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HEMATOPOIETIC STEM CELLS DIFFERENTIATE THROUGH A MULTIPOTENT PROGENITOR BEFORE GENERATING ALL MATURE HEMATOPOIETIC CELLS

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MOLECULAR, CELLULAR, AND DEVELOPMENTAL BIOLOGY

by

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ABSTRACT

Hematopoietic stem cells differentiate through a multipotent progenitor before generating all mature hematopoietic cells

Scott W. Boyer

Hematopoietic stem cells (HSC) are able to both self-renew indefinitely and differentiate into all the various mature hematopoietic cell types. The developmental pathways taken by hematopoietic stem cells as well as the point at which hematopoietic stem cells begin to commit to specific cell fates is highly controversial. We present evidence that the multipotent progenitor (MPP) is a critical developmental intermediate between HSC and mature hematopoietic cells using three different but complementary approaches. First, using a lineage tracing mouse model containing a Flk2-Cre transgene in conjunction with a dual-color fluorescent reporter we determined that all hematopoietic lineages develop through the MPP during steady-state hematopoiesis, upon hematopoietic stress, and upon hematopoietic stem cell transplantation. Second, we found the impaired propagation of the MPP stage in *Flk2*$^{-/-}$ mice negatively impacted the formation of all hematopoietic lineages. These two complementary approaches solidify a developmental pathway between HSC and mature hematopoietic cell types that requires the MPP as a developmental intermediate. Next, to assess lineage bias we quantified mature cell production from transplanted hematopoietic stem and progenitor cells, as well as performed single-cell *in vivo* assays to assess functional heterogeneity within these populations. The MPP produced mature cells in the same order of abundance as transplanted HSC, and as present during steady-state hematopoiesis. MPP also displayed multilineage reconstitution at the single-cell level, providing strong evidence that lineage commitment occurs after the loss of self-renewal and the subsequent formation of the MPP.

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CHAPTER 1: Introduction

In the clinic, transplantation of hematopoietic stem cells (HSC) is currently the only approved method of stem cell therapy and has been used since the 1950's. HSC transplantations have been used to treat autoimmune diseases, cancer, HIV, and genetic diseases such as sickle cell anemia. In research, the hematopoietic system has long served as a model to study stem cell differentiation, largely due to the strength of transplantation assays. Transplantation assays allow for the delineation of lineage potentials of purified cells with a unique phenotype. Upon transplantation of purified hematopoietic stem cells (HSC) or progenitors, these cells give rise to distinct mature cell types of the blood. The timing and magnitude of mature cell production has also been shown to be indicative of developmental maturity and hierarchal positioning (Forsberg et al 2006).

In addition to transplantation assays, another power of the hematopoietic system is the vast array of known cell surface markers used to isolate functionally distinct hematopoietic populations with high purity and viability. Since the 1980's, great strides have been made in identifying cell surface markers that allow for the isolation of HSC with increasing purity (Spangrude et al., 1988; Uchida et al., 1992; Morrison et al., 1997; Christensen and Weissman 2001, Kiel et al., 2005; Ooi et al., 2008). These endeavors have also led to the discovery of other unique progenitor cells that paved the way for the classical model of hematopoietic development (Figure 1A). Recently, the classical model has been tested as other reports have put forth an array of deviations from the model (Akashi et al., 2005, 2009; Hock and Orkin, 2005; Luc et al., 2008b). To fully understand hematopoietic development for therapeutic applications, it is important to map differentiation pathways taken by HSC, identify key points of lineage commitment, and determine the level of functional heterogeneity within hematopoietic populations. Here I will discuss recent advances and controversies in hematopoietic differentiation.

Hematopoietic Stem Cells

Although HSC are one of the most rare cell types in the body (~1:20,000 cells in the bone marrow), they give rise to one of the most abundant cell types, the red blood cell, among others. In a human, 2.5 million red blood cells must be generated every second to maintain homeostasis (Sackmann 1995). Even more surprising, most of the HSC are in a quiescent state, despite the high production demands (Passegue et al., 2005). The high proliferative pressure placed on the hematopoietic system renders it extremely sensitive to radiation or chemotherapeutic treatment. Radiation- or chemotherapy-induced host conditioning enables donor bone marrow cells to engraft into human patients to treat disease, as well as into mice to study hematopoiesis. In addition, human HSC can be transplanted into immunocompromised mice to form stable humanized mouse models to study human HSC biology and disease (McCune et al., 1988).

HSC have the ability to both self-renew and differentiate into all cell types of the blood, such as red blood cells (RBC), platelets (Plt), granulocytes and macrophage (GM), dendritic cells, natural killer cells, B-cells, and T-cells. Approximately 20% of single HSC transplanted into conditioned mice generate all mature hematopoietic cell types for the lifetime of that mouse, supporting that at least 20% of all phenotypic HSC have the ability to both self-renew and multilineage reconstitute at the single-cell level (Osawa et al., 1996; Wagers et al., 2004; Dykstra et al., 2007; Morita et al., 2010). The remaining single-HSC do not multilineage reconstitute in these assays, and have been deemed “committed” and thus it has been claimed that lineage commitment occurs at the HSC level (Dykstra et al., 2007; Morita et al., 2010). The conclusion that a fraction of HSC have lost multipotency is contradictory to the proposed existence of a non-self-renewing multipotent progenitor (MPP) downstream of the HSC (Christensen and Weissman 2001; Adolfsson et al., 2001).

The Multipotent Progenitor

The addition of the Flk2 receptor to the previous cell surface signature (Lineage-cKit+, Sca1+; KLS) allowed for the separation of the self-renewing HSC (Flk2-) from the non-self-renewing MPP (Flk2+) (Christensen and Weissman 2001; Adolfsson et al., 2001). While these initial reports found MPP to have a similar differentiation potential as HSC, more recent evidence has suggested MPP may not in fact be multipotent, but instead have reduced megakaryocyte and erythrocyte potential (Adolfsson 2005; Sitnicka et al., 2007; Buza-Vidas et al., 2009) (Figure 1B). These works support a model in which the Flk2 receptor functions to prime MPP toward a lymphoid fate, as Flk2 expression continues to be expressed during lymphoid development, but is quickly downregulated upon myeloid commitment. This model would support lineage commitment occurring at the HSC level (Dykstra et al., 2007; Morita et al., 2010). While it is clear MPP are able to generate erythrocytes and megakaryocytes upon transplantation, the level of functional heterogeneity and lineage bias within the MPP is still unclear (Forsberg et al., 2006; Lai and Kondo, 2006; Luc et al., 2008a). Adding to the controversy, other groups have suggested that HSC can skip the MPP stage all together, perhaps during times of hematopoietic stress (Takano et al., 2004). Despite the unclear contribution of MPP to hematopoiesis, it is clear that cells downstream of both the HSC and MPP have committed to either myeloid or lymphoid cell fates.

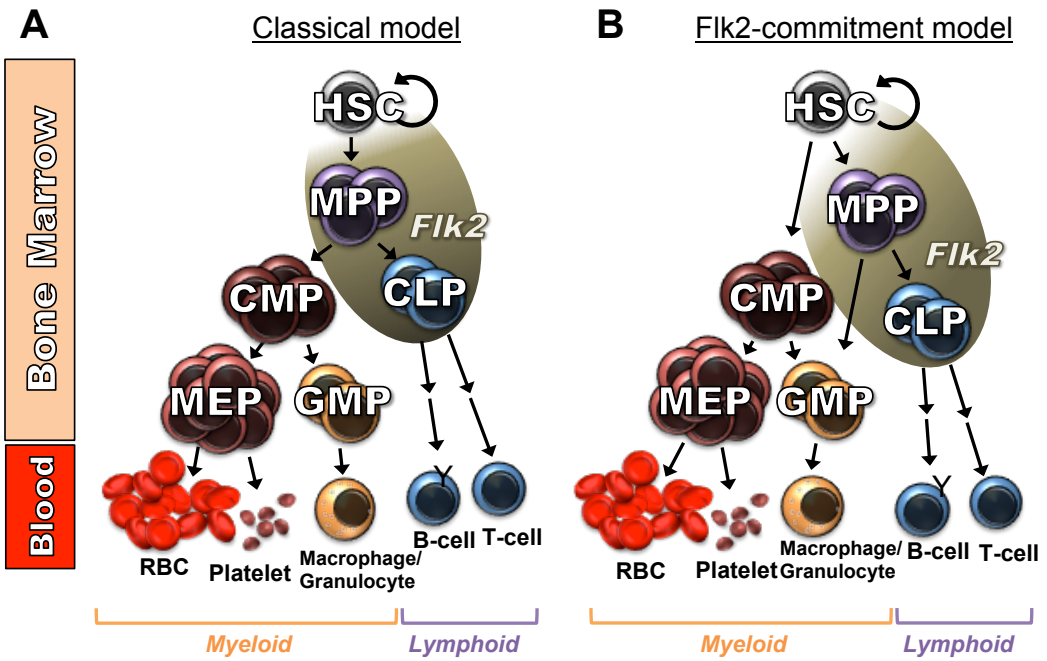


Figure 1. Models for HSC differentiation. (A) The classical model supports the Flk2+ MPP as critical developmental intermediate. (B) In the Flk2-commitment model, RBC and platelets don't differentiate through a Flk2+ stage.

Myeloid differentiation

The addition of two cell surface markers, FcγRα and CD34, allowed for the separation of distinct myeloid-committed progenitors (Akashi et al., 2000). The common myeloid progenitor (CMP) was found to give rise to RBC, Plt, and GM at the single-cell level *in vitro*, which contradicts the idea for alternative differentiation routes for erythrocytic/ megakaryocytic cells and granulocytic/myelomonocytic cells (Akashi et al., 2000). Beyond the CMP, progenitors restricted to either the megakaryocyte/erythrocyte lineages (MEP) or granulocyte/myelomonocyte lineages (GMP) have been identified (Akashi et al., 2000) (Figure 1). The MEP is thought to give rise to two unipotent progenitors, erythroid progenitors and megakaryocytic progenitors (Suzuki et al., 2003; Na Nakorn et al., 2003).

Further fractionation of myeloid cells by additional and alternative markers has not yielded a separation of functionally distinct myeloid progenitors with nonoverlapping potentials (Pronk et al, 2007; D'Amico and Wu, 2003, Nutt et al., 2005). Two subfractions of the CMP, Flk2+ and Flk2-, were found to have subtle, overlapping differences compared to the nonoverlapping lineage potentials of MEP and GMP. Flk2- CMP had more robust RBC and Plt potential *in vitro* relative to Flk2+ CMP (Nutt et al., 2005), while Flk2+ CMP had higher GM production than Flk2- CMP *in vivo* and *in vitro* (D'Amico and Wu, 2003, Nutt et al., 2005). Interestingly, Flk2+ CMP were shown to retain minor B-cell potential *in vivo* (D'Amico and Wu 2003), calling into question the clean separation of myeloid and lymphoid potentials originally proposed (Akashi et al., 2000; Kondo et al., 1997).

Lymphoid differentiation

Commitment to lymphoid lineages has been proposed to occur as MPP give rise to the common lymphoid progenitor (CLP) (Kondo et al., 1997) (Figure 1). However, lymphoid differentiation isn't without it's own controversies. *In vitro* assays have shown retention of myeloid potential in pro-T cells, while *in vivo* transplantation and lineage tracing data support restriction to lymphoid lineages occurring upon the upregulation of IL7Rα and the subsequent formation of the CLP (reviewed in Ichii et al., 2010 and Schlenner and Rodewald 2010). It is also debatable if CLP retain T-cell potential (Karsunky et al., 2008).

Future direction

The aforementioned controversies make it clear that *in vitro* lineage potentials can differ from the *in vivo* potential observed upon transplantation. It is of increasing importance to utilize *in vivo* assays capable of assessing the full lineage potential of single hematopoietic cells to give insight into the functional heterogeneity within a given phenotypic population. Fractionation of these populations with the addition of new cell surface markers may also

allow for the separation of functionally distinct cells. These methods have the potential to accurately pinpoint when lineage potentials are lost along hematopoietic development.

It will also be important to determine if the behavior of certain cell types upon transplantation is reflective of the endogenous capability of those cells *in situ*, as the majority of what is known about hematopoietic development is based upon their performance upon transplantation. Using methods that provide an accurately map of differentiation pathways taken by HSC will help clarify where lineage specification occurs along these paths. In addition, comparing cell behavior *in situ* to the behavior after transplantation would not only give weight to the use of transplantation assays in research, but will also be useful in clinical applications, as cell behavior upon transplantation may need to be adjusted to ensure cell engraftment and proper cell output to enhance patient outcome.

CHAPTER 2

All Hematopoietic Cells Develop from Hematopoietic Stem Cells through Flk2/Flt3-Positive Progenitor Cells

The text contained within this chapter of the dissertation includes reprints of the following previously published material: [Boyer SW, Schroeder AV, Smith-Berdan S, Forsberg EC. (2011). All hematopoietic cells develop from hematopoietic stem cells through Flk2/Flt3-positive progenitor cells. Cell Stem Cell. 9(1):64-73]. SWB performed all transplantations, BM analyses, and expression data in Figures 2-6. AVS is responsible for the data collected in Figure 5A. SSB optimized experimental protocols for FlkSwitch mice. SWB and ECF designed experiments and wrote the manuscript.

SUMMARY

While it is clear that a single hematopoietic stem cell (HSC) is capable of giving rise to all other hematopoietic cell types, the differentiation paths beyond HSC remain controversial. Contradictory reports on the lineage potential of progenitor populations have questioned the physiological contribution of progenitor populations to multilineage differentiation. Here, we established a lineage tracing mouse model that enabled direct assessment of differentiation pathways in vivo. We provide definitive evidence that differentiation into all hematopoietic lineages, including megakaryocyte/erythroid cell types, involves Flk2-expressing non-self-renewing progenitors. A Flk2+ stage was used during steady-state hematopoiesis, after irradiation-induced stress and upon HSC transplantation. In contrast, HSC origin and maintenance do not include a Flk2+ stage. These data demonstrate that HSC specification and maintenance are Flk2 independent, and that hematopoietic lineage separation occurs downstream of Flk2 upregulation.

INTRODUCTION

A core goal of stem cell biology is to understand how multipotent cells commit to specific cell fates. Comprehensive knowledge of differentiation pathways and the molecular regulation of fate decisions is necessary to efficiently guide uncommitted cells into specific fates; such directed differentiation serves as the basis for ESC differentiation into cell types of therapeutic value. Differentiation pathways must also be understood to enable rational drug design. Indeed, the concept of therapeutic targeting of cancer stem cells relies on understanding the cellular events underlying oncogenesis and distinguishing those from normal cell differentiation.

The well-studied hematopoietic system is at the forefront of lineage mapping during both normal and abnormal cell development. HSCs have been isolated to high purity and single-cell transplantation has demonstrated that clonal HSCs are capable of life-long reconstitution of all blood cell types (Ema et al., 2005; Kiel et al., 2005; Morita et al., 2010; Osawa et al., 1996; Wagers et al., 2002; reviewed in Hock, 2010). Although the concept of hierarchical hematopoietic differentiation from HSCs into lineage-restricted progenitor cells (Akashi et al., 2000; Kondo et al., 1997; Nakorn et al., 2003) is widely accepted, several recent reports have questioned the accuracy of schematic lineage maps (e.g., Figure 2A) (reviewed in Akashi, 2005, 2009; Hock and Orkin, 2005; Luc et al., 2008b). Recent questions have focused on when the initial lineage decision is made, which lineage (or lineages) is specified or lost first, and whether differentiation follows linear, obligatory paths, or whether there are alternative pathways to a specific fate. In particular, pathways of megakaryocyte and erythroid (MegE) development from HSCs are controversial (Adolfsson et al., 2005; Forsberg et al., 2006; Lai and Kondo, 2006; Lai et al., 2005; Nutt et al., 2005). Upregulation of Flk2 (Flt3), a tyrosine kinase receptor differentially expressed on functionally distinct hematopoietic subpopulations (Adolfsson et al., 2001; Christensen and Weissman, 2001; D'Amico and Wu, 2003; Karsunky et al., 2003, 2008) (Figure 2A), marks the loss of the self-renewal potential of hematopoietic cells; thus, HSCs reside within the Flk2– c-kit+Lin–Sca1+

(KLS) fraction of adult bone marrow (BM) (Adolfsson et al., 2001; Christensen and Weissman, 2001). Flk2 itself and Flk2+ lymphoid committed populations have been shown to promote lymphoid development, as genetic deletion of Flk2 leads to decreased numbers of B lineage cells (Mackarehtschian et al., 1995). In contrast, myeloid-committed progenitors do not express Flk2, and Flk2-deficient mice have normal numbers of mature myeloid cells. Flk2 expression on KLS cells has also been reported to correlate with loss of MegE potential and prime uncommitted cells for a lymphoid fate (Adolfsson et al., 2005). Extensive functional and molecular characterizations of Flk2+ KLS cells have demonstrated low MegE readout in vitro and increased expression of transcripts associated with lymphoid development, with retention of robust granulocyte/macrophage (GM) potential (Adolfsson et al., 2005; Luc et al., 2008a; Sitnicka et al., 2007). However, at least some cells within the Flk2+ KLS fraction retain MegE potential in vivo (Forsberg et al., 2006; Lai and Kondo, 2006; Luc et al., 2008a). Quantitative assessment of the lineage potential of multiple cell populations in parallel showed that MegE contribution from Flk2+ multipotent progenitors (MPP^F) was more robust than that from progenitor populations with undisputed MegE potential (Forsberg et al., 2006). These seemingly contradictory findings raise the possibility that MPP^F are capable of giving rise to MegE cells under conditions of acute need, but are normally dedicated to providing lymphoid cells. Transplantation assays, in combination with molecular characterization, have been unable to provide conclusive evidence for this model. Since no reports have ascertained the relative contribution of possible alternative pathways, the physiological relevance of different progenitor populations in development of distinct lineages remains uncertain (Figure 2A). Here, we have established a lineage tracing model that enabled us to determine the contribution of non-self-renewing MPPs, marked by Flk2 expression, to the distinct hematopoietic lineages during both steady-state and stress hematopoiesis in vivo.

RESULTS

Establishment of a Dual-Color Reporter Mouse Model

To determine which hematopoietic lineages develop through a Flk2+ stage, we generated ‘‘FlkSwitch’’ mice by crossing mice expressing Cre recombinase under the control of Flk2 regulatory elements (‘‘Flk2-Cre’’ BAC transgenic mice) (Benz et al., 2008) to mice containing a dual-color fluorescent reporter in the Rosa26 locus (‘‘mT/mG’’ mice) (Muzumdar et al., 2007) (Figure 2B). We expected that all cells in the resulting FlkSwitch mice would express the red-fluorescing protein Tomato (Tom), with the exception of cells expressing Cre because Cre-mediated excision of Tom would result in induction of GFP expression.

Importantly, because Tom excision is an irreversible genetic event, all progeny of Cre expressing cells would also express GFP regardless of whether these progeny themselves express Cre. Flow cytometry analysis revealed that >99% of platelets and nucleated BM and PB (peripheral blood) cells in both mT/mG and FlkSwitch mice express either the Tom or GFP reporter gene.

We envisioned two main experimental outcomes: if Flk2+ progenitors are significant physiological contributors to erythro- and megakaryopoiesis in vivo, MegE cells would inherit the floxed, GFP-expressing reporter allele and be GFP+, as indicated in the left schematic of Figure 2A. Alternatively, Flk2+ progenitors may be capable of providing MegE cells when transplanted into irradiated animals due to the acute requirement for erythrocytes and platelets, but may not normally contribute to MegE generation. In this model, Flk2– multipotent progenitors or HSCs would give rise to common myeloid progenitors (CMPs) or megakaryocyte/erythroid progenitors (MEPs) without differentiation through a Flk2+ stage, and mature MegE and/or GMs, and their progenitors, would remain Tom+ (Figure 2A, right model).

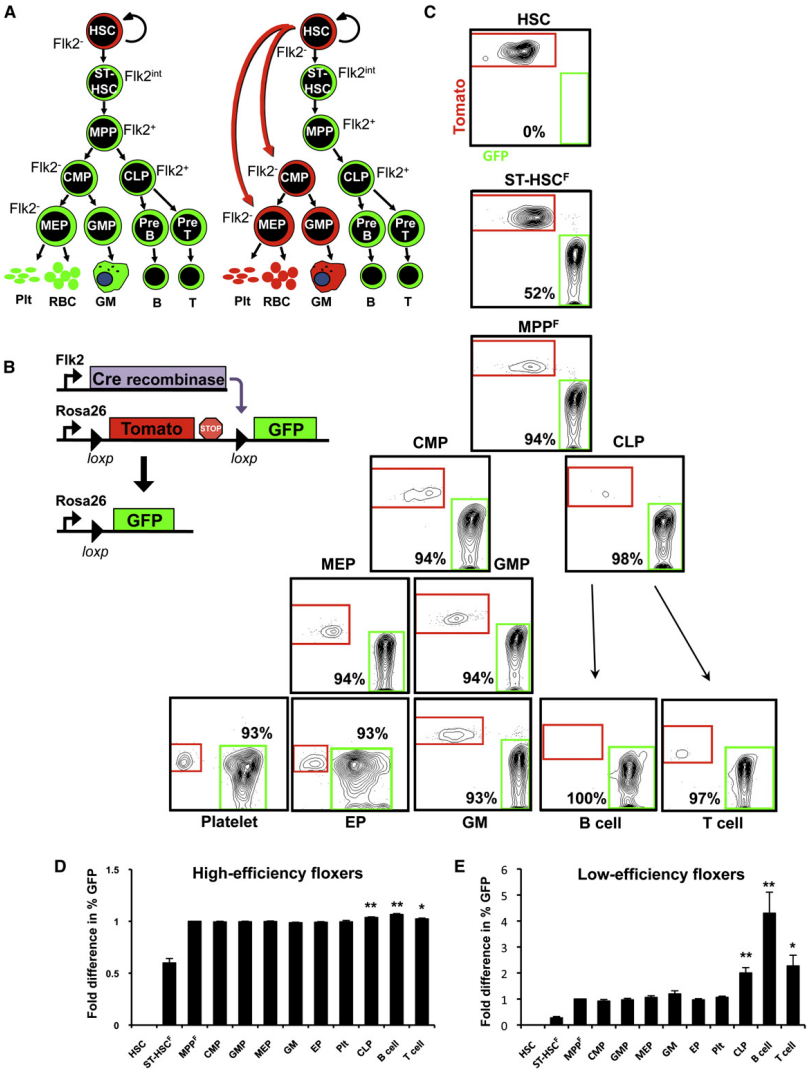


Figure 2. HSC Progeny Switch from Tom to GFP Expression in FlkSwitch Mice. (A) Two simplified alternative models for HSC differentiation. Cell types that are Flk2+ or derived from Flk2+ cells should express GFP, while Flk2– cells that have no history of Flk2 expression should express Tom. (B) Strategy for the generation of FlkSwitch reporter mice. (C) Representative flow cytometer data of Tom and GFP expression in multiple cell populations from a FlkSwitch mouse with high floxing efficiency (mouse number 8 in Table 1). (D) Fold difference in percent GFP+ cells compared to MPP^F in high-efficiency floxers (mice numbers 6–14 in Table 1; n = 9), and (E) low-efficiency floxers (mice numbers 1–5 in Table 1; n = 5). *p < 0.01, **p < 0.001 by Wilcoxon Signed Rank test, calculated using the raw GFP percentage (Table 1). GFP percentages in HSCs and $ST\text{-}HSC^F$ were significantly different from all other hematopoietic cell types. HSC, hematopoietic stem cells; $ST\text{-}HSC^F$, short-term hematopoietic stem cells; MPP^F, multipotent progenitors; CMP, common myeloid progenitors, GMP, granulocyte/macrophage progenitors; MEP, megakaryocyte/erythroid progenitors; GM, granulocyte/ macrophage cells; EP, erythroid progenitors; RBC, red blood cells; Plt, platelets; CLP, common lymphoid progenitors; B, B cells; T, T cells. Error bars indicate standard error of the mean (SEM). See also Figure S1.

All Hematopoietic Cell Types except HSCs Switch from Tom to GFP Expression

To distinguish between these possibilities, we analyzed the color of distinct cell types in FlkSwitch mice by multicolor flow cytometry. HSCs were defined as Flk2– BM cells that were also c-kit+, Lin–, Sca1+, CD150+, and CD48– (Figure S1A). This population contains all Flk2– KLS cells and is heterogeneous for CD34, and thus includes cells that have been referred to as ‘‘ST-HSC’’ in previous reports (Figure S1B). The entire remaining KLS fraction was divided into ST-HSC^{F} and MPP^{F} based on the level of Flk2 expression (intermediate for STHSC^{F} and high for MPP^{F}) and differential expression of CD150 and CD48 (Figure S1A). Importantly, HSCs expressed the Tom transgene, but not GFP (Figure 2C, Table 1), demonstrating that excision of GFP does not occur aberrantly in Flk2– populations. Lack of recombination in HSCs also demonstrated that all developmental precursors of HSCs are Flk2–. Analysis of ST-HSC^{F} and MPP^{F} revealed a dramatic color change from Tom to GFP as Flk2 and Cre expression was turned on (Figure 2C, Figure S2A, Table 1). This striking color switch (up to 97%) from Flk2– HSCs to Flk2+ MPPs established that the reporter model operated as expected. Strikingly, all cell populations of the myeloid lineage, including CMPs, granulocyte/macrophage progenitors (GMPs), MEPs, erythroid progenitors (EPs), and platelets (Plts), exhibited the same GFP percentage as MPP^{F} (Figures 1C–1E, Figure S2A, Table 1). Subfractionation of myeloid populations using alternative markers (Pronk et al., 2007) revealed similar results (Figure S2B). The almost complete color switch (up to 98%) in erythroid cells and Plts was particularly surprising given the uncertainty of Flk2+ cell contribution to MegE lineages. Even in mice with low floxing efficiency in Flk2+ populations (mice #1-5 in Table 1; Figure S2A), the GFP+ percentage in all myeloid and erythroid cell populations closely followed the GFP+ percentage in MPPF (Figures 2D and 2E, Table 1). To determine whether myeloid cells switch color due to differentiation through a Flk2+ progenitor stage or due to aberrant recombination, we investigated Cre expression and activity. Cre mRNA was readily detectable in MPP^{F}, but not in HSCs or myeloid progenitors, demonstrating a Flk2-dependent Cre expression pattern (Figure 3A). We then tested whether

myeloid progenitors exhibit Cre recombinase activity. Unfloxed (Tom+) and floxed (GFP+) CMPs and MPP^F were isolated from FlkSwitch mice and differentiated in vitro or transplanted into recipient mice. As expected, all progeny of GFP+ CMPs and MPP^F in vitro and in the PB and spleen of recipient mice were GFP+ (Figures 3B–3D). In contrast, Tom+ MPP^F gave rise to both Tom+ and GFP+ progeny in all three assays (Figures 3B– 3D). Importantly, we never detected GFP+ progeny from Tom+ CMPs, as they exclusively gave rise to Tom+ progeny in vitro and in vivo. The lack of detectable Cre mRNA and recombinase activity in myeloid progenitors and their progeny demonstrated that Cre is expressed and functional in a Flk2-dependent pattern, and support a model wherein myeloid cells are derived from Flk2-expressing progenitors.

