

In Vivo Repopulating Activity Emerges at the Onset of Hematopoietic Specification during Embryonic Stem Cell Differentiation

Stella Pearson,¹ Sara Cuvertino,¹ Maud Fleury,¹ Georges Lacaud,^{2,*} and Valerie Kouskoff^{1,*}

¹Stem Cell Hematopoiesis Group

²Stem Cell Biology Group

Cancer Research UK Manchester Institute, University of Manchester, Wilmslow Road, Manchester M20 4BX, UK

*Correspondence: georges.lacaud@cruk.manchester.ac.uk (G.L.), valerie.kouskoff@cruk.manchester.ac.uk (V.K.)

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SUMMARY

The generation of in vivo repopulating hematopoietic cells from in vitro differentiating embryonic stem cells has remained a long-standing challenge. To date, hematopoietic engraftment has mostly been achieved through the enforced expression of ectopic transcription factors. Here, we describe serum-free culture conditions that allow the generation of in vivo repopulating hematopoietic cells in the absence of ectopically expressed factors. We show that repopulating activity arises immediately upon the commitment of mesodermal precursors to the blood program, within the first wave of hematopoietic specification. We establish that the formation of these progenitors is extremely transient and exquisitely sensitive to the cytokine milieu. Our findings define the precise differentiating stage at which hematopoietic repopulating activity first appears in vitro, and suggest that during embryonic stem cell differentiation, all hematopoietic programs are unraveled simultaneously from the mesoderm in the absence of cues that restrict the coordinated emergence of each lineage as is normally observed during embryogenesis.

INTRODUCTION

Recent advances in the generation, propagation, and differentiation of pluripotent stem cells (PSCs) offer great promise in the field of regenerative medicine. Both embryonic stem cells (ESCs) and induced PSCs (iPSCs) provide limitless sources of self-renewing cells endowed with the potential to generate tissue-specific cell populations that can be used in transplantation therapy (Grabel, 2012; Keller, 2005). However, one major hurdle in realizing this potential is the lack of specific and efficient protocols for differentiating these PSCs to specific populations that can be used for therapeutic applications. Although stem-cell-based regenerative medicine is still a distant goal, outstanding progress has been made in generating and engrafting ESC-derived lineages such as dopamine neurones (Kriks et al., 2011) and cardiomyocytes (Shiba et al., 2012; Yang et al., 2008). In contrast, since the first report of blood cell generation from ESCs 30 years ago (Doetschman et al., 1985), progress in deriving hematopoietic cells that are able to engraft in vivo has been rather modest. To date, the most successful in vitro derivation of hematopoietic cells capable of repopulating mouse models has relied on the ectopic expression of transcription factors such as HOXB4 (Kyba et al., 2002), CDX4 (Wang et al., 2005b), LHX2 (Kitajima et al., 2011), and RUNX1a (Ran et al., 2013). However, although HOXB4 overexpression has been shown to confer reproducible engraftment capability in differentiating mouse ESCs (Bonde et al., 2008; Kyba et al., 2002; Lesinski et al., 2012; Matsumoto et al., 2009), this approach has not been successfully translated to hu-

man ESCs (Wang et al., 2005a). An alternative approach to the use of HOXB4 in differentiated human ESCs was recently reported by Doulatov et al. (2013), who showed that the ectopic expression of transcription factors (HOXA9, ERG, RORA, SOX4, and MYB) in differentiating ESCs promotes short-term erythroid and myeloid engraftment. Few reports have documented the in vitro generation of hematopoietic repopulating potential from unmanipulated ESCs (Burt et al., 2004; Hole et al., 1996; Müller and Dzierzak, 1993; Potocnik et al., 1997). However, these approaches have not been reproduced or pursued, suggesting that they involve serum-dependent conditions that cannot be easily replicated. The use of high serum concentrations (Wang et al., 2005a) and/or stroma cell lines (Ledran et al., 2008) to support the formation of repopulating hematopoietic cells derived from human ESCs has also shown promising results, but to date, no follow-up studies have further validated or extended these differentiation protocols. It is likely that the reported successes in deriving repopulating hematopoietic cells relied on specific factors present in rare batches of serum—parameters that are impossible to control for and thus are extremely difficult to reproduce.

It is thought that a better understanding of the molecular and cellular mechanisms that regulate the emergence and maintenance of long-term repopulating hematopoietic stem cells (HSCs) during embryonic development would aid in the development of optimal protocols to generate such cells in vitro from PSCs. HSCs have been shown to emerge first from the aorta-gonad-mesonephros (AGM) region around embryonic day 10.5 (E10.5) in murine



embryos (Medvinsky and Dzierzak, 1996). This occurs several days after the actual onset of hematopoietic activity, which is observed first in the yolk sac from E7.5 and next in the embryo proper from E9.0 (Palis et al., 1999). These early waves of hematopoiesis successively give rise to primitive erythroid, myeloid, definitive erythroid, and lymphoid progenitors (Costa et al., 2012; Lin et al., 2014). Several studies, including lineage tracing (Zovein et al., 2008) and in vivo imaging (Boisset et al., 2010) studies, have revealed the endothelial origin of HSCs emerging from a homogenic endothelium (HE) population within the AGM region. Similarly, earlier waves of hematopoietic progenitors were also shown to derive from the HE (Ema et al., 2006; Lancrin et al., 2010; Nishikawa et al., 1998).

The in vitro differentiation of ESCs has been widely used as a model system to dissect and understand the early events of hematopoietic specification in terms of both molecular mechanisms and cellular steps. The careful dissection of this in vitro program has demonstrated that, similarly to in vivo development, blood cells are generated from mesodermal hemangioblast precursors through an HE intermediate (Choi et al., 1998, 2012; Eilken et al., 2009; Fehling et al., 2003; Huber et al., 2004; Kennedy et al., 2007; Lancrin et al., 2009; Wang et al., 2004) and that the same network of transcription factors orchestrates both in vivo and in vitro processes (Moignard et al., 2013). Detailed studies of the generation of primitive erythroid, myeloid, and lymphoid progenitors have suggested a temporal emergence of these blood lineages in vitro, reflecting their sequential emergence in vivo during embryonic development (Irlion et al., 2010). This led to the concept that repopulating activity might emerge at late stages of the hematopoietic program during ESC differentiation (Kardel and Eaves, 2012; Lis et al., 2013; Sturgeon et al., 2013) and that the emergence of lymphoid potential marks the establishment of the definitive program (Kennedy et al., 2012; Slukvin, 2013). To date, however, attempts to derive in vivo repopulating hematopoietic cells from late stages of ESC differentiation have been largely unsuccessful. To revisit this long-standing challenge, we took an alternative approach and explored the very first step of hematopoietic specification from the mesoderm. We hypothesized that multilineage progenitors with in vivo repopulating ability might be specified very early upon commitment of mesoderm to the blood program, and might be difficult to maintain as such in the presence of serum or hematopoietic cytokines. We first evaluated the growth factor requirement for optimal specification of hemangioblast to HE. Next, defining the full hematopoietic potential of this emerging population, we observed the concomitant emergence of erythroid, myeloid, and lymphoid progenitors. Interestingly, this early population was also endowed with the capability to engraft immunocom-

promised mice and to confer multilineage, long-term engraftment. Further studies allowed us to define the temporal emergence of this repopulating ability and to determine the growth factor requirement and immunophenotypic characteristic of this population. Collectively, our findings demonstrate that in vitro repopulating cells emerge very rapidly from mesoderm precursors, are extremely transient, and are exquisitely sensitive to the growth factors present in the differentiating conditions.

RESULTS

BMP4, Activin A, and VEGF Are Critically Required for the Generation of HE and CD41⁺ Progenitor Cells

We previously showed that a combination of BMP4, Activin A, FGF, and VEGF was sufficient to efficiently drive the formation of blood precursors from differentiating ESCs in serum-free culture conditions (Pearson et al., 2008). However, optimal specification to each differentiation stage is likely to require precise temporal exposure to cytokine stimuli. Therefore, we set out to define which cytokines were specifically required for the transition from hemangioblast to HE, and then from HE to hematopoietic progenitors, with a particular emphasis on HE, from which repopulating cells are known to emerge in vivo (Bertrand et al., 2010; Boisset et al., 2010; Kissa and Herbomel, 2010). As depicted in Figure 1A, ESCs were differentiated via embryoid body (EB) for 3 days in serum-free culture with the successive addition of BMP4 at day 0, and then Activin A and FGF at day 2.5. This sequential exposure to growth factors was previously shown to promote hemangioblast specification efficiently in developing mesoderm (Pearson et al., 2008). At day 3 of the EB culture, FLK1⁺ cells enriched for hemangioblast were isolated and then further cultured with no added factors, a combination of four factors (BMP4 [B], Activin A [A], FGF [F], and VEGF [V]), or various combinations of these factors. The successful differentiation of FLK1⁺ cells into HE was measured at day 2 of the culture by the coexpression of TIE2 and cKIT, as previously described (Lancrin et al., 2009). The efficient generation of hematopoietic progenitors was assessed at day 3 by CD41 expression, which is known to mark emerging progenitors (Ferkowicz et al., 2003; Mikkola et al., 2003). In the absence of added factors, few cells coexpressed TIE2 and cKIT (Figure 1B), and the generation of CD41⁺ cells was limited (Figure 1C). In contrast, the addition of all factors (BAFV) led to the detection of a substantial TIE2⁺cKIT⁺ population and the enhanced generation of CD41⁺ cells. Dissecting the role of each factor individually or in combination revealed that individual factors on their own and most combinations were not able to generate or maintain an HE population (Figure 1B) and/or to produce a

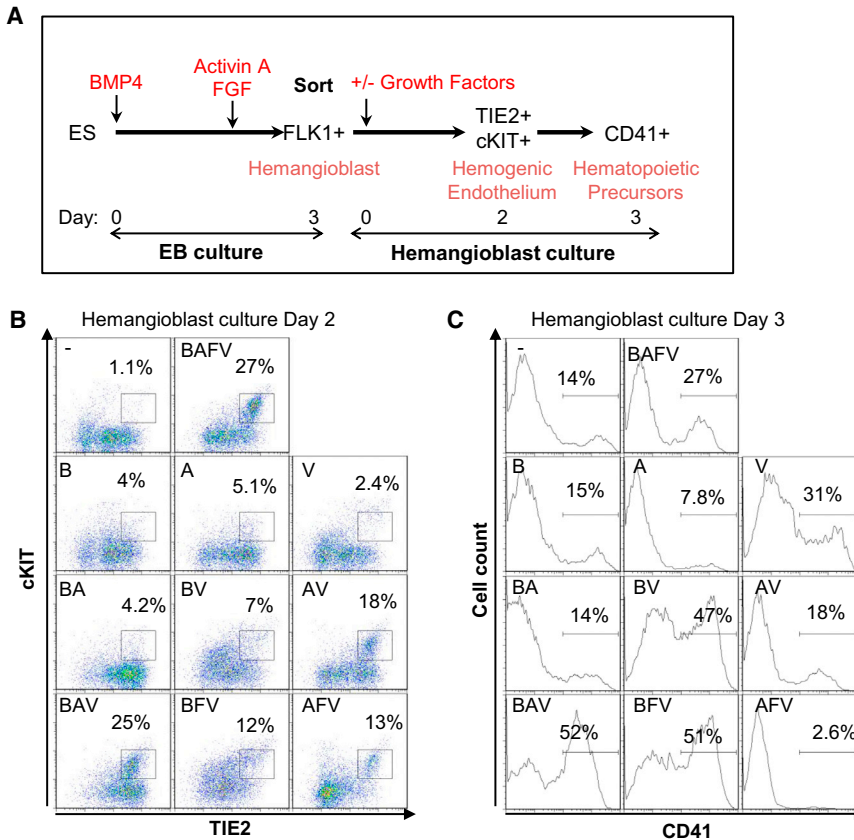


Figure 1. Optimal Cytokine Combinations for HE and Blood Cell Generation from FLK1⁺ Mesoderm Cells

(A) Schematic representation of the experimental strategy. ESCs were differentiated via embryoid body (EB) formation in serum-free culture supplemented with BMP4 at day 0 and with Activin A and FGF2 at day 2.5. FLK1⁺ cells sorted from day 3 EBs were seeded on gelatinized plates in serum-free media supplemented or not with cytokines (–, no cytokines; B, BMP4; A, Activin A; V, VEGF; F, FGF2).

