SPARC is dispensable for murine hematopoiesis, despite its suspected pathophysiological role in 5q- myelodysplastic syndrome.

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SPARC is dispensable for murine hematopoiesis, despite its suspected pathophysiological role in 5q- myelodysplastic syndrome

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All mature blood cell types arise from a limited number of hematopoietic stem cells (HSCs). HSCs sustain hematopoiesis throughout the lifetime of an individual and any insult to them can lead to severe pathologies. Elucidating the molecular mechanisms that govern the maintenance and differentiation of HSCs is critical to understanding their role in hematological pathologies. In a search for novel regulators of HSCs, we selected SPARC (Secreted Protein Acidic and Rich in Cysteine, also known as Osteonectin and BM40), because it was identified as one of the candidate regulators of HSCs by molecular profiling of differentiating hematopoietic cells in an in vitro model system (1). Several lines of evidence have also indicated an important role for SPARC in hematopoiesis. In zebrafish, a knockdown of SPARC resulted in lower numbers of circulating blood cells in developing embryos (2). In a murine model, SPARC deficiency resulted in thrombocytopenia and reduced erythroid colony formation (3). In humans, SPARC is transcriptionally silenced in acute myeloid leukemia patients and in cell lines with mixed lineage leukemia rearrangements (4). SPARC maps to the deleted region in 5q myelodysplastic syndrome (MDS), an HSC disorder. With indications that SPARC may have a role in the hematopoietic system, we aimed to elucidate its role in the regulation of HSCs, since HSCs have not been characterized in the SPARC null mice.

SPARC is a glycoprotein that binds calcium and interacts with members of the extracellular matrix including collagens and thrombospodin. SPARC is expressed in a variety of tissues undergoing rapid turnover and is a major component of the bone. SPARC expression is associated with cell proliferation, migration, changes in morphology, matrix-remodeling and cell-cell interactions, although the precise function of the gene still remains unknown (5). SPARC knockout mice (SPARC -/-) are viable and fertile but display a number of abnormalities, including increased deposition of fat, highly contractile skin, alterations in wound healing and an altered response to tumor growth, most severe of which are osteopenia and early onset cataract (6, 7). SPARC -/- mice generated previously by the targeted mutation of exon 6 that completely lacked SPARC RNA and protein (6), were used in this study to carry out functional assays for HSCs.

Steady state hematopoiesis was unperturbed in SPARC -/- mice as seen by normal peripheral blood counts (Supplementary Table I). A similar observation was made by Barlow et al. using a mouse model carrying a different mutation in SPARC (8). Also, the bone marrow of SPARC -/- mice showed normal morphology and cellularity (Supplementary Figure 1). A thorough phenotypic analysis of the bone marrow myeloerythroid compartment using a previously described FACS strategy (Supplementary Figure 2) (9) showed no significant differences in the frequencies of HSCs or myeloerythroid progenitors (Supplementary Table II). Since defects in HSC regulation may not always be reflected in steady state hematopoiesis (10), we tested if there was any functional impairment in SPARC -/- progenitors and HSCs. SPARC -/- hematopoietic progenitor cells showed normal proliferation and colony forming ability in vitro, in methylcellulose based cultures except for fewer burst forming units of the erythroid lineage (BFU-Es) (Supplementary Figure 3), as also shown previously (3). This defect in vitro did not translate to a defect in vivo as the mice were not anemic (Supplementary Table I) and had normal erythroid progenitors in the bone marrow (Supplementary Table II).

We performed bone marrow transplantations to analyse if bone marrow cells lacking SPARC could reconstitute the hematopoietic system of lethally irradiated mice.

Transplanted recipients, in a non-competitive setting, when followed for 16 weeks to assess donor contribution (schematic Figure 1A), showed efficient, long term multi-lineage reconstitution by SPARC -/- cells (Figure 1B). When bone marrow cells from primary recipients were transplanted into secondary recipients, the SPARC -/- cells continued to give rise to multi-lineage long-term hematopoiesis (Figure 1C). SPARC -/- bone marrow cells also performed similar to wild-type (WT) cells in competitive transplantations (Figure 1D, 1E), strongly indicating that HSCs lacking SPARC had no impairment in their function. To quantify frequency of HSCs, we carried out the limiting dilution competitive repopulation unit (CRU) assay. We found similar CRU frequencies in SPARC -/- mice compared to the WT (Figure 1F).

We further tested if the hematopoietic system of SPARC -/- mice recovered normally from radiation or chemically induced hematopoietic stress. For radiation-induced stress, we followed hematopoietic recovery of mice subjected to sub-lethal doses of gamma irradiation. We used the chemical, phenylhydrazine to specifically stress the erythroid compartment as SPARC -/- cells gave fewer BFU-Es in in vitro colony forming assay. We found no differences in the recovery of SPARC -/- mice compared to the controls in either radiation induced or phenylhydrazine induced stress of the hematopoietic system (Figure 2A, 2B).