Table 1. Percent GFP in Hematopoietic Populations from FlkSwitch Mice

Mouse #	HSC	ST-HSC^F	MPP^F	CMP	GMP	MEP	GM	EP	Plt	CLP**	B cell**	T cell
1	0	1	6	7	7	7	10	7	6	15	38	23
2	0	2	7	6	6	9	10	6	8	14	32	20
3	0	5	12	12	12	13	11	12	13	27	47	23
4	0	5	12	10	12	12	13	12	13	27	50	28
5	0	4	20	18	20	19	21	18	21	25	44	19
6	0	63	89	91	90	89	86	86	92	91	97	94
7	0	51	90	88	89	91	90	88	93	91	99	92
8	0	44	90	88	89	90	89	90	92	97	98	96
9	0	61	92	92	92	93	92	92	91	96	100	95
10	0	37	94	94	94	94	93	93	93	98	100	97
11	0	68	95	94	93	92	92	93	86	98	99	94
12	0	45	95	95	95	97	94	97	98	100	100	96
13	0	74	96	94	94	93	94	94	93	99	100	94
14	0	60	97	98	99	99	98	98	96	99	100	100

The differences between MPP^F versus CLPs, B cells, and T cells were significant (**$p < 0.001$, **$p < 0.001$, and *$p < 0.01$, respectively), by Wilcc Signed Rank Test. HSCs and ST-HSC^F were also significantly different from MPP^F and all other populations. No other comparisons to MPP^F v significantly different. Abbreviations are as in Figure 1. See also Figure S2.

Figure 3. Lack of Cre Expression and Activity in Myeloid Progenitors at Steady-State and upon In Vivo and In Vitro Differentiation. (A) Quantitative RT-PCR analyses of Cre recombinase mRNA levels in Flk2+ (MPP^F) and Flk2– (HSC; myeloid progenitors [MyPro; Lin–c-kit+Sca1– cells] and erythroid progenitors [EP; Ter119–Mac1–Gr1–B220–CD3–CD71+]) cell populations from FlkSwitch mice revealed that only MPP^F express Cre. Bar graph indicates the relative levels of Cre mRNA in cell populations sorted from individual (gray bar) or multiple (white bar; n = 3) FlkSwitch mice. b-actin was used as a positive control for all populations. Error bars indicate SEM. (B–D) Tom+ CMPs remain unfloxed during myeloid development. (B) Flow cytometry analysis of MPPF and CMP progeny after 10 days of in vitro methylcellulose culture (n = 6 in two independent experiments). (C) Tom and GFP analysis of donor-derived nucleated cells (total), GMs, B cells, T cells, and Plts in PB of sublethally irradiated mice transplanted with Tom+ or GFP+MPP^F (800 per mouse) or CMPs (10,000 per mouse) (n=5–7 in two independent experiments). (D) EP readout in spleens of lethally irradiated mice 11 and 9 days post-transplantation of Tom+ or GFP+ MPP^F or CMP (n = 10 in two independent experiments).

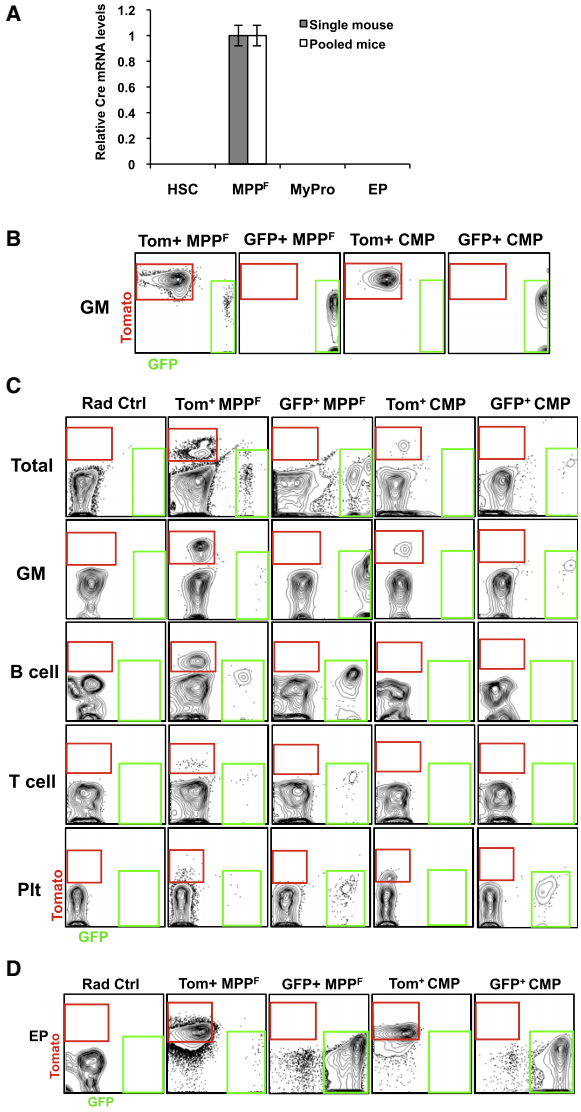


Figure S1. Flow cytometry gating strategies for identification of hematopoietic stem and progenitor cells. (**A**) Phenotypic definitions of HSC, ST-HSC^F, MPP^F, CMP, GMP, MEP and CLP. (**B**) The HSC gate in ***A*** contains both CD34-negative and CD34-positive cells. (**C**) Flow cytometry gates for distinct hematopoietic subpopulations from wt, mT/mG and FlkSwitch mice demonstrating the separation of Tomato and GFP fluorescence from each other and from non-fluorescent cells. Related to Figure 2.

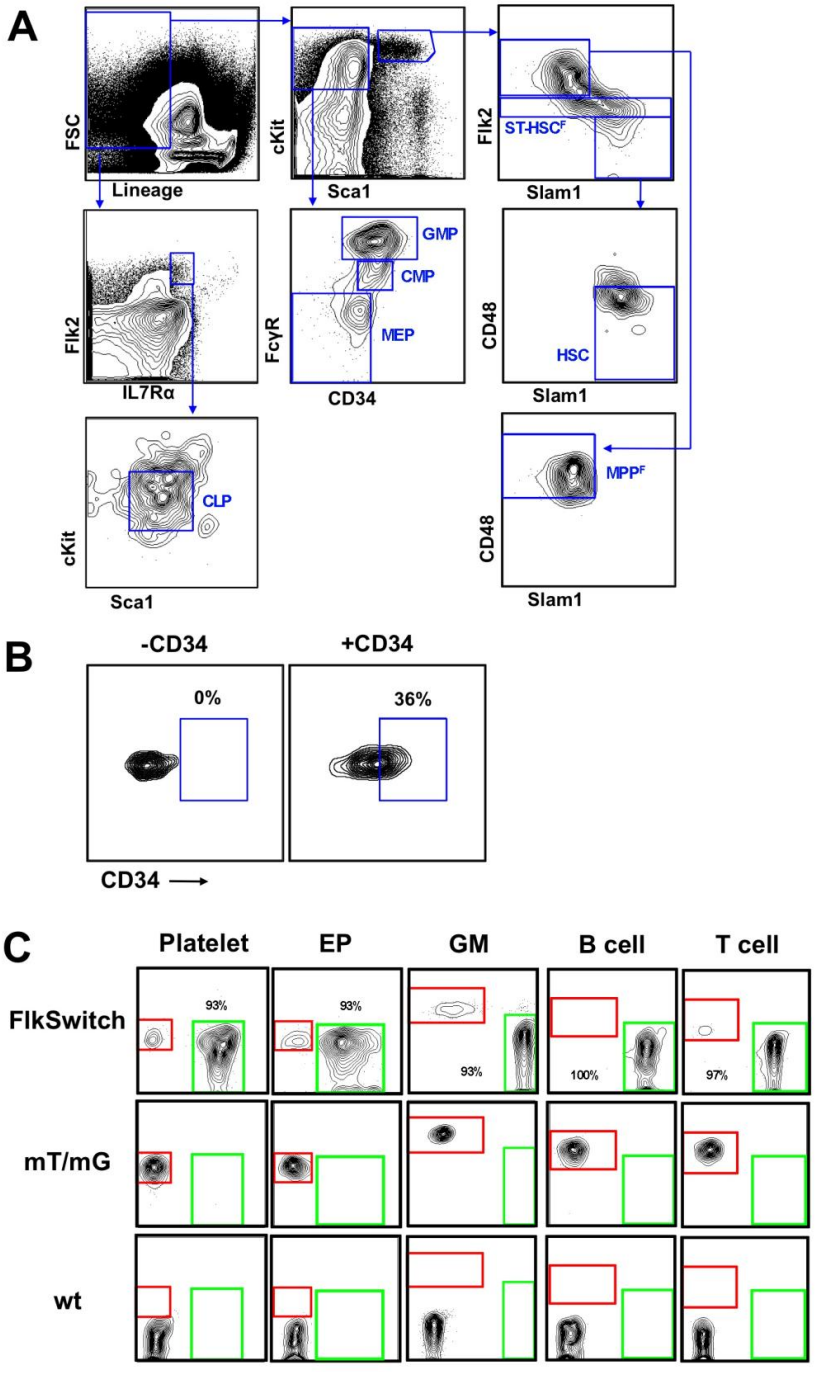
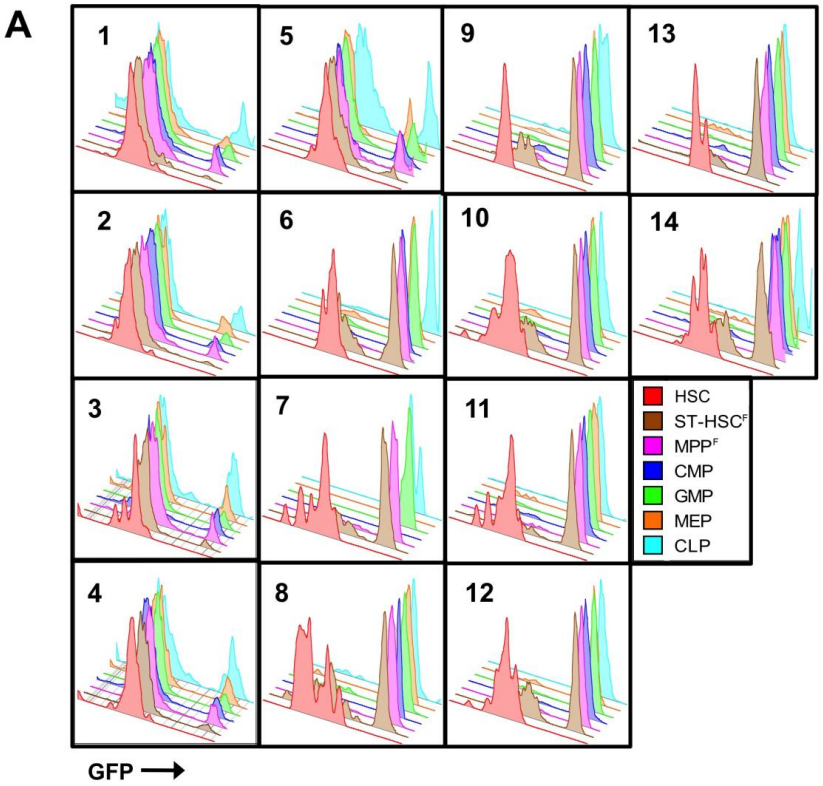
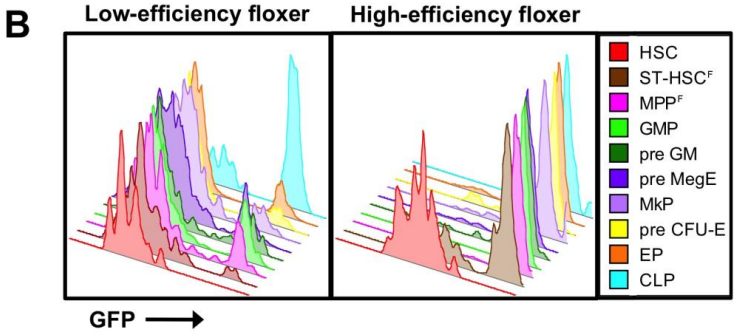


Figure S2. All myeloid progenitor populations have similar percentages of GFP-positive cells compared to MPP^F. (**A**) Flow cytometry histograms of GFP expression in stem and progenitor populations for 14 different FlkSwitch mice. Mouse numbers 1-14 correspond to those in Table 1. (**B**) Subfractionation of myeloid progenitors by alternative marker strategies resulted in similar GFP percentages as in MPP^F. One low-floxing (15% GFP in MPP^F) and one high-floxing (92% GFP in MPP^F) FlkSwitch mouse are shown. Cell populations in ***A*** were defined as in Figure S1. Alternative cell populations used in panel ***B*** were defined as follows: GMP (ckit+Lin-Sca1-CD41-FcgR+Slamf1-); PreGM (ckit+Lin-Sca1-CD41-FcgR-Endoglin-Slamf1-); preMegE (ckit+Lin-Sca1-CD41-FcgR-Endoglin-Slamf1+); MkP (ckit+Lin-Sca1-CD41+Slamf1+); preCFU-E (ckit+Lin-Sca1-CD41-FcgR Endoglin+Slamf1+); EP (ckit+Lin-Sca1-CD41-FcgR-Endoglin+ Slamf1-). Related to Table 1.

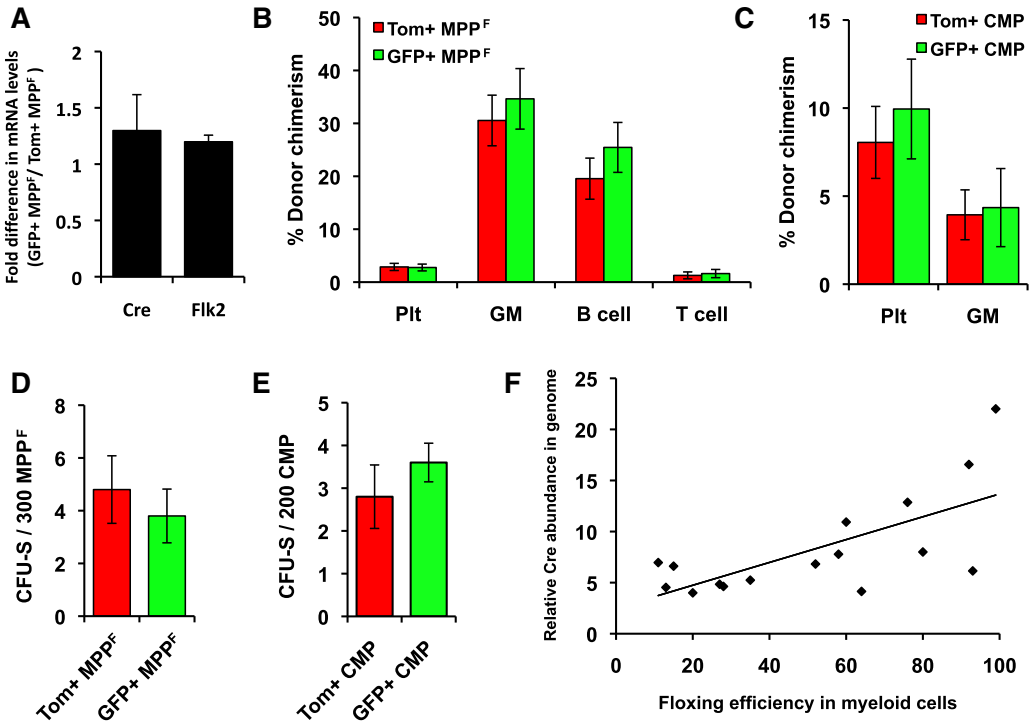




Tom-Expressing and GFP-Expressing Cells Are Functionally Equivalent

The highly variable floxing efficiency between different mice (from 6% to 97% in MPP^F) makes it unlikely that Tom+ and GFP+ cells within a phenotypic fraction are fundamentally different. Transcriptional analysis revealed a trend toward increased levels of Flk2 and Cre mRNA in GFP+ compared to Tom+ MPP^F (Figure 4A), consistent with floxing efficiency increasing with Flk2 levels. To directly test whether floxed and unfloxed cells are functionally equivalent, we compared the in vivo colony forming units spleen (CFU-S) and PB reconstitution abilities of purified Tom+ and GFP+ MPP^F and CMPs. We did not detect significant differences in PB reconstitution potential (Figures 4B and 4C) or CFU-S frequencies (Figures 4D and 4E) between Tom+ and GFP+ MPP^F or between Tom+ and GFP+ CMPs. We then analyzed the relative number of Cre transgenes between different mice by performing qPCR of genomic DNA. Indeed, mice with low floxing efficiency had fewer copies of the Flk2-Cre construct compared to high-floxing mice (Figure 4F). These data are consistent with the increase in floxing efficiency observed upon selective backcrossing of FlkSwitch mice. Collectively, these results led us to conclude that Tom+ and GFP+ cells within a phenotypic population are functionally equivalent and that the differential floxing efficiency between different mice is due to varying copy numbers of the Cre transgene.

Figure 4. Tom+ MPP^F and CMPs Exhibit Similar In Vivo Reconstitution Potential to That of Their GFP+ Counterparts. (A) Quantitative RT-PCR analyses of Cre recombinase and Flk2 mRNA levels in Tom+ and GFP+ MPP^F isolated from individual FlkSwitch mice (n = 4). (B–E) Tom+ and GFP+ MPP^F (B and D) and CMPs (C and E) give rise to similar numbers of Plts, GMs, B and T cells (B and C), and CFU-S (D and E). Purified Tom+ and GFP+ MPP^F (800 per mouse, n = 7 and 8) or Tom+ and GFP+ CMPs (10,000 per recipient, n = 5) were transplanted into sublethally irradiated hosts and the PB readout was analyzed weekly for 30 days posttransplantation (B and C). For CFU-S analysis, 300 Tom+ or GFP+ MPP^F (n = 10) or 200 Tom+ and GFP+ CMPs (n = 10 in two independent experiments) per recipient were transplanted into lethally irradiated hosts. CFU-S were enumerated 11 or 9 days posttransplantation (D and E). (F) The percentage of GFP+ cells in PB myeloid cells correlates with Cre transgene copy number. Error bars in (A)–(E) indicate SEM; no comparisons were statistically significantly different.



Analysis of Low-Efficiency Floxers Revealed Increased Reporter Switching in Lymphoid, but Not Myeloid, Cells

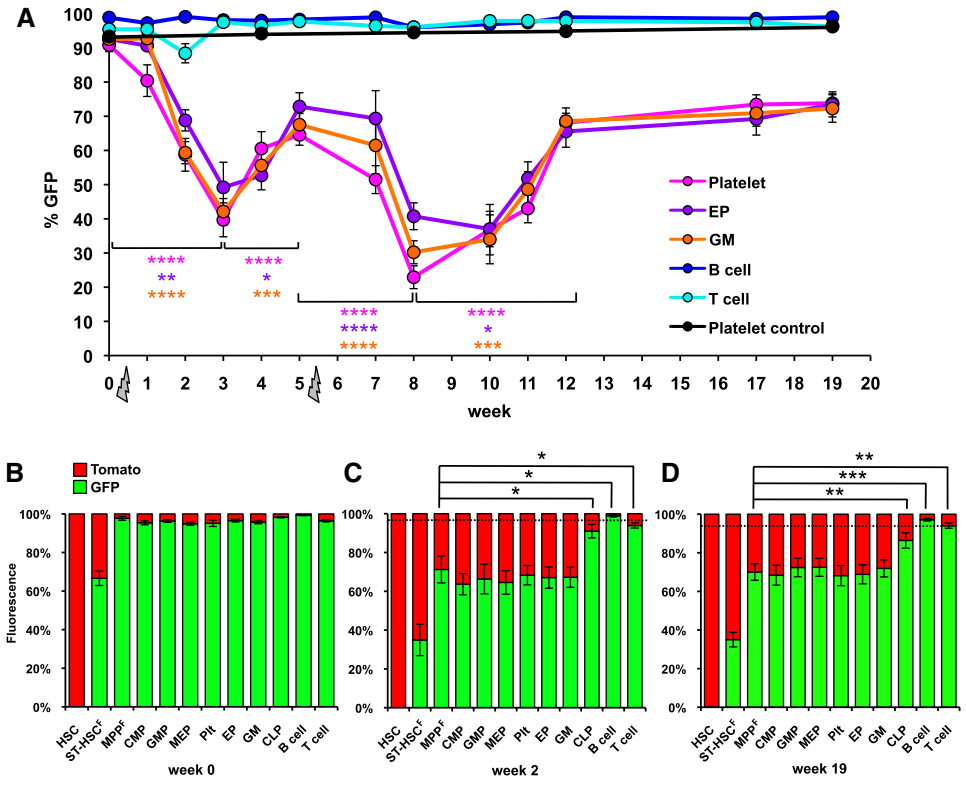
In contrast to MegE development, there is considerable agreement that MPP^F are capable of both GM and lymphoid differentiation (Adolfsson et al., 2001, 2005; Christensen and Weissman, 2001; Forsberg et al., 2006; Lai and Kondo, 2006; Lai et al., 2005). In addition, several populations with lymphoid-restricted potential, including common lymphoid progenitors (CLPs) and fractions of ProB and ProT cells, express Flk2 (Karsunky et al., 2003, 2008). It is therefore less surprising that cells of the lymphoid lineage also switch to GFP expression in the FlkSwitch mice (Figure 2C, Figure S1, Table 1). Indeed, statistical analysis revealed that Tom excision increased during lymphoid development (Table 1). Because it is not possible to substantially increase the percentage of floxed cells when it's already very high in MPP^F, this effect is most apparent in low-efficiency floxers (mice numbers 1–5 in Table 1; Figure 2E). The increased reporter switch is likely a result of sustained Cre expression during development through additional Flk2+ progenitor stages, such as CLP, ProB, and ProT cells (Karsunky et al., 2003, 2008). In contrast, myeloid cells did not display a detectable increase in percent GFP+ cells compared to MPP^F, consistent with Flk2+ cells not contributing significantly to myelopoiesis beyond the MPP^F stage.

Hematopoietic Differentiation Occurs through a Flk2+ Stage under Stress Conditions

To test whether alternative differentiation pathways can be used when there is an acute requirement for MegE cells, we analyzed the color composition of hematopoietic cells under stress conditions. First, we subjected FlkSwitch mice to sublethal irradiation and analyzed the color composition of mature cells in the PB over time. Intriguingly, the percentage of GFP+ Plts, EPs, and GMs, but not B and T cells, decreased for the first 3 weeks after irradiation (Figure 5A). Three weeks postirradiation, the percentage of GFP+ myeloid cells started to rebound. A second sublethal dose of irradiation at week 5 elicited a similar response, with GFP percentages in Plts, EPs, and GM cells decreasing for 3 weeks to

then increase over the next several weeks (Figure 5A). The magnitude of the changes in GFP percentages was similar for Plts, EPs, and GM cells, whereas neither B nor T cells displayed significant changes in the percent of GFP+ cells. The myeloid-specific decrease in GFP frequencies upon irradiation suggested that a proportion of the myeloid cells may be derived directly from Flk2– progenitors, ‘‘skipping’’ the Flk2+ intermediate stage used during steady-state hematopoiesis. Alternatively, overall floxing efficiency could be decreased in Flk2+ progenitors. To distinguish between these possibilities, we analyzed the color composition of stem and progenitor cells in the BM at 2 weeks (one sublethal dose) and 19 weeks (two sublethal doses) postirradiation. As shown in Figures 5C and 5D, the percentage of GFP+ MPP^F was the same as that of mature myeloid cells, and of all myeloid progenitor populations. These data are inconsistent with a bypass pathway and instead support an irradiation-induced decrease in floxing efficiency. Indeed, comparing GFP percentages of cells from unirradiated mice (Figure 5B) to cells from irradiated mice (Figures 5C and 5D) with similar floxing efficiencies in the PB prior to irradiation (~95%) indicated that irradiation caused a reduction in floxing efficiency in MPP^F. This effect was not detectable in B and T cells, presumably due to the multiple Flk2+ progenitor stages during lymphoid differentiation.

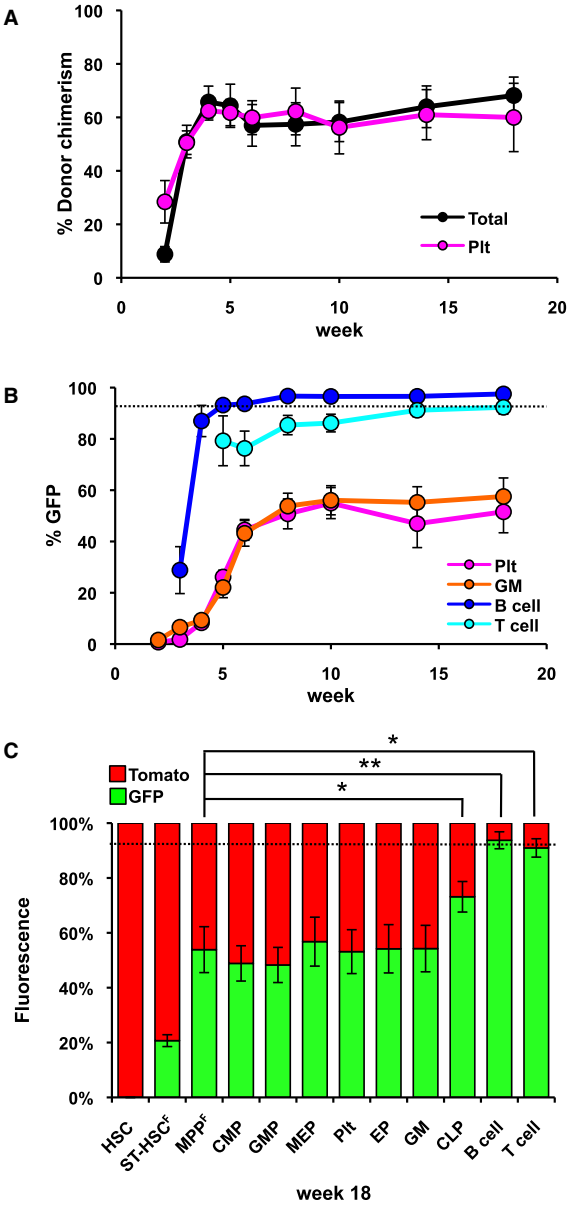
Figure 5. Hematopoietic Differentiation under Irradiation-Induced Stress Progresses through a Flk2+ Stage. (A) Analysis of PB cells of FlkSwitch mice before and after irradiation-induced stress revealed a myeloid-specific decrease in the percentage of cells expressing the GFP reporter gene, with a concomitant increase in the percentage of cells expressing Tom. GFP percentages in Plts (black line), GMs, and B and T cells (not shown) remain unchanged over time in unirradiated mice. (B–D) Ratio of GFP to Tom expression in BM cell populations without irradiation (B), 2 weeks after one sublethal irradiation dose (C), and 19 weeks after two sublethal doses administered at weeks 0 and 5 (D), demonstrating that the decrease in MPPF floxing efficiency is reflected in myeloid populations. The black dotted line indicates average GFP percentages of myeloid PB cells of the same mice prior to irradiation. Error bars indicate SEM. p values were determined using a paired two-tailed t test. *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.00005. GFP percentages in HSCs and ST-HSCF were significantly different from all other hematopoietic cell types (B–D).



HSCs Differentiate through a Flk2+ Stage upon Transplantation

We then tested differentiation pathways upon transplantation. HSCs isolated from high-floxing (~95% GFP+ myeloid PB cells) FlkSwitch mice were transplanted into sublethally irradiated wild-type mice, followed by PB analysis of donor-derived progeny. Figure 6A shows the robust, long-term reconstitution of nucleated blood cells and Plts in recipient mice. Analysis of the color composition of PB progeny revealed that the percentages of GFP+ Plts, GM, and B cells increased with time after transplantation (Figure 6B). B cells quickly reached >90% GFP+ cells, whereas the increase in the GFP+ percentage of Plts and GM cells plateaued at ~50% 8 weeks after transplantation. Robust T cell readout was first detected at 5 weeks posttransplantation, with T cells displaying high GFP percentages throughout the time course. Analysis of the BM cell populations from the same mice 16–18 weeks after transplantation revealed that the GFP percentages were similar in MPP^F, myeloid progenitors, Plts, and mature GM cells (Figure 6C). These results are similar to those from the sublethal irradiation experiments and prompted us to conclude that myeloid cells, including MegE cells, are derived through a Flk2+ progenitor stage even under hematopoietic stress conditions.

