the corresponding time points. (C) Thrombospondin-1 effects on c-Myc mRNA in WT Jurkat and CD47-deficient (JinB8) T lymphoma cells. (D) CD47 re-expression in JinB8 cells alters expression c-Myc compared with WT Jurkat cells. (E) Effects of CD47-binding TSP1 peptide 7N3 and control peptide 604 on c-Myc mRNA expression in Jurkat T cells. (F and G) mRNA levels in thrombospondin-1-null vs. WT lung and spleen. (H) CD47 over-expression in Rat1 fibroblasts and B16 melanoma cells does not suppress growth. (I) Deregulation of translocated c-Myc in Raji Burkitt's lymphoma cells prevents growth regulation by CD47 over-expression. (\* $p < 0.05$ , \*\* $p < 0.01$ ).

Sox2-positive cells in several organs of CD47-null mice, may contribute to the remarkable ability of tissues in these mice and in thrombospondin-1 null mice to recover from various injuries<sup>2,58</sup>. In addition to the potential therapeutic utility of CD47 antagonists for treating such injuries, the present data suggest that antagonists of CD47 signaling could be used to increase the ex vivo expansion of tissue stem cells for cell-based therapies and tissue engineering. We propose that CD47 antagonists may also be useful to enhance the generation of lineage-committed or multipotent stem cells and to circumvent the requirement for ectopic expression using plasmids or integrating retroviruses encoding tumor promoting genes such as c-Myc.

c-Myc expression greatly increases the frequency of iPS cells induced by combined ectopic expression of Oct3/4, Sox2, and Klf4<sup>32</sup>. Because our data show that CD47 limits c-Myc expression and other studies have shown that thrombospondin-1 inhibits endothelial progenitor cell function via CD47<sup>59</sup>, it is remarkable that CD47 expression is elevated on hematopoietic stem cells<sup>25</sup>. CD47 in this context was proposed to prevent clearance of stem cells by NK cells or macrophages expressing the CD47 counter-receptor SIRP $\alpha$ <sup>16,25</sup>, but we propose that such stem cells must adapt to the cell-autonomous inhibitory effects of high CD47 expression that suppress c-Myc and other stem cell transcription factors. Myc expression is presumably maintained through other regulatory pathways to preserve viability. The loss of viability that we observe following the acute withdrawal of c-Myc due to CD47-ligation or restoring CD47 expression in null cells may be an example of

oncogene addiction in normal cells. These results indicate that a downward excursion of Myc must be carefully managed to prevent cell death or senescence.

Previous studies have implicated thrombospondin-1 as an inhibitor of certain stem cell functions but have not invoked CD47 as the relevant receptor. Thrombospondin-1 null mice exhibited 5-fold more circulating endothelial lineage-committed stem cells (EPCs, CD13<sup>+</sup>/VEGFR-2<sup>+</sup>/CD45<sup>-</sup>/CD117<sup>+</sup>) than WT mice<sup>60</sup>. Because the elevation in EPCs was suppressed when the null mice were treated with a drug targeting the thrombospondin receptor CD36, the increased number of EPCs was attributed to loss of anti-angiogenic thrombospondin-1 signaling via CD36 in the null. Conversely, elevated thrombospondin-1 levels in diabetes and peripheral artery disease have been associated with suppression of vascular wound repair mediated by EPCs<sup>59,61</sup>. Notably, EPCs highly express CD47, and suppression of CD47 by RNAi enhanced their proliferation and angiogenic potential<sup>59</sup>. The authors attributed this to increased activity of the SDF-1/CXCR4 pathway, but the present data reveal a novel and much broader role of CD47 to limit stem cell function by suppressing c-Myc and other stem cell transcription factors. Furthermore, because CD47 null stem cells show an enhanced capacity to differentiate along diverse lineages, we argue that the inhibitory function of CD47 in stem cell maintenance is not restricted to the endothelial lineage.

These findings add new significance to the previously reported inhibition of thrombospondin-1 transcription and mRNA stability



by elevated expression of c-Myc<sup>62,63</sup>. Combined with our results, thrombospondin-1 and c-Myc can be seen to form a negative feedback loop in normal cells that limits the expression of both genes. This feedback would normally limit the expression of inhibitory thrombospondin-1 and thereby promote tissue renewal and regeneration. This feedback regulation is disrupted in Burkitt's lymphoma where transcriptional control of Myc expression by thrombospondin-1 is lost. Thus, c-Myc dysregulation may allow tumor cells to maintain the elevated surface expression of CD47 that was first identified in ovarian carcinoma<sup>64</sup> and subsequently extended to other cancers<sup>3,17,18,21,25</sup>. Conversely, aging and chronic diseases such as diabetes and pulmonary hypertension where thrombospondin-1 expression is elevated should limit c-Myc expression<sup>65–67</sup>, which could contribute to pathogenesis of these diseases by limiting tissue stem cell maintenance and function.

