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Dissociation of Survival, Proliferation, and State Control in Human Hematopoietic Stem Cells

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SUMMARY

The role of growth factors (GFs) in controlling the biology of human hematopoietic stem cells (HSCs) remains limited by a lack of information concerning the individual and combined effects of GFs directly on the survival, Mitogenesis, and regenerative activity of highly purified human HSCs. We show that the initial input HSC activity of such a purified starting population of human cord blood cells can be fully maintained over a 21-day period in serum-free medium containing five GFs alone. HSC survival was partially supported by any one of these GFs, but none were essential, and different combinations of GFs variably stimulated HSC proliferation. However, serial transplantability was not detectably compromised by many conditions that reduced human HSC proliferation and/or survival. These results demonstrate the dissociated control of these three human HSC bio-responses, and set the stage for future improvements in strategies to modify and expand human HSCs ex vivo.

INTRODUCTION

The discovery of transplantable hematopoietic cells with stem cell properties in mice half a century ago (Siminovitch et al., 1963; Till and McCulloch, 1961; Wu et al., 1967) was rapidly translated into a clinical therapeutic modality. Transplants of human hematopoietic stem cell (HSC)-containing products now form a key component of curative treatments for many diseases (Thomas, 1993). New applications are becoming increasingly feasible due to the widening availability of cord blood (CB) units and advances in the genetic modification of human HSCs (Naldini, 2015). The field has been further galvanized by increasing evidence of early transforming events in human leukemogenesis that target HSCs (Fearon et al., 1986; Lindsley et al., 2015; Prchal et al., 1978; Shlush et al., 2014).

In mice, it has been possible to show that individual HSCs with durable regenerative activity can be greatly expanded in vivo with lifetime retention of their original functional potential (Dykstra et al., 2007; Harrison, 1979; Iscove and Nawa, 1997; Keller et al., 1985). Years of persisting hematopoiesis in patients given gene-marked autologous cells (Aiuti et al., 2013; Biffi et al., 2013; Cartier et al., 2009; Cavazzana-Calvo et al., 2010) indicate human HSCs maintained ex vivo for a few days can also remain active for many years post-transplant. We have previously shown that the survival, proliferation, and maintenance of the regenerative potential of mouse HSCs able to produce serially transplantable progeny can be differentially and directly regulated ex vivo by different combinations of external cues (Wohrer et al., 2014). In contrast, a detailed analysis of the direct effects of similarly defined human HSCs to external factors has remained elusive. However, this situation has recently changed with the identification of the $CD34^+CD38^-CD45RA^-CD90^+CD49f^+$ subset of human CB cells (hereafter referred to as $CD49f^+$ cells) as a highly enriched source of HSCs with long-term repopulating potential in transplanted immunodeficient mice (~10% purity) (Notta et al., 2011).

Combinations of five human growth factors (GFs), i.e., stem cell factor (SCF), Flt3-ligand (FLT3L), interleukin-3 (IL-3), IL-6, and granulocyte colony-stimulating factor (G-CSF), were previously shown to expand the number of primitive adult human hematopoietic cells identified in vitro as long-term culture-initiating cells when maintained in vitro for up to 10 days (Petzer et al., 1996a, 1996b, Zandstra et al., 1997, 1998). Subsequent experiments showed the same five-GF combination modestly expanded (2-fold) CB cells that could regenerate multi-lineage hematopoiesis for a few weeks in sublethally irradiated NOD/SCID mice in 7-day cultures (Conneally et al., 1997). We now report the differential effects of the same five GFs, analyzed alone and in various combinations on the survival, proliferation, and serial regenerative activity of purified human $CD49f^+$ CB cells. The results establish the ability of the five-GF combination to promote every viable cell to divide while retaining serially transplantable human HSC numbers over a 4- to 21-day period in vitro. Additional

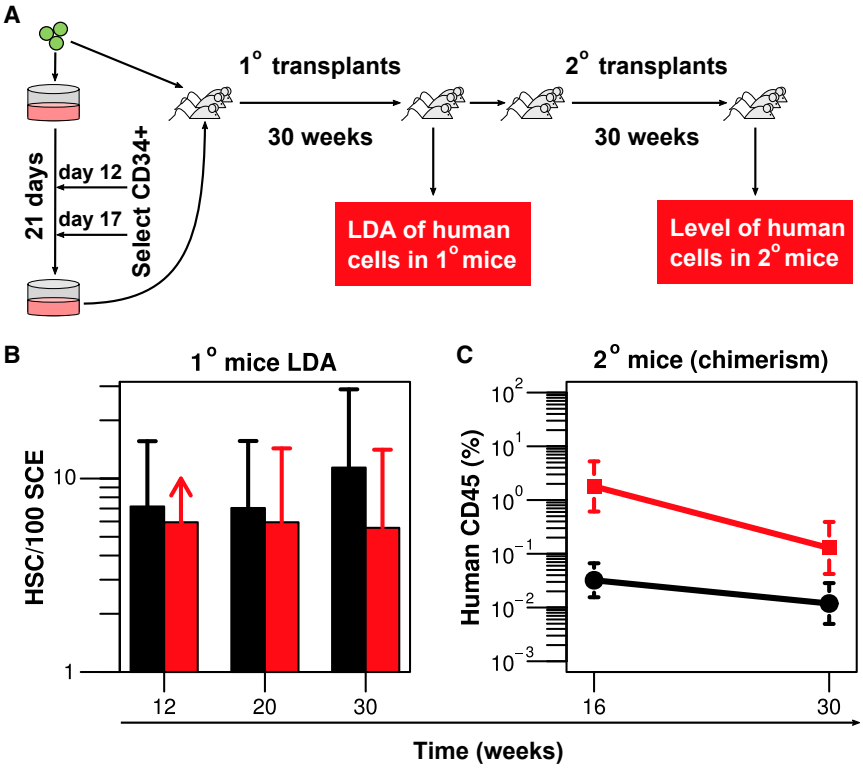


Figure 1. Five GFs Maintain Human HSC Numbers in 21 Day Cultures Initiated with CD49f$^+$ CB Cells

