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Identification of factors promoting ex vivo maintenance of mouse hematopoietic stem cells by long-term single-cell quantification

Short title: Dermatopontin for ex vivo HSC maintenance

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Abstract

The maintenance of hematopoietic stem cells (HSCs) during ex vivo culture is an important prerequisite for their therapeutic manipulation. However, despite intense research, culture conditions for robust maintenance of HSCs are still missing. Cultured HSCs are quickly lost, preventing their improved analysis and manipulation. Identification of novel factors supporting HSC ex vivo maintenance is therefore necessary. Co-culture with the AFT024 stroma cell line is capable of maintaining HSCs ex vivo long-term, but the responsible molecular players remain unknown. Here, we use continuous long-term single-cell observation to identify the HSC behavioral signature under supportive or non-supportive stroma co-cultures. We report early HSC survival as a major characteristic of HSC-maintaining conditions. Behavioral screening after manipulation of candidate molecules revealed that the extracellular matrix protein dermatopontin (*Dpt*) is involved in HSC maintenance. DPT knock-down in supportive stroma impaired HSC survival, while ectopic expression of the *Dpt* gene or protein in non-supportive conditions restored HSC survival. Supplementing defined stroma- and serum-free culture conditions with recombinant DPT protein improved HSC clonogenicity. These findings illustrate a previously uncharacterized role of *Dpt* in maintaining HSCs ex vivo.

Key Points

- AFT024-induced HSC maintenance correlates with early survival/proliferation while early death is a major reason for HSC loss in culture
- Dermatopontin is required for ex vivo HSC maintenance, and also improves HSC clonogenicity in stroma-based and stroma-free cultures

Introduction

Hematopoietic stem cells (HSCs) regenerate the blood system by constantly producing differentiating blood cells, while self-renewing long-term to maintain the HSC pool. HSC/bone-marrow transplantations have been used since 1955[1–4] against blood disorders, injury or non-hematopoietic conditions. The extremely low HSC frequency limits their improved clinical application. For their improved analysis and therapeutic manipulation, ex vivo cultivation is required.[5] However, even short-term culture significantly reduces HSC numbers.

Survival and stemness retention are key requirements for quantitative and qualitative HSC maintenance ex vivo. Extensive research has been done to identify culture conditions supporting HSC survival/proliferation while favoring self-renewal versus differentiation. A plethora of different hematopoietic cytokines (stem cell factor, thrombopoietin, Flt3-ligand, interleukin-3, -6 and -11[6–10]), growth factors (pleiotrophin[11], insulin-like growth factor[12], fibroblast growth factor[13], angiopoietin-like proteins[14]) and their combinations have been extensively tested over the last decades. However, robust ex vivo maintenance of repopulating cells has not yet been achieved[15,16] or is limited to extremely short culture periods[10,17,18], mainly due to pleiotropic effects of those factors in cell-fate decisions[19]. Ectopic overexpression of intrinsic factors (e.g. Polycomb family *Ezh2*[20], *Hoxb4*[21], Nucleoporin98-*Hoxa10*/Nucleoporin98-Homeodomain[22], *Hoxb6*[23]) or chemical inhibitors[24,25] prevented exhaustion or achieved considerable HSC expansion. Yet, direct genetic manipulation has limited clinical applicability, due to high risk of oncogenic transformation[26,27], reduced stability of virus-free delivery systems[5,28] or non-predictable off-target effects.

Mimicking the interaction between HSCs and niche cells offers a potentially less invasive alternative for ex vivo culture. Only few cell lines are capable of maintaining co-cultured HSCs.[29,30] Among those, the clonal AFT024 line qualitatively and quantitatively maintains murine[29] and human HSCs[31–36] for several weeks. However, the exact behavior of co-cultured HSCs remains obscure. For example, both increased proliferation, and/or reduced cell death, and/or quiescent cells

surviving without division, and/or repeated asymmetric cell division, could all result in the reported maintenance of HSC numbers. Population-based snapshot analyses are insufficient to describe single-cell behaviors over time, especially when studying heterogeneous or impure populations such as isolated HSCs.[37–40] Similarly, low-temporal resolution[41] or short-term imaging[42,43] often fail to preserve single-cell identity after initial divisions in long-term cultures. Unraveling the biology behind such events requires continuous long-term observation of living cells with a temporal resolution allowing precise reconstruction of colony genealogy.

Identifying the responsible maintenance-promoting factor(s) could improve HSC culture towards better defined and clinically applicable stroma-free conditions. In-depth genomic mRNA expression analysis revealed over 1000 candidate factors preferentially or exclusively expressed by supportive "AFT024" compared to intermediate ("2012") or non-supportive cells ("2018").[44,45] However, this high number of candidates, in combination with lengthy functional readouts required upon their manipulation made it impossible to comprehensively screen individual molecules improving HSC maintenance.

To better filter the long list of candidate factor(s) responsible for AFT024-mediated HSC maintenance, we aimed at first identifying HSC behaviors specific for their maintenance. These would then be used to filter relevant molecular candidates and functional screening upon their molecular manipulation. We therefore performed continuous long-term time-lapse imaging of individual primary murine HSCs and their progeny for up to two weeks in stroma co-cultures. This allowed identification of HSC behavior under maintenance conditions, and of dermatopontin (DPT) as a key factor for HSC survival and proliferation. DPT improves non-supportive HSC cultures both, in the presence and absence of stromal cells. It therefore plays a critical and previously unanticipated role in maintaining HSCs in culture.

Methods

Mice

Twelve-week old male wildtype C57Bl/6J-*Ly5.2*, C57Bl/6J-*Ly5.1* or transgenic B6J;129-Tg(CAG-EYFP)7AC5Nagy/J[46] (>10 backcrosses) mice were used to isolate

hematopoietic cells and C57Bl/6J-*Ly5.1* or immunocompromised Kit^{W-41J} as transplantation recipients. All procedures were approved by veterinary office of Canton Basel-Stadt, Switzerland (numbers 2655, 2707) and Regierung von Oberbayern (AZ55.1-2-54-2531-59-08).

Stroma culture and generation of manipulated stromal lines

Stroma lines obtained from K.Moore were cultured as previously described.[29,47] For stroma manipulation, third-generation lentiviruses expressing gene-specific shRNAs (Table S1) or AFT024-derived candidate genes followed by fluorescent reports were used (Figure S4A). shRNA knockdown efficiency quantified by flow cytometry (membrane proteins, working antibodies, Figure S4B) or qRT-PCR (Figure S4C-D). Transduced stroma was isolated based on fluorescent-marker expression and analyzed frequently for expression stability.

Hematopoietic-cell isolation

Bone marrow cells suspended in PBS (1mM EDTA, 2% FCS) and stained with biotinylated CD3e(145-2C11), CD19(eBio1D3), CD41(eBioMWRag30), Ter119(TER-119), B220(RA3-6B2), Ly-6G(RB6-8C5) and CD11b(M1/70) lineage antibodies (eBioscience) followed by addition of streptavidin-labeled magnetic beads (Roth). Lineage cells were depleted by immune-magnetic removal (EasySep magnet) and lineage-depleted cells were stained with CD34-e450(RAM34), Sca1-PerCPCy5.5(D7), CD48-APC(HM48-1), cKit-PECy7(2B8), Streptavidin-APCe780 (eBioscience) and CD150-PE(TC15-12F12.2, Biolegend) for 60 minutes on ice and sorted with FACSAriaIII.

Hematopoietic cultures with or without stroma

Stroma cells grown to confluency in 0.1% gelatin-coated plates (Nunc) were irradiated (20Gy) one day prior to co-culture. Media was changed to "Dexter-type": 10% FCS (PAA), 10% Horse serum (Gibco), $5x10^{-5}$M β-mercaptoethanol, 10^{-6}M Hydrocortisone and penicillin/strepromycin in DMEM (Gibco). Conditioned media was collected, centrifuged and filtered (0.22um) before use. Alternatively, HSCs were cultured in stroma/serum-free media (SFEM, StemCell Technologies) supplemented with 100ng/mL stem cell factor and thrombopoietin (Peprotech).

