Supplementary Material (ESI) for Integrative Biology

Supplementary Information:

Elucidation of Single Hematopoietic Stem Cell Fate in Artificial Niches

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1. Detailed Experimental Procedures

Isolation and purification of hematopoietic stem/progenitor cells by flow cytometry: Bone marrow donors were 8- to 12-week-old GFP+C57BL/6-Ly5.1. After isolating the bone of the hind legs, the bone marrow of the femurs and tibias was extensively flushed with several ml of FACS buffer consisting of PBS (pH 7.4) containing 12.5% fetal bovine serum (FBS) (Omega Scientific, USA Cat# FB-01) and 2mM EDTA. The cell suspension was filtered through a 70µm nylon cell strainer (BD Falcon, USA), filled to 40 ml with the above FACS buffer, supplemented with 10 ml FBS and centrifuged for 10 min at 1400 rpm. The remaining pellet was resuspended in 5 ml red blood cell (RBC) lysis buffer, incubated on ice for 5 min, filled to 40 ml with FACS buffer, supplemented with 10 ml FBS and centrifuged for 10 min at 1400 rpm and again resuspended in 1 ml FACS buffer. $Lin^{-}ckit^{+}Sca1^{+}$ (LKS) cells (Spangrude et al., 1988), and a subpopulation of LKS expressing the SLAM receptor CD150 (LKS-CD150+) cells (Kiel et al., 2005) were isolated using an adaptation of published methods. Briefly, a mouse lineage panel (BD Biosciences, USA, used according to manufacturer's instructions) was employed to stain differentiated cells and 3 µl Fc-block (antiCD16/CD32 BD, Bioscience) and 20 µl of each lineage panel antibody (anti-CD3e biotin, anti-CD45R/B220 biotin, anti-CD11b biotin, anti-Ly-6G biotin and anti4 TER-119 biotin) were added to the cell suspension and incubated on ice for 20 min. Then, 10 ml FACS buffer were added to the cell suspension and centrifuged at 1400 rpm for 10 min. The remaining cell pellet was resuspended in 900 µl FACS buffer and stained by adding 100 µl streptavidin magnetic microbeads (Miltenyi Biotech, Germany), 5 µl anti-c-Kit-PE/Cy7 (eBioScience, USA), 10 µl anti-Sca1-PE (BD Bioscience, USA), 10 µl anti-CD150-APC (Bio Legend, USA) and 5 µl TexasRed-Streptavidin (Molecular

Probes, USA). After an incubation time of 30 min at 4°C with gentle shaking using a rotating plate, 10 ml FACS buffer were added and centrifuged at 1400 rpm for 10 min. The suspension was resuspended in 1 ml FACS buffer and separated using a MidiMACS magnetic column (Miltenyi Biotech, Germany). The eluted cell suspension was centrifuged at 1400 rpm for 10 min and resuspended in 3 ml FACS buffer, to which 3 µl propidium iodide (PI) was added. The lineage depleted cell population was further separated by Flow cytometry on a Vantage SE FACS instrument (BD Bioscience, USA) at the Stanford FACS core facility. Single viable (propidium iodide negative) Lin^- $ckit^+Sca1^+CD150^+$ cells were triple sorted using the gates shown in the FACS plots in Figure 1 and directly deposited in microwell arrays. FACS data were plotted using FlowJo (TreeStar Inc., USA).

Long-Term reconstitution assays: In order to assess self-renewal potential of HSCs, long-term blood reconstitution assays were conducted in mice lethally irradiated to deplete their endogenous HSCs. We transplanted 10, 20, 40, 100 or 500 GFP^+ cells (C57BL/6, Ly5.1) of the LKS or LKS-CD150+ population per animal together with 500,000 $GFP^-Sca1^-CD150^-$ bone marrow 'helper' cells incapable of long-term reconstitution (C57BL/6, Ly5.1) into lethally irradiated wild-type host mice (C57BL/6, Ly5.2) as previously described (Corbel et al., 2003). All transplant recipients were Ly5-congenic and a split dose irradiation, i.e. two sequential doses of 4.8Gy were used. After sorting the donor population, the number of cells to be injected per mouse was resorted into individual wells of a 96-well plate containing 500,000 helpers. Helpers were efficiently HSC depleted from RBC lysed bone marrow stained with anti-Sca1-biotin (1/200 BD Bioscience, USA) and anti-CD150-biotin (1/200, Biolegend, USA) antibodies, followed by a streptavidin-magnetic bead sort (Miltenyi Biotech, Germany). The contents of individual wells were injected into the tail veins of individual lethally irradiated recipients. Reconstitution was measured by assessment of $GFP^+Ly5.1^+$ cells in the CD45 gated peripheral blood derived from retro-orbital bleeding 4, 8, 12, and 24 weeks after transplantation. Blood was subjected to RBC lysis with ammonium chloride, and WBCs stained with directly conjugated antibodies to monitor engraftment: CD45.1-PE (A20), CD45-PE(30F11), B220-biotin(6B2), CD3-biotin (KT31.1), Mac1-biotin (M1/70), Gr-1-biotin (8C5) and Streptavidin-APC (BD Bioscience, USA). Six to seven months post-transplant, BM was harvested from reconstituted primary recipients. The cell