(B) Representative flow cytometry of cells analyzed at day 2 of the culture for the coexpression of TIE2 and cKIT marking HE. (C) Representative flow cytometry of cells analyzed at day 3 of the culture for CD41 expression marking the emergence of blood cells. Data are representative of four independent experiments.

See also [Figure S1](#).

substantial frequency of CD41⁺ cells (Figure 1C). Both Activin A and VEGF appeared to be critically required for the generation and maintenance of HE cells, since only culture conditions containing both factors led to the formation of a clear TIE2⁺cKIT⁺ population (AV, AFV, and BAV). As observed in V, BV, and BFV culture conditions, the absence of Activin A in the culture led to CD41 cell production associated with a decrease in TIE2⁺cKIT⁺ frequency that was already observed at day 2 (Figures 1B and 1C). In contrast, the absence of BMP4 in the culture led to a dramatic decrease in CD41⁺ cell production, as observed in A, AV, and AFV culture conditions, suggesting that while this factor is dispensable for the generation or maintenance of a TIE2⁺cKIT⁺ population, BMP4 is required for the emergence of CD41⁺ cells. To address this issue, we supplemented AV culture with BMP4 at day 2 and assayed for CD41 expression at day 3 (Figures S1A and S1B). However, the delayed addition of BMP4 did not enhance the generation of CD41⁺ cells, suggesting that although it does not impact the generation of a TIE2⁺cKIT⁺ immunophenotypic population from FLK1⁺ cells, BMP4 exposure is nonetheless critical for shaping the hematopoietic potential of this population at the onset of FLK1 differentiation. The expression of a panel of endothelial markers, such as ICAM2, FLK1, and CD144 (VE-cadherin), further revealed that the pres-

ence of both BMP4 and Activin A was critical to maintain the endothelial identity of the cKIT⁺ population at day 2 of the culture (Figure S1C). Only a fraction of cKIT⁺ cells maintained the expression of these endothelial markers when cultured in the presence of AFV or BFV. Altogether, these data revealed that the combination of BMP4, Activin A, and VEGF is critical for the generation of both HE and CD41⁺ cells. Interestingly, early exposure to BMP4 appears to confer hematopoietic potential to the TIE2⁺cKIT⁺ population.

Inhibitory Effect of FGF and Activin A on Further Hematopoietic Commitment

We next compared the emergence and frequency of HE when FLK1⁺ cells were cultured with BAV and BAFV, as these two conditions were the most effective for generating HE (Figure 1B). In both cases, a low frequency of TIE2⁺cKIT⁺ cells was observed at day 1 of the culture; the frequency of this population peaked at day 2 and decreased thereafter (Figure 2A). No noticeable differences were observed in the temporal emergence and frequency of this population regardless of whether FGF was added to the culture or not (Figures 2A and 3D). In contrast, the formation of CD41⁺ cells was negatively affected by the presence of FGF in the culture, with on average a 2-fold increase in the frequency

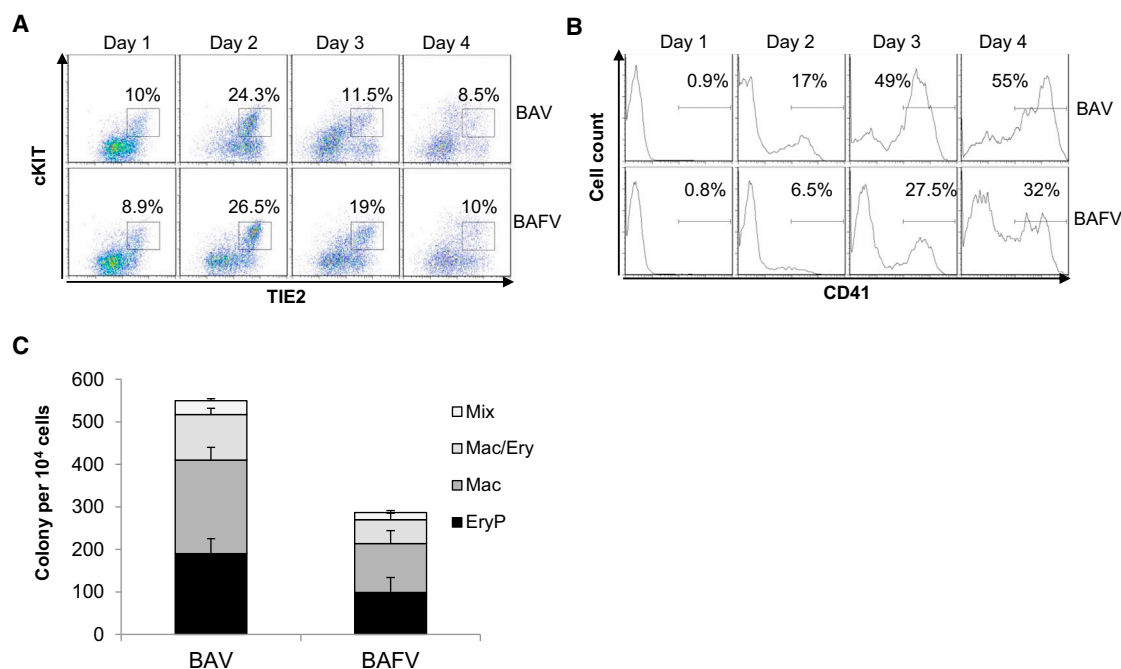


Figure 2. FGF2 Impairs the Generation of CD41⁺ Cells from HE

FLK1⁺ cells sorted from day 3 EBs were seeded on gelatinized plates in serum-free media supplemented with a BAV or BAFV cytokine combination (B, BMP4; A, Activin A; V, VEGF; F, FGF2).

(A and B) Cells were analyzed daily by flow cytometry for (A) coexpression of TIE2 and cKIT marking HE and (B) CD41 expression marking blood cells.

(C) At day 2 of the culture, cells were plated in clonogenic assay for hematopoietic precursors, and colonies were counted at day 5 for primitive erythroid (EryP) and day 8 for all other colonies (Mac, macrophages; Mac/Ery, macrophages and erythroid; Mix, multilineage myeloid and erythroid). Data shown are representative of at least three experiments. In (C), data are presented as the mean number of colonies from three dishes from one representative experiment; bars represent SEM.

of CD41⁺ cells produced in the absence of FGF from day 2 onward (Figures 2B and 3E). Both cultures gave rise to primitive erythroid and definitive colonies upon replating in clonogenic assays; however, FLK1⁺ cells cultured in the BAV condition resulted in the production of higher frequencies of hematopoietic precursors (Figure 2C), in agreement with the CD41 flow cytometry data. Altogether, these data suggest that although FGF does not affect the temporal emergence and frequency of HE, this growth factor negatively impacts the formation of hematopoietic progenitors.

We next assessed the influence of Activin A on the emergence of CD41⁺ progenitor cells because this factor was previously shown to negatively affect the generation of definitive hematopoiesis (Kennedy et al., 2012). For this purpose, sorted FLK1⁺ cells were cultured for 1 day in the presence of BMP4, Activin A, and VEGF (BAV), and then switched to media containing only BMP4 and VEGF (BV) as depicted in Figure 3A. Flow cytometric analysis revealed that removing Activin A after 1 day of culture significantly enhanced the frequency of TIE2⁺cKIT⁺ cells (Figures 3B and

3D), as well as the generation of CD41⁺ cells (Figures 3C and 3E). Time-lapse imaging of these cultures over a 3-day period illustrated the formation of adherent colonies of endothelial cells followed by the emergence of individual floating blood cells (Figures 3F and 3G), as previously shown in serum-supplemented cultures (Lancrin et al., 2009). The BAV-BV culture condition led to the emergence of large and healthy clusters of round floating cells (Figures 3F and S2A). In contrast, maintenance of Activin A in the culture (BAV) appeared to reduce the viability and size of the clusters of round floating cells (Figures 3G and S2B). This was further highlighted by the overall growth of the cultures in which a change of media from BAV to BV led to a marked increase in cell confluency (Figure S2C). Altogether, these data reveal that restricting the temporal exposure to Activin A is critically important for optimal specification of HE and hematopoietic precursors.

Multilineage Hematopoietic Potential of cKIT⁺ Cells

Having defined the optimal serum-free culture condition for the early steps of hematopoietic specification, we next

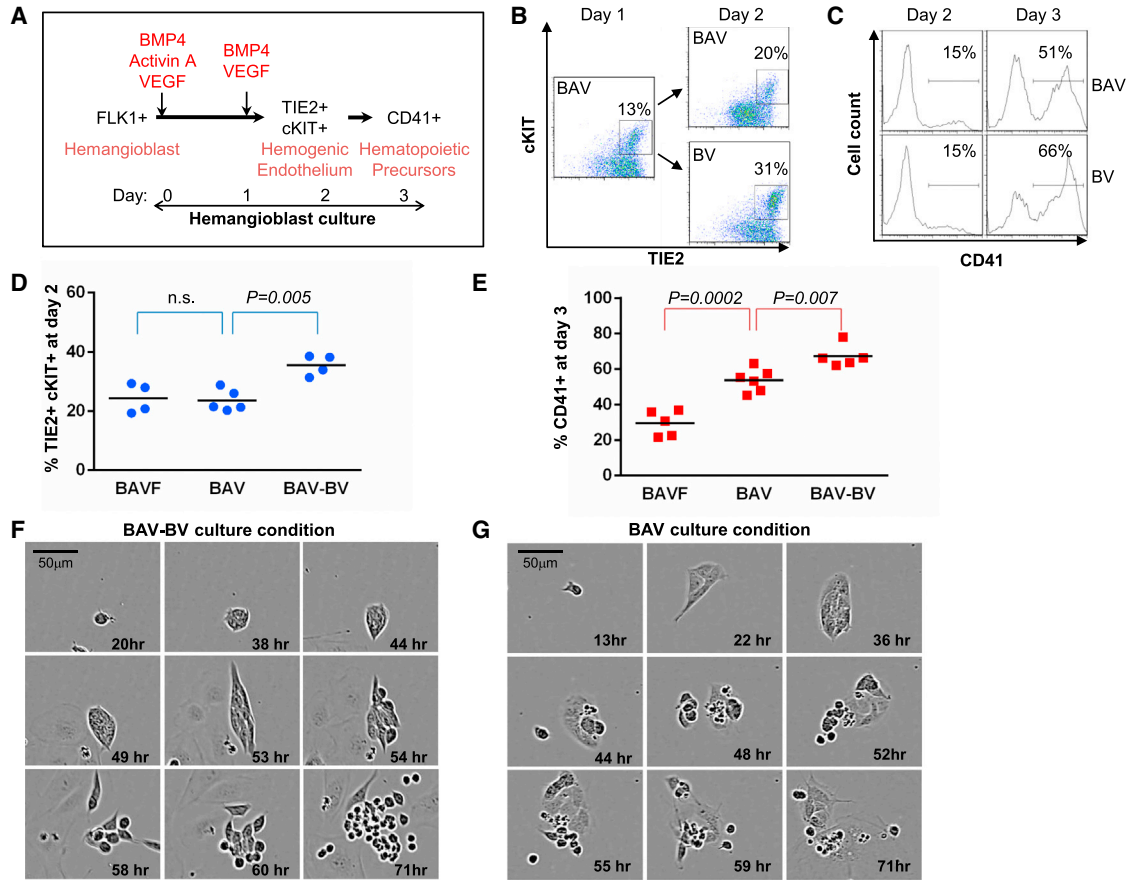


Figure 3. Activin A Impairs the Maintenance of HE

(A) Schematic representation of the experimental strategy. FLK1⁺ cells sorted from day 3 EBs were seeded on gelatinized plates in serum-free media supplemented with BAV for the first day and then with BV from day 1 onward (B, BMP4; A, Activin A; V, VEGF).