SPARC is a critical regulator of bone remodeling (11). We therefore asked if the niche might be compromised in the absence of SPARC, by testing whether the niche could support normal hematopoiesis. We transplanted WT cells into lethally irradiated SPARC deficient mice and found that the WT cells could give rise to long-term multi-lineage reconstitution (Figure 2C). We found no skewing of myeloid and lymphoid lineages (Figure 2D) and the peripheral blood counts of SPARC -/- recipients after 16 weeks of transplantation was found to be similar to WT recipients (Supplementary Table III).

In humans, SPARC is expressed in bone marrow CD34+ cells; a compartment enriched in stem and progenitor cells. In 5q MDS patients, a deletion on chromosome 5 results in the loss of one copy of SPARC among other genes. Studies have shown that there is 70% down regulation in the expression of SPARC in the progenitor compartment (3, 12). Lenalidomide is a drug that is used for the treatment of the disease. It inhibits growth of the malignant hematopoietic progenitors but its mechanism of action is not known. Although this drug has been shown to dramatically up-regulate SPARC, evidence to support a direct role for this gene in the pathophysiology of the disease is limited. We examined the role of SPARC in hematopoiesis using a knockout mouse model and found it to be dispensable for normal hematopoiesis.

Steady state hematopoiesis was normal in SPARC -/- mice and transplantation assays showed that absence of SPARC did not affect the reconstitution ability of HSCs. We have also tested few other conditions of stress including gamma irradiation and phenylhydrazine. Our results show that SPARC does not have any cell intrinsic role in the regulation of hematopoiesis. There may be other complications of the hematopoietic system that have not been tested in this study, where SPARC may play a role. For instance, a complex situation could be the case of leukemias, which can result in more profound stress to the HSCs. It has been shown in cancers of other tissues that aberrant SPARC expression can lead to bad prognosis (13). On the other hand, SPARC belongs to a family of proteins, few of which could potentially

compensate for its function. Some members of the SPARC family have been shown to compensate for SPARC function in other organ systems. For example, SC-1 plays a compensatory role for SPARC in angiogenesis related to foreign body response. Knockout mice for either SPARC or SC-1 show normal vessel densities on foreign body capsules but a double knockout of both the genes shows a significant increase in vessel density (5).

Our data from reverse transplantation experiments indicated that the lack of SPARC in the niche does not affect normal hematopoiesis from WT HSCs. SPARC may not be an important component of the hematopoietic niche even though its absence can lead to severe osteopenia. There are known molecules like Rac, signaling through which is required for normal bone development but dispensable for hematopoietic development (14). Some components of the bone remodeling machinery like osteoclasts are also dispensable for hematopoiesis (15).

In summary, in vitro, lack of SPARC did not impair colony-forming capacity of BM hematopoietic cells, except for BFU-Es. In transplantation experiments SPARC -/- BM cells gave rise to long-term multi-lineage reconstitution. These cells competed normally with WT cells in competitive transplantation assays and responded as well as their WT counterparts to experimentally induced stress; indicating that SPARC is not essential for murine hematopoiesis. Furthermore, even in the absence of SPARC, the BM niche supported normal hematopoiesis assessed by transplantation of WT cells into SPARC -/- recipients. In conclusion, we tested the role of SPARC in murine HSC function and found that it is dispensable for both normal and stress hematopoiesis.

The mouse model used in our study had only the SPARC gene missing, unlike in 5q MDS where multiple genes are in the hemizygous state. It is possible that the change in dosage of multiple genes together can account for a strong disease-like phenotype. It is also possible that SPARC has more diverse functions in the human system. Therefore, before ruling out a role for SPARC in the disease, its role needs to be tested in human hematopoiesis to provide insights into the function of SPARC, if any, in the regulation of human hematopoietic stem and progenitor cells.