content equivalent to one femur was injected into 1-2 secondary lethally irradiated C57Bl/6 Ly5.2 recipients.

Hematopoietic stem cell culture: LKS-CD150+ were cultured under sterile conditions in a serum-free basal media containing Stemline II hematopoietic expansion medium (Sigma, USA) supplemented with 100 ng/ml SCF and 2 ng/ml Flt-3 ligand in 10% CO2 at 37°C in a humidified incubator. In a typical experiment, 300 individual LKS-CD150+ cells were seeded per well of a 96-well plate containing a total of 400 microwells in 200µµl of medium (or else 1000 to 2000 cells per well of a 24-well plate containing 4000 microwells in 1 ml of medium to facilitate micromanipulation). After 1 hour during which individual cells randomly sedimented to the bottoms of microwells, the plate was transferred to the incubator of the microscope and further cultured under the same sterile conditions for at least four days.

Selection of putative soluble HSC regulatory proteins. The soluble proteins listed in Table 1 below were tested for their effects on HSC fate. These factors were added to the above basal medium at the specified concentrations, which were selected based on previous reports. 10% FBS served as an enriched medium. When cultured for 7 days, new factors were added at Day 4.

Table 1: Tested soluble HSC regulatory proteins

Candidate Protein	References	Suggested role in the niche	Suggested niche	Source	Employed concentration
Wnt3a	(Willert et al., 2003) (Reya et al., 2003)	HSC self-renewal	Endosteal	(mouse) R&D cat# 1324-WN-002	100 ng/ml
IL-11	(Neben et al., 1994)	Cytokines stimulating HSC expansion	NA	(mouse) R&D cat# 418-ML	20 ng/ml
FGF-1	(de Haan et al., 2003)	Maintenance of function HSC *in vitro*	NA	(human) R&D cat# 232-FA	10 ng/ml
TPO	(Kimura et al., 1998; Tong et al., 2007)	Maintenance of HSC activity and self-renewal	NA	(mouse) R&D cat# 488-TO	100 ng/ml
IGF-2	(Zhang and Lodish, 2004)	Stimulate HSC expansion	NA	(mouse) R&D cat# 792-MG	20 ng/ml
Ang-1	(Arai et al., 2004; Cheng et al., 2000)	HSC quiescence and cell cycle regulation	NA	(human) R&D cat# 923-AN	1 ug/ml
Shh	(Bhardwaj et al., 2001; Trowbridge et al., 2006)	Proliferation of HSC	NA	(mouse) R&D cat# 461-SH	100 ng/ml

Fabrication of hydrogel microwell arrays for high-throughput analysis of single HSC behavior:

Poly(ethylene glycol) (PEG). 8arm-PEG-OH (mol. wt. 40000g/mol) and linear PEG-(SH)2 (mol. wt. 3400g/mol, 100% substitution as indicated by manufaturer) were purchased from Nektar (Huntsville, AL, USA). Divinyl sulfone was purchased from Aldrich (Buchs, Switzerland). 8arm-PEG-vinylsulfones (8arm-PEG-VS) were produced and characterized as described elsewhere (Lutolf and Hubbell, 2003). The final product was dried under vacuum and stored under argon at -20 C. The degree of end group conversion, confirmed with 1H NMR (CDCl3): 3.6 ppm (PEG backbone), 6.1 ppm (d, 1H, =CH2), 6.4 ppm (d, 1H, =CH2), and 6.8 ppm (dd, 1H, -SO2CH=), was found to be 87%.