(B) Flow cytometry analysis of TIE2 and cKIT coexpression at day 1 and 2 of FLK1⁺ cell culture grown in a BAV or BAV-BV cytokine combination.

(C) Flow cytometry analysis of CD41 expression at days 2 and 3 of the same cultures.

(D and E) Graph of data obtained from BAVF, BAV, and BAV-BV cultures, showing the frequencies of TIE2⁺cKIT⁺ cells at day 2 (D) and CD41⁺ cells at day 3 (E). Each point represents an independent experiment.

(F and G) Representative time-lapse imaging of FLK1⁺ sorted cells cultured in serum-free media supplemented with a BAV-BV (F) or BAV (G) cytokine combination. Data shown are representative of at least three independent experiments (n.s., nonsignificant).

See also [Figure S2](#).

evaluated the biological characteristics of cKIT-expressing cells generated in this condition. As shown by multiparameter flow cytometry analysis, cKIT⁺ cells coexpressed all endothelial markers tested ([Figure 4A](#)), including ICAM2 and CD40, which were previously shown to mark HE ([Pearson et al., 2010](#)). At this stage of the culture, a small fraction of cKIT⁺ cells also coexpressed low levels of CD41, but none expressed CD45 ([Figure 4A](#); both of these markers are indicative of a further commitment to hematopoiesis). We next evaluated the biological potential of this cKIT⁺ population sorted at day 2 of BAV-BV culture. When cKIT⁺ cells were plated in clonogenic assays for hematopoietic progenitors, we observed the formation of both primitive erythroid col-

onies and definitive colonies ([Figures 4B, S3A, and S3B](#)). After 1 week, the culture of cKIT⁺ cells on OP9 or OP9-DL1 stroma in lymphoid-promoting conditions led to the formation of clusters of free-floating cells in the culture media and cobblestone-like areas underneath the stromal layer ([Figure 4C](#)). Analysis of cells derived from the OP9-DL1 cocultures after 3–4 weeks revealed the generation of T lymphocytes as defined by the expression of T cell-specific genes ([Figure S3C](#)); CD4, CD8, and CD3 expression; an immature CD4⁺CD8⁺ population; and low frequencies of more mature CD4⁺ and CD8⁺ cells ([Figure 4D](#)). Similarly, analysis of cells derived from OP9 cocultures demonstrated the generation of B lymphocytes as marked by the

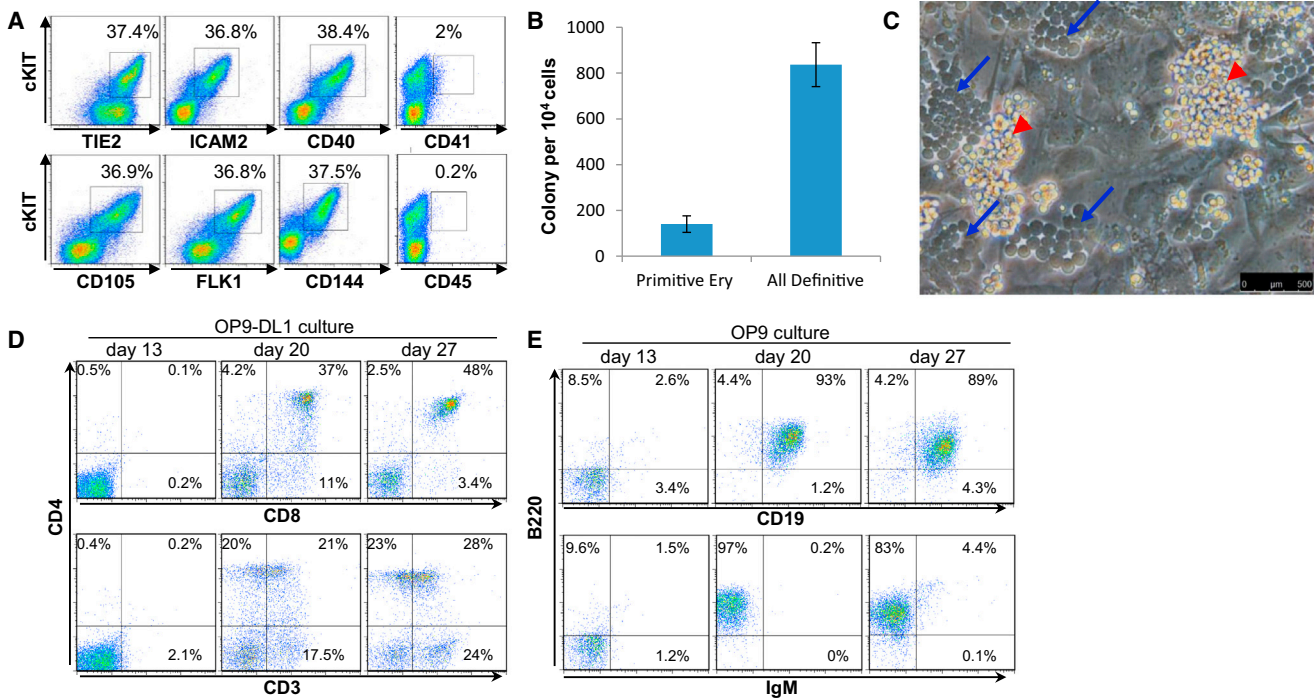


Figure 4. The cKIT⁺ Cell Population Derived from FLK1⁺ Hemangioblast Contains Erythroid, Myeloid, and Lymphoid Potential (A and B) FLK1⁺ cells sorted from day 3 EBs were seeded on gelatinized plates in serum-free media supplemented with BAV for the first day and then with BV from day 1 onward (B, BMP4; A, Activin A; V, VEGF). At day 2 of culture, cells were analyzed for the coexpression of cKIT with a panel of endothelial and hematopoietic cell-surface markers. (B) Day 2 sorted cKIT cells were plated in clonogenic assay for hematopoietic precursors. Primitive Ery: primitive erythrocytes; all definitive colonies: macrophages, macrophages/erythrocytes, GM, and GEMM colonies. Data are presented as the mean number of colonies from three dishes; bars represent SEM. (C) Bright-field picture taken at 1 week of culture. Blue arrows mark cobblestone areas; red arrowheads mark free-floating hematopoietic clusters. (D) Cells derived from OP9-DL1 culture were stained at the indicated time for the coexpression of CD4, CD8, and CD3 marking T lymphocytes. (E) Cells derived from OP9 culture were stained at the indicated time for the coexpression of B220, CD19, and IgM marking B lymphocytes. Data shown are representative of at least three experiments. See also [Figure S3](#).

expression of B cell-specific genes ([Figure S3D](#)) and the co-expression of B220 and CD19, with a small fraction of these cells expressing immunoglobulin M (IgM; [Figure 4E](#)). Altogether, these data reveal that FLK1⁺ mesodermal progenitors that have grown for 2 days in the sequential presence of BMP4, Activin A, and VEGF (BAV) followed by BMP4 and VEGF (BV) give rise to a population of cKIT⁺ cells endowed with the capacity to generate erythroid, myeloid, and lymphoid cells.

Long-Term and Multilineage Engraftment Potential of the cKIT⁺ Population

Given the in vitro multilineage capacity of the cKIT⁺ population isolated from FLK1⁺ cells that had differentiated for 2 days, we next investigated whether this population con-

tained cells that were able to engraft in vivo. For this purpose, cKIT⁺ cells were isolated from BAV-BV culture at day 2 and injected into sublethally irradiated recipient mice as depicted in [Figure 5A](#). Blood samples were taken every 4 weeks over a 16-week period and analyzed by flow cytometry for the expression of CD45.1 (marking recipient cells) and CD45.2 (marking in vitro-derived donor cells). In the initial experiments, the persistence of a small CD45.2⁺ population was observed in four out of ten mice over the 16-week period ([Figures 5B and 5C](#)). The in vitro origin of the CD45.2⁺ cells was further confirmed by detection of the *Brachyury-GFP* knockin allele, which was present in the starting ESC line but absent from the recipient mice ([Figure 5D](#)). Similar in vivo engraftment results were also obtained with cKIT⁺ cells derived from the in vitro

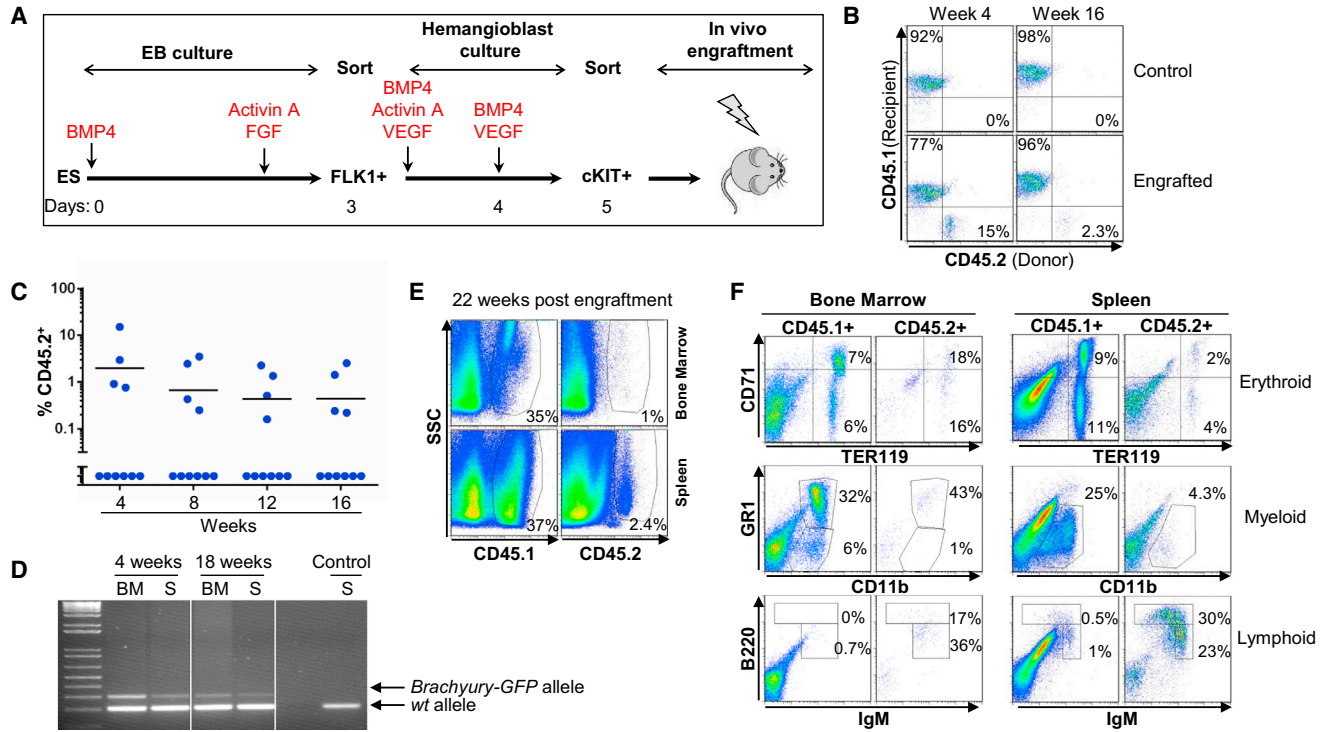


Figure 5. The cKIT⁺ Population Contains In Vivo Repopulating Activity

- (A) Schematic representation of the experimental strategy.
 (B) Blood cells from control and engrafted mice were stained for CD45.1 expression (marking recipient cells) and CD45.2 expression (marking ESC-derived cells).
 (C) Frequency of CD45.2⁺ cells (blue circles) in the blood at the indicated week. Each point represents a mouse (n = 2, with 5 mice per experiment).
 (D) PCR detection of the *Brachyury* endogenous and *Brachyury-GFP* knockin alleles carried by ESC-derived blood cells (BM, bone marrow; S, spleen).
 (E) Bone marrow and spleen cells harvested 22 weeks after engraftment were stained for CD45.1 and CD45.2.
 (F) Staining for lineage analysis is shown for CD45.1⁺ recipient and CD45.2⁺ ESC-derived cells from bone marrow and spleen at 22 weeks after engraftment. CD71 and TER119 mark erythroid cells, CD11b and GR1 mark myeloid cells, and IgM and B220 mark B lymphocytes. See also Figure S4.