Time-lapse imaging and single-cell tracking

Time-lapse imaging was performed using Zeiss Axiovert200M or AxioObserver.Z1 microscopes equipped with motorized stages and temperature incubators (37°C). YFP was detected by 46HE filter (Zeiss, Excitation BP500/25, BS FT515HE, Emission 535/30). Sorted cells and their progeny were monitored for up to two weeks and tracked up to third generation. Phase-contrast and fluorescent pictures were acquired every 6-12 and 7-15 minutes respectively by 5xPlanNeoFluar objective (0,3NA) and AxioCamHRm camera (Zeiss, Germany, 1388x1040 or 692x520 pixel resolution) using Zeiss AxioVision4.8 software. Mercury lamps or light-emitting diode-based systems (Lumencore, Laser 2000) were used for fluorescent illumination. Single-cell tracking was performed as previously described[48,49] and cell behaviors were displayed in tree pedigrees.

RNA extraction and quantitative real time PCR (qRT-PCR)

RNA isolation was performed using RNeasy Mini Kit (Qiagen). Intron-separated gene-specific primers were used (Table S2) and all samples performed in triplicates. Dissociation/melting curves always generated as quality controls. Normalization was based on GAPDH expression of each line.

Transplantation experiments and chimerism analysis

In vivo transplantations were performed using CD45.1/CD45.2 congenic mouse system. For knockdown experiments, 1250 HSCs (CD45.1) were co-cultured with wildtype AFT024, 2018 or AFT024-knockdown stroma for 7 days. The contents of each well were transplanted into sub-lethally irradiated CD45.2 recipient (4Gy). For rescue experiments, 125 HSCs were co-cultured with AFT024, 2018 or DPT-expressing 2018 stroma for 7 days before injected into sub-lethally irradiated immunocompromised W41 primary and later secondary recipients (more sensitive for assessing HSC potential). Peripheral blood was collected on defined timepoints to assess chimerism levels. Erythrocytes were lysed by ammonium-chloride-potassium buffer (LifeTechnologies). White blood cells (WBC) were stained with CD45.1-FITC(A20), CD45.2-APC(104), Ter119-APCe780(TER-119), B220-PE(RA3-6B2), CD11b-PECy7(M1/70) and Ly-6G-PECy7(RB6-8C5). Donor-derived single, living cells positive

for CD11b/Ly-6G and negative for B220 were classified as myeloid cells and lineage contribution was calculated over the total WBC of that lineage.

Statistical analysis

Results were analyzed with GraphPad Prism using non-parametric Mann-Whitney test for non-normally distributed data, unless otherwise stated. Bars represent mean and error bars standard deviation. Statistically significant differences were: *P<.05,** P<.01,*** P<.001.

Results

Long-term single-cell quantification of HSC behavior

To identify the HSC-specific behavior in different stroma co-cultures, we followed single cells and their progeny for up to two weeks using time-lapse imaging and single-cell tracking (Figure 1). Highly enriched HSCs (CD150+CD34-CD48-KSL)[50] and multipotent progenitors (MPPs)[50] were isolated from mice ubiquitously expressing yellow fluorescent protein (YFP+) to facilitate hematopoietic-cell identification in complex co-cultures. Sorted cells cultured with stroma lines previously reported to fully (AFT024), intermediately (2012) or unable to support (2018) HSC maintenance.[29] Fates of individual cells and their progeny were followed for three generations and up to two weeks. Quantification included three distinct fates: division, death or survival without division until the end of the imaging period.

Initial survival and early proliferation correlate with stroma-induced HSC maintenance

We hypothesized that HSC behavior would differ depending on stroma's capacity to maintain their numbers. To identify HSCs-specific behavior under maintenance conditions, we compared genealogy trees from cells cultured on AFT024, 2012 or 2018 stroma. Most of founder HSCs co-cultured with AFT024 stroma survived and displayed high proliferation, whereas the majority died on 2012 and 2018 stroma (Figure 2A). Less than 5% of founder HSCs survived without division for two weeks in all conditions. Since at least 50% of the purified founder cells were HSCs at culture initiation[51], the non-dividing cell compartment (<5%) cannot contain all HSCs, illustrating that most HSCs proliferated over the culture period. Importantly,

high proliferation levels were maintained for the first three generations on AFT024 stroma (Figure 2B). These data suggest that the stroma's reported capacity to maintain repopulating cells directly correlates with and can be quantified by founder-HSC survival and proliferation rates.

To assess whether the effects of AFT024 stroma were HSC specific, different MPPs were analyzed (early MPPs: CD150+CD48-CD34+KSL, late MPPs: CD150-CD48+CD34+KSL). Indeed, we find decreased levels of founder MPP survival/proliferation on AFT024 stroma (Figure 2C), which was gradually increased in later generations (data not shown). The vast majority of founder MPPs died on non-supportive stroma (Figure S1). HSCs divided slower than early and late MPPs when cultured on AFT024 (Figure 2D) or other stroma. These results highlight the presence of an early AFT024-mediated selection mechanism based on the primitiveness of co-cultured hematopoietic cells. After initial selection, the progeny of surviving cells is highly supported by AFT024.

Tree analysis reveals distinct HSC and MPP colony types

Continuous time-lapse imaging and single-cell tracking allowed retrospective reconstruction of HSC and MPP colony genealogies. To identify cell-type specific tree patterns, we quantified 290 HSC, 257 early and 184 late-MPP colonies cultured on AFT024 stroma (Figure 2E). For HSC colonies, the most frequent patterns include symmetric (43%, both daughters divide or die) or asymmetric fates of the two daughters (17%, one dies, one divides). Colonies without surviving progeny (22% total) had lower frequencies than those with at least one surviving daughter (22% and 60%, respectively). In contrast, the most frequent pattern for MPP trees were dying colonies. From those surviving, 30% of early MPPs show symmetric, whereas 11% asymmetric fates. Tree analysis up to the third generation can be found in Figure S2. These results illustrate considerable hematopoietic-colony heterogeneity and the need for continuous single-cell analysis.

Cell adhesion is required for self-renewal specific behavior

To identify whether adhesion or secreted factors are responsible for HSC behavior under self-renewing conditions, we performed conditioned-media

experiments. No proliferation differences were observed for HSCs cultured on 2018 stroma supplemented with AFT024-conditioned media or vice versa (Figure 3A). To exclude conditioned-media stability issues, HSCs were co-cultured on a physically separated area of 2018 stroma, while being exposed to media conditioned by surrounding AFT024 cells (Figure 3B). Under those conditions, founder HSC proliferation slightly increased, but reduced again in later generations, suggesting that AFT024-secreted factor(s) have only transient effects on HSC proliferation. 2108-conditioned media did not affect HSCs cultured on AFT024 stroma suggesting that 2018 do not secrete cell-death promoting factors. All HSCs died in stroma-free cultures in Dexter-type media, also with AFT024-conditioned media, but the latter doubled time until death (Figure S3). These results illustrate that adhesion to stroma components is required for the self-renewing-specific HSC behavior.

Non-supportive stroma lacks factor(s) promoting HSC proliferation

To further investigate the relevance of cell-contact for HSC behavior, we performed mixed-stroma experiments. HSC proliferation rates were similar to controls when AFT024 was mixed with 10% 2018 stroma (or vice versa, Figure 3C). Equal mixing of both stroma lines led to intermediate proliferation levels suggesting dose-dependent stroma effects. Differential fluorescent stroma labelling allowed us to quantify the absolute time individual HSCs adhere to each stroma and correlate this with their future fates (Figure 3D). HSCs that mostly adhered to AFT024 (75% of their life time) proliferated, despite their transient adherence to 2018 cells (Figure 3E), again suggesting that 2018 stroma does not actively promote HSC death. No active migration towards AFT024 stroma was observed. Comparing cell-death kinetics revealed that 2018-cultured HSCs exhibit the longest cell-lifetime of all groups with almost half of founder cells surviving over 100 hours (Figure 3F). In combination, those data demonstrate that non-supportive stroma does not actively promote HSC death, but probably lacks mitogenic and/or pro-survival factor(s) expressed on AFT024 cells.