Gelation of PEG precursors (Fig. 1A). A previously developed mild and versatile chemistry was adapted to form hydrogels from the above PEG precursors in stoichiometrically balanced amounts (Lutolf and Hubbell, 2003). Both precursors were dissolved at a solid concentration of 10% (w/v) in 0.3 M triethanolamine (8-arm-PEG-VS) and in ultra pure water (PEG-(SH)2), respectively, and mixed to form crosslinked gel networks by Michael-type addition. In order to avoid batch-to-batch variability, each precursor solution was prepared in large quantities (2.5 ml), filter sterilized (0.22 µm) and aliquoted in amounts for the synthesis of 250 µµl PEG hydrogel.

Hydrogel microwell array formation (Fig. 1B). Hydrogel microwell arrays were fabricated by a multistep soft lithography process. PDMS microwell array masters the size of an entire Si wafer were a generous gift from Dr. Marc Dusseiller (ETH Zurich, Switzerland). Prior to PEG gel casting, the PDMS master was cut to a size matching a desired well format (96-, or 24-well), thoroughly cleaned, and then modified with a surface layer of 1H,1H,2H,2H-Perfluorodecyltrichlorosilane (Oakwood Chemicals, USA). Immediately after mixing of the above precursors in an Eppendorf tube, the PEG precursor solutions (80 µl for the 24-well size) was pipetted on the PDMS surface positioned on a hydrophobic glass slide (precoated with *SigmaCote*, Sigma, USA). Appropriate spacers of the thickness of the PDMS master plus 0.7 mm were placed at both ends of the glass slide and a second hydrophobic slide was placed on top. The two slides were clamped with binder clips on both ends, ensuring an optimal wetting of the PDMS microstructures with the precursor solution. Curing of the gel network was conducted for 30 min at 37°C in a humidified incubator. The PEG hydrogel microwell arrays were then peeled off using a pair of blunt forceps, washed at least 4 times 15 min with 4 ml PBS, and left to swell

overnight in PBS. Prior to cell culture, the swollen PEG hydrogel microwell arrays were fixed on the bottom of plastic wells of a desired well plate using the above solution of gel precursors as efficient 'glue', and the arrays were equilibrated at 37°C in cell culture medium.

Time-lapse microscopy and image analysis: LKS-CD150+ cells were directly sorted into wells containing the above microwell surfaces. The plate was then placed in the environmental chamber of an inverted microscope (Zeiss Axiovert 200) equipped with a motorized stage. After cells were randomly distributed and trapped in microwells, the XYZ stage was programmed to repeatedly raster across the microwell array surface, acquiring phase contrast images at 5X (in some cases 10X) magnification of multiple locations in defined time intervals for a period of up to 7 days. The entire surface of the microwell arrays were scanned and the resulting images of the time-lapse experiment were then automatically compiled into a stack (library) using the Volocity software (Improvision). Cells were scored dead when they ceased to move on the microwell surface. PI staining was utilized to confirm the death read-out. Scoring of time-lapse movies was facilitated by a Matlab program designed to take advantage of high-throughput automated image analysis while maintaining the high accuracy of manual counting. Starting with a master image containing all 400 microwells within an array, edge detection is used to locate all microwells and then segment each microwell into its own image, yielding 400 separate images per array. For each microwell containing a single cell, a series of images corresponding to that microwell was generated auomatically at each time point (every 24 hours). This program allowed us to rapidly select the microwells for analysis and automatically visualize successive frames of time-lapse movies of the same microwell on the computer screen, enabling rapid and precise visual evaluation. The raw data containing the cell count and the region location were then compiled on an Excel spreadsheet for further statistical analysis of the growth kinetics of individual live cells.

Analysis of HSC maintenance and self-renewal *in vitro* by transplantation of cultured cells into mice: In a first experimental paradigm, the progeny of 100 stem cells, after at least 4 days of culture in basal media containing SCF and Flt-3, were pooled and then transplanted into lethally irradiated mice as described above. In other

sets of experiments, micromanipulation was employed to pick progeny of single stem cells that underwent a defined number of divisions in culture. Single cells, doublets or larger colonies (> 3 divisions) were collected with a glass micropipette using an inverted microscope equipped with a micromanipulator and micro-injector. The micromanipulated cells were transferred to Eppendorf tubes and then transplanted as described above.