differentiation of two other ESC lines (Figures S5D and S5G–S5J). Analysis of the lineage contribution in bone marrow and spleen at 22 weeks after engraftment revealed the presence of CD45.2⁺ cells expressing cell-surface markers characteristic of erythroid (TER119 and CD71), myeloid (Gr1 and CD11b), and B lymphoid (IgM and B220) lineages (Figures 5E and 5F). The B lymphocytes generated upon engraftment coexpressed IgM, CD19, and B220 (Figure S4A), and were able to secrete immunoglobulins (Figure S4B). It has been shown that embryonic hematopoietic precursors preferentially give rise to B-1 B lymphoid cells involved in innate immunity (Montecino-Rodriguez and Dorshkind, 2012). In addition to IgM expression, these B lymphocytes are characterized by the expression of CD11b and CD5 for the B-1a subset and CD11b for the B-1b subset. Given the embryonic origin

of the cKIT⁺ donor cells, we further assessed the immunophenotype of the B lymphoid population generated upon engraftment. None of the IgM⁺ cells expressed CD11b or CD5, suggesting that these cells are B-2 type B lymphocytes (Figure S4C). Altogether, these data demonstrate that cKIT⁺ cells isolated from ESCs that have differentiated in serum-free culture with restricted temporal exposure to specific growth factors are able to confer long-term and multilineage engraftment in vivo.

Temporal Emergence of Hematopoietic Repopulating Activity

The repopulating activity observed upon engraftment of cKIT⁺ cells isolated from day 2 culture was reproducible but remained low in terms of both the chimerism level and frequency of mouse repopulation (Table 1). Therefore,



Table 1. Summary of the In Vivo Engraftment Experiments

Population Tested	Cytokines in FLK1 Culture	n	Engrafted Mice	Chimerism Frequency at 4 Weeks (Percentage CD45.2 ⁺)
FLK1 ⁺	no further culture	2	0/8	0
cKIT ⁺ day 1	BAFV	6	14/26	4, 5.9, 6.5, 0.37, 4.5, 2.32, 0.77, 21, 3.36, 2.17, 10.04, 15.66, 5.04, 16.36
	BAV	2	3/8	0.4, 19.9, 15.65
	AV	1	1/4	0.43
	BNVF	1	0/4	0
	BNV	1	1/4	0.09
	BFV	1	0/4	0
	cKIT ⁺ day 2	BAV(day 1)-BV(day 2)	7	9/28
BAV(day 1)-A(day 2)		1	2/4	0.12, 2.9
BAV(day 1)-SB(day 2)		1	2/4	0.19, 2.5
BAV(day 1)-B(day 2)		1	1/4	1.5
BAV(day 1)-none(day 2)		1	1/3	0.24
BAFV		2	5/12	0.78, 1.04, 1.3, 2.9, 6.1
BAV		1	1/8	1.42
BNVF		1	0/4	0
BNV		1	0/4	0
cKIT ⁺ day 3		BAV(day 1)-BV(day 2)	2	0/12

B, BMP4; A, Activin A; V, VEGF; F, FGF2; N, Nodal; SB, SB-431542. See also [Figure S5](#).

we explored whether we could achieve a higher repopulation activity by changing the timing of cKIT⁺ cell isolation during the course of FLK1 differentiation to hematopoiesis. The cKIT⁺ population isolated from day 3 culture was unable to engraft in vivo, whereas day 1 cKIT⁺ cells gave rise to reproducible engraftment capability ([Figure 6A](#)). The overall level of chimerism observed with cKIT⁺ cells derived from day 1 culture was higher than that observed with cells derived from day 2 culture. Given this very rapid onset of repopulating activity upon culture of FLK1⁺ mesodermal precursors, we also evaluated the potential of FLK1⁺ cells to engraft directly without further culture. However, FLK1⁺ cells isolated from EBs and directly injected in vivo did not give rise to any repopulation activity ([Figure 6A](#)). Altogether, these findings suggest that the repopulating ability of ESC-derived hematopoietic precursors emerges rapidly upon mesoderm specification and is very transient.

Growth Factor Requirement for the Emergence of Hematopoietic Repopulating Activity

We next evaluated how altering the cytokine combination during the differentiation of FLK1⁺ mesoderm might impact the generation of in vivo repopulating cells. In a

first set of experiments, we assessed the growth factor requirement for the generation of this repopulating activity. We tested combinations of cytokines in which FLK1⁺ cells were cultured for the first day with BAV cytokines and then either maintained with BAV or changed to Activin A, BMP4, TGFβ inhibitor (SB-431542), or no cytokines for the second day of culture. In addition, cKIT⁺ cells isolated after 2 days of culture with BAFV were also tested in engraftment experiments. In all conditions tested, no improvement was observed in either the chimerism level or frequency of engrafted mice when compared with the BAV-BV cytokine combination ([Table 1](#)). Altogether, these data suggest that a variation in cytokine exposure during the second day of FLK1 culture does not affect the repopulating activity of the cKIT⁺ population isolated at day 2. Additionally, cKIT⁺ cells isolated from day 1 culture gave higher engraftment levels, suggesting that the first day of FLK1 culture is critical for determining in vivo repopulating competency. We therefore focused on the cytokine requirement for the first day of culture. Data presented in [Figure 1A](#) show that the cytokine combinations BAV, BAFV, and AV were the best conditions for generating a TIE2⁺cKIT⁺ HE population at day 2. When analyzed at day 1 of culture,

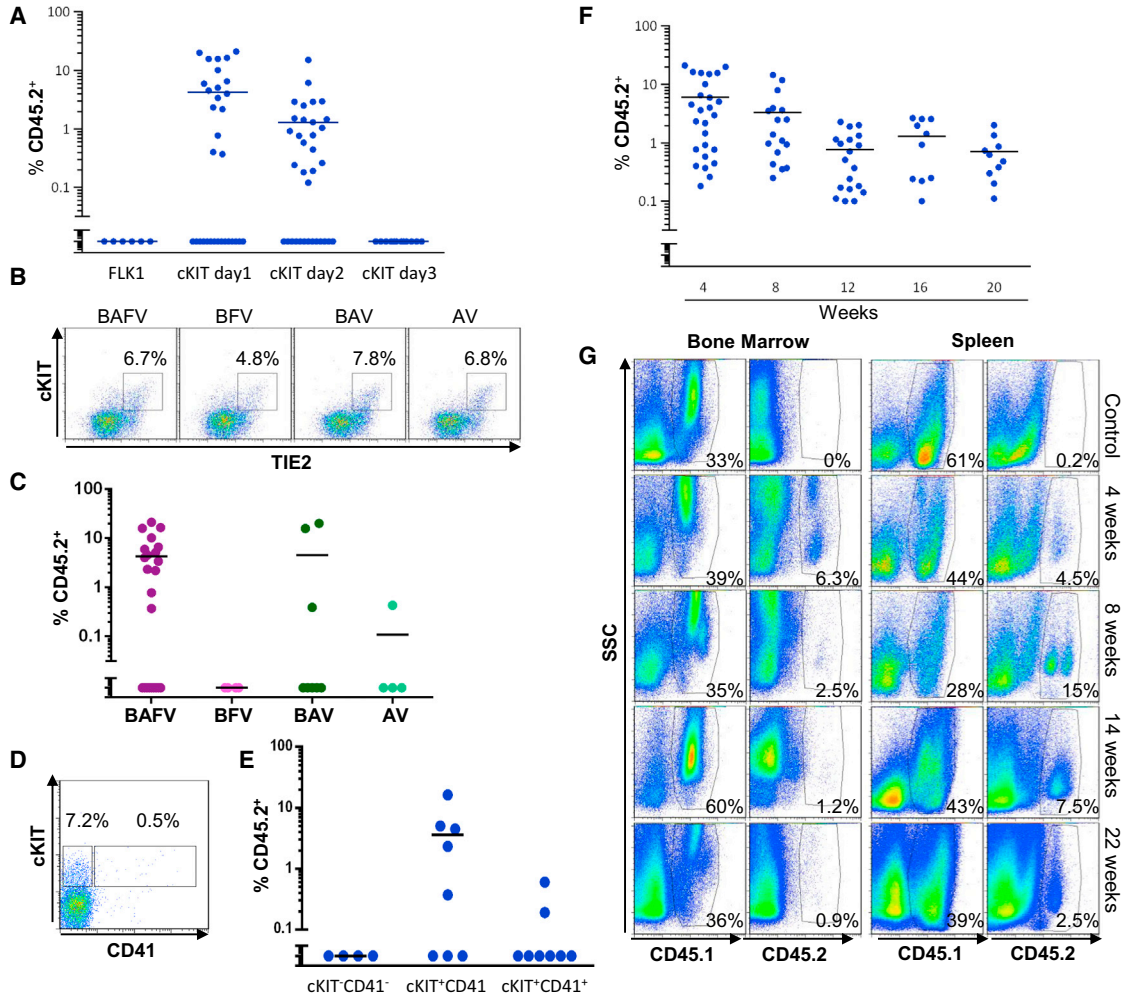


Figure 6. Successful Engraftment Is Highly Dependent on Cytokine Exposure

(A) Frequency of CD45.2⁺ cells (blue circles) in the blood of recipient mice 4 weeks after engraftment with the indicated population. The data presented in this graph for cKIT⁺ cells at days 1 and 2 were obtained under BAFV, BAV, and BAV-BV culture conditions. Each point represents one mouse; the number of experiments conducted for each population is shown in Table 1 (B, BMP4; A, ActivinA; V, VEGF; F, FGF2).

(B) Representative flow cytometric analysis of cKIT and TIE2 expression for cells obtained from day 1 FLK1 culture grown in the indicated cytokines.

(C) Frequency of CD45.2⁺ cells in the blood of recipient mice 4 weeks after engraftment with cKIT⁺ cells isolated from day 1 FLK1 culture grown in the indicated cytokine mix (BAFV, purple circles; BFV, pink circles; BAV, dark green circles; AV, light green circles). Each point represents one mouse; the numbers of mice and experiments are detailed in Table 1.

(D) Representative flow cytometric analysis of cKIT and CD41 expression for cells obtained from day 1 FLK1 culture grown in the presence of BAFV cytokines.

(E) Frequency of CD45.2⁺ cells (blue circles) in the blood of recipient mice 4 weeks after engraftment with the indicated cells isolated from day 1 FLK1 culture grown in the presence of the BAFV cytokine mix. Each point represents one mouse (n = 2, with 4 mice per experiment, except for group CD41⁻cKIT⁻ with 2 mice per experiment).

