TITLE PAGE

EX VIVO EXPANSION POTENTIAL OF MURINE HEMATOPOIETIC STEM CELLS: A RARE PROPERTY ONLY PARTIALLY PREDICTED BY PHENOTYPE

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Running title: HSC expansion *in vitro*

Key point:
Ex vivo self-renewal is an intrinsic property of rare candidate HSCs, with implications for assessments of HSC activity by transplantation.

ABSTRACT

The scarcity of hematopoietic stem cells (HSCs) restricts their use in both clinical settings and experimental research. Here, we examined a recently developed method for expanding rigorously purified murine HSCs *ex vivo.* After three weeks of culture, only 0.1% of cells exhibited the input HSC phenotype, but these accounted for almost all functional long-term HSC activity. Input HSCs displayed varying potential for *ex vivo* self-renewal, with alternative outcomes revealed by single cell multimodal RNA- and ATAC-seq profiling. While most HSC progeny offered only transient *in vivo* reconstitution, these cells efficiently rescued mice from lethal myeloablation. The amplification of functional HSC activity allowed for long-term multilineage engraftment in unconditioned hosts that associated with a return of HSCs to quiescence. Thereby, our findings identify several key considerations for *ex vivo* HSC expansion, with major implications also for assessment of normal HSC activity.

INTRODUCTION

Hematopoietic stem cells (HSCs) are the functional units in bone marrow transplantation (BMT) and gene therapy approaches via their ability for multilineage differentiation and long-term self-renewal[1]. However, restrictions in HSC numbers, the requirement of harsh host conditioning and challenges with how to genetically manipulate HSCs with retained function are barriers for BMT/gene therapy[2]. Due to the lack of reliable models for accurate assessment of human HSCs, mice continue to be the preferred experimental model for studying HSC biology. However, studying murine HSC biology also poses challenges due to the scarcity of HSCs.

Recently, culture conditions supporting efficient maintenance and expansion of murine HSCs were reported[3]. However, the expansion of functional *in vivo* HSC activity over a 28-day period (estimated to 236-899-fold) was not on par with the total cell expansion (>10^4-fold), demonstrating that large amounts of differentiated progeny were generated along with self-renewing HSC divisions[3]. Moreover, candidate HSCs (cHSCs) by phenotype represented only a minor portion of the cells at the culture end-point[3], although assessment of HSC activity based on this might be obscured by previous observations that HSCs change some phenotypes in culture[4]. While *in vitro* differentiation complicates assessments of HSC expansion, it might also be beneficial. For example, while HSCs can support lifelong hematopoiesis after bone marrow transplantation (BMT), they have limited capacity to rescue the host from lethal conditioning[5]. To address this limitation, there is a requirement for progenitor cells that can efficiently supply the host with mature blood cells, such as erythrocytes and platelets[6,7].

When transplanted, *in vivo* HSC activity is typically determined using both quantitative and qualitative parameters[8]. Most commonly, this is achieved by comparing to a known amount of competitor cells[9]. However, although the competitive repopulation assay (CRA) represents a mainstay in experimental HSC biology, it associates with shortcomings often neglected. First, while clinical BMT aims at transplanting large numbers of HSCs[10,11], experimental murine BMT typically use limited HSC grafts, which can strongly influence on HSC function[12]. Secondly, the CRA is from a quantitative perspective rather blunt, with sigmoidal rather than linear characteristics[9]. This limits the boundaries of the effects that can be measured. Thirdly, the CRA assesses HSC activity based on overall hematopoietic chimerism, which might reflect poorly on the actual HSC activity given the vastly different turnover rates

of different blood cells lineages[13]. At the same time, alternative assays such as limit dilution assays or single-cell transplantation assays are cumbersome, expensive and associated with ethical concerns. Newer developments in DNA barcoding technology have overcome many of these issues[14].

Another concern with BMT is the use of aggressive host conditioning to permit engraftment. While eradicating malignant cells is an important part of treatment regimens for leukemic patients, the fates of HSCs in conditioned hosts versus native hematopoietic contexts are dramatically different[15]. But transplantation into unconditioned recipients is inefficient and requires large amounts of transplanted HSCs to saturate available niches[16,17]. To achieve even limited HSC chimerism in unconditioned hosts can have strong long-term therapeutic effects[18] and many experimental approaches might benefit from avoiding host conditioning.

Here, we provide molecular and functional details on HSC differentiation and self-renewal following culture and define a set of parameters critical for *in vivo* assessment of HSC activity. Importantly, we find that HSC heterogeneity was a key influencing factor for HSC expansion even among rigorously purified cHSCs. This determinism has broad implications for experiments involving *in vitro* manipulation of HSCs, but also when assessing normal HSC function by BMT.

RESULTS

In vivo HSC activity is restricted to cells expressing high levels of EPCR

We first assessed the *in vivo* reconstituting activity of cHSCs with different phenotypes. The endothelial protein C receptor (EPCR or PROCR/CD201) is highly expressed on HSCs[19], while CD41 (ITGA2B) was suggested to be functionally relevant for HSC maintenance and homeostasis[20]. Based on expression of EPCR and CD41, we isolated 4 subfractions within BM SLAM LSK cells ($Lin^{-}Sca1^{+}cKit^{+}CD150^{+}CD48^{-/low}$) (Fig 1A) and competitively transplanted them at equal numbers into lethally irradiated recipients (Fig 1B). The reconstitution from the three $EPCR^{high}$ fractions was restricted to the PB myeloid lineages 4-week post-transplantation (Fig 1C), and most of these recipients displayed robust long-term (beyond 16 weeks) multilineage reconstitution with equivalent cHSC chimerism in the BM (Fig 1C-D). $EPCR^{high}CD41^{-}$ cells produced slightly more lymphoid cells than the $EPCR^{high}CD41^{+}$ cells at the endpoint (Fig S1), but the cHSC chimerism in the BM was very similar to other tested $EPCR^{high}$ fractions (Fig 1D). In contrast to Gekas et al.[20], we failed to observe that staining with the CD41 antibody (MWreg30 clone) compromises HSC engraftment. Transplantation of EPCR negative cells failed to associate with either long-term PB or BM cHSC chimerism, demonstrating lack of HSC activity within this fraction (Fig 1C-D).

Next, single $EPCR^{+}$ SLAM LSKs were index sorted (Fig S1B) and expanded *ex vivo* in an F12-Polyvinyl alcohol (PVA) based culture media[21]. At an early stage (8 days, Fig S1C), cultures initiated with SLAM LSK cells with higher EPCR expression proliferated less compared to those with lower EPCR expression. However, after longer expansion (25 days, Fig S1C), the EPCR higher cells generated on average a larger amount of progeny. To evaluate the HSC activity following *ex vivo* expansion and to correlate this directly to EPCR expression levels, the expanded cells from index sorted cultures of cHSCs were collected and transplanted into individual lethally irradiated mice (Fig S1D). While freshly and stringently isolated HSCs normally fail to rescue mice from lethal irradiation[5], we here entertained that culturing of HSCs in addition to inducing self-renewal, would also generate progenitors that could radioprotect the hosts. If so, survival could be used as a proxy for combined hematopoietic stem and progenitor cell (HSPC) activity. Indeed, mice receiving progeny from SLAM LSK EPCR higher cells were more efficiently radioprotected (Fig 1E). Thereafter, we initiated cultures with $EPCR^{high}$ or $EPCR^{lower}$ SLAM LSK cells fractions (Fig S1E). Mice transplanted with cells expanded from $EPCR^{high}$ cells

124 radioprotected 5/5 recipients, while only two out of 5 animals receiving the progeny of
125 EPCR^{low} cells survived long-term. As expected, these surviving mice displayed
126 exclusive test-cell derived myelopoiesis long-term after transplantation (Fig 1F).
127 Taken together, these experiments established that HSC activity was restricted
128 to EPCR^{high} HSCs, and such cells could effectively generate progeny *in vitro* that
129 rescue mice from lethal irradiation. Therefore, we operationally defined our input
130 cHSCs by their EPCR^{high} SLAM phenotype.
131
132 **Phenotypic heterogeneity from *ex vivo* expanded cHSCs**
133 The PVA-dependent cell culture system was reported to efficiently support HSC
134 activity *ex vivo* over several weeks[3]. However, the functional HSC frequency did not
135 appear on par with the total amount of cells generated by the end of the culture period,
136 suggesting the generation also of many differentiated cells[3]. To detail this, we
137 expanded aliquots of cHSCs *ex vivo* for 14-21 days, followed by multicolor
138 phenotyping. From 50 cHSCs, an average of 0.5 million (14 days) and 13.6 million
139 cells (21 days) were generated (Fig S2A) and which associated with a phenotypic
140 heterogeneity that increased over time (Fig S2B). With the assumption that HSCs
141 might retain their cell surface phenotype in cultures, we quantified EPCR^{high} SLAM
142 cells and observed a decrease in their frequency over time, although this was
143 numerically counteracted by the overall proliferation in cultures (Fig 2A). Thus,
144 quantification of cHSCs translated into an average expansion of 54-fold (26- to 85-fold)
145 and 291-fold (86- to 1,273-fold) at 14 and 21 days, respectively (Fig 2A).
146 To gain a deeper understanding of the cells generated in our *ex vivo* cultures,
147 we collected cells from the entire culture and isolated nuclei for single cell multiome
148 sequencing using a commercial platform from 10x Genomics. This approach combines
149 single-cell RNA sequencing and single-cell ATAC sequencing, allowing for integrated
150 analysis of these modalities. Data was analyzed in R[22] using mainly the Seurat[23] and
151 Signac[24] packages, with the SAILERX package[25].
152 Based on the transcriptomic signatures and distal motif identities, we were able
153 to identify not only early hematopoietic stem and progenitor cells (HSPCs), but also
154 many differentiated myeloid cells in the expansion cultures (Fig 2B), however with little
155 evidence for lymphoid differentiation. Assessment of a condensed HSC signatures
156 (Fig S2C) confirmed that the cHSCs were primarily located to cluster 5 (Fig 2D), with

157 trajectory analyses suggesting multiple differentiation pathways from early HSPCs to
158 differentiated myeloid progeny (Fig 2C).