REFERENCES

1. Bruno L, Hoffmann R, McBlane F, Brown J, Gupta R, Joshi C, et al. Molecular signatures of self-renewal, differentiation, and lineage choice in multipotential hemopoietic progenitor cells in vitro. Mol Cell Biol. 2004 Jan;24(2):741-56.
2. Eckfeldt CE, Mendenhall EM, Flynn CM, Wang TF, Pickart MA, Grindle SM, et al. Functional analysis of human hematopoietic stem cell gene expression using zebrafish. PLoS Biol. 2005 Aug;3(8):e254.
3. Lehmann S, O'Kelly J, Raynaud S, Funk SE, Sage EH, Koeffler HP. Common deleted genes in the 5q- syndrome: thrombocytopenia and reduced erythroid colony formation in SPARC null mice. Leukemia. 2007 Sep;21(9):1931-6.
4. DiMartino JF, Lacayo NJ, Varadi M, Li L, Saraiya C, Ravindranath Y, et al. Low or absent SPARC expression in acute myeloid leukemia with MLL rearrangements is associated with sensitivity to growth inhibition by exogenous SPARC protein. Leukemia. 2006 Mar;20(3):426-32.
5. Yan Q, Sage EH. SPARC, a matricellular glycoprotein with important biological functions. J Histochem Cytochem. 1999 Dec;47(12):1495-506.
6. Gilmour DT, Lyon GJ, Carlton MB, Sanes JR, Cunningham JM, Anderson JR, et al. Mice deficient for the secreted glycoprotein SPARC/osteonectin/BM40 develop normally but show severe age-onset cataract formation and disruption of the lens. EMBO J. 1998 Apr 1;17(7):1860-70.
7. Norose K, Clark JI, Syed NA, Basu A, Heber-Katz E, Sage EH, et al. SPARC deficiency leads to early-onset cataractogenesis. Invest Ophthalmol Vis Sci. 1998 Dec;39(13):2674-80.
8. Barlow JL, Drynan LF, Hewett DR, Holmes LR, Lorenzo-Abalde S, Lane AL, et al. A p53-dependent mechanism underlies macrocytic anemia in a mouse model of human 5q-syndrome. Nat Med. 2010 Jan;16(1):59-66.
9. Pronk CJ, Rossi DJ, Mansson R, Attema JL, Norddahl GL, Chan CK, et al. Elucidation of the phenotypic, functional, and molecular topography of a myeloerythroid progenitor cell hierarchy. Cell Stem Cell. 2007 Oct 11;1(4):428-42.
10. Brun AC, Bjornsson JM, Magnusson M, Larsson N, Leveen P, Ehinger M, et al. Hoxb4-deficient mice undergo normal hematopoietic development but exhibit a mild proliferation defect in hematopoietic stem cells. Blood. 2004 Jun 1;103(11):4126-33.
11. Delany AM, Hankenson KD. Thrombospondin-2 and SPARC/osteonectin are critical regulators of bone remodeling. J Cell Commun Signal. 2009 Dec;3(3-4):227-38.
12. Pellagatti A, Jadersten M, Forsblom AM, Cattan H, Christensson B, Emanuelsson EK, et al. Lenalidomide inhibits the malignant clone and up-regulates the SPARC gene mapping to the commonly deleted region in 5q- syndrome patients. Proc Natl Acad Sci U S A. 2007 Jul 3;104(27):11406-11.
13. Tai IT, Tang MJ. SPARC in cancer biology: its role in cancer progression and potential for therapy. Drug Resist Updat. 2008 Dec;11(6):231-46.
14. Lane SW, De Vita S, Alexander KA, Karaman R, Milsom MD, Dorrance AM, et al. Rac signaling in osteoblastic cells is required for normal bone development but is dispensable for hematopoietic development. Blood. 2012 Jan 19;119(3):736-44.
15. Miyamoto K, Yoshida S, Kawasumi M, Hashimoto K, Kimura T, Sato Y, et al. Osteoclasts are dispensable for hematopoietic stem cell maintenance and mobilization. J Exp Med. 2011 Oct 24;208(11):2175-81.

