In vitro differentiation of Gata2 and Ly6a reporter embryonic stem cells corresponds to in vivo waves of hematopoietic cell generation

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**INTRODUCTION**

Since the first report of blood cell production from embryonic stem cells (ESCs) (Doetschman et al., 1985), it has been a long-term goal to use in vitro cultures to produce definitive hematopoietic progenitor cells (HPCs) and hematopoietic stem cells (HSCs). With the advent of patient-specific induced pluripotent stem cells (iPSCs), this approach could be used for treating blood disorders without the adverse effects of rejection. Some progress toward differentiation into distinct blood lineages has been made through addition of growth factors to ESC/iPSC differentiation cultures (Doulatov et al., 2013; Kennedy et al., 2012; Pearson et al., 2015), and limited in vivo repopulation has been achieved by overexpression of transcription factors in ESC/iPSCs/endothelial cells (Li et al., 2014; Sugimura et al., 2017). More fundamental studies on ESC hematopoietic differentiation have provided some insight into whether such ex vivo cultures recapitulate in vivo hematopoietic development (Choi et al., 1998; Huber et al., 2004; Lancrin et al., 2009).

The natural development of the hematopoietic system occurs in spatiotemporally distinct waves (reviewed in Dzierzak and Speck, 2008; Kauts et al., 2016). The first blood cell production occurs in the yolk sac (YS) at mouse embryonic day 7 (E7), producing a transient cell population of mainly primitive erythrocytes (Palis et al., 1999). Definitive erythrocytes and myeloid cells appear in the YS starting at E8.25 and originate from erythroid-myeloid progenitors (EMPs) (Frame et al., 2013). Shortly thereafter, HPCs with erythroid-myeloid-lymphoid potential (Godin et al., 1995), lymphoid progenitors (Boiers et al., 2013; Li et al., 2014), and neonatal-engrafting hematopoietic cells arise (Yoder et al., 1997). HSC production is initiated in the final wave starting at E10.5 in the aorta-gonads-mesonephros (AGM) (Medvinsky and Dzierzak, 1996; Muller et al., 1994).

The Gata2 transcription factor plays a pivotal intrinsic role in EMP, HPC, and HSC generation in the embryo (de Pater et al., 2013; Gao et al., 2013; Ling et al., 2004; Tsai et al., 1994). Gata2−/− mouse and human (h) ESCs show defective hematopoietic differentiation (Huang et al., 2015; Tsai et al., 1994), and most hESC-derived HPCs are marked by Gata2 reporter expression, although it is uncertain whether this reporter parallels endogenous GATA2 expression (Huang et al., 2016). In our Gata2Venus (G2V) mice, in which levels of Gata2 expression are normal, it was found that Gata2 marks all HSCs and majority of HPCs. Gata2 is expressed in endothelial cells of the E8.5 YS and dorsal aorta (Minegishi et al., 1999; Robert-Moreno et al., 2005), E10.5 aortic hemogenic endothelial cells (HECs) (Eich et al., 2017), and C-KIT+/CD31+ intra-aortic hematopoietic cluster cells (IAHCs) (Kaimakis et al., 2016). Thus, the G2V reporter facilitates the examination of HPCs and HSCs as they emerge in the mouse embryo. Ly6a(SCA1)/GFP (LG) is another reporter that marks embryonic aortic cells. All HSCs, some HECs, IAHCs (Chen et al., 2011; de Bruijn et al., 2002; Li et al., 2014; Solaimani Kartalaei et al., 2015), and embryonic cells with lymphoid potential (de Bruijn et al., 2002; Li et al., 2014) are LG expressing. Vital imaging of the E10.5 mouse aorta shows endothelial-to-hematopoietic transition (EHT) of
Figure 1. G2V-Expressing Cells in Early Differentiation Culture Possess Primitive Erythroid and Endothelial Potential

(A) Scheme of ESC differentiation. BMP4 was present on days 0–3. FACS-analyzed/sorted embryoid body (EB) cells were tested in CFU-C (colony-forming unit—cell) and Matrigel assays.

(B) Dot plots for FLK1 (F) and Venus (V) expression from day 3 (d3) to day 6 (n = 3).

(C) Quantification of FACS data in (B) showing percentage of FLK1+/V−, FLK1+/V+, and FLK1−/V+ cells at days 3–6 (n = 3).

(D) Bar graphs showing CFU-C/10^4 FLK1+/V−, FLK1+/V+, FLK1−/V+, and FLK1−/V− cells at days 4–6 (n = 4). Colony types are indicated by color. CFU-M, macrophage; BFU-E, definitive erythroid; CFU-EryP, primitive erythroid.
these cells (Boisset et al., 2010). However, unlike the G2V reporter, LG+ cells are found in the AGM only beginning at late E9 (Mascarenhas et al., 2009), and thus, this distinguishes the later induction of an intraembryonic definitive HPC/HSC program.

Taking into account the complex spatiotemporal organization of in vivo blood development, it is likely that the ability to robustly generate definitive HPC/HSC in vitro depends on the spatiotemporal programs occurring during ESC differentiation, and requires enrichment methodologies with pivotal reporters to identify/isolate the cells of interest. Such reporters are a powerful tool for studying the dynamics of functional HPC/HSC generation in vitro (Huber et al., 2004; Lancrin et al., 2009; Ng et al., 2016) and their relationship to normal HPC/HSC development.