159 Since the EPCR expression also condensed to cluster 5 (Fig S2D), we further
160 investigated whether the expression of EPCR marks cHSCs *ex vivo* by collecting *ex*
161 *vivo* expanded EPCR^{+} cells for single-cell multiome sequencing. These cells displayed
162 less heterogeneity than what we observed for the total culture (Fig 2B), which included
163 a more even distribution of the condensed HSC signature (Fig 2E) as well as the
164 expression of EPCR (Fig S2E). Here, the most pronounced separator for the
165 interrogated cells attributed to the cell cycle position (Fig 2F).

166 To explore if other markers could provide additional phenotypic information for
167 genuine HSCs in cultures, we applied an Fgd5 reporter mouse model previously
168 shown to selectively mark HSCs *in situ*[26]. While the Fgd5 reporter only marked a
169 subset of cells in culture (Fig S2F), we noted heterogeneous expression of CD48
170 within the Fgd5 positive fraction (Fig S2F-G) and that we failed to clearly demarcate
171 in our single-cell multimodal data.

172 Collectively, these results demonstrated robust expansion of cHSCs using the
173 PVA-based culture system, albeit with additional parallel generation of a large number
174 of more differentiated cells. While the identities of most of the cells in cultures could
175 be clearly deduced by their combined gene expression and chromatin accessibility
176 profiles, this approach failed to inform on the heterogeneous expression pattern of
177 CD48 on cHSCs (Fig S2).

178

179 **Functional HSC activity associates to a minor LSK SLAM EPCR^{high} fraction**
180 **within *ex vivo* cultures**

181 Given our inability to distinctly identify cHSCs by their molecular profiles, we next
182 explored the possibility to prospectively isolate HSCs from the cHSC cultures and
183 instead rely on their functional ability to long-term repopulate lethally irradiated hosts
184 for readout. cHSCs were isolated and cultured *ex vivo* for 21 days and the expanded
185 cells were separated into two equal portions: one portion was kept unfractionated,
186 while three subpopulations of CD150^{+}LSKs: EPCR^{high}CD48$^{-/low}$, EPCR^{high}CD48^{+} and
187 EPCR^{-} were sorted from the other portion. The fractions were competitively
188 transplanted into lethally irradiated mice, such that each recipient received fractions

189 equivalent to the expansion from 50 cHSCs (EE50) along with 500,000 competitor
190 WBM cells (Fig 3A).

191 Stable and very high long-term multilineage reconstitution was observed in all
192 recipients of unfractionated cultured cells. By contrast, test-cell derived reconstitution
193 was not recovered from EPCR^{-} cells (Fig 3B and Fig S3). EPCR^{high}CD48^{+} cells
194 produced very high reconstitution levels short-term after transplantation, but the
195 chimerism provided by these cells dropped considerably over time (Fig 3B and Fig S3).
196 This differed strikingly from recipients receiving the minor fraction of EPCR^{high}CD48^{-}
197 $^{/low}$ cells, which presented with robust long-term multilineage reconstitution (Fig 3B and
198 Fig S3). While almost no BM cHSC chimerism was observed from EPCR^{high}CD48^{+}
199 cells, EPCR^{high}CD48$^{-/low}$ cells produced very high chimerism in all evaluated progenitor
200 fractions, including for cHSCs (Fig 3C).

201 These data established that functional *in vivo* HSC activity following culture is
202 restricted to the minor fraction of SLAM LSK EPCR^{high} cells.

203

204 **Quantification of HSC activity in *ex vivo* expansion cultures**

205 Expanded cHSCs presented with a vastly higher reconstitution activity compared to
206 freshly isolated cHSCs, and mostly fell out of range for accurate quantification (Fig 3B).
207 We therefore assessed the impact of lowering the amount of input cHSCs and
208 enhancing the amount of competitor cells. Aliquots of 10 cHSCs were cultured and
209 expanded for 21 days. Cells from each culture (EE10) were next mixed with 2 or 20
210 million (2M or 20M) competitor WBM cells, followed by transplantation (Fig 4A,
211 'individual'). In parallel, we also included a group in which EE50 cHSCs were mixed
212 with 10 million WBM cells and split among 5 lethally irradiated recipients (Fig 4A,
213 'pooled'). This allowed us to assess the variability among input cHSCs,

214 Although robust reconstitution was observed in most recipients of 'individual'
215 cultures (Table 1, Fig 4B-C, Fig S4A-B), the levels of test-cell derived cells varied
216 extensively among these recipients. To quantify the HSC activity in each recipient, we
217 calculated the repopulating units (RUs)[9] for each lineage and recipient separately at
218 the experimental endpoint (Table 1). Short-lived myeloid cells has been proposed as
219 a better indicator of ongoing HSC activity[27], and in agreement with this, we observed
220 a high correlation between the RU^{myeloid} and RUs for cHSCs in the BM (Table 1). A
221 much more consistent HSC activity was observed in between recipients of 'pooled'

222 cells (Table 1, Fig 4B-C). This confirmed that the differential chimerism in recipients of
223 limited cHSC numbers relates to an unresolved cellular heterogeneity of input cHSCs.
224 RUs per initial cHSC were calculated for freshly isolated BM cHSCs (the three
225 EPCR^{high} groups from Fig 1B) and following 3-week culture ('pooled' group). This
226 revealed 461- and 70-fold increases in PB myeloid and BM cHSC chimerism in
227 recipients of *ex vivo* expanded cells, respectively. This is an estimate of the increase
228 of functional HSC activity following *ex vivo* culture (Fig S4G).

229 We next assessed HSC clonality using a lentiviral barcoding approach. Five
230 aliquots of 1,000 cHSCs were transduced with a barcode library[28] 48 hours after
231 culture and then expanded for 19 days. Half of the expanded cells from each well were
232 transplanted into one 'parental' recipient, respectively, while the other half was mixed
233 with each other, with each 'daughter' recipient receiving 1/5 of the mixed cells (Fig 4D-
234 i). As a proxy for the HSC activity, barcodes were extracted from BM test-derived
235 myeloid cells 16-weeks post-transplantation. Following stringent filtering, we retrieved
236 223 unique barcodes in the 'parental' recipients, with a highly variable contribution (0.1
237 - 26.0%, Fig 4E-G). Representation of the same barcode in 'parental' and 'daughter'
238 recipients demonstrates a shared clonal/HSC origin, where more robustly expanding
239 HSCs should have a higher chance of reconstituting more 'daughter' recipients. Our
240 data agreed well with this presumption (Fig 4E), and the most actively shared clones
241 also associated with larger outputs in 'daughter' recipients (Fig 4F).

242 While our initial barcode experiments were designed to assess the *in vitro*
243 expansion potential of cHSCs, a next set of experiments were executed to assess the
244 clonal HSC activity following expansion. For this, cHSCs were *ex vivo* expanded for
245 13 days, provided with barcodes[29] overnight, and transplanted into 4 lethally irradiated
246 recipients (Fig 4D-ii). 16-weeks post-transplantation, we recovered 573 unique
247 barcodes. The median clone sizes of HSCs post-culture were significantly smaller than
248 the pre-culture clones (Fig 4G), which was expected as more clones (573 vs 223) were
249 evaluated in the 'post-culture' setting. However, the frequency of dominantly
250 contributing clones was significantly less abundant (Fig 4H), demonstrating a more
251 even contribution from individual clones in this setting. This is in line with the
252 interpretation that expanded HSCs are functionally more equivalent than input cHSCs.