Siva et al. Figure 1

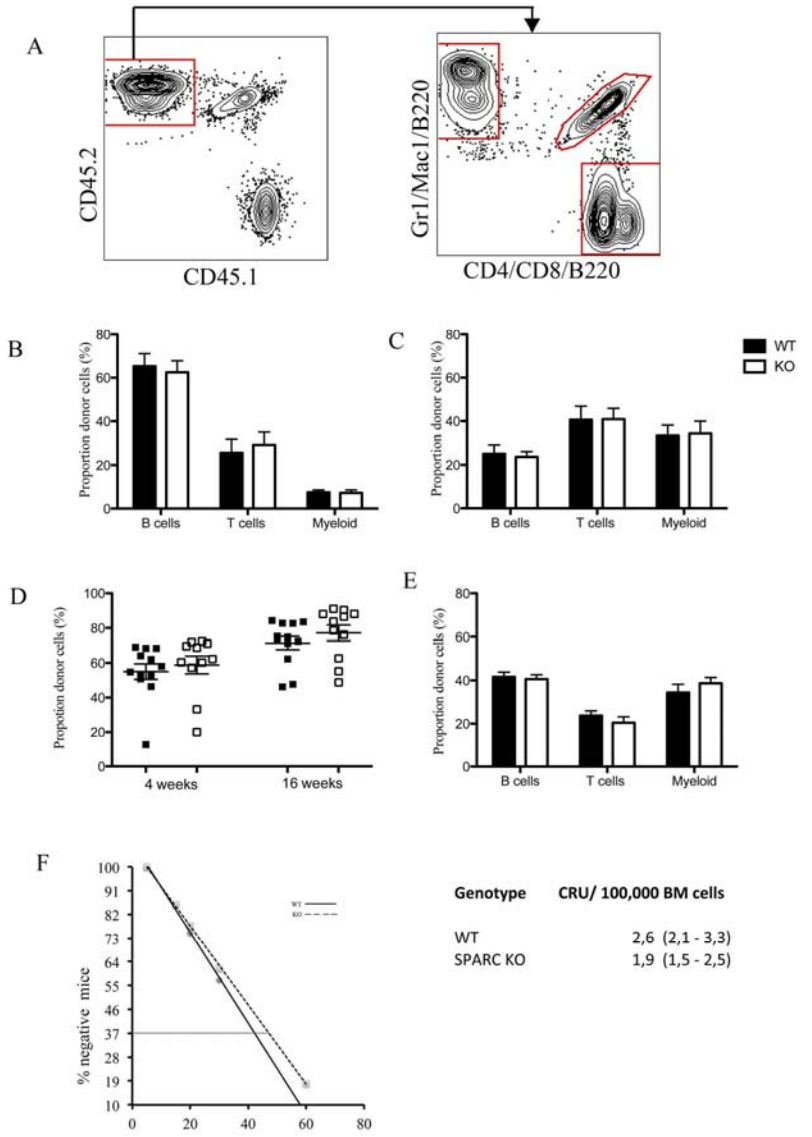

Figure 1. SPARC -/- cells show no disadvantage in transplantation assays.

Representative FACS plots from peripheral blood analysis in transplantation assays (A). CD45.1-PE-Cy7 and CD45.2 FITC were used to distinguish donor cells and Gr-1-APC, Mac-1-APC, B220-APC, B220-PE, CD4-PE and CD8-PE were used to identify myeloid and lymphoid cells. Lineage distribution of donor cells in primary (B) and secondary (C) non-competitive transplantations (n=3 donors per genotype and 2-6 recipients per donor). Donor contribution in competitive repopulation assay at 4 and 16 weeks post transplantation (D) and lineage distribution of the donors (E) (n=2 donors per genotype). Frequency of HSCs in BM cells of SPARC -/- mice compared to WT littermates estimated by Limiting dilution CRU assay (F), n=3 donors and 2-7 recipients per dose.

Siva et al. Figure 2

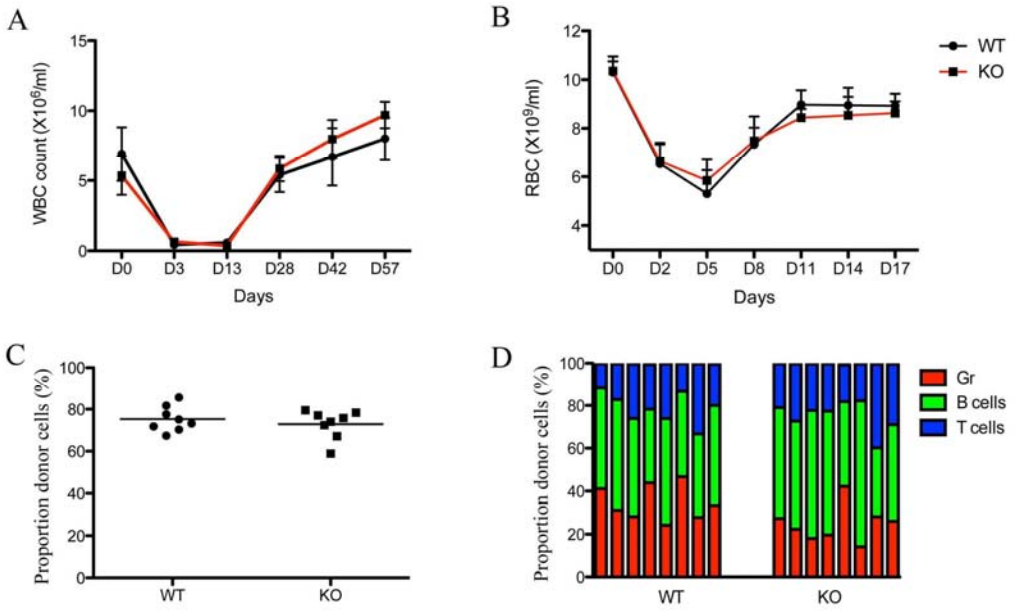

Figure 2. Lack of SPARC neither perturbs recovery from hematopoietic stress nor disturbs normal hematopoiesis from the niche.