(F) Frequency of CD45.2⁺ cells (blue circles) in the blood of recipient mice at the indicated number of weeks after engraftment. Data in this graph represent a summary of all recipients that successfully engrafted. Each point represents one mouse, n = 18 with 4 mice per experiment as detailed in Table 1.

(G) Flow cytometric analysis of CD45.1 and CD45.2 expression relative to side scatter (SSC) for bone marrow and spleen cells at the indicated weeks after engraftment.

See also Figure S6.



the TIE2⁺cKIT⁺ population was detected at equivalent frequencies in those three cytokine mixes (Figure 6B). However, when tested in an in vivo repopulation assay, the absence of BMP4 was detrimental to engraftment, as shown by a comparison of engraftment for cKIT⁺ cells derived from AV and BAV (Figure 6C). Interestingly, cKIT⁺ cells derived from BFV culture did not give rise to any repopulating activity, even though this cytokine combination gave rise to a high frequency of blood progenitors (Figure S5A). Furthermore, although the chimerism levels observed in mice repopulated with cKIT⁺ cells derived from BAV and BAFV were similar, the number of mice engrafted was substantially higher in the presence of FGF (14/26; 53.8%) than in its absence (3/8; 37.5%; Figure 6C). It was recently shown that in vitro differentiated, Nodal-derived endoderm contributed in vivo to embryonic endoderm much more efficiently than Activin A-derived endoderm (Chen et al., 2013). Therefore, we assessed the in vivo repopulating ability of cKIT⁺ cells obtained from cultures in which Activin A was replaced by Nodal. Although Nodal was able to induce the formation of a TIE2⁺cKIT⁺ population at days 1 and 2 of FLK1⁺ cell culture (Figure S5B), we only observed a very low level of engraftment (0.09%) in one mouse out of 12 for all conditions tested (Figure S5C). Finally, to ensure the reproducibility and specificity of the differentiation process that is promoted by the BAFV cytokines, we performed the differentiation and engraftment protocol using BAFV cytokines from another commercial supplier. We observed similar levels of engraftment and contributions to erythroid, myeloid, and lymphoid lineages with these cytokines (Figures S5D–S5F). Altogether, these data demonstrate that very specific growth factor exposure during FLK1⁺ differentiation to cKIT⁺ is critically important to generate in vivo repopulating cells. Changes in a single cytokine can dramatically alter the potential of these in vitro-generated cells to engraft in vivo.

Immunophenotype of In Vitro-Derived Cells Endowed with Repopulation Activity

In order to refine our analysis of the cell population that harbored in vivo engraftment potential, we aimed to further define the immunophenotypic characteristics of this population. All endothelial cell-surface markers tested were coexpressed by cKIT⁺ cells and therefore could not be used to subfractionate the cKIT⁺ population. In contrast, CD41, which is known to be expressed on early embryonic HSCs (McKinney-Freeman et al., 2009; Robin et al., 2011), showed a distinct expression in a small subset of cKIT⁺ cells (Figure 6D). Surprisingly, however, when tested in in vivo engraftment, the cKIT⁺CD41⁻ fraction was more enriched in repopulating activity than the cKIT⁺CD41⁺ fraction, whereas, as expected, the cKIT⁻CD41⁻ subset was devoid of this activity (Figure 6E). These results establish that as

soon as TIE2⁺cKIT⁺ HE cells acquire CD41 expression, their in vivo engrafting ability is dramatically decreased, further reinforcing the hypothesis that the potential for in vitro-derived hematopoietic cells to repopulate recipient mice is extremely transient.

Long-Term and Multilineage Engraftment

The two most fundamental characteristics of HSCs are their abilities to self-renew and to give rise to all lineages of the blood system. As shown above in Figure 5F, blood progenitor contribution to the myeloid, erythroid, and lymphoid lineages was observed at 22 weeks after engraftment, but also at all stages analyzed (Figure S6). To address the self-renewing property of these in vitro-derived repopulating cells, we followed the frequency of CD45.2⁺ cells in the peripheral blood of recipients for up to 20 weeks (Figure 6F), considering that engraftment past 16 weeks is a readout of long-term repopulation. From 4 to 12 weeks after engraftment, we observed a progressive decline in the contribution of donor cells, with the frequency of these cells plateauing from 12 weeks onward. Analysis of donor cell contributions in the bone marrow and spleen revealed that initially the frequency of CD45.2⁺ cells was high in the bone marrow and low in the spleen (Figure 6G), a ratio that was reversed over time. By 22 weeks, the contribution to the bone marrow was low but still clearly detectable. Altogether, these data suggest that in vitro-derived repopulating cells are able to provide long-term multilineage engraftment, but their self-renewing ability is not as robust as that of in vivo-derived repopulating cells. In the future, it will be important to determine whether this is an intrinsic property of in vitro-derived repopulating cells or whether self-renewal can be modulated by the culture conditions.

DISCUSSION

The present study defines the temporal emergence of repopulating activity in vitro, identifying the precise step at which this population is generated. Altogether, our data establish that the presence of this repopulating activity is remarkably transient and the emergence/maintenance of this cell population is critically dependent on a precise set of growth factors.

To investigate the presence of long-term in vivo repopulating hematopoietic progenitor cells during ESC differentiation, we made the assumption that this activity might emerge rapidly upon specification of the hematopoietic program in mesoderm precursors, and we implemented several experimental strategies to test this hypothesis. First, the differentiating conditions were designed to avoid exposure to serum and cytokines such as SCF, IL6, and IL3 to prevent further differentiation of newly emerging



hematopoietic progenitors as much as possible. Second, we only considered cKIT expression as a potential marker of repopulating activity, since to date it is the only cell-surface marker that has been shown to be expressed on all HSCs throughout embryonic development and adulthood (all other markers are either expressed transiently during embryonic development or are only expressed on adult HSCs). Finally, to test for engraftment potential, we used sublethally conditioned recipients, reasoning that, in contrast to lethal irradiation, the remaining endogenous hematopoietic system would allow recipient mice to survive even with very low levels of contribution from the donor cells. A possible drawback to this approach is that the full engraftment potential might be underestimated due to competition between recipient and donor cells. However, the combination of these various experimental settings allowed us to reproducibly monitor the serum- and stroma-free generation of ESC-derived repopulating hematopoietic cells.

Our data demonstrate that both the emergence and maintenance of *in vivo* repopulating cells are extremely sensitive to the cytokine milieu: a change in a single factor will completely abolish the detection of engrafting cells. When we compared the outcome of FLK1 cells cultured in the presence of BAFV versus BFV, the absence of Activin A dramatically affected the detection of *in vivo* engrafting cells, as they were most likely rapidly pushed toward differentiation when exposed only to BFV. Taken together, our results strongly suggest that the signaling pathway activated by Activin A, but not Nodal, is critical, but not sufficient, for maintaining the repopulating ability of the TIE2⁺cKIT⁺ population. The addition of FGF seems to reinforce the role of Activin A; however, on its own, FGF is not able to maintain the population of engrafting hematopoietic progenitors. Finally, BMP4 signaling appears to be critical for conferring hematopoietic competency to the HE, an observation that is consistent with the known role of BMP4 in the regulation of *Runx1* (Burns et al., 2005; Pimanda et al., 2007). Although our study identifies differentiating conditions that allow the detection of engrafting hematopoietic cells, further work will be required to improve and optimize the culture conditions for the maintenance and expansion of these repopulating cells.

An important conceptual aspect of our findings relates to the relationship between ESC-derived repopulating cells and their *in vivo* counterparts. Based on their immunophenotypic characteristics, the ESC-derived cells more closely resemble the VE-cad⁺CD45⁻CD41^{low} pre-HSC type I population identified in the AGM region at E11.5 (Rybtsov et al., 2011). However, these type I pre-HSCs express a low level of CD41 and do not engraft recipients unless they are cocultured for 4 days with OP9, in contrast to ESC-derived repopulating cells, which do not express CD41 and are able to

engraft directly, albeit when injected intrafemorally. Based on their limited self-renewal characteristics, the ESC-derived engrafting cells might correspond to lineage-committed progenitors with a repopulating ability in which the multilineage potential is dissociated from the self-renewal capacity, as recently described for adult mouse bone marrow progenitors (Yamamoto et al., 2013). An interesting finding in our study is the concomitant emergence of primitive erythroid, myeloid, definitive erythroid, and lymphoid potential very early upon mesoderm specification. This is in contrast to previous studies that reported the sequential generation of these progenitors during *in vitro* differentiation of ESCs (Keller et al., 1993; Kennedy et al., 2012; Rafii et al., 2013). In those studies, serum-supplemented factors may have conditioned or altered the timing of differentiation and delayed the emergence of specific progenitor subsets. During embryonic development, the emergence of hematopoietic progenitors occurs in successive waves, with primitive erythroid progenitors emerging first around E7.25, followed by erythro-myeloid progenitors from E8.25 and lymphoid progenitors from E9, whereas definitive HSCs are only detected from E10.5 onward (Costa et al., 2012; Lin et al., 2014). One possible explanation to account for our findings is that during serum-free ESC differentiation, all hematopoietic programs unravel simultaneously because there are no extrinsic factors restricting or altering the developmental timing for the emergence of each lineage. During embryonic development, these cues are provided by the microenvironment in which these precursors reside.

The present study establishes a first critical step toward the generation of *in vitro*-derived, repopulating hematopoietic cells that might be suitable for therapeutic applications. Further work will need to be carried out to translate this protocol to the differentiation of human ESCs and iPSCs.

EXPERIMENTAL PROCEDURES

ESC Growth and Differentiation

Unless specified otherwise, the ESC line used in this study is an E14.1 (129/ola) carrying a GFP reporter cassette knocked in the Brachyury locus (Fehling et al., 2003). The F1 (129/B6) and RI (129/sv) ESC lines were also tested for engraftment potential. ESCs were maintained on irradiated mouse embryonic fibroblasts in Dulbecco's modified Eagle's medium (DMEM) supplemented with 50 µg/ml penicillin-streptomycin (GIBCO), 15% fetal calf serum (FCS; PAA Laboratories), 1% leukemia inhibitory factor (conditioned medium from Chinese hamster ovary cells), and 1.5×10^{-4} M monothioglycerol (Sigma). Prior to differentiation, ESCs were passaged twice on gelatinized tissue-culture-grade plates to remove the mouse embryonic fibroblasts. For the first passage, ESCs were grown in DMEM supplemented as above, and the second passage was performed in Iscove's modified Dulbecco's medium



supplemented as above. For gelatin treatment, dishes were coated for 20 min with 0.1% w/v gelatin in ddH₂O. For EB generation, ESCs were trypsinized and plated at 50,000 cells/ml in petri-grade dishes in StemPro-34 SFM (GIBCO) supplemented with 2 mM L-glutamine (GIBCO), transferrin (Roche), 0.5 mM ascorbic acid (Sigma), and 4.5×10^{-4} M monothioglycerol (Sigma). BMP4, bFGF, Activin A, Nodal, and VEGFa (R&D Systems or PeproTech) were used at 5 ng/ml unless otherwise stated. For hemangioblast culture, FLK1⁺ sorted cells were seeded on gelatinized plates in StemPro-34 SFM supplemented as above with the addition of cytokines as stated for each experiment.

Mice Engraftments

NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ mice were purchased from The Jackson Laboratory and bred in-house or at Harlan. Male mice were irradiated with a 125 cGy sublethal dose and injected intraperitoneally with sorted cells. Depending on the experiment, between 10⁵ and 5 × 10⁵ sorted cells were injected per mouse. All animal work was performed in accordance with regulations established by Home Office Legislation under the 1986 Animal Scientific Procedures Act.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2015.01.003>.