DLK1, DPT and FAP knockdown reduce HSC/MPP proliferation on AFT024-based cultures

Knowing that cell contact is essential for the observed HSC behavior, we selected cell-surface and extracellular matrix-related candidates from AFT024-specific gene lists. We first confirmed differential gene expression between AFT024 and 2018 by quantitative real-time PCR (qRT-PCR). The expression of 152 genes, which include 115 genes previously described to be preferentially expressed by AFT024[44,45] was compared. From those, 27 were differentially expressed between AFT024 and 2018 stroma (Figure 4A). To mimic co-culture conditions, we also examined irradiated stroma, where fold differences were slightly reduced.

Gene-specific shRNA vectors (4-5 per gene) were then designed to knock-down the following cell-adhesion related genes expressed by AFT024 stroma: *Slc38a4*, *Slc02a1*, *Dlk1*, *Igfbp6*, *Ptx3*, *Bgn*, *Thbs2*, *Mmp9*, *Col6a3*, *Dpt*, *Arhgdib*, *Fap*, *Dcn*, *Vcam1*, *Tgfbi*, *Loxl1*, *Plaur*, *Tm4sf1*.

Founder HSCs cultured on DLK1-knockdown AFT024 stroma (DLK1^{KD}) exhibit 1.4-fold reduced proliferation, while DPT^{KD} or FAP^{KD} stroma reduced HSC proliferation by 1.8-fold (Figure 4B). Importantly, co-culture with 'scrambled' shRNA control lines or less efficient constructs (20% DLK1 knockdown) had no effect on HSC behavior. Double knockdown DLK1/DPT^{DKD} or DLK1/FAP^{DKD} stroma did not further decrease HSC proliferation, whereas DPT/FAP^{DKD} or DLK1/DPT/FAP^{TKD} reduced it to 2018 levels. Thus, DPT and FAP are independently important for HSC survival and proliferation. Analysis of HSC progeny revealed that the reduced proliferation levels were maintained throughout the first three generations (data not shown), suggesting that DLK1, DPT and FAP permanently impair HSC survival/proliferation ex vivo.

No significant proliferation defects were detected upon early MPPs co-culture with DLK1^{KD} or FAP^{KD} stroma (Figure 4C). However, DPT^{KD} stroma co-culture resulted in marked decrease of both early MPPs' (4.3-fold) and HSCs' proliferation capacity (1.9-fold), suggesting that DPT affects fates of both HSCs and early MPPs.

DPT is essential for maintenance of short and long-term repopulating cells

To confirm DLK1, DPT and FAP effects on HSC maintenance, we transplanted CD45.1 HSC cultured on wildtype or knockdown stroma into CD45.2 recipients (Figure 4D). Peripheral-blood analysis revealed significantly higher chimerism from AFT024-cultured HSCs compared to 2018 cultures (Figure 4E). Chimerism levels of $DLK1^{KD}$ or FAP^{KD} cultured HSCs were similar to AFT024 levels for the first 16 weeks (Table S3, short/intermediate-term repopulation), but decreased by 32 weeks (Table S3, long-term repopulation). Importantly, HSCs cultured on DPT^{KD} stroma had similar kinetics, chimerism levels and lineage contribution with 2018-cultured cells (Figure 4E and S5A-B). These results validate that DLK1 and FAP have intermediate effects on long-term HSCs, whereas DPT is essential for maintenance of both short and long-term repopulating cells.

DPT restores HSC behavior under non-supportive conditions

Next, we ectopically expressed DLK1/DPT/FAP or their combination on 2018 stroma. Ectopic DPT expression alone fully restored founder HSCs' proliferation to AFT024 levels (Figure 5A), while co-culture with 2018^{DLK1} or 2018^{FAP} stroma resulted in 2-fold increase. Although slightly decreased in later generations, proliferation rates were maintained at significantly higher levels than wildtype 2018 stroma (Figure 5B). Notably, all combinations (except $2018^{DLK1/DPT}$) led to intermediate HSC proliferation suggesting a potential antagonistic interaction between DPT and FAP. DPT overexpression also restored early MPP proliferation to AFT024 levels (Figure 5C). Transplantation experiments confirmed that HSCs cultured on 2018^{DPT} or AFT024 stroma (Figure S6A) exhibit equally high chimerism in primary and secondary recipients' peripheral blood and bone marrow (Figure 5D and S6B-D), and almost exclusively outcompeted recipients' HSC/MPP populations (Figure 5E). In contrast, HSCs cultured on non-supportive stroma showed lower contribution in peripheral blood (Figure 5F and S6B-D) and bone marrow, where residual HSCs and early MPPs were not outcompeted. Taken together, DPT is sufficient to convert non-supportive stroma to supportive being indispensable for maintaining HSC potential under stroma co-cultures.

Recombinant DPT improves HSC clonogenicity in both serum/stroma-free conditions

To examine whether recombinant protein could efficiently replace virus-mediated gene-delivery methods, we supplemented 2018 co-cultures with murine (mDPT) or human DPT (hDPT). Indeed, HSC proliferation was increased by 2.3 fold in their presence, an effect that was maintained for the first three generations (Figure 6A-B).

Finally, we assessed the effect of recombinant mDPT in defined stroma/serum-free conditions supporting short-term self-renewal.[10] Supplementing SCF, TPO with mDPT consistently increased the number of proliferating HSCs (80%, Figure 6C) without influencing their proliferation kinetics (Figure 6D). Therefore, recombinant DPT can supplement standard serum/stroma-free culture conditions to improve HSC clonogenicity.

Discussion

Long-term imaging and single-cell analysis provide quantitative data of HSC fates

Despite characterization of the AFT024 molecular milieu, the large number of candidate factors and effort, duration and high cost required for their functional assessment by conventional methodologies (transplantations) discouraged high throughput screenings. In the current study, we utilized long-term imaging coupled with cell-fate quantification[52–55] to establish a sensitive screening platform with single-cell resolution. This technology allowed to continuously follow fates of single HSCs directly after isolation and for the first time over three generations in vitro, recording both early and late effects. We reconstructed HSC colony history under different stroma co-cultures and identified the HSC-specific behavior under self-renewing conditions.

We report for the first time that the balance between HSC survival and death in vitro quantitatively correlates with their reported repopulating potential in vivo. High HSC proliferation rates were characteristic of self-renewing conditions (AFT024 stroma), whereas intermediate and high cell-death rates marked cultures with

reduced or no supportive capacity. Similar survival rates were reported when human CD34+CD38-HSCs were cultured with AFT024 stroma.[41] Notably, initial proliferation cannot always be coupled with stemness retention, especially under stroma-free conditions.[56] No differences in HSC cell-cycle progression were observed between different stroma suggesting that no active regulation of proliferation occurs.

Our data illustrate that differences in HSC behavior between supportive and non-supportive conditions occur early, before the first cell division. Since the majority of founder HSCs divides only under supportive conditions, early proliferation was used as a fast indicator of stroma's potential to maintain repopulating cells. This allowed us to minimize the overall length of molecular screening from several months required for typical in vitro (long-term culture initiating-cell, cobblestone area-forming assay) or in vivo experiments (transplantations) to few days.

Taking advantage of our imaging and single-cell analysis pipeline, we report precise cell-death rates and kinetics per generation over two weeks. Our data show that death occurring at the initial or later generations is a major reason for loss of stemness under non-supportive culture conditions. This had been overlooked in previous studies based on snapshot analyses of bulk populations, which analyzed only surviving and not all starting HSCs. Our findings expand previous data linking HSC clonogenicity with repopulation capacity during short-term culture[56], by providing data over two weeks in vitro (Figure 3F and S7).

Continuous long-term imaging revealed a small proportion of cells that survive without division, under all conditions. It is likely that some of those cells have repopulating capacity, as indicated by low chimerism under non-supporting conditions and previous studies.[57] However, due to their low frequency (5%), the possibility repopulating cells exclusively reside in this deeply quiescent compartment can be excluded.