Here we examine the expression of G2V and LG reporters and the emergence of functional hematopoietic cells in a stepwise in vitro system of induction, enrichment, and differentiation of ESCs. We show that the temporal wave-like G2V reporter expression corresponds to waves of primitive and definitive hematopoietic emergence. Gata2 is co-expressed in these cells with hematopoietic transcription factors and marks functional HPCs emerging in the sequential waves. LG expression is specific to HPCs that emerge/persist in later differentiation stages, marking definitive progenitors with erythroid, myeloid, and/or B-lymphoid potential. This is confirmed in double reporter ESCs to show that in vitro differentiation occurs in stages that approximate the in vivo hematopoietic cell generation in mouse embryos.

RESULTS

Hematopoietic and Endothelial Potential of Gata2+ ESCs

Hematopoiesis is initiated by FLK1+ cells, which are able to generate endothelial and definitive hematopoietic progenitors (Choi et al., 1998; Huber et al., 2004). In hESC differentiation cultures, KDR+ cells develop in response to bone morphogenetic protein 4 (BMP4) between 72 and 96 hr and represent a transient population that precedes the onset of primitive hematopoiesis (Kennedy et al., 2007). To directly examine the relationship of Gata2 expression and hematopoietic differentiation of mouse ESCs, we used a G2V reporter ESC line (Kaimakis et al., 2016) that facilitates tracing and isolation of live Gata2+ cells by Venus fluorescence (Figure S1), while preserving normal Gata2 endogenous levels.

This is critical, since altered Gata2 levels severely affect the production and expansion of embryonic HSCs and HPCs and affect HSC robustness in the adult (Ling et al., 2004; Rodrigues et al., 2005), and dysregulation leads to leukemic syndromes (Katsumura et al., 2017). To examine whether Gata2+ cells possess endothelial or hematopoietic cell characteristics, we induced G2V ESC differentiation in the presence of BMP4 (Figure 1A) and analyzed them at days 3–6. FLK1+V+, FLK1+V+, FLK1+V+, and FLK1+V− cells sorted by fluorescence-activated cell sorting (FACS) were evaluated in hematopoietic colony-forming unit—cell (CFU-C) and Matrigel endothelial assays.

At days 3 and 4, the majority of V+ cells co-expressed FLK1 (67% and 76%, respectively) (Figure 1B). As the FLK1+V+ cell frequency decreased at day 5, the V+ single positive population became more prominent and FLK1+ cell frequency decreased (Figure 1C). Only the V+ fraction produced CFU-C (Figure 1D). CFU-Cs were detected from day 4 onward, with the main colony type being CFU-EryP (—primitive erythroid). Few CFU-M (—macrophages) and burst-forming units—definitive erythroid (BFU-E) progenitors were observed. Although the majority of colonies were scored in the FLK1+V+ population (24 ± 13, 16 ± 11, and 23.2 ± 10 CFU-C/10⁴ cells at days 4, 5, and 6, respectively), CFU-C were also detected in the FLK1+V+ cells at day 4.

To test the day-4 endothelial potential of ESCs (time of highest CFU-C potential in FLK1+V+ fraction), we quantified tubule formation of sorted cells (Figures 1E and 1F). The highest frequency was in FLK1+V+ cells (100 tubes/2 × 10⁴ cells). FLK1+V+ cells also showed endothelial potential (30 tubes/2 × 10⁴ cells), demonstrating that in addition to hematopoietic potential, some FLK1+V+ cells are capable of endothelial differentiation. This transient Gata2-expressing FLK1+ population emerges at day 4 and rapidly decreases thereafter. Thus, Gata2-expressing FLK1+ cells give rise to the earliest blood cells.

Temporally Defined Gata2 Expression Waves Are Detected in ESC Cultures

The temporal dynamics of Gata2 expression was characterized in ESC hematopoietic differentiation from days 4 to 14 by FACS (Figures 2 and S1D). V+ cells were detected throughout the time course and showed wave-like dynamics with peaks of increased V+ cell frequency. Compared with day 4, day-6 V+ cell percentages were significantly higher (5.5-fold) (Figure 2A) and then
decreased at days 7–10. V+ cell percentages increased again at day 11, decreased at day 13, and increased again at day 14 (RNA analysis in Figure S1E). Gata2 RNA expression (Figure 2B) showed increases at days 6 and 14 that correspond with the V+ cell frequency (Figure 2A; no Gata2 RNA analysis performed at day 11). To exclude the possibility that the later Gata2 expression peaks are due to the remaining undifferentiated ESCs, we analyzed the expression of the Nanog pluripotency factor. Nanog RNA was significantly decreased after day 3 of differentiation and no protein was detected at d12 (Figures S1F and S1G). Also, Brachyury expression, indicative of primitive streak/mesoderm, showed the expected dynamics with increased expression at days 3–6, and was downregulated thereafter (Figure S1H), thus supporting the likelihood of independent induction waves of Gata2+ hematopoietic cells in ESC cultures.