AUTHOR CONTRIBUTIONS

S.P., S.C., and M.F. performed the research and analyzed the data. V.K. and G.L. designed and supervised the research, analyzed the data, and wrote the manuscript.

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Stem Cell Reports

Supplemental Information

**In Vivo Repopulating Activity Emerges
at the Onset of Hematopoietic Specification
during Embryonic Stem Cell Differentiation**

Stella Pearson, Sara Cuvertino, Maud Fleury, Georges Lacaud, and Valerie Kouskoff

Supplemental Experimental Procedures

Gene expression analysis. For gene specific PCR, total RNA was extracted with an Rneasy plus kit (Qiagen) and reverse-transcribed with random hexamer using a SuperscriptIII kit (Life Technologies). The PCR reactions were performed using Biomix Taq (Bioline) and 0.2 mM of each primer. Real time PCR was performed on an ABI 7900 system (Applied Biosystem) using Exiqon universal probe library and primer designer (Roche): *β-actin* (F: tgacaggatgcagaaggag, R: cgctcaggaggagcaatg, probe #106) , *E47* (F: aagaggacaagaaggacctgaa, R: ttattggccatacgcctctc, probe #25) , *Pre-Tα* (F: ctgctttccggagcctct, R: gaggagcaggcgtag, probe #5) , *Rag1* (F: gaactgaagctcagggtagacg, R: gaactgaagctcagggtagacg, probe #16) , *Ebf1* (F: gaaacctctcggctccagta, R: caggattcttcgcaccatt, probe #17) , *Pax5* (F: acgctgacagggatggtg, R: ggggaacctccaagaatcat, probe #83). Expression data were calculated relative to *actin* controls as $2^{-\Delta\text{ct}}$.

Flow Cytometry. Cells were blocked with FcRγII/III antibody (24G2 supernatant) prior to staining with various combinations of antibodies as indicated in specific experiments (CD45.1-APC (17-0453), CD45.2-PE (12-0454), CD71-bio (13-0711), TER119-FITC (11-5921), GR1-bio (13-5931), CD11b-FITC (11-0112) , B220-bio (13-0452), IgM-FITC (11-5890), CD4-FITC (11-0041), CD8-PE (12-0081), CD3-bio (13-0032), CD19-bio (13-0193), CD5-PE-CY7 (25-0051-81), TIE2-bio (13-5987), cKIT-APC (17-1171-83), CD41-PE (12-0411), ICAM2-FITC (53-1021), FLK1-bio (13-5821-85), CD144-APC (17-1441-82), CD105-bio (13-1051-81), CD40-bio (13-0401), CD45-FITC (11-0451), strep-PE-CY7 (25-4317) all from eBioscience). Analyses were performed on a FACSCalibur and cell sorts on a FACSria (BD Biosciences). Data were analyzed with Flowjo (TreeStar), gating first on the forward scatter versus side scatter to exclude non-viable cells. Unstained and isotype controls were used for gating strategies.

Clonogenic assays. Cells were plated at a density of 2×10^4 cells/ml in 1% methylcellulose containing 10% plasma-derived serum (PDS; Antech), 5% protein-free hybridoma medium (PFHM-II; Gibco-BRL) and KL (1% conditioned medium), TPO (5 ng/ml), Erythropoietin (2 U/ml), IL-11 (25 ng/ml), IL-3 (1% conditioned medium), GM-CSF (3 ng/ml), G-CSF (30 ng/ml), M-CSF (5 ng/ml) and IL-6 (5 ng/ml). LIF, IL3 and c-kit ligand were derived from media as described (Fehling et al., 2003). GM-CSF, M-CSF, G-CSF, TPO, IL-6 and IL-11 were purchased from R&D Systems.

Time lapse imaging. Sorted FLK1⁺ cells were seeded on gelatinized Image-lock plates (Essen BioScience) in StemPro supplemented with the indicated cytokines, placed in the IncuCyte incubator system (Essen BioScience) and imaged every 15 minutes over a 4-day period in 5% CO₂ at 37° C.

Lymphoid cells derivation. Sorted cKIT⁺ cells were plated on irradiated OP9 or OP9-DL1 in IMDM supplemented with serum, IL7, KL and FLT3 as previously described (Schmitt et al., 2004). Cells were passaged weekly on fresh irradiated OP9.

Supplemental References

Fehling, H.J., Lacaud, G., Kubo, A., Kennedy, M., Robertson, S., Keller, G., and Kouskoff, V. (2003). Tracking mesoderm induction and its specification to the hemangioblast during embryonic stem cell differentiation. *Development* 130, 4217-4227.

Schmitt, T.M., de Pooter, R.F., Gronski, M.A., Cho, S.K., Ohashi, P.S., and Zuniga-Pflucker, J.C. (2004). Induction of T cell development and establishment of T cell competence from embryonic stem cells differentiated in vitro. *Nat Immunol* 5, 410-417.

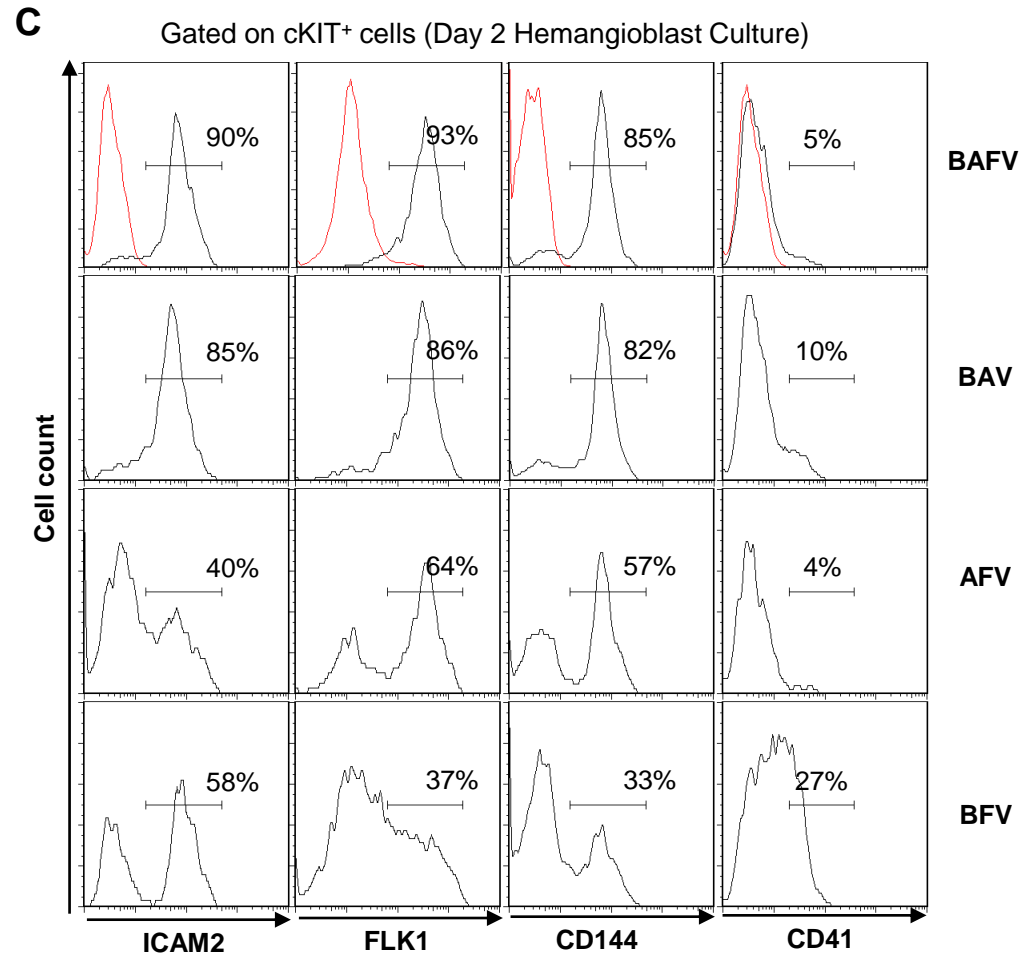
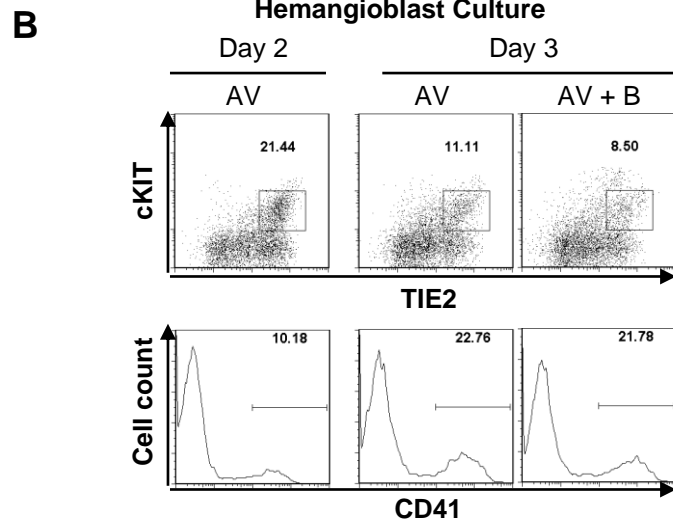
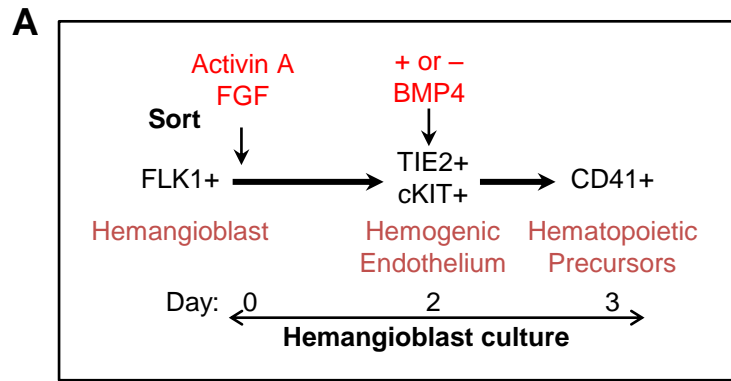


Figure S1, relating to figure 1: Endothelial characteristic of cKIT⁺ cells

(A) Schematic representation of the experimental strategy. FLK1⁺ cells sorted from day 3 EBs were seeded on gelatinized plate in serum-free media. Activin A, FGF and BMP4 were added or not as indicated. (B) Representative flow cytometry of cells analyzed after 2 or 3 days of culture in the indicated cytokines for the co-expression of TIE2 and cKIT which marks the hemogenic endothelium population and for CD41 expression marking the emergence of blood cells. (C) Representative flow cytometric analysis of the indicated cell surface markers on the cKIT⁺ population cultured for two days in the indicated cytokines. Red histograms indicate unstained controls. Data are representative of 3 independent experiments (B: BMP4, A: ActivinA, V: VEGF, F: FGF2).