We also extended our analysis to MPPs and reported for the first time that their survival is also favored under maintenance-promoting conditions. Given that MPPs are responsible for short-term reconstitution of hematopoiesis upon

transplantation, such finding might have important clinical applications. Comparing kinetics of co-cultured HSCs and MPPs, we confirm that longer cell-cycle length over the first three generations correlates with more primitive/immature hematopoietic cells extending previous reports limited to short-term imaging.[57–59]

Identification of novel players for improved cultivation of HSC ex vivo

Little was known about the underlying mechanism governing the interaction between AFT024 stroma and murine HSCs, whereas studies using human cells led to contradictory results suggesting prevalence of either adhesion[31,34,35] or secreted factors.[33,36] We therefore performed conditioned-media and stroma-free experiments and found that cell adhesion was essential for HSC fate regulation, despite transient synergistic effect of secreted factor(s). Notably, mechanisms might vary between different stroma lines, as aorta-gonads-mesonephros-derived lines (UG26-1B6) regulate HSC self-renewal via secreted factors[60,61], while embryonic (EL08-1D2)[60] and fetal liver-derived (AFT024) require direct contact.

Functional screening of adhesion-related molecules revealed that two transmembrane proteins delta-like homolog 1 (DLK1) and fibroblast activation protein (FAP) are involved in maintenance of HSC self-renewal in vitro. DLK1 is an epidermal growth factor (EGF)-like transmembrane protein and the best known non-canonical Notch ligand.[62] Cleavage of the extracellular domain yields a soluble 50-kDa protein[63] with potentially distinct function.[64] The precise role of *Dlk1* in hematopoiesis is not fully understood. It has been reported that *Dlk1* is necessary for normal B-cell differentiation[65], and is involved in inhibiting Notch1 receptor[66] expressed by adult HSCs although dispensable for their in vivo maintenance.[67] In addition, there is growing evidence that *Dlk1* might play a role in ERK/MAPK[68] and FGF signaling.[69] Here we report that DLK1 knockdown decreases survival and repopulation capacity of HSCs but not short-term repopulating progenitors, while ectopic overexpression in non-supportive stroma partially restores HSC behavior. These results confirm previous studies reporting similar intermediate effects of DLK1 on HSC maintenance[70], thus validating our approach while also suggesting that additional factors are necessary for AFT024-mediated HSC maintenance ex vivo. Recent studies used DLK1 as a marker to isolate fetal-liver hepatic progenitors

capable of maintaining HSCs in direct-contact cultures[71,72], but provided no evidence on its role on the observed HSC maintenance. Interestingly, it is reported that *Dlk1* can also negatively regulate HSCs when co-cultured with stroma cells from the aorta-gonad mesonephros (AGM) region[73], suggesting that its role is context-dependent and might reflect functional differences between in vivo niches.

FAP is a transmembrane glycoprotein with peptidase activity.[74] Similar to DLK1, extracellular domain cleavage yields a soluble form (APCE[75] or soluble FAP[76]). FAP is involved in matrix remodeling[77], but its precise function in homeostatic conditions is unknown, since *Fap* knockout mice showed no abnormalities.[78] Recent data showed that depletion of *Fap* expressing bone marrow stroma reduced committed progenitors in vivo[79], but its role in the maintenance of cultured HSCs has not been assessed. We report that FAP is important for HSC maintenance ex vivo, similar to DLK1. The fact that both proteins have biologically active soluble forms might explain the transient positive effect of AFT024-conditioned media on HSC proliferation. However, overexpression of single or combination of both proteins only partially restored HSC behavior ex vivo.

We also identified dermatopontin (*Dpt*) as a key factor for maintaining HSC self-renewal ex vivo. DPT, an extracellular matrix-located protein regulates cell-matrix interactions and matrix assembly.[80,81] No role in hematopoiesis was previously reported in *Dpt* knockout mice.[82] We show that DPT knockdown significantly impairs HSC and early MPP proliferation and repopulation capacity, whereas ectopic overexpression reverts effects of non-supportive conditions. Also, exogenous DPT addition increases HSC clonogenicity under stroma/serum-free conditions.

The exact mechanism through which DPT interacts with hematopoietic cells is unknown. Its high content of sulfated tyrosine residues enables interaction with secreted factors.[83] In the presence of decorin (also expressed by AFT024), DPT enhances the binding and activity of transforming growth factor β1 (TGF-β1) to target cells.[84,85] TGF-β signaling regulates HSC maintenance, proliferation and dormancy through SMAD2/3 activation both in vivo[86] and in vitro[87]. In addition to

niche cells (non-myelinating Schwann cells), HSCs themselves secrete latent TGF-β1, but the niche is required for its biological activation.[87] However, TGF-β deficient mice exhibit no defects in HSC maintenance or quiescence[88], illustrating that its role in hematopoiesis is not essential or compensated by other factors in vivo. DPT has a characteristic peptide sequence functioning as a potential integrin binding site.[85] HSCs express a wide range of integrins, such as integrin α4/β1, α7, α9 and β1 which bind to the ECM proteins fibronectin[89], laminin[90], tenasin-c[91] and osteopontin[92] respectively, ensuring adhesion to the niche thus maintaining stem-cell properties. It is therefore possible that DPT facilitates HSC contact with supportive stroma/niche through integrin binding.

In summary, this study provides quantitative continuous data of single HSCs and their progeny under self-renewing conditions. To our knowledge, this is the first study using long-term single mammalian stem cell behavior quantification for screening of molecular candidates. This new approach identified DPT as a niche factor which is both, essential for maintaining HSCs in stromal co-culture, and able to improve stem-cell survival in stroma- and serum-free cultures. These results have important implications in improving ex vivo HSC culture and clinical applicability.

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Authorship Contributions

K.K. designed and performed experiments, collected and analyzed data; E.D. performed experiments; M.E. provided support with transplantations with C.H. and M.K., maintained flow cytometry with P.S.H.; M.T. provided support with molecular biology; K.M. provided stroma lines and advised the study with I.L.; T.S. designed and supervised the study, developed and maintained long-term bioimaging with D.L. and K.K., single-cell tracking with O.H., analysis software with B.S. and S.S., and wrote the manuscript with K.K..

Disclosure of Conflicts of Interest

Authors declare no conflict of interest. TS is President Elect of the International Society for Experimental Hematology (ISEH).

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Figure Legends

Figure 1. Long-term single cell quantification of HSCs/MPPs behavior in complex stroma co-cultures for up to two weeks. HSCs, early and late MPPs were isolated from B6J;129-Tg(CAG-EYFP)7AC5Nagy/J mice ubiquitously expressing YFP and co-cultured with stroma lines differentially supporting HSC maintenance. Cells were observed for the first three generations (two weeks).

Figure 2. Continuous single-cell analysis reveals cell-fate differences between supportive and non-supportive conditions. (A) Cell-fate quantification of founder HSCs co-cultured with different stroma: supportive AFT024 (dark grey bars, n=7 independent experiments, 290 trees), intermediately supportive 2012 (n=3 independent experiments, 129 trees) and non-supportive 2018 (white bars, n=5 independent experiments, 264 trees). (B) Quantification of dividing HSC rates on different stroma over the first three generations. (C) Cell-fate quantification of founder early (n=5 independent experiments, 274 trees) and late MPP (n=4 independent experiments, 211 trees) compared with HSCs cultured on supportive AFT024 stroma. (D) Cumulative time curves representing absolute time required for HSC/MPP division on AFT024 stroma. (E) Quantification of most representative HSC/MPP tree genealogies cultured on AFT024 stroma (up to generation 1).