Figure 6. HSCs Differentiate into All Lineages through a Flk2+ Stage upon Transplantation. (A) Donor chimerism in recipient mice transplanted with 100 HSCs from FlkSwitch mice demonstrates robust long-term engraftment of nucleated (GMs, B cells, and T cells) PB cells, as well as Plts. (B) GFP percentages of donor-derived B cells, Plts, and GMs increase for the first few weeks after transplantation and then remain stable. (C) BM analysis of recipient mice >16 weeks posttransplantation revealed that MPP^F displayed similar GFP percentages as myeloid progenitors, Plts, and GMs. The black dotted line indicates the GFP percentage of MPP^F of the same mice prior to transplantation. (n = 7 in two independent experiments.) Error bars indicate SEM. p values were determined using a two-tailed paired t-test. *p < 0.005, **p < 0.001. GFP percentages in HSC and $ST\text{-}HSC^F$ were significantly different from all other hematopoietic cell types.



DISCUSSION

All Hematopoietic Cells Differentiate through a Flk2+ Stage

Our data clearly demonstrate that upregulation of Flk2 is a shared feature of HSC differentiation into all hematopoietic cell types. Thus, the FlkSwitch lineage tracing model definitively rules out differentiation of HSCs directly into Flk2– myeloid-committed progenitors or mature myeloid cells. The model also provides definitive evidence that HSC specification and maintenance do not involve Flk2+ developmental stages. During steady-state, irradiation-induced stress, and upon HSC transplantation, all myeloid subpopulations displayed reporter recombination, with up to 98% of Plts and EPs being GFP labeled. Although we did not observe a 100% color switch in myeloid populations, we showed that this is the result of imperfect floxing efficiency as opposed to bypass of the Flk2+ stage (Figure 2, Figure 3, Figure 5, and Figure 6). MPP^F, by definition, do not bypass the Flk2+ stage and displayed <100% floxing efficiency. If it was favorable to ‘‘escape’’ the Flk2+ stage during myeloid differentiation, a proportion of myeloid progeny would be generated through the bypass pathway and remain Tom+. This scenario may be more readily detectable in low-efficiency floxers and in mice under hematopoietic stress, but was not observed under any condition examined. Furthermore, we ruled out Cre misexpression or activity as significant contributors to reporter switching in myeloid cells (Figure 3). This allowed us to conclude that all hematopoietic cells, including cells of the MegE lineage, are derived from HSCs through a Flk2+ progenitor. Böiers et al. (2010) recently made similar conclusions for granulocyte differentiation when analyzing granulocytic and MegE development; however, they did not report their findings on erythroid cell or platelet origins.

Robustly Flk2+ Cells Are the Likely Immediate Progenitors of Myeloid-Committed Cells

Our observation that all myeloid populations exhibit the same floxing efficiency as MPP^F in FlkSwitch mice under both steady-state and dynamic conditions, and the lack of identification of a downstream Flk2+ cell type with MegE capability, points to MPP^F as the

likely intermediate Flk2+ cell population (Figure 2A, left). Importantly, multipotent progenitors with previously demonstrated MegE potential (Flk2–CD34+ KLS cells) and our ST-HSC^F (here defined by intermediate Flk2 cell surface expression) lack (Figure S1B) or display significantly lower (Figures 2C–2E, Figures 5B–4D, Figure 6C, Table 1) floxing efficiency compared to myeloid progenitors and mature cells. Because CMPs lacked any detectable recombinase expression or activity, we find it unlikely that these ST-HSC populations are the immediate progenitors of myeloid-committed cells. In contrast, MPP^F and all myeloid populations exhibited the same proportion of floxed cells in every mouse examined (Figures 2C–2E, Figures 5B–5D, Figure 6C, Table 1). This concordance in GFP percentages between MPP^F and myeloid populations was remarkably conserved even during the highly dynamic conditions of hematopoietic stress (Figure 5 and Figure 6). Known marker heterogeneity within Flk2+ populations (as well as HSCs; see below) raises the possibility that lineage biases occur during or prior to Flk2 upregulation. Thus, while it is possible that the cells within the Flk2+ fraction that give rise to MegE lineages are different from the cells that give rise to lymphoid cells, loss of MegE ability before the onset of Flk2 expression (Figure 2A, right) is incompatible with the data we present here. Instead, our results support a model wherein all hematopoietic lineages develop through a robustly Flk2+ intermediate.

Comparison of Steady-State Hematopoietic Differentiation to Pathways in Transplantation Models

Given the relatively poor MegE readout in vitro and low CFU-S frequency in vivo from Flk2+ progenitors (Adolfsson et al., 2005; Forsberg et al., 2006), one might have expected that at least a fraction of MegE cells would be derived from a Flk2-independent pathway. In contrast, our data revealed that all MegE cells are derived via a Flk2+ stage. Flk2 clearly plays a role in lymphoid development (Mackarehtschian et al., 1995). However, it is important to distinguish between the function of Flk2 itself and the role of cell populations defined by Flk2 expression. Our data show that upregulation of Flk2, and other lymphoid characteristic

genes, does not exclude MegE development in vivo, and suggest that in vitro assays may underestimate MegE potential.

Hematopoietic differentiation pathways have been modeled primarily on hematopoietic reconstitution upon transplantation, and we therefore considered the possibility that steady-state hematopoiesis may differ from transplantation hematopoiesis. The FlkSwitch model is uniquely suited to assess differences between unmanipulated, steady-state hematopoiesis and differentiation that occurs under high-stress conditions. However, our lineage tracing results showed that hematopoietic pathways are similar during steady-state and transplantation, and consistent with the hierarchical differentiation model inferred from transplantation assays (Figure 2A, left). The increased floxing efficiency observed in the lymphoid lineage with GFP percentages increasing from MPP^F to CLP and again from CLP to B and T cells (e.g., Figure 2E) is consistent with MPP^F giving rise to B and T cells via a CLP intermediate. In contrast to lymphoid cells, GMs did not display a detectable increase in percent GFP+ cells compared to MPP^F, possibly indicating that any physiological contribution of Flk2+ myeloid progenitors to GMs (Böiers et al., 2010; Nutt et al., 2005) is relatively low compared to the contribution by Flk2– CMPs. In addition, neither high- nor low-efficiency floxers showed an increase in GFP percentages in CMPs, GMPs, MEPs, EP, or Plts compared to MPP^F, consistent with Flk2+ cells not contributing significantly to those lineages beyond the MPP^F stage. These observations justify a focus exclusively on Flk2– cell types when further investigating myeloid-committed progenitor cells. Intriguingly, the dynamics and magnitude of the changes in GFP percentages were strikingly similar for Plts, EPs, and GM cells during irradiation-induced stress and recovery. These data are consistent with the existence of a shared myeloid progenitor cell, as concluded upon the identification of CMPs (Akashi et al., 2000). By establishing the physiological contribution of specific progenitors to distinct hematopoietic lineages, our lineage tracing results eliminate several question marks from pathway models based on transplantation assays.

HSC Specification and Maintenance Do Not Include Flk2+ Stages

Our data also provide important clues on the developmental origin and maintenance of HSCs themselves. First, because adult HSCs are Tom+ in FlkSwitch mice, all developmental precursors to BM HSCs must be Flk2–. Second, because HSCs remain Tom+ in adulthood, we conclude that dedifferentiation of Flk2+ progenitor cells back to the stem cell stage is extremely low or nonexistent. The recently reported heterogeneity of cells that fits the classical definition of HSCs (reviewed by Hock, 2010; Schroeder, 2010) has put into question the hierarchy of different ''HSC'' populations. Importantly, previous studies have shown that HSCs separated by cell cycle status show differential reconstitution ability (Fleming et al., 1993; Passegue´ et al., 2005). Because these cells are arguably HSCs even though their reconstitution ability (and likely at least some markers) is altered by cell cycling, some degree of heterogeneity must be allowed. This transient difference in reconstitution ability also reminds us that transplantation models are dependent on the engraftability of cells at any given time, and that the behavior of transplanted cells may differ from unmanipulated resident cells with the same marker phenotype. It is not yet clear whether HSCs cycle back and forth between different marker phenotypes. Here we show that such possible HSC differentiation/ dedifferentiation does not include cells beyond the Flk2+ stage, establishing a clear hierarchy between HSCs and non-self-renewing progenitors.

Stem Cell Fate Decisions

Do stem cells, themselves, make fate decisions? While we addressed this question in a specific system (hematopoiesis) with a specific tool (Flk2-driven Cre expression), the concept of when cell fate decisions are made is relevant for all stem cell types and developmental systems. Even in the intensely investigated hematopoietic system, it is clear from the many proposed versions of hematopoietic lineage maps (Akashi, 2009; Ceredig et al., 2009; Hock and Orkin, 2005; Laiosa et al., 2006; Luc et al., 2008b) that fundamentally important questions remain unresolved. Our results clearly demonstrate that ''multipotent''

progenitors are not only capable of providing all hematopoietic lineages upon transplantation under acute stress conditions, but that they do contribute to all lineages under both steady-state and stress conditions in vivo. This definitive demonstration of the physiological significance of the multilineage capability of a progenitor subpopulation justifies further efforts into targeting MPP, and analogous populations in other stem cell systems, to enable efficient manipulation of cell fate decisions.

EXPERIMENTAL PROCEDURES

Mice

Mice were maintained in the UCSC vivarium according to IACUC approved protocols. mT/mG reporter mice containing a loxP-flanked Tom transgene followed by enhanced GFP in the Rosa26 locus, and Flk2-Cre mice generated by Cre recombinase expression from a Flk2 BAC transgene, were described previously (Benz et al., 2008; Muzumdar et al., 2007). FlkSwitch mice were generated by breeding mT/mG reporter mice and Flk2-Cre mice. Early generations displayed low floxing efficiency, while subsequent backcrossed generations displayed increasing floxing efficiencies nearing 100%.

Cell Isolation and Analysis

BM and PB cells were isolated and processed as described previously (Forsberg et al., 2005, 2006; Smith-Berdan et al., 2011) using a four-laser FACSAria or LSRII (BD Biosciences, San Jose, CA). Flowjo Software (Ashland, OR) was used for data analysis and display. Cell populations were defined by the following cell surface phenotypes: HSC (Lin–Sca1+c-kit+ CD48–Slamf1+Flk2–), ST-HSCF (Lin–Sca1+ c-kit +Flk2intermediate), MPPF (Lin–Sca1+ c-kit+CD48+Slamf1–Flk2+), CMP (Lin–Flk2–Sca1– c-kit+FcgRmid CD34mid), GMP (Lin–Flk2–Sca1– c-kit+FcgRhiCD34hi), MEP (Lin–Flk2–Sca1– c-kit+FcgRloCD34lo), CLP (Lin–Sca1mid c-kitmidFlk2+IL7Ra+), GM (Ter119– Mac1+Gr1+CD3–B220–), EP (Mac1–Gr1–CD3–B220–Ter119+CD71+), Plts (FSCloTer119–CD61+), B cell (Ter119–Mac1–Gr1–CD3–B220+), and

T cell (Ter119–Mac1–Gr1–CD3+B220–). The lineage cocktail consisted of CD3, CD4, CD5, CD8, B220, Gr1, Mac1, and Ter119.

Statistics

A Wilcoxon Signed Rank Test, appropriate for application to data with nonparametric distribution, was used to calculate the significance of the differences in percent GFP+ cells between cell types at steady-state (Table 1). Paired two-tailed t tests were used to calculate the significance of the difference in percent GFP+ cells between hematopoietic cell types after sublethal irradiation and HSC transplantation. Unpaired two-tailed t tests were used for all other statistical analyses.

Transplantation Assays

Double-sorted HSCs (100 per mouse), Tom+ or GFP+ MPPF (800 per mouse), and CMPs (10,000 per mouse) were injected into sublethally irradiated mice (770 rads). The BM and PB of recipients were analyzed by flow cytometry for GFP and Tom fluorescence posttransplantation. The data in Figure 2 and Figure 3 display PB reconstitution of Plts and GM at 9 days posttransplantation; at 9 days for total nucleated cells (CMPs only); 17 days for total nucleated cells (MPP only); and at 30 days posttransplantation for B and T cells (MPP only). For CFU-S analysis, Tom+ or GFP+ MPPF or CMPs were injected into lethally irradiated mice (1036 rads). Spleens were dissected, colonies were enumerated, and cells were analyzed by flow cytometry for GFP and Tom fluorescence 9 days (CMPs) and 11 days (MPPF) posttransplantation.

Sublethal Irradiation Assays

Mice with high floxing efficiency (>95%) were given a half-lethal dose (518 rads) of irradiation. Consecutive doses were given 5 weeks apart. PB and BM cells were analyzed by flow cytometry for GFP and Tom fluorescence.

In Vitro Culture

Twenty to fifty Tom+ or GFP+ MPPF and CMPs from FlkSwitch mice with high floxing efficiency (>90% GFP in mature myeloid cells) were plated in Methocult M3231 (Stem Cell Technologies) supplemented with SCF (25 ng/ml), IL-11 (25 ng/ml), IL-3 (10 ng/ml), Tpo (25 ng/ml), Epo (2.5 U/ml), and GM-CSF (10 ng/ml). Cells were harvested and analyzed by flow cytometry for GFP and Tom fluorescence after 10 days of culture.

CHAPTER 3

Mapping differentiation pathways from hematopoietic stem cells using Flk2/Flt3 lineage tracing

The text contained within this chapter of the dissertation includes reprints of the following previously published material: [Boyer SW, Beaudin AE, Forsberg EC. (2012). Mapping differentiation pathways from hematopoietic stem cells using Flk2/Flt3 lineage tracing. Cell Cycle. 11(17):3180-8]. SWB performed experiments. SWB, AEB, and ECF designed experiments, analyzed data and wrote the manuscript.

SUMMARY

Genetic fate-mapping approaches provide a unique opportunity to assess differentiation pathways under physiological conditions. We have recently employed a lineage tracing approach to define hematopoietic differentiation pathways in relation to expression of the tyrosine kinase receptor Flk2 (Boyer et al., 2011). Based on our examination of reporter activity across all stem, progenitor and mature populations in our Flk2-Cre lineage model, we concluded that all mature blood lineages are derived through an Flk2+ intermediate, both at steady-state and under stress conditions. Here, we reexamine in depth our initial conclusions and perform additional experiments to test alternative options of lineage specification. Our data unequivocally support the conclusion that onset of Flk2 expression results in loss of self-renewal but preservation of multilineage differentiation potential. We discuss the implications of these data for defining stem cell identity and lineage potential among hematopoietic populations.

INTRODUCTION

Understanding the mechanisms that drive multipotent stem cells to self-renew or to

commit to specific cell fates is a central goal of regenerative medicine. Accurate maps of differentiation pathways are not only critical for directed differentiation of pluripotent and multipotent cells for therapeutic use, but also for understanding disease pathogenesis and enabling targeting of the cells and molecules that are at the core of aberrant behavior. The hematopoietic system can be considered a model paradigm for dissecting stem cell differentiation pathways, as it has been established that a single, multipotent hematopoietic stem cell (HSC) can both self-renew and give rise to all mature blood cell types. Furthermore, progressively restricted progenitor cells capable of giving rise to unilineage-committed precursors and, ultimately, mature cells have been identified. Our knowledge of hematopoietic differentiation has benefitted greatly from an array of assays capable of measuring the lineage potential of defined cell populations both in vitro and in vivo. Unfortunately, recent advances in technical capability combined with development of more sensitive assays have generated more confusion than consensus. Previously defined cell populations have been further subdivided, and the lineage potential of both myeloid and lymphoid populations has been contested in iterations of classical and novel assays. Transplantation assays have long been considered the highest standard for measuring the functional capacity of phenotypically distinct populations. Most in vivo reconstitution experiments are based on CD45 allelic discrimination between host- and donor-derived cells. Because the mature megakaryocyte/erythroid (MegE) cells, platelets (Plt) and red blood cells (RBC) do not express CD45, many studies on hematopoietic lineage potential, including early identification of “multipotent” populations capable of giving rise to granulocytes/macrophages (GM), B and T cells, did not include analysis of in vivo MegE potential (Adolfsson et al., 2001; Christensen et al., 2001; Morrison et al., 1997). Many studies have instead relied heavily on in vitro assays to assess whether defined progenitor populations give rise to MegE cells. Interestingly, in vitro differentiation assays have reported both lack and gain of lineage potential compared with readout from in vivo transplantation experiments (reviewed in Ichii et al., 2010 and Schlenner and Rodewald 2010). While it is clear that the assay conditions can

have a profound impact on the outcome, it is unclear which assays are insufficiently sensitive and what conditions induce lineage readout that does not normally occur. Thus, the true role of several distinct progenitor populations in development of mature hematopoietic cells remains uncertain. To enable interrogation of hematopoietic differentiation pathways under unperturbed, physiological conditions, we recently established a Cre/lox-based lineage tracing model (Figure 7A) (Boyer et al., 2011). We found two properties of fate mapping models particularly appealing: the irreversibility of the genetic excision of the floxed locus and the opportunity to examine steady-state hematopoiesis. We reasoned that steady-state differentiation pathways would enable us to determine the physiological relevance of specific differentiation steps, and that the irreversible change in reporter expression would provide definitive information on the hierarchical relationship between distinct cell populations. In addition, inducing stress and performing transplantations would enable us to determine whether steady-state paths change under different conditions. We are particularly interested in whether fate decisions are made by stem cells themselves or deferred until later differentiation steps. Surface expression of the tyrosine kinase receptor Flk2 (Flt3) within the "KLS" ($cKit^{hi}$, Lin^{-}, $Sca1^{+}$) stem and progenitor cell compartment corresponds with loss of long-term self renewal ability (Adolfsson et al., 2001; Christensen et al., 2001) and therefore separates KLS cells into multipotent, self-renewing HSC and non-self-renewing, multipotent progenitor cells (MPP^{F}). Flk2 expression is sustained during lymphoid differentiation but is not detectable on myeloid committed progenitors or mature cells (Figure 7C). This off-on-off expression pattern provides an excellent opportunity to determine whether Flk2+ progenitors represent an obligatory or optional stage in myeloid differentiation, a question that has been debated in particular for the MegE lineage (Forsberg et al., 2006). Thus, we established an in vivo lineage tracing model that enabled us to determine the relative contribution of Flk2+ cells to the distinct hematopoietic lineages during steady-state hematopoiesis (Boyer et al., 2011). Our group used a mouse model expressing Cre under Flk2 promoter elements (Benz et al., 2008) combined with a dual-fluorescent reporter mouse model (Muzumdar et al.,

2007), whereby Flk2-Cre expression resulted in reporter switching from Tomato to GFP expression ("FlkSwitch" mice; Figure 7A). Due to the permanent removal of the Tomato allele induced by Cre expression, a cell's Flk2 expression history can be determined in FlkSwitch mice (Figure 7B). In our previous report, we made the simple observation that mature cells of all major hematopoietic lineages, including MegE lineages, switch from Tomato to GFP expression when Cre recombinase is driven by the Flk2 locus (Boyer et al., 2011). Because HSC do not express detectable cell surface levels of Flk2 (Adolfsson et al., 2001; Christensen et al., 2001) and remain Tomato+ in the FlkSwitch model, we concluded that all hematopoietic lineages differentiate through a Flk2+ stage (Boyer et al., 2011). Here, we consider alternative explanations to the conclusions drawn in our previous report, provide additional experiments to test these alternative options and discuss our new insights in the context of related studies in the field.

Results and Discussion

Both the reporter and Cre transgenes are expressed as intended and do not alter endogenous Flk2 expression or population size. A basic requirement of lineage tracing models is that the Cre transgene is expressed in the intended pattern. Several different methods to regulate transgene expression can be utilized, including targeted gene replacement, knock-ins, fusion proteins or randomly integrated genes driven by small or large regulatory elements. Each approach has specific advantages, but no strategy guarantees perfect recapitulation of the intended expression pattern. For cell-ubiquitous reporter expression, we utilized a previously characterized mT/mG dual-color cassette inserted into the Rosa26 locus (Muzumdar et al., 2007). The Rosa26 locus is a well-characterized and very commonly used site for targeted transgene integration with no effects on hematopoietic function. The Flk2-Cre mice were generated by BAC transgenic methods (Benz et al., 2008), thereby leaving both endogenous Flk2 loci intact, yet allowing regulation of the Cre transgene by a very large region of the Flk2 locus. We previously demonstrated that Cre expression

parallels endogenous Flk2 expression: MPP^F display high levels of both Flk2 and Cre expression, whereas the Flk2-negative HSC and CMP do not express Cre (Boyer et al., 2011). These analyses were greatly facilitated by the fact that Flk2 is a surface marker, allowing FACS isolation of clearly Flk2+ and Flk2-populations. To also test for residual Cre activity in the myeloid lineage, the fraction of common myeloid progenitors (CMP) that remained Tom+ in FlkSwitch mice were isolated and subjected to in vitro and in vivo differentiation. Reporter switching (e.g., from Tomato to GFP expression) was not observed in progeny derived from in vitro differentiated or transplanted Tom+ CMP, indicating that neither Cre expression nor activity persist in myeloid progenitors (Boyer et al., 2011).

To be confident in our conclusions regarding lineage specification in the FlkSwitch model, we must also be certain that expression of endogenous Flk2 is unchanged by insertion of the Flk2-Cre transgene. Since BAC transgenesis was used to integrate the Flk2-Cre construct randomly into the genome, both of the endogenous Flk2 loci remain intact. However, it is possible that the Flk2-Cre construct(s) may alter endogenous Flk2 expression by other mechanisms, such as promoter competition for limiting factors, subsequently affecting hematopoiesis. We therefore compared Flk2 surface levels in KLS cells derived from FlkSwitch, $Flk2^{-/-}$, $Flk2^{+/-}$ and wt mice. Flk2 cell surface levels on FlkSwitch KLS cells were equal to the levels on wt KLS but significantly higher compared with KLS from $Flk2^{-/-}$ and $Flk2^{+/-}$ mice (Figure 8A). Additionally, we also found no differences in the cell frequency of stem and progenitor cell populations in the bone marrow of wt and FlkSwitch mice (Figure 8B). Together, these data show that both the reporter and Cre transgenes are expressed as intended without effects on Flk2 expression levels or lineage specification. Theoretically, the ideal lineage tracing model is one in which 100% of parent cells are unfloxed, and 100% of Cre-expressing cells and their progeny are floxed (Figure 7B). Unexpectedly, however, we found that the “imperfect,” highly variable floxing efficiency between different individual mice was an advantageous feature of the FlkSwitch model (Boyer et al., 2011). While mice with high reporter-switching at the MPP^F stage have a “ceiling effect” that prevents additional

reporter switching, low-floxing mice allow for the detection of differences in reporter activity downstream of MPP^F. In our model, low-floxing mice enabled us to demonstrate that both B and T cells utilized multiple Flk2+ developmental stages (Boyer et al., 2011). GM and MegE lineages, in contrast, did not exhibit increased reporter switching compared with MPP^F, as illustrated in Model 1 (Figure 7C). It is important to note that although the floxing efficiency between different mice was highly variable, the Tom/GFP ratios of different populations within individual mice always followed the same pattern. This unintended but highly beneficial feature of the FlkSwitch model enabled a more quantitative assessment of the relationship between different lineages than an all-or-none floxing response, as illustrated below.

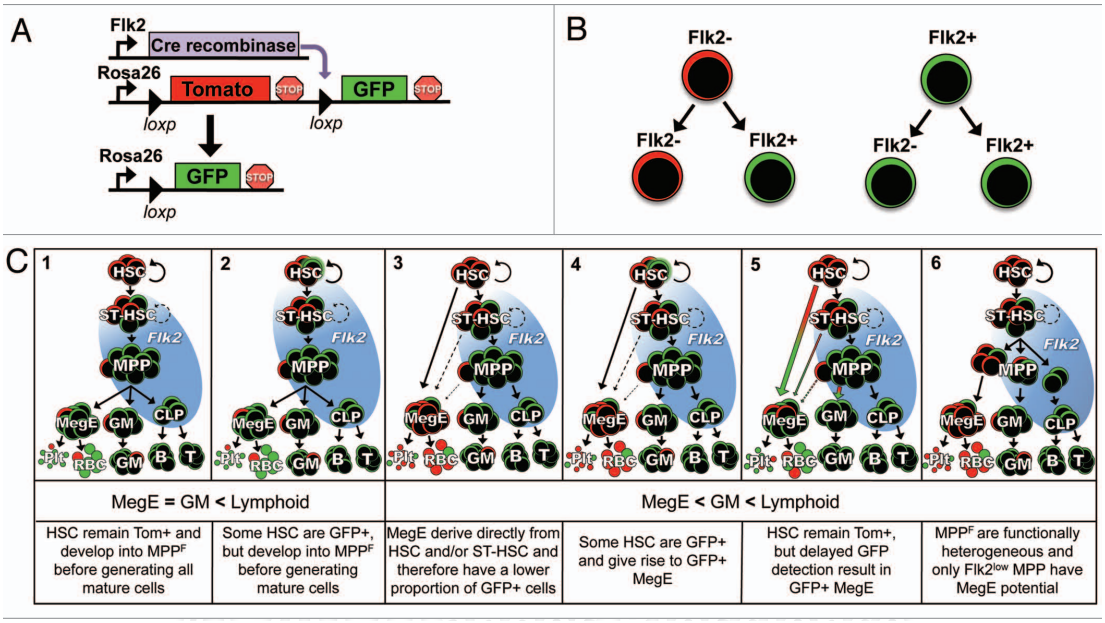


Figure 7. Modeling hematopoiesis with Flk2-Cre lineage tracing. (A) Flk2-Cre mice were crossed to mT/mG dual-color reporter mice to generate Flk-Switch mice. (B) Simplified model of lineage tracing strategy for FlkSwitch mice. (C) Six possible outcomes from FlkSwitch lineage tracing. Flk2 expression is indicated by the blue background circle. The second row indicates the relative proportion of GFP+ cells in MegE, GM and lymphoid lineages. The third row describes a hypothetical scenario for each model. In Models 1, 3, 5 and 6, all HSC remain Tomato+. In Models 2 and 4, Flk2 expression is initiated in a fraction of HSC resulting in reporter floxing and GFP expression. Although additional versions of these models can be drawn, only Model 1 fits the results from lineage tracing studies. HSC (hematopoietic stem cell), ST-HSC (short-term hematopoietic stem cell), MPP (multipotent progenitor),. MegE (megakaryocyte/erythrocyte progenitor), GM (granulocyte/myelomonocyte), Plt (platelet), RBC (red blood cell), B (B cell), T (T cell).