In situ patterning of biomolecules on microstructured gels by 'reactive microcontact printing': In order to control both topography and localized presentation of transmembrane HSC regulatory proteins on gel microwell arrays, protein immobilization was restricted to selected areas on the gel surface via a novel hydrogel microfabrication process termed 'reactive microcontact printing' described briefly below and in more detail elsewhere (Lutolf et al., in preparation). The above PDMS masters were first 'inked' using PEGmodified ProteinA (PIERCE, USA; a protein that can strongly bind engineered Fc-chimeric proteins) to adsorb the ProteinA just on the tip of the positive PDMS template features (i.e. pillars). For this purpose, ProteinA was pre-reacted for 30 min at room temperature with a 10-fold molar excess of a heterofuntional NHS-PEG-VS PEG linker (Nektar, Huntsville, AL, USA). This allowed the free VS-groups to be covalently attached to the gel surface in the next step. Hydrogel microwell casting was then conducted as described above on this ProteinA-adsorbed template, transferring and locally covalently immobilizing ProteinA from the PDMS surface to the microstructured gel matrix. Subsequently, ProteinA-modified PEG hydrogel microwells were incubated with 400 µl and 50µl (in the case of a 24-well and 96-well plate, respectively) of a solution of a desired Fc-chimeric protein (at 10 µg/ml) in PBS. After an incubation time of 1 hour at 37°C in a humidified incubator, the microwell samples were washed 4 times 15 min with PBS to remove non-immobilized Fc-chimeric proteins. Nonspecific protein adsorption on the sample was minimized by incubating with 4 ml of a solution of 1% BSA (w/v) in PBS (0.22 µm filter sterilized) for 1 hour at room temperature prior to the immobilization of the Fc-chimeric proteins.

Selection of putative tethered HSC regulatory proteins. The Fc-chimeric proteins listed in Table 2 were tested for their effect on HSC fate.

Table 2: Tested transmembrane (Fc-chimeric) HSC niche regulatory proteins

Candidate Protein	References	Suggested role in the niche	Suggested niche	Source

Jagged-1	(Duncan et al., 2005; Varnum-Finney et al., 2000)	Notch ligand self-renewal and clonal expansion	Endosteal	(rat) Fc-chimeric R&D cat# 599-JG
N-Cadherin	(Arai et al., 2004; Zhang et al., 2003)	Homotypic interaction anchorage/quiescence role (?)	Endosteal	(human) Fc-chimeric R&D cat# 1388-NC
VE-Cadherin	(Avecilla et al., 2004)	Interaction of megakaryocytes with sinusoidal bone marrow endothelial cells (BMEC); promotion of megakaryocyte maturation	Vascular	(human) Fc-chimeric R&D cat# 938-VC
ICAM-1	(Jung et al., 2005)	Physical contact with osteoblast for HSC survival	Endosteal	(mouse) Fc-chimeric R&D cat# 796-IC
VCAM-1	(Avecilla et al., 2004)	Heterotypic interaction with VLA4 HSC adhesion to osteoblasts	Vascular Endosteal	(mouse) Fc-chimeric R&D cat# 643-VM
P-Selectin	(Eto et al., 2005)	Homing and engraftment into the niche; expansion of hematopoietic progenitors	Vascular	(mouse) Fc-chimeric R&D cat# 737-PS

Qualitative assessment of efficiency of microwell protein tethering using confocal laser scanning microscopy: Confocal laser scanning microscopy was utilized to test the extent, uniformity, and stability of the microcontact printing process. FITC-BSA (Invitrogen, USA) was functionalized with VS groups as described above and was tethered to the bottom of the microwell surface using the microcontact printing method described above. As a positive control, FITC-BSA was also mixed directly with the precursor solution to achieve bulk fuctionalization of the microwell array. Images were acquired using a LSM 510 META confocal laser-scanning microscope (Zeiss, Germany). Typically, z-stacks were acquired with a constant slice thickness of 1.5 - 2 µm, reconstructing a cross section profile of 150 µm. Crosssectional analysis, 3D-reconstructions and image processing were done using Volocity (Improvision, USA) and Photoshop CS (Adobe, USA).

Tethering of selected Fc-chimeric proteins listed in Table 2 was also assessed via immunostaining. For example, N-Cadherin-functionalized PEG hydrogel microwells were fabricated by tethering ProteinA to the bottom of the microwells and then incubating the array with Fc-N-Cadherin as described above. After blocking in 4 ml PBS containing 1% BSA for 1 hour (RT), the samples were washed 4 times for 15 min in 4 ml PBS. The hydrogels were then incubated for 1 hour (RT) with 1 ml of a solution of mouse monoclonal anti-N-Cadherin IgG (BD Biosciences, USA) at 1:1000 in PBS containing 3% goat serum, followed by subsequent washing for 4 times for 15 min in 4 ml PBS. The secondary antibody incubation was conducted for 1 hour (RT) using 1 ml of an Alexa

Fluor 488 labeled goat anti-mouse IgG (Invitrogen, USA) dissolved 1:500 in PBS plus 3% goat serum. Afterwards the samples were washed 4 times for 15 min in 4 ml PBS and imaged via confocal microscopy as described above.