Gata2 functions in combination with other key (heptad) hematopoietic transcription factors to direct HPC/HSC development (Wilson et al., 2010). The expression of Gata2, Tal1, Lyl1, Lmo2, Fli1, Runx1, and Erg was tested (by qRT-PCR) in V+ and V− sorted cells (Figure 2C). Gata2, Tal1, Lyl1, Lmo2, Runx1, and Fli1 were significantly higher in the day-6 V+ compared with V− cells. At day 12, Gata2, Tal1, Lyl1, and Lmo2 were significantly higher in V+ compared with V− cells. Similar expression levels of Erg were found in V+ and V− cells at day 6/day 12, and Runx1 and Fli1 expression levels in these fractions were similar at day 12. As a control, the expression of a non-heptad ubiquitous transcription factor, Cbfβ (Runx1 binding partner), was assessed (Figure S1I) and found to be similarly expressed in day-6 V+ and V− cells. Thus, the expression of the relevant heptad hematopoietic transcription factors in ESC-derived Gata2+ cells indicates a developmental hematopoietic gene expression profile consistent with that found in mouse embryos (Eich et al., 2017).

Sequential Waves of Gata2+ Cells Correlate with Functional Primitive and Definitive Hematopoietic Potential

Hematopoiesis in the mouse embryo occurs in stage-specific waves, with the generation of cells with progressively greater multipotent hematopoietic properties. To elucidate whether V+ cells in the sequential waves express genes indicative of primitive (Hbb-y and βH1; early erythroid) and/or definitive (βmajor; adult erythroid) programs (Palis et al., 2010; Sankaran et al., 2010), we performed qRT-PCR (Figure 2D). Gata1 (pan-erythroid factor) showed expression at days 6 to 14 (Figure 2B, right). Hbb-y was expressed from days 6 to 12 and decreased at day 14 (Figure 2D, upper), and βH1 expression was detected from day 8 onward (Figure 2D, middle). Expression of both embryonic globins preceded the onset of βmajor (adult globin) expression that was detectable from day 10 onward (Figure 2D, lower), suggestive of definitive erythropoiesis.

In the mouse embryo, CD31+ HPCs/HSCs emerge from CD31+ HECs (Boisset et al., 2010; Yokomizo et al., 2012). CD31+, V+, and CD31+V+ cells were detected in day-6 and day-12 embryoid bodies (EBs) (Figures 2E and 2E′). The morphology and localization of V+ cells in CD31 immunostained whole EBs was analyzed by confocal microscopy. At day 6, CD31+ cells were dispersed throughout the EBs with no distinct structural organization, and flat endothelial-like V+CD31+ cells were found (Figure 2E). At day 12, round hematopoietic-like V+CD31+ cells were closely associated with the CD31+ cells lining the EB cavities (Figures 2E [lower] and 2E′). These imaging data indicate that V+ cells in the day-6 and day-12 EBs are morphologically distinct, and thus may possess different functions.

To test the relationship between Gata2 expression, stage of differentiation, and hematopoietic function, we harvested cells from day-6, -12, and -14 EBs, sorted V+ and V− cells, and analyzed the expression of hematopoietic markers. CD31+ and V+ cells were detected at days 6 and 12 (Figure 2F). At day 14, V+ cells were not detected. The expression of βmajor and βH1 was detected at day 12, but not at day 14. The expression of Hbb-y was detected at day 6 and decreased at day 14. These results suggest that CD31+ V+ cells in the sequential waves express genes indicative of definitive hematopoietic potential.

Figure 2. G2V Temporal Expression Defines Primitive and Definitive Hematopoietic Stages of ESC Differentiation

(A) Percentage of Venus+ (V+) cells in day 4–14 G2V ESC cultures (n = 3).

(B) Relative Gata2 (left) and Gata1 (right) mRNA as measured in qRT-PCR analysis of differentiated (total unsorted) cells from G2V ESC at days 4, 6, 8, 10, 12, and 14. Normalization to β-actin (n = 3).

(C) Relative Gata2, Tal1, Lyl1, Lmo2, Runx1, Fli1, and Erg mRNA as measured in qRT-PCR analysis of V+ and V− cells at days 6 (upper) and 12 (lower) of G2V ESC culture. Normalization to β-actin (n = 3).

(D) qRT-PCR analysis of relative Hbb-y (embryonic globin), βH1 (embryonic globin), and βmajor (adult globin) mRNA of cells from G2V differentiated ESCs at days 4, 6, 8, 10, 12, and 14. Normalization to β-actin (n = 3).

(E) Confocal images (representative) of whole-mount stained day-6 (upper) and day-12 (lower) EBs. Venus, green; CD31, red. Arrows indicate round endothelial-like V+ cells in day-6 EBs and round hematopoietic-like V+ cells/clusters in day-12 EBs. Scale bars, 40 μm. (E′) Magnification of whole-mount stained day-12 EB showing round V+ cells within the EB cavity close to CD31+ endothelial cells. Scale bar, 40 μm.

(F) Bar graphs showing CFU-C per 10^6 V+ and V− cells from day-6 and day-12 G2V ESCs (n = 3). CFU-GEMM, granulocyte-erythrocyte, monocyte-macrophage; CFU-GM, granulocyte-macrophage; CFU-M, macrophage; CFU-G, granulocyte; BFU-E, definitive erythroid; CFU-EryP, primitive erythroid.

Data are shown as means ± SEM. *p < 0.05, **p < 0.01. See also Figures S1 and S2.
Figure 3. Temporal Phenotypic Analysis of Reporter ESC-Derived Cells during Hematopoietic Differentiation

(A–C) Percentage of C-KIT+ cells in total viable cells from differentiated (A) G2V (n = 6), (B) LG (n = 3), and (C) LGGV (day 6, n = 3; day 8 and day 12, n = 4; day 10, n = 7; day 14, n = 5) ESCs at days 6, 8, 10, 12, and 14.