253 Finally, we assessed how the culture system supports the *in vivo* activity of fetal
254 liver (FL) cHSCs. E14.5 FL-cHSCs were sorted using the same immuno-phenotype
255 as BM cHSCs and cultured *ex vivo*. We observed an average 188-fold increase of

256 cHSCs following 21-days culture (Fig S4D). We used the 'pooled' approach for
257 functional evaluation and competed EE10 cells with either 2 or 20 million WBM cells
258 (Fig S4C). While *ex vivo* expanded FL-cHSCs contributed considerably to multilineage
259 reconstitution (Fig S4E), we observed compared to adult cHSCs 5.7- and 6.6-fold
260 lower reconstitution from FL-cHSCs within PB myeloid cells and cHSCs, respectively
261 (Fig S4F-G).
262 Taken together, these experiments demonstrated robust increases in HSC
263 activity following culture of cHSCs, albeit slightly less for FL-cHSCs. However, clonal
264 barcode assessments revealed substantial variation in *ex vivo* expansion potential of
265 even stringently purified input cHSCs.
266
267 ***Ex vivo* expanded cHSCs returns to a quiescent state following engraftment in**
268 **unconditioned recipients**
269 Successful engraftment in an unconditioned setting requires large numbers of
270 HSCs/HSPCs[16], which reportedly can be accommodated by PVA-cultured HSCs[3]. To
271 test this, we transplanted EE100 CD45.1$^+$ cHSCs into lethally irradiated or
272 unconditioned CD45.2$^+$ recipients (Fig S5A). In lethally irradiated hosts, cultured cells
273 reconstituted >90% of PB cells, including with robust contribution of donor cells to the
274 myeloid lineages. By contrast, most of the unconditioned recipients lacked long-term
275 reconstitution (Fig S5B). Given that CD45 mismatching might be a sufficient
276 immunological barrier to prevent HSPC reconstitution[30], we instead transplanted
277 expanded cells into unconditioned F1 CD45.1$^+$/CD45.2$^+$ hosts (Fig 5A). Clear long-
278 term multilineage engraftment was observed in all these hosts (Fig 5B).
279 In the BM, cHSCs exist for the most part in a quiescent state, which contrasts
280 the situation in cultures (Fig S5C). To investigate to what extent *ex vivo* expanded
281 cHSCs could return to quiescence following transplantation, we expanded 100
282 CD45.2$^+$ cHSCs for 21 days and stained all their progeny with the proliferation-tracking
283 dye Cell Trace Violet (CTV) prior to transplantation into CD45.1$^+$/CD45.2$^+$ hosts. As a
284 control, we transplanted CTV labelled unmanipulated CD45.2$^+$ cKit-enriched BM cells
285 (Fig 5C). In these animals, splenic CD4$^+$ enriched cells were co-transplanted as a
286 control for non-dividing cells (Fig S5D). Reconstitution and cHSC fates were monitored
287 biweekly for up to 8-weeks post-transplant. Although the phenotypic cHSC contribution
288 in recipients receiving *ex vivo* expanded cells outnumbered the contribution from
289 unmanipulated cells two-weeks post-transplantation, the levels became comparable

290 later (Fig 5D). Recipients from both groups showed high levels of CTV signal two-
291 weeks post-transplantation, with the majority of donor EPCR^{+} cHSCs having
292 undergone fewer than 3 cell divisions. Over time, increasing numbers of cHSCs had
293 proliferated within both groups and with similar patterns (Fig 5E-F, Fig S5E), with a
294 slightly higher number of undivided cHSCs retrieved from *ex vivo* expanded HSCs at
295 all time points (Fig 5E). The most quiescent cHSCs associated with higher EPCR
296 levels (Fig S5F-G), again attesting to the usefulness of EPCR to predict robust HSC
297 activity.

298 Therefore, *ex vivo* expanded cHSCs can efficiently engraft immunologically
299 matched hosts without conditioning. Under such conditions, expanded cHSCs can
300 rapidly return to quiescence following transplantation.

301 **DISCUSSION**

302 Although many efforts have aimed to *in vitro* expand murine HSCs, the results have
303 for the most part been disappointing[31]. A recent study suggested that the substitution
304 of undefined serum products with the synthetic polymer PVA is one key determinant
305 towards this goal[3]. Here, we detailed this culture system further. We focused on the
306 requirements of candidate input HSCs, the reproducibility of the system, the cellular
307 output and the functional *in vivo* performance of the *in vitro* replicating HSCs. Several
308 observations that we believe are valuable not only for future applications of the PVA
309 culture system, but also for HSC biology in general, emerged from our work.

310 While the PVA-system supports HSC activity even from unfractionated BM cell
311 preparations[32], the long-term repopulating HSCs (LT-HSCs) in cruder input cell
312 preparations is very low. While having its own advantages, this makes it difficult to
313 track the fates of cHSCs. Therefore, we here consistently initiated cultures with more
314 stringently purified cHSCs. As a starting point, we used cHSCs isolated based on a
315 current standard SLAM phenotype ($Lin^{-}Sca^{+}cKit^{+}CD48^{-}CD150^{+}$)[33]. In agreement with
316 previous reports[34], we observed that CD201/EPCR further enriches for LT-HSCs, a
317 knowledge that has so far not been adopted as a standard in the field[33]. This applied
318 both to cHSCs isolated directly from the BM and following culture. In fact, despite that
319 HSCs change many phenotypes in culture[4], the LT-HSC activity following culture was
320 remarkably enriched not only in the EPCR positive fraction, but also for other
321 phenotypic attributes of unmanipulated LT-HSCs. Importantly, such cHSCs
322 represented only a very minor fraction in cultures (0.6% and 0.1% of 2- and 3-week
323 cultures, respectively) but contained all LT-HSC activity, with the massive overall
324 expansion in cultures leading to substantial net expansions of such cells (100- to
325 1,200-fold).

326 While we gained insights into the dynamics of *ex vivo* differentiation of cHSCs
327 into mature lineages by simultaneously measuring ATAC signal and RNA transcripts
328 in individual nuclei using single-cell multiome sequencing, the limited sensitivity of this
329 method hindered the identification of more homogeneous populations of bona fide
330 HSCs within the sorted EPCR+ cells. Therefore, we assessed the functional *in vivo*
331 HSC activity in most of our subsequent work. While our clonal barcoding experiments
332 unequivocally demonstrated HSC self-renewal, the large spectra in expansion
333 potential of individual cHSCs was noteworthy. This variation was reduced when
334 assessing HSC activity following culture and aligns with the interpretation that the

335 offspring of cHSCs that self-renew robustly in cultures exhibit less differences.
336 Nonetheless, the demonstrated *in vitro* self-renewal refutes alternative views in which
337 the culture process would mainly act to enhance the reconstitution capacity of
338 individual HSCs, for instance by altering their homing properties and/or by the
339 conversion of more differentiated progenitors into cells with LT-HSC activity. Therefore,
340 our data support the view that this (or other) culture systems might not support all
341 types of LT-HSCs previously alluded to[35]. Further support to this notion can be derived
342 from our data on FL-cHSCs, which did not expand better than adult HSCs in the PVA
343 system, despite that FL HSCs repopulate mice more efficiently than adult HSCs when
344 unmanipulated[36]. In a broader sense, this associates also with the general concept of
345 self-renewal and that has recently been highlighted in studies on native hematopoiesis,
346 where distinct but only slowly contributing LT-HSCs co-exist with more active
347 progenitors that can presumably also execute self-renewal divisions[37-39]. It appears
348 that many aspects of this structure are re-created in cultures and, as we show, this
349 requires as input the normally very slowly dividing and presumably most primitive LT-
350 HSCs.

351 While the parallel self-renewal and differentiation in HSC cultures represent a
352 fundamental difference compared to other *in vitro* stem cell self-renewal systems (e.g.,
353 for ES/iPS cells), where preferential self-renewal can be achieved, the differentiation
354 of HSCs can be taken advantage of. We observed that even single cultured HSCs can
355 enhance the speed of donor reconstitution and alleviate the effects of myeloablative
356 conditioning, and the parallel self-renewal and differentiation can also be harnessed
357 to approach HSC fate decisions at a molecular level[40]. This was exemplified by our
358 single-cell multimodal experiments, which allowed for determinations of the
359 differentiation trajectories of cHSCs in the culture system and their associated
360 molecular features. However, as the expansion of genuine LT-HSC activity is far from
361 unlimited, a shortage of HSCs is by all likelihood still an obstacle for larger-scale
362 screening efforts such as genome-wide CRISPR screens and larger small molecule
363 screens.

364 During our studies, we encountered several aspects of the CRA that are often
365 neglected. First, the HSC activity when transplanted at limited numbers highlighted
366 large variations in between recipients, which is in line with previous extensive single
367 cell transplantation experiments[41-45]. While raised previously[46], taking into account not
368 only the repopulating activity but also clonal aspects has not reached wide use.

369 Emerging barcoding technologies, by which many clones can be evaluated in a single
370 mouse, makes this easier. Secondly, the progeny from a relatively small number of
371 cultured cHSCs (10-50 cells) vastly outcompeted “standard” doses of competitor cells,
372 making quantification unreliable/impossible. This contrasted results from freshly
373 isolated cHSCs transplanted at the same doses used to initiate cultures. While this
374 concern could be overcome by adjusting the graft composition (e.g. enhancing the
375 number of competitor cells), extensive variation still existed among cultures that could
376 be eliminated by using larger amounts of input cells and splitting the contents into
377 individual recipients. This should have relevance also for normal HSC biology by
378 highlighting intrinsic rather than stochastic regulation as the central determinant for
379 persistent HSC function. A third consideration relates to the definition of ongoing HSC
380 activity. We and others have previously established that this is best mirrored by the
381 HSC contribution to myelopoiesis[47,48]. When we assessed cultured EPCR^{+}CD48^{+} cells
382 (with predominantly transient multilineage activity), we observed prominent long-term
383 T cell contribution but limited myeloid reconstitution. This should not be confused with
384 the concept of HSC lineage-bias, but rather reflects that the pool of T cells *in vivo* can
385 be maintained by homeostatic proliferation and thus do not require continuous input
386 from HSCs. Thus, short-lived non-self-renewing multipotent progenitors, which are
387 generated in large numbers in culture, can contribute extensive (lymphoid) offspring
388 in the long-term but should not be mistaken for ongoing HSC activity. This
389 consideration is valid also for competitor grafts in the CRA (in the form of WBM cells),
390 which contain numerous progenitor cells that can rapidly and persistently contribute to
391 lymphopoiesis. Thus, as opposed to the classic CRA, where the overall donor
392 contribution is assessed[9], the contribution to short-lived lineages reflects better the
393 ongoing HSC activity, with the contribution to lymphoid lineages serving as a
394 qualitative parameter for multipotency. In agreement with previous viral barcoding
395 studies on HSCs[14] we observed that when cultured HSCs are barcoded and assessed
396 long-term, there is a strong correlation between barcodes in BM progenitors and
397 myeloid cells, while the pool of mature B cells contain clones that cannot be recovered
398 in other lineages (data not shown).