2. Supplemental Figures

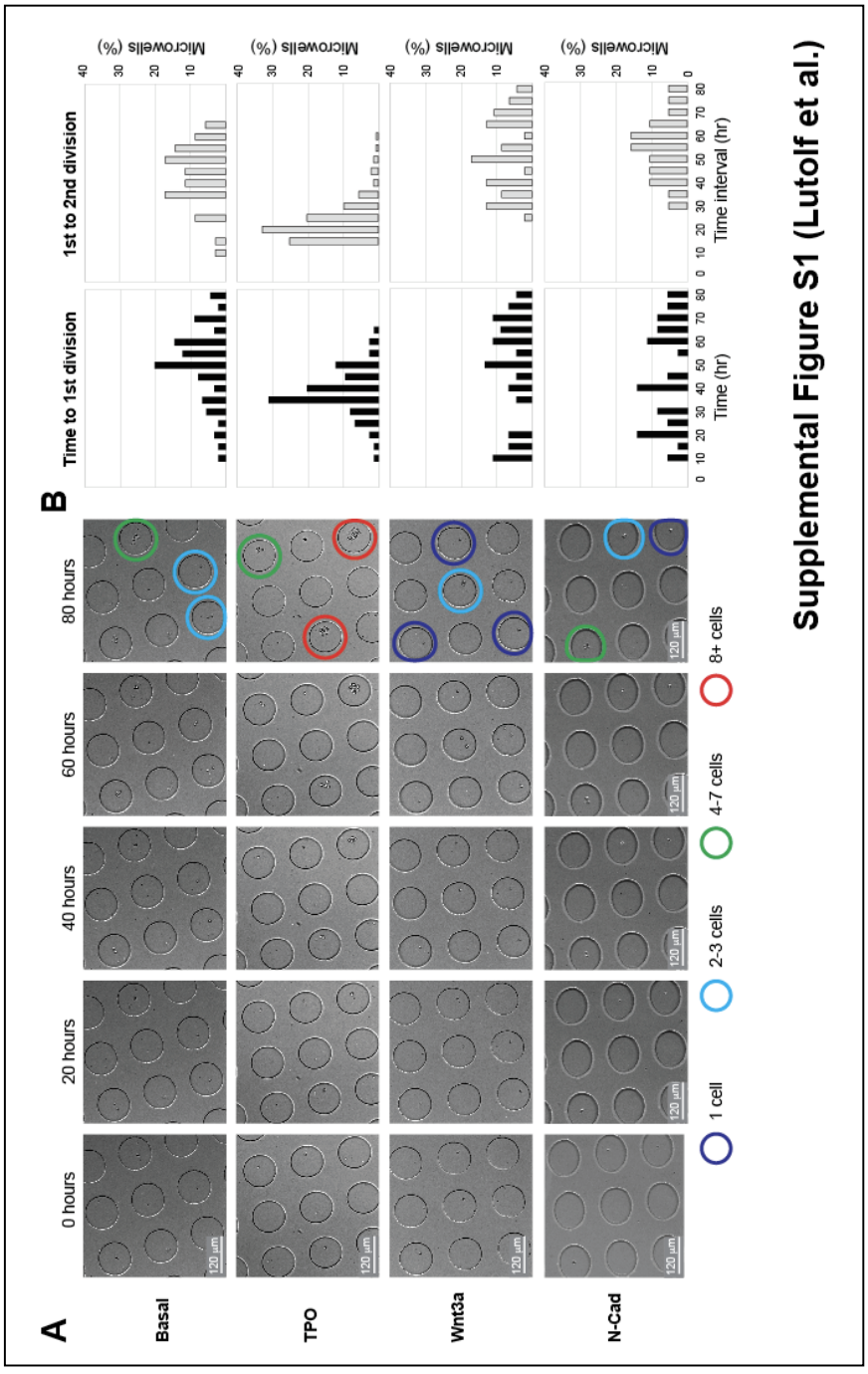


Figure S1 – Analyses of single cell proliferation kinetics of TPO-, Wnt3a-or N-Cad-exposed HSCs reveals marked differences.