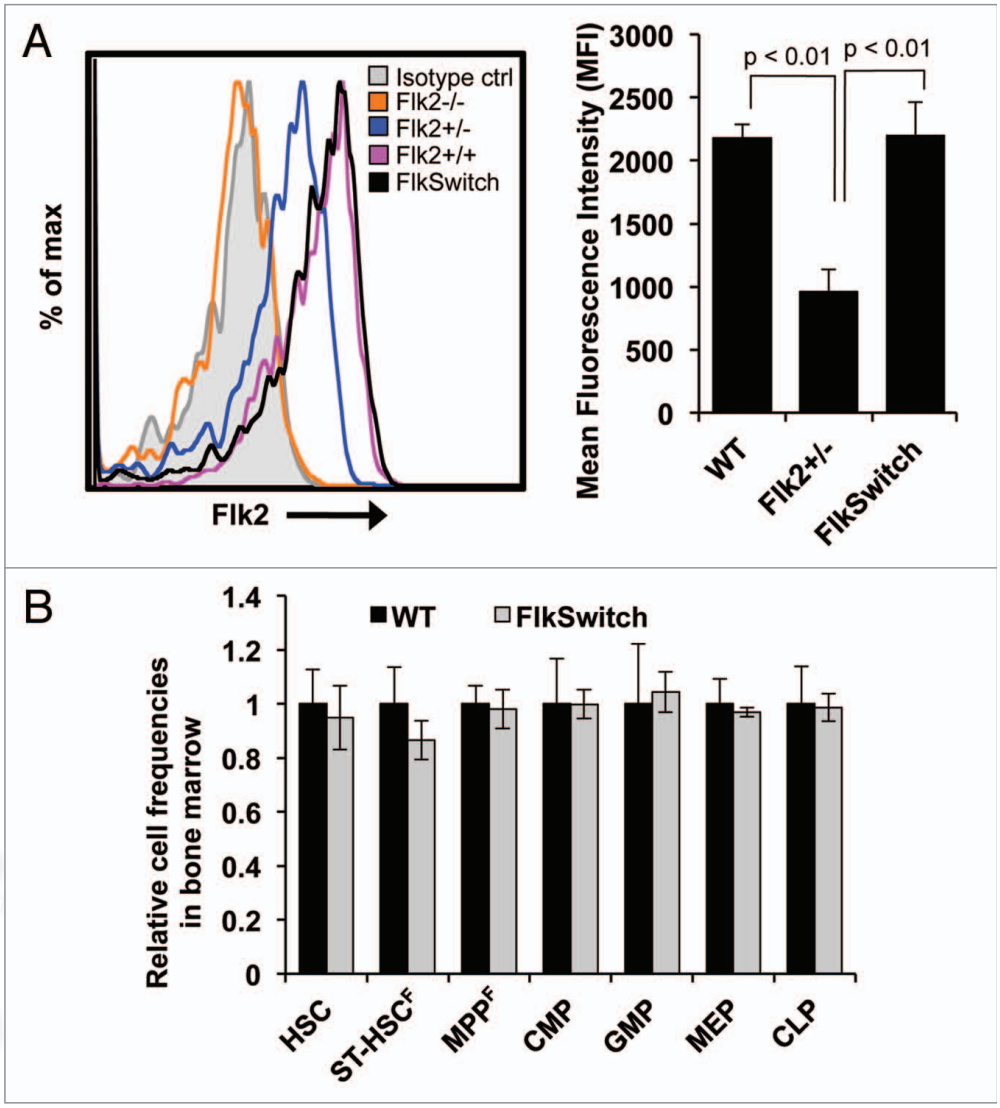


Figure 8. Flk2 expression and lineage specification is not altered in Flk2-Cre reporter mice. (A) Flow cytometric analysis (left) showing Flk2 expression within the KLS compartment of Flk2-/-, Flk2+/-, Flk2+/+ (wt) and FlkSwitch (Flk2+/+) mice. Isotype control is shown in gray. Mean fluorescence intensity for Flk2 surface levels from multiple mice (right). (B) Cell frequencies of hematopoietic stem and progenitors in FlkSwitch bone marrow relative to wt mice. n = 3–5 mice per experiment.

All functional HSC remain Tomato+

While it is clear that the vast majority of mouse HSC lack detectable Flk2 cell surface protein (Adolfsson et al., 2001; Christensen et al., 2001) it is possible that Flk2 (and therefore Cre) expression occurs in HSC prior to display of the protein on the cell surface (Models 2 and 4, Figure 7C). Low level Cre expression in HSC could lead to Tomato excision and result in GFP+ progeny without differentiation through a truly Flk2+ stage (Model 4, Figure 7C). Such "premature" excision could mean that our conclusion that all lineages derive through a Flk2+ stage is erroneous. In our previous analyses, we were unable to detect Cre mRNA in HSC, and we also showed that all phenotypic HSC are Tom+ in FlkSwitch mice (Boyer et al., 2011). However, phenotypic analysis does not exclude the possibility that HSC also exist in the GFP+ fraction in FlkSwitch mice. We therefore tested this directly by subfractionating KLS cells from FlkSwitch mice based on Tomato and GFP expression and measuring their functional capacity upon transplantation into recipient mice. Long-term, multilineage reconstitution was observed when either 100 or 500 Tomato+ KLS cells were transplanted (Figure 9). In contrast, contribution from GFP+ KLS cells to B-cells (Figure 9C) and T-cells (Figure 9D) peaked at week 4 and 8, after which readout steadily declined. Similarly, Plt and GM production from GFP+ KLS cells declined to undetectable levels by week 8, consistent with retention of multilineage potential but loss of self-renewal ability in GFP+ cells (Figure 9A and B). Thus, transplantation of either 100 or 500 GFP+ KLS cells was unable to sustain myeloid (Figure 9A and 9B) or lymphoid (Fig. 9C and 9D) output for the longterm, even when 500 cells were transplanted. Consistent with the lack of detectable Cre expression in phenotypic HSC, these data demonstrate that all, or virtually all, long-term reconstituting HSC in FlkSwitch mice remain Tomato+, and therefore that GFP expression observed in mature lineages cannot be attributed to direct generation from a GFP+ HSC. The possibility of delayed floxing has to be applied equivalently to all progeny. We then considered the possibility that a high proportion of myeloid cells are GFP+ due to delayed detection of floxing (Model 5, Figure 9C). In this model, a precursor cell expresses Cre but remains Tom+ due to

the lag between Cre expression and excision of the first reporter and transcription/translation of the second reporter. If low levels of Cre led to initiation of floxing in HSC, they could remain GFP-negative (and Tom+) due to the delay in GFP protein detection. In this scenario, all HSC progeny could turn GFP+, even if these progeny do not express Cre themselves. Such delayed detection of floxing could lead us to erroneously conclude that the Flk2- myeloid cells—or maybe more likely, only the contested MegE lineage of the myeloid branch—are derived through a Flk2+ stage, while in reality they are derived directly from HSC. When evaluating reporter expression in MegE progeny only, it is difficult to rule out the possibility that GFP expression is due to delayed floxing in HSC. When considering this in the bigger context of other hematopoietic lineages, however, it is clear that this explanation is highly unlikely. First, cell populations of the MegE and GM lineages have the same proportion of GFP+ cells, not only on average, but in every individual mouse.1 If delayed floxing results in MegE cells that have an increased proportion of GFP+ cells compared with their immediate progeny, analogous increases in GFP+ proportion should also be observed for the GM lineage (Model 5, Figure 7C). Because MPP^F possess strong and undisputed GM potential, delayed floxing predicts that GM populations would exhibit a higher proportion of GFP+ cells than MPP^F, in addition to a higher GFP+ proportion than MegE cells (Model 5, Figure 7C). This is not what we observe. Furthermore, the consistently higher proportion of GFP+ cells in lymphoid populations demonstrates that a “ceiling” of possible floxing efficiency has not been reached in GM cells (Boyer et al., 2011). The fact that neither MegE nor GM lineages express Flk2 (or Cre) and yet exhibit equivalent levels of floxing indicates that they are either derived from the same progenitor population or from two different populations with equivalent floxing efficiencies. The identity of these precursor populations is discussed in the next section. Another argument against the possibility of delayed floxing in our model stems from the fact that HSC are exclusively Tomato+. Due to self-renewal, HSC are also their own progeny. If all progeny turn GFP+ due to initiation of floxing in preexisting HSC, newly derived HSC would also be GFP+. This is not what we observe at steady-state or even after hematopoietic

stress. We have shown that all HSC remain Tom+ after stress induced by irradiation or transplantation into irradiated recipients. These conditions induce HSC proliferation but do not lead to detection of GFP+ HSC (Boyer et al., 2011). Furthermore, since floxing is irreversible, we would expect that low level Cre activity in HSC would lead to an accumulation of GFP+ HSC over time. Analysis of aging FlkSwitch mice revealed that all phenotypic BM HSC remained Tom+ in mice as old as 22 months, even with GFP+ MPP^F proportions as high as 92% (data not shown). Combined with the lack of detectable Cre mRNA in HSC and lack of HSC activity in GFP+ KLS fraction, we are compelled to conclude that Flk2 and Cre are only expressed at functionally significant levels upon differentiation, but not in HSC themselves. These results strongly support our conclusion that all hematopoietic lineages differentiate through a Flk2+ stage (Model 1, Figure 7C). Flk2+ KLS cells must be MegE progenitors.

Having concluded that all myeloid lineages must differentiate through a progenitor that expresses Flk2, thereby excluding Flk2- HSC as an immediate precursor, we revisit our conclusion that MPP^F is the Flk2+ intermediate stage for all hematopoietic lineages. In part, this conclusion was based on our observation that the proportion of GFP+ cells within all myeloid populations was strikingly similar to that of MPP^F in each individual mouse across a wide range of floxing efficiencies (6–97% in MPP^F) (Boyer et al., 2011). Thus, two distinct populations, for example MPP^F and MEP, each consisted of the same proportion of Tom:GFP cells. Clearly, this does not in itself necessarily mean one population gives rise to the other. Since we had already established that HSC do not express Flk2 or exhibit reporter-switching whereas downstream progenitors upregulate Flk2, the options were quickly narrowed down to Flk2+ cells within the KLS fraction: no other Flk2+ cell type with MegE potential has been identified. Quite contrary, increased Flk2 expression has been proposed to correlate with loss of MegE capability (Adolfsson et al., 2005; Nutt et al., 2005). Thus, the remaining challenge is to determine whether Flk2+ progenitors are multipotent at the population and/or clonal levels, and whether the MegE and GM lineages derive from different or overlapping populations

within this fraction.

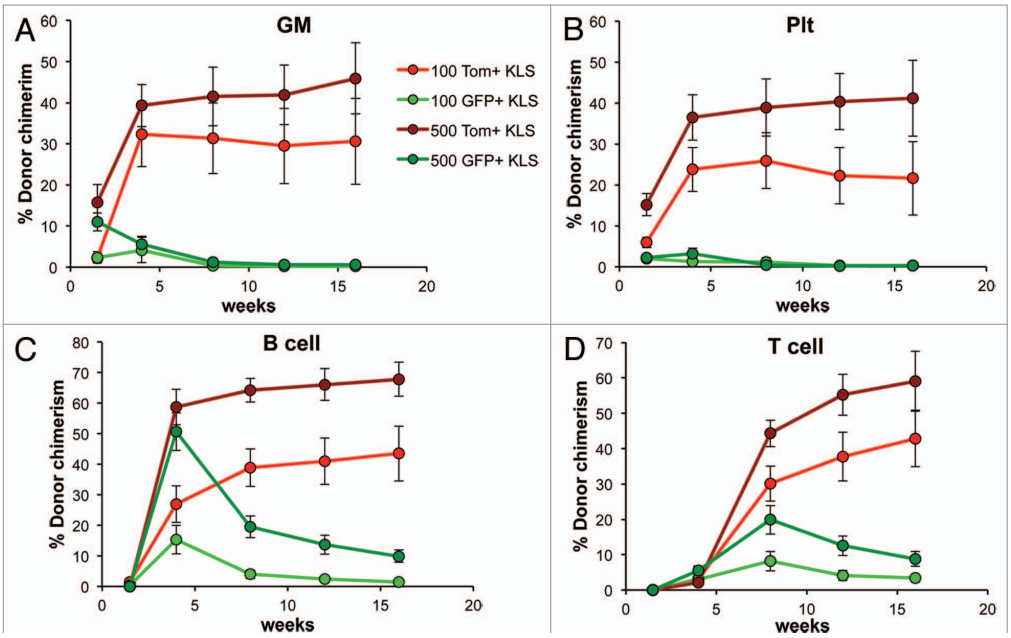


Figure 9. HSC are contained within the Tomato+ fraction in FlkSwitch mice. Percent donor chimerism of (A) GM, (B) platelet, (C) B cell and (D) T cell lineages in the peripheral blood from mice injected with 100 or 500 Tomato+ or GFP+ KLS cells from FlkSwitch mice. Data are from 12–15 recipients per cell type and dose from three independent experiments.

Multipotency at the clonal vs. population level: Heterogeneity of cell populations.

To determine which Flk2+ KLS cells give rise to the MegE lineage and whether these same cells also are GM progenitors, we considered three main models representing complete population overlap to no overlap at all. Figure 4 illustrates the range of possibilities using, for simplicity, only the three main lineages (GM, MegE and lymphoid) discussed in this report. In the "multipotent" model, the vast majority of Flk2+ KLS cells are multipotent at the single cell level. Because any one cell can give rise to any lineage, this model represents complete overlap between MegE, GM and lymphoid progenitors. At the opposite end, there is very little or no overlap in the "commitment" model, in which most cells have committed to a specific lineage. Here, the "MPP" compartment consists of three lineage-committed cell populations despite sharing the defining Flk2+ cell surface phenotype. The "hybrid" model consolidates the two extremes by including both true multipotent cells as well as substantial numbers of oligopotent and lineage-committed cells. The distinction between these models may depend on both the phenotypic definition and functional heterogeneity of the cell populations involved. It is clear from our previous analyses that Tom/GFP expression does not segregate functionally distinct MPP^F: Tom+ and GFP+ MPP^F gave rise to similar levels of Plt, GM, B and T cells in the PB upon transplantation as well as to similar numbers of CFU-S colonies (Boyer et al., 2011). Analogous results were obtained for Tom+ and GFP+ CMP. Thus, if these populations consist of a mix of lineage-committed cells, their relative numbers are roughly equivalent between Tom+ and GFP+ fractions in both MPP^F and CMP.

To address the impact of the phenotypic definition of cell populations, we previously tested whether alternatively defined myeloid progenitor populations12 had different Tom/GFP proportions that correlated with commitment toward MegE or GM lineages. Regardless of how the myeloid progenitor fraction ($cKit^+$ Lin^- $Sca1^-$ cells) was subdivided, the different populations consisted of similar proportions of Tom+ and GFP+ cells. These data demonstrated that Tom/GFP reporter expression does not correlate with commitment into MegE vs. GM lineages, consistent with the unipotent (EP) or mature (Plt and GM) cells of

each lineage displaying similar levels of floxing.

In contrast to the myeloid progenitor pool, dividing the KLS fraction in different ways results in clear differences in Tom/GFP proportions. This is consistent with the variable Flk2 (and Cre) expression levels in KLS. Therefore, slicing the KLS fraction in different ways results in different proportions of Tom/GFP cells in different populations, with implications on progenitor-progeny relationships. One model has proposed that MegE potential is lost in cells with the highest levels of Flk2 expression (Adolfsson et al., 2005). Do MPP that are less Flk2+ have more MegE potential than MPP that express high levels of Flk2? Because GM potential is retained in the highly Flk2+ fraction, adherence to this model would result in higher proportions of GFP+ cells in GM compared with MegE populations (Model 6, Figure 7C). This is not what we observe. Thus, it seems that if MPP^F are functionally heterogeneous with regards to MegE and GM potential, these distinct MegE and GM precursor populations have the same proportion of GFP:Tom cells, suggesting that they cannot be distinguished by Flk2 expression levels alone.

Time as a factor in floxing efficiency.

So far, we have focused on the level of Flk2 expression as the major factor of floxing efficiency. However, it is also highly likely that time spent within a Flk2+ stage also significantly influences the extent of floxing. The consistently higher GFP+ proportion of lymphoid cells, known to differentiate through additional Flk2+ stages, provides strong evidence for this. In addition, the floxing efficiency of MPP^F after irradiation- or transplantation-induced stress is decreased (Boyer et al., 2011). The stress-induced increase in proliferation rates likely causes cells to transition faster through the Flk2+ stage, resulting in a reduction of overall floxing efficiency. Thus, if one considers the “time spent” parameter in addition to the “Flk2 level” parameter, it is conceivable that MegE cells could derive from a KLS cell with lower Flk2 levels than the KLS that give rise to GM. This scenario would be consistent with models proposing loss of MegE, but retention of GM, potential in highly Flk2+

progenitors (Models 3 and 4, Figure 7C). However, if Flk2 levels are lower in the KLS population that give rise to MegE than in KLS cells generating GM, the time MegE precursors spend at this Flk2^{low} stage must be prolonged to achieve the equivalent levels of reporter switching observed for these two lineages. Although it is difficult to measure whether MegE precursors spend more time at a Flk2+ stage, the notion seems inconsistent with the very rapid appearance of Plt and EP after HSC transplantation (Forsberg et al., 2006). It is also important to note that the ratio of Tom:GFP cells in MPP^{F} and all myeloid populations were similar, and equally reduced, after stress (Boyer et al., 2011). Thus, if MegE- and GM-destined progenitor cells spend a differential amount of time at a Flk2+ stage during differentiation, this time is equally reduced upon two different stress conditions. We find it much more likely that all MegE and GM cells follow a shared path of differentiation through the MPP^{F} stage, with subsequent divergence into lineage committed progenitor cells.

Segregation into lineage committed cells.

Our conclusions in favor of the multipotency model based on our FlkSwitch data are supported by several lines of additional evidence. Using the same Flk2-Cre model coupled to a single-color reporter (Rosa26-eYFP), Buza-Vidas and colleagues demonstrated high reporter activity in MegE progenitors, consistent with a significant contribution of Flk2+ progenitors to MegE lineages (Boyer et al., 2011). Although there were some differences between their study and ours (see below), both reports demonstrated that GM and MegE progenitors had similar frequencies of reporter activity. As described above, these data support the existence of common myeloid progenitors (CMP), originally isolated based on combined MegE and GM potential in functional assays in vitro and in vivo (Akashi et al., 2000). Because phenotypic MPP^{F} have much higher burst size relative to downstream progenitors, and the timing of CFU-S or mature cell readout in PB is distinctly different between MPP^{F} and CMP/MEP (Forsberg et al., 2006), MPP^{F} cannot simply consist of a mix of CMP, MEP, GMP and CLP, but rather precursors to such populations.

The multipotency model has been challenged by subfractionation of MPP and other progenitor populations into phenotypically distinct populations by use of both cell surface and intracellular markers. However, identification of novel phenotypic subpopulations has not led to clean separation of functional activity in vivo. Quite the contrary, subfractionation of cells using new marker combinations has supported the existence of cell populations with oligo- or bilineage potential, sometimes also demonstrated clonally (Pronk et al., 2007; Arinobu et al., 2007; Karsunky et al., 2008). These data highlight the notion that differentiation is gradual, and that marker heterogeneity is not synonymous with functional heterogeneity. A simple example is a protein expressed in a cell cycle-dependent pattern; both Ki67- and Ki67+ MPP are still MPP, even though the marker distinction may be accompanied by transient differences in functional readout (Passegue et al.,2005). We therefore find little evidence for a strict commitment model at the level of MPP. If the commitment model is nonetheless correct, we expect that new marker combinations will very soon enable clear separation of the functionally distinct subsets within current cell compartments. Instead of MPP being comprised of cells with clearly distinct functional capabilities, MPP may consist of two or more populations of lineage-biased cells. Lineage bias and recent claims of novel lineage combinations, such as GM/T- or GM/B-restricted progenitors, seem to support the hybrid model (Figure 10). However, novel lineage combination claims have relied heavily on in vitro assays and have been rapidly refuted (Boyer et al., 2011; Schlenner et al., 2010(1); Forsberg et al., 2006; Karsunky et al., 2008; Richie Ehrlich et al., 2011; Schlenner et al., 2010(2); Serwold et al., 2009). A recent IL7R-Cre fate mapping study provided particularly convincing evidence for a shared T/B cell differentiation path and clear separation of myeloid and lymphoid lineages (Schlenner et al., 2010(1); Schlenner et al 2010(2)) as proposed upon isolation of CLP and CMP (Akashi et al., 2000; Kondo et al., 1997). Our own data, as well as the results from Buza-Vidas et al., in 2011, also support this model, as all myeloid-committed cells have similar Flk2-Cre-mediated floxing efficiencies that are lower than those of lymphoid-committed cells. These studies also show that the contribution of putative Flk2+

myeloid restricted progenitors, proT cells or “lymphoid-biased” MPP (Adolfsson et al., 2005; Nutt et al., 2005; Bell et al., 2008; Wada et al., 2008) to the total GM output is either minimal or includes equivalent MegE production. Because in vitro assays can both underestimate and induce lineage potential (reviewed in Ichii et al., 2010 and Schlenner et al., 2010), we are strongly inclined to favor models based on genetic lineage tracing experiments performed under unperturbed, physiological conditions. Reassuringly, the three fate mapping studies described above (Boyer et al., 2011; Schlenner et al., 2010; Buza-Vidas et al., 2011) all support a closer relationship between GM and MegE than between GM and T or GM and B cell lineages.

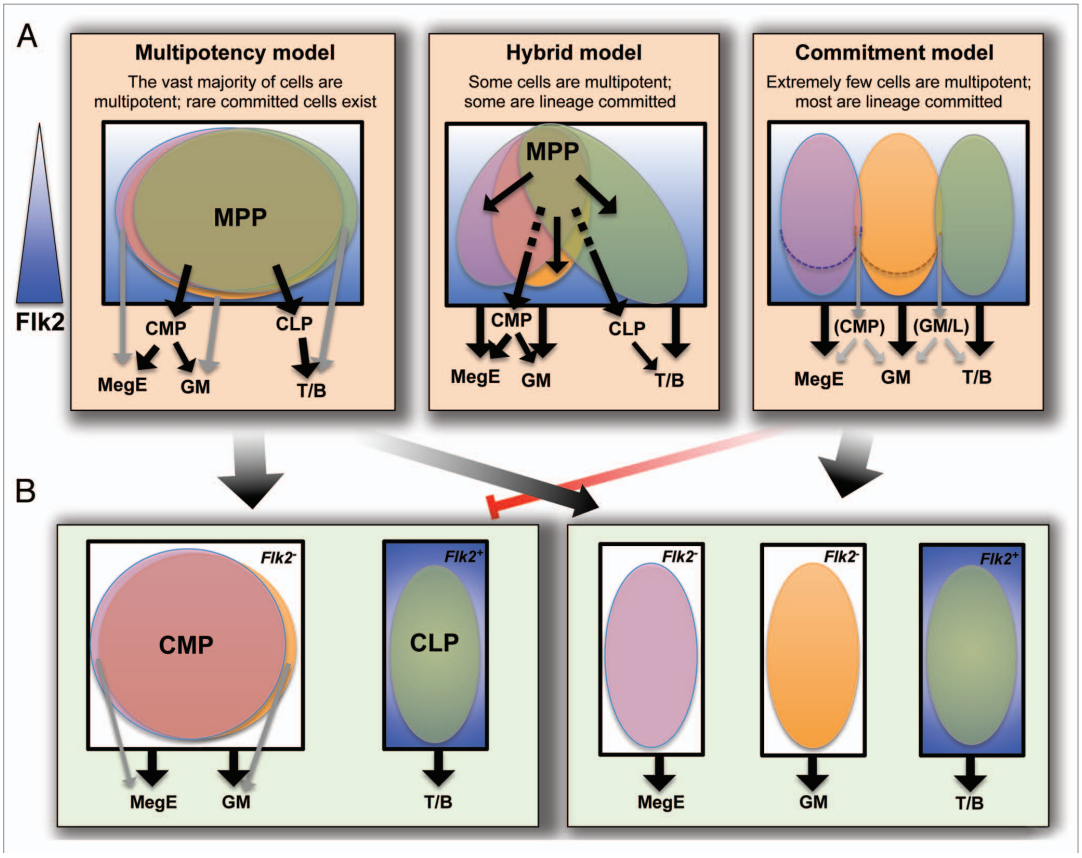


Figure 10. Models for lineage potential at the clonal or population level. (A) The phenotypic MPP^F compartment (Flk2low-high KLS cells, with Flk2 levels indicated by a gradually intensifying blue background) may consist of cells with clonal multilineage potential (Multipotent model), a mix of phenotypically similar cells that are lineage committed (Commitment model), or a mix of clonally multipotent and lineage biased cells (Hybrid model). If different cells within the phenotypic MPP^F fraction give rise to MegE and GM progeny, these two cell populations must have equivalent levels of Flk2 (dotted lines in Commitment model to delineate lower Flk2 levels) as MegE and GM lineages have the same proportion of floxed cells. (B) Lineage-committed progenitor populations may consist of cells with clonal, oligopotent potential (left), or a mix of lineage biased cells (right). If lineage decisions are made by MPPF (or by HSC prior to differentiating into MPP^F) as depicted in the Commitment model, it is unlikely that clonal, oligopotent progenitor cells such as CMP exist, as indicated by the red inhibition arrow. Cell populations within a black box share defining cell surface markers, whereas cell populations in different black boxes are phenotypically distinct. Major differentiation paths are indicated by black arrows, whereas alternative, infrequently used paths are shown in gray.

Flk2 expression delineates loss of self-renewal but is compatible with multipotency.

We previously established that all phenotypic HSC in the FlkSwitch model are Tom+ and therefore must be derived from a Flk2- precursor (Boyer et al., 2011). In this report, we established that both the reporter and Cre transgenes are inert, without effects on endogenous Flk2 expression or the size or function of populations defined by Flk2 expression (Figure 8). Using an unbiased approach of transplanting Tom+ or GFP+ KLS cells, we further demonstrated that all self-renewing cells with long-term, multilineage capacity do not express Cre or Flk2 (Figure 9). In contrast to our findings, Buza-Vidas and colleagues reported that a fraction of HSC exhibited reporter activity, as many as 23% of phenotypic HSC ($CD150^+$ $CD48^-$ KLS cells) were reporter-positive (Buza-Vidas et al., 2011). However, a serial transplantation assay demonstrated that the self-renewal capability of the floxed KLS cells was far inferior to that of unfloxed KLS (Buza-Vidas et al., 2011). Combined with our transplantation data (Figure 9), it is clear that all cells expressing Flk2 and therefore Cre and GFP in FlkSwitch mice, have lost the ability to self-renew. These results also show that addition of Flk2 to the KLS CD150/CD48 marker combination eliminates a fraction of cells that have lost self-renewal potential. Thus, in the mouse, Flk2 is a valuable marker for isolation of highly pure functional HSC.

Conversely, while upregulation of Flk2 marks loss of self-renewal, Flk2 expression does not result in loss of multilineage potential. Data from our previous transplantation experiments in which we quantitatively assessed multilineage reconstitution, including MegE generation, across multiple stem and progenitor populations support a model in which multilineage capability is retained as Flk2- HSC develop into Flk2+ MPP^F (Forsberg et al., 2006). Despite this clear demonstration of MegE potential, it remained possible that MPP^F only give rise to MegE cells under conditions of acute stress in transplantation models, whereas the physiological role of MPP^F under normal conditions is to promote lymphoid development. The FlkSwitch model clearly demonstrates multilineage differentiation through

a Flk2+ intermediate under physiological conditions, and that Flk2 expression does not restrict lineage potential. Expression of Flk2 in human HSC provides additional evidence that Flk2 expression is compatible with multilineage potential (Doulatov et al., 2010; Kikushige et al., 2008).

Definitive conclusions and questions that remain.

Our fate mapping data allows us to definitively conclude that Flk2 is not expressed during HSC development or maintenance, but that a Flk2+ stage is obligatory for differentiation into all lineages at both steady-state and under stress conditions. However, despite the strong evidence for a shared Flk2+ progenitor for all lineages, definitive demonstration for this is still imperative. An essential missing clue is what proportion of MPP are multipotent at the clonal level. Importantly, conclusions regarding multipotency of one population have clear implications for the lineage potential of its progeny: if the "MPP" compartment in reality is composed of several unilineage committed cell types that happen to share a handful of surface markers (commitment model, Figure 10A), it seems highly unlikely that clonal, oligopotent CMP exist (Fig. 10B). Likewise, if lineage commitment is made by HSC, the argument for the existence of clonal, multipotent MPP is severely undermined. While a growing number of reports have established multilineage, long-term readout from transplanted single HSC (Ema et al., 2005; Kiel et al., 2005; Morita et al., 2010; Osawa et al., 1996; Wagers et al., 2002), recent evidence also points toward significant lineage bias between different types of HSC (Benveniste et al., 2010; Challen et al., 2010; Dykstra et al., 2007; Weksberg et al., 2008; Hock et al., 2010; Schroeder et al., 2010). For MPP, we believe that the most convincing data converge on a slightly imperfect version of the multipotency model (Figure 10A). Cells with exclusive GM/T or GM/B potential may exist, but our data indicate that their numbers are vanishingly small and likely of little physiological relevance. Establishment of assays capable of efficiently measuring physiologically accurate lineage potential at the clonal level seems necessary to resolve conflicting views. This will be

extremely challenging, but strikes us as an issue of high significance to the field as the very definition of a stem cell— multilineage and self-renewal potential at the single-cell level—is under debate.