399 Successful repopulation of non-conditioned hosts is based on the suggestion
400 that large numbers of HSCs are needed to saturate those few BM niches available for
401 engraftment[18,49]. Avoiding toxic myeloablation has apart from evident clinical
402 implications also a precedence in experimental murine HSC biology, where lethal

403 myeloablation enforces vigorous HSC proliferation following transplantation[12], but
404 which contrasts native contexts[12,37,38]. We observed that the CD45.1/CD45.2
405 differences are sufficiently immunogenic to mediate long-term graft failure in the non-
406 conditioned setting, which in hindsight has been alluded to previously[18,30,50]. Thus, by
407 matching hosts for CD45-isoforms, we could successfully obtain low-level (2-5%) long-
408 term multilineage chimerism in adult non-myeloablated hosts from only 100 cHSCs.
409 Intriguingly, despite being activated to proliferate *in vitro*, many cHSCs rapidly returned
410 to a quiescent state *in vivo*, with the most dormant cHSCs exhibiting the most stringent
411 HSC phenotype (EPCR^{high} SLAM LSKs). The possibility for non-conditioned
412 transplantation should complement genetic lineage tracing models aimed at exploring
413 both normal and malignant hematopoiesis in more native/physiological contexts[12,37,38].
414 In summary, we here characterized and detailed murine HSCs as they self-
415 renew and differentiate *in vitro*. Although several aspects of the PVA system remain
416 to be explored, a powerful *in vitro* self-renewal system has been a long-sought goal in
417 HSC biology where HSCs have been notoriously difficult to uphold in an
418 undifferentiated/self-renewal state. The merger of this system with recent advances in
419 transplantation biology, genome engineering and single-cell techniques holds promise
420 for many exciting discoveries of relevance to both basic and more clinically oriented
421 hematopoietic research.

422 **MATERIALS AND METHODS**

423 **Mice**

424 Adult (8-10-week-old) C57Bl/6N (CD45.2^{+}) female mice were purchased from Taconic.
425 Transgenic Fgd5-ZsGreen-2A-CreERT2 mice[26] (JAX:027789) was a kind gift from
426 Derrick Rossi. Mice were maintained in the animal facilities at the Biomedical Center
427 of Lund University and kept in environment-enriched conditions with 12-hour light-dark
428 cycles and water and food provided *ad libitum*. All experimental procedures were
429 approved by a local ethical committee (permits M186-15 and 16468/2020). All efforts
430 were made to reduce the number of experimental animals and suffering.

431

432 **BM transplantation**

433 All mice used as recipients were 8 - 12 weeks of age. For conditioned recipients, mice
434 were lethally irradiated (950 cGy) at least 4 h prior transplantation. The conditioned
435 mice received prophylactic ciprofloxacin (HEXAL, 125 mg/L in drinking water) for 2
436 weeks beginning on the day of irradiation. All transplantations were performed through
437 intravenous tail vein injection. The amount of transplanted test cells and
438 competitor/support cells is described in the results section.

439

440 ***In vitro* HSC culture**

441 *In vitro* HSC cultures were performed using F12-PVA based culture conditions as
442 previously described[3]. In brief, cHSCs (HSCs (EPCR^{high}CD150^{+}CD48$^{-/low}$ LSKs) were
443 sorted into 96-well flat-bottom plates (Thermo Scientific) coated with 100 ng/ml
444 fibronectin (Sigma) for >1 hour at 37 °C prior to sorting. HSCs were sorted into 200 µl
445 HSC media (Supplemental Table 1) and expanded at 37°C with 5% CO_2 for up to 21
446 days. The first media changes were performed 5 days (for ⩾ 50 initial HSCs), 10 days
447 (for 10 initial HSCs) or 15 days (for single-cell cultures) after initiation of cultures and
448 then every 2 days. For pre-cultures associated with lentiviral transduction, the first
449 media change was performed 24h after transduction and then as above. Cells were
450 split at a 1:2-1:4 ratio into new fibronectin coated plates when reaching 80-90%
451 confluency (normally every 4 days after the first split). In order to expand cHSCs to
452 reach a workable number as well as avoiding vast numbers of differentiated cells
453 generated after very long-term of culture (f.i. up to 4 weeks), the cHSCs were kept
454 expanding *ex vivo* for 3 weeks as a standard protocol. Following 14- and/or 21-day

455 expansion, cellularity was assessed using an Automated Cell Counter (TC20, BioRad)
456 and used for flow cytometric analyses and/or transplantation.
457
458 **Cell preparation**
459 Mice were euthanized by cervical dislocation after which bones (both right and left
460 tibias, femurs, and pelvis) or spleens were extracted. Fetal cells were extracted from
461 livers at E14.5. Bones were crushed using a mortar and pestle and BM cells were
462 collected in ice-cold phosphate-buffered saline (PBS, Gibco) with 1% fetal bovine
463 serum (Sigma-Aldrich) (FACS buffer), filtered (100 µm mesh) and washed. FL or
464 spleen cells were brought into single cell suspension by grinding through a 100 µm
465 mesh. BM or FL cells were cKit-enriched by anti-cKit-APCeFluor780 (eBioscience)
466 staining and spleen cells were CD4-enrihced by anti-CD4-APC-Cy7 staining, followed
467 by incubation with anti-APC conjugated beads (Miltenyi Biotec). Magnetic separation
468 was performed using LS columns according to manufacturer's instructions (Miltenyi
469 Biotec). cKit-enriched BM or FL cells were washed and stained with fluorescently
470 labeled antibodies for analysis or sorting.
471 Peripheral blood (PB) chimerism analysis after transplantation was done as
472 previously described[39]. In brief, blood was drawn from tail vein into FACS buffer
473 containing 10 U/ml Heparin (Leo). After incubating with an equal volume of 2% dextran
474 (Sigma) in PBS at 37 °C, the upper phase was collected and erythrocytes were lyzed
475 using an ammonium chloride solution (STEMCELL Technologies) for 3 min at room
476 temperature, followed by washing.
477 For analysis and/or sorting after *ex vivo* expansion, cultured cells were
478 resuspended by pipetting and collected using FACS buffer. An aliquot was taken for
479 cell counting using an Automated Cell Counter (TC20, BioRad).
480
481 **Flow cytometry analysis and FACS sorting**
482 Cells were kept on ice when possible, with the FACS buffer kept ice-cold. Staining,
483 analysis and sorting were performed as previously described[12]. In brief, cells were
484 resuspended in Fc-block (1:50, 5 x 10^6 cells/50 µl, InVivoMab) for 10 min and then for
485 20 min with a twice concentrated antibody staining mixture (Supplemental Tables 2-
486 10) at 4 °C in dark. In case biotinylated lineage markers were included, a secondary
487 staining with streptavidin BV605 was performed for 20 min (1:400, 5 x 10^6 cells/100 µl,

488 Sony) at 4 °C in dark. After a final wash, the cells were resuspended in FACS buffer
489 containing propidium iodide (1:1000, Invitrogen).
490 All FACS experiments were performed at the Lund Stem Cell Center FACS
491 Core Facility (Lund University) on Aria III and Fortessa X20 instruments (BD). Bulk
492 populations were sorted using a 70-micron nozzle, 0.32.0 precision mask, and a flow
493 rate of 2 - 3K events/s. HSCs for single cell culture were index sorted. FACS analysis
494 was performed using FlowJo version 10.8.0 (Tree Star Inc.).
495
496 **Multiome single-cell sequencing**
497 ***Library preparation:*** Multiome sequencing experiments were performed at the
498 Center for Translational Genomics (Lund University) using the Chromium Next GEM
499 Single Cell Multiome ATAC + Gene Expression Reagent Bundle kit according to
500 manufacturer's instructions (10x Genomics). 40,000 viable cells or EPCR$^+$ viable cells
501 were sorted from *ex vivo* expanded cultures for multiome single-cell sequencing. Data
502 has been uploaded to GEO under accession number GSE234906
503 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE234906) and will be
504 available upon publication.
505
506 ***Bioinformatic analysis:***
507 All accompanying code for the single-cell multiome analysis post Cell Ranger can be
508 found at this Github repository (https://github.com/razofz/DB_QZ_multiome), including
509 conda specifications for the environments used with exact versions of all packages.
510
511 *i. Preprocessing*
512 The count matrices were generated with the Cell Ranger ARC tool (v2.0.0), aligned to
513 the reference genome mm10. The aggregated dataset (containing data for both
514 samples) were then loaded into Signac/Seurat in R where non-standard chromosomes
515 were removed and the Seurat object split into the two samples, here named Diverse
516 and Immature, which corresponds to whole culture or sorted EPCR$^+$ cells, respectively.
517 The data were then filtered on UMI, gene and peak counts, as well as nucleosome
518 signal and TSS enrichment, with separate thresholds for the two samples. The ATAC
519 features were classified as either distal or proximal peaks, and split accordingly.
520 Proximal peaks were defined as a ± 2 kbp region around the transcription start site
521 (TSS). The TSS positions were retrieved from GREAT's website, and GREAT v4

