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Reprogramming Committed Murine Blood Cells to Induced Hematopoietic Stem Cells with Defined Factors

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SUMMARY

Hematopoietic stem cells (HSCs) sustain blood formation throughout life and are the functional units of bone marrow transplantation. We show that transient expression of six transcription factors Run1t1, Hlf, Lmo2, Prdm5, Pbx1, and Zfp37 imparts multilineage transplantation potential onto otherwise committed lymphoid and myeloid progenitors and myeloid effector cells. Inclusion of Mycn and Meis1 and use of polycistronic viruses increase reprogramming efficacy. The reprogrammed cells, designated induced-HSCs (iHSCs), possess clonal multilineage differentiation potential, reconstitute stem/progenitor compartments, and are serially transplantable. Single-cell analysis revealed that iHSCs derived under optimal conditions exhibit a gene expression profile that is highly similar to endogenous HSCs. These findings demonstrate that expression of a set of defined factors is sufficient to activate the gene networks governing HSC functional identity in committed blood cells. Our results raise the prospect that blood cell reprogramming may be a strategy for derivation of transplantable stem cells for clinical application.

INTRODUCTION

Within the hematopoietic system, Hematopoietic stem cells (HSCs) are the only cells with the functional capacity to differentiate to all blood lineages, and to self-renew for life. These properties, in combination with the ability of HSCs to engraft conditioned recipients upon transplantation, have established the paradigm for stem cell use in regenerative medicine. Allogeneic and autologous HSC transplantation is used in the treatment of ~50,000 patients/year for congenital and acquired hematopoietic diseases and other malignancies (Gratwohl et al., 2010). Despite wide clinical use, HSC transplantation has inherent risks with transplantation outcomes impacted by multiple factors including relapse of primary disease, the numbers of HSCs transplanted, graft failure, and opportunistic infection. Moreover, allogeneic transplantation often leads to graft versus host disease (GVHD), a devastating T-cell-mediated condition resulting from minor histoincompatibility differences between donor and recipient. In spite of advances in HLA-typing to identify histocompatible donors, GVHD remains a significant cause of morbidity and mortality for ~60%–80% of patients receiving grafts from unrelated donors (Petersdorf, 2013). De novo generation of isogenic HSCs from patient-derived cells would obviate these issues, and extend transplantation to patients for whom a histocompatible donor cannot be identified. Furthermore, deriving HSCs from patients with hematological diseases would be invaluable for gaining insights into disease etiology through in vitro and in vivo disease modeling, as well as providing a cell-based platform for therapeutic screening. Deriving HSCs from alternative cell types has thus been a long sought goal in regenerative medicine.

Considerable effort has been mounted toward developing strategies for generating transplantable HSCs from alternative cell types, such as pluripotent (ES/iPS) stem cells (Choi et al., 2009; Kennedy et al., 2012). The advantages of using pluripotent cells to derive HSCs are many and include the ease by which iPS cells can be derived from patient cells, thereby putting autologous cell-based therapies within reach if HSCs can be successfully generated. However, despite considerable progress in defining the developmental pathways leading to HSCs from pluripotent cells (Sturgeon et al., 2013), the generation of

robustly transplantable definitive HSCs from pluripotent cells remains elusive. The developmental plasticity of fibroblasts and success in converting them to other cell types has prompted efforts to generate HSCs from these cells as an alternative strategy to pluripotent stem-cell-based methodologies. In one study, ectopic expression of OCT4 combined with the instructive signals of hematopoietic cytokines led to the generation of blood cell progenitors from human fibroblasts, though the resulting cells showed limited self-renewal potential and were unable to give rise to all blood cell lineages (Szabo et al., 2010). More recently, expression of *Gata2*, *Gfi1b*, *cFos*, and *Etv6* in murine fibroblasts led to the production of hematopoietic progenitors through an endothelial-like cell intermediate, though the resulting cells ultimately did not possess HSC potential (Pereira et al., 2013). In another study, expression of five transcription factors *HOXA9*, *RORA*, *ERG*, *SOX4*, and *MYB* imparted transient myeloerythroid engraftment potential onto iPS-derived blood cell progenitors, but were unable to instill the multilineage differentiation and self-renewal potential characteristic of HSCs (Doulatov et al., 2013).