(A) Experimental design. One-milliliter cultures were initiated with 1,000 freshly isolated CD49f$^+$ cells in SFM containing five GFs. An additional 1 mL of GF-supplemented medium was added on days 9, 10, 11, 16, and 20. On days 12 and 17, CD34$^+$ cells were isolated immunomagnetically (thereby removing CD34$^-$ cells) and used to initiate further cultures in fresh SFM plus the five GFs. An aliquot of the freshly isolated CD49f$^+$ cells was injected into sublethally irradiated NSG mice at doses of 10, 50, and 200 cells each (black). Cultured cells were injected at doses equivalent to the output of 35 and 175 initial CD49f$^+$ cells (red). Starting cell equivalent (SCE) doses were determined based on the proportion of the final culture volume that would have contained the indicated number of input cells (e.g., from an input of 1,000 cells, 100 SCE = 1/10th of the final culture assuming no losses during the interim CD34$^+$ cell selection steps).

(B) HSC numbers per 100 starting CD49f$^+$ cells were derived by LDA of the proportions of primary recipients in two independent experiments (37 mice transplanted total) in which the level of human CD45$^+$ cell chimerism in the bone marrow was below the limit of detection (<0.005%). Error bars show 95% confidence intervals. None of the primary recipients of the cultured cells were negative 12 weeks post-transplant; hence minimum values at this time were calculated assuming one of the primary recipients of the minimum cell dose tested was negative.

(C) Levels of total human CD45$^+$ cell chimerism in the bone marrow of secondary mice transplanted with human cells regenerated from the equivalent of 450 freshly isolated CD49f$^+$ cells, or the corresponding cells regenerated from the cultured progeny of the equivalent of 362 initial CD49f$^+$ cells (three mice per arm). At 16 weeks, p = 0.04, and at 30 weeks, p = 0.17 (Student's t test). See also Figures S1 and S2.

single-cell tracking studies demonstrate that these GFs regulate the short-term (4 day) survival and proliferation of human HSCs directly in a tunable and combinatorial fashion, but independently of the maintenance of their long-term regenerative activity in vivo.

RESULTS

Five GFs Alone can Maintain Serially Transplantable Human HSCs for 21 Days In Vitro

Figure 1A shows the protocol used to evaluate the ability of a five-GF cocktail without further additives to sustain the HSC activity of CD49f$^+$ CB cells in 21-day cultures. As previous studies had indicated that the production of sufficient mature granulocytes and macrophages (GMs) can inhibit HSC maintenance (Csaszar et al., 2012), we used three strategies to try to minimize such an effect. The first was to initiate each culture with 1,000 fluorescence-activated cell sorting (FACS)-purified CD49f$^+$ cells in 1 mL of medium to maximally delay the production of GMs. The second was to increase the culture volume on days 9, 10, 11, 16, and 20 by adding 1 mL of fresh GF-supplemented medium. The third was to isolate the CD34$^+$ cells present in the cultures on days 12 and 17 and transfer them into fresh GF-containing medium.

The calculated total cell output after 21 days per 1,000 initial input CD49f$^+$ cells was >17 × 10^6 cells in both experiments performed (Figure 1, Table 1). This infers a minimum of 14 divisions per input CD49f$^+$ cell, assuming maximum survival and the continuing division of every cell produced (i.e., an average rate of one division every ~36 hr). Limiting dilution analysis (LDA) experiments were used to measure the number of transplantable HSCs present in the input CD49f$^+$ population and again after the 21 days in vitro. The results showed that ~10% of the input CD49f$^+$ cells had a 30-week repopulating activity and there was no change in their numbers in the 21-day cultures, despite the large change in their frequency (Figure 1B, Table 2). Secondary recipients of transplants of human CD34$^+$CD38$^{low/-}$

Table 1. Total Numbers of Cells Produced from 1,000 CD49f$^+$ Cells in 21-Day Cultures and Cell Numbers Removed by EasySep Selection Steps Performed on Days 12 and 17

Experiment	Time in Culture (Days)	Number of Cells per Culture ($\times 10^6$)		Number of Cells Removed by EasySep ($\times 10^6$)		% of Cells Removed by EasySep	
		Total	CD34$^+$	Total	CD34$^+$	CD34$^-$	CD34$^+$
1	12	6.0	0.7	3.8	0.04	70	5
	17	16.8	1.1	10.9	0.10	69	9
	21	17.6	ND	NA	NA	NA	NA
2	12	5.3	0.3	4.1	0.02	83	6
	17	23.0	2.1	12.4	0.05	59	2
	21	19.4	ND	NA	NA	NA	NA

ND, not determined; NA, not applicable.

cells harvested from the primary mice provided even more stringent evidence that the culture-derived HSCs possessed the same or better functional activity by comparison with CD49f$^+$ cells isolated directly from CB (Figure 1C).

Five GFs Support Full Survival and Mitogenesis of CD49f$^+$ Human CB Cells but with Different Concentration Dependencies

A next series of experiments were designed to assess the rate and extent of recruitment of the CD49f$^+$ cells into division when incubated in the presence of the five GFs. Accordingly, we set up 141 single-cell cultures of CD49f$^+$ cells in five GFs and monitored the survival and division timing for up to 11 days or until at least nine cells were produced from each input cell (four divisions, Figure 2A). Of the initial 141 cells, 96% survived. All of the survivors completed at least three divisions, and 99% completed at least four divisions within 9 days (Figure 2B). These findings demonstrate the ability of the five GFs in combination to directly and rapidly stimulate functional human HSCs to divide in vitro. This precludes the observed maintenance of functional HSC numbers in the 21 day cultures being explained by their remaining quiescent.