522 mm10 version was used. All peaks not classified as proximal received the
523 classification "distal". Motifs were then identified for distal and proximal peaks
524 separately[51].
525
526 *ii. Downstream processing and joint-modality dimensionality reduction*
527 The samples were processed separately. Since SAILERX uses pre-existing
528 dimensionality reduction and clusters for the RNA modality, the RNA data was
529 normalized and scaled, highly variable genes (HVGs) were found, principal component
530 analysis (PCA) was run and clustering was performed, all with the Seurat standard
531 functions with default parameters. After that, relevant data was extracted (metadata
532 including RNA clusters, PCA embeddings, a whitelist of non-filtered out cell barcodes,
533 ATAC peak count matrix) and with it an hdf5 file similar to the example files provided
534 by the SAILERX repository was created (https://github.com/uci-cbcl/SAILERX). A
535 SAILERX model was then trained on the generated hdf5 files (still sample-wise)
536 according to their provided instructions. The resulting embeddings from the SAILERX
537 joint-modality dimensionality were then exported and imported into the respective
538 Seurat objects. In Seurat, the embeddings were used for graph construction, clustering
539 and constructing UMAPs. Scores for cell cycle gene signatures were calculated and
540 visualized (Fig 2E for the Immature sample) using Seurat's corresponding standard
541 functions with default parameters.
542
543 *iii. Differential gene expression*
544 Differential gene expression testing was performed on the resulting clusters using
545 Seurat's FindAllMarkers function with the HVGs found above. This analysis was used
546 to calculate HSC signature score as described below (v. HSC signature).
547
548 *iv. ATAC processing and cluster annotation*
549 Motif activity scores were identified with chromVAR[52] for distal and proximal peaks
550 respectively, still sample-wise. Differentially active motifs (DAMs) were then identified
551 for each cluster. Annotation of clusters in terms of cell type was then performed
552 manually with the help of DEGs and DAMs (Fig 2B), guided by preexisting data on the
553 expression profiles of cell types using the Enrichr platform
554 (https://maayanlab.cloud/Enrichr/).
555

556 *v. HSC signature*

557 A 13 gene condensed HSC signature list (Fig S2C) was obtained by mining genes
558 associated to cluster 5 with publicly available gene expression data of HSCs from the
559 ImmGen consortia and Bloodspot (https://servers.binf.ku.dk/bloodspot/). This
560 signature was then used to calculate a score with Seurat's AddModuleScore function.
561 A classification for expression of this gene signature was performed with a slightly
562 modified version of Seurat's CellCycleScoring function, where instead of three
563 classifications (S, G2M or Undecided/G1) two classifications were used (expressed or
564 not expressed). This classification was then used to create the contours used in Fig
565 2D-E, and the signature score used for coloring the points in the same plots.

566

567 *vi. Trajectory inference in the Diverse sample*

568 Trajectory inference was performed for the Diverse sample with the slingshot
569 package[53]. As initiating cluster, the cluster 5 was assigned (the earliest cluster), and
570 end clusters chosen were clusters 4, 1, 6 and 7. The slingshot function of slingshot
571 was used with otherwise default parameters. A joint UMAP with the four resulting
572 trajectories was constructed (Fig 2C). To circumvent the problem of interfering color
573 scales, only the top 20% expressing cells for each marker was chosen for coloring,
574 and overlapping cells between these sets were excluded.

575

576 **Assessment of HSC heterogeneity via DNA barcoding**

577 Lentiviral barcode libraries were purchased from AddGene (No. 115645 and No.
578 83993) and amplified according to instructions[28,29]. AddGene 83993 is a Crispri gRNA
579 library that was repurposed here for the purpose of lentiviral barcoding (e.g. no Cas9
580 was co-expressed). These libraries were kind gifts from Samantha Morris and
581 Jonathan Weissman.

582 For pre-culture labelling, five wells containing 1000 CD45.1^{+} HSCs each were
583 sorted and cultured for 48 h. Cells were then transduced with the AddGene 115645
584 library (containing >5737 unique barcodes) overnight with approximately 35%
585 transduction efficiency, which was followed by regular culture procedures. After 19
586 days, expanded cells were collected from each initial wells separately. Half of the
587 expanded cells were transplanted into individual 'parental' CD45.2^{+} recipients and the
588 rest were mixed together and transplanted into five 'daughter' CD45.2^{+} recipients.
589 Each 'parental' or 'daughter' recipient received an estimated expansion equivalent to

590 500 cHSCs. For post-culture labelling, 3000 cHSCs were sorted from CD45.1$^+$ mice
591 and expanded for 13 days. Thereafter, the cells were transduced with the AddGene
592 83993 library (containing >10090 unique barcodes) overnight at an approximate
593 density of 100,000 cells/well. Half of the cultured and transduced cells were
594 transplanted into 5 CD45.2$^+$ WT recipients. Each recipient received an estimated
595 expansion equivalent of 300 cHSCs, of which approximately 15% were transduced
596 according to assessments of transduction efficiencies. Barcode labelled cells were
597 collected and analyzed from four recipients. For both pre- and post-culture labeling
598 experiments, recipients were lethally irradiated and provided with 500,000
599 unfractionated CD45.2+ WBM cells each as support. At the experimental end points
600 (16 weeks after transplantation), barcode labelled BM myeloid cells were sorted into
601 200 µl RNA lysis buffer (Norgen Biotek Corp) ("pre-culture" barcoding) or pelleted for
602 genomic DNA extraction ("post-culture" barcoding) and proceeded to library
603 preparation and sequencing analysis.
604 RNA was isolated according to the protocol of Single Cell RNA Purification Kit
605 (Norgen Biotek Corp) and cDNA were synthesis using qScript cDNA SuperMix
606 (Quantabio). Genomic DNA was extracted using PureLink Genomic DNA Mini Kit
607 (Invitrogen). The 8- (pre-culture) and 21-base pair (bp) barcodes (post-culture) were
608 amplified by PCR using Q5 High-Fidelity DNA Polymerase (New England Biolabs).
609 Primers used are listed in Supplemental Table 11. Adapters containing index
610 sequences (Supplemental Table 10) were added by PCR for 7 cycles. After each PCR
611 amplification step, the products were cleaned up using SPRIselect beads (Beckman
612 Coulter). Purified libraries were run on Agilent High Sensitivity DNA Kit chip (Agilent
613 Technologies) to verify the expected size distribution, quantified by Qubit dsDNA HS
614 Assay Kit (Thermo Fisher Scientific), and pooled at equimolar concentrations. Pooled
615 libraries were loaded on an Illumina MiSeq Reagent Nano Kit v2 flow cell following
616 protocols for single-end 100-cycle sequencing. FASTQ files were further assessed
617 with FastQC (v.0.11.2), including read count, base quality across reads, and guanine
618 and cytosine (GC) content per sequence.
619 After extracting the reads of each barcode, the frequency in each recipient was
620 calculated and barcodes with a frequency less than 0.1% were considered background
621 reads. This is an arbitrary cut-off. Barcodes present in more than one 'parental'
622 recipient were excluded from analysis (7 % and 2% of barcodes pre- and post-culture,
623 respectively) to avoid the possibility of including HSCs transduced independently with

624 the same barcode or potential over-represented barcodes. Reads were normalized to
625 10^6 total reads per recipient followed by statistical analyses.
626
627 **Cell Trace Violet labelling for *in vitro* and *in vivo* HSC proliferation tracing**
628 CD45.2$^+$ cultured cells, cKit-enriched BM cells or CD4-enriched spleen cells were
629 collected, washed and pelleted. Cells were resuspended to a final concentration of 5
630 x 10^6 cells/ml and labelled with 1 µM CTV (Invitrogen) in PBS for 10 min at 37 °C. The
631 reaction was stopped by adding same volume of ice-cold FACS buffer and washing
632 again with FACS buffer. For *in vitro* proliferation tracing, 10^5 CTV labeled cKit-enriched
633 BM cells were plated into each well containing F12-PVA culture media (Supplemental
634 Table 1). At each time point, half of the cultured cells were collected and stained for
635 evaluating cHSC CTV signals (Supplemental Table 8) while the remaining cells were
636 kept in culture for continue expansion. For *in vivo* proliferation tracing, CTV labeled
637 EE100 cultured cells or 5 x 10^6 cKit-enriched BM cells were mixed with 2 x 10^6 CTV
638 labeled CD4-enriched spleen cells and transplanted into one CD45.1$^+$/CD45.2$^+$
639 unconditioned recipients. For analysis, BM cells were cKit-enriched and stained
640 accordingly (Supplemental Table 9). CD4 positive spleen cells were collected from the
641 same recipient as a control for positive signal/undivided cells (Supplemental Table 10).
642
643 **Statistical test**
644 Statistical analyses were performed using Microsoft Excel and Graphpad Prism 9.
645 Significance was calculated by Mann-Whitney tests. Statistical significances are
646 described in figure legends for the relevant graphs. In all legends, n denotes biological
647 replicates.