Materials and Methods

Mice.

Mice were maintained by the UCSC animal facility according to approved protocols. Flk2-/- mice have been described previously (Mackarehtschian et al., 1995). Flk2-Cre mice8 were crossed to mT/mG (Muzumdar et al., 2007) mice to generate FlkSwitch mice.1 FlkSwitch mice with high floxing efficiency (> 80% GFP in myeloid cells) were used for all experiments.

Flow cytometry to determine cell frequencies and Flk2 surface levels.

Whole bone marrow samples were stained with antibody cocktails, as described previously (Boyer et al., 2011; Smith-Berdan et al., 2011; Smith-Berdan et al., 2012) to identify HSC (Lin^- $Sca1^+cKit^+CD48^-Slam1^+Flk2^-$), ST-$HSC^F$ ($Lin^-Sca1^+cKit^+Flk2^{intermediate}$), MPP^F ($Lin^-Sca1^+cKit^+CD48^+Slam1^-Flk2^+$), CMP ($Lin^-Flk2^-Sca1^-cKit^+FcgR^{mid}CD34^{mid}$), GMP ($Lin^-Flk2^-Sca1^-cKit^+FcgR^{hi}CD34^{hi}$), MEP ($Lin^-Flk2^-Sca1^-cKit^+FcgR^{lo}CD34l^o$) and CLP ($Lin^-Sca1^{mid}cKit^{mid}Flk2^+IL7Ra^+$).

Transplantation assays.

100 or 500 Tomato-positive and GFP-positive KLS cells were double sorted from cKit-enriched bone marrow from individual FlkSwitch mice and transplanted by retroorbital transfer into sublethally irradiated mice. Peripheral blood samples from recipient mice were analyzed by flow cytometry, as described previously (Boyer et al., 2011; Smith-Berdan et al., 2011) after staining with B220, CD3, Gr1, Mac1 and Ter119 antibodies. GM ($Ter119^-$, $B220^-$, $CD3^-$, $Mac1^+$, $Gr1^{hi}$), Plt (SSC^{lo}, $CD61^+$, $Ter119^-$), B-cell ($Ter119^-$, $B220^+$, $CD3^-$, $Mac1^-$, $Gr1^-$), T-cell ($Ter119^-$, $B220^-$, $CD3^+$, $Mac1^-$, $Gr1^-$).

CHAPTER 4

Flk2/Flt3 promotes both myeloid and lymphoid development by expanding non-self-renewing multipotent hematopoietic progenitor cells

The text contained within this chapter of the dissertation is currently in review in the *Blood* journal. AEB is the major contributor to this work. SWB is responsible for the data and analysis presented in Figure S3A and 14A. SWB contributed to Figures 11 and 12. AEB, SWB and ECF designed experiments, analyzed data and wrote the manuscript.

SUMMARY

Defining differentiation pathways is central to understanding the pathogenesis of hematopoietic disorders, including leukemia. The function of the receptor tyrosine kinase Flk2 (Flt3) in promoting myeloid development remains poorly defined, despite being commonly mutated in acute myeloid leukemia. Here, we investigated the effect of Flk2 deficiency on myelopoiesis, focusing on specification of progenitors between HSC and mature cells. We provide evidence that Flk2 is critical for proliferative expansion of multipotent progenitors (MPP) that are common precursors for all myeloid and lymphoid lineages. Flk2 deficiency impaired the generation of both lymphoid and myeloid progenitors by abrogating propagation of their common upstream precursor. At steady-state, but not upon transplantation, downstream compensatory mechanisms masked the effect of Flk2 deficiency on mature myeloid output. Flk2 deficiency did not affect lineage choice, revealing a dissociation between the role of Flk2 in promoting cell expansion and regulating cell fate. Surprisingly, despite impairing myeloid development, Flk2 deficiency afforded protection against myeloablative insult. This survival advantage was attributed to reduced cell proliferation in Flk2-deficient mice. Our data support the existence of a common Flk2+ intermediate for all hematopoietic

lineages and provide insight into how activating Flk2 mutations promote hematopoietic malignancy in non-Flk2-expressing myeloid cells.

INTRODUCTION

The receptor tyrosine kinase Flk2 (Flt3) has been implicated in many cellular processes in normal hematopoiesis, including proliferation, self-renewal, survival, and lineage specification, particularly of the lymphoid lineages (Adolsson et al., 2005; Hunte et al., 1996; Hudak et al., 1995; Hirayama et al., 1995; Adolfsson et al., 2001; Hudak et al., 1998). Activating mutations in Flk2 are present in >30% of patients with acute myeloid leukemia (AML) and the use of Flk2 inhibitors for the treatment of AML is under intense investigation (Swords et al., 2012). In humans, Flk2-driven myeloid leukemias could be explained by Flk2 expression by HSC (Doulatov et al., 2012; Kikushige et al., 2008). However, mouse models of Flt3-ITD also develop myeloproliferative disease (Li et al., 2008; Chu et al., 2012), despite lack of Flk2 expression by HSC or myeloid-restricted progenitors. Thus, determining the role of Flk2 in myelopoiesis is critical for understanding normal differentiation pathways, as well as leukemia origin and propagation.

Controversies regarding the role of Flk2+ populations in myeloid development were clarified recently by Flk2-Cre lineage tracing approaches, demonstrating that all hematopoietic lineages, including MegE lineages, develop through a Flk2+ intermediate population (Boyer et al., 2011; Boyer et al 2012; Buza-Vidas et al., 2011). The role of Flk2 itself in myeloid differentiation, however, remains unclear as previous examination of hematopoiesis in both the $Flk2^{-/-}$ mouse and the Fl-/- mouse has led to opposite conclusions with regards to regulation of myelopoiesis by Flk2 signaling (Sitnicka et al., 2002; Buza-Vidas et al., 2009, Boiers et al., 2010; Mackarehtschian et al., 1995). The more obvious defects on B cell development and the robust expression of Flk2 on lymphoid-competent progenitors have contributed to the notion that Flk2 signaling drives lymphoid specification, potentially at the expense of myelopoiesis.

Because it is difficult to reconcile these data, we aimed to provide more definitive answers to the role of Flk2 in multilineage differentiation. We took advantage of advances in understanding hematopoietic development that have been made since the original report of hematopoiesis in $Flk2^{-/-}$ mice (Mackarehtschian et al., 1995), including the discovery that mouse HSC do not express Flk2 (Boyer et al., 2011; Boyer et al., 2012; Christensen and Weissman 2001; Forsberg et al., 2006), identification of additional functionally distinct progenitor populations (Pronk et al., 2007; Kondo et al., 1997; Akashi et al., 2000), and more sensitive methods for lineage readout that also enable assessment of erythroid progenitors and platelets. The data from these studies expand our understanding of the function of Flk2 in regulating self-renewal and lineage specification, and support a critical role of Flk2 in driving expansion of a non-self-renewing multipotent progenitor.

RESULTS

Separation of stem and progenitor cells into Flk2- and Flk2+ fractions in the absence of Flk2

To investigate the effect of Flk2 deficiency on specific stem and progenitor cells within the KLS compartment, an alternative staining strategy was devised that did not rely on Flk2 as a surface marker. Both CD150 (SLAMf1) (Forsberg et al., 2005; Kiel et al., 2005) and ESAM1 (Forsberg et al., 2005, Ooi et al., 2008; Yokota et al., 2008) have been previously used to positively identify the HSC population, as neither CD150- nor ESAM1- cells support long-term reconstitution. Surface staining of KLS progenitors with antibodies to CD150 and ESAM1 identified three distinct populations with cleanly separable Flk2 expression; Flk2 expression analysis in WT mice revealed that $CD150^{+}/ESAM1^{+}$ KLS did not express Flk2 at the cell surface, whereas $CD150^{-}/ESAM1^{+}$ and $CD150^{-}/ESAM1^{-}$ KLS expressed intermediate and high cell surface levels of Flk2, respectively (Figure 11A,B). These phenotypic data, combined with functional analysis upon transplantation (below), led us to designate these populations as HSC ($CD150^{+}/ESAM1^{+}$ KLS), ST-HSC ($CD150^{-}/ESAM1^{+}$ KLS), and multipotent progenitors (MPP; $CD150^{-}/ESAM1^{-}$ KLS).

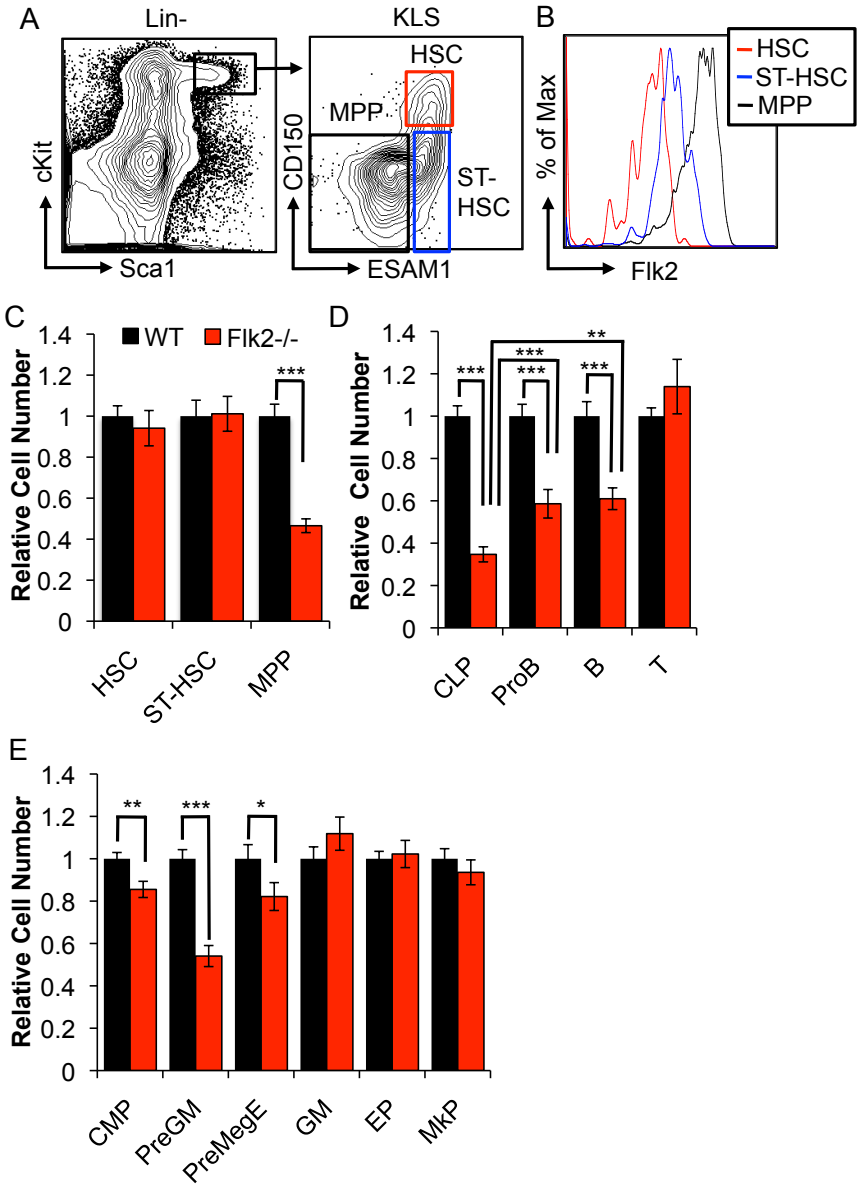


Figure 11. Flk2 deficiency decreases cellularity of multipotent, lymphoid, and myeloid progenitors. A) Flow cytometry gating strategy used to identify Flk2^{-} HSC, Flk2^{lo} ST-HSC, and Flk2^{hi} MPP in Flk2$^{-/-}$ mice. B) Relative distribution of Flk2 expression levels among the three populations gated in A. C) Relative cellularity of multipotent stem and progenitor cells in WT and Flk2$^{-/-}$ mice. D) Relative cellularity of the lymphoid compartment in WT and Flk2$^{-/-}$ mice. E) Relative cellularity of the myeloid compartment in WT and Flk2$^{-/-}$ mice. Data represent mean ± SEM of total cell number normalized to WT average for each cell type. Differences were analyzed by Student's T-test. N = 11/genotype representing 3 independent experiments. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$.

Flk2 deficiency results in decreased cellularity of multipotent, lymphoid, and myeloid progenitors

To determine the effect of Flk2 deficiency on hematopoiesis at steady-state, we examined the cellularity of stem, progenitor, and mature cells in $Flk2^{-/-}$ mice and WT controls, with a focus on early progenitors. Flk2 deficiency did not significantly affect whole bone marrow (WBM) cellularity (data not shown). Examination of the KLS compartment revealed that the relative cellularity of both HSC and ST-HSC were unaffected by Flk2 deficiency (Figure 11C), whereas cellularity of MPP, typically defined by high surface levels of Flk2, were significantly decreased by approximately 60% ($p<0.0001$, Figure 11C). Flk2 deficiency also significantly decreased the cellularity of the lymphoid compartment (Figure 11D), consistent with the well-documented role for Flk2 signaling in lymphoid differentiation [2,4,15]. Common lymphoid progenitors (CLP) (Kondo et a., 1997) exhibited an approximate 70% reduction in $Flk2^{-/-}$ mice ($p<0.0001$), as observed previously (Sitnicka et al., 2002). Cellularity of proB progenitors and mature B cells was also significantly reduced in response to Flk2 deficiency ($p<0.001$, Figure 1D), as reported previously (Buza-Vidas et al., 2009; Mackarehtschian et al., 1995) although the reduction was not as severe as that observed for the CLP (ProB vs. CLP, $p<0.001$; B cell vs CLP, $p<0.01$). Despite the significantly reduced numbers of MPP and CLP, the likely precursors to mature T cells (Schlenner et al., 2010) T cell cellularity in the BM was unaffected in $Flk2^{-/-}$ mice.

Interestingly Flk2 deficiency also affected the generation of myeloid-committed progenitors (Figure 11E). Using two alternative staining strategies for myeloid progenitor subpopulations (Akashi et al., 2000; Pronk et al., 2007) we found that the total cell numbers for phenotypically defined CMP, PreGM and PreMegE progenitors were significantly reduced by 20%, 60% and 20%, respectively, as compared to WT controls ($p<0.01$, $p<0.0001$ and $p<0.05$, respectively; Figure 11E). Cellularity of unipotent and mature myeloid cells was unaffected in $Flk2^{-/-}$ mice. Thus, despite similar numbers of stem and mature myeloid cells, our examination of intermediate differentiation stages at the population level uncovered that

Flk2$^{-/-}$ mice have significantly reduced capacity to generate MPP as well as both myeloid- and lymphoid-committed progenitors at steady-state. These data provide evidence for compensatory mechanisms that regulate production of mature myeloid, T and, to a lesser extent, B cells.

Transplanted Flk2-deficient HSC exhibit impaired reconstitution of all lineages

Previous WBM transplant experiments yielded contradictory conclusions with regards to myeloid output from Flk2$^{-/-}$ cells (Buza-Vidas et al., 2009; Makarehtschian et al., 1995). We hypothesized that transplantation of purified stem and progenitor cells, by allowing kinetic analysis, would provide a more definitive answer on the role of Flk2 in myeloid differentiation and also help to pinpoint the differentiation stage at which Flk2 exerts its effects. To assess the effect of Flk2 deficiency on MegE production, we crossed Flk2$^{-/-}$ mice with the mT/mG reporter mice to label plts, as well as all nucleated cells (Muzumdar et al., 2007). We transplanted highly purified Tomato-expressing WT and Flk2$^{-/-}$ HSC, ST-HSC, and MPP into sublethally irradiated recipients.

Examination of multilineage reconstitution in the peripheral blood (PB) at 4, 8, 12, and 16 weeks after transplantation of highly purified Flk2$^{-/-}$ or WT HSC revealed that donor contribution to platelets and GM cells was significantly different between Flk2$^{-/-}$ and WT cells throughout the 4-16 week analysis period (Figure 12A, B). Reduced contribution to B and T cell lineages in the PB was also evident at 8 weeks and beyond. Lineage distribution analysis of donor-derived cells at the 16-week timepoint showed that Flk2$^{-/-}$ HSC were drastically more defective in their ability to generate B lineages ($p<0.001$) as compared to the GM lineage ($p<0.05$) (Figure 12B-C).

Examination of BM reconstitution 17-18 weeks after transplantation revealed that, similar to steady-state hematopoiesis, chimerism within the HSC and ST-HSC compartments was not significantly different for transplanted Flk2$^{-/-}$ HSC as compared to WT HSC. In contrast, stark differences were observed in chimerism of MPP and all downstream myeloid

and lymphoid lineages (Figure 12D). Collectively, these data imply that cell expansion downstream of Flk2$^{-/-}$ HSC was impaired, thereby resulting in a reduction in the number of progeny in all mature lineages.

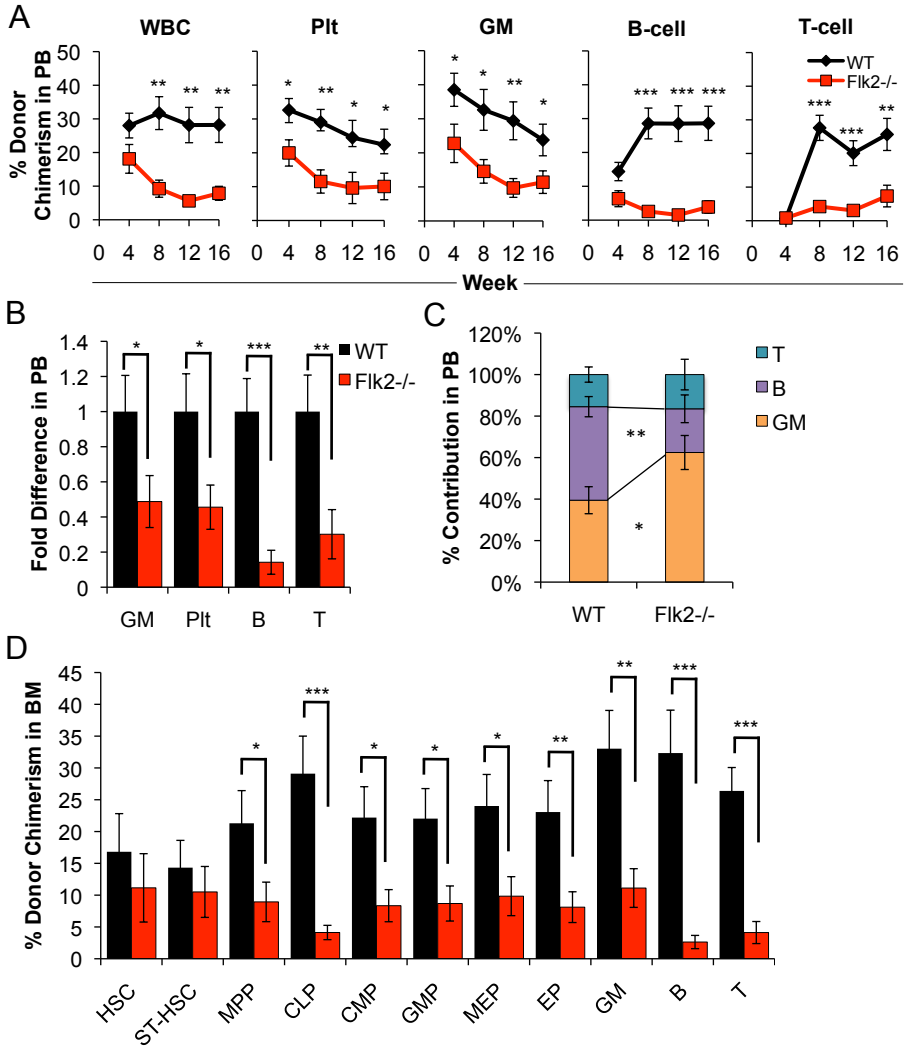


Figure 12. Flk2 deficiency impairs HSC reconstitution capacity and lineage output following transplantation. A) Quantitative and kinetic analysis of donor-derived Tomato+ WBC and Plt production in PB of sublethally irradiated mice transplanted with 100 WT or Flk2$^{-/-}$ HSC. B) Fold difference in GM, B cell, T cell, and Plt PB reconstitution measured 16 weeks post-transplantation for the same mice as in (A). C) Relative contribution of each mature WBC lineage (GM, B, and T) to total PB reconstitution measured 16 weeks post-transplantation for same mice as in (A). D) Total donor chimerism of each stem, progenitor, and mature cell population in the BM 17-18 weeks after transplantation in the same mice as in (A). Data represent mean ± SEM of the frequency of donor-derived cells for each population. N = 16/genotype representing 3 independent experiments. Differences were analyzed by Student's T-test. * $P < 0.05$; **$P < 0.01$. *** $P < 0.001$.

Flk2$^{-/-}$ ST-HSC exhibit significantly impaired reconstitution

As the numbers of HSC were unaffected by Flk2 deficiency both at steady-state and after transplantation, we aimed to identify the stage at which Flk2 deficiency impaired multilineage output. We therefore investigated PB reconstitution from Flk2$^{-/-}$ and WT ST-HSC. Readout from WT ESAM+/CD150- KLS cells closely resembled previous reports of PB readout from previously identified "ST-HSC" populations (Yang et al., 2011), and we therefore chose to refer to them as such. Flk2$^{-/-}$ ST-HSC displayed an immediate impairment in readout of all mature lineages, including platelets (Figure 13A). This robust difference was sustained at four weeks, but disappeared at 8 weeks for GM and Plt lineages, at which point reconstitution from both WT and Flk2$^{-/-}$ ST-HSC was negligible. Differences in B cell repopulation were sustained through 8 weeks, after which B cell readout was exhausted from both WT and Flk2$^{-/-}$ ST-HSC. Similarly, T-cell reconstitution from Flk2$^{-/-}$ ST-HSC was significantly impaired through 12 weeks.

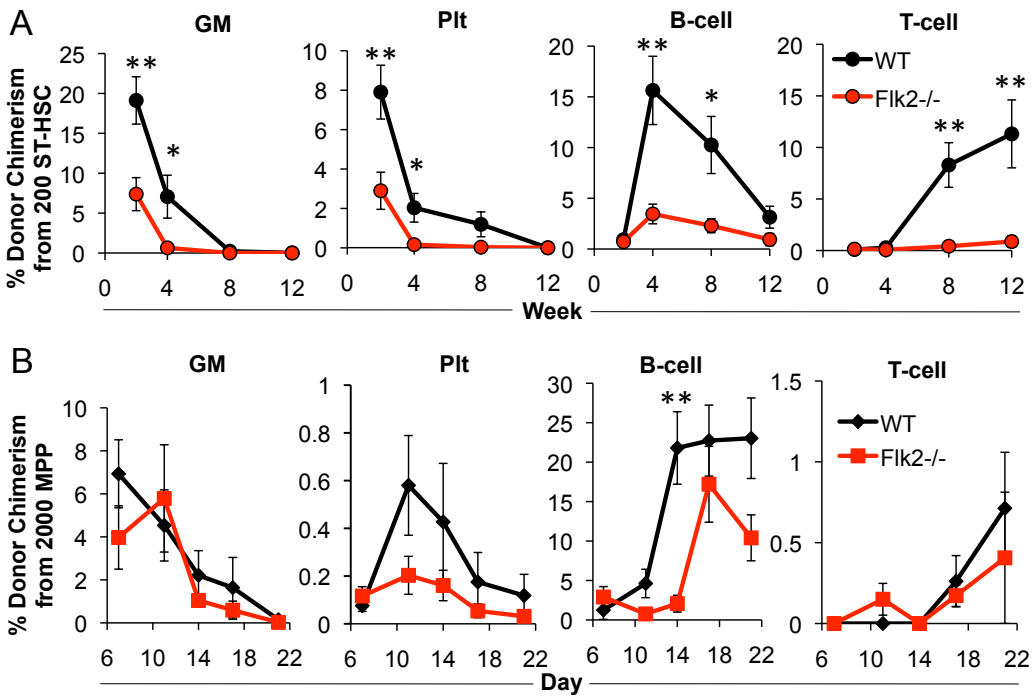


Figure 13. Flk2 deficiency impairs multilineage reconstitution from ST-HSC but not MPP. Multilineage reconstitution kinetics of transplanted WT and Flk2$^{-/-}$ progenitors. A) Quantitative and kinetic analysis of Tomato+ GM, Plt, B, and T cells derived from 200 WT or Flk2$^{-/-}$ ST-HSC transplanted into sublethally irradiated recipients (N = 14 representing 2 independent experiments). B) Quantitative and kinetic analysis of Tomato+ total GM, Plt, B, and T cells derived from 2500 WT or Flk2$^{-/-}$ MPP transplanted into sublethally irradiated recipients. N = 9/genotype representing 2 independent experiments. Note the difference in x-axis scale in panel A (weeks) and B (days). Differences were analyzed by Student's T-test. * P < 0.05. **P < 0.01. *** P < 0.001.

B cell readout, but not total reconstitution, is impaired from transplanted Flk2$^{-/-}$ MPP

Our results above suggested that the effect of Flk2 deficiency on multilineage repopulation from HSC and ST-HSC was due primarily to an inability to generate adequate numbers of Flk2+ MPP, a critical developmental intermediate for all myeloid and lymphoid lineages. To test this hypothesis, we transplanted equivalent numbers (2500) of WT and Flk2$^{-/-}$ MPP into sublethally irradiated hosts and monitored PB reconstitution. Remarkably, readout from Flk2$^{-/-}$ MPP closely resembled that of WT MPP (Figure 13B), with the exception of a transient difference in B-cell readout at two weeks ($p<0.01$). Neither GM nor platelet readout was significantly different between WT and Flk2$^{-/-}$ MPP at any timepoint examined. Very little T cell readout was observed at these early timepoints due to the extended time required for generation of mature T cells; however, examination over four months revealed that Flk2 deficiency did not significantly impair T or B cell readout from MPP at later timepoints (data not shown). These data suggest that although Flk2 is important for generating MPP from HSC, once cells reach the MPP stage only B cell production is significantly affected by Flk2 deficiency.

Flk2 deficiency does not affect CFU-S efficiency of HSC or MPP

To further examine the effect of Flk2 deficiency on generation of MegE progenitors and mature cells, we examined colony-forming-unit of the spleen (CFU-S) efficiency of transplanted Flk2-deficient stem and progenitor cells. We confirmed that the CFU-S assay is a clonal measure of erythroid potential, as cell frequency analysis revealed that the vast majority (>99%) of HSC- and MPP-derived CFU-S cells are erythroid (Figure S3A). Flk2 deficiency had no effect on the frequency of CFU-S derived from either HSC, ST-HSC, or MPP (Figure S3B). However, consistent with previous results (Forsberg et al., 2006) we observed a lower frequency of CFU-S arising from MPP as compared to HSC and ST-HSC, regardless of Flk2 genotype.