(D and E) Graphs showing the percentage of C-KIT+CD31+, C-KIT+CD41+, C-KIT+CD45+, and C-KIT+CD16/32+ cells in the reporter+ and reporter− cells at days 6, 8, 10, 12, and 14. (D) G2V (V+, green; V−, black; n = 3); (E) LG ESCs (G+, red; G−, black; n = 4).

(legend continued on next page)
V− cells, and analyzed CFU-C. HPCs were found exclusively in the V+ cell fractions (Figure 2F). Day-6 V+ cells gave rise to 50.1 ± 13 CFU-C/10^4 cells (1 HPC:198 cells), whereas day-12 V+ cells generated 36.0 ± 25 (1:278) and day-14 V+ cells 94.7 ± 20 CFU-C/10^4 cells (1:278). The majority (67%) of day-6 cells had CFU-EryP potential. Only a low frequency of macrophage, granulocyte, and definitive erythropoietic (CFU-M, CFU-G, BFU-E) colonies were observed. In contrast, day-12 and day-14 cells gave rise to a variety of erythropoietic and myeloid colony types, including mixed multipotent colonies (CFU-GEMM), suggesting that day-12, but not day-6 V+ cells possess EMP potential. Moreover, the day-14 progenitor frequency was increased 2.6-fold compared with day 12, and more mixed multipotent colonies were detected (Figure 2F). CFU-C derived from day-12 to -14 progenitors exhibited significantly increased proliferation capacity compared with the CFU-C from day-6 EBs. Together these data indicate that Gata2+ cells represent at least two different stages of induction in ESC cultures that correlate with primitive and erythropoietic-myeloid definitive functional cell potentials and molecular programs.

Gata2-Expressing C-KIT+ Cells Show Phenotypic Markers Characteristic of Definitive Hematopoiesis
G2V ESCs were examined for C-KIT expression (marker of cells undergoing transition to the hematopoietic lineage) during the ESC differentiation culture. A high frequency of C-KIT+ cells was found at day 6 ( ~64%) and decreased to ~16% by days 10–14 (Figure 3A). The co-expression of C-KIT with CD31 (endothelial and hematopoietic marker), CD41 (HPC and megakaryocyte marker), CD45 (pan-hematopoietic), and CD16/32 (EMP marker co-expressed with C-KIT) was measured together with Venus expression on differentiated cells by FACS (Figures 3D and S3). CD31+C-KIT+ cells were detected predominantly in V− fraction, whereas very few V− cells expressed this phenotype. The percentages of CD31+C-KIT+ cells in the V− fraction increased steadily at days 6–10, with a low point at day 12, and then rose again, suggesting a late wave of hematopoietic emergence. Four percent of day-6 V−C-KIT+ cells were CD41+ and this increased to 41% ± 3.3% at day 8, followed by decreases at days 10–14. Interestingly, C-KIT was mainly detected on V+CD41+ and not V+CD41− cells, in line with published data showing that AGM HP/SCs express CD41 at low/intermediate levels (Robin et al., 2011). Very few V− cells were CD41+ throughout all time points analyzed, thus suggesting that co-expression of Gata2, CD41, and C-KIT define an HP/SC population during ESC differentiation. CD45 expression was barely detectable in V+CD-KIT+ cells at day 6 (1.3%). There was a profound increase and peak at day 10 with 46% ± 2.2% of V+ cells CD45+C-KIT+ (as compared with the peak of CD41+C-KIT+ cells at day 6), and this was followed by decreased CD45+C-KIT+ cell percentages at days 12 and 13. C-KIT was expressed also in V− cells (15% ± 2.3% and 21% ± 5.0% at day 6). This is expected and is in line with the in vivo results showing Gata2-independent EMPs (Kaimakis et al., 2016). When the viability of day-10 to -14 V+ cells was tested (Figure S1J) no profound change in cell viability was found, indicating that the decreased frequency in phenotypic HPCs is not related to cell death. Together, these data demonstrate that Gata2 expression defines most phenotypic HPCs generated throughout ESC differentiation (both early and later) and that these markers reveal wave-like cell kinetics.

The LG Reporter Distinguishes a Late Hematopoietic Wave with Multilineage Potential
Unlike G2V reporter embryos in which Gata2 expression marks almost all hematopoietic cells from the earliest time of generation, the SCA1 (LG) reporter in vivo is expressed in fewer hematopoietic cells and in cells generated at later developmental stages (Boisset et al., 2010; de Bruijn et al., 2002). Analysis of day-10 and day-14 G2V ESC cultures showed respectively that 3.7% and 2.2% of cells expressed SCA1 (Figure 4A). Of the day-10 SCA1+ cells, less than 1% were C-KIT+CD41+. Although a slightly higher percentage of SCA1+ cells was found in day-10 cultures, the day-14 cultures contained a 6-fold higher percentage of C-KIT+CD41+ cells in the SCA1+ fraction, indicating that the LG reporter may be a relevant tool to aid enrichment for hematopoietic progenitors late in ESC differentiation cultures.

An LG ESC line was established from the LG mouse model to examine reporter expression kinetics during ESC differentiation. FACS analysis (Figure 4B) showed the presence of GFP+ (G+) cells already at days 3 (7%) and 4
(10%), followed by a rapid 5-fold decrease. G+ cell percentages increased at day 7 and peaked at days 9–10 of differentiation (3-fold increase; 6% at day 10).