(A,B) – Stress experiments. Peripheral blood WBC count of mice following sublethal irradiation (A). Peripheral blood RBC count following phenylhydrazine-induced anemia (B). Data represents mean values ± SEM (n=7 of each genotype, for both experiments).

(C, D) – Reverse transplantation assay. Percentage donor contribution (C). Lineage distribution of the donor cells (D). Each recipient is represented on the plot as a filled circle or dotted square (A) or as a bar (B). Donor cells were assayed as shown in Figure 1A.

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Conflict of interest statements

The authors declare no competing financial interests.

SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS

Mice

129 Sv SPARC -/- mice were a kind gift from Prof. Martin Evans, Cardiff School of Biosciences, Cardiff University, Wales, UK and have been described previously (1). The mice have a targeted disruption at exon 6 that causes premature termination of transcription. Neither *mRNA* nor protein was detected when analysed by Northern and Western blot analysis respectively (1). The mice were maintained by mating heterozygous individuals, which gave SPARC -/- and WT littermates. Genomic DNA from ear or tail biopsies were used for genotyping the mice. Genotyping for detection of the targeted allele was done using polymerase chain reaction (PCR) with the following primer sets-

Ex6 F 5' TGC AGC AAT GAC AAC AAG ACC 3'

Ex6 R 5' TTG CAT GGT CCG ATG TAG TCC 3'

Mut F 5'GCTCGCGGTTGAGGACAAAC□3'

Mut R 5' TCTTCGCTATTACGCCAGCT 3'

Quantitative real-time (RT) PCR was done on cDNA from cells dissociated from the femur to confirm the absence of SPARC in the knockout mice (data not shown). The mice were housed in individual ventilated cages and the Lund University ethics committee for animals approved all animal experiments.

Cell preparations

Peripheral blood was collected from the tail vein in heparin or in EDTA coated tubes from SARSTEDT. SYSMEX KX-21N hematology analyser was used for determining blood cell counts. For FACS, blood cells were stained with antibodies, and red blood cells (RBCs) were lysed with ammonium chloride. Femur and tibiae were crushed and passed though a 70 μm cell strainer to obtain single cell suspension of bone marrow cells. Cellularity of bone marrow was measured by counting cells from two femurs and two tibiae.

Histology

Organs for histopathological analysis were fixed in 4% paraformaldehyde buffered with phosphate buffered saline (PBS), followed by paraffin embedding and sectioning. Sections were stained with eosin and hematoxylin for microscopic examination. Microscope used was Nikon E50i and the imaging system used was CellSens Standard.

In vitro culture and colony forming assays

Freshly isolated bone marrow cells were plated in 35 mm Petri dishes in methycellulose medium containing 50 ng/mL mSCF, 10 ng/mL mIL-3 and 10 ng/mL hIL-6. Colonies were scored by viewing under a microscope; CFU-E on day 3 of culture and CFU-G, CFU-M, CFU-GM and CFU-GEMM on day 8. BFU-E was scored on day 14 after growing cells in Methocult M3436 and CFU-Mk were scored on day 7 following culture in collagen based Megacult medium. All methylcellulose and collagen based media were bought from STEMCELL technologies Inc.

Flow Cytometry

Fluorochrome-conjugated antibodies against the following cell surface receptors were purchased from BD Biosciences: B220, CD3, CD4, CD8, Gr-1, Mac-1, CD45.1, CD45.2, CD105, CD150, CD44, CD41, cKit, Ter119 and Sca-1. Dead cells were excluded through staining with 7-aminoactinomycin D or propidium iodide. Cells were analyzed using a FACS CantoII and sorted using the FACSAria (BD Biosciences). Analysis was done using the FlowJo software (TreeStar).

Transplantations

All transplantations were done as described earlier (2). In brief, all transplantations involved the use of lethally irradiated (900 cGy) recipient mice. Donor, recipient and support cells were tracked based on expression of different isoforms of the pan-hematopoietic marker CD45 (CD45.1, CD45.2) or green fluorescent protein (GFP) expression. For all transplantations, donor cells were tracked by their expression of CD45.2 except in reverse transplantations where the donors were tracked using GFP. Peripheral blood was collected at different time points to determine reconstitution from donor cells and their lineage distribution.

Non-competitive transplantations - 1 X 10^6 fresh bone marrow cells from SPARC -/- or WT mice were transplanted into lethally irradiated C57BL/6 recipients. For secondary transplantations, 1 X 10^6 bone marrow cells from primary recipients was used to transplant a new set of recipients.