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AUTHORSHIP CONTRIBUTIONS

Q.Z. designed the research, performed experiments, analyzed data, and wrote the manuscript; R.O. performed bioinformatic analysis and wrote the manuscript; A.K-C. and O.Y. performed experiments and analyzed data; D.B. supervised the project, was responsible for funding acquisition, designed the research, analyzed data and wrote the manuscript.

CONFLICT OF INTEREST DISCLOSURES

The authors report no conflict of interest.

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839

840 **TABLES**

841 **Table 1. RUs for each lineage in PB and for BM cHSCs of each recipient.**

Recipients		*PB*	*B cells*	*T cells*	*Myeloid* cells	*BM cHSCs*
Individual BM EE10 + 2M	***#1***	6	6	6	4	12
	#2	31	35	19	27	75
	#3	21	23	17	35	70
	#4	98	144	46	57	21
	#5	1	1	2	0.03	0.4
Pooled BM EE10 + 2M	***#1***	101	78	173	583	49
	#2	110	84	120	516	82
	#3	87	68	103	248	130
	#4	101	70	149	384	187
	#5	65	127	186	331	112

842 ** 1 RU equals to the average reconstitution activity of 1 x10 ^{5} WBM cells.*

Figure 1

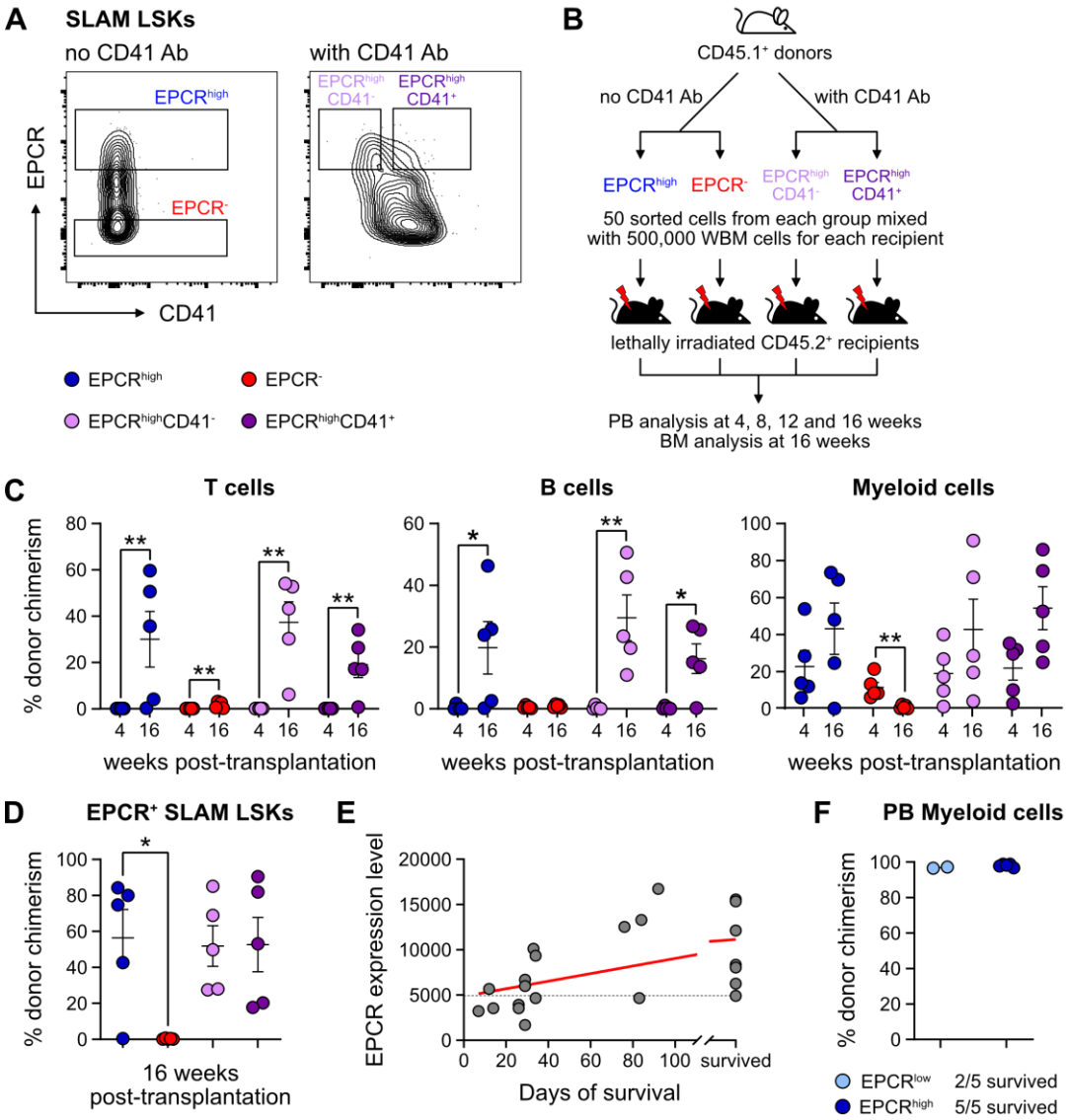

A
SLAM LSKs
no CD41 Ab
with CD41 Ab
EPCRhigh
EPCR-
EPCRhigh CD41-
EPCRhigh CD41+
EPCR
CD41
EPCRhigh
EPCR-
EPCRhigh CD41-
EPCRhigh CD41+
B
CD45.1+ donors
no CD41 Ab
with CD41 Ab
EPCRhigh
EPCR-
EPCRhigh CD41-
EPCRhigh CD41+
50 sorted cells from each group mixed with 500,000 WBM cells for each recipient
lethally irradiated CD45.2+ recipients
PB analysis at 4, 8, 12 and 16 weeks
BM analysis at 16 weeks
C
T cells
B cells
Myeloid cells
% donor chimerism
weeks post-transplantation
D
EPCR+ SLAM LSKs
% donor chimerism
16 weeks post-transplantation
E
EPCR expression level
Days of survival
survived
F
PB Myeloid cells
% donor chimerism
EPCRlow 2/5 survived
EPCRhigh 5/5 survived

Figure 1. EPCR expression within the BM SLAM LSK fraction is a high-confidence predictor of transplantation-associated HSC activity.

(A) Expression patterns of EPCR and CD41 within the BM LSK SLAM fraction. Gates depict the assessed cell fractions.

(B) Strategy used to assess the correlation of EPCR and CD41 expression to the *in vivo* HSC activity.

(C) Test-cell derived chimerism 4 and 16weeks post-transplantation.

(D) Test-cell derived chimerism in BM $EPCR^{+}$ SLAM LSKs 16 weeks post-transplantation.

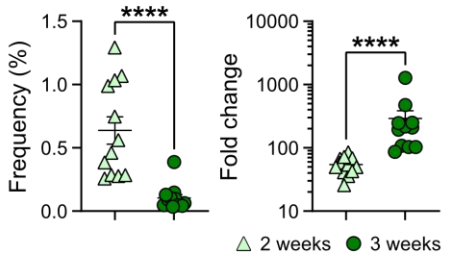
(E) Correlation between duration of radioprotection and EPCR expression levels. BM LSK SLAM cells were co-stained with EPCR and index-sorted at one cell per well, cultured for 21 days, and the content of each well transplanted to individual recipients (n = 22). Correlation to mortality of individual mice was made by assessing which well was transplanted into which mouse and coupling this to the index-sorting information. The grey dash line indicated the separation of EPCR higher (>4900) or lower expression (<4900) cHSCs.

(F) Donor contribution in PB myeloid cells. Mice were transplanted with *ex vivo* expanded cells from either 50 SLAM LSK $EPCR^{low}$ (n = 5) or 50 SLAM LSK $EPCR^{high}$ cells (n = 5) and assessments made 16 weeks after transplantation.

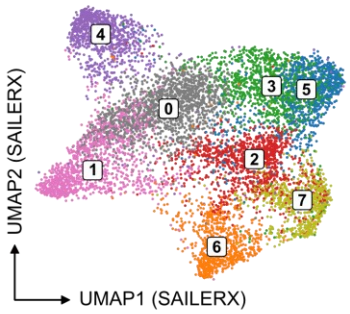
All data points depict values in individual recipients. Error bars denote SEM. The asterisks indicate significant differences. *, $p < 0.05$; **, $p < 0.01$. In E) a regression line was generated based on an end-point survival of 150 days (the time at which the experiment was terminated).