The percentage of C-KIT+ cells in cultures at days 6–14 averaged ~17% with little change (Figure 3B), indicating less efficient hematopoietic differentiation of the LG line compared with G2V line (Figure 3A). Next, C-KIT and co-expression of CD31, CD41, CD45, and CD16/32 within the G+ and G− fractions of day-6 to -14 LG ESC cultures was determined (Figures 3E and S4). At day 6 the percentages of CD31+C-KIT+ cells in G+ and G− fractions, respectively (6.9% ± 0.4% and 6.9% ± 1.3%), were similar. From day 10 onward, the percentage of CD31+C-KIT+ cells increased in the G+ (rising to 26.0% ± 6.2%) compared with the G− fraction. CD41+C-KIT+ cells were detected beginning at day 8 in G+ cells (0.9% ± 0.2%) and gradually increased to 14% ± 3.0% at day 14. Percentages of CD45+C-KIT+ showed a trend similar to that of CD41+C-KIT+ cells and were detected in the G+ fraction from day 8 (0.2% ± 0.1%) onward. The frequency increased significantly after day 12 and rose to 18% ± 5.1% at day 14. The G− fraction contained no to very few CD45+C-KIT+ cells. C-KIT+CD16/32+ cells were detected from day 8 onward in both G+ and G− fractions, with the frequency of C-KIT+CD16/32+ cells increasing considerably in the G+ fraction at days 10–12. These data indicate that LG reports
phenotypic hematopoietic cells predominantly in the later stages of ESC differentiation.

The relationship of GFP expression with hematopoietic activity in the LG ESC culture was tested by blast colony-forming assay. At early differentiation stages (day 3), significantly more blast colonies (BL-CFC) per $10^4$ cells were found in the $G^-$ fraction ($215 \pm 11$) than in the $G^+$ fraction ($17 \pm 12$) (Figure 4C). Accordingly, very few FLK1+$G^+$ cells were found ($1\% \pm 0.1\%$) (Figure 4D). CFU-C assays from day-7 EBs revealed a small number of HPCs in both $G^+$ and $G^-$ fractions (Figure 4E). However, at days 12 and 14 significantly more CFU-Cs, including all the CFU-GEMMs, were found in the $G^+$ fraction. CFU-C frequencies increased from day 7 to day 14 (Figures 4E and 4F) but were similar in the $G^+$ and $G^-$ fractions at days 7 and 10. A significantly higher frequency of CFU-C was in the $G^+$ fraction than the $G^-$ fraction at day 12, and at day 14 virtually all CFU-Cs were in the $G^+$ fraction. This 2.3-fold increase in CFU-C frequency from day 12 to day 14 indicates that the LG reporter enriches for late appearing HPCs.

To examine whether late appearing cells have B-lymphoid potential, we co-cultured day-14 LG EB-derived $G^+$ cells for 22 days on OP-9 cells with lymphoid-promoting factors (Figure 4G). FACS analysis of B cell markers CD19 and B220 revealed that 4.3% $\pm$ 0.6% of the cells were CD19$^+$CD45$^+$ and 4.9% $\pm$ 0.8% were B220$^+$CD45$^+$. Seven percent of CD45$^+$ were positive for both CD19 and B220 (Figures 4H and S5). Thus, some LG reporter-expressing cells possess B-lymphoid potential.

Double Reporter Expression Defines Most HPCs in Late-Stage ESC Differentiation

To assess whether $G2V$ and LG reporter expression is overlapping, we established a Ly6a(SCA1)$GFP$:Gata2Venus ($LGGV$) double ESC reporter line and examined the kinetics and frequency of reporter-expressing hematopoietic cells during differentiation (Figures S6, 5B, and 5C).

The efficiency of $LGGV$ ESC hematopoietic differentiation was determined by C-KIT FACS analysis at days 6, 8, 10, 12, and 14 (Figure 3C). The percentage of C-KIT$^+$ cells in the total viable cell population of day-6 and -8 $LGGV$ cultures was 37% and 34% respectively. At days 10–14, the cultures contained an average of 18% C-KIT$^+$, thus indicating that the temporal hematopoietic differentiation efficiency of the $LGGV$ line is similar to that of the $G2V$ line (Figure 3A). Next, analysis for co-expression of C-KIT and CD31, CD41, CD45, or CD16/32 within the $V^+$ and $V^+$ fractions of day-10, -12, and -14 ESC differentiation cultures was performed (Figures 3F and S7). In general, the highest percentage of C-KIT$^+$CD31$, C$-K$IT^+$CD41$, C$-K$IT^+$CD45$, and C-KIT$^+$CD16/32$ cells was in the V$^+$ fraction at all three time points. Moreover, at day 14 for all the markers except for C-KIT$^+$CD41$, significant increases in the percentages of marker-positive cells were found in the V$^+$ fraction as compared with the V$^-$ and/or V$^-$ fractions, indicating a late shift in phenotypic hematopoietic progenitors.