Competitive transplantations – 2 X 10^5 fresh bone marrow cells from SPARC -/- mice or WT littermates were transplanted together with 2 X 10^5 WT competitor cells into lethally irradiated recipients.

Limiting dilution CRU - Different cell doses ranging between 2 X 10^3 and 6 X 10^4 cells per donor genotype, together with 2 X 10^5 WT competitor cells were transplanted into recipients. 3 donors each for SPARC -/- and WT cells were used and a total of 2-7 recipients per dose.

A cell dose was considered to contain at least one CRU if donor chimerism was >1% for both the myeloid and the lymphoid lineages. The CRU frequency was calculated using L-calc software (STEMCELL technologies Inc).

Reverse transplantations - 2 X 10^6 bone marrow cells from GFP expressing mice (actin-GFP) were transplanted into lethally irradiated SPARC -/- or WT littermate recipients.

Stress experiments

Irradiation induced stress - Mice were sub-lethally irradiated with 700 cGy of gamma irradiation and bled every other week to follow changes in leukocyte counts.

Phenylhydrazine induced anemia - Mice were injected intra-peritoneally with 120mg/kg Phenylhydrazine and bled every 3 days to monitor RBC count and hemoglobin levels using SYSMEX.

Statistics

Statistical significance was determined by calculating two-tailed p-values using t-test.

SUPPLEMENTARY TABLES

Supplementary Table I. SPARC -/- mice carry normal peripheral blood cell counts.

Peripheral blood counts of un-manipulated SPARC -/- mice and age matched WT controls between 8-14 weeks of age are shown (Mean±SEM).

	WT (n=20)	SPARC -/- (n=20)	P value
RBC	10.6 ± 1.0	10.4 ± 0.7	0.46
WBC	8.2 ± 4.2	7.9 ± 4.4	0.86
Platelets	698.5 ± 227.8	761.0 ± 216.0	0.35

Supplementary Table II. SPARC -/- mice show normal frequencies of HSCs and myeloerythroid progenitors.

Frequencies of viable progenitors within the bone marrow, assessed by flow cytometry, are shown (Mean ± SEM). HSCs and myeloid progenitor frequencies were calculated from within total nucleated cells, while erythroid frequencies include mature RBCs. HSC and progenitors were as represented in the schematic (Suppl Fig 2).

	WT (n=10)	SPARC -/- (n=10)	P value
HSC	0.04 ± 0.01	0.04 ± 0.02	0.97
Pre GM and GMP	0.98 ± 0.22	1.07 ± 0.32	0.48
MkP	0.08 ± 0.03	0.09 ± 0.05	0.48
PreMegE	0.13 ± 0.02	0.16 ± 0.07	0.22
Pre CFU-E and CFU-E	0.29 ± 0.05	0.34 ± 0.19	0.41
Pro-erythroblasts	0.88 ± 0.31	1.03 ± 0.45	0.39
Basophilic erythroblasts	5.12 ± 2.13	5.50 ± 2.11	0.68
Late basophilic and chromatophilic erythroblasts	22.29 ± 3.52	23.18 ± 4.41	0.62
Orthochromatic and non-erythroblasts	33.78 ± 4.98	33.35 ± 9.18	0.90