Figure 3. Direct contact is required for HSC survival/proliferation on AFT024 co-cultures. (A) Schematic representation of the experimental procedure for the conditioned media exchange approach (upper panel): irradiated AFT024 stroma was cultured on Dexter-type media for 2 or 4 days, before conditioned media was transferred to 2018 stroma (or vice versa). Percentage of dividing founder HSCs cultured on 2018 cells in the presence of AFT024 media conditioned for 2 (n=4 independent experiments, 168 trees) or 4 days (n=3 independent experiments, 162 trees) are compared to the 2018 control (no media exchange, white bar). In addition, the respective percentage of dividing HSCs cultured on AFT024 stroma with 2018-conditioned media for 2 (n=3 independent experiments, 149 trees) and 4 days (n=3 independent experiments, 175 trees) is compared to the AFT024 control (dark grey bar). (B) Schematic representation of the experimental procedure for continuous media conditioning (upper panel): AFT024 stroma surrounding a physically separated (silicon insert) island of 2018 cells (or vice versa). Area covered by the surrounding stroma is approximately 8 times larger. HSCs were exclusively cultured in contact with the inner stroma compartment, but exposed to media mainly conditioned by the outer stroma (approximately 8x more cells). Generation-based analysis of dividing HSCs cultured on 2018 stroma while exposed to AFT024 conditioned media (n=3 independent experiments, 194 trees) or vice versa (n=3 independent experiments, 141 trees). White and dark grey bars represent control conditions. (C) Quantification of HSC divisional rates after culture on different ratios of AFT024 and 2018 stroma: 100%-0% (AFT024 control, dark grey bar), 90%-10% (n=5 independent

experiments, 180 trees), 50%-50% (n=4 independent experiments, 122 trees), 10%-90% (n=4 independent experiments, 120 trees) and 0%-100% (2018 control, white bar) respectively. (D) Snapshots from time-lapse imaging experiment showing the different channels acquired. Stroma cells were differentially transduced with lentiviral vectors expressing distinct fluorescent proteins fused with the c-HA-Ras farnesylation signal domain for membrane anchoring allowing visualization of the entire cell volume (including cell protrusions). (E) Bar chart representing the percentage of cell lifetime, for which dividing (left panel) or dying HSCs (right panel) were adherent to AFT024 (black bar), 2018 (white bar) or both stroma (grey bar) (n=3 independent experiments, 47 trees). (F) Violin plots depicting cell lifetime of dying founder HSCs cultured on AFT024 (n=7 independent experiments, 49 trees), 2018 (n=5 independent experiments, 184 trees) or 2012 stroma (n=3 independent experiments, 75 trees). Black lines represent the median. Data were compared using the rank-based non-parametric Kruskal-Wallis test with Dunn's post-hoc test.

Figure 4. Dermatopontin (DPT) is essential for ex vivo proliferation and maintenance of long-term repopulating cells. (A) Fold difference in the expression of membrane-bound or extracellular matrix genes between non irradiated or irradiated AFT024 and 2018 stroma at the RNA level based on quantitative real time PCR (ΔΔCt method). (B) Comparison of proliferation rates of founder HSCs cultured on different knock-down AFT024 lines (grey bars) or wild type stroma (AFT024 dark grey bar, 2018 white bar). AFT024 knock down lines included scrambled shRNA control (n=3 independent experiments, 103 trees), single $DLK1^{KD}$ (20% knock down efficiency, n=3 independent experiments, 133 trees), single $DLK1^{KD}$ (90% knock down by FACS, n=4 independent experiments, 163 trees), single DPT^{KD} (99% knock down, RNA level, n=6 independent experiments, 211 trees), single FAP^{KD} (95% knock down, RNA level, n=4 independent experiments, 109 trees), double $DLK1DPT^{KD}$ (n=3 independent experiments, 111 trees), double $DLK1FAP^{KD}$ (n=4 independent experiments, 150 trees), double $DPTFAP^{KD}$ (n=3 independent experiments, 120 trees) and triple $DLK1DPTFAP^{KD}$ line (n=5 independent experiments, 196 trees). (C) Proliferation rates of founder HSCs or early MPPs upon co-culture with wildtype, $DLK1^{KD}$ (n=3 independent experiments, early MPP 108 trees), DPT^{KD} (n=3 independent experiments, 91 early MPP trees) or FAP^{KD} stroma (n=4 independent experiments, 184 early MPP trees). (D) Experimental approach for in vivo transplantation of sorted HSCs cultured on knockdown cell lines prior to injection into sub-lethally irradiated recipients. (E) 1250 CD45.1 HSCs were sorted and co-cultured with different stroma cell lines. After seven days of co-culture, the content of each well was transplanted into a CD45.2 sub-lethally irradiated recipient. The peripheral blood (PB) contribution of donor CD45.1 cells was analyzed at several timepoints up to 32 weeks post transplantation.

Figure 5. Dermatopontin (DPT) is sufficient to improve ex vivo maintenance of long-term repopulating cells. (A) Comparison of proliferation rates of founder HSCs cultured on wildtype (2018: n=5 independent experiments, 264 trees, AFT024: n=7

independent experiments, 290 trees) or virally transduced 2018 stroma overexpressing tdTOMATO – $2018^{tdTOMATO}$ (mock, n=3 independent experiments, 120 trees), DLK1 – 2018^{DLK1} (n=4 independent experiments, 162 trees), DPT – 2018^{DPT} (n=4 independent experiments, 202 trees), FAP – 2018^{FAP} (n=4 independent experiments, 138 trees) or combinations, such as DLK1 and DPT – $2018^{DLK1DPT}$ (n=4 independent experiments, 132 trees), DLK1 and FAP – $2018^{DLK1FAP}$ (n=3 independent experiments, 103 trees), DPT and FAP – 2018^{DPTFAP} (n=4 independent experiments, 150 trees) or all three – $2018^{DLK1DPTFAP}$ (n=4 independent experiments, 216 trees). (B) Generation-based analysis of HSC and their progeny cultured on the same conditions as in A. (C) Similar analysis for early MPPs on wildtype (2018: n=3 independent experiments, 270 trees, AFT024: n=5 independent experiments, 279 trees) or virally transduced 2018 stroma overexpressing DLK1 (n=5 independent experiments, 150 trees), DPT (n=3 independent experiments, 194 trees), FAP (n=3 independent experiments, 90 trees), DLK1 and DPT (n=5 independent experiments, 169 trees), DLK1 and FAP (n=3 independent experiments, 90 trees), DPT and FAP (n=3 independent experiments, 93 trees) or all three (n=3 independent experiments, 90 trees). (D) 125 CD45.1 HSCs were sorted and co-cultured with wildtype lines (AFT024, 2018) or DPT expressing 2018 stroma for seven days before transplanted into sub-lethally irradiated W41 recipients. Donor contribution was calculated in the peripheral blood (PB) and bone marrow (BM) 20 weeks post transplantation. (E) Cell type-specific contribution of donor cells in recipient's bone marrow. (F) Lineage-specific donor contribution in recipients' peripheral blood 20 weeks post-transplant.

Figure 6. Exogenous addition of dermatopontin enhances HSC clonogenicity in both stroma/serum-based and stroma/serum-free culture conditions without influencing cell-cycle progression. (A) Effect of exogenous addition of 1.67ug/mL mouse (mrp, n=4 independent experiments, 166 trees) or human recombinant DPT (hrp, n=4 independent experiments, 155 trees) on proliferation rates of founder HSCs cultured on 2018 stroma. (B) Similar analysis showing the effect of exogenous DPT addition on the HSC progeny for the first three generations. (C) Founder HSC proliferation rates in stroma/serum-free cultures supplemented with 100ng/mL SCF, 100ng/mL TPO without (n=5 independent experiments, at least 30 trees per experiment, 153 trees total) or with 1.67ug/mL mouse DPT (n=5 independent experiments, at least 30 trees per experiment, 190 trees total). (D) HSC proliferation kinetics in stroma/serum-free conditions in the presence of 100ng/mL SCF, 100ng/mL TPO and 1.67ug/mL mDPT. Values indicate the time at which 50% of the cells have divided. Dividing cells from three independent experiments were pooled.