We then designed a second series of single-CD49f$^+$ cell tracking experiments using different GF conditions to determine whether the regulation of human CD49f$^+$ cell survival and mitogenesis are tightly linked. Monitoring of the cells in each culture was limited to 4 days. This time line was chosen because it is long enough to detect the death of >96% of CD49f$^+$ cells in the absence of GFs (see below), as well as a first division of 98.5% of cells in the presence of five GFs (Figures 2B and 2D, see also next section and Figure 3). Decreasing the concentration of all five GFs resulted in progressive decreases in both the 4 day survival and proliferation responses of the individually tracked CD49f$^+$ cells (Figures 2C and 2D), although their survival was clearly less GF concentration-dependent than their mitogenesis. Thus, at 1% of the original GF concentration, survival was only minimally affected (84% versus 96%; false discovery rate [FDR] = 0.005), while second- and third-division frequencies were already substantially reduced using a 10% GF concentration (FDR = 0.007). These results show that both CD49f$^+$ cell survival and proliferation can be modulated by the strength of an applied GF stimulus, with a higher threshold required for proliferation than for survival, as also seen for mouse HSCs (Audet et al., 2002).

Human CD49f$^+$ CB Cell Survival and Proliferation Are Differentially Controlled by Specific GFs

A next series of 4 day single-CD49f$^+$ cell cultures was designed to analyze the roles of the individual components of the five-GF cocktail on CD49f$^+$ cell survival and proliferation (Figure 3A). A total of 2,625 single CD49f$^+$ cells were monitored either in Terasaki plates or in microfluidic arrays (for greater temporal resolution). As the overall survival and proliferation dynamics were the same using either system, the results were pooled.

In these experiments, the overall 4 day survival of the CD49f$^+$ cells was slightly lower at 84%, but this level of survival was not significantly altered by the removal of any one of the five GFs (FDRs $\geq$ 0.15, Figures 3B and S3A, Table S1). Moreover, each of the five GFs alone (except for G-CSF) increased the survival of CD49f$^+$ cells significantly above the no-GF condition (<4%, FDRs $\leq$ 0.002). As single factors, SCF and FLT3L had the most prominent pro-survival effects (43% and 51%, respectively) and the combination of any two of FLT3, SCF, IL-3, and IL-6 had even greater pro-survival effects than any of these on their own (FDRs $\leq$ 0.05). However, all of these conditions were less effective than all five GFs together (FDRs $\leq$ 0.02).

The five-GF cocktail also stimulated 82% of the same CD49f$^+$ cells to complete two divisions within 4 days

Table 2. HSC Frequencies in CD49f$^+$ Cells and Their Progeny in 21 Day Cultures Derived by LDA of Primary Transplanted Mice, See Figure 1

Culture Condition	Weeks Post-transplant	Cell Dose Per Mouse	Engrafted/Total	HSC Frequency (95% CI)	p Value
Day 0	12	200	6/6	1 in 14 (1 in 30–1 in 6.4)	0.32
		50	8/8		
		10	3/7		
5 GFs	12	175	8/8	1 in <17 (1 in 30–1 in <17)	
		35	8/8		
Day 0	20	200	6/6	1 in 14 (1 in 32–1 in 6.4)	0.78
		50	7/7		
		10	3/7		
5 GFs	20	175	8/8	1 in 17 (1 in 41–1 in 7)	
		35	7/8		
Day 0	30	200	6/6	1 in 8.8 (1 in 22–1 in 3.5)	0.28
		50	7/7		
		10	4/6		
5 GFs	30	175	5/5	1 in 18 (1 in 46–1 in 7.1)	
		35	6/7		

CI, confidence intervals.

(median = 66 hr, interquartile range [IQR] = 58–72 hr) with the second division occurring much faster than the first (median = 25 hr later, IQR = 19–28 hr later) (Figures 3C, 3D, S3B, and S3C, Table S1). These dynamics are very similar to those observed in the previous GF concentration experiments (Figure 2B). Interestingly, these proliferation kinetics are also similar to those recently reported for the same phenotype of cells stimulated with a slightly different GF cocktail (Laurenti et al., 2015).

The fraction of CD49f$^+$ cells that any single GF stimulated to divide within 4 days was very low, with the strongest effects (~10% divided cells) elicited by either SCF or IL-3. SCF + IL-3 was the only two-GF combination that was as mitogenic as all five GFs together (Figure 3C), although the timing of the divisions stimulated by any of these, including SCF + IL-3, was significantly delayed compared with the five-GF cocktail (FDRs $\leq$ 0.01 except for IL-3 + IL-6, where the number of divided cells was too low for significance assessment, Figure 3D; Table S1). Removal of G-CSF slightly increased the proportion of CD49f$^+$ cells that were induced to divide within 4 days (by 1.2- and 1.9-fold for the first and second divisions with FDRs of 0.006 and $\ll$0.001, respectively). This suggests that G-CSF may inhibit mitogenic pathways in these cells elicited by the other four GFs. Conversely, elimination of SCF from the five-GF cocktail significantly reduced the proportion of CD49f$^+$ cells that responded within 4 days (1.8-fold fewer completing a first division, and 3.1-fold fewer completing a second division, FDRs $\ll$0.001), despite their high survival or lack of effect on the time to complete a first division (FDR = 0.11). On the other hand, removal of IL-3, IL-6, or FLT3L from the five-GF cocktail had no significant effect on the recruitment of CD49f$^+$ cells into division within 4 days. Removal of IL-3, however, caused a slight delay in the time the cells take to complete a first division (median = 71.6, IQR = 64.8–77.9 hr; a 5.6-hr delay, Kolmogorov-Smirnov test FDR = 0.008). Removal of FLT3L had a similar delaying effect (median time to complete a first division = 70.6, IQR = 62.5–77.9; a 4.6-hr delay, Kolmogorov-Smirnov test FDR = 0.009). Thus, the five GFs, both individually and in combination, differentially activate (or suppress) mechanisms that control not only the survival and mitogenesis of very primitive human hematopoietic cells, but also their rate of entry into (or passage through) the cell cycle.

