

Long-term ex vivo haematopoietic-stem-cell expansion allows nonconditioned transplantation

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Multipotent self-renewing haematopoietic stem cells (HSCs) regenerate the adult blood system after transplantation¹, which is a curative therapy for numerous diseases including immunodeficiencies and leukaemias². Although substantial effort has been applied to identifying HSC maintenance factors through the characterization of the in vivo bone-marrow HSC microenvironment or niche^{3–5}, stable ex vivo HSC expansion has previously been unattainable^{6,7}. Here we describe the development of a defined, albumin-free culture system that supports the long-term ex vivo expansion of functional mouse HSCs. We used a systematic optimization approach, and found that high levels of thrombopoietin synergize with low levels of stem-cell factor and fibronectin to sustain HSC self-renewal. Serum albumin has long been recognized as a major source of biological contaminants in HSC cultures⁸; we identify polyvinyl alcohol as a functionally superior replacement for serum albumin that is compatible with good manufacturing practice. These conditions afford between 236- and 899-fold expansions of functional HSCs over 1 month, although analysis of clonally derived cultures suggests that there is considerable heterogeneity in the self-renewal capacity of HSCs ex vivo. Using this system, HSC cultures that are derived from only 50 cells robustly engraft in recipient mice without the normal requirement for toxic pre-conditioning (for example, radiation), which may be relevant for HSC transplantation in humans. These findings therefore have important implications for both basic HSC research and clinical haematology.

To optimize HSC cultures, we initially titrated thrombopoietin (TPO) against stem-cell factor (SCF) in seven-day CD34⁺c-KIT⁺SCA1⁺Lineage(Lin)[−](CD34[−]KSL) HSC cultures (Extended Data Fig. 1a, b) and determined the consequences by competitive transplantation into lethally irradiated recipient mice, against 1×10^6 bone-marrow competitor cells. The highest peripheral-blood chimerism (about 30%) at 16 weeks was observed with 100 ng ml^{−1} TPO and 10 ng ml^{−1} SCF (Fig. 1a), perhaps owing to the increased c-KIT internalization at higher SCF concentrations causing a loss of sensitivity to SCF (Extended Data Fig. 1c, d).

These conditions (100 ng ml^{−1} TPO and 10 ng ml^{−1} SCF) preferentially induced proliferation from bone-marrow-derived CD150⁺CD34[−]KSL HSCs rather than bone-marrow-derived CD34⁺KSL haematopoietic progenitor cells (Fig. 1b). We therefore determined whether longer-term ex vivo HSC expansion was possible by attempting one-month-long cultures. Because 50 starting HSCs expanded by approximately 13,000-fold during culture (Fig. 1c), we transplanted 1×10^4 cells per recipient—approximately 1/50th of the culture or about 1 starting HSC equivalent (hereafter termed ‘HSCeq’). Using half-medium changes, we only detected short-term reconstitution (Fig. 1d). However, by performing complete medium changes on the HSC cultures, we achieved cellular expansion similar to

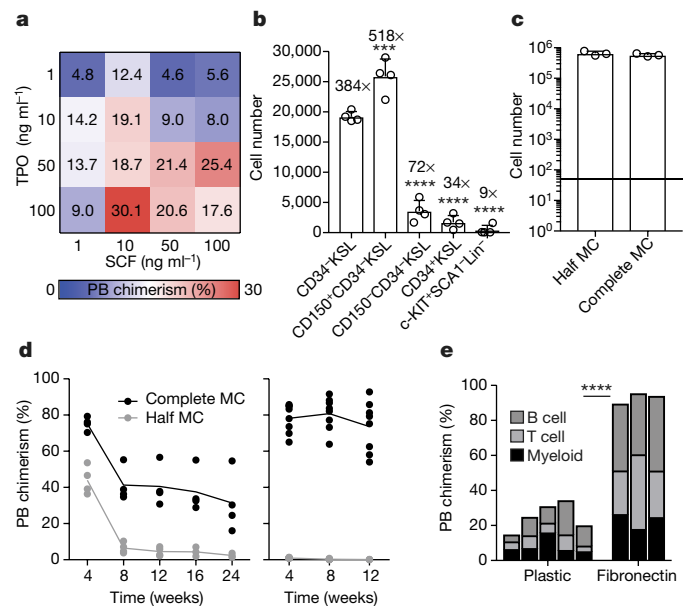


Fig. 1 | High levels of TPO synergize with low levels of SCF and fibronectin to enhance HSC expansion. **a**, Mean donor peripheral-blood (PB) chimerism at week 16, from 50 CD34[−]KSL HSCs after a 7-day-long culture in mouse TPO (1–100 ng ml^{−1}) and mouse SCF (1–100 ng ml^{−1}), as described in Extended Data Fig. 1a. Competitive transplantation against 1×10^6 bone-marrow competitor cells. **b**, Cell number derived from 50 CD34[−]KSL, 50 CD150⁺CD34[−]KSL, 50 CD150[−]CD34[−]KSL, 50 CD34⁺KSL or 50 c-KIT⁺SCA1[−]Lin[−] bone-marrow cells after a 7-day-long culture in 100 ng ml^{−1} TPO and 10 ng ml^{−1} SCF. Statistical significance was calculated using analysis of variance (ANOVA). *** $P = 0.004$. **** $P < 0.0001$. Mean \pm s.d. of four independent cultures. **c**, Twenty-eight days of growth of 50 CD34[−]KSL HSCs in 100 ng ml^{−1} TPO and 10 ng ml^{−1} SCF, and with half or complete medium changes (MC) every 3 days. Mean \pm s.d. of four independent cultures. **d**, Donor peripheral-blood chimerism in recipient mice from 1×10^4 HSC-derived cells (about 1 HSCeq), after a 28-day-long culture (started from 50 CD34[−]KSL), as described in **c**. Competitive transplantation against 1×10^6 bone-marrow competitor cells. Donor peripheral-blood chimerism at weeks 4 to 24, in primary recipients (left) and at weeks 4 to 12 in secondary recipients (right). **e**, Donor peripheral-blood chimerism at 16 weeks, from 1×10^4 HSC-derived cells (1.25 HSCeq) following a 28-day-long culture (started from 50 CD34[−]KSL) on plastic ($n = 5$ mice) or fibronectin ($n = 3$ mice) plates cultured in 100 ng ml^{−1} TPO and 10 ng ml^{−1} SCF with complete medium changes. Competitive transplantation against 1×10^6 bone-marrow competitor cells. Each column represents an individual mouse. Statistical significance was calculated using an unpaired two-tailed *t*-test. **** $P < 0.0001$.

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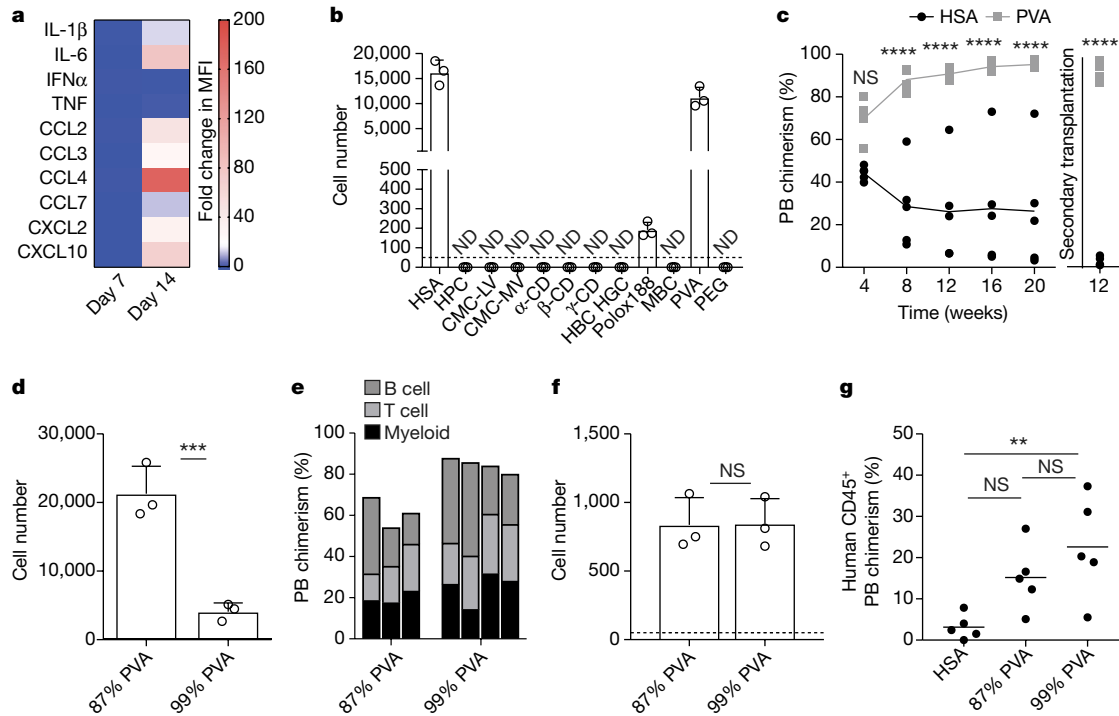


Fig. 2 | PVA can replace serum albumin for ex vivo HSC expansion.