Figure 1

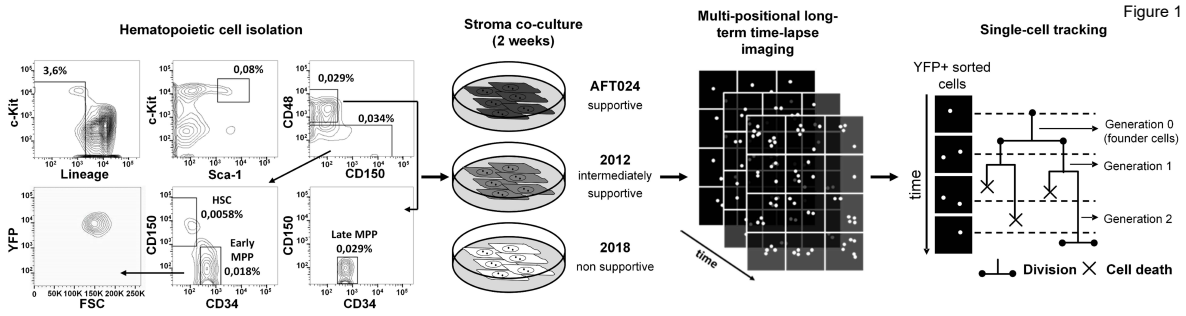

Hematopoietic cell isolation
Stroma co-culture (2 weeks)
Multi-positional long-term time-lapse imaging
Single-cell tracking
3,6%
c-Kit
Lineage
0,08%
c-Kit
Sca-1
0,029%
0,034%
CD48
CD150
HSC 0,0058%
Early MPP 0,018%
CD150
CD34
Late MPP 0,029%
CD150
CD34
YFP
FSC
AFT024 supportive
2012 intermediately supportive
2018 non supportive
time
YFP+ sorted cells
time
Generation 0 (founder cells)
Generation 1
Generation 2
Division
Cell death

Figure 2

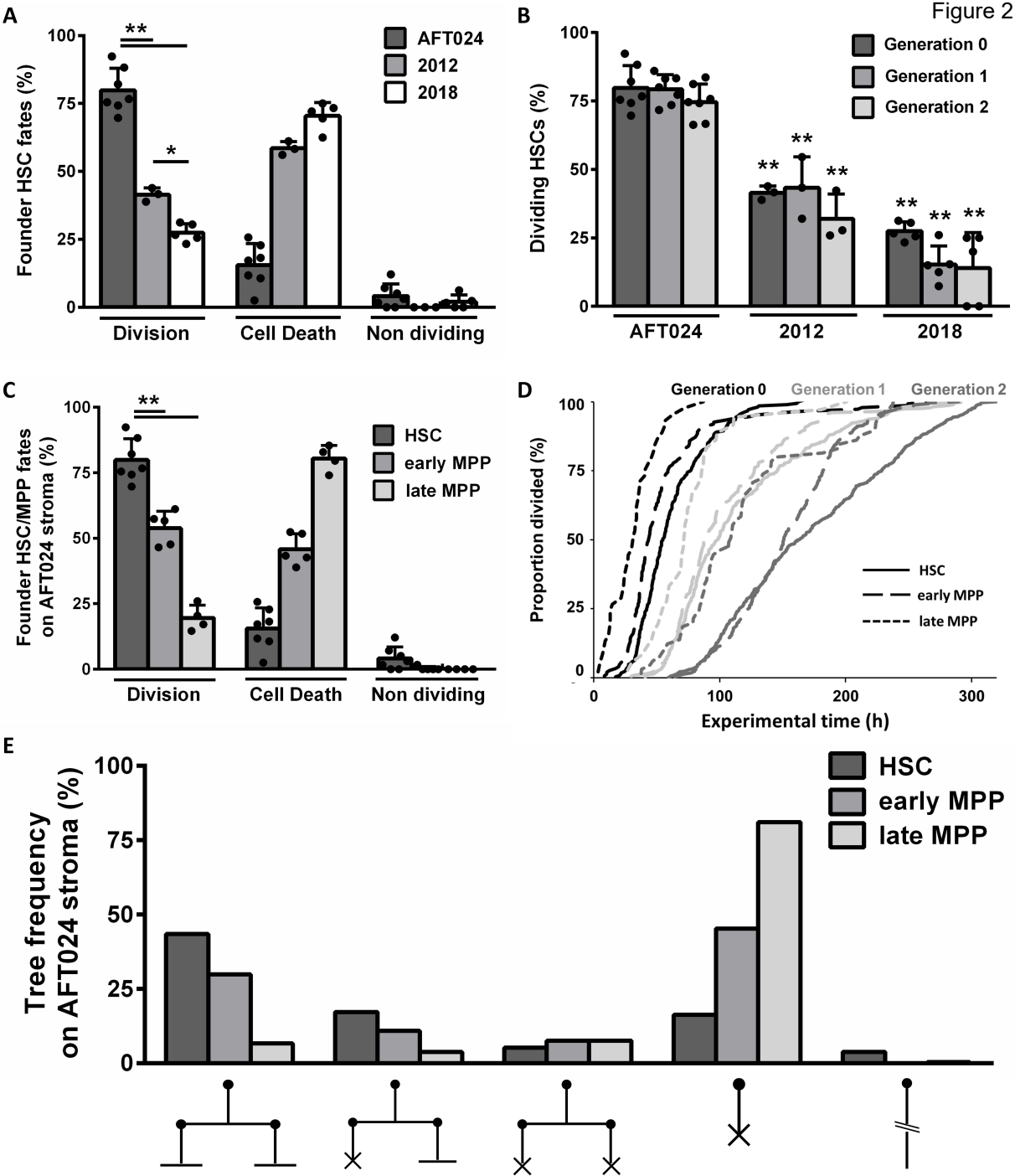
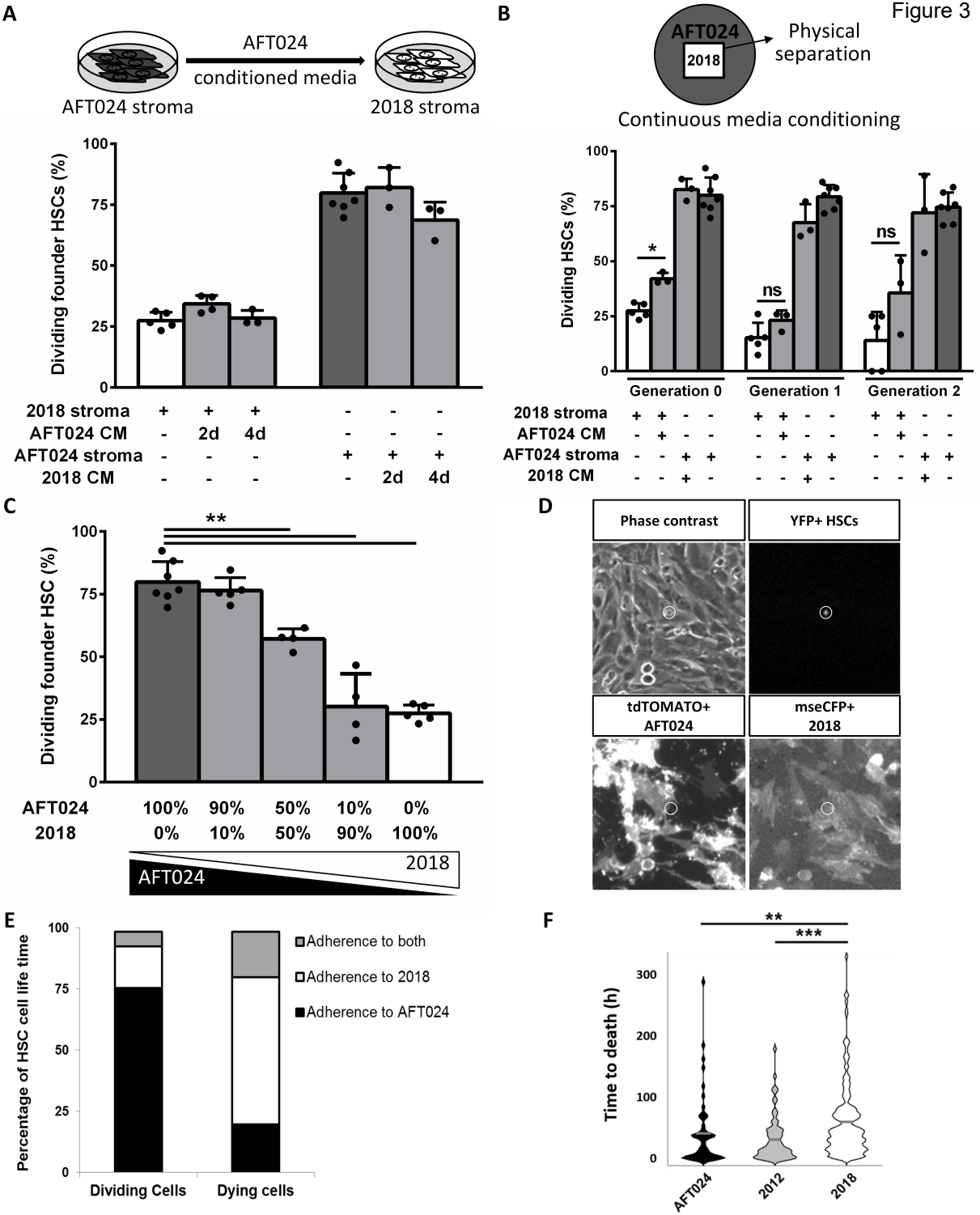
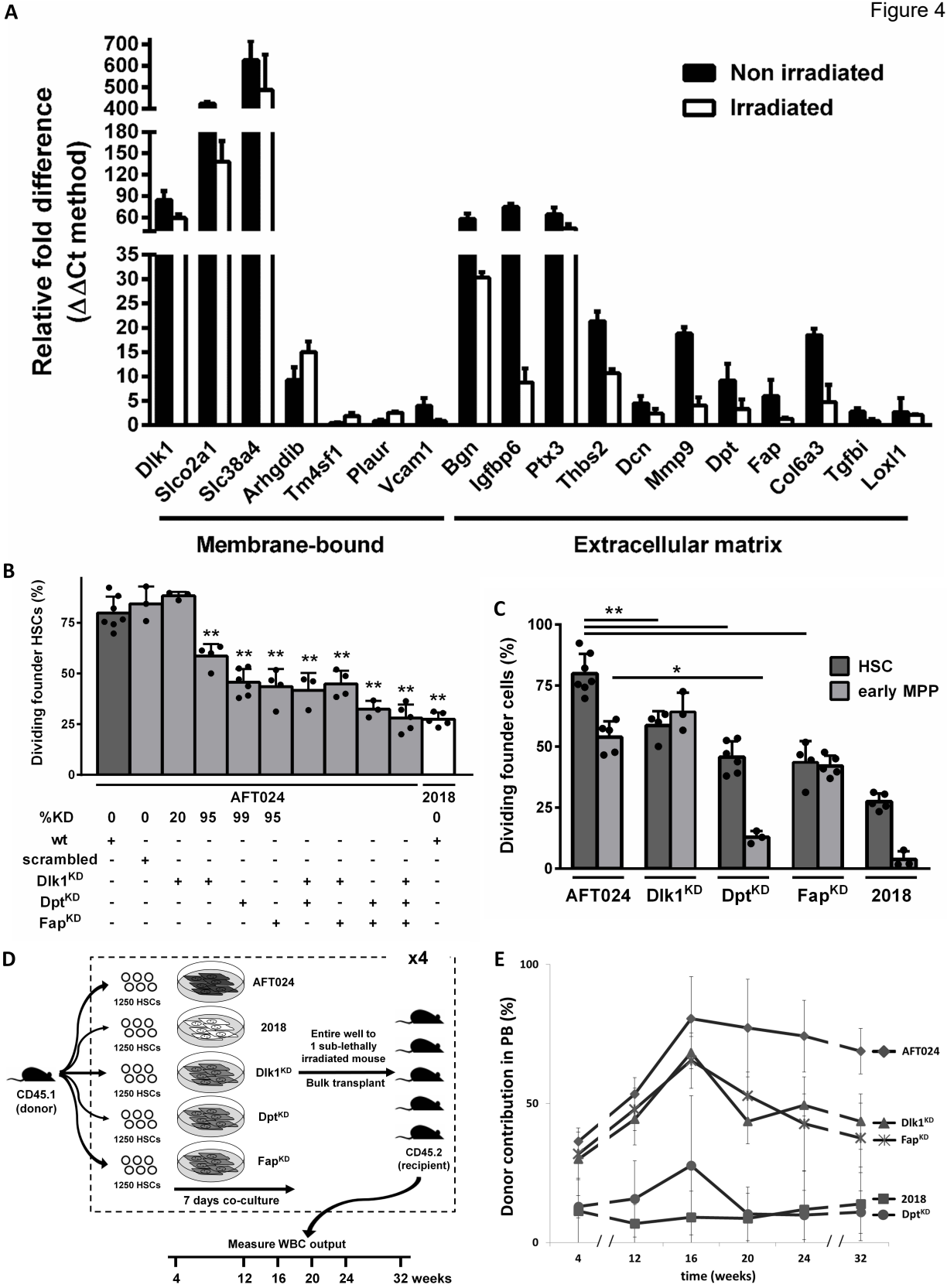