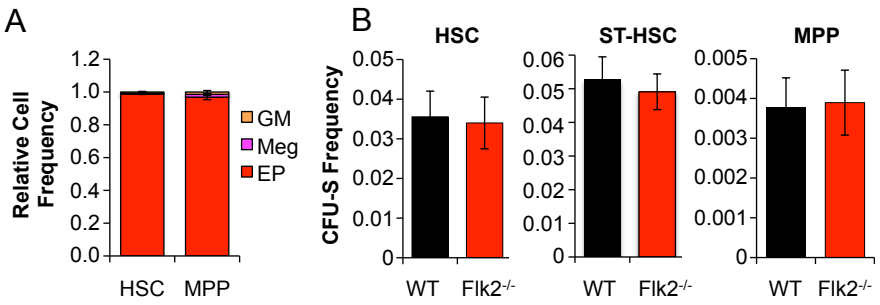


Figure S3. A) Frequency of GM, megakaryocytes (Meg) and erythroid progenitors (EP) in Day 11 CFU-S formed from transplanted HSC or MPP. B) CFU-S frequency was not significantly different between transplanted Flk2$^{-/-}$ and WT HSC (100 cells, N = 9-10 representing 2 independent experiments), ST-HSC (200 cells, n = 22 representing 3 separate experiments) or MPP (500 cells, N = 18-19 representing 3 independent experiments).

Homing to the spleen partially accounts for the differential CFU-S efficiency between HSC and MPP

The difference in CFU-S formation between HSC and MPP could be due to differences in the ability to home to the spleen. We tested this possibility in two different assays. First, we tested spleen homing efficiency by performing short-term localization analysis after injection of HSC and MPP into lethally irradiated hosts. As donor cells, we used KLS cells isolated from the recently characterized FlkSwitch model (Boyer et al., 2011; Boyer et al., 2012); in mice with high floxing efficiency, almost all (>96%) MPP express GFP, whereas all HSC express Tomato. Although HSC (Tomato+ cells) and MPP (GFP+ cells) were recovered at similar frequencies from the BM and blood three hours later, significantly fewer MPP were recovered from the spleen ($p = 0.02$; Figure 14A).

We then compared CFU-S frequency of HSC and MPP injected either IV or directly into the spleen (intra-splenic, IS), with the assumption that direct spleen injection would circumvent inherent differences in the ability to home to the spleen. Direct spleen injection resulted in significantly higher CFU-S frequency for both HSC and MPP (Figure 14B,C). Importantly, CFU-S frequency from MPP injected IS (1/37; Figure 14C) was comparable to the frequency observed for HSC injected IV (1/28; Figure 14B), indicating that MPP do indeed possess significant MegE potential in the CFU-S assay. Despite the increase observed for MPP upon IS injection, however, a substantial difference in CFU-S frequency was still observed between HSC and MPP (Figure 14D,E). Together, these two assays clearly show that although homing ability significantly impacts CFU-S frequency, other mechanisms contribute to the differences in CFU-S readout between HSC and MPP.

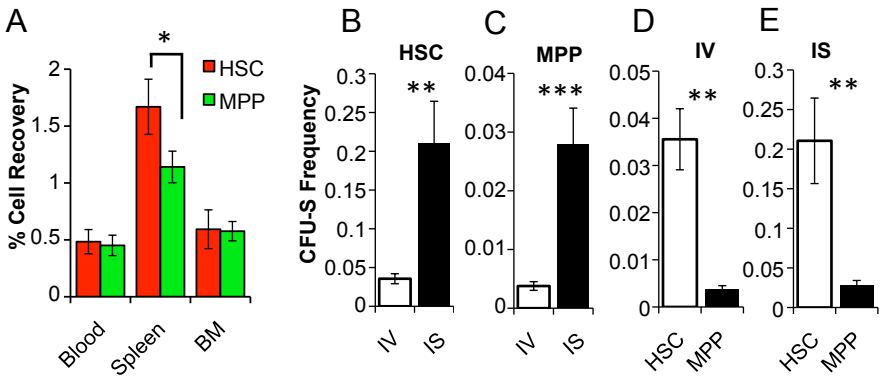


Figure 14. Flk2 expression does not account for differences in CFU-S efficiency between HSC and MPP. A) Percent cell recovery in blood, spleen, and BM three hours after transplantation of HSC (Tom+) and MPP (GFP+) into lethally-irradiated recipients (N = 13 representing 3 independent experiments) B) Frequency of CFU-S observed on day 12 following injection of WT HSC IV (100 cells) or intra-splenic (IS, 10 cells) into lethally irradiated recipients. C) Frequency of CFU-S observed on day 11 following injection of WT MPP IV (500 cells) or IS (50 cells) into lethally irradiated recipients. D) Comparison of colony frequency of WT HSC and MPP following IV injection. E) Comparison of CFU-S frequency of WT HSC and MPP following IS injection. N – 12-14/condition representing three independent experiments. Differences were analyzed by Student's T-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Flk2 deficiency improves survival in response to 5-FU treatment

Our finding that Flk2 deficiency affects myeloid cell generation both at steady-state and after HSC transplantation prompted us to examine whether Flk2 deficiency influences the ability of HSC to rapidly generate myeloid cells under conditions of hematopoietic stress. WT and $Flk2^{-/-}$ mice were treated with 110 mg/kg of the myeloablative agent 5-fluorouracil (5-FU) weekly for four weeks; a dose that allows for survival of approximately 50% of WT mice. Survival was examined over the course of treatment and for four weeks thereafter. Surprisingly, Flk2 deficiency did not impair survival of mice in response to 5-FU (Figure 15A). In contrast, binary analysis at the last timepoint using the Fisher's Exact Test revealed enhanced survival of $Flk2^{-/-}$ mice compared to WT mice ($p= 0.05$). Thus, not only did Flk2 deficiency fail to sensitize mice to 5-FU, but lack of Flk2 expression afforded protection following the termination of treatment.

Flk2 deficiency impairs proliferation of stem and progenitor cells

As levels of proliferation influence the sensitivity to chemotherapeutic agents such as 5-FU (Yang et al., 2011; Winkler et al., 2012), the finding that $Flk2^{-/-}$ mice were less sensitive to 5-FU prompted us to examine the cell cycle status of $Flk2^{-/-}$ progenitors. We reasoned that $Flk2^{-/-}$ mice may be less sensitive to 5-FU, which targets proliferating cells, if they have fewer stem and progenitor cells in cycle. We therefore investigated cell cycle status by quantifying DNA content of sorted HSC, ST-HSC, MPP, and myeloid progenitors. Flk2 deficiency significantly reduced the proportion of HSC, ST-HSC, and MPP in S-G2-M phase compared to WT cell populations (Figure 15B-C), but did not affect the cell cycle status of myeloid progenitors. Cycling of MPP was most affected by Flk2 deficiency (40% reduced) as compared to HSC and ST-HSC (21% and 14% reduced, respectively), consistent with the highest level of Flk2 expression in WT MPP. These data suggest that $Flk2^{-/-}$ mice may exhibit higher survival because more quiescent KLS cells remain to support hematopoiesis after 5-FU treatment.

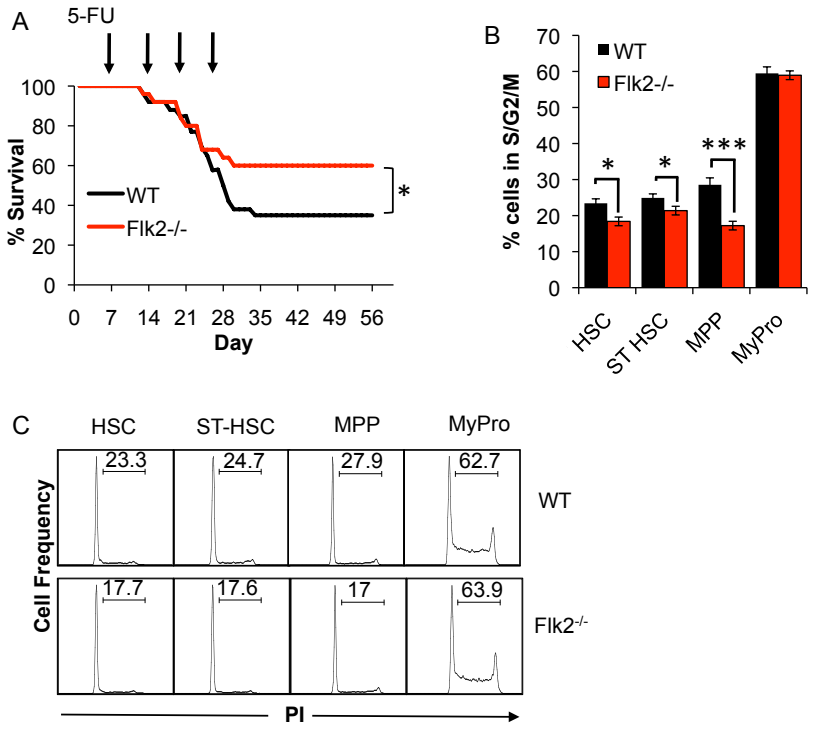


Figure 15. Flk2 deficiency enhances survival in response to 5-FU but impairs proliferation. A) Survival of Flk2$^{-/-}$ and WT mice in response to treatment with 110 mg/kg 5-fluorouracil (5-FU). Arrows indicate time of dosing. More Flk2$^{-/-}$ mice survived for 6 weeks as compared to WT (Fisher exact test, p = 0.05). N = 32-33/genotype representing 4 independent experiments. B) Quantification of the percentage of cells in S/G2/M phase for each progenitor subtype. N = 5-7 mice/genotype representing 3 independent experiments. C) Representative FACS plots of DNA content for WT and Flk2$^{-/-}$ HSC, ST-HSC, MPP, and myeloid progenitors (MyPro). Brackets demarcate the percentage of cells in S/G2/M phase. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

DISCUSSION

Flk2 deficiency affects both myeloid and lymphoid lineages

Although the role of Flk2 signaling in hematopoietic specification has received considerable attention, conflicting findings in both the Flk2-/- and the Fl-/-(Flt3L) mouse models have left several key aspects unresolved. In the present study, thorough investigation of hematopoiesis in Flk2-deficient mice with a focus on myelopoiesis has clarified the role of Flk2 in multilineage differentiation downstream of HSC. Separation of the stem and progenitor cells using a novel surface staining strategy (Figures 11A-B) enabled us to assess the effects of Flk2 deficiency on Flk2- HSC and Flk2+ MPP without the confounding use of Flk2 as a surface marker, as well as myeloid and lymphoid progenitors and mature cells. Both steady-state and transplantation experiments revealed impairment across all myeloid lineages (Figures 11-13); as Flk2 is not expressed in myeloid progenitors or mature cells, the observed impairment in myeloid development can be attributed to attenuated MPP expansion in $Flk2^{-/-}$ mice. Impaired expansion of the MPP stage is consistent with the observed reduction in proliferation of stem and progenitor cells in $Flk2^{-/-}$ mice (Figure 15), as well as the well-documented role for Flk2 in cell proliferation during normal hematopoiesis and in leukemia's (Stubbs et al., 2007; Brasel et al., 1996; Karsunky et al., 2007). These results indicate that Flk2 promotes generation of all hematopoietic lineages, and that its role is particularly important during acute needs for cell replacement, such as transplantation into irradiated hosts.

Compensatory mechanisms mitigate the effects of Flk2 deficiency

Several previous reports that investigated the role of Flk2 signaling in hematopoiesis in both the $Flk2^{-/-}$ and the Fl-/- mouse models (Buza-Vidas et al., 2009; Mackarehtschian et al., 1995; Sitnicka et al., 2007; McKenna et al., 2000) were consistent with each other and with our results in reporting impaired B cell lineage readout. However, both steady-state

analyses and WBM transplantation in previous reports resulted in contradictory results with regards to myeloid and T cell readout (Buza-Vidas et al., 2009; Mackarehtschian et al., 1995; Boiers et al., 2010; McKenna et al., 2000). Our comprehensive and quantitative analyses of stem, progenitor, and mature cells revealed that Flk2 deficiency does affect generation of both myeloid and lymphoid populations, including MegE progenitor cells. Interestingly, the significant decrease in early myeloid-restricted progenitors did not translate into a reduction in the cellularity of unipotent and mature myeloid cells at steady-state (Figure 11E). These data indicate that mechanisms exist downstream of early progenitor specification to normalize production of mature cells necessary for survival, such as red cells and platelets. Similarly, we also observed downstream compensation during lymphoid specification, as cellularity of ProB, B and T cells was increased relative to their common precursor, the CLP (Figure 11D). Whereas T cells reached normal levels, the B cell recovery was only partial. The more drastic effects of Flk2 deficiency on B cell generation is consistent with the sustained expression and function of Flk2 during B cell development (Karsunky et al., 2008; Dolence et al., 2011).

In contrast to the downstream compensation observed at steady-state, transplantation of purified Flk2$^{-/-}$ HSC led to decreased reconstitution of all investigated cell types from MPP and beyond, including erythroid progenitors and platelets (Figure 12). More severe deficiency upon transplantation as compared to steady-state are common, and was also observed in the original report of hematopoiesis in Flk2$^{-/-}$ mice (Mackarehtschian et al., 1995). Acute need may be harder to adapt to, whereas germline loss leads to more permanent and gradual adjustments in order to maintain homeostasis. Nevertheless, our results demonstrate that compensatory mechanisms can be revealed by more comprehensive analysis of intermediate progenitors and by assessing cell output under increasingly challenging conditions. Although the compensatory mechanisms are not fully understood, previous investigation indicates that the c-kit receptor is important for offsetting the impact of Flk2 loss (Mackarehtschian et al., 1995).

Flk2 is critical for progenitor expansion, but does not instruct lineage choice

In addition to revealing downstream compensation, analysis and transplantation of purified stem and progenitor cells allowed us to pinpoint the cellular stage at which Flk2 deficiency is manifested. Not surprisingly, significant decreases in cell numbers were first observed at the MPP stage (Figures 11C, 12), coinciding with establishment of robust Flk2 cell surface levels (Figure 11B). Whereas comparison of reconstitution from equivalent numbers of $Flk2^{-/-}$ and WT HSC and ST-HSC revealed significant impairment in PB reconstitution of all matures lineages, including platelets (Figures 12 and 13A), transplantation of equivalent number of $Flk2^{-/-}$ and WT MPP revealed only subtle reconstitution defects that were restricted to the B lineage (Figure 13B). The abrogation of impairment in B-cell readout and the absence of an effect of Flk2 deficiency on myeloid readout when MPP numbers are normalized upon transplantation suggests that impaired reconstitution from Flk2-deficient HSC and ST-HSC is due primariliy to an inability to expand the MPP stage.

The continued dependence on Flk2 signaling during lymphoid development, in particular in the absence of readily detectable effects on mature myeloid cells, could easily lead to the conclusion that Flk2 signaling regulates the decision between lymphoid and myeloid fates. If Flk2 promotes lymphoid development at the expense of myeloid cells, Flk2 deficiency would be expected to decrease lymphoid generation with a corresponding increase in the generation of myeloid cells. Although a subtle increase in mature myeloid cellularity at steady state has been reported previously in the Fl-/- mouse (Boiers), Fl-/- WBM cells are not impaired in their ability to generate GMs upon transplantation (Sitnicka 2002; Boiers 2010). Our observation of reduced MPP, myeloid and lymphoid restricted progenitors in Flk2-/- mice clarifies that Flk2 promotes expansion of Flk2-expressing cells, including MPP, that then contribute to all downstream cell types, including MegE cells. Together, these data indicate that Flk2 is critical for expanding the pool of available MPP, which in turn must give

rise to sufficient numbers of both myeloid and lymphoid lineages, but does not regulate lineage choice.

Trafficking to the spleen and CFU-S frequency are Flk2-independent

Despite robust MegE generation from MPP upon transplantation *in vivo,* MPP have reduced CFU-S readout as compared to Flk2- cells including HSC, ST-HSC, and myeloid progenitors (Forsberg et al., 2006; Yang et al., 2005). We reported that Flk2 expression itself does not influence CFU-S frequency from Flk2+ cells, consistent with our conclusion that Flk2 does not direct lineage specification downstream of HSC (Figure S3). Both the short-term homing assay (Figure 14C) and the IV vs IS CFU-S assays (Figures 14D-G) indicated that homing efficiency plays an essential role in CFU-S readout. However, despite significant differences in the ability of HSC and MPP to home to the spleen, homing alone did not account for differences in CFU-S frequency, since direct spleen injections still resulted in decreased CFU-S frequency from MPP relative to HSC. Whether factors such as survival, burst size, and ability to stay within the spleen differentially affect CFU-S generation from MPP as compared to HSC remains unclear. Interestingly, even though all HSC possess erythroid potential, IS injection of HSC resulted in CFU-S frequency of approximately 1 of 5 cells. These data indicate that the CFU-S assay underestimates erythroid potential by at least 5-fold when performed IS and more than 30-fold upon IV injection, and also provide direct evidence for robust erythroid potential of MPP in vivo.

Flk2 impacts cell proliferation and animal survival in response to chemotherapy

Despite reduced numbers of MPP and myeloid progenitors, $Flk2^{-/-}$ mice were less sensitive than WT mice to the myeloablative agent 5-FU (Figure 15A); specifically, more Flk2-/- mice survived following the termination of treatment as compared to WT mice. How can mice with demonstrated impairments in the propagation of myeloid progenitors be less sensitive to myeloablation? Examination of cell cycle status showed that fewer HSC, ST-

HSC, and MPP were in cycle in Flk2$^{-/-}$ mice as compared to WT mice (Figures 15B-C), with the greatest differences observed in the MPP compartment. Reduced proliferation of HSC despite a lack of Flk2 expression is mostly explained by the presence of additional, indirect cues; for example, substantial expansion of HSC has been observed in mice and humans in response to G-CSF, despite HSC lacking expression of the G-CSF receptor (for review see Greenbaum et al., 2010). 5-FU mainly targets proliferating cells, this relative quiescence may have spared a greater fraction of KLS cells in Flk2$^{-/-}$ mice, with the higher number of remaining cells conferring enhanced survival. Although we did not directly examine whether altered proliferation rates contributed to reduced sensitivity to 5-FU in Flk2-/- mice, reduced 5-FU sensitivity has also been observed in other models with reduced HSC proliferation [33]. As cells with activating Flk2 mutations may be more sensitive to 5-FU, and activating Flk2 mutations are present in 30-35% of AML cases (Langdon et al,, 2012), these findings bear significant implications for therapeutic approaches to treatment of Flk2-driven leukemias.

Flk2+ progenitors contribute to all mature blood lineages

Our new data converge with previous reports on a model where Flk2 is dispensable for HSC maintenance, including self-renewal, consistent with our recent functional validation that HSC do not express detectable surface levels of Flk2 (Boyer et al., 2013). Gradually increased Flk2 expression as HSC differentiate into ST-HSC and then MPP coincides with increased dependence on Flk2 to promote cell proliferation. Thus, Flk2 acts to promote expansion of progenitor cells that gives rise to both myeloid and lymphoid lineages, rather than mediating fate decisions between lineages. Once a sufficient number of MPP have been established, Flk2 expression is no longer needed to generate myeloid or T cells. However, optimal B cell generation is dependent on sustained Flk2-mediated proliferation, and B cell numbers are therefore particularly affected by Flk2 loss. These conclusions are consistent with recent reports using a Flk2-Cre lineage-tracing approach, demonstrating that all lymphoid and myeloid lineages, including MegE cells, derive from Flk2+ progenitors *in vivo*

(Boyer et al., 2011; Boyer et al., 2012; Buza-Vidas et al., 2011). Together, these data underscore the importance of understanding differentiation pathways and examining effects on intermediate precursors, as they reveal novel insights into mechanisms regulating HSC function, hematopoietic specification, and how constitutively active mutations in Flk2 contribute to the development of leukemia in non-Flk2-expressing myeloid cells.

METHODS

Mice. Mice were housed in the UCSC vivarium and all animal experiments were approved by the UCSC IACUC. Flk2$^{-/-}$, mT/mG, and FlkSwitch mice (Flk2-Cre crossed to mT/mG mice), all on C57BL/6 background, were described previously (Boyer et al., 2011; Buza-Vidas et al., 2009; Pronk et al., 2007). Flk2$^{-/-}$ mice were maintained as a homozygous breeding colony unless crossed to mT/mG reporter mice for experiments.

Cell isolation and analysis. BM and PB cells were isolated and processed as previously described (Boiers et al., 2010; Kondo et al., 1997) using a four-laser FACSAria or LSRII (BD Biosciences, San Jose, CA). Analysis and display of FACS data was accomplished using FlowJo analysis software (Ashland, OR). Cell populations were defined using phenotypes defined in Supplemental Methods.

Transplantation assays. HSC, ST-HSC, and MPP transplants were performed by retroorbital injection of 100, 200, or 2500 cells, respectively, double-sorted from Flk2$^{-/-}$ or wt mice expressing the mT/mG allele into sub-lethally irradiated recipients (750 rads). BM and PB chimerism of recipients was analyzed by flow cytometry for Tom fluorescence post-transplantation. For CFU-S analysis, MPP, ST-HSC, and HSC (500, 200, or 100 cells per mouse) were double-sorted from the same mice and injected retro-orbitally into lethally irradiated mice (1036 rads). For direct spleen injections, mice were anesthetized with Avertin and spleens were exposed through the peritoneal cavity. Ten HSC or 50 MPP were injected directly into the spleen in a 50 μL volume. Spleens were dissected and colonies were enumerated 11 (for MPP) or 12 (for ST-HSC and HSC) days later.

Short-term homing. 100,000 single-sorted KLS cells from FlkSwitch mice (Boyer et al., 2011) were injected retro-orbitally into mice 24 hours after administration of a lethal dose of

irradiation (1037 rads, split dose). Cell localization was analyzed 3 hours post-injection as described previously (Kondo et al., 1997).

5-fluorouracil treatment. 8-10 week-old WT and Flk2$^{-/-}$ mice received weekly injections of 110 mg/kg 5-fluorouracil (Sigma) dissolved in 5-methylbutane and distilled water. Injection volume was determined individually for each mouse based on weight taken weekly. Mice received treatment weekly for four weeks and survival was monitored daily for six weeks.

Cell cycle analysis. BM cells were isolated from Flk2$^{-/-}$ and WT mice, stained with antibodies and fixed with 1% PFA. HSC, ST-HSC, MPP, and myeloid progenitors were isolated by FACS and incubated with 40μg/mL propidium iodide (PI) with 100μg/mL RNase A in PBS supplemented with 0.03% Saponin for 30 minutes at 37°C. Following incubation, sorted cell populations were analyzed for DNA content by FACS.

Supplemental Methods:

Cell populations defined as follows: HSC (Lin- Sca1+ c-kit+ SLAMf1+ ESAM1+ CD48-), ST-HSC (Lin- Sca1+ c-kit+ SLAMf1- ESAM1+), MPP (Lin- Sca1+ c-kit+ SLAMf1- ESAM1- CD48+), CMP (Lin- Sca1- c-kit+ FcgR^{mid} CD34^{mid}), GMP (Lin- Sca1– c-kit+ FcgR^{hi} CD34^{hi}), MEP (Lin- Sca1- c-kit+ FcgR^{lo} CD34^{lo}), CLP (Lin- Sca1^{mid} c-kit^{mid} IL7Ra+), GM (Ter119- Mac1+ Gr1+ CD3- B220-), EP (Mac1- Gr1- CD3- B220- Ter119+ CD71+), Plts (FSC^{lo} Ter119- CD61+), B cell (Ter119- Mac1- Gr1- CD3- B220+), and T cell (Ter119- Mac1- Gr1- CD3+ B220-). The lineage cocktail consisted of CD3, CD4, CD5, CD8, B220, Gr1, Mac1, and Ter119.

CHAPTER 5

Multilineage reconstitution from single hematopoietic progenitors *in vivo*

SUMMARY

Single-cell assays are a powerful tool for assessing the lineage potential of stem and progenitor cells. Single hematopoietic stem cell (HSC) transplantation has demonstrated that at least a fraction of HSC have the ability to reconstitute all hematopoietic lineages for the long term. However, it has yet to be definitively established where the first lineage commitment event occurs during hematopoietic differentiation and which lineage potentials are lost first. Quantitative analysis of mature cell production from hematopoietic stem and progenitors has revealed that both HSC and multipotent progenitor (MPP) generate more red blood cells and platelets than white blood cells upon transplantation. These results provide a new perspective of the lineage potential of MPP and clearly demonstrate that MPP can reconstitute all hematopoietic lineages at the population level. However, it has yet to be determined if MPP are multipotent at the single cell level, or consist of a heterogeneous mixture of lineage-committed cells. To test this, we assessed the multilineage reconstitution from single MPP *in vivo* using CFU-S assays. Single transplanted MPP were able to reconstitute erythroid, megakaryocytic, myeloid, and lymphoid lineages. These results demonstrate that MPP are essential contributors to all hematopoietic lineages and suggest that a significant fraction of MPP have multilineage potential at the single-cell level.

INTRODUCTION

Understanding when and how stem cells commit to specific cell fates is important for directing specific cell fates to treat disease. The hematopoietic system serves as a powerful tool to study stem cell fate decisions, largely due to the ability to transplant phenotypically distinct cell types from the bone marrow and determine their cell potential by examining their progeny in the blood. A developmental hierarchy for the various hematopoietic stem and progenitor cells can be deduced from transplantation assays, as a progressive loss of lineage potential would place cells upstream or downstream of each other. The timing and magnitude of mature cell production is also indicative of hierarchal position and developmental maturity (Forsberg et al 2006). As a testament to the robustness of transplantation assays, a single hematopoietic stem cell (HSC) transplanted into a conditioned mouse is able to reconstitute all hematopoietic lineages for that mouse's lifetime, supporting both the multipotent and self-renewal ability of HSC (Osawa et al., 1996; Wagers et al., 2002; Dykstra et al., 2007; Morita et al., 2010). However, it has yet to be definitively established where the first lineage commitment event occurs during hematopoietic differentiation and which lineage potentials are lost first.

Transplantation data in combination with *in situ* lineage-tracing support HSC differentiating through a non-self-renewing multipotent progenitor (MPP) before generating all hematopoietic cell types (Figure 16A) (Forsberg et al., 2006; Boyer et al., 2011, 2012). Although it has been shown that MPP can generate all mature hematopoietic cell types, the lineage bias within this population is controversial (Adolfsson et al., 2005, Forsberg et al., 2006; Lai et al., 2005; Sitnicka et al., 2007; Luc et al., 2008a; Buza-Vidas et al., 2009). Moreover, the level of functional heterogeneity within the MPP is unknown, as single cell *in vivo* assays have not yet been performed to test multipotency, and no single *in vitro* conditions is able to simultaneously assess the full spectrum of both myeloid and lymphoid potential from hematopoietic cells.

Conventionally, lineage assessment from transplanted cells is limited to Granulocyte/Macrophage (GM), B-cell, and T-cell lineages, as these nucleated cells readily express a transgene or congenic marker to distinguish host- from donor-derived cells. Contribution of transplanted cells to red blood cells (RBC) and platelets (Plt) is more difficult to assess, and is thus typically not assayed *in vivo.* Previously, platelet-forming potential from hematopoietic stem and progenitors cells was directly measured, coupled with a qualitative assessment of red blood cell potential using CFU-S and radioprotection assays (Forsberg et al., 2006). Comprehensive analysis of mature RBC production from transplanted hematopoietic stem and progenitors has not been directly assessed, nor has RBC potential been assessed simultaneously with other hematopoietic cell potentials. Simultaneous assessment of multiple hematopoietic lineages being derived from purified hematopoietic stem and progenitor cells in a quantitative fashion will help delineate when commitment occurs in hematopoietic development. Here we address issues of lineage bias by quantifying mature cell production from transplanted hematopoietic subtypes, and assess functional heterogeneity using single cell *in vivo* assays.