To determine in which reporter-expressing populations HPC function was localized, we differentiated...
LGGV ESCs, and analyzed day-12 and day-14 single (V^G^− and V^G^+) and double (V^G^+^G^+) reporter-expressing cells along with reporter-negative (V^G^−) cells (Figure 5A). At day 12, 119 CFU-C/10^4^ were detected, of which 57 were derived from V^G^− and 62 from V^G^+. Negligible to no CFU-Cs were present in the V^G^+ and V^G^−^G^− fractions. The CFU-C frequency at day 14 increased to 200/10^4^ cells, and all CFU-GEMM were in the V^G^+ fraction (Figure 5A). Interestingly, when taking into account the frequency of reporter-expressing cells in the total cell population derived from LGGV EBs (Figures 5A and 5B), overall CFU-C frequency in the day-14 V^G^+ versus V^G^− fraction increased 4-fold (from 5% to 20% of the total colonies) compared with day 12 (Figure 6A, lower). This is in line with the increase in CFU-C frequency in the G^+ versus G^− fractions derived from day-12 and day-14 LG cultures (Figure 6A, middle). Colonies derived from G2V cultures were found only within the V^+ fraction throughout differentiation (Figure 6A, upper).

Together, these data suggest that a subset of progenitors is generated and/or gradually acquires the LG marker later in the culture and possesses more complex hematopoietic potential, thereby distinguishing and allowing specific enrichment of a more potent definitive wave of ESC hematopoietic emergence.

DISCUSSION

In this study, we show that ESC differentiation progresses through stages of functional hematopoietic cell production that can be discriminated by the G2V and/or LG reporters (Figure 6B). The fact that these reporters are validated in vivo indicators of normal temporal and spatial generation of functional hematopoietic cells in the mouse embryo (de Bruijn et al., 2002; Kaimakis et al., 2016; Li et al., 2014; Solaimani Kartalaei et al., 2015) ensures high fidelity in their use to examine the stages of hematopoietic emergence in vitro. The reporter-expressing ESC-derived cells exhibit functional hematopoietic properties that correspond to cells generated in the in vivo waves of hematopoietic emergence.

Hematopoiesis in the mouse embryo starts in the YS with a transient wave of primitive hematopoiesis, and is characterized by FLK1^+^ cells that give rise to the earliest blood cells and endothelium (Choi et al., 1998; Kennedy et al., 1997; Palis et al., 1999; Shalaby et al., 1997). Our analysis of early G2V EB differentiation (days 4–6) reveals a transient FLK1^+^V^+^ population that differentiates into endothelial and/or primitive erythroid cells. In contrast, early LG EB differentiation shows that most FLK1 expression is in the G^−^ cell fraction and blast colony-forming activity is in the FLK1^−^G^+^ cell fraction. Thus, G2V but not LG reports the earliest onset of hematopoietic potential in ESCs.
V+ cells, albeit very few, are already present at day 4 of G2V ESC differentiation. It was difficult to detect Gata2, Gata1, and globin gene expression at day 4 (Figure 2B) by qRT-PCR of total G2V EB RNA. Others have shown, by single cell transcriptomics performed on pseudotemporally ordered E7.5–7.75 FLK1+ mesodermal cells, an upregulation of Gata1, βH1, and Itga2b (CD41) following Brachyury expression during primitive streak specification (Scialdone et al., 2016). In total EB RNA, we found Brachyury expression at days 3 and 6 (Figure S1H), indicating that at least some differentiating EBs closely follow the formation of early mesoderm in gastrulating embryos. Gata1 and Gata2 expression was initiated at days 4–6. Embryonic globin Hbby and βH1 expression began at days 4–6 and 6–8, respectively, and adult globin (δmajor) expression began at days 8–10 (Figure 2D) although its levels were relatively low. These data are somewhat in agreement with those found in the mouse embryo (Sankaran et al., 2010), βH1 expression begins in the YS at E7.5 (Kingsley et al., 2006), with only very low levels of Hbby and δmajor expression. Hbby expression is high in the E10.5 and E15.5 peripheral blood, and δmajor expression is high in the enucleated E15.5 erythrocytes. Thus, while the general onset of expression of the embryonic globins prior to δmajor expression is similar to in vivo expression, the other differences seen in the ESC differentiation cultures are likely due to heterogeneous cell growth and frequency.

Interestingly, a decreased frequency of CFU-EryP in the V+ fraction corresponded with the onset of the second Gata2 expression wave at days 10–11 that is accompanied by expression of adult globin and the appearance of EMFs (Figure 2F). Thus, the Gata2 expression peaks discriminate functional primitive and definitive stages in the ESC differentiation. It is likely that these waves derive from independent cohorts of cells as supported by transcriptomic (Scialdone et al., 2016) and hESC differentiation data (Ditadi et al., 2015; Sturgeon et al., 2014), but contrasts with data suggesting that all waves of hematopoietic activity and in vivo repopulating cells arise as early as day 3 (Pearson et al., 2015). Additionally, the temporal dynamics of Gata2-defined primitive/definitive stages are consistent with studies reporting hemogenic endothelium and hematopoietic cell generation in mouse ESC and hESC differentiation cultures (Nakano et al., 1996; Ng et al., 2016; Zambidis et al., 2005). Although all CFU-Cs were obtained in the V+ cell fraction, FACS analysis revealed some phenotypic HPCs in the V− fraction. Hence, we cannot rule out that some early HPCs may be at low frequency in the V− fraction as was found in vivo (Kaimakis et al., 2016).