a, Heat map displaying the fold change in cytokine immunoassay mean fluorescence intensity (MFI), using conditioned medium from HSC cultures at day 7 and day 14. Mean of four independent cultures with fold change relative to unconditioned medium. **b**, Cellular expansion of 50 CD34⁺ KSL HSCs after a seven-day-long culture in serum-albumin-free conditions, supplemented with various potential chemically defined serum albumin replacements (see ‘Serum-albumin-free mouse-cell cultures’ in Methods for definitions of abbreviations). Recombinant HSA used as a positive control. Mean \pm s.d. of three independent cultures. ND, not detected. **c**, Mean donor peripheral-blood chimerism in primary recipients ($n = 5$ mice per group) and secondary recipients ($n = 4$ mice per group) from 50 CD34⁺ KSL HSCs following a 7-day-long culture in HSA-based or PVA-based medium, supplemented with 100 ng ml⁻¹ TPO and 10 ng ml⁻¹ SCF in U-bottomed plates. Competitive transplantation against 1×10^6 bone-marrow competitors in primary recipients. Statistical significance

was calculated using ANOVA. **** $P < 0.0001$; NS, not significant. **d**, Seven-day-long expansion of 50 CD150⁺CD34⁺ KSL HSCs in medium containing 87% hydrolysed PVA or >99% hydrolysed PVA. Mean \pm s.d. of three independent cultures. Statistical significance was calculated using *t*-test. *** $P = 0.0021$. **e**, Donor peripheral-blood chimerism at 16 weeks, from 1×10^4 cells from day 28 in 87% PVA ($n = 3$ mice) and 99% PVA ($n = 4$ mice) cultures (see Extended Data Fig. 2k for cell counts at 28 days). Each column represents an individual mouse. **f**, Seven-day-long expansion of 50 human umbilical-cord-blood-derived CD34⁺CD38⁺CD90⁺CD49f⁺ HSCs in HSA- or PVA-based cultures, supplemented with 10 ng ml⁻¹ human SCF and 100 ng ml⁻¹ human TPO. Mean \pm s.d. of three independent cultures. **g**, Mean human CD45⁺ peripheral-blood chimerism at week 16, within sub-lethally irradiated NOG (NOD.Cg-Prkdc^{scid}Il-2r γ^{null}/Szj) mice ($n = 5$ mice per group) following transplantation with 7-day-long cultures derived from 2×10^3 CD34⁺ cells. Statistical significance was calculated using ANOVA. ** $P = 0.0098$.

half-medium change cultures but also sustained long-term HSC activity from about 1 HSCeq (1×10^4 cells) (Fig. 1c, d).

Given the need for complete medium changes during the culture, we hypothesized that HSC plate attachment may help to retain HSCs during medium changes. Of the 5 plate coatings that we tested, fibronectin led to the most improved peripheral-blood chimerism at 16 weeks (Extended Data Fig. 1e). Although HSC proliferation on fibronectin was similar to that on plastic (Extended Data Fig. 1f), 1×10^4 cells (1.25 HSCeq) from fibronectin cultures at day 28 gave almost 100% peripheral-blood chimerism at 16 weeks (Fig. 1e). This is consistent with recent suggestions that fibronectin is a bone-marrow niche factor⁹ and that fibronectin signalling improves HSC maintenance^{10,11}.

Similar to human haematopoietic stem and progenitor cell cultures¹², several cytokines and chemokines (for example, IL-6 and CCL2, CCL3 and CCL4) were abundant in cultures at day 14 (Fig. 2a, Extended Data Fig. 2a) and suggested mechanisms of haematopoietic progenitor cell contamination; as little as 3 ng ml⁻¹ IL-6 enhanced ex vivo CD34⁺KSL haematopoietic progenitor cell proliferation (Extended Data Fig. 2b). The secretion profile also suggested the activation of an innate immune response¹³. Consistent with this idea, cytokine secretion was reduced from *Tlr4*^{-/-} HSCs or by the addition of dexamethasone (Extended Data Fig. 2c, d). Conditioned medium also induced loss of HSC activity, which suggests that the differentiation-inducing factors were soluble (Extended Data Fig. 2e).

Our HSC cultures used yeast-derived recombinant human serum albumin (HSA). We hypothesized that recombinant protein contaminants might be responsible for the inflammatory phenotype^{14,15} and sought replacements for HSA. Serum albumin could have several possible functions in HSC cultures, including as a ‘carrier molecule’ or as a source of amino acids. As HSCs do not grow in low amino acid media containing albumin^{16,17}, we focused on replacing the carrier-molecule function. Of the 11 chemically synthesized potential replacements that we screened (listed in Methods), only polyvinyl alcohol (PVA) supported HSC survival and growth, and the maintenance of phenotypic haematopoietic stem and progenitor cells (Fig. 2b, Extended Data Fig. 2f). In addition, HSCs cultured with PVA outperformed HSCs cultured with HSA in competitive transplantation assays (Fig. 2c). We also observed significantly lower concentrations of secreted factors in the PVA cultures (Extended Data Fig. 2g). Senescence-associated gene expression^{18,19} (*Trp53*, and the *Cdkn2a* isoforms *p16Ink4a* and *p19Arf*) was also reduced in PVA cultures and accumulation of γ -histone 2A.X phosphorylation²⁰ was not detected (Extended Data Fig. 2h, i). Consistent with the role of TLR4 in the HSA phenotype, the addition of the TLR4 agonist lipopolysaccharide to PVA cultures caused a similar induction of the *Cdkn2a* isoforms *p16* and *p19*, as well as the secretion of IL-6 (Extended Data Fig. 2j).

Although PVA has previously been used for culturing embryonic cell types^{21,22}, its mechanistic role is poorly understood. To investigate the properties of PVA that enable it to act as an albumin replacement,

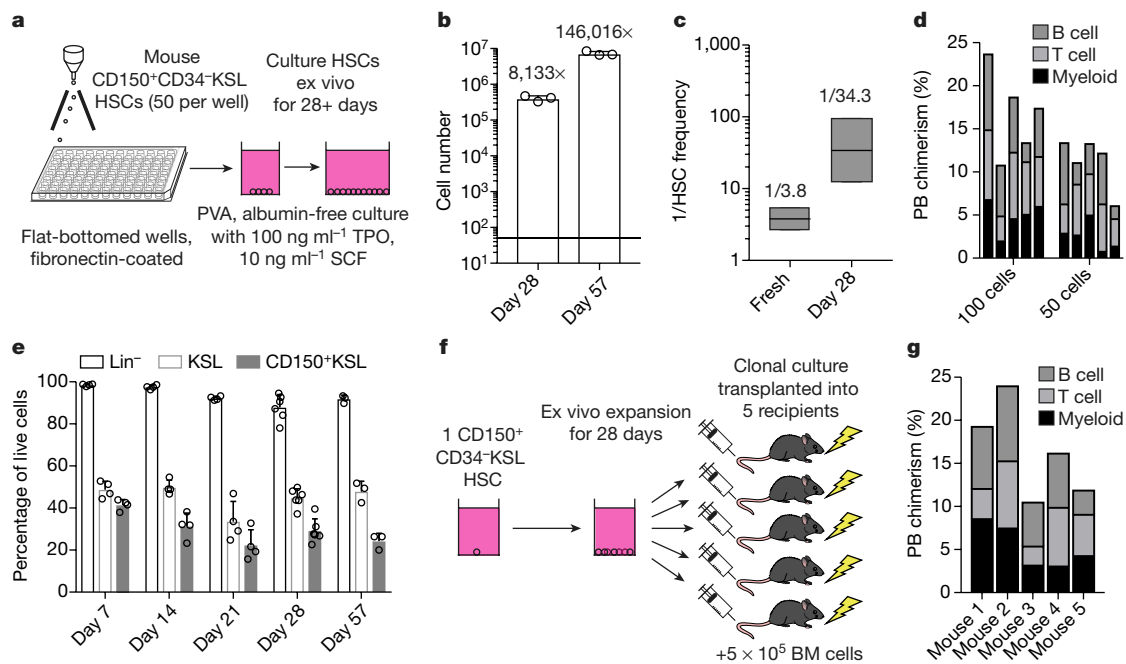


Fig. 3 | Long-term ex vivo expansion of functional HSCs. **a**, Schematic of the optimized mouse HSC expansion culture: 50 CD150⁺CD34⁻KSL HSCs were sorted into flat-bottomed fibronectin-coated plate wells, containing albumin-free F12 medium supplemented with 1 mg ml⁻¹ PVA, 100 ng ml⁻¹ TPO and 10 ng ml⁻¹ SCF. **b**, Mean number of live cells after culturing 50 CD150⁺CD34⁻KSL HSCs for 28 days ($n = 6$ cell cultures) or 57 days ($n = 3$ cell cultures) on fibronectin-coated plates in PVA-based culture medium. Error bars denote s.d. **c**, Box plots representing limiting dilution analysis of fresh CD150⁺CD34⁻KSL (a total of 138 mice; data have previously been published^{24,25}) and 28-day-long HSC cultures (total of 16 mice; Extended Data Fig. 3a) calculated with ELDA software²³, using a positive cut-off of >1% multilineage peripheral-blood chimerism at 16 weeks. Box plots denote calculated mean, and upper and lower limits. **d**, Donor peripheral-blood chimerism at week 12 in secondary recipient mice ($n = 5$ mice per group), from 100 and 50 donor cells from PVA cultures at day 28 (using primary recipients in Extended Data Fig. 3b).

we compared states of PVA hydrolysis. Our initial screen used 87% hydrolysed PVA (87% PVA), an amphiphilic polymer that contains acetate and alcohol domains. By contrast, more-than-99% hydrolysed PVA (99% PVA) lacks acetate domains. HSCs survived in medium that contained either form of PVA, but proliferation was fivefold-less in 99% PVA cultures (Fig. 2d, Extended Data Fig. 2k). However, competitive transplantation of 1×10^4 cells from 87% PVA and 99% PVA cultures at day 28 demonstrated that both types of PVA supported HSCs ex vivo (Fig. 2e).

As an inexpensive albumin replacement that is compatible with good manufacturing practice, PVA may also have important implications for the expansion of human HSCs. As proof-of-concept, we confirmed that PVA can replace serum albumin in human umbilical-cord-blood-derived CD34⁺ haematopoietic stem and progenitor cell cultures (Extended Data Fig. 2l). However, human CD34⁺CD38⁻CD90⁺CD49f⁺ HSCs proliferated to a similar extent in both 87% PVA and 99% PVA (Fig. 2f), which suggests that—unlike in mouse—human HSC proliferation is not sensitive to amphiphilic PVA. Both types of PVA could maintain functional HSC activity ex vivo (Fig. 2g).