HSC Regenerative Activity Is Retained under GF Conditions that Variably Support CD49f$^+$ CB Cell Survival or Proliferation

We next investigated the effects that specific GFs, either alone or in combination, would have on the maintenance

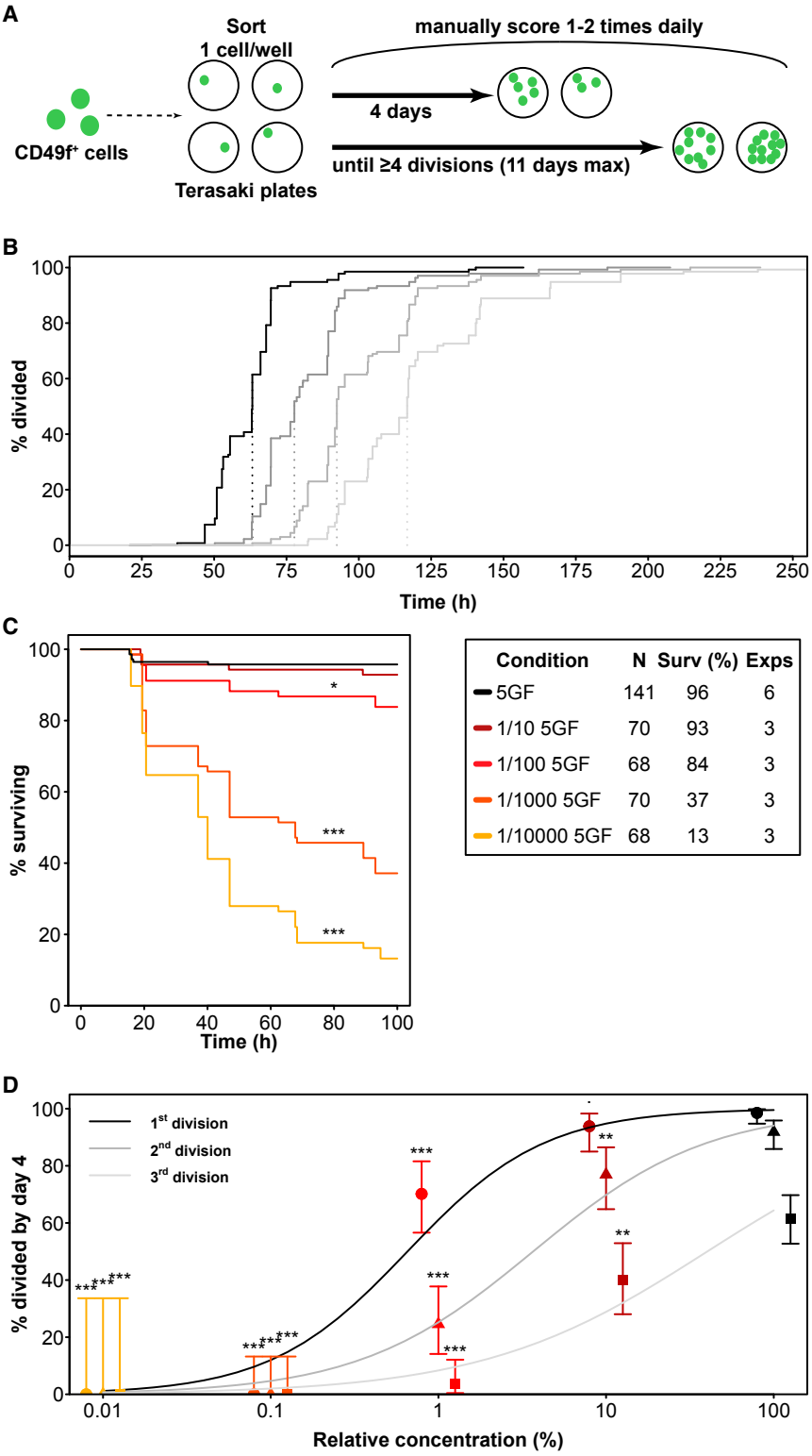


Figure 2. GF Concentrations Differentially Affect CD49f+ Cell Survival and Proliferative Responses In Vitro

(A) Experimental design (100% GF = 100 ng/mL SCF and FLT3L, and 20 ng/mL each of IL-3, IL-6, and G-CSF).