As an inhibitory cell surface receptor that controls self-renewal, CD47 may be critical for understanding how the microenvironment in the stem cell niche regulates stem cell differentiation<sup>68</sup>. CD47 may directly transmit a negative signal from the environment that inhibits self-renewal or proliferation, or lateral cross talk of CD47 with integrins and growth factor receptors in the plasma membrane<sup>3,69</sup> may negatively modulate these signals in stem cells. Our studies identify thrombospondin-1 as a key environmental signal that inhibits stem cell self-renewal via CD47.

## Methods

**Animals.** Thrombospondin-1 null<sup>70</sup> CD47 null mice<sup>71</sup> extensively back-crossed onto a C57Bl/6J background and WT mice were housed in a specific pathogen-free environment and had ad libitum access to standard rat chow and water. Care and handling of animals and all experimental procedures used here were approved under protocol LP-012 and in compliance with standards established by the Animal Care and Use Committee of the National Cancer Institute.

**Cells.** Mouse lung endothelial cells were isolated and their purity verified as described previously<sup>50</sup>. These conditions were previously documented to reproducibly yield >95% pure endothelial cells at passage 2 (CD31<sup>+</sup>, smooth muscle actin<sup>−</sup>). Mouse lung endothelial cells were cultured at 37°C with 5% CO<sub>2</sub> using Endothelial Growth Medium-2 (EGM2, Fisher Scientific). Cell populations from mouse spleens were separated using Pan T cell Isolation, CD4(L3T4), CD8a(Ly-2) CD11b, and CD19 microbead kits (Miltenyi Biotec).

V6.5 mouse embryonic stem (ES) cells were cultured on gelatin-coated dishes with mouse embryonic fibroblast (MEF) feeder cells using standard mES medium containing DMEM (high glucose), 15% ES cell-qualified FBS, 200 mM L-glutamine, non-essential amino acids (LifeTechnology), Pen/Strep, 0.1 mM 2-mercaptoethanol, and 1000 unit/ml LIF.

**Western blots.** Equal numbers of lung endothelial cells from WT and CD47 null were plated in 6-well plates overnight. Cell lysates were made from washed cells using NP-40 lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl and 1% NP-40 along with ProteoBlock Protease inhibitor Cocktail (Fermentas)). The lysates were centrifuged, and equal volumes of supernatant were boiled with 4× NuPAGE-LDS sample buffer (Invitrogen) for 5 min at 95°C. Proteins were separated using 4–12% Bis-Tris gels (Invitrogen). N-terminal c-Myc antibody (Epitomics), human CD47 -B6H12, V5-peroxidase (Invitrogen) and β-actin (Sigma) 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 (Fisher Scientific) was used to detect bound antibodies. For protein normalization, the blots were reprobed using a β-actin antibody (Sigma Aldrich).

**Sox2 immunohistochemistry.** Lung and spleen tissues from groups of 3 WT and CD47<sup>−/−</sup> mice were fixed in 10% formalin. Tissue was paraffin embedded and 5 μm thick sections were immunostained using a Sox2 antibody (1 : 100) or a non-specific control antibody and detected using the DAKO LASB Universal Kit. Stained sections were visualized and photographed under light microscope using the Q-Imaging system.

**Transient CD47 re- or over-expression.** Isolated mouse lung endothelial cells, Raji Burkitt's lymphoma cells, B16 melanoma cells, and Myc null Rat1 fibroblast cells were plated overnight in 6-well plates. The cells were transfected with CD47-FLAG expression plasmid<sup>72</sup> and/or human c-Myc-GFP plasmid<sup>26</sup>. The cells were transfected using Opti-MEM<sup>®</sup> I Reduced Serum Medium (Invitrogen) and the FuGENE HD Transfection kit (Roche). The serum free medium was replaced with complete RPMI medium 5 h after transfection. The cells were analyzed at 24–36 h post transfection. The supernatants were analyzed for lactate dehydrogenase (LDH) release using CytoTox 96<sup>®</sup> Non-Radioactive Cytotoxicity Assay (Promega).