Figure 2

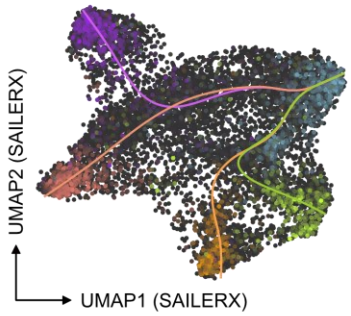
A Lin⁻Fcer1a⁻Sca1⁺cKit⁺CD150⁺EPCR⁺CD48⁻/low

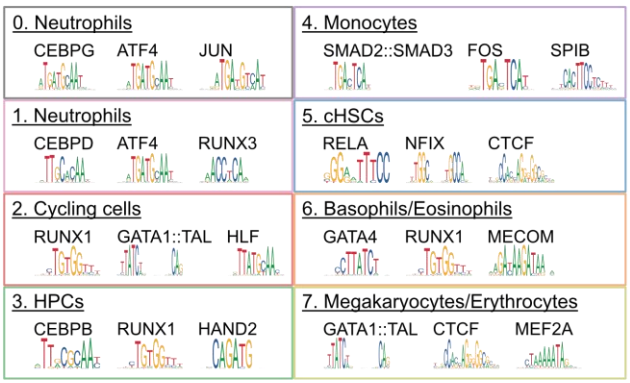


B Whole culture

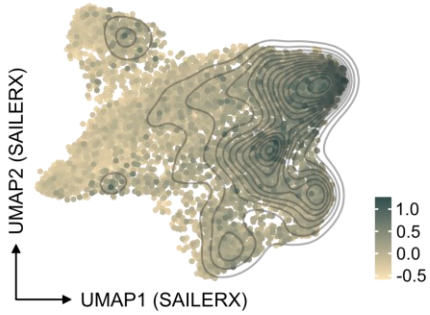


C Trajection of whole culture

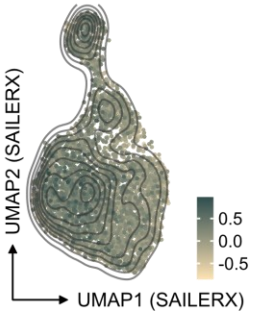




D HSC signatures



E HSC signatures of EPCR⁺ cells



F Cell cycle signatures of EPCR⁺ cells

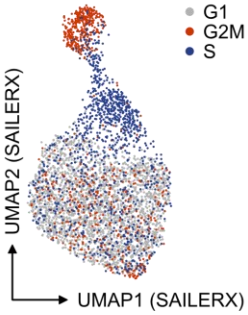


Figure 2. The phenotypic properties of HSC expansion cultures.

(A) Frequency and fold change of phenotypic cHSCs (EPCR^{high} SLAM LSKs) in *ex vivo* cultures after 14 or 21 days of culture. Data points depict values from individual cultures initiated from 50 cHSCs. Error bars denote SEM. The asterisks indicate significant differences. ****, $p < 0.0001$.

(B) UMAP (based on SAILERX dimensionality reduction) of single-cell multiome profiling of cells expanded *ex vivo* for 21 days. Cell type annotations were derived using marker gene signatures and distal motif identities.

(C) Trajectory analysis of lineage differentiation for cells expanded *ex vivo* (left), with the top 3 scoring TF motifs of each cluster (right).

(D) Expression of HSC signature on whole culture.

(E) Expression of HSC signature of EPCR^{+} cells sorted from *ex vivo* cultures.

(F) Cell cycle phase classification of EPCR^{+} cells sorted from *ex vivo* cultures.

Figure 3

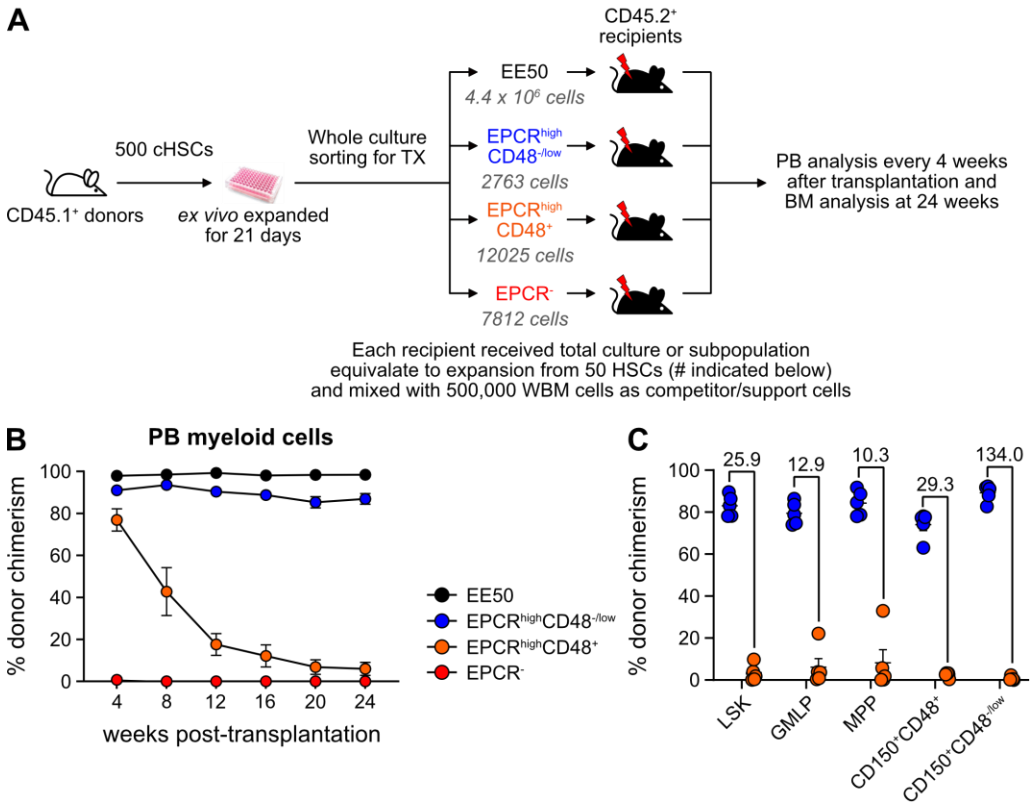


Figure 3. HSC activity can be prospectively isolated from cHSC cultures and associates to a minor EPCR$^+$ SLAM LSK fraction.

(A) Strategy to assess the *in vivo* HSC activity of subfractions from *ex vivo* cultures.

(B) Test-cell derived chimerism in PB myeloid lineages over 24-weeks post-transplantation. Data represent mean values ± SEM (n = 5 for each group).

(C) Test-cell derived chimerism in BM progenitor subsets 24-weeks post-transplantation. Numbers indicate fold differences between the EPCR$^+$ CD48$^{-/low}$ and EPCR$^+$CD48$^+$ fractions, and data points depict chimerism levels in individual recipients.