RESULTS

Quantifying mature cell production to assess lineage bias

To accurately assess the lineage potential of transplanted hematopoietic stem and progenitor cells, mature cell output was measured as donor-derived cells per microliter of blood, taking into account the different ratios of mature cells (Figure 16B and C) as well as the distribution of these mature cells that exist within the body (Figure 16D). The quantities of mature cells found at steady-state in Figure 16 are consistent with previous murine blood cell analyses (Seebach et al., 1995; Wilkinson et al., 2001; Nemzek et al., 2001; Kakumitsu et al., 2005). Donor cells were isolated from UBC-GFP mice (Schaefer et al., 2001), in which GFP expression is driven from ubiquitin C promoter elements, to allow for the simultaneous detection of donor-derived RBC, Plt, GM, B-cells, and T-cells (Figure 16E). HSC were transplanted to serve as an upper limit control for mature cell production, as the self-renewal ability and multipotent potential of HSC is well established. Progenitors restricted to megakaryocyte/erythrocyte (MEP), granulocyte/myelomonocyte (GMP), and lymphocyte lineages (CLP) were transplanted to serve as lower-limit controls for either RBC and Plt, GM, or B-cells and T-cell readout (Figure 16A).

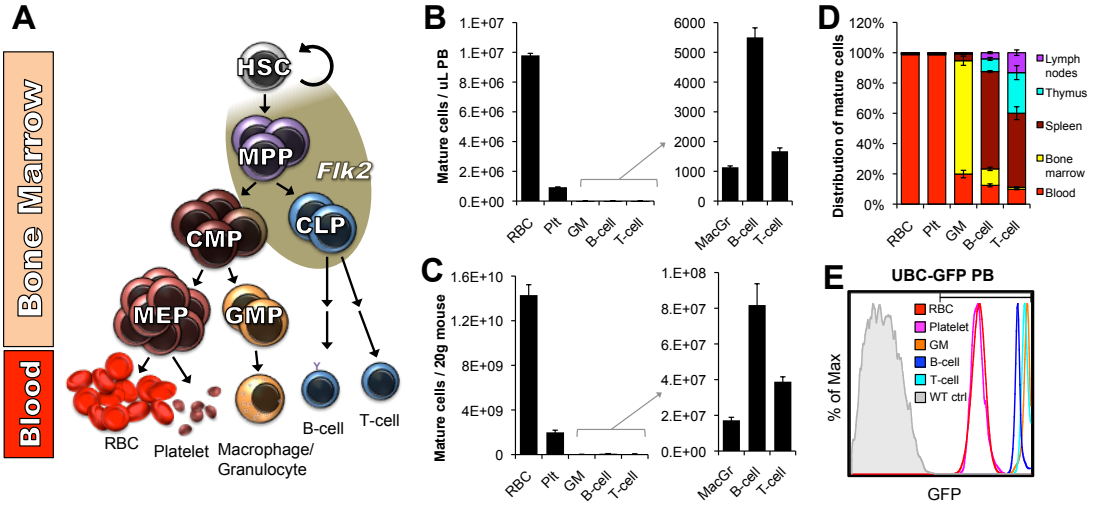


Figure 16. (A) Schematic of hematopoietic development. (B) Number of mature hematopoietic cells in a microliter of peripheral blood (PB) at steady-state, n=10. (C-D) The total (C) and distribution of (D) mature hematopoietic cells in a 20g mouse as measured in blood (perfused), bone marrow (2X femur+tibia), spleen, thymus, and lymph nodes (inguinal+axillary+superficial cervical), n=7 . (E) GFP levels in UBC-GFP PB cells compared to wt control. HSC = Hematopoietic Stem Cell; MPP = Multipotent Progenitor; CMP = Common Myeloid Progenitor; CLP = Common Lymphoid Progenitor; MEP = Megakaryocyte/Erythocyte Progenitor; GMP = Granulocyte/Myelomonocyte Progenitor.

As expected, transplanted HSC gave rise to all mature hematopoietic cells investigated and was stable throughout the 110 day timecourse, in accordance with the long-term multilineage reconstituting ability of HSC (Figure 17A). In addition, we found that HSC produced mature cells in the peripheral blood (PB) in the same order of abundance after transplantation as found during steady-state hematopoiesis (i.e. RBC > Plt > B-cell > T-cell > GM) (Figure 17B-C, 16B). Interestingly, transplantation of the non-self-renewing progenitor downstream of the HSC, the multipotent progenitor (MPP), gave rise to high numbers of RBC and Plt, with lower numbers of white blood cells being produced (Figure 17E), even when the data were normalized to the distribution of mature cells in all hematopoietic tissues (Figure 17F). Despite a low Plt chimerism (Figure 17D), Plt were the second most abundantly produced cell type by MPP (Figure 17E-F). MPP-derived Plt and GM disappeared by day 34 and 42, in accordance with the inability of MPP to self-renew upon transplantation (Christensen 2001, Adolfsson 2001), and is also consistent with the short half-life of

peripheral blood Plt and GM (van Furth and Cohn 1968; Lord et al., 1991; Baker et al., 1997; Basu et al., 2002). In addition to being the most highly produced mature cell by MPP, RBC were also the longest-lived myeloid cell, as MPP-derived RBC remained detectable until day 110. The longevity of measureable MPP-derived RBC is consistent with the previously reported murine RBC half-life of ~40 days (Van Putten 1958). The number of MPP-derived B-cells and T-cells in the PB remained stable after their initial peak (Figure 17E-F), despite a decrease in percent donor chimerism over time (Figure 17D). This decrease in donor chimerism is likely due to the increase in host cells after recovering from irradiation (Figure S4). The alternative enumeration of donor-derived cells per microliter of blood is not affected by host cell presence, and is only dependent on the quantity of donor-derived cells (Figure 12, 2^{nd} and 3^{rd} columns). The prolonged presence of MPP-derived lymphoid cells is likely due to the long half-life and/or proliferation of mature lymphoid cells (Hao and Rajewsky, 2001; Bourgeois et al., 2008) and not due to self-renewal of MPP after transplantation, as similar readout was detected from the lymphoid-restricted CLP (Figure 17 S-U). In summary, MPP give rise to mature cells in the same order of abundance as HSC (Figure 17B and E) and as found during steady-state hematopoiesis (Figure 16A).

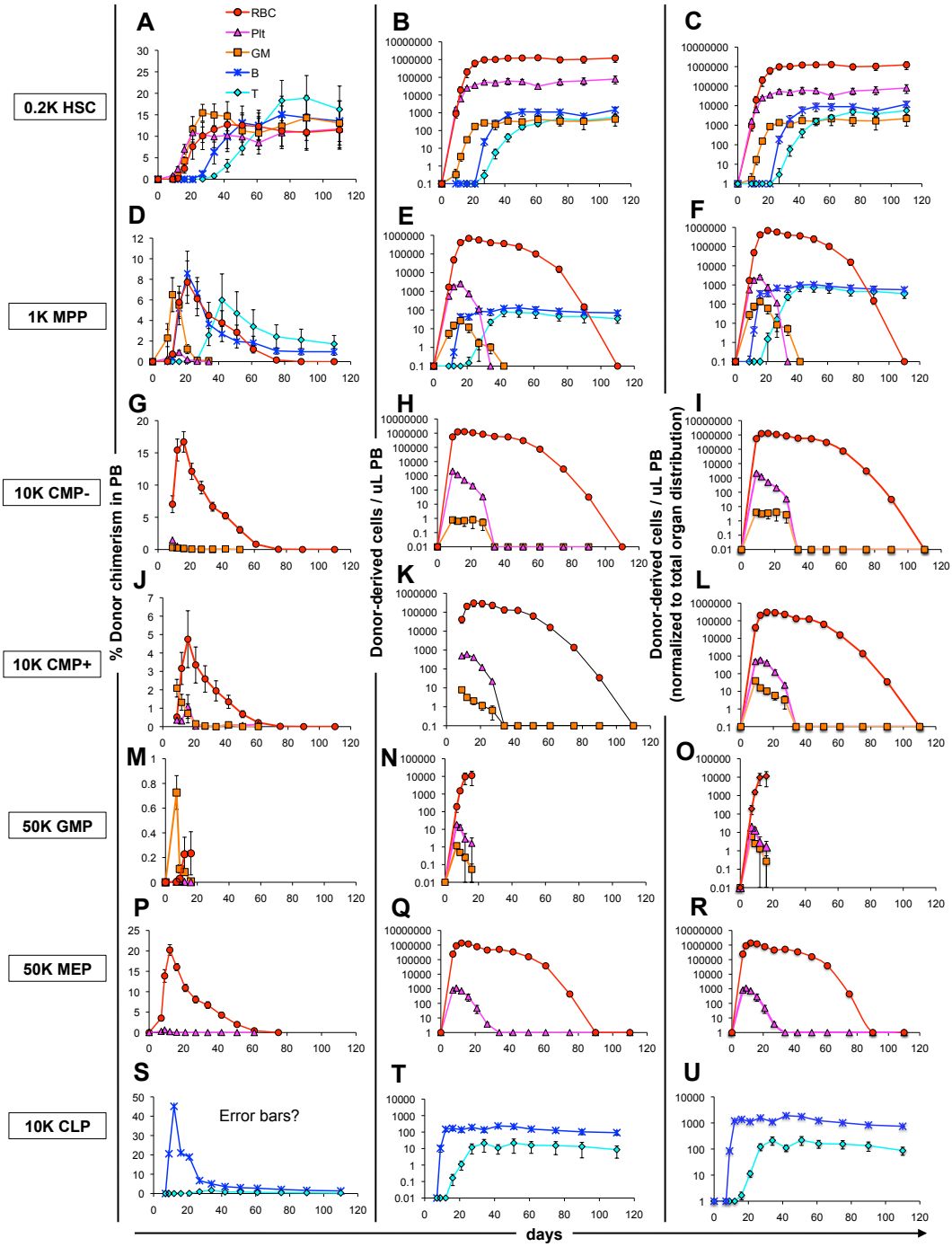


Figure 17. Mature cell production from transplanted hematopoietic stem and progenitors. Sublethally irradiated mice were transplanted with either 200 HSC (n=7), 1,000 MPP (n=8), CMP- (n=8), CMP+ (n=7), 50,000 MEP (n=8), 50,000 GMP (n=4), or 10,000 CLP (n=8) double sorted from UBC-GFP mice. Donor-derived mature cells were assessed at days 9 to 110 days post-transplantation, with the addition of a 7 day timepoint for MEP, GMP and CLP. RBC (red), Plt (pink), GM (orange), B-cell (blue), T-cell (teal).

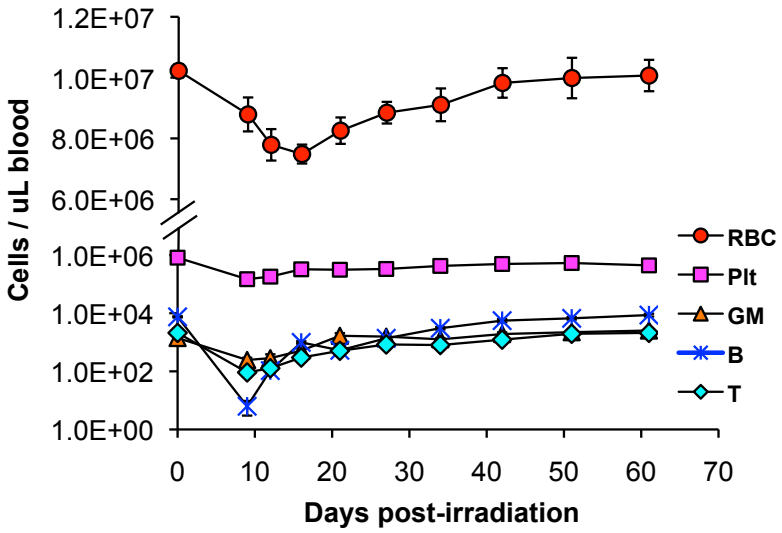


Figure S4. Kinetics of mature blood cells after a subethal dose of irradiation over time. Mice were given a sublethal dose of irradiation and mature cells per microliter of blood was measured over time. n=7.

To clarify lineage potentials downstream of the MPP, we transplanted two subpopulations of the common myeloid progenitor (CMP), the Flk2- and Flk2+ CMP, whose full *in vivo* lineage potentials are unclear (D'Amico and Wu 2003; Nutt et al., 2005; Yang et al., 2007). Although unfractionated CMP are capable of giving rise to RBC, Plt, and GM at the single-cell level *in vitro* (Akashi et al., 2000), the function and hierarchical position of Flk2- CMP (CMP-) and Flk2+ CMP (CMP+) in hematopoiesis has not been established. Upon transplantation, both CMP- and CMP+ produced large numbers of RBC with fewer Plt, and even fewer GM, being produced (Figure 17 G-L). Neither B-cell nor T-cell readout was detected in the blood from either CMP- or CMP+, despite previous evidence for retention of B-cell potential in CMP+ as determined by spleen analysis post-transplantation (D'Amico and Wu 2003; Yang et al., 2007).

As expected, transplantation of high numbers of MEP only gave rise to detectable RBC and Plt (Figure 17P-R). Surprisingly, transplantation of high number of GMP not only

gave rise to GM, as expected, but also produced RBC and Plt upon transplantation (Figure 17M-R). While the total contribution of GMP-derived erythroid and megakaryocytic cells to the host's total cells was minor compared to CMP and MEP (Figure 17G, J, and M), enumeration of GMP-derived mature cells revealed more RBC and Plt were produced from GMP per microliter of blood compared to GM (Figure 17N-O). In summary, CMP-, CMP+, MEP, and GMP have different yet overlapping lineage potentials.

Comparing cell production between hematopoietic stem and progenitors to establish hierarchical positioning

To determine hierarchical positioning from the data presented in Figure 17, the magnitude and timing of peak mature cell production was compared between the transplanted hematopoietic subtypes (Figure 18). More developmentally advanced progenitors will not produce as many mature cells as their upstream counterparts, but will give rise to them earlier, as they require less developmental steps to reach full maturity (Forsberg et al., 2006). Per transplanted cell, MPP gave rise to more RBC, Plt, GM, B-cells, and T-cells than any other progenitor (Figure 18 A-E). The peak timing of mature cell production by MPP was in between the HSC and downstream progenitors (Figure 18 A-E), consistent with previous reports (Forsberg et al., 2006). These transplantation data combined with previous transplantation data and lineage tracing data support MPP residing immediately downstream of the HSC, but upstream of CMP, CLP, and other committed progenitors (Forsberg et al., 2006; Boyer et al., 2011, 2012).

Despite similar Plt production between the two CMP subpopulations, CMP- gave rise to more RBC than CMP+ (Figure 18A-B). Conversely, GM output from CMP- was lower compared to CMP+ (Figure 18C), consistent with previous data (D'Amico and Wu 2003; Nutt et al., 2005), although *in vivo* RBC and Plt readout was not determined in these works. Although there were differences in the magnitude of RBC and GM production between CMP- and CMP+, the timing of mature myeloid cell production was comparable (Figure 18 A-E).

Important to note is CMP- and CMP+ contribution to RBC, Plt, and GM was lower per cell than both HSC and MPP (Figure 18A-C).

Our previous characterization of Flk2 lineage tracing mice, which express Cre under the control of Flk2 regulatory elements (Benz et al., 2008) and also contain a dual-color fluorescent reporter (Muzumdar et al., 2007), has revealed that RBC, Plt, and GM develop through the highly Flk2+ MPP (Boyer et al., 2011; 2012). In these mice, Flk2 expression promotes Cre-mediated removal of the Tomato allele, resulting in a switch from Tomato expression to GFP expression. The CMP transplantation data presented here would suggest that GM would be more likely to develop through two Flk2+ intermediates, the MPP and CMP+. This would result in a higher proportion of cells with reporter switching in the GM lineage in Flk2 lineage tracing mice compared to erythroid or megakaryocytic (MegE) lineages, which are more likely to develop through the MPP followed by CMP-. Quantification of Flk2 mRNA levels revealed significantly reduced Flk2 expression in CMP+ compared to MPP and CLP (Figure S5A). To supplement the Flk2 expression data we performed additional analysis of Flk2 lineage tracing mice with low recombination at the MPP stage, which can be used to assess differentiation through additional Flk2+ developmental stages beyond the Flk2+ MPP, as observed by a further increase in GFP levels (Boyer et al., 2011). In accordance with the Flk2 expression data presented in Figure S5A, the proportion of GFP+ cells in both CMP- and CMP+ mirrored the proportion of GFP+ MPP, while CLP had a substantial increase (Figure S5B). In addition to Flk2 expression, other factors may also affect recombination, such as the time spent at each Flk2+ developmental stage (discussed in Chapter 3; Boyer et al., 2012). Combined, expression analysis and additional lineage tracing reconcile the transplantation data presented here with previous reports (Boyer et al., 2011; Boyer et al., 2012).

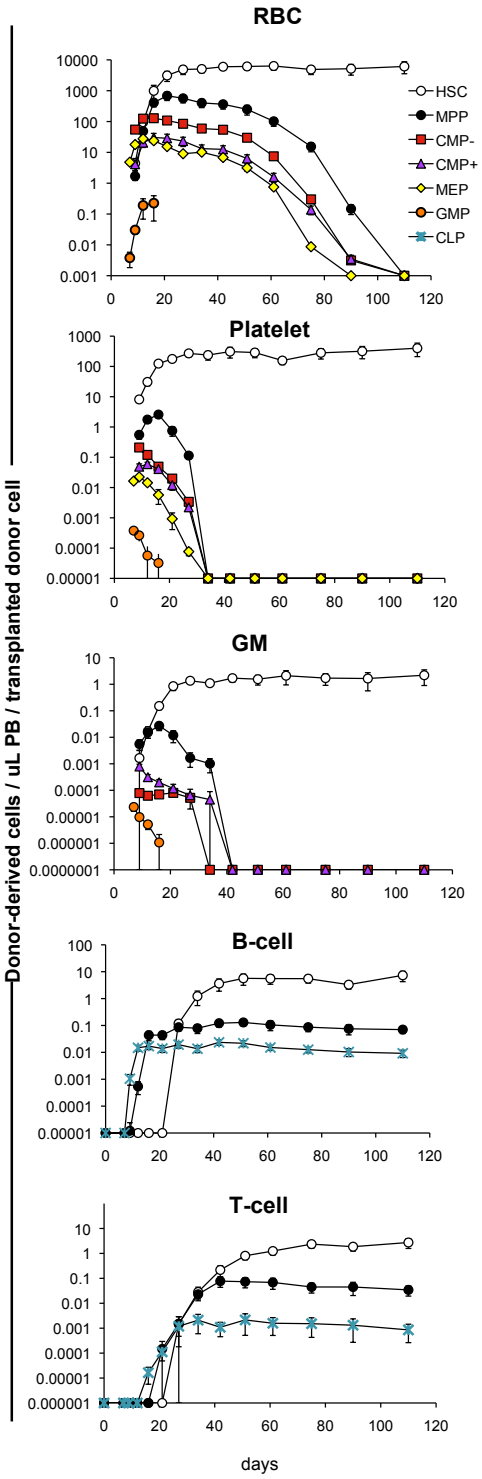


Figure 18. Comparing mature cell output between transplanted hematopoietic stem and progenitors. The transplantation data from Figure 17 was re-plotted to compare production of RBC (A), Plt (B), GM (C), B-cell (D), T-cell (E). The data is displayed as the number of donor-derived cells per microliter of peripheral blood (PB) per transplanted cell present during a 110 day timecourse posttransplantation.

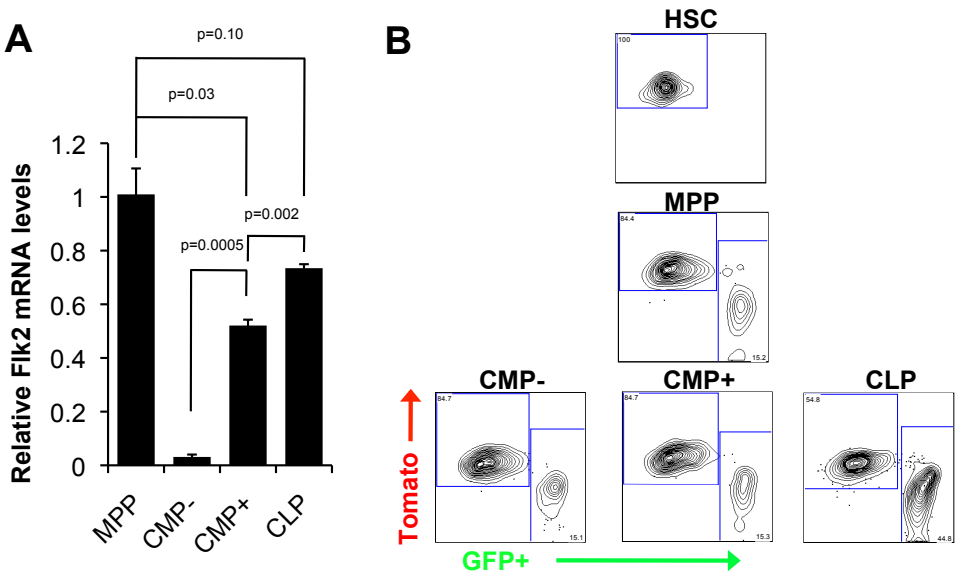


Figure S5. CMP+ have reduced Flk2 expression. (A) CMP+ display reduced Flk2 mRNA levels compared to MPP and CLP, as determined by RT-qPCR of FACS sorted cells. P-values were determined using an unpaired two-tailed t-test. Error bars indicate SEM. (B) Compared to MPP, CMP+ do not have additional reporter activity in Flk2 lineage tracing mice (Flk2-Cre X mT/mG; n=4).

Single MPP display multilineage reconstitution *in vivo*

Although is it clear from these data that transplantation of multiple MPP has a similar mature cell production profile compared to HSC (Figure 17), it cannot be deduced if MPP are functionally homogeneous, each containing a similar differentiation potential as HSC (Figure 19A, Model #1), or if MPP are a heterogeneous population of committed progenitors that share a common phenotype (Figure 19A Model #2). To differentiate between these two possibilities, we analyzed the lineage potential of single hematopoietic cells *in vivo*. Colonies formed from splenic colony forming assays (CFU-S), which are derived from a single transplanted hematopoietic stem or progenitor cell with erythroid potential (Till and McCulloch 1961; Weber et al., Nature Medicine, 2011), were analyzed for multilineage reconstitution. To ensure that colonies analyzed were derived from a single cell, we transplanted a mixture of Tomato+ (mT/mG; Muzumdar et al., 2007) and GFP+ (UBC-GFP; Schaefer et al., 2007) cells of the same cell type. Colonies comprised of mixed colors were never observed (data not shown). T-cell output was not assessed, as T-cell development requires an extended development in the thymus. It is well established that HSC and MPP give rise to T-cells, while other myeloid-committed cells that give rise to CFU-S do not. Out of the 13 HSC-derived CFU-S, 6 (46%) contained observable erythroid (Ery), megakaryocytic (Meg), GM, and B-cell lineages (Figure 19B). Similarly, 6 of the 18 (33%) single MPP-derived colonies also produced Ery, Meg, GM, and B-cell lineages (Figure 19B). In contrast, single CFU-S derived from CMP-, CMP+, and MEP only had observable Ery cells. These data support at least some MPP retain multilineage reconstitution at the single cell level.

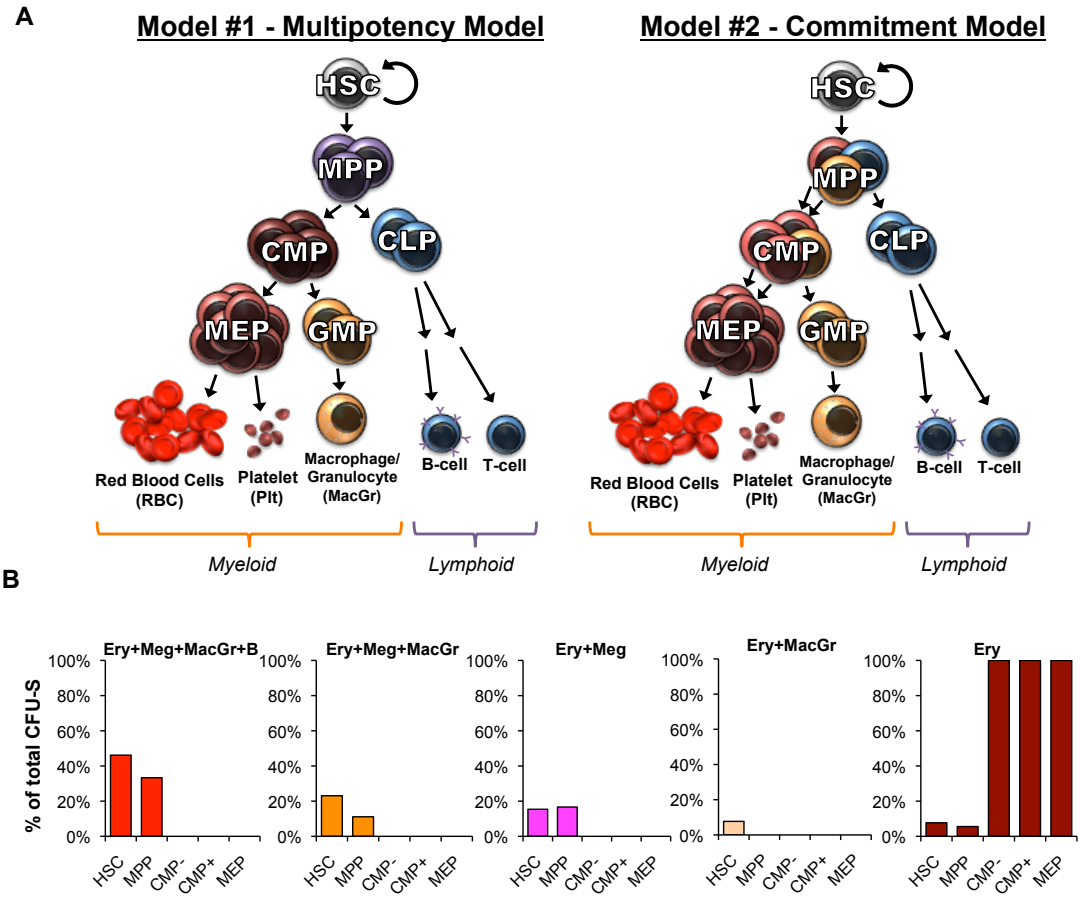


Figure 19. Multilineage readout from MPP-derived CFU-S. (A) Two competing models for lineage commitment in hematopoiesis. (B) The lineage contribution of single-cell derived CFU-S. HSC n=13, MPP n=18, CMP- n=16, CMP+ n=8, MEP n=9.

DISCUSSION

MPP display robust erythroid and megakaryocytic potential *in vivo*

Evidence exists for both the lack of and the retention of MegE potential in MPP (Adolfsson et al., 2005; Forsberg et al., 2006; Arinobu et al., 2007; Boyer et al., 2011, 2012). Previous *in vivo* evidence supporting retention of MegE potential in MPP found elevated platelet output from MPP compared to downstream myeloid-committed progenitors, such as CMP/MEP, which have undisputed platelet potential (Forsberg et al 2006). MPP were also found to protect from a lethal dose of irradiation with fewer transplanted cells than myeloid-committed progenitors, providing evidence of the robust RBC potential by MPP (Forsberg et al., 2006). For the first time, we quantified mature RBC production from transplanted hematopoietic stem and progenitor cells alongside the production of other mature hematopoietic cell types (Figure 17 and 18). At the population level, MPP generated ~3,500x more RBC than WBC, and ~10x more Plt than WBC in the blood (Figure 17E), demonstrating that not only do MPP retain MegE potential, but MPP are a vital source of RBC and Plt. Despite the large numbers of MegE cells produced from transplanted MPP (Figure 17E and 18A-B), MPP exhibit reduced MegE potential *in vitro* (Adolsson et al., 2005, Arinobu et al., 2007). Similar results can be found with CMP+, which do not generate MegE colonies *in vitro* (D'Amico and Wu 2003), despite the robust MegE *in vivo* readout reported here (Figure 17K-L).