Morphological observation of V+ cells in the differentiating EBs corroborates both developmental and functional stage changes. Day-12 (but not day-6) EBs show CD31+ cells lining vascular tubules and the close association of clusters of V+CD31+ cells, mainly near the EB cavities (Figures 2E and 2F). This is supported by FACS data showing an increased frequency of C-KIT+CD31+V+ cells after day 6 (Figure 3D). Most probably it represents the EHT detected in E8.25–11 circulation-deficient YS where EMPs emerge in C-KIT+ cell clusters (Frame et al., 2016). Although a recent study with day-23 to -33 differentiated hESCs showed similar aortic and cluster-like structures (Ng et al., 2016), it is as yet uncertain, due to the heterogeneity of our cultures, whether they contain the CD31+V+ aortic endothelial and CD31−C-KIT+V+ intra-aortic hematopoietic cluster cells equivalent to E10.5 G2V AGM tissue (Kaimakis et al., 2016).

Surface marker analysis of G2V ESC-derived cells revealed that the highest frequency of phenotypic V+C-KIT+CD41+ hematopoietic cells is at day 8, whereas V+C-KIT+CD45+, V+C-KIT+CD16/32+, and V+C-KIT+CD31+ cells peak at day 10 (Figure 3D). Thereafter their frequency is greatly reduced. These dynamics may reflect early stages of myeloid differentiation. However, our FACS data (not shown) indicate that the differentiated blood cells are within the V− cell compartment, supporting the view that the V+ cell waves reflect different progenitor emergence stages. This is in line with several hESC expression profiling and surface marker studies that suggest waves of hematopoietic generation followed by blood cell maturation (Irion et al., 2010; Nakano et al., 1996; Zambidis et al., 2005). Thus, the second wave of ESC V+ hematopoietic cell production functionally, morphologically, and phenotypically resembles in vivo definitive hematogenesis in the YS.

HPC potential in our ESC differentiation cultures continues at day 12 and thereafter, and LG reporter expression identifies all definitive HPCs at these late time points. LG did not enrich for primitive stage blast-cell progenitors, nor for HPCs during the first stage of EB differentiation (e.g., days 6–10). This contrasts with G2V ESCs where all HPCs are V+ throughout the differentiation. From day 12 onward, HPC activity is significantly enriched in G+ fraction, and at day 14 some G+ cells possess B-lymphoid potential. Distribution of HPC activity in the LG EBs is consistent with the distribution in the LG mouse embryos. In the LG YS, most HPCs are G− whereas AGM lymphoid progenitors at E10 and multipotent HPCs/HSC are at much higher frequency in the E10.5/11.5 G+ than in the G− fraction (de Bruijn et al., 2002; Li et al., 2014; Solaimani Kartalaei et al., 2015). In the E10.5 aorta, LG expression reports hemogenic endothelial cells transitioning to HSCs (Boisset et al., 2010). Thus, LG expression in vivo specifically reports aortic HSCs and cells with lymphoid potential, but not the YS-stage progenitors. Although there is consistency between our in vitro LG ESC results and in vivo LG mouse data, it is as yet uncertain whether the ESC-derived definitive G+ progenitors that have B-lymphoid potential represent an aorta-like or YS-definitive program, as lymphoid potential has also
been demonstrated in the YS independently from arterial identity (Frame et al., 2016; Yoshimoto et al., 2011).

Importantly, our LGνV double reporter ESC line shows that these late G+ HPC co-express Gata2, thus facilitating high enrichment of multipotent progenitors that are generated in late ESC differentiation cultures. In some preliminary short-term transplantation experiments (4 × 10⁴ V+ cells from day-12 or -14 differentiated G2V ESCs) we were unable to detect donor-derived spleen colonies. Such short-term and long-term transplantation experiments with the LGGV ESC line are planned after further optimization of the differentiation cultures and flow-cytometric enrichment.

In conclusion, we demonstrate that different stages of HPC generation can be prospectively isolated by the G2V and/or LG reporter expression in ESC differentiation cultures. Such findings facilitate a better understanding of the hematopoietic development in ESC differentiation cultures, and should ultimately enable the recapitulation of robust physiologic HSC emergence for the de novo generation of transplantable HSCs.

EXPERIMENTAL PROCEDURES

ESC In Vitro Differentiation

ESCs (WT; G2V, LG, LGνV) were established from 129/Ola, Gata2Venus (Kaimakis et al., 2016), Ly6a(SCA1)GFP (de Bruijn et al., 2002), and (Gata2Venus × Ly6a(SCA1)GFP)F1 day-3.5 blastocystcs cultured on irradiated mouse embryonic fibroblasts (MEFs) in SR-ES medium (KO DMEM + 20% KO Serum Replacement + 1% penicillin/streptomycin [P/S] + 2 mM L-glutamine + 1× non-essential amino acids [NEAA] [all Gibco] + 100 mM β-mercaptoethanol [Sigma] + recombinant mouse leukemia inhibitory factor [LIF; 1,000 U/mL; Chemicon International]). To allow for inner cell mass expansion the medium was changed every 2 days and cells were trypsinized, transferred to 12-well ME-coated plates, and maintained in DMEM (Lonza) + 15% fetal bovine serum (FBS; HyClone) + 2 mM GlutaMAX + 1 mM Na-pyruvate + 1% P/S + 50 μM β-mercaptoethanol (all Gibco) + 0.1 mM NEAA (Lonza) + 1,000 U/mL LIF (Sigma) at 37°C and 5% CO₂. MEFs were depleted by 30-min incubation in EB medium (Iscoev’s modified Dulbecco’s medium [IMDM] + 15% fetal calf serum [HyClone] + 1% P/S [Gibco]). EB formation was induced (40 rpm; 25 × 10⁵ cells/mL) in EB medium + 2 mM GlutaMAX (Gibco) + 50 μg/mL ascorbic acid (Sigma) + 4 × 10⁻⁴ M monothioglycerol (Sigma) + 300 μg/mL transferrin (Roche) and supplemented with 5% proteome-free hybridoma medium (Gibco) on day 3. From day 6 onward, 100 ng/mL stem cell factor (SCF) + 1 ng/mL interleukin-3 (IL-3) + 5 ng/mL IL-11 were added. For blast-cell cultures, 5 ng/mL BMP4 was added on days 0–3. All factors were from PeproTech.