From the results above, we defined the optimal mouse HSC culture conditions as: 100 ng ml⁻¹ TPO, 10 ng ml⁻¹ SCF and 87% PVA on fibronectin (Fig. 3a). Under these conditions, 50 CD150⁺CD34⁻KSL HSCs expanded approximately 8,000-fold over 28 days (Fig. 3b). In limiting dilution assays of cultures at 28 days (transplanted against 2×10^5 bone-marrow competitors), only 50 cell aliquots displayed more than 1% multilineage peripheral-blood chimerism at 16 weeks,

Bone marrow from 3 primary recipients was pooled, and 1×10^6 cells were transplanted into secondary recipients. Each column represents an individual mouse. **e**, Mean percentage of phenotypic Lin⁻, KSL and CD150⁺KSL cells during cultures, as described in a, at day 7 ($n = 4$ cell cultures), day 14 ($n = 4$ cell cultures), day 21 ($n = 4$ cell cultures), day 28 ($n = 6$ cell cultures) and day 57 ($n = 3$ cell cultures). Error bars denote s.d. **f**, Schematic of the single HSC expansion assay: single CD150⁺CD34⁻KSL HSCs were expanded for 28 days and then transplanted into 5 lethally irradiated recipient mice against 5×10^5 bone-marrow (BM) competitors. Single HSCs expanded into $\sim 5 \times 10^5$ cells, which means that each recipient received $\sim 1 \times 10^5$ cells (0.2 HSCeq). Ten single HSC-derived cultures were transplanted. **g**, Donor peripheral-blood chimerism at week 16, from one fifth of a 28-day-long culture derived from a single CD150⁺CD34⁻KSL HSC ($n = 5$ mice), as described in f. Each column represents an individual mouse. Representative data for three independent single HSC cultures (out of ten transplanted).

in 2 out of 3 recipients (Extended Data Fig. 3a, b). Using extreme limiting dilution analysis²³, we calculated the HSC frequency as 1:34.3 cells, which is equivalent to 1.2×10^4 functional HSCs in the culture at day 28 (Fig. 3c). This compares to a 1:3.8 frequency of functional HSCs within the freshly isolated CD150⁺CD34⁻KSL population (on the basis of previous transplantation data²⁴). We therefore estimate that the expansion of functional HSCs is between 236-fold (assuming all 50 starting cells were functional HSCs) and 899-fold (assuming 1:3.8 starting cells (about 13 cells) were functional HSCs).

Secondary transplantation was performed by pooling bone marrow from the primary limiting dilution assay recipients (3 of these recipients survived to 16 weeks): all secondary recipients of 1×10^6 bone-marrow cells from 100-cell and 50-cell primary transplants displayed donor peripheral-blood chimerism at 12 weeks (Fig. 3d). Because these were pooled secondary transplants, we can only conclude that at least 1 in 150 cells at day 28 were serially transplantable long-term HSCs. We therefore estimate that the expansion of serially engraftable HSCs, at a minimum, is between 54-fold (assuming all starting cells were functional HSCs) and 204-fold (assuming only 1:3.8 of starting cells (about 13 cells) were functional HSCs).

Consistent with the high functional activity at day 28, senescence markers were not increased and *Trp53* remained unmutated (Extended Data Fig. 3c, d). The KSL population also remained negative for senescence-associated β -galactosidase (Extended Data Fig. 3e, f). In addition, engrafting HSCs were karyotypically normal (Extended Data Fig. 3g). Ex vivo phenotypic KSL populations remained fairly stable during culture (Fig. 3e, Extended Data Fig. 3h), and most cells in the

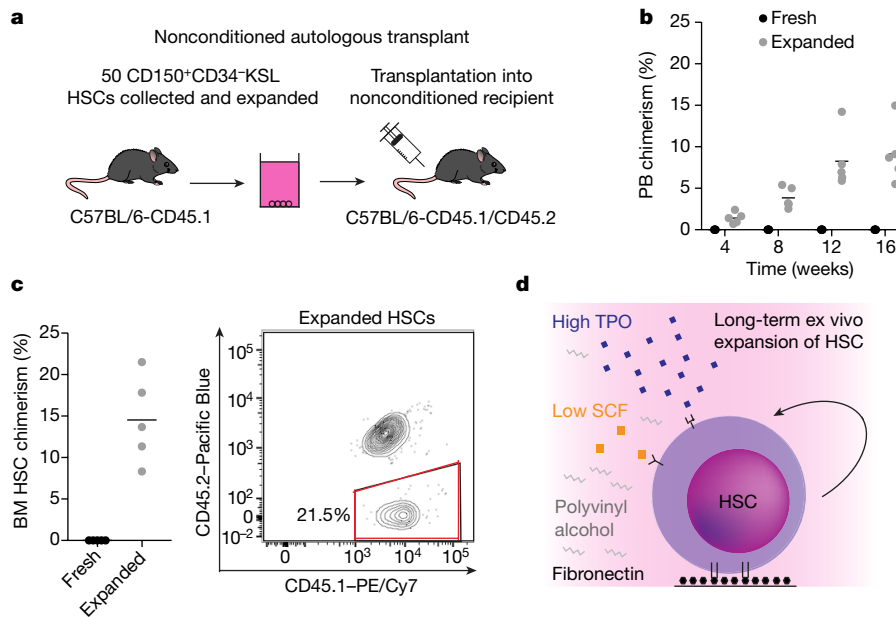


Fig. 4 | Ex vivo expanded HSCs engraft in nonconditioned recipients.

a, Schematic of nonconditioned autologous transplantation. Fifty CD150⁺CD34⁻KSL cells from C57BL/6-CD45.1 mice (C57BL/6 mice expressing *Ptprc^d*) were expanded for 28 days, before being transplanted into nonconditioned C57BL/6-CD45.1/CD45.2 (C57BL/6 mice expressing *Ptprc^d* and *Ptprc^b*) recipients. **b**, Mean donor peripheral-blood chimerism at weeks 4–16, from 50 fresh HSCs ($n = 5$ mice) or

cultures remained Lin⁻ (based on a LY-6G/LY-6C, TER119, CD45RA, CD4, CD8 and CD127 antibody lineage cocktail; Fig. 3e). The Lin⁺ cells were LY-6G/LY-6C⁺, although additional analysis identified FCER1⁺ cells at similar frequencies (Extended Data Fig. 3i). HSC cultures could also be continued longer term: by day 57 of culture, the 50 starting HSCs had generated approximately 7.3×10^6 cells, while retaining a stable phenotypic KSL population and functional HSC activity (Fig. 3b, e, Extended Data Fig. 3j).

Although mouse bone-marrow HSCs can be highly enriched on the basis of surface-marker expression, purified CD150⁺CD34⁻KSL cells exhibit substantial functional heterogeneity in single-cell transplantation assays^{24,25}. Consistent with this, considerable variability in the expansion of single HSCs was observed; some cells generated less than 100 cells, whereas others expanded to approximately 5×10^5 cells (although more than 90% generated colonies) (Extended Data Fig. 4a). The most proliferative clones generated cell numbers similar to those of cultures from 50 or 500 HSCs (Extended Data Fig. 4a), which suggests that surface area limited growth. Phenotypic heterogeneity was also observed in clonally derived cultures; some clones retained about 90% KSL (Extended Data Fig. 4b), whereas others almost exclusively generated c-KIT⁺SCA1⁻Lin⁻ cells. Transplantation of clonal cultures resulted in high-level multipotent activity in 4 out of 14 recipients (Extended Data Fig. 4c). We also detected robust (approximately 15%) peripheral-blood chimerism when clonally derived cultures were divided and transplanted into 5 recipients, against 5×10^5 bone-marrow competitors (for 3 out of 10 single HSC cultures tested) (Fig. 3f, g). These experiments confirmed bona fide HSC self-renewal ex vivo, but suggested that the self-renewal capacity is unevenly distributed within the phenotypic HSC compartment.

Radiation-based bone-marrow conditioning is normally required to make space for donor HSCs in HSC transplantation. Donor engraftment in nonconditioned recipients is possible but normally not feasible, because very large numbers of HSCs are required for the transplant^{26,27}. By expanding 50 HSCs for 28 days before transplantation, we could achieve long-term donor peripheral-blood and bone-marrow HSC chimerism in nonconditioned immunocompetent mice (Fig. 4a–c). This approach could also be used to engraft immunodeficient

a 28-day-long culture derived from 50 HSCs (50 HSCeq; $n = 5$ mice), transplanted as described in **a**. **c**, Mean donor bone-marrow CD34⁻KSL HSC chimerism at week 16 ($n = 5$ mice) for the assay described in **a** (left), and an example flow cytometry plot displaying CD45.1 and CD45.2 expression within the bone-marrow CD34⁻KSL compartment of recipient mice (right). **d**, Summary of the optimized conditions for expansion of functional mouse HSCs.

NOD/SCID mice, a model of congenital immunodeficiency; multilineage peripheral-blood chimerism was observed in all recipients with donor lymphoid B and T cells detected in the long term (Extended Data Fig. 5a–c).

In summary, we have developed an albumin-free ex vivo culture condition that expands functional mouse HSCs (Fig. 4d), which has the potential for broad applications in HSC research. Although we have been able to isolate HSCs at high purity for over 20 years¹, stable ex vivo expansion of functional HSCs has not been achievable. Our results suggest that poor optimization of existing culture constituents, combined with medium supplement impurities, have been a major barrier to ex vivo HSC expansion.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at <https://doi.org/10.1038/s41586-019-1244-x>.

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Author contributions A.C.W. conceptualized the research, performed experiments, analysed data and wrote the manuscript. R.I., M.K., K.S., M.M., R.V.C. and R.Y. performed experiments and analysed data. K.M.L. conceptualized the research and edited the manuscript. M.W. and Y.N. supervised experiments and edited the manuscript. H.N. conceptualized the research, supervised experiments, analysed data and wrote the manuscript. S.Y. conceptualized the research, performed and supervised experiments, analysed data and wrote the manuscript.

Competing interests H.N. is a co-founder and shareholder of ReproCELL. Inc.

Additional information

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METHODS

Data reporting. No statistical methods were used to predetermine sample size. The experiments were not randomized and the investigators were not blinded to outcome assessment.