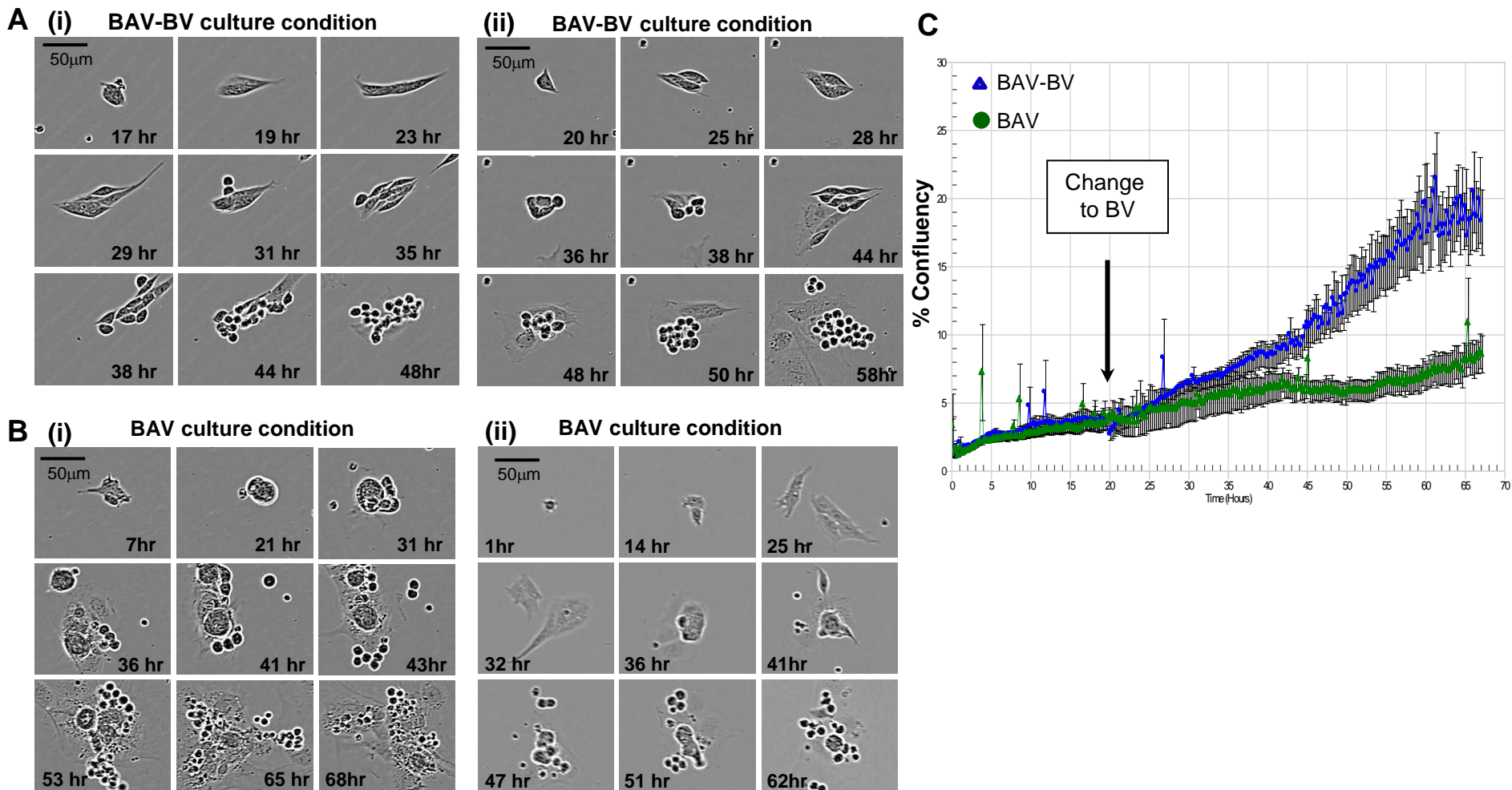


Figure S2, related to figure 3: Activin A impairs the expansion of emerging round floating hematopoietic cells

Further representative time lapse imaging of FLK1⁺ sorted cells cultured in serum-free media supplemented with **(Ai and ii)** BAV for the first day then switched to BV for the remaining time or with **(Bi and ii)** BAV for the entire culture. **(C)** Confluency over a 3-day period of FLK1⁺ cells cultured with BAV for the first day then BV for the remaining time (BAV-BV) or with BAV for the entire culture. Data were acquired with the IncuCyte software and are presented as a mean +/- SD of 30 individual measurements at each time point for each culture condition using a 15-minute time frame. Data are representative of 3 independent experiments (B: BMP4, A: ActivinA, V: VEGF, F: FGF2).

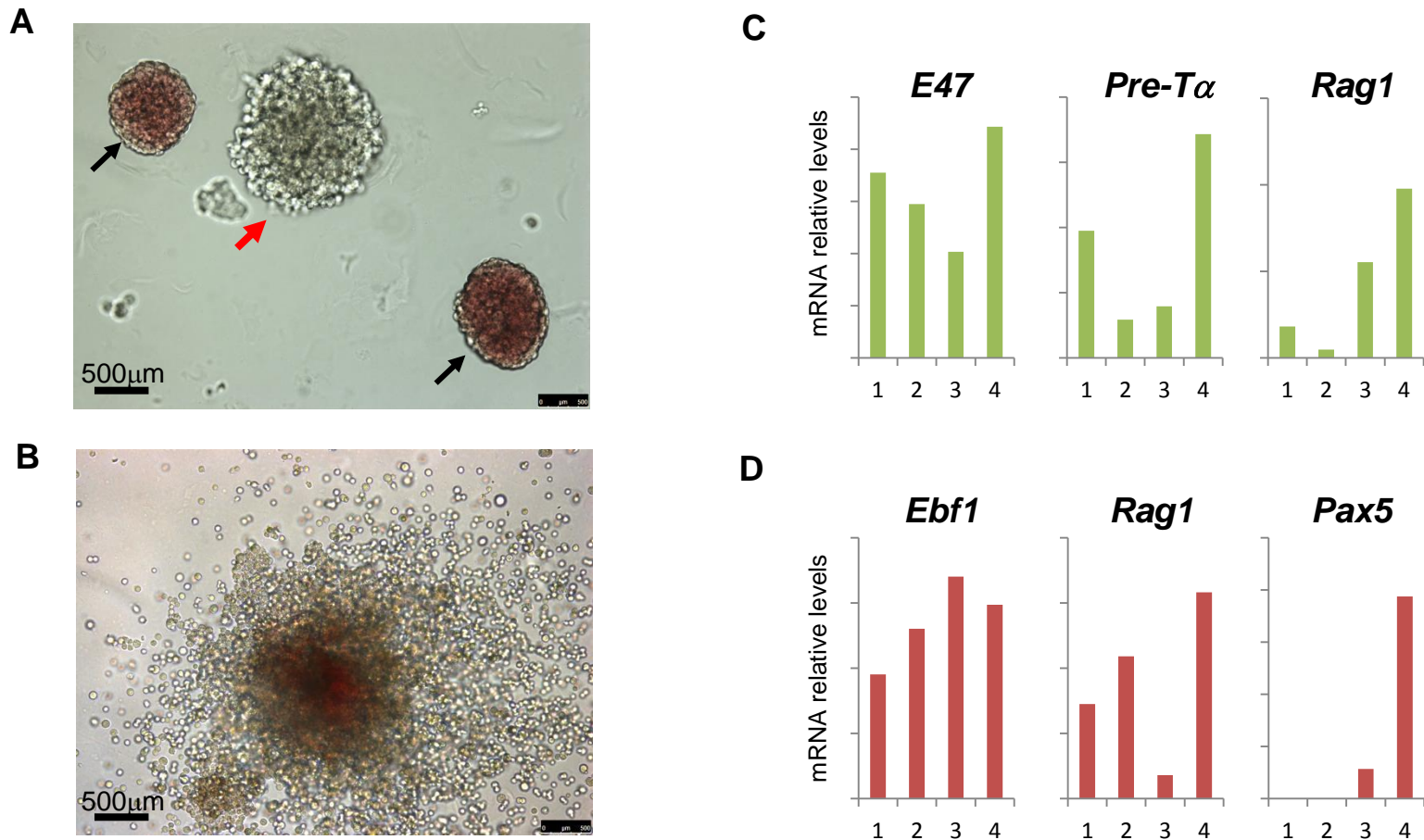


Figure S3, related to figure 4: Hematopoietic output of cKIT⁺ cells

(A) Representative picture of primitive colonies (black arrows) and definitive colonies (red arrow) taken 5 days after replating of cKIT⁺ cells. At this stage of the culture, cells within the definitive colonies are highly proliferative and not yet differentiating toward myeloid or erythroid lineages. (B) Representative picture of a multilineage definitive colony taken 10 days after replating of cKIT⁺ cells. (C) Real time PCR for the expression of the indicated genes in T cells derived from cKIT⁺ cultured on OP9-DL1. (D) Real time PCR for the expression of the indicated genes in B cells derived from cKIT⁺ cultured on OP9. Note that in two cases, the level of *Pax5* expression was below detection limit. Data are shown for 4 independent experiments (1 to 4) and mRNA expression data were calculated relative to β -actin controls as $2^{-\Delta Ct}$.

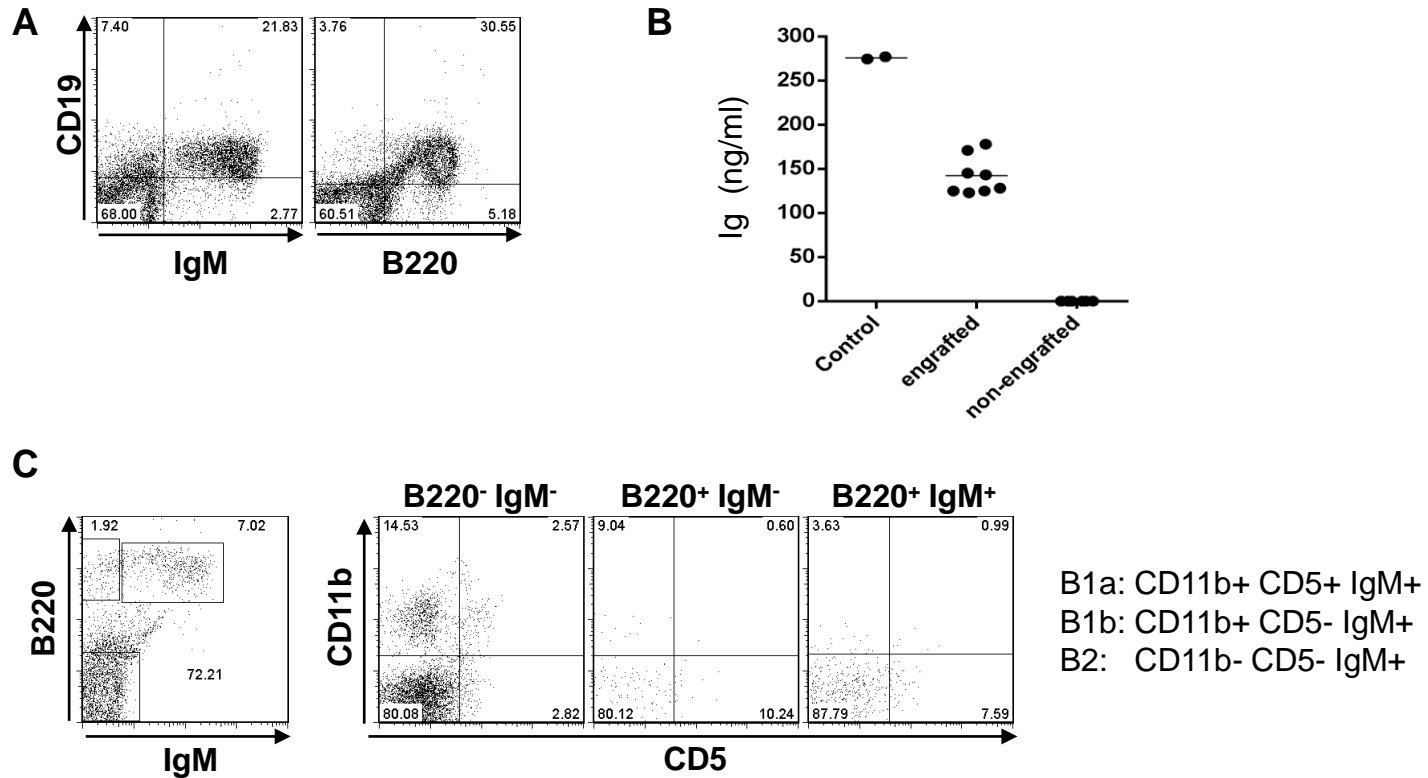


Figure S4, related to figure 5: B lymphocytes generated upon engraftment of ES-derived cKIT⁺ cells are of the adaptive B2 lineage
 (A) B cells in the spleen of engrafted mice co-express CD19, B220 and IgM. (B) Immunoglobulin concentration in the serum of control C57Bl/6 mice and NSG mice engrafted or not with ES-derived hematopoietic repopulating cells as measured by ELISA. Each dot represents an individual mouse. (C) Spleen cells of engrafted mice were co-stained for the expression of IgM, B220, CD11b and CD5. The expression of CD11b and CD5 which defines B1 B cell subsets was analyzed on B220-IgM⁻, B220⁺IgM⁻ and B220⁺IgM⁺. Data are representative of four independent mice .