When evaluating lineage potential, it is commonplace to compare mature cell output from HSC to other progenitors within the KLS (cKit+, Sca1+, Lineage-) fraction, including the MPP. Previous data and the data presented here make it clear MPP do not generate as many mature cells as HSC, including MegE cells, due to the MPP's lack of self-renewal upon transplantation (Figure 17 and 18) (Forsberg et al., 2006; Christensen and Weissman 2001; Adolfsson et al., 2001). In combination with the lack of *in vitro* MegE potential from MPP, this observation has led to the conclusion that MPP have reduced MegE potential compared to HSC. When hematopoietic output from transplanted progenitors is compared to cells both

upstream and downstream of the progenitor type being tested, it is clear that more developmentally mature cells will not produce as many mature cells as their upstream counterpart (Figure 18) (Forsberg et al., 2006). This type of comparison can be used to place cells developmentally relative to each other. Here we observe that MPP generate greater numbers of RBC and Plt per transplanted cell than any other progenitor tested, but not as many cells as HSC (Figure 18A-B). Similar results were observed with GM, B-cell, and T-cell output from MPP. Thus, we conclude that the MPP lie immediately downstream of the HSC developmentally.

When assessing lineage bias, however, the number of mature cells being generated per transplanted cell type should be measured, then compared to the total output from other transplanted cell types, as done in Figure 17. Due to the early onset and rapid depletion of progenitor-derived mature myeloid cells, and the more delayed but sustained output of mature lymphoid cells, donor-derived mature cells should be measured at multiple time points, both early and late. Otherwise, lineage output might seem myeloid biased early on, and lymphoid biased at later time points (Figure 17E). Utilizing quantitative analysis at multiple time points, we observe that transplanted MPP produce mature cells in a similar ratio as HSC and as present at steady-state (Figure 17B-C, E-F and Figure 16B-C). Thus, at the population level, MPP retain RBC and Plt potential and is not lymphoid biased.

MPP are developmentally upstream of CMP and CLP

While it is undisputed that the MPP is the parental progenitor of the CLP, it remains controversial if the MPP is the sole precursor to CMP, and therefore MegE lineages. Conditional bypass of the MPP stage by HSC feeding directly into the myeloid progenitor pool that has been proposed previously (Takano et al., 2004), but is inconsistent with Flk2 lineage tracing in which the MPP is a critical developmental stage for both myeloid and lymphoid cells during both steady-state and stress hematopoiesis, as well as post-transplantation (Boyer et al., 2011; 2012) Similar to previous findings, we report a prolonged timing and increased

quantity of mature cells produced from MPP compared to CLP, CMP-, CMP+, MEP, or GMP (Forsberg et al 2006) (Figure 18). Together, transplantation data and lineage tracing data support HSC giving rise to MPP, then subsequently the CMP and CLP.

Because the CMP develops from the MPP, it seems unlikely that the MPP is lymphoid bias, as a lymphoid biased progenitor would not give rise to myeloid-committed progenitors responsible for all myeloid cell production. In addition, given the existence of an oligopotent CMP capable of giving rise to RBC, Plt, and GM at the single cell level (Akashi et al., 2000) and that the CMP is derived from the MPP (Boyer et al 2011, 2012), it is unlikely that MPP have lost MegE potential while retaining GM and lymphoid potential.

CMP- and CMP+ are parallel populations

As discussed above, *In vivo* lineage tracing data and transplantation data support the HSC giving rise to MPP, and subsequently the CMP and CLP (Boyer et al., 2011, 2012; Forsberg et al., 2006) (Figure18). Since CMP- and CMP+ are subfractions of the CMP, this places both subfractions downstream of the MPP. Per transplanted cell, both CMP- and CMP+ produce similar numbers of platelets in a similar timeline (Figure 18B). Despite similar Plt production between the two populations, we were able to observe a preferential ability of CMP- to produce RBC, and CMP+ to produce GM (Figure 18A & C). Although lymphoid production was not detectable in the peripheral blood using a sublethal does of irradiation (Figure 17G-L), a low level of CMP+-derived B-cells have been found in the spleen after a lethal dose was used (D'Amico et al., 2003; Yang et al., 2007). However, neither CMP- nor CMP+ generate cells of the T-cell lineage that are able to seed the thymus (D'Amico et al., 2003). Despite these partially overlapping differences in lineage output, the timing of RBC, Plt, and GM production suggests the CMP- and CMP+ are parallel populations, and are not upstream or downstream of each other (Figure 17G-L and Figure 18A-C). In other words, CMP+ is not the precursor to CMP-, or vice versa.

Lineage commitment occurs after formation of MPP

HSC have been previously shown to long-term multilineage reconstitute upon single-cell transplantation (Osawa et al., 1996; Wagers et al., 2002; Dykstra et al., 2007; Morita et al., 2010). However, a fraction of single HSC lack long-term and/or multilineage potential upon transplantation, and thus has been concluded that lineage decisions are made at the HSC level. Given that the MPP stage is a necessary developmental intermediate between the HSC and all mature hematopoietic cells (Boyer et al., 2011, 2012), it might be inferred from these data that MPP have committed to specific cell fates, as they are derived from HSC. Here we report that a fraction of MPP have multilineage capacity, making it unlikely that a portion of HSC have already committed to specific cell fates prior to giving rise to the MPP, a progenitor with multilineage potential. Alternatively, the observed heterogeneity in lineage readout from both single HSC reported previously and single MPP reported here may be a technical limitation, as these cells are transplanted into a highly perturbed environment (Osawa et al., 1996; Wagers et al., 2002; Dykstra et al., 2007; Morita et al., 2010). Because MPP have not been shown to self-renew upon transplantation, we conclude that loss of multilineage potential occurs after the loss of self-renewal and the subsequent generation of the MPP.

Although some MPP were shown to be multipotent, we cannot exclude the possibility that a fraction of MPP have begun to commit, or are fully committed, to either myeloid or lymphoid fates. This is a feasible model, as commitment must occur somewhere in between the generation of the MPP and the generation of the downstream CMP and CLP. It can be concluded, however, that MPP are not lymphoid biased as a population, given the vast number of RBC and Plt produced form transplanted MPP (Figure 17D-F).

Lineage potentials of progenitors beyond MPP

Previous modeling supports a clear separation of myeloid and lymphoid cell fates with the formation of the CMP and CLP (Akashi et al., 2000; Kondo et al., 1997). More recent data have presented evidence for the retention of B-cell potential in a subfraction of CMP, the CMP+ (D'Amico et al., 2003; Yang et al., 2007), rendering the previous commitment model to a more overlapping model than initially proposed. However, evidence for the retention of myeloid potential in lymphoid progenitors, as determined by *in vitro* data, is inconsistent with *in vivo* transplantation and lineage tracing data which supports restriction of these progenitors to lymphoid lineages (reviewed in Schlenner 2010B).

CMP have been shown to differentiate into MEP and GMP *in vitro*, in support of CMP being the precursor to MEP and GMP (Akashi et al 2000). Further fractionation of the CMP revealed CMP+ preferentially gave rise to GMP *in vitro*, while CMP- produced both MEP and GMP (Nutt et al., 2005). It is unlikely that CMP- and CMP+ cannot simply be comprised of MEP and GMP, given the higher number and delayed timing of mature cell production from CMP- and CMP+ compared to MEP and GMP. This model would also be incompatible with *in vitro* evidence supporting RBC, Plt, and GM potential of single CMP (Akashi et al., 2000).

Here we report retention of MegE potential in GMP, which has only been shown to produce GM both *in vitro* and *in vivo* (Akashi et al., 2000; Na Nakorn et al., 2002; Na Nakorn et al., 2003). When our results are displayed as the percent donor contribution to the total mature cells in the host (Figure 17M), RBC and Plt readout appears negligible, consistent with the inability of GMP to radioprotect or form significant erythroid precursors in the spleen upon transplantation (Na Nakorn et al., 2002; Na Nakorn et al., 2003). When we quantified the total cell output from GMP, we find significant MegE production, albeit lower per cell than CMP-, CMP+, or MEP (Figure 18A-B). One way to explain these discrepancies in data is the reduced ability of hematopoietic cells to form MegE colonies *in vitro*. For instance, MPP give rise to mostly GM *in vitro*, and rarely give rise to MegE colonies (Adolfsson et al., 2005;

Arinobu et al., 2007), yet MegE cells comprise the vast majority of mature cells produced by MPP upon transplantation. Perhaps a similar phenomenon occurs with GMP, but GMP lack the burst size of MPP to detect significant MegE production *in vitro* and *in vivo*. Although other reports have not detected mature Plt (Na Nakorn 2003; Forsberg et al., 2006) or erythroid precursors (Na Nakorn et al., 2002) from transplantation of lower numbers of GMP, this is the first enumeration of donor-derived RBC and Plt from transplanted hematopoietic stem and progenitors, including high numbers of transplanted GMP (Figure 17 and 18). In contrast to CMP+, GMP have not been found to give rise to B-cells upon transplantation (Yang et al., 2007).

Modeling lineage commitment throughout hematopoiesis

For the first time, we directly measured mature RBC production from transplanted hematopoietic stem and progenitors. We found that RBC were the most highly produced cell type from HSC, MPP, CMP-, CMP+, MEP, and even GMP (Figure 17). RBC are also the most abundant hematopoietic cell type during steady-state hematopoiesis (Figure 16B-C), and are necessary for the immediate survival of the organism. Therefore, it can be postulated that the primary goal of the HSC is to maintain proper RBC numbers, as modeled in Figure 20. While other mature cell types also have important functions in the body, these cells are needed at much lower numbers in the body (Figure 16B-C), placing less developmental pressure on the hematopoietic system toward these lineages.

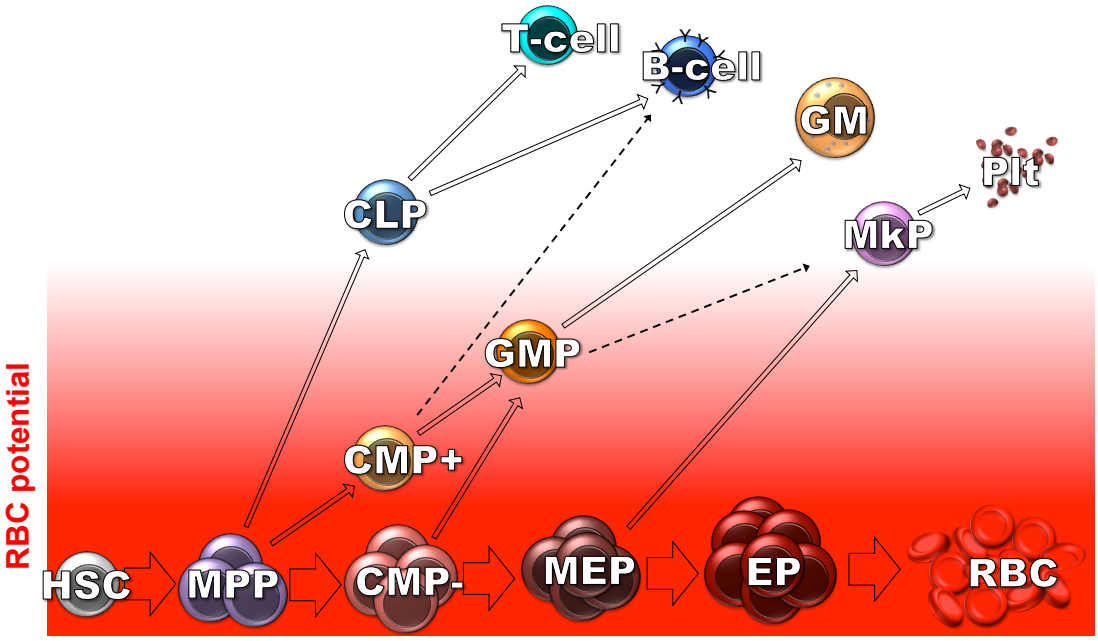


Figure 20. Modelling RBC potential throughout hematopoietic development. The driving force for the HSC is the generation of RBC, represented by the heavily weighted arrows. As other specialized cell are produced, RBC potential is gradually lost, as indicated by the fading of the red background.

From combining previous lineage tracing data, transplantation data, and the data presented here, a model for lineage commitment to specific hematopoietic fates can be constructed (Boyer et al., 2011, 2012; Schlenner 2010A; Forsberg et al., 2006; Na Nakorn et al., 2002; Na Nakorn et al., 2003; Nutt et al., 2005; Yang et al., 2007). Upon commitment to myeloid lineages, MPP give rise to CMP, in which T-cell potential is lost first, followed by B-cell potential (Figure 21A). CMP- are likely to differentiate into MEP, where GM potential is lost, followed by restriction to either RBC or Plt potential by forming erythroid progenitors (EP) and megakaryocyte progenitors (MkP). Upon commitment to the GM lineage, CMP+ give rise to GMP, in which B-cell potential is completely lost followed by diminished RBC potential, then finally diminished Plt potential (Figure 21B). Commitment to lymphoid lineages appears more straightforward, as myeloid potentials are lost as MPP gives rise to CLP (Figure 21C). The model in Figure in 21 represents the likely progenitor source of each mature cell, but is

not limited to other progenitors as CMP-, CMP+, and GMP have overlapping lineage potentials. Given the existence of other unipotent progenitors, such as EP, MkP, proT, and proB cells, it is likely there exists an unidentified, unipotent progenitor for the GM lineage, as shown in Figure 21B.

In summary, lineage commitment must occur after the formation of the MPP. With regard to myeloid lineages, commitment is more gradual and promiscuous than has been proposed in previous models. In the future it will be important to identify functionally distinct progenitors with non-overlapping potentials to further clarify issues of lineage commitment, as well as utilize sensitive single-cell *in vivo* assays to interrogate functional heterogeneity within phenotypically distinct populations.

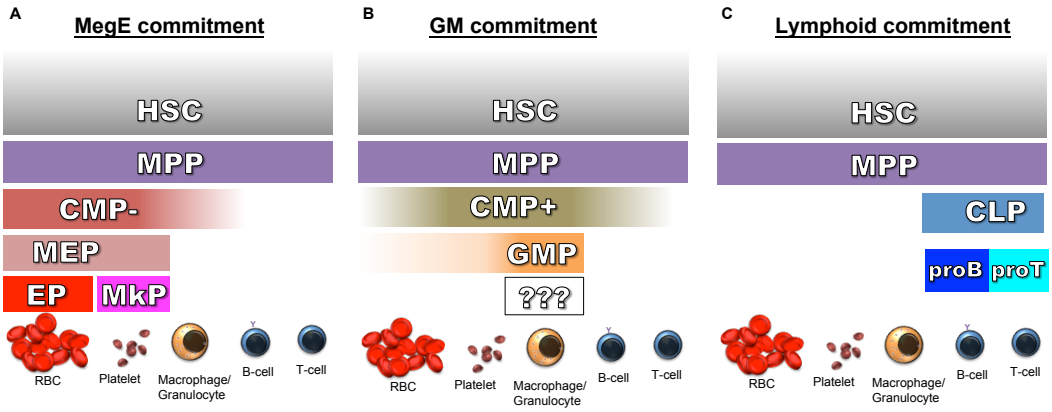


Figure 21. Modeling lineage commitment in hematopoiesis. Three models of HSC differentiation toward the MegE (A), GM (B), and Lymphoid (C) lineages. HSC, hematopoietic stem cell; MPP, multipotent progenitor; CMP-, Flk2- common myeloid progenitor; CMP+, Flk2+ common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte/erythrocyte progenitor; GMP, granulocyte/myelomonocyte progenitor; EP, erythroid progenitor; MkP, megakaryocyte progenitor.

MATERIALS AND METHODS

Transplantation assay

Donor cells were isolated and FACS sorted from UBC-GFP mice (Shaefer et al., 2007; The Jackson Laboratory, Stock # 004353) and transplanted into sublethally irradiated (500 rads) WT mice. 200 HSC (Lineage-, cKit+, Sca1+, Slamf1+, Flk2-), 1,000 MPP (Lineage-, cKit+, Sca1+, Slamf1-, Flk2+), CMP^{-} (Lineage-, $cKit^{+}$, $Sca1^{-}$, $FcγRα^{mid}$, $CD34^{mid}$, $Flk2^{-}$), 10,000 CMP^{+} (Lineage-, $cKit^{+}$, $Sca1^{-}$, $FcγRα^{mid}$, $CD34^{mid}$, $Flk2^{+}$), 50,000 MEP (Lineage-, $cKit^{+}$, $Sca1^{-}$, $FcγRα^{lo}$, $CD34^{lo}$), 50,000 GMP (Lineage-, $cKit^{+}$, $Sca1^{-}$, $FcγRα^{hi}$, $CD34^{hi}$), and 10,000 CLP (Lineage-, $cKit^{mid}$, $Sca1^{mid}$, $IL7Rα^{+}$, $Flk2^{+}$) were transplanted. The Lineage cocktail was comprised of CD3, CD4, CD5, CD8, Ter119, Mac1, Gr1, B220.

Mature cell quantitation

A known volume of peripheral blood was mixed with antibodies (Ter119, CD61, Mac1, Gr1, B220, CD3) and a known quantity of Calibrite-APC beads (Catalog no. 340487) prior to analysis. For tissues, a known quantity of Calibrite-APC beads was added to each tissue preparation prior to antibody staining and analysis. RBC (FSC^{lo-mid}, Ter119+, CD61-, Mac1-, Gr1-, B220-, CD3-), Platelets (SSC^{lo}, Ter119-, CD61+, Mac1-, Gr1-, B220-, CD3-), GM (FSC^{mid-hi}, Ter119-, CD61-, Mac1+, Gr1+, B220-, CD3-), B-cell (FSC^{mid}, Ter119-, CD61-, Mac1-, Gr1-, B220+, CD3-), T-cell (FSC^{mid}, Ter119-, CD61-, Mac1-, Gr1-, B220-, CD3+).

CFU-S analysis

Lethally irradiated WT mice were transplanted with an equal mixture of FACS sorted cells isolated from mT/mG (Muzumdar) mice and UBC-GFP mice (Schaefer et al., 2007; The Jackson Laboratory, Stock # 004353). 25/25 HSC, 75/75 MPP, 100/100 CMP-, 200/200 CMP+, 150/150 MEP. On day 8.5 (MEP), 9.5 (CMP- and CMP+), MPP (11.5) and HSC (13.5) post-transplantation, mice were sacrificed and perfused to remove peripheral blood. Individual CFU-S were isolated using a fluorescent dissecting scope and labeled with the

following antibodies: Ter119, CD41, Mac1, Gr1, and B220. Erythroid Progenitor (EP; FSC^{mid-hi}, Ter119+, CD41-, Mac1-, Gr1-, B220-); Megakaryocyte (Meg; FSC^{mid-hi}, Ter119-, CD41+, Mac1-, Gr1-, B220-); GM (FSC^{mid-hi}, Ter119-, CD41-, Mac1+, Gr1+, B220-); B-cell (FSC^{mid-h},Ter119-, CD41-, Mac1-, Gr1-, B220+)

RT-qPCR for Flk2

Cells were double sorted by FACS. mRNA was isolated using TRIZOL reagent. cDNA synthesis was performed with a High Capacity cDNA Reverse Transcription Kit (SKU # 4374966). 300 cells per reaction were used for qPCR of the cDNA, and were performed in triplicate. Flk2 RT-qPCR Forward primer (5'- ATCCCCAGAAGACCTCCAGT-3'); Flk2 RT-qPCR Reverse primer (5' - CACTTGCAGGGTGATGGAC).

CHAPTER 6: Conclusion

In this dissertation I have presented evidence for the existence of a multipotent progenitor (MPP) that serves as a necessary developmental intermediate for all hematopoietic lineages. Here I will describe conclusions that can be drawn from the results contained within these chapters, as well as discuss the future direction for advancing our knowledge of hematopoietic stem cell fate decisions.

MPP is a critical developmental stage for both myeloid and lymphoid lineages

Using various approaches, we have mapped differentiation pathways throughout hematopoiesis. Using Flk2 lineage tracing mouse models (FlkSwitch mice) in chapters 2 and 3, it is clear that myeloid lineages develop through a highly Flk2+ intermediate. Given that the MPP is the only highly Flk2+ cell type thought to give rise to at least some myeloid cells, we concluded that all HSC utilize the MPP stage prior to the generation of all hematopoietic lineages. This data was substantiated when analyzing $Flk2^{-/-}$ mice in chapter 4. When propagation of the MPP stage is inhibited by Flk2 deficiency, both myeloid and lymphoid progenitors were also reduced at steady-state hematopoiesis. Performing steady-state analysis of hematopoietic stem and progenitors in FlkSwitch and $Flk2^{-/-}$ mice allowed for the mapping of differentiation pathways used by both myeloid and lymphoid lineage *in vivo* under non-transplantation settings. These models were also able to compare differentiation paths utilized during steady-state to differentiation paths during hematopoietic stress and after transplantation. In FlkSwitch mice, the MPP stage was critical for all hematopoietic lineages during all conditions tested (Chapter 2) (Boyer et al., 2011; 2012). Transplanted $Flk2^{-/-}$ HSC were unable to sufficiently propagate the MPP population, leading to a decrease not only in hematopoietic progenitors as seen at steady-state, but a global decrease was seen in all mature hematopoietic cells. However, transplantation of equal numbers of wt and $Flk2^{-/-}$ MPP

had similar myeloid reconstitution, supporting propagation of the MPP stage as a requirement for establishing a healthy hematopoietic system. This finding also alludes to the relative insignificance of Flk2 expression during myelopoiesis, as most myeloid progenitors lack Flk2 expression or have expression that is significantly lower than MPP (Figure S4A). In summary, the mapping of differentiation paths inferred from FlkSwitch and $Flk2^{-/-}$ transplantation data was in accordance with *in situ* differentiation.

Chapter 6 provided additional evidence that the MPP is developmentally in between the HSC and committed progenitors. By quantifying mature cell output upon transplantation, MPP were found to (1) produce mature cells in the same order of abundance as transplanted HSC and as found during steady-state hematopoiesis, and (2) generate mature cells with prolonged timing but increased numbers compared to progenitors with undisputed myeloid and lymphoid potential. Therefore, timing and burst size provide indirect evidence for the MPP giving rise to CMP and CLP. With the data from chapters 2,3,4 and 5 combined, it is clear the MPP is the developmental precursor to committed progenitors.

Assessing functional heterogeneity in the MPP

In addition to mapping differentiation pathways, we have provided increased resolution into where the first commitment step could possibly occur in hematopoiesis. In Chapter 5, we showed that at least a fraction of MPP are multipotent at the single cell level, in support of multipotency being lost after the formation of the MPP (Figure 19B). However, it is not known if all MPP are multipotent, or if some have begun to commit to specific cell fates (Figure 19A). The level of functional heterogeneity could be clarified by single-cell transplantation of MPP and the monitoring of donor-derived mature cells in the blood, as has been done for HSC (Osawa et al., 1996; Wagers et al., 2002; Dykstra et al., 2007; Morita et al., 2010). Since MPP produce less total cells than HSC, and MPP do not self-renew upon transplantation, these assays must be highly sensitive, and samples will need to be taken at various time points after transplantation to assess multilineage reconstitution (as discussed in

Chapter 5). By assessing the mature cells derived from single MPP transplanted into lethally irradiated hosts, we can calculate the percent of truly multipotent MPP. Unfortunately, HSC appear to be functionally heterogeneous upon single-cell transplantation, which has lead to the conclusion that the level of commitment occurs at the HSC (Dykstra et al., 2007; Morita et al., 2010). This conclusion is inconsistent with the multilineage reconstitution observed from single MPP in our CFU-S assays (Figure 19B), as a lineage-restricted HSC population would likely not give rise to a multipotent progenitor cell. Similar to previous single-HSC transplantation data, we also found that not all single HSC formed multilineage CFU-S. Therefore, single-cell transplantation may under-estimate the full potential of the cell being transplanted, but can give insight into the functional heterogeneity within MPP and still has the potential to increase our understanding of which hematopoietic potentials are lost first upon commitment to specific lineages (e.g., are RBC the least likely cell type to be found alongside T-cells?). Other methods to assess functional heterogeneity include but are not limited to; (1) transplantation of multiple MPP which have been individually barcoded with a unique DNA sequence (Lu et al., 2011), or (2) transplanting single MPP into minimally conditioned mice to reduce off effects of non-specific host conditioning, such as irradiation and chemotherapy. Perhaps specific depletion of endogenous host MPP and/or HSC will allow for the acceptance of donor MPP and provide a cellular output that mimics output *in situ*.

Alternatively, heterogeneity could be assessed by the addition of cell surface markers to subfractionate and purify MPP with distinct differences in hematopoietic output. Given that (1) MPP give rise to CMP and CLP (as discussed in Chapter 5), and (2) a single CMP is capable of giving rise to RBC, Plt, and GM (Akashi et al., 2000), and (3) at least a fraction of CLP are bitpotent for both B- and T-cell lienages (Karsunky et al., 2008), it is unlikely that this committed fraction within the MPP population have committed to any extent beyond the capability of the CMP or CLP. Determining the level of functional heterogeneity

within the MPP fraction has the potential to reveal which hematopoietic lineages are lost first upon commitment to either lymphoid or myeloid cell fates.

Hopefully, similar *in vivo* techniques as described above can be applied to validate the oligpotency of CMP and CLP seen *in vitro* (Akashi et al., 2000; Karsunky et al., 2008). Although a single CMP in culture is capable of generating RBC, Plt, and GM (Akashi et al., 2000), *in vitro* assays have been shown to under- and over-estimate lineage potentials *in vivo* (as discussed in Chapter 5 and in Schlenner et al., 2010A, 2010B). Further fractionation of myeloid progenitors using alternative markers has not fully separated populations with unique, non-overlapping functions (Pronk et al., 2007).

In the future, it is imperative to determine if subtle differences in cell output between hematopoietic progenitors is due to differences in lineage decisions, or if those cell types are heterogeneous in function. Identifying functionally distinct progenitors with unique lineage potentials will provide valuable therapeutic targets for increasing cell numbers of those particular lineages during hematopoietic deficiency, either during a disease state or post-transplantation.

In situ self-renewal of MPP

It is well established that MPP do not continuously self-renew upon transplantation, nor are they capable of de-differentiating into the pool of self-renewing HSC (Christensen and Weissman 2001, Adolfsson et al., 2001; Forsberg et al., 2006; Boyer et al., 2011, 2012). It remains unclear to what extent MPP, or any other progenitor, self-renew under unperturbed steady-state conditions. Endogenous MPP may in fact self-renew to the same extent as HSC *in situ*, but transplantation of MPP into the damaged host environment of a conditioned mouse may place stress on MPP to choose differentiation at the expense of self-renewal in order to replenish depleted host cells necessary for the mouse's survival. Due to the reduced burst size of MPP compared to HSC, there may be more pressure on transplanted MPP to produce more mature cells at the expense of generating more MPP. The retention of *in situ*

self-renewal ability from MPP may not be a surprising discovery given the high proportion of HSC that are quiescent (Passegue et al., 2005).

An optimal method for assessing endogenous self-renewal potential would be to specifically eliminate HSC and monitor hematopoietic maintenance over time. Can MPP sustain hematopoiesis in the absence of HSC? Alternatively, transplantation of MPP into minimally conditioned mice may exhibit similar behavior as endogenous MPP. A mouse model in which endogenous MPP and /or HSC have been selectively removed at a specific time may serve as host for transplanted MPP, as it is thought that competition for niche space is a barrier to hematopoietic cell engraftment. Transplantation of hematopoietic stem and progenitors cells into minimally conditioned mice such as this may not only reveal endogenous self-renewal ability, but also has the potential to give insight into mature cell production kinetics under steady-state hematopoiesis.

In summary, the use of three different but complementary approaches allowed for the careful mapping of HSC differentiation pathways *in vivo*, both *in situ* and upon transplantation.

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