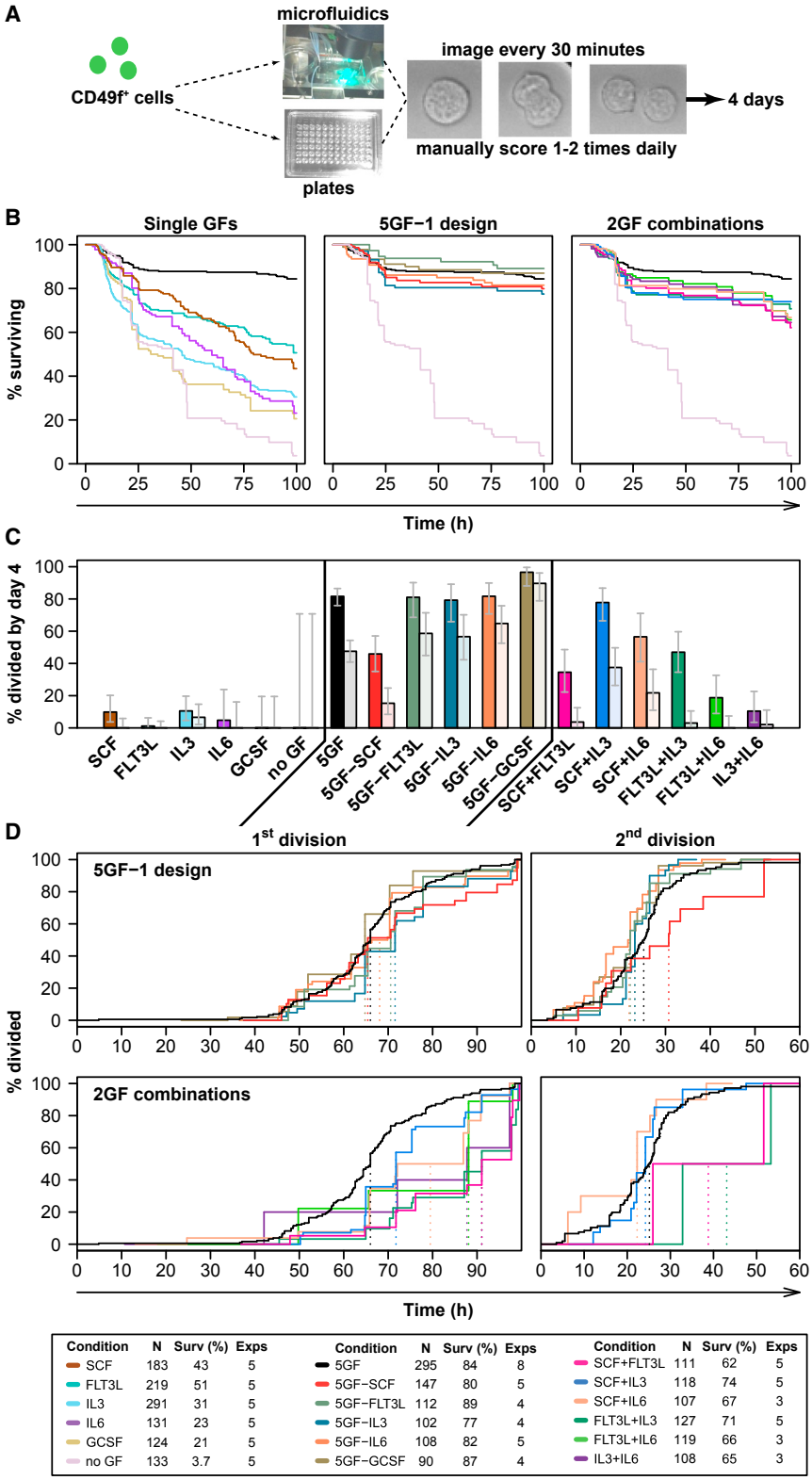
(B) Cumulative divisions of CD49f+ cells exposed to the five GF cocktail over a period of up to 11 days. First divisions are shown in black, second in dark gray, third in medium gray, and fourth in light gray. Median division times are indicated by dotted lines.

(C) Kaplan-Meier survival curves for FACS-purified CD49f+ CB cells tracked over 4 days in single-cell cultures containing serial 10-fold dilutions of the five GF cocktail. All conditions were compared with the 100% five GF cocktail.

(D) Percent of surviving single cells observed to undergo first, second, and third divisions within 4 days when cultured in the different GF concentrations shown (together with binomial 95% confidence intervals). Logistic regression fits for division recruitment for each concentration of five GFs are shown as black (first), gray (second), or light gray (third) lines. An FDR correction for multiple testing was applied where relevant.

p = 0.1, *p = 0.05, **p = 0.01, ***p ≤ 0.001. The legend in the middle right panel indicates the color used to indicate each condition, the number of cells analyzed and the number of experiments over which they were collected.

of the serially transplantable regenerative potential of HSCs also after being maintained under similar conditions in 4-day cultures initiated with CD49f+ cells (<1 cell/μL, Figure 4A). To minimize possible confounding effects of Poisson sampling of the HSCs present, each culture was initiated with 330 CD49f+ cells; i.e., ~33 transplantable



Condition	N	Surv (%)	Exps
SCF	183	43	5
FLT3L	219	51	5
IL3	291	31	5
IL6	131	23	5
GCSF	124	21	5
no GF	133	3.7	5

Condition	N	Surv (%)	Exps
5GF	295	84	8
5GF−SCF	147	80	5
5GF−FLT3L	112	89	4
5GF−IL3	102	77	4
5GF−IL6	108	82	5
5GF−GCSF	90	87	4

Condition	N	Surv (%)	Exps
SCF+FLT3L	111	62	5
SCF+IL3	118	74	5
SCF+IL6	107	67	3
FLT3L+IL3	127	71	5
FLT3L+IL6	119	66	3
IL3+IL6	108	65	3

Figure 3. Survival and Proliferative Responses of CD49f+ Cells Are Independently and Combinatorially Regulated

(A) Experimental design.

(B) Kaplan-Meier survival curves for FACS-purified CD49f+ CB cells tracked over 4 days in single-cell cultures containing a single GF (left), four GFs (middle), or the two GFs combinations shown (right). Survival of the cells in the five GF cocktail (black), or no GFs (thistle), are shown for reference. Cells from one experiment were censored at 40 hr.

(C) Percent of surviving single cells observed to undergo first and second divisions (darker and lighter bars respectively). Binomial 95% confidence intervals are shown.

(D) Cumulative distribution functions for the timing of first and second cell divisions over time for each GF combination. First division distributions are shown on the left. Differences between the first and second division were calculated on a clone by clone basis. Median division times are indicated by the dotted lines. At the bottom of the figure are shown the total number of cells and experiments from which the data were generated. See also Figure S3, Table S1.