A
Founder HSC fates (%)
AFT024
2012
2018
Division
Cell Death
Non dividing
B
Dividing HSCs (%)
Generation 0
Generation 1
Generation 2
AFT024
2012
2018
C
Founder HSC/MPP fates on AFT024 stroma (%)
HSC
early MPP
late MPP
Division
Cell Death
Non dividing
D
Generation 0
Generation 1
Generation 2
Proportion divided (%)
HSC
early MPP
late MPP
Experimental time (h)
E
Tree frequency on AFT024 stroma (%)
HSC
early MPP
late MPP

Figure 3


A
AFT024
conditioned media
AFT024 stroma
2018 stroma
Dividing founder HSCs (%)
100
75
50
25
0
2018 stroma + + + - - -
AFT024 CM - 2d 4d - - -
AFT024 stroma - - - + + +
2018 CM - - - - 2d 4d
B
AFT024
2018
Physical separation
Continuous media conditioning
Dividing HSCs (%)
*
ns
ns
Generation 0
Generation 1
Generation 2
2018 stroma + + - - + + - - + + - -
AFT024 CM - + - - - + - - - + - -
AFT024 stroma - - + + - - + + - - + +
2018 CM - - + - - - + - - - + -
C
**
Dividing founder HSC (%)
AFT024 100% 90% 50% 10% 0%
2018 0% 10% 50% 90% 100%
AFT024
2018
D
Phase contrast
YFP+ HSCs
tdTOMATO+
AFT024
mseCFP+
2018
E
Percentage of HSC cell life time
Adherence to both
Adherence to 2018
Adherence to AFT024
Dividing Cells
Dying cells
F
**
***
Time to death (h)
300
200
100
0
AFT024
2012
2018


A
Relative fold difference (ΔΔCt method)
Non irradiated
Irradiated
Dlk1
Slco2a1
Slc38a4
Arhgdib
Tm4sf1
Plaur
Vcam1
Bgn
Igfbp6
Ptx3
Thbs2
Dcn
Mmp9
Dpt
Fap
Col6a3
Tgfbi
Loxl1
Membrane-bound
Extracellular matrix
B
Dividing founder HSCs (%)
AFT024
2018
%KD 0 0 20 95 99 95 0
wt + - - - - - +
scrambled - + - - - - -
Dlk1KD - - + + - - -
DptKD - - - - + - -
FapKD - - - - - + -
C
Dividing founder cells (%)
HSC
early MPP
AFT024
Dlk1KD
DptKD
FapKD
2018
D
x4
AFT024
2018
Dlk1KD
DptKD
FapKD
1250 HSCs
CD45.1 (donor)
Entire well to 1 sub-lethally irradiated mouse
Bulk transplant
CD45.2 (recipient)
7 days co-culture
Measure WBC output
4 12 16 20 24 32 weeks
E
Donor contribution in PB (%)
AFT024
Dlk1KD
FapKD
2018
DptKD
time (weeks)