Mice. C57BL/6-CD45.2 and C57BL/6-CD45.1 (Pepboy) mice were purchased from Japan SLC, Sankyo-Laboratory Service, Jackson Laboratories (000664, 002014) or bred in-house. For congenic transplantation experiments, 8–12-week-old male mice were used as donors and 8–12-week-old female mice were used as recipients. *Thr2* knockout mice, *Thr4* knockout mice, and NOD/SCID (NOD.Cg-Prkdc^{scid}) mice were purchased from Jackson Laboratories (004650, 007227 and 001303, respectively). NOG (NOD.Cg-Prkdc^{scid} IL-2r^γnull/SzJ) mice were purchased from In Vivo Science. All mice were housed in specific-pathogen-free conditions, with free access to food and water. All animal protocols were approved by the Animal Care and Use Committee of the Institute of Medical Science University of Tokyo, the Animal Care and Use Committee of RIKEN Tsukuba Branch and/or the Administrative Panel on Laboratory Animal Care at Stanford University.

Cell collection by fluorescence-activated cell sorting. Mouse bone-marrow cells were isolated from the tibia, femur and pelvis, stained with APC-c-KIT antibody and c-KIT⁺ cells enriched using anti-APC magnetic beads and LS columns (Miltenyi Biotec). The c-KIT-enriched cells were then stained with a lineage antibody cocktail (biotinylated CD4, CD8, CD45RA, TER119, LY-6G/LY-6C and CD127), before being stained with anti-CD34, anti-c-KIT, anti-SCA1 and streptavidin-APC/eFluor 780 (as detailed in Extended Data Table 1) for 90 min. Where indicated, cells were also stained with anti-CD150. Cell populations were then purified using a FACS AriaII (BD) by direct sorting into wells containing media using propidium iodide as a dead stain (Extended Data Fig. 1b).

Serum-albumin-based mouse-cell cultures. Serum-albumin-based cultures were performed using F12 medium (Life Technologies), 1% insulin-transferrin-selenium-ethanolamine (ITSX; Life Technologies), 1% penicillin/streptomycin/glutamine (P/S/G; Life Technologies), 10 mM HEPES (Life Technologies), 0.1% recombinant HSA (Albumin Biosciences), at 37°C with 5% CO₂. Cultures were supplemented with recombinant mouse SCF and recombinant mouse TPO (Peprotech), as indicated. All long-term cultures used 10 ng ml⁻¹ SCF and 100 ng ml⁻¹ TPO, with medium changes made every 3 days after the first 5 days, by manually removing conditioned medium by pipetting and replacing fresh medium as indicated. In a 96-well plate, wells containing 200 μl of medium, this involved gentle removal of 190–200 μl of the conditioned medium using a pipette to avoid disturbing cells that were lightly adherent to the well bottom, and then gently pipetting in 200 μl of pre-warmed and freshly prepared medium down the side of the well to minimize disturbing the cell layer. Any cells removed from the well in the conditioned medium were discarded. Long-term cultures were performed using flat-bottomed plates, tissue-culture-treated and/or coated with fibronectin (Corning; 354409), collagen 1 (Corning; 354407), collagen 4 (Corning; 354429), gelatin (Sigma; G2500) or laminin 511 (iMatrix; 892018). Where indicated, various concentrations of recombinant mouse IL-6 (Peprotech) were added to cultures.

Serum-albumin-free mouse-cell cultures. HSCs were cultured in medium composed of F12 medium, 1% ITSX, 10 mM HEPES, 1% P/S/G, 100 ng ml⁻¹ mouse TPO, 10 ng ml⁻¹ mouse SCF and 0.1% of one of the following chemicals (all from Sigma): hydroxypropyl cellulose (HPC; 435007), low-viscosity carboxymethylcellulose sodium salt (CMC-LV; C5678), medium-viscosity carboxymethylcellulose sodium salt (CMC-MV; 21902), α-cyclodextrin (α-CD; C4642), β-cyclodextrin (β-CD; C4767), γ-cyclodextrin (γ-CD; C4930), 2-hydroxypropyl-β-L-cyclodextrin (HBC; H107), 2-hydroxypropyl-γ-cyclodextrin (HGC; H125), methyl-β-cyclodextrin (MBC; C4555), poloxamer 188 (Polox188; P5556), PVA (P8136, 363081 or 363146) or polyethylene glycol (PEG), at 37°C with 5% CO₂. For long-term cultures in PVA-based cultures, complete medium changes were made every 2–3 days after the first 5–6 days, as described above for albumin-based cultures. Long-term cultures were split 1:3 at ~90% confluency. Where indicated, lipopolysaccharide (Sigma L2762) or IL-6 (Peprotech) were added to cultures.

Analysis of cultured cells. Following ex vivo culture, cells were counted (using a haemocytometer, a CYTORECON cytometer or a Nucleocounter NC-3000). For flow cytometric analysis, cells were stained with a lineage cocktail (biotinylated CD4, CD8, CD45RA, TER119, LY-6G/LY-6C and CD127) and then with antibodies detailed in Extended Data Table 1 for 30–90 min. Following a wash step, flow cytometric analysis was performed using a FACS AriaII (BD), LSRFortessa (BD) or FACS Canto (BD) using propidium iodide as a dead stain.

Competitive transplantation assay. Cultured HSCs from C57BL/6-CD45.1 mice were transplanted alongside 1 × 10⁶ whole bone-marrow competitor cells from C57BL/6-CD45.1/CD45.2 (F1) mice into C57BL/6-CD45.2 mice, following irradiation at a lethal dose (9.5 Gy). Donor chimerism was tracked by collecting peripheral-blood cells and staining with anti-CD45.1, anti-CD45.2, anti-CD11b, anti-LY-6G/LY-6C, anti-CD45RA, anti-CD4 and anti-CD8 antibodies (detailed in Extended Data Table 1) for 30 min. Following a wash step, cells were

analysed by flow cytometry (as above) using propidium iodide as a dead stain. Secondary bone-marrow transplantation assays were performed by transferring 1 × 10⁶ bone-marrow cells from the primary recipient mice into lethally irradiated C57BL/6-CD45.2 mice, with donor chimerism analysed as above.

Limiting dilution assays. For limiting dilution assays, set numbers of cultured C57BL/6-CD45.1 cells were aliquoted by fluorescence-activated cell sorting (FACS) after culture, and transplanted into lethally irradiated C57BL/6-CD45.2 recipient mice, together with 2 × 10⁵ F1 bone-marrow competitor cells. Donor chimerism was analysed as above. Limiting dilution analysis was performed using ELDA software²³, based on a 1% peripheral-blood multilineage chimerism (at least 0.2% myeloid donor chimerism) as the threshold for positive engraftment. To calculate fresh HSC frequency, the same criteria were applied to a total of 138 transplantation assays from freshly isolated CD150⁺CD34⁻ KSL cells that were previously published^{24,25}. Where indicated, secondary transplantation assays were performed as described above.

Nonconditioned transplantation assays. CD150⁺CD34⁻ KSL cells were purified from C57BL/6-CD45.1 or C57BL/6-CD45.2 mice and expanded, as described above. Freshly isolated or expanded bulk-cell cultures were then transplanted into non-irradiated C57BL/6-CD45.1/CD45.2 recipient mice or NOD/SCID (CD45.1) recipient mice, split into three doses over consecutive days.

Cytokine immunoassays. Conditioned medium was collected at day 7 (before any medium change) or day 14 (following a medium change at day 10), or as indicated in the figure legends. Mouse 39-plex kits (eBiosciences) were used according to the manufacturer's recommendations, with modifications described below. In brief, beads were added to a 96-well plate and washed in a Biotek ELx405 washer. Samples were added to the plate containing the mixed antibody-linked beads and incubated at room temperature for 1 hour, followed by overnight incubation at 4°C with shaking. Cold and room-temperature incubation steps were performed on an orbital shaker at 500–600 r.p.m. Following the overnight incubation, plates were washed in a Biotek ELx405 washer and then biotinylated detection antibody was added for one hour at room temperature with shaking. The plate was washed as above, and streptavidin-PE was added. After incubation for 30 min at room temperature, the wash was performed and reading buffer was added to the wells. Plates were read using a Luminex Flex3D instrument with a lower bound of 50 beads per sample per cytokine. Custom control beads (Assay CHEX) by Radix Biosolutions were added to all wells.

Human cell cultures and xenograft assays. Human umbilical-cord-blood-derived CD34⁺ cells (purchased from Lonza or Stem Cell Technologies) or FACS-purified CD34⁺CD38⁻CD90⁺CD49f⁺ cells (following staining with APC-CD34 (Biolegend, 560940), PE-CD38 (BD, 347687), FITC-CD90 (Biolegend, 328113) and APC/Cy7-CD49f (Biolegend, 313611)) were cultured in IMDM (Life Technologies) containing 0.1% HSA or 0.1% PVA (Sigma P8136, 363081, or 363146), supplemented with 1% ITSX, 1% P/S/G and 10 mM HEPES. For proliferation assays, 50 cells were seeded per well and supplemented with 10 ng ml⁻¹ human SCF and 100 ng ml⁻¹ human TPO (Peprotech). During the cultures, medium was refreshed every three days and counted at day seven. For xenograft assays, 2 × 10³ cells were expanded for 7 days before being injected intravenously into sub-lethally irradiated (1.5 Gy) NOG mice. Human cell chimerism in the peripheral blood was calculated at 16 weeks post-transplantation, using PE/Cy7-CD45.1 (Biolegend; 110730) and V450-human CD45 antibodies (BD; 560367).

Senescence analysis. Total RNA of KSL cells was extracted using the RNeasy Mini Kit (Qiagen) and reverse-transcribed using SuperScript III First-Strand Synthesis System and Oligo (dT) primers (Invitrogen). Quantitative PCR was performed on a Thermal Cycler Dice Real Time System (Takara) using SsoAdvancedTM Universal SYBR Green Supermix and the following primer sets: *p19Ink4a* (GAATCTTTCGGTTCGTACCC and CGAATCTGCACCGTATTGA), *p19Arf* (GGGTTTTCTTGGTGAAGTTCG and TTGCCATCATCATCACCT) and *Trp53* (CAGTCTACTTCCCGCCATAA and GTCTCAGCCCTGAGTCATAAG). The reaction conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 20 s. Gene expression of genes was normalized relative to *Gapdh* expression (using primer set: CGACTCAACAGCAACTCCCCTCTTCC and TGGGTGGTCCAGGGTTTCTTACTCCTT). Senescence β-galactosidase staining (Cell Signaling, Kit 9860S) was performed according to the manufacturer's instructions, following cell attachment to poly-L-lysine-coated plates. For Sanger sequencing, *Trp53* cDNA was PCR-amplified (using primer set: CATCTGGCTGTAGGTAGCG ACCCTATGAGGGCCCAAGAT) and sequenced using nested sequencing primers: AAAAGTCTGCCTGTCTCCAG; TGATGGCCTGGCTCCTCC; CACGTACTCTCCTCCCCTCA; and CTCTGTACGGCGGTCTCTC. Sanger sequencing was outsourced to FASMAC, and visualized and aligned using SnapGene software.