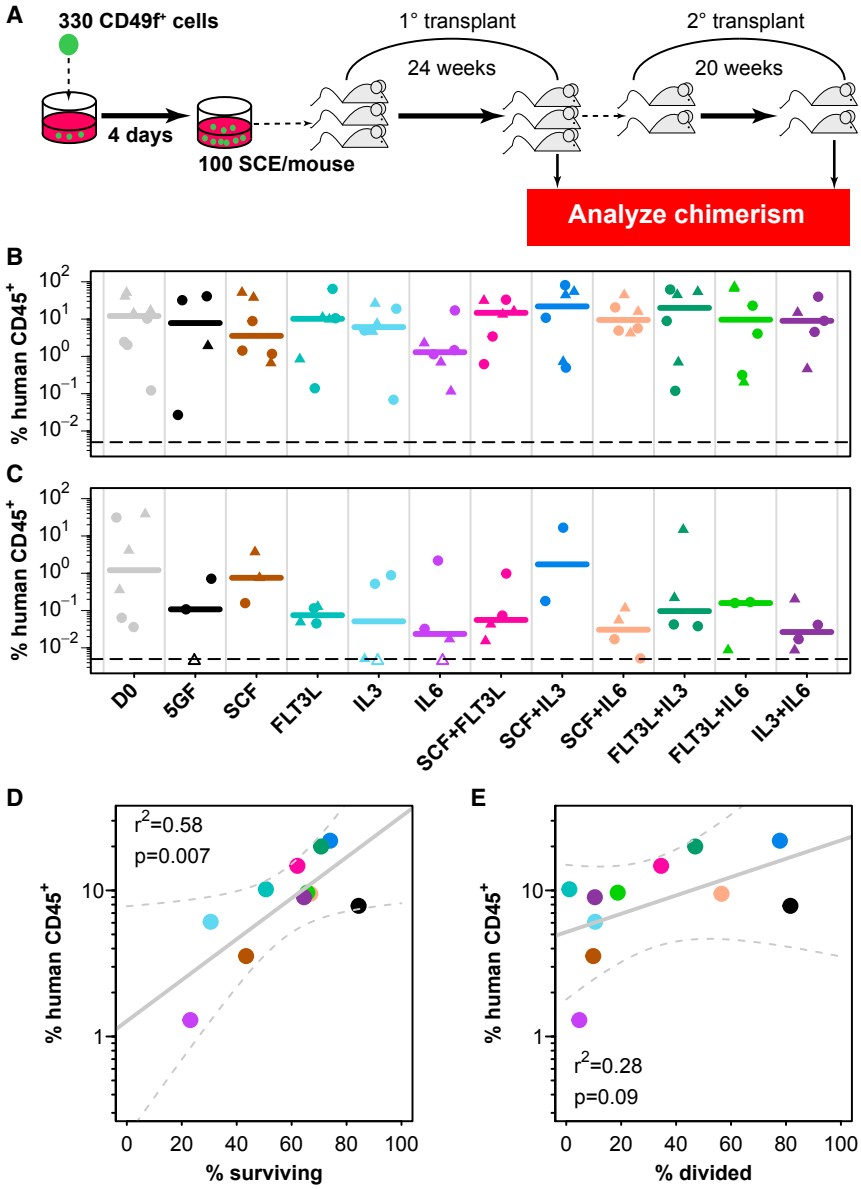


Figure 4. Multiple GF Combinations Maintain Serially Transplantable HSC Activity for at Least 4 Days In Vitro

(A) Experimental design. A total of 30% of the total culture harvested was injected per mouse (=100 SCE).

(B) Total human CD45$^+$ cell chimerism levels in the bone marrow of mice assessed 6 months after being transplanted with the cells present after 4 days in cultures containing the indicated GFs. Each point indicates an individual recipient. Results from two different experiments are shown as circles and triangles. The limit of detection of human cells is shown as a dotted line. Solid symbols indicate measured chimerism values and open symbols indicate mice where no evidence of chimerism was detectable.

(C) Total human CD45$^+$ cell chimerism in the bone marrow of secondary recipients of cells transferred from the primary mice shown in (B) and assessed 5 months later.

(D and E) Median ($\log_{10}$) chimerism in primary recipients for each condition compared with the 4-day survival (D) or division (E) of the cells pre-transplant. Points are colored by condition. The thick gray lines indicate linear regression fits. Dotted gray lines show the 99th confidence intervals. Regression p and r^2 values are indicated. See also Figure S4.

HSCs, assuming ~10% purity (Figure 1). In each of two experiments, HSC activity was assessed by injecting the cells from 30% of the total culture volume per mouse (the post-culture equivalent of 100 input CD49f$^+$ cells). Another group of mice was injected with 100 of the CD49f$^+$ freshly isolated cells used to initiate the cultures to serve as pre-culture controls. These cell doses were selected to try to balance between obtaining consistent engraftment from the input assays (thus avoiding skewing by Poisson sampling), and preventing their generation of saturating levels of chimerism (to enable both positive and negative effects of the cultures to be detected). Counts of final cell numbers obtained in each culture confirmed these were similar to those predicted from the 4 day maintenance of single CD49f$^+$ cells under the same conditions (data not shown). Accordingly, in some cultures, mice were actually transplanted with fewer cells than the recipients of the fresh CD49f$^+$ cells (e.g., recipients of cells from the cultures that contained only IL-6 would have been injected with only ~23 viable cells corresponding to the 23% survival and no proliferation of the input cells determined from the single-cell cultures).

The median levels of chimerism measured in the bone marrow of the transplanted mice 6 months later showed no significant differences between any of the test groups of cultured cells and the freshly isolated CD49f$^+$ cells, or

of cells that had been maintained in five GFs for 4 days (Kruskal-Wallis rank-sum test p = 0.91, Figures 4B and S4). Transfer of human cells from the primary recipients to secondary mice that were then followed for another 5 months also showed no intergroup differences in the levels of human $CD45^+$ cell chimerism obtained (p = 0.32, Figure 4C). However, a highly significant log-linear relationship was evident between the median level of human $CD45^+$ cell chimerism obtained in the primary recipients and the extent of total $CD49f^+$ cell survival in the 4 days of culture pre-transplant (Figure 4D, p = 0.007). Taken together, these results suggest that the effects of the GFs tested on the recovery of functional HSCs mirror the survival of the total $CD49f^+$ cell subset. In contrast, there was no significant relationship between the median level of chimerism obtained and the proportion of initial $CD49f^+$ cells recruited into division pre-transplant (Figure 4E, p = 0.09).