(**A**) Still images of time-lapse movies (see corresponding Supplemental Movie4-6) of protein-exposed single HSCs leading to the most pronounced differences in cell division kinetics according to the analyses conducted above. (**B**) Proliferation kinetics quantified by distributions of the time to the 1st and times between 1st and 2nd divisions of single TPO-, Wnt3a-or N-Cad-exposed cells (100-220 microwells/condition analyzed; n=90 and 40 cells/histogram for basal, n=74 and 143 cells/histogram for TPO, n=64 and 53 for Wnt3a and n=45 and 27 cells/histogram for N-Cad, respectively).

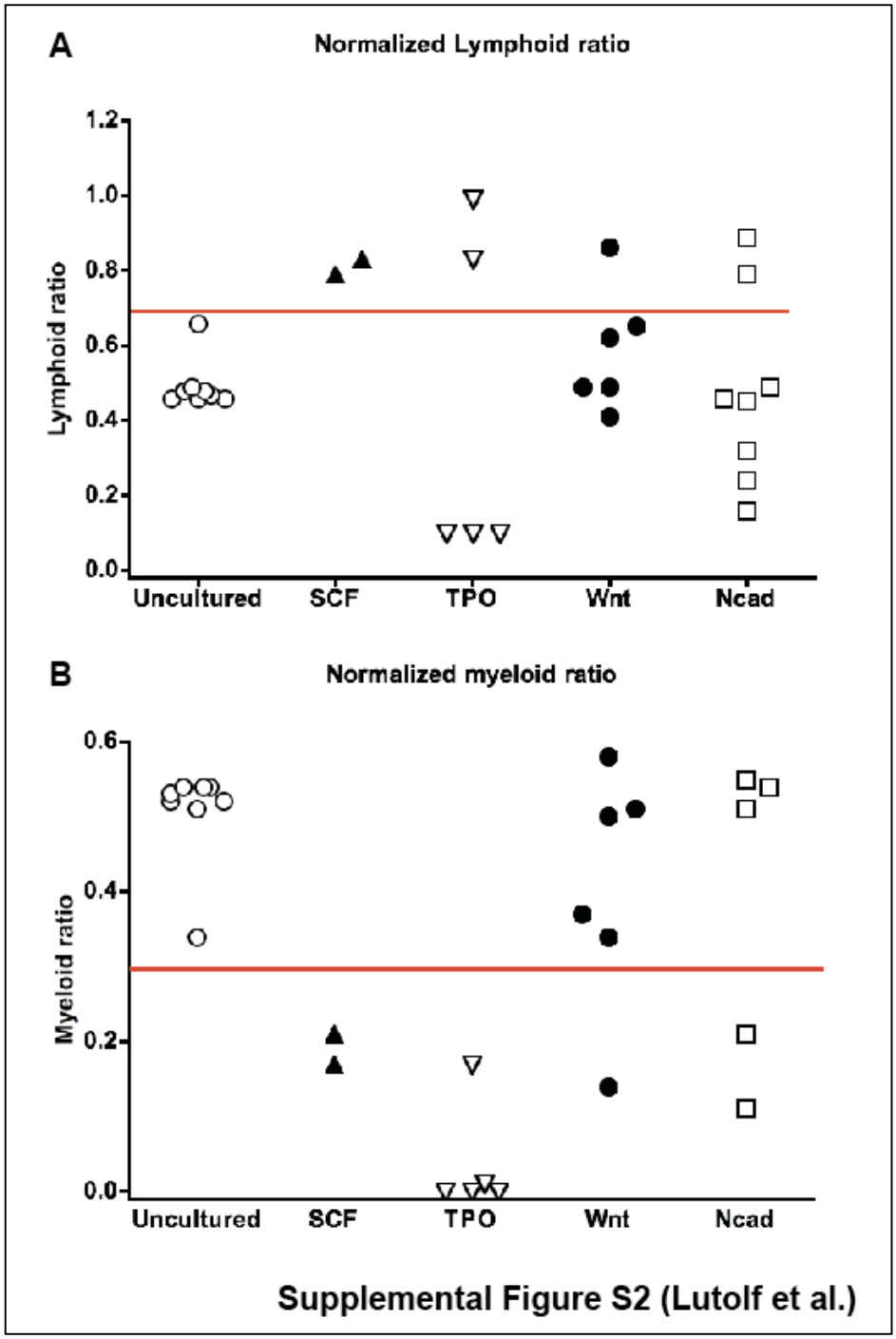
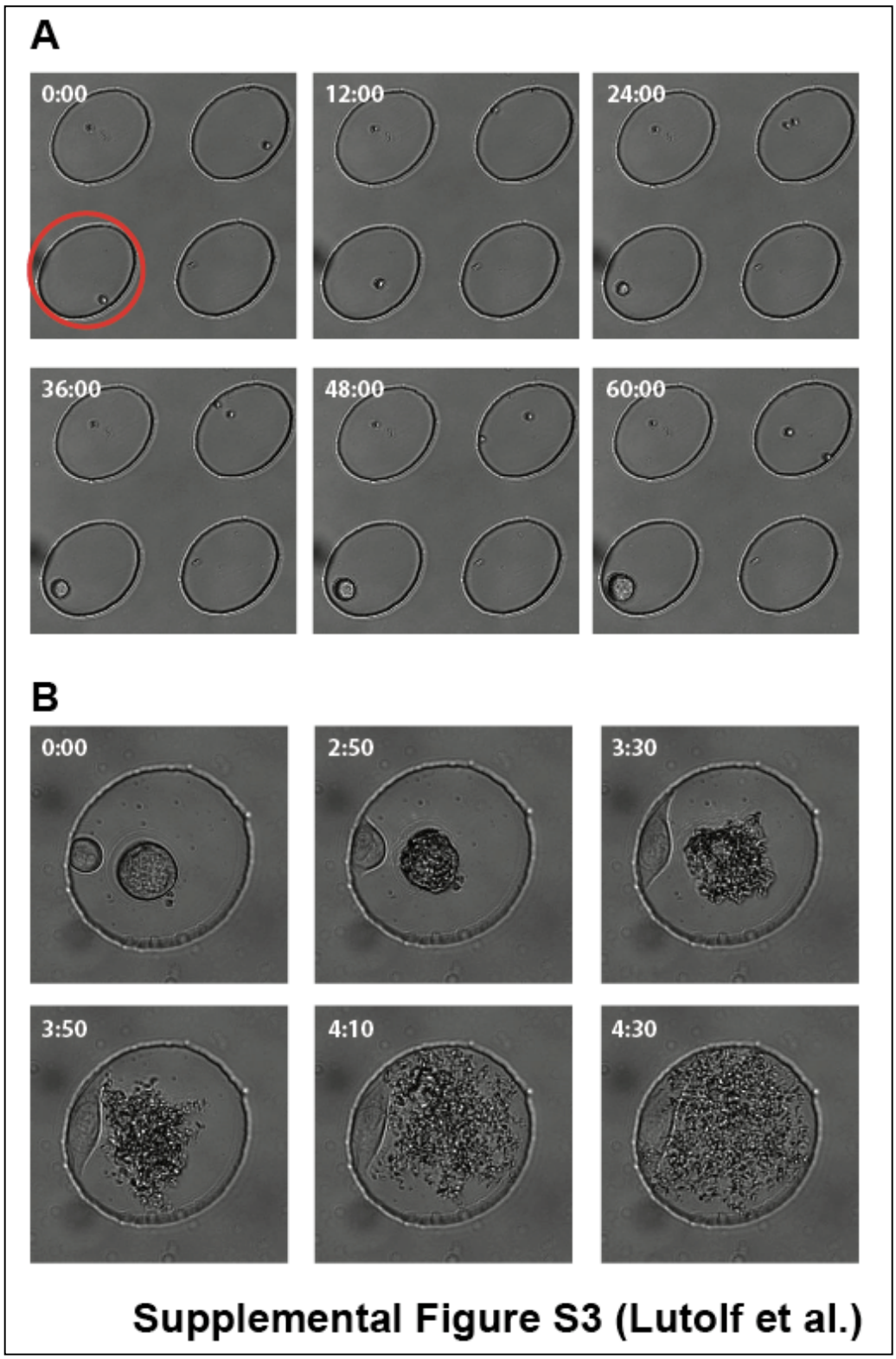