Phospho-protein fluorescence immunostaining. Fluorescence immunostaining was performed by attaching, fixing and staining cells on poly-L-lysine-coated glass slides (Matsunami Glass), with imaging and quantification performed with

a Cellomics ArrayScan VTI HCS Reader (Thermo Scientific), using previously described methods^{20,28}.

Karyotyping analysis. Karyotyping was performed on CD45.1⁺ bone-marrow cells, FACS-purified from primary recipients of 28-day-long PVA-cultured HSCs at 16 weeks post-transplantation, and performed by Nihon Gene Research Laboratories.

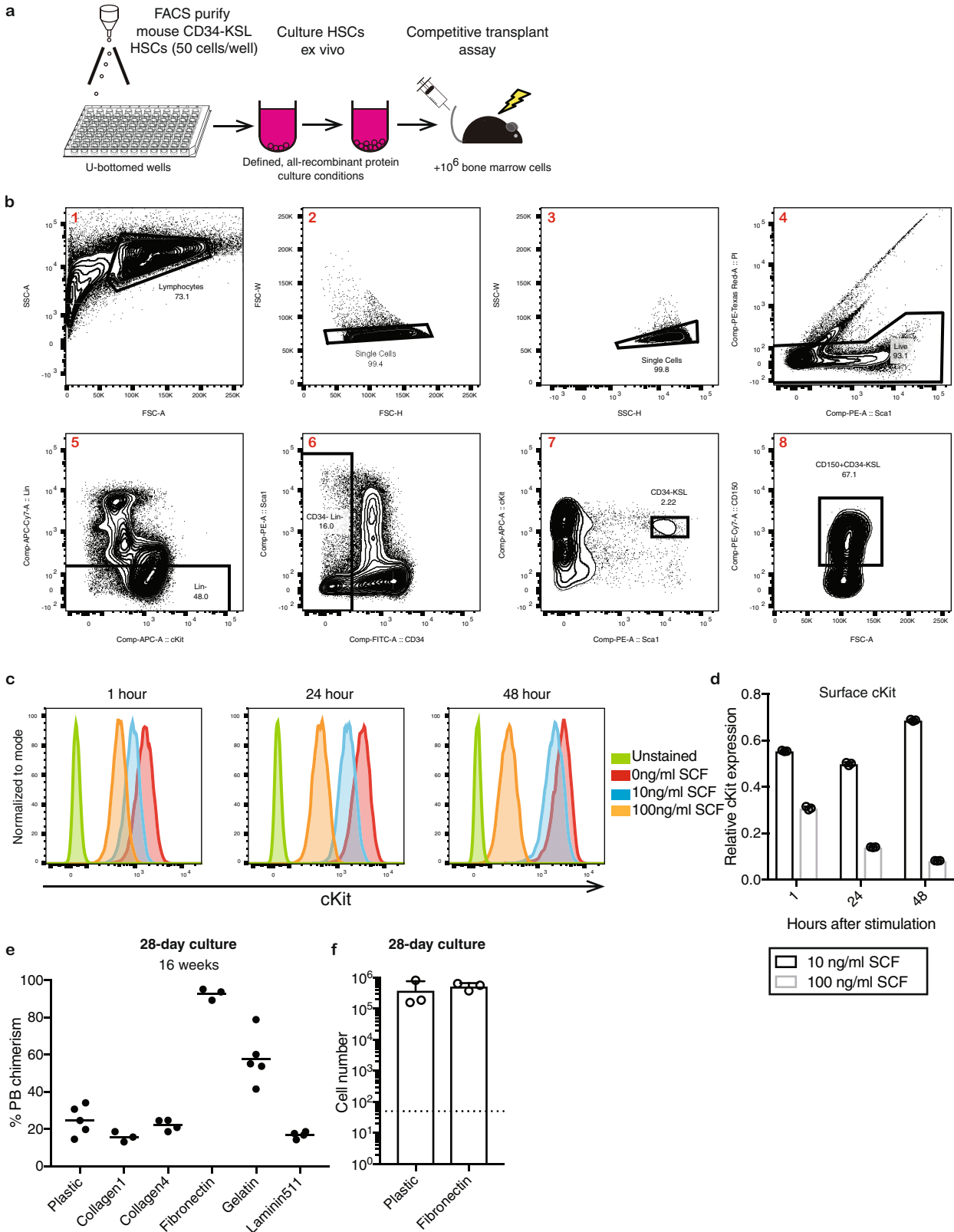
Statistical analysis. One-way and two-way ANOVA tests and unpaired two-tailed *t*-tests were performed as indicated in the figures, using Prism 7 software.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability

All graphed datasets can be found in the Supplementary Data. Additional data files will be made available by the corresponding authors upon reasonable request. A detailed protocol is available at the Protocol Exchange²⁹.

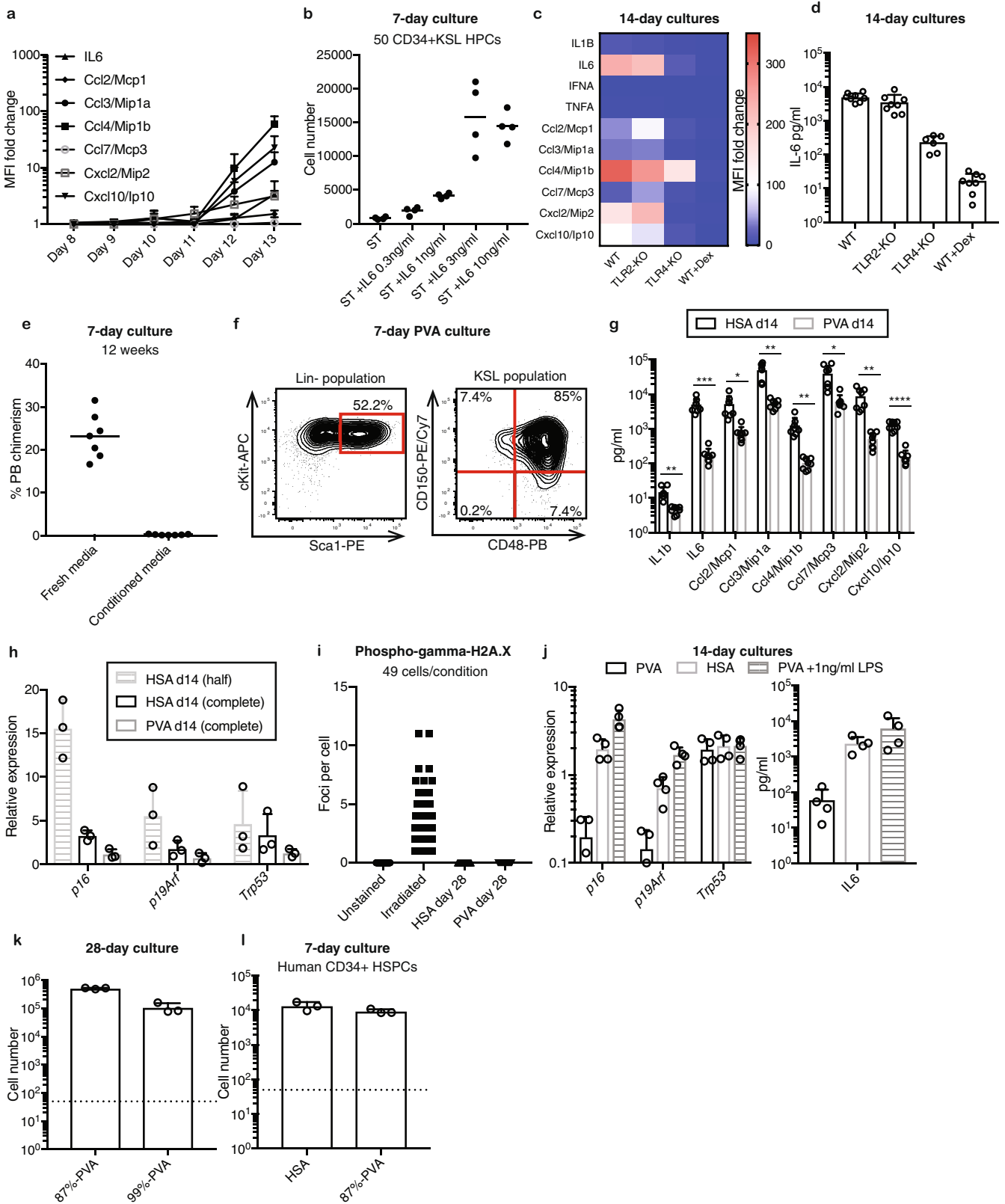
28. Seita, J. et al. Lnk negatively regulates self-renewal of hematopoietic stem cells by modifying thrombopoietin-mediated signal transduction. *Proc. Natl Acad. Sci. USA* **104**, 2349–2354 (2007).
29. Wilkinson, A. C. et al. Ex vivo mouse hematopoietic stem cell expansion using polyvinyl alcohol. *Protoc. Exch.* <https://doi.org/10.21203/rs.2.9270/v1> (2019).



Extended Data Fig. 1 | See next page for caption.