The JIN8 cells were transfected with CD47-V5 construct using Amaxa (Lonza). The transfection efficiency was determined by flow Cytometry. The CD47-V5 transfected cells were purified using magnetic beads preincubated with human CD47 antibody (B6H12). The transfected cells were loaded onto MACS column which was placed in the magnetic field of a MACS separator. The magnetic bead labeled CD47<sup>+</sup> cells were retained on the column. The unbound cells were depleted from CD47<sup>+</sup> cells. After removal of the column, the CD47<sup>+</sup> cells bound to magnetic beads were eluted. The Pure population of CD47<sup>+</sup> was stably cultured using G418 drug (250 μg/ml). The Pure population of CD47<sup>+</sup> and Jurkat cells were centrifuged and re-suspended in RPMI + 1% FBS at 10<sup>6</sup>/ml. Cells were plated in 12 well plates and treated with 1 μg/ml (2.2 nM) thrombospondin-1, and total RNA was isolated using TRIzol. The relative gene expression of c-MYC was measured using GAPDH as a control.

**CD47 knockdown in T cells and in WT mice.** A translation-blocking antisense morpholino oligonucleotide complementary to human and murine CD47 (CGTCA CAGGAGGACCCACTGCCCA) and a 5-base mismatch control morpholino (CGTgACAGcCacGACcGACTGCGcA) were obtained from GeneTools as previously described<sup>48</sup>. Primary T cells isolated from c-Myc EGFP knock-in mouse were transfected using morpholinos (2.5 μM) along with Endoport according to manufacturer's instructions. Mice were treated by injection of 750 μl of 10 μM morpholino in saline as described<sup>8</sup>. Organs were harvested for mRNA isolation after 48 h.

**Modulation of stem cell transcription factors by thrombospondin-1.** Jurkat and Jin8 cells were centrifuged and re-suspended in RPMI + 1% FBS at 10<sup>6</sup>/ml. Cells were plated in 12 well plates and treated with 1 μg/ml (2.2 nM) thrombospondin-1 using the indicated times and concentrations, and total RNA was isolated using TRIzol.

**Cell proliferation assays.** Equal numbers of mouse lung endothelial cells from WT and CD47 (Passage 1) were plated in 96 well plates using RPMI + 1% FBS. After 72 h, net cell proliferation was assessed by the increase in formazan absorbance versus controls assessed at time 0 using Cell Titer 96R aqueous MTS kit (Promega). DNA synthesis was measured using a BrdU Assay (EMD Biosciences).

**Senescence-associated β-galactosidase assay.** Senescent cells were detected in WT and CD47 null lung endothelial cells (Passage 3) by staining for a senescence-associated β-galactosidase<sup>73</sup>. Positive cell numbers were expressed as a percentage of the total cells.

**Statistical analysis.** Two-way ANOVA with replication was used for analyzing real time PCR. Student t-test was used for cell proliferation, cell cytotoxicity and cell senescence assays, which were performed in triplicate. All results are presented as mean ± SD.

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## Acknowledgements

This work was supported by the Intramural Research Programs of the NIH/NCI (D.D.R., D.L.) and NHGRI (A.G.E.), 1R01HL108954-01 (NIH/NHLBI), 1P01HL103455-01 (NIH), 11BGIA7210001 (AHA), the Institute for Transfusion Medicine, and the Western Pennsylvania Hemophilia Center (J.S.I.). We thank Dr. Yosuke Mukoyama for providing reagents, Judith Horn and Maryann Williamson (Laboratory of Pathology, NCI) for their help for preparing and staining sections, Weiwei Wu for microarray analysis, and Susan Garfield, Poonam Manan and Tonya Huang (NCI) for confocal microscopy.

## Author contributions

S.K., D.S.P., J.S.I., D.L. and D.D.R. conceived and designed the experiments. S.K., D.S.P., E.V.S., C.L., A.G.E., M.L.P., S.P.S., A.N., Z.N. and J.S.I. performed experiments and analyzed





data. S.K., D.S.P., E.V.S., C.L. A.G.E., D.L., J.S.I. and D.D.R. wrote the manuscript. All authors reviewed the manuscript.

### Additional information

**Supplementary information** accompanies this paper at <http://www.nature.com/scientificreports>

**Competing financial interests:** The authors declare no competing financial interests.

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**How to cite this article:** Kaur, S. *et al.* Thrombospondin-1 Signaling through CD47 Inhibits Self-renewal by Regulating c-Myc and Other Stem Cell Transcription Factors. *Sci. Rep.* 3, 1673; DOI:10.1038/srep01673 (2013).