These results demonstrate that stringently defined HSC regenerative activity in vivo is not differentially affected for at least 4 days under a number of GF conditions that variably affect HSC survival or proliferative activity. Thus, it appears that at least the short-term maintenance of the full functional abilities of HSCs is not critically dependent on the stimulus afforded by any one of the five GFs tested here, nor closely correlated with a GF-induced mitogenic response.

DISCUSSION

Human HSC Divisions Are Rapidly and Directly Stimulated In Vitro by GFs Alone without Loss of Their In Vivo Regenerative Potential

Limitations in various sources of HSCs for adults requiring a transplant and the advent of efficient genome-editing techniques, such as CRISPR (Hsu et al., 2014) for gene or immunotherapeutic applications, are accelerating interest in protocols to expand functionally intact HSC numbers ex vivo. Here we provide definitive evidence that purified CB HSCs can be stimulated by five GFs alone to directly and rapidly (within 9 days) divide without loss of their regenerative activity monitored for over a year in serially transplanted mice. The present study also now demonstrates that this level of durable human HSC activity can be maintained for up to 3 weeks from cells that are dividing rapidly and simultaneously producing thousands of more differentiated cells. These results markedly extend previous evidence suggesting the ability of these five GFs to elicit such a response in 7 day cultures assessed for the maintenance of cells with a much shorter period of repopulating ability (Conneally et al., 1997). They are, however, very different from the well-documented failure of similar GFs to maintain the serial transplantable activity of HSCs from adult mice (Antonchuk et al., 2002; Kent et al., 2008; Wohrer et al., 2014). Loss of HSC activity has also been repeatedly observed in clinical trials of cells cultured in various GF combinations (Glimm et al., 2005; de Lima et al., 2012; McNiece et al., 2000; Shpall et al., 2002) and even in recent experiments where less pure $CD34^+$ CB cells were used to initiate expansion cultures (Fares et al., 2014). The present findings thus reinforce the importance of other elements of the ex vivo protocol used for maintaining human HSCs, besides the GFs used.

A Combinatorial Rheostat Regulates GF-Stimulated Survival and Proliferation of Human HSCs

Our single-cell tracking studies show that GF concentrations required to promote HSC survival appear to be lower than those required to promote their proliferation. These findings are similar to findings for mouse HSCs that survive under lower concentrations of SCF than are required to stimulate their proliferation (Kent et al., 2008). However, other aspects of human HSC behavior appear to be regulated differently from mouse HSCs, as suggested by the finding that a partial $CD49f^+$ cell-survival benefit elicited by multiple single GFs was improved by the addition of a second GF. This finding is notable as we have also recently found that the same GFs used here activate different signaling pathways (Knapp et al., 2016), despite their similar abilities as individual factors to support HSC survival, and the additional survival benefit obtained with multiple combinations of these GFs. They also show that multiple signaling pathways can be integrated to promote the survival human HSCs.

In contrast, the stimuli able to elicit a proliferative response in human $CD49f^+$ CB cells were more limited. Similarly, any combination of four of the five GFs tested supported the survival of virtually every initial $CD49f^+$ cell, whereas removal of SCF alone had a significant detrimental impact on their mitogenic response. Taken together, human HSCs appear to be able to efficiently integrate a variety of distinct signals activated by multiple different GFs to effectively maintain their viability, but proliferation requires a higher threshold of these and is also more dependent on the specific signals activated by component GFs of the five tested here.

Distinct Mechanisms Regulate Human HSC Regenerative Potential

Examination of the effect of different GF combinations on the retention of HSC regenerative activity revealed some surprises. Overall, significant losses were not detected for any of the GF combinations tested, even when these caused a marked reduction in total $CD49f^+$ cell survival. Thus, HSC maintenance could not have been additionally compromised by conditions that poorly supported their mitogenesis or even survival. Maintenance of the regenerative function of human HSCs thus seems likely to involve

mechanisms distinct from those that regulate their survival and proliferation, although the possibility of biochemical heterogeneity within the human CD49f$^+$ CB population has not yet been ruled out.

The relative lack of sensitivity of the regenerative function of human HSCs to GF conditions that compromise their survival and mitogenesis also differs from historical data for adult mouse HSCs. For mouse HSCs, maintenance of regenerative activity has been found to be more dependent on GF stimulation than their proliferative response or survival (Audet et al., 2002; Kent et al., 2008). This species difference suggests that certain aspects of human HSC homeostasis are regulated in a fundamentally different manner and hence cannot be anticipated from analyses of mouse models. A mechanistic dissociation between GF-mediated control of human HSC survival, proliferation, and durable regenerative activity does, however, raise new questions as to how the latter function is regulated at a molecular level. Paracrine-negative effects of factors released from mature myeloid cells have been implicated (Csaszar et al., 2012), and likely contribute to the poorer recovery of transplantable HSCs observed previously in 9 day expansion cultures initiated with less-purified starting populations (Bhatia et al., 1997; Fares et al., 2014).

A dissociation of these mechanisms also has implications for human leukemogenesis. Mutations or epigenetic alterations activating survival or proliferative response control pathways could be envisaged to stimulate abnormal HSC expansion without compromising their ability to activate the execution of unperturbed differentiation programs. Such a pattern is seen in normal aging (Steensma et al., 2015) and in myeloproliferative diseases (Raskind and Fialkow, 1987). Interestingly, mutations causing acute myeloid leukemias tend to block differentiation without necessarily disrupting downstream loss of proliferative programmability, resulting in an accumulation of non-dividing blasts (Minden et al., 1978). The dissociation of HSC state maintenance from survival and mitogenesis control allows for a number of alternative pathways by which pre-leukemic clones could arise and/or progress.