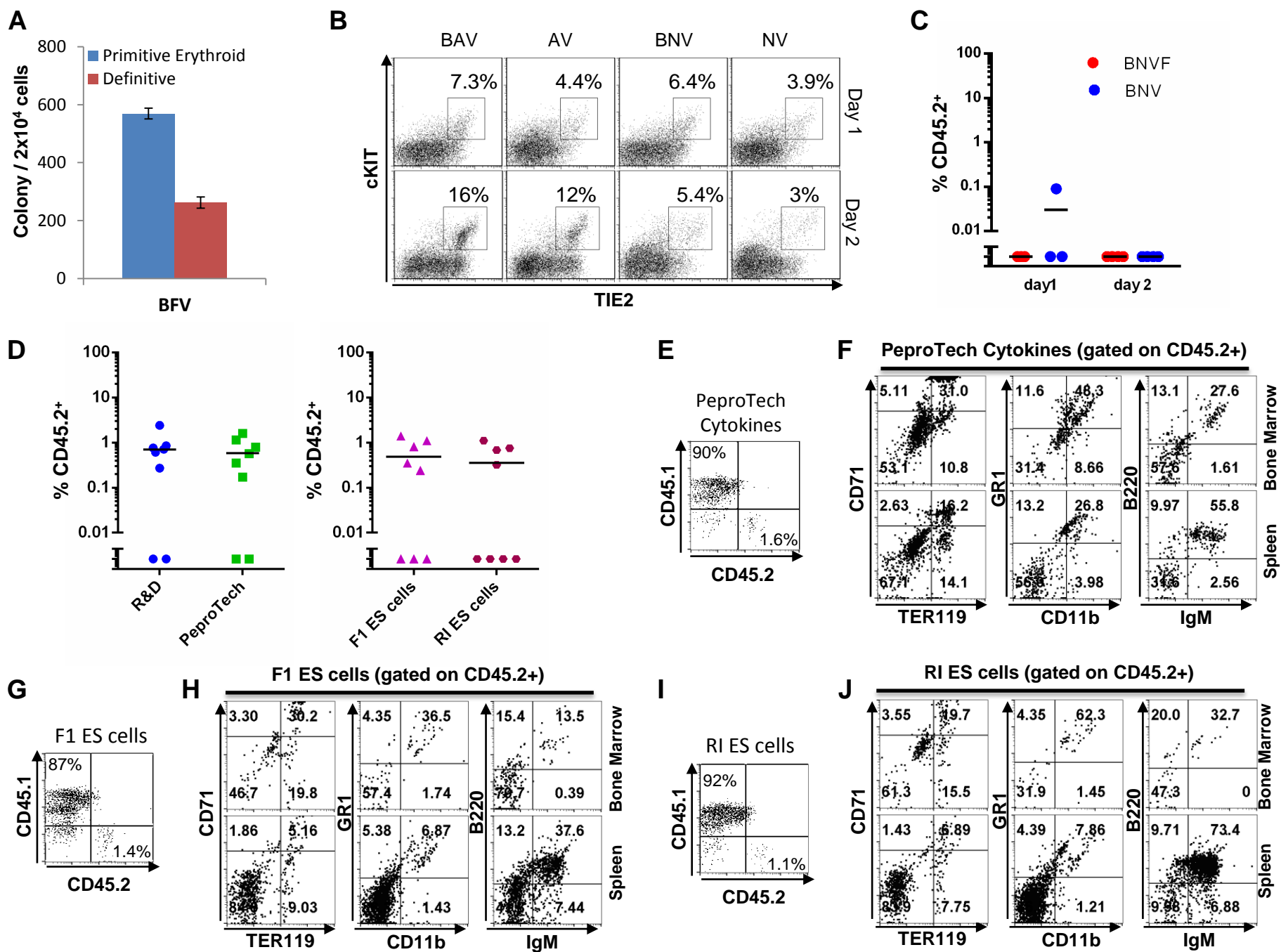


Figure S5, related to Table 1: Importance of cytokines in the formation of ES-derived repopulating cells

(A) At day 2 of culture in the indicated cytokines, cells were plated in clonogenic assay for hematopoietic precursors. Primitive colonies: primitive erythrocytes. Definitive colonies: all definitive colonies (macrophages colonies, macrophages/erythrocytes colonies, mix colonies and GM colonies). Data shown are representative of at least three experiments. Data are presented as the mean number of colonies from three dishes; bars represent SEM. (B) FLK1⁺ cells sorted from day 3 EBs were seeded on gelatinized plate in serum-free media supplemented with the indicated cytokine combinations (B: BMP4, A: ActivinA, V: VEGF, N: Nodal). Cells were analyzed by flow cytometry at day 1 and 2 for the co-expression of TIE2 and cKIT marking hemogenic endothelium. (C) Frequency of CD45.2⁺ cells in the blood of recipient mice 4 weeks after engraftment with cKIT⁺ cells derived from day 1 or day 2 culture containing BNV (blue circles) or BNVF (red circles) cytokines. Each point represents one mouse; number of mice and experiments are detailed in Table 1. (D) To assess the reproducibility of the protocol, *in vitro* differentiation followed by *in vivo* engraftment experiments were performed with cytokines obtained from a different supplier (R&D: blue circles; PeproTech: green squares) or with different ES cell lines F1 ESCs (purple triangles) and RI ESCs (brown circles). Frequency of CD45.2⁺ cells in the blood of recipient mice at 4 weeks is presented after engraftment with cKIT⁺ cells derived from day 1 in the indicated conditions. CD45.1 versus CD45.2 staining of peripheral blood samples at 4 weeks post engraftment using PeproTech cytokines for the *in vitro* differentiation (E), from *in vitro* differentiated F1 ESCs (G), and from *in vitro* differentiated RI ESCs (I). Each point represents one mouse; n=2, 4 mice per experiments. Bone marrow and spleen were harvested 12 weeks after engraftment and single cell suspensions were stained for CD45.1, CD45.2 and specific lineages markers. Flow cytometric analysis for lineage marker expression is shown on CD45.2⁺ ES-derived gated cells for bone marrow and spleen. CD71 and TER119 mark erythroid cells, CD11b and GR1 mark myeloid cells, IgM and B220 mark B lymphocytes. Data are presented for mice engrafted with cKIT cells derived using PeproTech cytokines for the *in vitro* differentiation (F), from *in vitro* differentiated F1 ESCs (H), from *in vitro* differentiated RI ESCs(J). For all reproducibility experiments n=2.

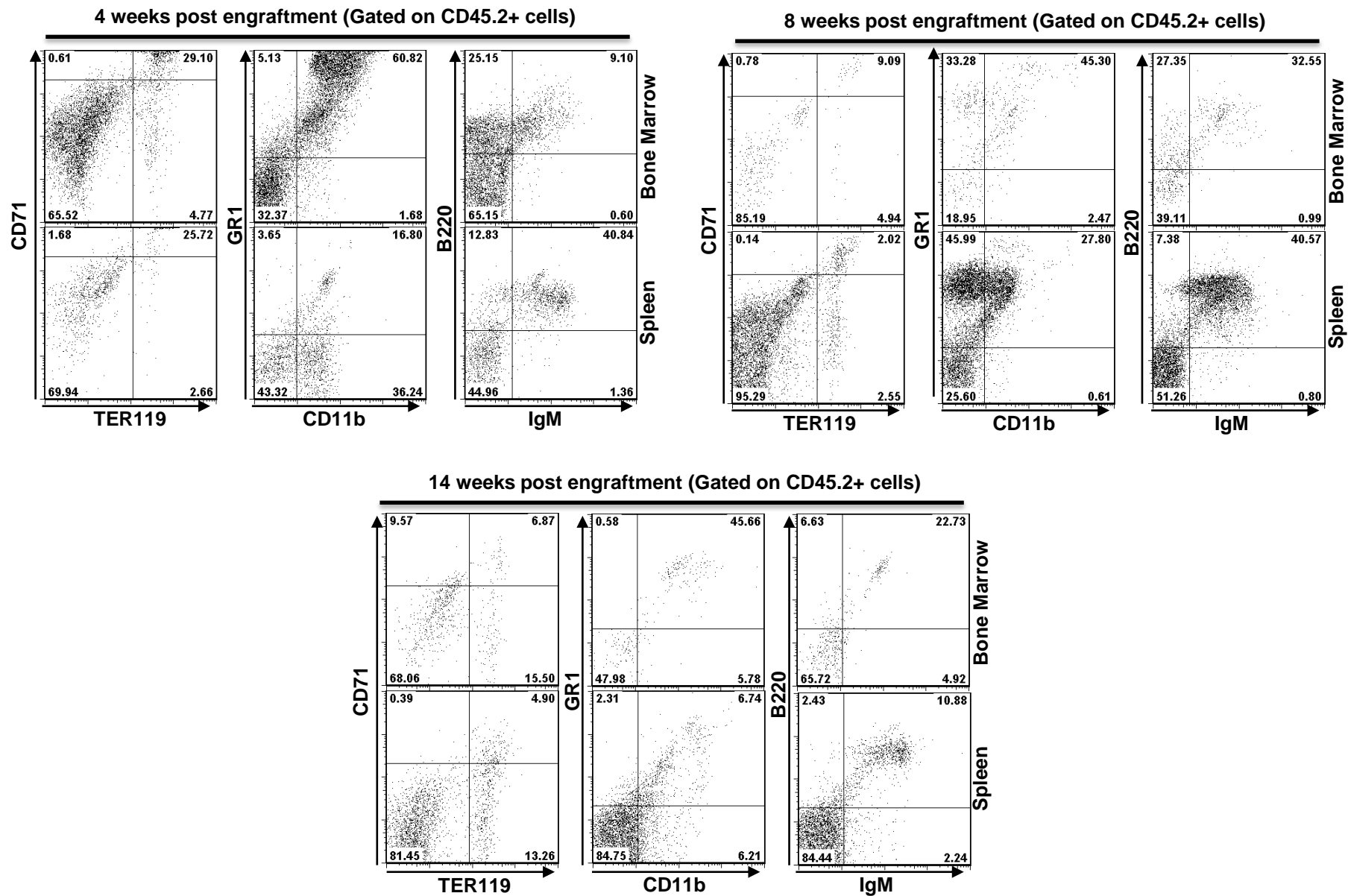


Figure S6, related to figure 6: Multilineage engraftment Bone marrow and spleen cells harvested 4, 8 or 14 weeks after engraftment were stained for CD45.1, CD45.2 and specific lineages markers. Flow cytometric analysis for lineage marker expression is shown on CD45.2+ ES-derived cells for bone marrow and spleen. CD71 and TER119 mark erythroid cells, CD11b and GR1 mark myeloid cells, IgM and B220 mark B lymphocytes.