Extended Data Fig. 1 | Optimizing conditions for long-term HSC culture. **a**, Schematic of the standard HSC culture assay. Bone-marrow CD34⁻ KSL HSCs of C57BL/6-CD45.1 mice were sorted (50 cells per well) into U-bottomed 96-well plate wells (**b** for sorting scheme). HSC growth can be observed during culture by counting or flow cytometry, with medium changes made every three days (after an initial seven days in culture). After 7–28 days, functional HSC activity was determined using competitive transplantation into irradiated C57BL/6-CD45.2 mice, against 1×10^6 bone-marrow competitor cells from C57BL/6-CD45.1/CD45.2 (F1) mice. Donor chimerism within peripheral-blood myeloid, T cell and B cell lineages was determined after 4–16 weeks, or longer. Where indicated, secondary transplantation assays were performed by transplanting 1×10^6 bone-marrow cells from primary recipients into irradiated C57BL/6-CD45.2 mice. **b**, FACS gating strategy for sorting CD34⁻ KSL cells (gates 1–7) and CD150⁺CD34⁻ KSL cells (gates 1–8) from c-KIT-enriched mouse bone marrow. Representative of at least five experiments. **c**, Flow cytometric histograms for cell-surface c-KIT staining

of HSCs following stimulating with 100 ng ml⁻¹ TPO and 0, 10 or 100 ng ml⁻¹ SCF for 1, 24 and 48 h. Representative of three independent cultures. **d**, Mean fluorescence intensity of c-KIT antibody staining on HSCs cultured in 100 ng ml⁻¹ TPO supplemented with 10 ng ml⁻¹ or 100 ng ml⁻¹ SCF, analysed after 1–72 h in culture, relative to cultures containing 100 ng ml⁻¹ TPO without SCF. Mean of three independent cultures. Error bars denote s.d. **e**, Mean donor peripheral-blood chimerism at week 16, from 1×10^4 HSC-derived cells following a 28-day-long culture on plastic ($n = 5$ cell cultures), collagen 1 ($n = 3$ cell cultures), collagen 4 ($n = 4$ cell cultures), fibronectin ($n = 3$ cell cultures), gelatin ($n = 5$ cell cultures) or laminin 511 ($n = 4$ cell cultures) culture plates (cultured in 100 ng ml⁻¹ TPO and 10 ng ml⁻¹ SCF with complete medium changes). Competitive transplantation against 1×10^6 bone-marrow competitors. **f**, Number of live cells after culturing 50 CD34⁻ KSL HSCs for 28 days on plastic (tissue-culture-treated) plates or fibronectin-coated plates. Mean of three independent cultures. Error bars denote s.d.

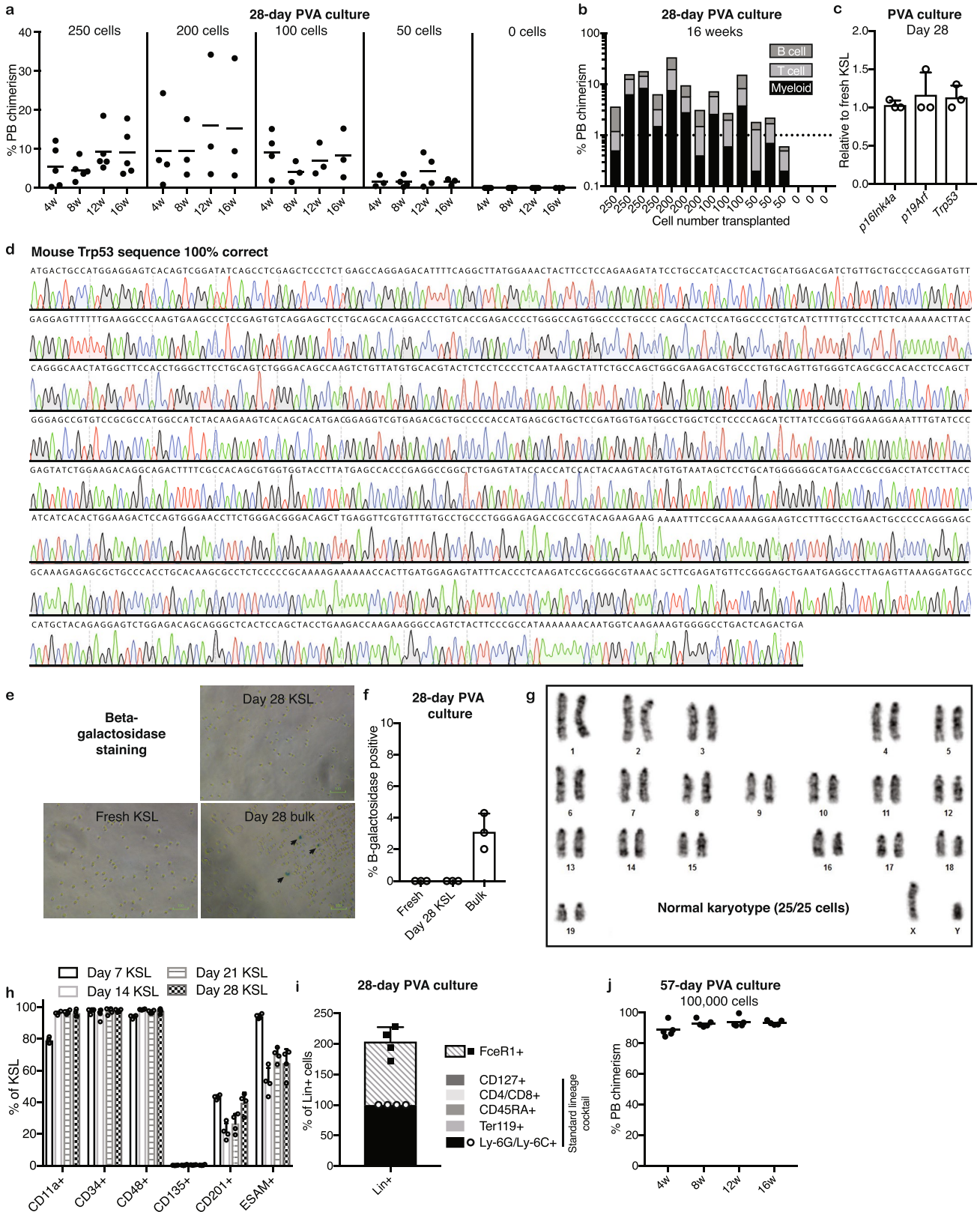


Extended Data Fig. 2 | See next page for caption.

Extended Data Fig. 2 | Identification of PVA-based HSC culture conditions.

a, Fold change in MFI from cytokine immunoassays performed on HSA-based HSC cultures between day 8 and day 13. Medium changes performed at day 7 and day 10. Mean of four independent cultures with fold change relative to unconditioned medium. Error bars denote s.d. **b**, Mean expansion of 50 CD34⁺KSL haematopoietic progenitor cells at day 7, in 100 ng ml⁻¹ TPO and 10 ng ml⁻¹ SCF with or without addition of 0.3 ng ml⁻¹ to 10 ng ml⁻¹ mouse IL-6 ($n = 4$ cell cultures). **c**, Heat map displaying the MFI fold change from cytokine immunoassays using conditioned medium from HSC cultures at day 14. CD34⁻ KSL HSCs were isolated from C57BL/6 wild-type (WT), *Tlr2* knockout (TLR2-KO) or *Tlr4* knockout (TLR4-KO) mice, and cultured in HSA-based cultures. Dexamethasone (+Dex) at 50 nM was added, where indicated. Mean of four independent cultures with fold change relative to unconditioned medium. **d**, Concentration of IL-6 observed in HSA-based cultures at day 14 of wild-type HSCs ($n = 8$ cell cultures), *Tlr2* knockout HSCs ($n = 8$ cell cultures), *Tlr4* knockout HSCs ($n = 6$ cell cultures), or wild-type HSCs + dexamethasone ($n = 8$ cell cultures). Error bars denote s.d. **e**, Mean donor peripheral-blood chimerism at week 12, from HSCs cultured for 7 days, in fresh medium ($n = 7$ mice) or in medium composed of 50% medium collected from a 12-day-long HSC culture and 50% fresh medium (termed 'conditioned media', $n = 7$ mice). Competitive transplantation against 1×10^6 bone-marrow competitors. **f**, Example flow cytometry plots displaying c-KIT and SCA1 expression on the Lin⁻ progeny (left), and CD150 and CD48 expression in the KSL population (right) after a PVA-based HSC culture for seven days. Representative of four independent cultures. **g**, Concentration of various