Overall, the evidence presented here that GFs can differentially control human HSC survival, proliferation, and maintenance of regenerative activity provides a new foundation for further optimizing the ex vivo expansion and genetic modification of human HSCs and for understanding mechanisms of their leukemic transformation.

EXPERIMENTAL PROCEDURES

Human CB Cells

Anonymized heparinized CB was obtained from consenting mothers undergoing normal full-term deliveries in accordance with procedures approved by the Research Ethics Board of the University of British Columbia and samples from a single day pooled for further processing and cryopreservation as described in Supplemental Experimental Procedures. To isolate CD49f$^+$ cells from thawed suspensions, cells were resuspended in blocking buffer and stained with appropriate antibodies for 1–2 hr (Supplemental Experimental Procedures) prior to being sorted on a BD Influx II, a BD FACSAria II or III, or a Fusion sorter (Becton Dickinson) using purity or single-cell modes (as relevant).

Bulk CD49f$^+$ Cell Cultures

For the 21 day HSC expansion culture, 1,000 CD49f$^+$ CB cells were delivered directly into 1 mL of serum-free medium (SFM, Supplemental Experimental Procedures) containing a cocktail of five recombinant human GFs. These consisted of SCF (gifted by Amgen) and FLT3L (gifted by Immunex) at 100 ng/mL each, and IL-3 (gifted by Novartis), IL-6 (gifted by Cangene), and G-CSF (from STEMCELL Technologies) at 20 ng/mL each. Subsequent media additions and CD34$^+$ cell-enrichment methods are detailed in Supplemental Experimental Procedures.

For the 4 day cultures, CD49f$^+$ cells were sorted into SFM and then split by volume into each medium condition such that each condition had a total of 330 starting CD49f$^+$ cells in 0.5 mL (i.e., 1 cell per 1.5 μL = 150× more dilute than 10^5 cells per mL) to provide conditions unlikely to promote paracrine effects. Individual GF concentrations were the same as used in the five GF cocktail.

HSC Quantification in Immunodeficient Mice

Test cell suspensions were injected intravenously into female 8- to 16-week-old NOD.Cg-*Prkdc*^{scid}*Il2r*γ^{tm1Wjl}/SzJ (NSG) mice pretreated with 315 cGy whole-body ^{137}Cs γ-irradiation for primary LDA. In all other studies, recipients were female NOD.Cg-*Rag1*^{tm1Mom} *Il2rg*^{tm1Wjl}/SzJ (NRG) mice pretreated with 900 cGy whole-body ^{137}Cs γ-irradiation delivered over 3 hr. Human chimerism was tracked by flow-cytometric analysis of sequential bone marrow aspirates (using a BD LSRFortessa analyzer, Becton Dickinson; Supplemental Experimental Procedures). A detection threshold of ten cells that showed positive staining with each of two independent anti-human CD45 antibodies was used. This made it possible to reproducibly detect a level of human cell chimerism of ~0.005% for the identification of "positive" mice from 200,000 cells analyzed (Figure S1). Calculations for cell dosage and conditions for secondary transplantations are detailed in Supplemental Experimental Procedures. No blinding or randomization was performed in any of the animal experiments. Mice were bred and maintained in the Animal Resource Center of the British Columbia Cancer Research Center under specific pathogen-free conditions and used according to protocols approved by the Animal Care Committee of the University of British Columbia.

Single-Cell Cultures of CD49f$^+$ CB Cells

Cells were cultured and monitored in microfluidic arrays using a protocol similar to that described by Lecault et al. (2011), but adapted for human CD49f$^+$ CB cells and the GF additions relevant here, with acquisition of bright-field images every 30 min and fluorescent images daily (see also Supplemental Experimental Procedures). Single-cell cultures of CD49f$^+$ CB cells performed in Terasaki

plates were initiated using the FACS to deposit individual cells directly into each well pre-filled with 20 μL SFM with indicated GFs (Robbins Scientific or Greiner Bio-One). The plates were assessed an hour later to identify any well that did not contain just a single, viable (refractile) cell. Images from the microfluidics arrays were assessed manually to determine if and when a cell had died based on its loss of refractility or movement (change in shape and relative location in the microfluidic well between frames), and then rapid and continuous reduction in volume and/or disintegration. Terasaki plates were visually assessed once or twice daily based on the same criteria. These criteria were validated in an independent experiment in which cells identified as dead were not able to proliferate following supportive GF addition. The first observation of two viable cells was used to denote the timing of a first cell division, three to four for a second division, etc. When two or more divisions occurred between observations, division timing was linearly interpolated with the final division occurring at the observation point and other divisions occurring at equal intervals between adjacent observation points.

Data Analysis

All statistical testing and data analysis was performed in R (The R Foundation for Statistical Computing; https://www.R-project.org/). All LDA calculations were performed using the "elda" function in the package "statmod." Analysis of survival was performed using the package "survival." FDR corrections for multiple testing were applied where relevant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.12.003.

AUTHOR CONTRIBUTIONS

D.J.H.F.K. and C.J.E. designed the experiments and wrote the manuscript. D.J.H.F.K. and C.A.H. performed the experiments. D.J.H.F.K. performed the final data analysis. P.H.M., P.A.B., and A.M.C. assisted with experimental designs and interpretation. D.P. helped with data analysis and interpretation. G.M.R. assisted with mouse analyses. M.R., V.L., D.D., and M.V. assisted with microfluidics cell cultures. J.P. and C.H. assisted with the single-cell culture experimental design and interpretation of the results. All authors read and approved the final manuscript.

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