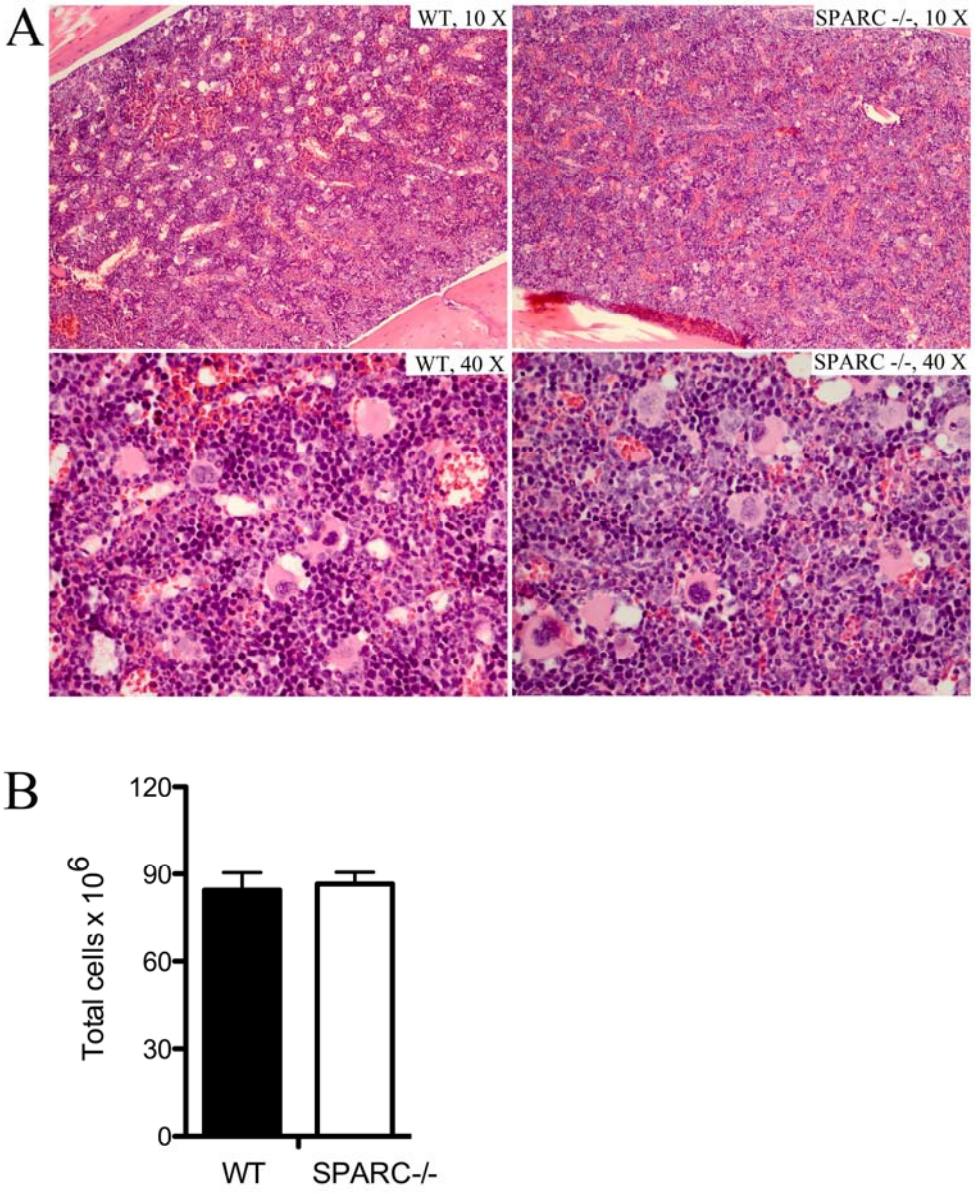
Supplementary Table III. SPARC -/- niche supports normal hematopoiesis.

Peripheral blood counts of SPARC -/- mice and of their age matched WT controls, both of which received GFP+ WT cells following irradiation are shown (Mean±SEM).

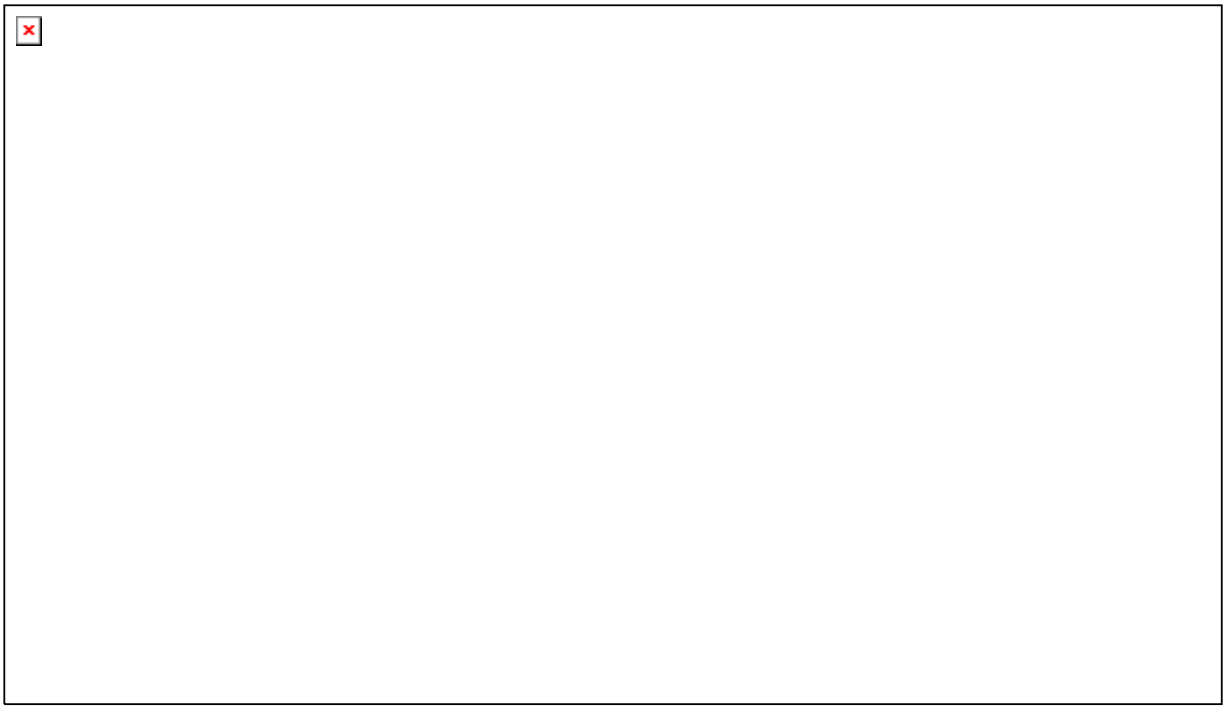
	WT (n=8)	SPARC -/- (n=8)	P-value
RBC	9.16 ± 0.29	9.2525 ± 0.63	0.72
WBC	9.38 ± 3.82	7.825 ± 2.46	0.38
Platelets	1037.38 ± 274.34	835.75 ± 216.12	0.15