Figure 4

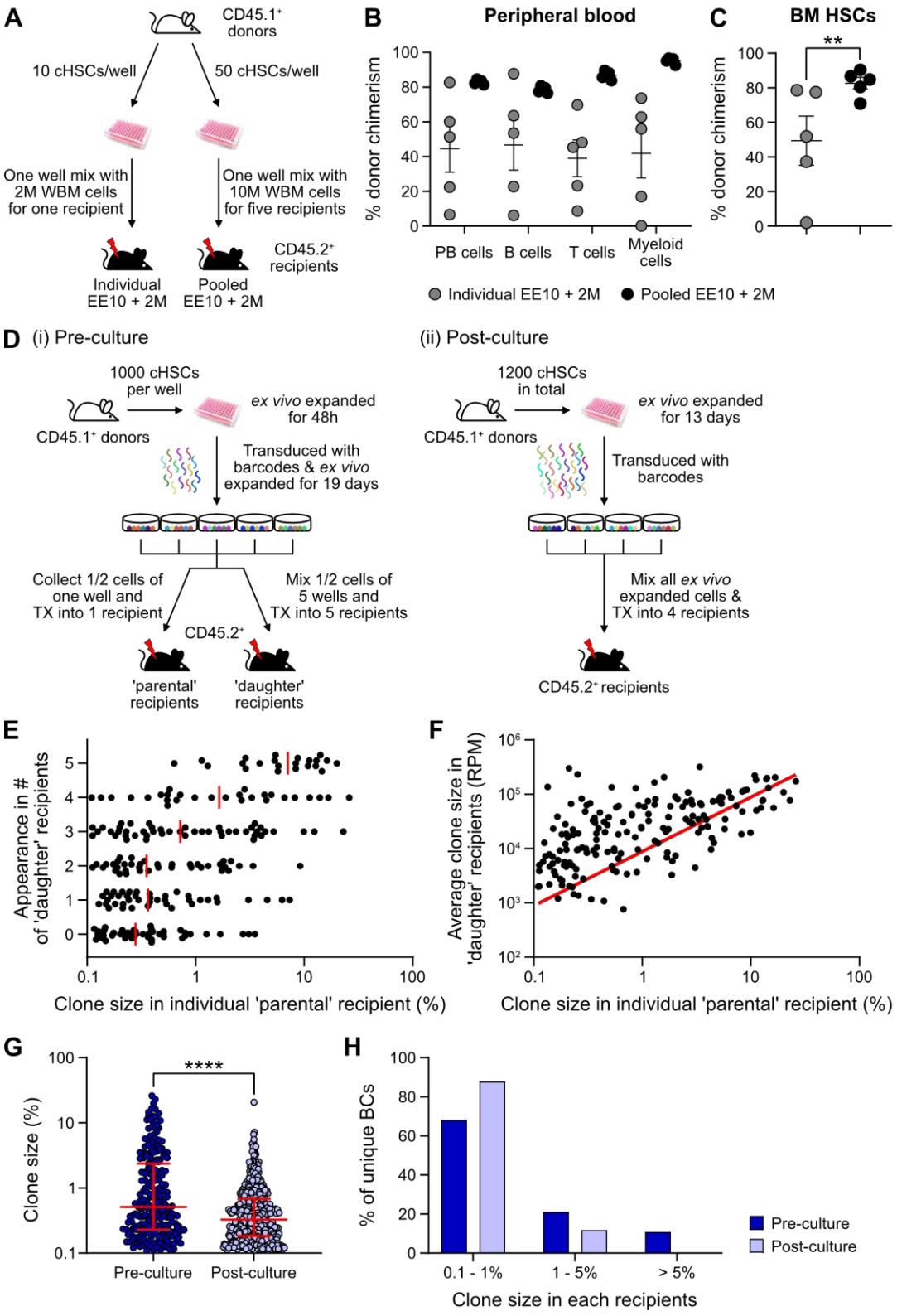

A
CD45.1⁺ donors
10 cHSCs/well
50 cHSCs/well
One well mix with 2M WBM cells for one recipient
One well mix with 10M WBM cells for five recipients
CD45.2⁺ recipients
Individual EE10 + 2M
Pooled EE10 + 2M
B
Peripheral blood
% donor chimerism
PB cells
B cells
T cells
Myeloid cells
Individual EE10 + 2M
Pooled EE10 + 2M
C
BM HSCs
**
% donor chimerism
D
(i) Pre-culture
1000 cHSCs per well
ex vivo expanded for 48h
CD45.1⁺ donors
Transduced with barcodes & ex vivo expanded for 19 days
Collect 1/2 cells of one well and TX into 1 recipient
Mix 1/2 cells of 5 wells and TX into 5 recipients
CD45.2⁺
'parental' recipients
'daughter' recipients
(ii) Post-culture
1200 cHSCs in total
ex vivo expanded for 13 days
CD45.1⁺ donors
Transduced with barcodes
Mix all ex vivo expanded cells & TX into 4 recipients
CD45.2⁺ recipients
E
Appearance in # of 'daughter' recipients
Clone size in individual 'parental' recipient (%)
F
Average clone size in 'daughter' recipients (RPM)
Clone size in individual 'parental' recipient (%)
G
****
Clone size (%)
Pre-culture
Post-culture
H
% of unique BCs
0.1 - 1%
1 - 5%
> 5%
Clone size in each recipients
Pre-culture
Post-culture

Figure 4 Quantification of repopulating activity from expanded cHSCs.

(A) Competitive transplantation strategies used to assess the repopulation of *ex vivo* expanded cHSCs.

(B) Test-cell derived PB reconstitution 16-weeks post-transplantation. Symbols denote individual mice and means ± SEM.

(C) BM cHSCs chimerism 16 weeks post transplantation. Symbols denote individual mice, and means ± SEM.

(D) Barcode approaches used to assess the clonal HSC contribution of *ex vivo* expanded HSCs before (i) or after (ii) *ex vivo* expansion.

(E) Clone sizes of unique barcodes in 'parental' recipients and their appearance in 'daughter' recipients, demonstrating extensive variation in expansion capacity among individual HSCs. Red lines indicate the median clone size in 'parental' recipients.

(F) Clone sizes of unique barcodes detected in BM myeloid cells of 'parental' recipients and their corresponding contribution in 'daughter' recipients. The red line denotes the correlation/linear regression.

(G) Clone sizes in 'parental' recipients transplanted with 'pre-culture' barcoded cells, or in recipients of 'post-culture' barcoded cells. Median clone sizes are shown with interquartile ranges.

(H) Frequency of barcodes and their clone sizes in recipients of pre- or post-cultured barcoded HSCs. BCs, barcodes.

All data points depict values in individual recipients or barcodes. Asterisks indicate significant differences. *, $p < 0.05$; ****, $p < 0.0001$.

Figure 5

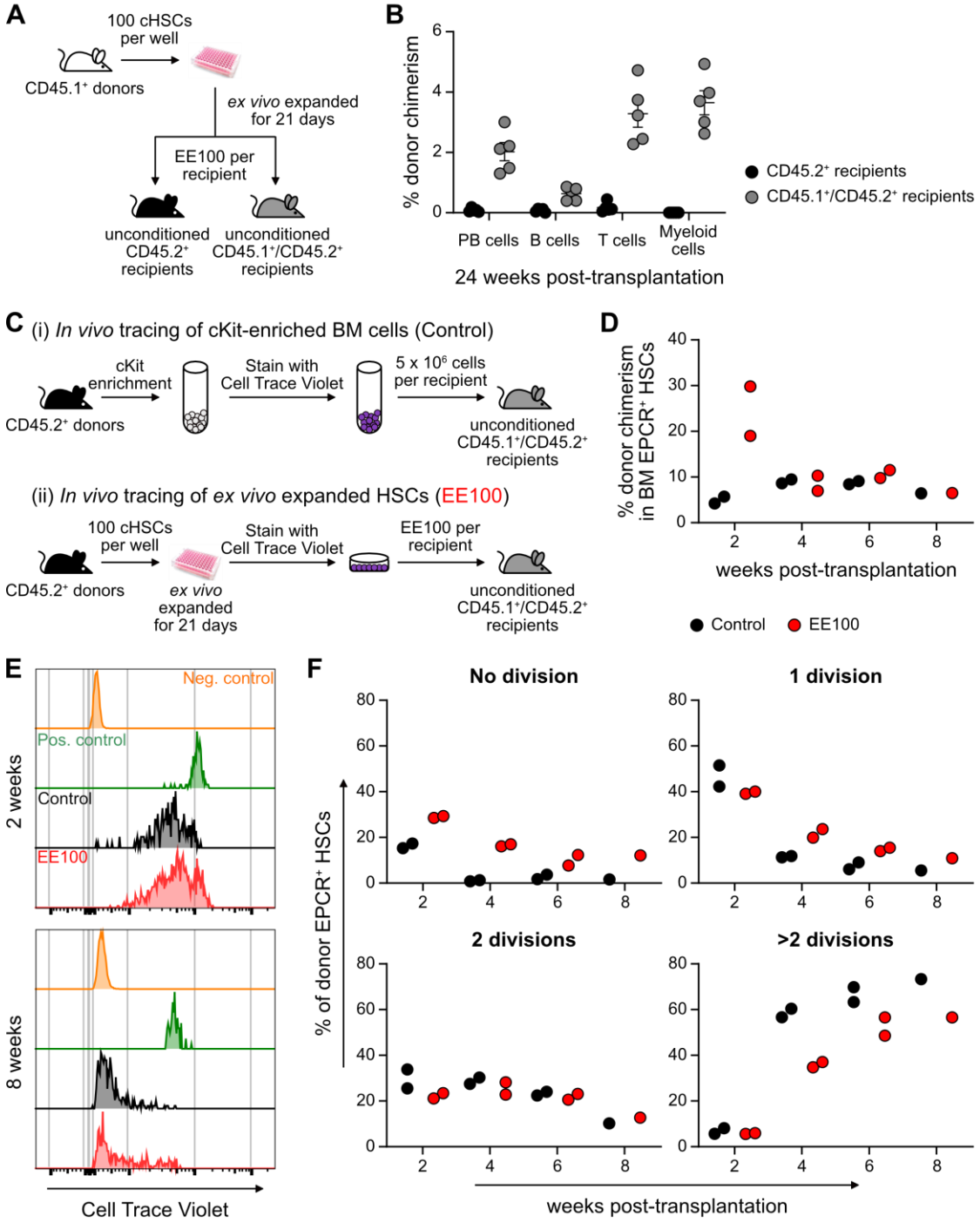

A
100 cHSCs per well
CD45.1+ donors
ex vivo expanded for 21 days
EE100 per recipient
unconditioned CD45.2+ recipients
unconditioned CD45.1+/CD45.2+ recipients
B
% donor chimerism
PB cells
B cells
T cells
Myeloid cells
24 weeks post-transplantation
CD45.2+ recipients
CD45.1+/CD45.2+ recipients
C
(i) In vivo tracing of cKit-enriched BM cells (Control)
cKit enrichment
Stain with Cell Trace Violet
5 x 10^6 cells per recipient
CD45.2+ donors
unconditioned CD45.1+/CD45.2+ recipients
(ii) In vivo tracing of ex vivo expanded HSCs (EE100)
100 cHSCs per well
Stain with Cell Trace Violet
EE100 per recipient
CD45.2+ donors
ex vivo expanded for 21 days
unconditioned CD45.1+/CD45.2+ recipients
D
% donor chimerism in BM EPCR+ HSCs
weeks post-transplantation
Control
EE100
E
Neg. control
Pos. control
Control
EE100
2 weeks
8 weeks
Cell Trace Violet
F
No division
1 division
2 divisions
>2 divisions
% of donor EPCR+ HSCs
weeks post-transplantation

Figure 5. Cultured cHSCs allow for transplantation into non-conditioned syngeneic recipients.

(A) Strategy to assess the ability of *ex vivo* expanded cHSCs to engraft unconditioned recipients.

(B) Test-cell derived PB reconstitution 24-weeks post-transplantation. Symbols denote individual mice and means ± SEM.

(C) Strategy used to assess the *in vivo* proliferation of *ex vivo* expanded cHSCs.

(D) BM cHSC chimerism 2-8 weeks post transplantation.

(E) Representative CTV label profiles of donor EPCR$^+$ cHSCs compared to negative control signal (host EPCR$^+$ cHSCs) and positive signal (donor CD4$^+$ spleen cells) at 2 or 8 weeks post-transplantation.

(F) Donor EPCR$^+$ cHSCs were evaluated for the number of cell divisions they had undergone through 8 weeks post-transplantation.

All data points depict values in individual recipients.