FACS

PBS-washed EBs were incubated (37°C, 3–5 min) in TrypLE Express (Gibco). PBS + 10% FBS + 1% P/S was added and single cells suspended (P1000 pipette). 10⁶ cells per 100 μL were immunostained (30 min on ice) (Table S1). Dead cells were Hoechst 33342 (Invitrogen) excluded. LSRFortessa, FACSscan, FACSaria III/Fusion (Becton Dickinson) and FlowJo software (TreeStar) were used. Strategy for detection of GFP and/or Venus is described in Supplemental Experimental Procedures and Figure S6.

CFU-C Assays

For BL-CFC, day-3 EB cells were plated in methylcellulose (STEMCELL Technologies) + 10% FCS + vascular endothelial growth factor (5 ng/mL) + IL-6 (10 ng/mL) (PeproTech). After 4 days, BL-CFC (grape-like multicellular structures [Kennedy et al., 2007]) were microscopically counted. D6-14 EB cells were plated in methylcellulose (1 mL/dish, M3434; STEMCELL Technologies) + IMDM + FBS + BSA + recombinant human insulin + human transferrin (iron-saturated) + 2-mercaptoethanol + recombinant SCF + recombinant IL-3 + recombinant IL-6 + recombinant human erythropoietin. After 12–14 days, colonies were scored (Zeiss Axiovert25).

Matrigel Assay

Day-4 EB cells (2 × 10⁴) from each FLK1/Venus fraction (Figure 1B) were pre-cultured in gelatin-coated 96-well plates (DMEM + 20% FBS + 1% P/S; 20 hr). Cells were trypsinized, transferred to Matrigel-coated (60 μL Matrigel/well, 30 min, 37°C; Qiagen) 96-well plates and cultured in EGM-2 (Lonza) for 4 hr. Tubes were quantified by the Angiogenesis Analyzer (Gilles Carpentier Image News, 2012) plugin for Fiji.

Transcription Analysis

Up to 10⁶ cells were lysed and total RNA isolated (TRI-Reagent, MRC). RNA (1 μg) was DNase (Invitrogen) treated. RNA from sorted cells was isolated using an RNeasy kit (Qiagen). cDNA was synthesized with oligo(dT) (Invitrogen) and SuperScriptIII (Life Technologies). qRT-PCR was performed with FastSybrGreen master mix (Life Technologies) and primers (Table S2).

B-Lymphoid Culture

At day 6 of ESC differentiation, 100 ng/mL SCF + 1 ng/mL IL-3 + 5 ng/mL IL-11 + 5 ng/mL FLT3L were added and medium replaced every other day. On day 14, EBs were dissociated and 5–10 × 10⁴ FACS-sorted cells plated on OP9 (plated at ~1 day in a 6-well plate) in 2 mL of z-minimal essential medium + 20% FBS + 1% P/S + 5 ng/mL IL-7 + 5 ng/mL FLT3L. Every 4–6 days, cells were replated on OP9 and harvested on day 22. The FACS strategy is displayed in Figure S5.

Whole-Mount Staining

EB staining was performed as in Yokomizo et al. (2012). EBs were washed in PBS, fixed in PBS + 2% paraformaldehyde (Sigma) for 10 min, washed in PBS (×3), and dehydrated in 50% methanol + PBS and 100% methanol (10 min, ×2). EBs were rehydrated in 1:1 methanol/PBS (10 min) and 100% methanol (10 min), washed in PBS (10 min on ice), and blocked in PBS + 1% skimmed milk + 0.005% Tween 20 (Sigma) (PBS-MT) + 10% BSA (Sigma) + 0.1% goat serum (4–6 hr). Antibody (Table S1) staining was performed (4°C) in PBS-MT and EBs washed in...
PBS-MT (>2 hr ×3), in 100% methanol (30 s, ×4) in a fast well, cleared in 1:1 benzyl alcohol benzyl benzoate (BABB)/methanol (30 s) followed by 100% BABB, overlaid with a coverslip, and imaged (Leica SP5).

**Statistics**

Unpaired Student’s t test or one-way ANOVA with Bonferroni correction for multiple comparisons were used (GraphPad). Results were statistically significant at p < 0.05 (**p < 0.01, ***p < 0.001 in figures). All data are shown as mean ± SEM. n denotes the number of biological replicates.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at [https://doi.org/10.1016/j.stemcr.2017.11.018](https://doi.org/10.1016/j.stemcr.2017.11.018).

**AUTHOR CONTRIBUTIONS**


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