Figure S2 - Assessment of myeloid and lymphoid ratios of the blood in lethally irradiated mice reconstituted with HSCs exposed to proteins in microwells.

The overall donor repopulation level of each transplanted mouse was analyzed by assessing $GFP^{+}Ly5.1^{+}$ circulating white blood cells along both lymphoid (B220/CD3) and myeloid (Mac1/Gr1) lineages 24 weeks post transplant. These values were then plotted as normalized relative myeloid (**A**) and lymphoid (**B**) ratios: % donor myeloid = (% myeloid donor derived cells / (% myeloid donor derived cells + % myeloid non-donor derived cells); % donor lymphoid = (% lymphoid donor derived cells / (% lymphoid donor derived cells + % lymphoid non-donor derived cells). Normalized relative myeloid ratio = % donor myeloid / (% donor myeloid + % donor lymphoid); Normalized relative lymphoid ratio = % donor lymphoid / (% donor myeloid + % donor lymphoid). Wnt3a- and N-Cad-treated cells yielded lymphoid and myeloid ratios similar to uncultured stem cells, whereas SCF and TPO-treated cells gave rise primarily to lymphoid lineages.



Supplemental Figure S3 (Lutolf et al.)

Figure S3 – Rare megakaryocyte development from TPO or Wnt3a-cultured LKS-CD150+ cells.

On our microwell array platform, the rare formation of megakaryocytes from LKS-CD150+ over a few days in culture was apparent. (**A**) In the basal medium supplemented with TPO approximately 1-2 of 100 single cells developed into megakaryocytes that were very easily distinguishable from 'normal' HSC progeny due to their vast size (red circle indicates single cell that develops to form a megakaryocyte). Megakaryocytes developed mostly upon a first division of the initial mother cell. Still images from time-lapse movies captured every 2 hours for 72 hours (see corresponding Supplemental Movie7). (**B**) Time-lapse experiments over longer time periods with shorter time intervals (10min) reveal formation of proplatelets from single megakaryocytes (see corresponding Supplemental Movie8). The cytoplasm is remodeled into large pseudopodia (proplatelet extensions) that elongate and become thinner over time (Italiano J.E., The Journal of Cell Biology, Volume 147, Number 6, 1299–1312 (1999)).

3. Supplemental Movies

Supplemental Movie1 – High-throughput proliferation analysis of single hematopoietic stem/progenitor cells in microwell arrays. In transparent hydrogel microwells, proliferation behavior of single stem/progenitor cells can readily be monitored via time-lapse microscopy. Proliferating cells remain confined within individual microwells. LKS cells were imaged for 4 days with 1-hr time intervals in serum-free medium supplemented with a cocktail of cytokines/growth factors (SCF, or c-kit ligand, Flt-3 ligand, IL-3 and IL-6).

Supplemental Movie2 - Tracking of single LKS in microwell arrays (representative example). Single LKS (see Fig. 2 for purification strategy) cultured in basal serum-free medium supplemented with only stem cell factor (SCF, or c-kit ligand; 100 ng/ml) and Flt-3 ligand (2 ng/ml) were tracked by automated time-lapse microscopy over 4 days with 1-hr time intervals. LKS proliferated rapidly compared to LKS-CD150+ (Supplemental Movie3)

Supplemental Movie3 - Tracking of single LKS-CD150+ in microwell arrays (representative example). Single LKS-CD150+ (Fig. 2) cultured in basal medium were tracked by automated time-lapse microscopy over 4 days with 1-hr time intervals. LKS-CD150+ proliferated slowly compared to LKS-CD150+ (Supplemental Movie2).

Supplemental Movie4 - Tracking of single LKS-CD150+ exposed to soluble Wnt3a in microwell arrays (representative example). Single LKS-CD150+ cultured in basal medium supplemented with *TPO* were tracked by automated time-lapse microscopy over 4 days with 1-hr time intervals.

Supplemental Movie5 - Tracking of single LKS-CD150+ exposed to soluble Wnt3a in microwell arrays (representative example). Single LKS-CD150+ cultured in basal medium supplemented with *Wnt3a* were tracked by automated time-lapse microscopy over 4 days with 1-hr time intervals.

Supplemental Movie6 - Tracking of single LKS-CD150+ exposed to soluble TPO in microwell arrays (representative example). Single LKS-CD150+ cultured in microwell

arrays that were functionalized to display immobilized Fc-chimeric *N-Cadherin* (see Fig. 4 for microwell array fabrication) were tracked by automated time-lapse microscopy over 4 days with 1-hr time intervals (basal medium).

Supplemental Movie7 - Megakaryocyte formation of single LKS-CD150+. Time-lapse microscopy revealed the rare development of megakaryocytes from single LKS-CD150+ cultured in basal medium supplemented with TPO. A representative movie is shown. Images were acquired every 2 hours for 3 days.

Supplemental Movie8 – Proplatelet formation imaged via time-lapse microscopy. Over longer periods of time (>4 days) proplatelet formation from single megakaryocytes were detected. Time intervals: 10minutes, Duration: 60hr.

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