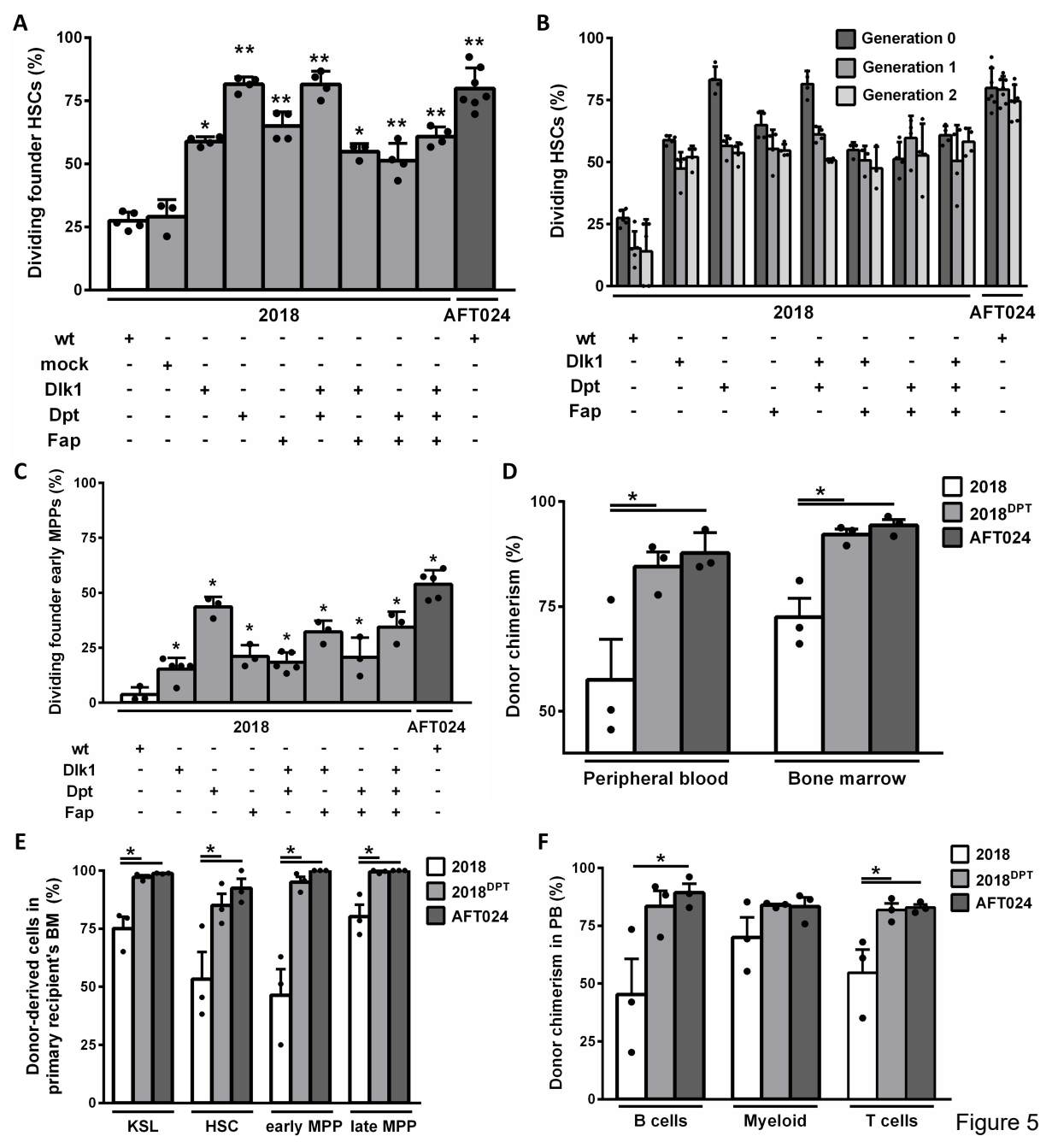

A
Dividing founder HSCs (%)
2018
AFT024
wt
mock
Dlk1
Dpt
Fap
B
Dividing HSCs (%)
Generation 0
Generation 1
Generation 2
2018
AFT024
wt
Dlk1
Dpt
Fap
C
Dividing founder early MPPs (%)
2018
AFT024
wt
Dlk1
Dpt
Fap
D
Donor chimerism (%)
2018
2018DPT
AFT024
Peripheral blood
Bone marrow
E
Donor-derived cells in primary recipient's BM (%)
2018
2018DPT
AFT024
KSL
HSC
early MPP
late MPP
F
Donor chimerism in PB (%)
2018
2018DPT
AFT024
B cells
Myeloid
T cells

Figure 5

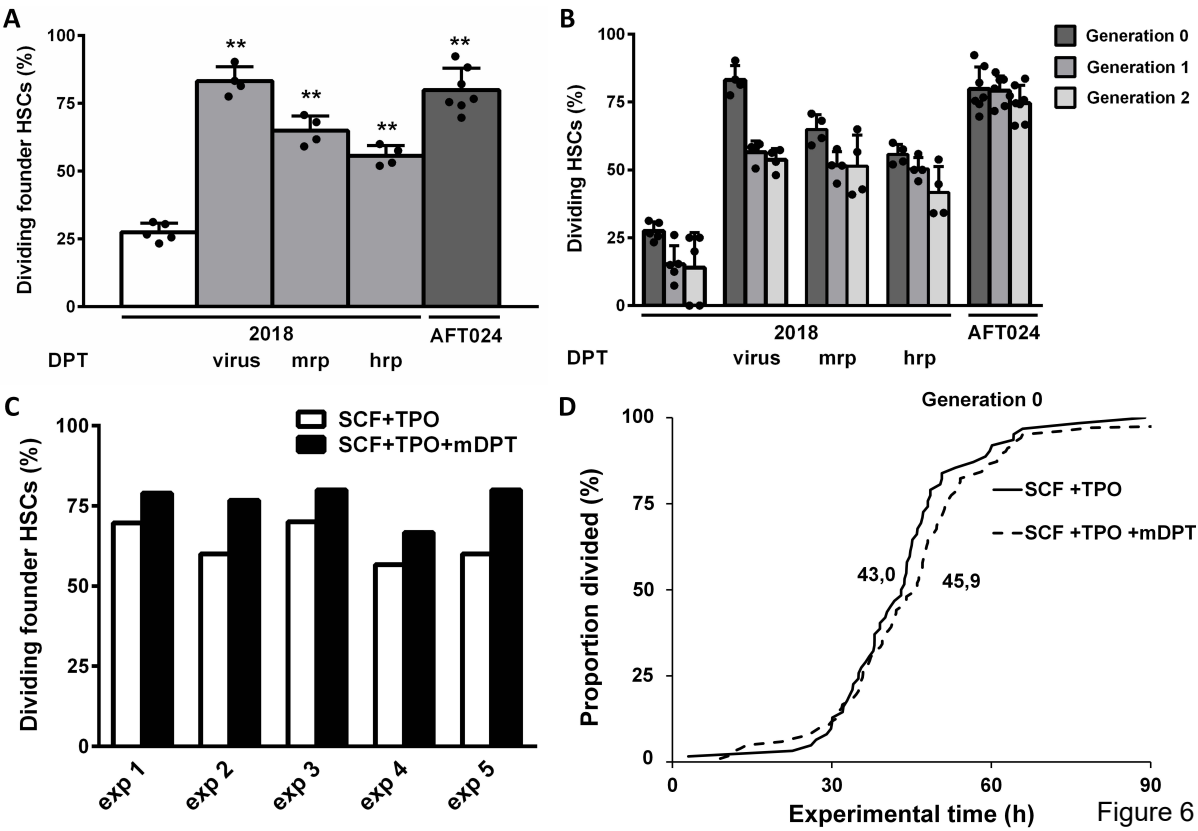

A
Dividing founder HSCs (%)
100
75
50
25
0
**
**
**
**
2018
AFT024
DPT
virus
mrp
hrp
B
Dividing HSCs (%)
Generation 0
Generation 1
Generation 2
C
Dividing founder HSCs (%)
SCF+TPO
SCF+TPO+mDPT
exp 1
exp 2
exp 3
exp 4
exp 5
D
Generation 0
Proportion divided (%)
SCF +TPO
SCF +TPO +mDPT
43,0
45,9
Experimental time (h)

Figure 6

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