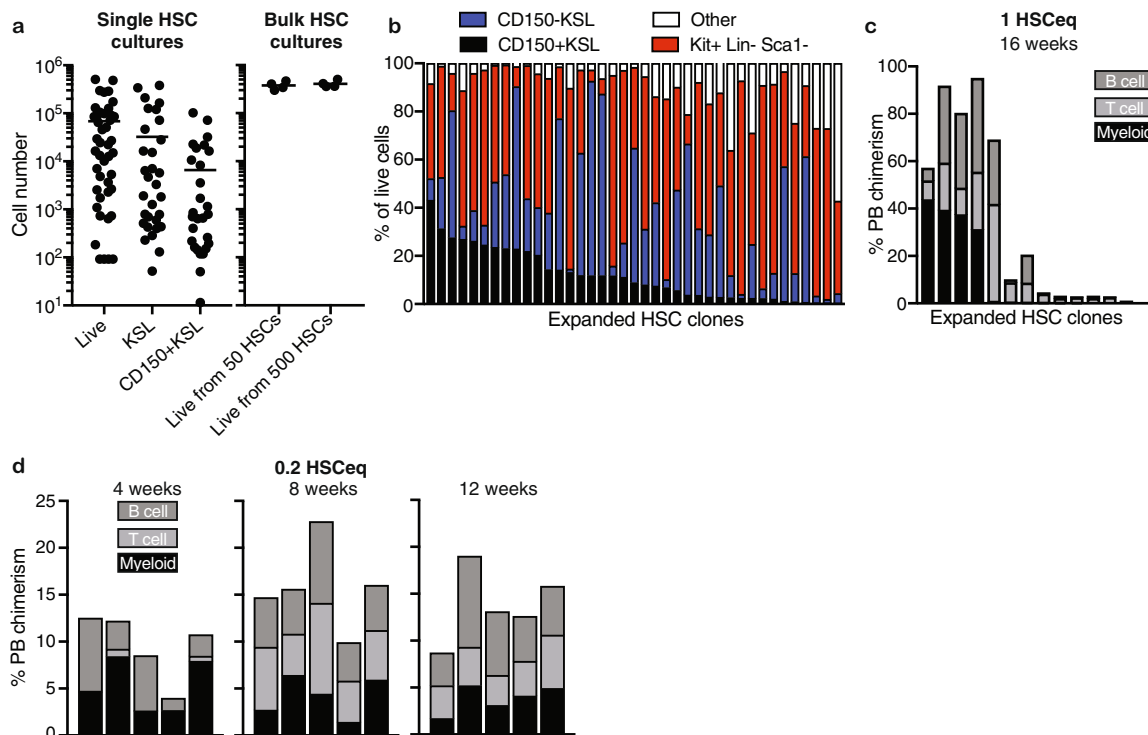
cytokines in conditioned medium at day 14, from HSA- or PVA-based CD34⁻KSL HSC cultures. Mean of eight independent cultures. Error bars denote s.d. Statistical significance was calculated using *t*-tests. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. **h**, Relative expression of *p16Ink4a*, *p19Arf* and *Trp53* in KSL cells collected from cultures at day 14 (HSA-based cultures with half-medium changes, HSA-based cultures with complete medium changes and PVA-based cultures with complete medium changes), relative to expression in freshly isolated KSL cells. Mean of three independent cultures, with gene expression normalized to *Gapdh* expression. Error bars denote s.d. **i**, Number of phospho- γ -histone 2A.X (H2A.X) nuclear foci in KSL cells at day 28, from HSA-based or PVA-based HSC cultures. Irradiated cells were included as a positive control. Forty-nine cells quantified per condition. **j**, Relative expression of *p16Ink4a*, *p19Arf* and *Trp53* in KSL cells collected from cultures at day 14 (left): HSA-based cultures, PVA-based cultures and PVA-based cultures supplemented with 1 ng ml⁻¹ lipopolysaccharide. Mean of technical quadruplets, with gene expression normalized to *Gapdh* expression. The concentration of IL-6 observed in these culture conditions is shown on the right. Mean of four independent cultures. Error bars denote s.d. **k**, Twenty-eight-day-long expansion of 50 CD150⁺CD34⁻ KSL HSCs in medium containing 87% hydrolysed PVA or >99% hydrolysed PVA. Ten thousand cells at day 28 represent ~1 HSCeq for 87% PVA and ~5 HSCeq for 99% PVA. Mean of three independent cultures. Error bars denote s.d. **l**, Seven-day-long expansion of 50 human cord-blood CD34⁺ cells in HSA- or PVA-based cultures supplemented with 10 ng ml⁻¹ human SCF and 100 ng ml⁻¹ human TPO. Mean of three independent cultures. Error bars denote s.d.



Extended Data Fig. 3 | See next page for caption.

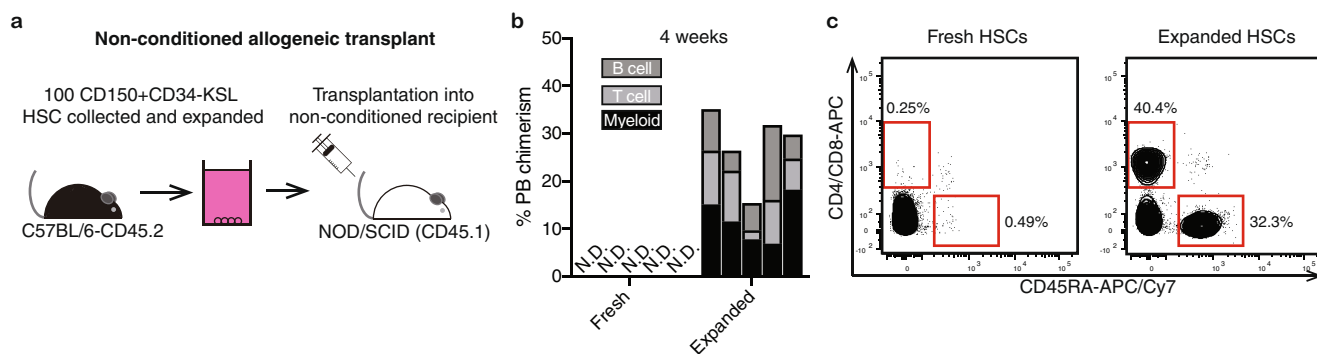
Extended Data Fig. 3 | Characterization of long-term PVA-based HSC cultures. **a**, Mean donor peripheral-blood chimerism at weeks 4–16, from 28-day-long PVA-based (CD150⁺CD34⁻KSL) HSC cultures using 100 ng ml⁻¹ TPO and 10 ng ml⁻¹ SCF in fibronectin-coated wells with complete medium changes. Indicated cell numbers transplanted against 2×10^5 bone-marrow cells. Data from two independent transplantation experiments. **b**, Multilineage donor peripheral-blood chimerism at week 16, for each individual mouse in **a**. **c**, Expression of *p16Ink4a*, *p19Arf* and *Trp53* in 28-day-long PVA-cultured KSL cells, relative to expression in freshly isolated KSL cells. Mean of three independent cultures, with gene expression normalized to *Gapdh* expression. Error bars denote s.d. **d**, Sanger sequencing trace of *Trp53* cDNA amplified from KSL cells collected from 28-day-long PVA-based HSC cultures ($n = 1$ cell culture). **e**, Representative images of β -galactosidase activity staining of freshly isolated KSL, KSL isolated from 28-day-long PVA-based cultures and bulk 28-day-long PVA-based cultures. Representative of two biological replicates. **f**, Percentage of β -galactosidase-positive cells in conditions described in **e**. Mean of technical triplicates (50–100 cells counted per

replicate). Error bars denote s.d. **g**, Karyotype of CD45.1⁺ bone-marrow repopulating progeny of expanded functional HSCs at day 28, in PVA-based media at 16 weeks after transplantation. All chromosomes that were analysed were normal in 25 out of 25 cells (performed by Nihon Gene Research Laboratories). **h**, Frequency of CD11a⁺, CD34⁺, CD48⁺, CD135⁺, CD201⁺ and ESAM⁺ cells within the phenotypic KSL population during ex vivo HSC culture (derived from 50 CD150⁺CD34⁻KSL cells). Mean of four independent cultures. Error bars denote s.d. **i**, Composition of the Lin⁺ compartment of HSC cultures at day 28. The lineage antibody cocktail used in this study comprised CD4, CD8, CD45RA, TER119, LY-6G/LY-6C and CD127. A non-overlapping FCER1⁺ cell population was also identified within the culture, and is quantitated relative to the Lin⁺ population. Mean of four independent cultures. Error bars denote s.d. **j**, Mean donor peripheral-blood chimerism at weeks 4–16, from 1×10^5 cells from a 57-day PVA-based HSC culture using fibronectin-coated plates and supplemented with 100 ng ml⁻¹ TPO and 10 ng ml⁻¹ SCF ($n = 5$ mice). Competitive transplantation against 1×10^6 bone-marrow competitors.



Extended Data Fig. 4 | Characterization of clonally derived HSC expansion cultures. **a**, Mean number of live cells, KSL cells and CD150⁺KSL cells derived from single CD150⁺CD34⁻KSL HSCs after 28 days of culture ($n = 48$ single cell cultures) (left), and mean number of live cells from bulk (50 and 500) CD150⁺CD34⁻KSL HSC cultures after 28 days of culture ($n = 4$ cell cultures) (right). **b**, Proportion of phenotypic cell types that constitute cultures at day 28 derived from single CD150⁺CD34⁻KSL HSCs. Only cultures with >10,000 cells were analysed

(39 wells of 84 wells analysed). **c**, Donor peripheral-blood chimerism at week 16, from 28-day expanded single CD150⁺CD34⁻KSL HSC cultures, transplanted into lethally irradiated recipients against 2×10^5 bone-marrow cells. Each column represents an individual mouse. **d**, Donor peripheral-blood chimerism at weeks 4–12, from one fifth of a 28-day-long culture derived from a single CD150⁺CD34⁻KSL HSC, as described in Fig. 3f, g. Each column represents an individual mouse. Representative data for three independent single HSC cultures (out of ten transplanted).



Extended Data Fig. 5 | Nonconditioned transplantation into immunodeficient recipients. **a**, Schematic of nonconditioned allogeneic transplantation. One hundred CD150⁺CD34⁻KSL cells from C57BL/6-CD45.2 mice were expanded for 28 days before being transplanted into nonconditioned immunodeficient NOD/SCID recipient mice. **b**, Donor peripheral-blood chimerism at week 4, from 100 fresh

HSCs ($n = 5$ mice) or a 28-day-long HSC culture derived from 100 HSCs ($n = 5$ mice), transplanted as described in **a**. Each column represents an individual mouse. **c**, Example flow cytometry plots displaying T cell (CD4 and CD8) and B cell (CD45RA, also known as B220) peripheral-blood lineages within nonconditioned NOD/SCID mice at 16 weeks after transplantation (representative of 5 mice), as described in **a**.