SUPPLEMENTARY FIGURES

Siva et al. Supplementary Figure 1

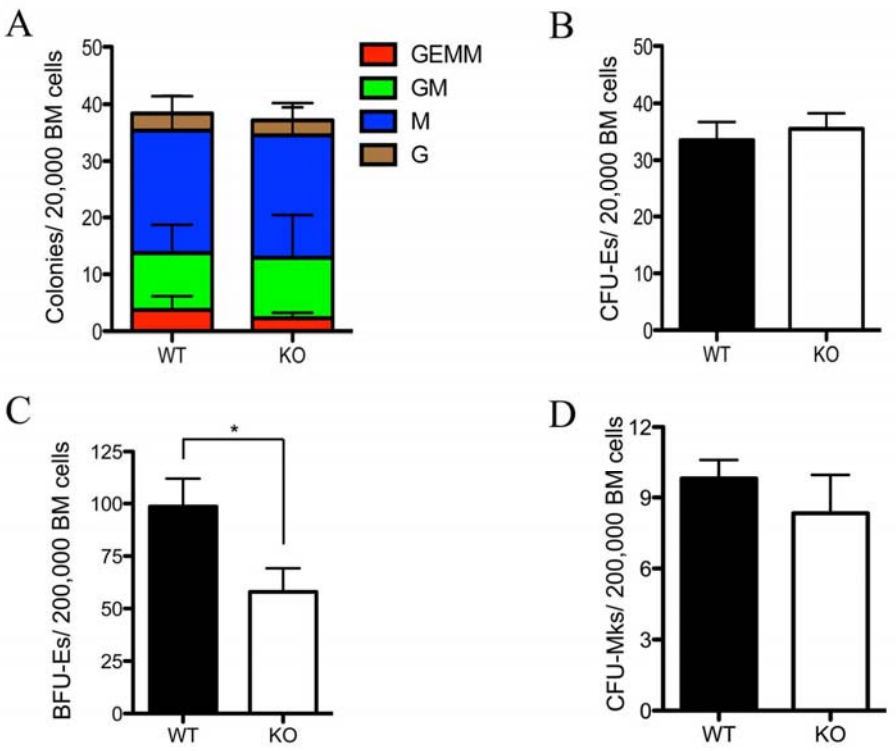


Supplementary Figure 1. Bone marrow in SPARC -/- mice is phenotypically normal. (A) Images of bone marrow from SPARC -/- mice and WT littermate is depicted at two different magnifications. (B) Plot depicting cellularity of bone marrow.



Supplementary Figure 2. Schematic of FACS strategy used for myeloerythroid lineage analysis of bone marrow cells.

Siva et al. Supplementary Figure 3



Supplementary Figure 3. SPARC -/- BM cells have a reduced frequency of BFU-Es. Colony counts from CFU assays on freshly isolated bone marrow cells from SPARC -/- and WT mice are plotted. (A) Differential colony count, n=6. (B) CFU-E, n=4, (C) BFU-E, n=7 (P value = 0.04) and (D) CFU-Mk, n=5.

REFERENCES

1. Gilmour DT, Lyon GJ, Carlton MB, Sanes JR, Cunningham JM, Anderson JR, et al. Mice deficient for the secreted glycoprotein SPARC/osteonectin/BM40 develop normally but show severe age-onset cataract formation and disruption of the lens. EMBO J. 1998 Apr 1;17(7):1860-70.
2. Karlsson G, Blank U, Moody JL, Ehinger M, Singbrant S, Deng CX, et al. Smad4 is critical for self-renewal of hematopoietic stem cells. J Exp Med. 2007 Mar 19;204(3):467-74.