Extended Data Table 1 | List of antibodies

Antibody	Source	Identifier
Biotin anti-CD4	eBioscience	Cat# 13-0041-85
Biotin anti-CD8	eBioscience	Cat# 13-0081-86
Biotin anti-CD45RA/B220	eBioscience	Cat# 36-0452-85
Biotin anti-TER-119	eBioscience	Cat# 13-5921-85
Biotin anti-Ly-6G/Ly-6C (RB6-8C5)	eBioscience	Cat# 13-5931-85
Biotin anti-CD127 (A7R34)	eBioscience	Cat# 13-1271-85
APC anti-c-Kit (2B8)	eBioscience	Cat# 17-1171-83
PE-Cy7 anti-c-Kit (2B8)	Biolegend	Cat# 105814
FITC anti-CD34 (RAM34)	eBioscience	Cat# 11-0341-85
PE-Cy7 anti-CD150 (TC15-12F12.2)	BioLegend	Cat# 115914
PE anti-Ly-6A/E (Sca-1) (D7)	BioLegend	Cat# 122508
PE anti-Ly-6A/E (Sca-1) (D7)	eBioscience	Cat# 12-5981-83
FITC anti-Ly-6A/E (Sca-1) (D7)	Biolegend	Cat# 108105
Streptavidin-APC/eFluor 780	eBioscience	Cat# 47-4317-82
PE-Cy7 anti-CD45.1	BioLegend	Cat# 110730
BrilliantViolet421 anti-CD45.2 (104)	BioLegend	Cat# 109832
eFluor450 anti-CD45.2 (104)	eBioscience	Cat# 48-0454-82
FITC anti-Ly-6G/Ly-6C (RB6-8C5)	eBioscience	Cat# 11-5931-85
FITC anti-CD11b (M1/70)	eBioscience	Cat# 11-0112-41
PE anti-Ly-6G/Ly-6C (RB6-8C5)	eBioscience	Cat# 12-5931-82
PE anti-CD11b (M1/70)	eBioscience	Cat# 12-0112-82
APC-eFluor780 anti CD45R (RA3-6B2)	eBioscience	Cat# 47-0452-82
APC anti-CD4 (RM4-5)	eBioscience	Cat# 17-0042-83
APC anti-CD8 (53-6.7)	eBioscience	Cat# 17-0081-83
Pacific Blue anti-Ter119 (TER119)	eBioscience	Cat# 48-5921-82
PE-Cy7 anti-Ly-6G/Ly-6C (RB6-8C5)	eBioscience	Cat# 25-5931-82
FITC anti-CD127 (A7R34)	eBioscience	Cat# 11-1271-85
PE anti-FcεR1 (MAR-1)	eBioscience	Cat# 12-5898-81
BrilliantViolet421 anti-CD135 (A2F10)	Biolegend	Cat# 135353
PE anti-CD11a (M17/4)	eBioscience	Cat# 12-0111-081
APC anti-CD201 (eBio1560)	eBiosciences	Cat# 17-2012-82
Pacific Blue anti-CD48 (BCM1)	Biolegend	Cat# 103418
APC anti-ESAM (1G8/ESAM)	Biolegend	Cat# 136207

Table of anti-mouse antibodies used in this study, including supplier and identifier.

Reporting Summary

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- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated
- Clearly defined error bars
State explicitly what error bars represent (e.g. SD, SE, CI)

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Software and code

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Data collection

BD FACS Diva 8

Data analysis

FlowJo 10, Prism 7, SnapGene 4, Extreme Limiting Dilution Assay (ELDA) software (<http://bioinf.wehi.edu.au/software/elda/>)

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All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All graphed datasets can be found in the Supplementary Source Data Files. All datasets available upon request.

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

Life sciences Behavioural & social sciences Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/authors/policies/ReportingSummary-flat.pdf](https://www.nature.com/authors/policies/ReportingSummary-flat.pdf)

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	No statistical methods were used to predetermine the experimental sample size.
Data exclusions	No data were excluded.
Replication	For all figures, multiple independent experiments were performed and all attempts at replicating observation as described in the manuscript were successful.
Randomization	Recipient mice were randomly selected for transplantation groups.
Blinding	Blinding was not performed.

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a	Involved in the study
<input checked="" type="checkbox"/>	<input type="checkbox"/> Unique biological materials
<input type="checkbox"/>	<input checked="" type="checkbox"/> Antibodies
<input checked="" type="checkbox"/>	<input type="checkbox"/> Eukaryotic cell lines
<input checked="" type="checkbox"/>	<input type="checkbox"/> Palaeontology
<input type="checkbox"/>	<input checked="" type="checkbox"/> Animals and other organisms
<input checked="" type="checkbox"/>	<input type="checkbox"/> Human research participants

Methods

n/a	Involved in the study
<input checked="" type="checkbox"/>	<input type="checkbox"/> ChIP-seq
<input type="checkbox"/>	<input checked="" type="checkbox"/> Flow cytometry
<input checked="" type="checkbox"/>	<input type="checkbox"/> MRI-based neuroimaging

Antibodies

Antibodies used

Biotin anti-mouse CD4 eBioscience Cat#13-0042-85 (1:2800)
 Biotin anti-mouse CD8 eBioscience Cat# 13-0081-86 (1:2800)
 Biotin anti-mouse CD45RA/B220 eBioscience Cat# 13-0452-85 (1:1400)
 Biotin anti-mouse TER-119 eBioscience Cat# 13-5921-85 (1:700)
 Biotin anti-mouse Ly-6G/Ly-6C (RB6-8C5) eBioscience Cat# 13-5931-85 (1:700)
 Biotin anti-mouse CD127 (A7R34) eBioscience Cat# 13-1271-85 (1:1400)
 APC anti-mouse c-Kit (2B8) eBioscience Cat# 17-1171-83 (1:100)
 PE-Cy7 anti-mouse c-Kit (2B6) Biolegend Cat# 105814 (1:100)
 FITC anti-mouse CD34 (RAM34) eBioscience Cat# 11-0341-85 (1:100)
 PE-Cy7 anti-mouse CD150 (TC15-12F12.2) BioLegend Cat# 115914 (1:350)
 PE anti-mouse-Ly-6A/E (Sca-1) (D7) BioLegend Cat# 122508 (1:700)
 PE anti-mouse-Ly-6A/E (Sca-1) (D7) eBioscience Cat# 12-5981-83 (1:700)
 FITC anti-mouse Ly-6A/E (Sca-1) (D7) Biolegend Cat# 108105 (1:100)
 Streptavidin-APC/eFluor780 eBioscience Cat# 47-4317-82 (1:700)
 PE-Cy7 anti-mouse CD45.1 BioLegend Cat# 110730 (1:500)
 BrilliantViolet421 anti-mouse CD45.2 (104) BioLegend Cat# 109832 (1:400)
 eFluor450 anti-mouse CD45.2 (104) eBioscience Cat# 48-0454-82 (1:500)
 FITC anti-mouse Ly-6G/Ly-6C (RB6-8C5) eBioscience Cat# 11-5931-85 (1:2000)
 FITC anti-mouse CD11b (M1/70) eBioscience Cat# 11-0112-41 (1:2000)
 PE anti-mouse Ly-6G/Ly-6C (RB6-8C5) eBioscience Cat# 12-5931-82 (1:2000)
 PE anti-mouse CD11b (M1/70) eBioscience Cat# 12-0112-82 (1:2000)
 APC-eFluor780 anti-mouse CD45R (RA3-6B2) eBioscience Cat# 17-0452-83 (1:1000)
 APC anti-mouse CD4 (RM4-5) BioLegend Cat# 100516 (1:2000)
 APC anti-mouse CD8 (53-6.7) eBioscience Cat# 17-0081-83 (1:2000)

Pacific Blue anti-mouse Ter119 (TER119) eBioscience Cat# 48-5921-82 (1:100)
 PE-Cy7 anti-mouse Ly-6G/Ly-6C (RB6-8C5) eBioscience Cat# 25-5931-82 (1:1000)
 FITC anti-mouse CD127 (A7R34) eBioscience Cat# 11-1271-85 (1:100)
 PE anti-mouse FcεR1 (MAR-1) eBioscience Cat# 12-5898-81 (1:800)
 BrilliantViolet421 anti-mouse CD135 (A2F10) Biolegend Cat# 135313 (1:200)
 PE anti-mouse CD11a (M17/4) eBioscience Cat# 12-0111-081 (1:800)
 APC anti-mouse CD201 (eBio1560) eBiosciences Cat# 17-2012-82 (1:400)
 Pacific Blue anti-mouse CD48 (BCM1) Biolegend Cat# 103418 (1:200)
 APC anti-mouse ESAM (1G8/ESAM) Biolegend Cat# 136207 (1:400)
 APC anti-human CD34 (8G12) BD Cat# 560940 (1:10)
 PE anti-human CD38 (HB7) BD Cat# 347687 (1:10)
 FITC anti-human CD90 (5E10) Biolegend Cat# 328113 (1:10)
 APC-Cy7 anti-human CD49f (GoH3) Biolegend Cat# 313611 (1:10)
 V450 anti-human CD45 (HI30) BD Cat# 560367 (1:10)
 AlexaFluor488 anti-phospho-histone H2A.X (20E3) Cell Signaling Technology Cat# 9719S (1:200)

Validation

All antibodies were validated by manufacturers for the applications and species used in this study. See manufacturers websites for validation statements (www.biolegend.com; www.thermofisher.com/us/en/home/life-science/antibodies/ebioscience; wwwbdbiosciences.com; www.cellsignal.com).

Animals and other organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research

Laboratory animals

All mice were 8-12 weeks when experiments were started. Male and female mice were used in this study.
 C57BL/6 mice - Jackson Laboratories (000664), Japan SLC, or Sankyo-Lab Service
 PepBoyJ mice - Jackson Laboratories (002014)
 TLR2-knockout mice - Jackson Laboratories (004650)
 TLR4-knockout mice - Jackson Laboratories (007227)
 NOD/Scid (NOD.Cg-Prkdcscid/SzJ) mice - Jackson Laboratories (001303)
 NOG (NOD.Cg-Prkdcscid Il2rgTm1Sug/ShiJic) mice - In Vivo Sciences Inc

Wild animals

This study did not involve wild animals.

Field-collected samples

This study did not involve field-collected samples

Flow Cytometry

Plots

Confirm that:

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Bone marrow, peripheral blood, and in vitro cell cultures were prepared into a single cell suspension in PBS. Bone marrow cells were cKit-enriched using an MACS LS column (Miltenyi). Red blood cell lysis using aqueous 140 mM ammonium chloride was performed on peripheral blood cells. Cells were filters (40µm) before FACS.

Instrument

BD FACS Ariall, BD LSRFortessa, BD FACS Canto.

Software

FACS Diva for data collection, FlowJo for data analysis.

Cell population abundance

FACS machine cell sorting efficiency was confirmed by flow cytometric analysis of post-sorted cells.

Gating strategy

FSC-A/SSC-A for mononuclear cells, FSC-H/FSC-W followed by SSC-H/SSC-W for singlets, PI/Sca1 for PI- live cells, Lineage-cocktail/cKit for Lin- cells, Sca1/CD34 for CD34-/lo Lin-, cKit/Sca1 for CD34-/lo cKit+ Sca1+ Lin-, CD150/blank channel for CD150+ CD34-/lo Kit+ Sca1+ Lin-. See Extended Data Figure 1b for details. For peripheral blood analysis, live cells were gated based on positive and negative markers.

- Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.