The reasons underlying the current inability to generate transplantable HSCs from fibroblasts or pluripotent stem cells may be many but likely include the failure of current ex vivo conditions to support maintenance and propagation of HSCs. Moreover, the epigenetic landscape underlying HSC functional identity may be difficult to establish from divergent lineages such as fibroblasts or pluripotent stem cells. An alternative strategy that has the potential to surmount such challenges would be to reprogram differentiated blood cells back to HSCs. Striking examples in which hematopoietic cells have been experimentally reprogrammed to alternative blood cell fates by forced expression or ablation of lineage-affiliated transcription factors (Choi et al., 1990; Hanna et al., 2008; Iwasaki et al., 2006; Laiosa et al., 2006; Rolink et al., 1999; Taghon et al., 2007; Xie et al., 2004) demonstrate that cells of the hematopoietic system are amenable to reprogramming to alternative fates. Indeed, studies by Busslinger and colleagues have shown that ablation of a single transcription factor, Pax5, in early B cell progenitors (Nutt et al., 1999; Rolink et al., 1999), or terminally differentiated B cells (Cobaleda et al., 2007) was sufficient to dedifferentiate these cells to a primitive progenitor state that possessed multilineage myeloid and T cell potential but ultimately did not possess HSC activity. Pax5 ablation in peripheral B cells also led to the formation of highly penetrant and aggressive lymphomas in vivo (Cobaleda et al., 2007) thus limiting prospects for translating Pax5 ablation as a strategy to derive engraftable multilineage progenitors.

HSCs rely on complex gene regulatory networks to enable their full functional potential (Orkin and Zon, 2008). Cell fate reprogramming studies including those mentioned above have however demonstrated that ectopic expression of surprisingly few regulators is often sufficient to rewrite the gene networks regulating cell identity for many cell types. We hypothesized that the regulatory networks governing HSC identity might similarly be accessed through the action of a small number of genes, and if so, that the functional identity of HSCs might be endowed to downstream blood cells upon ectopic expression of such factors. In this study, we have used the approach of transcription-factor-mediated cellular reprogramming within the hematopoietic system to derive transplantable HSC-like cells that possess the functional and molecular properties of HSCs.

RESULTS

Identification of Factors Capable of Imparting Alternative Lineage Potential In Vitro and Multilineage Engraftment Potential on Committed Progenitors In Vivo

Experimental strategies for reprogramming diverse cell types generally rely on the action of one or more genes to impart the cellular and molecular properties of one cell type onto a different cell type. We reasoned that regulatory factors with relatively restricted expression in HSCs in relation to their downstream hematopoietic progeny are likely to be involved in defining the functional identity of HSCs through regulation of the gene networks underlying their fundamental properties. We therefore hypothesized that transient ectopic expression of such factors in committed blood cells might therefore endow them with the functional potential of HSCs and potentially stably reprogram them back to an HSC-like state. To identify such factors in an unbiased manner, we analyzed microarray data of 40 different hematopoietic cell types that others and we have generated comprising the majority of hematopoietic progenitor and effector cells in addition to HSCs. These data sets (142 arrays in total) were normalized into a single database through which we identified 36 regulatory factors with relatively restricted expression in HSCs (Figure 1A, Table S1). These included 33 genes encoding transcription factors, and 3 genes encoding translational regulators, many of which have been previously identified in studies profiling different hematopoietic cell types to define HSC transcriptional signatures (Chambers et al., 2007; Gazit et al., 2013). In addition to genes with known roles in regulating HSCs such *Ndn*, *Evi1*, *Meis1*, and *Egr1*, we also identified several genes that remain unstudied in HSC biology. The 36 factors were then cloned into doxycycline-inducible lentiviruses bearing a reporter cassette (Zs-Green) and high-titer viruses produced (Figure 1B).

It has been recognized that one of the challenges to reprogramming mature cells is that they are inherently stable. This is not necessarily true of oligo-potent and lineage-committed hematopoietic progenitors, which are in the process of differentiation. Moreover, since progenitor cells proximal to HSCs are more epigenetically related to HSCs (Bock et al., 2012), we reasoned that such proximity might be leveraged to lower the epigenetic barriers to HSC derivation. We first sought to determine if we could impart myeloid lineage potential onto otherwise B cell restricted progenitors by expression of the 36 factors. Toward this, we purified pro-/pre-B cells ($CD19^+B220^+AA4.1^+IgM^-$) from mice expressing the reverse tetracycline-controlled transactivator (rtTA) from the Rosa26 locus ($Rosa26^{rtTA}$) (Figure S1 available online), and transduced them with control virus (Zs-green), or the 36-factor viral cocktail. Transduced cells were then cultured in the presence of doxycycline for 2 days followed by plating into methylcellulose in the presence of myeloid promoting cytokines, as well Flt3L and IL7 to promote B cell survival (Figure 1C). These experiments showed that, whereas control-transduced pro-/pre-B cells were unable to form myeloid

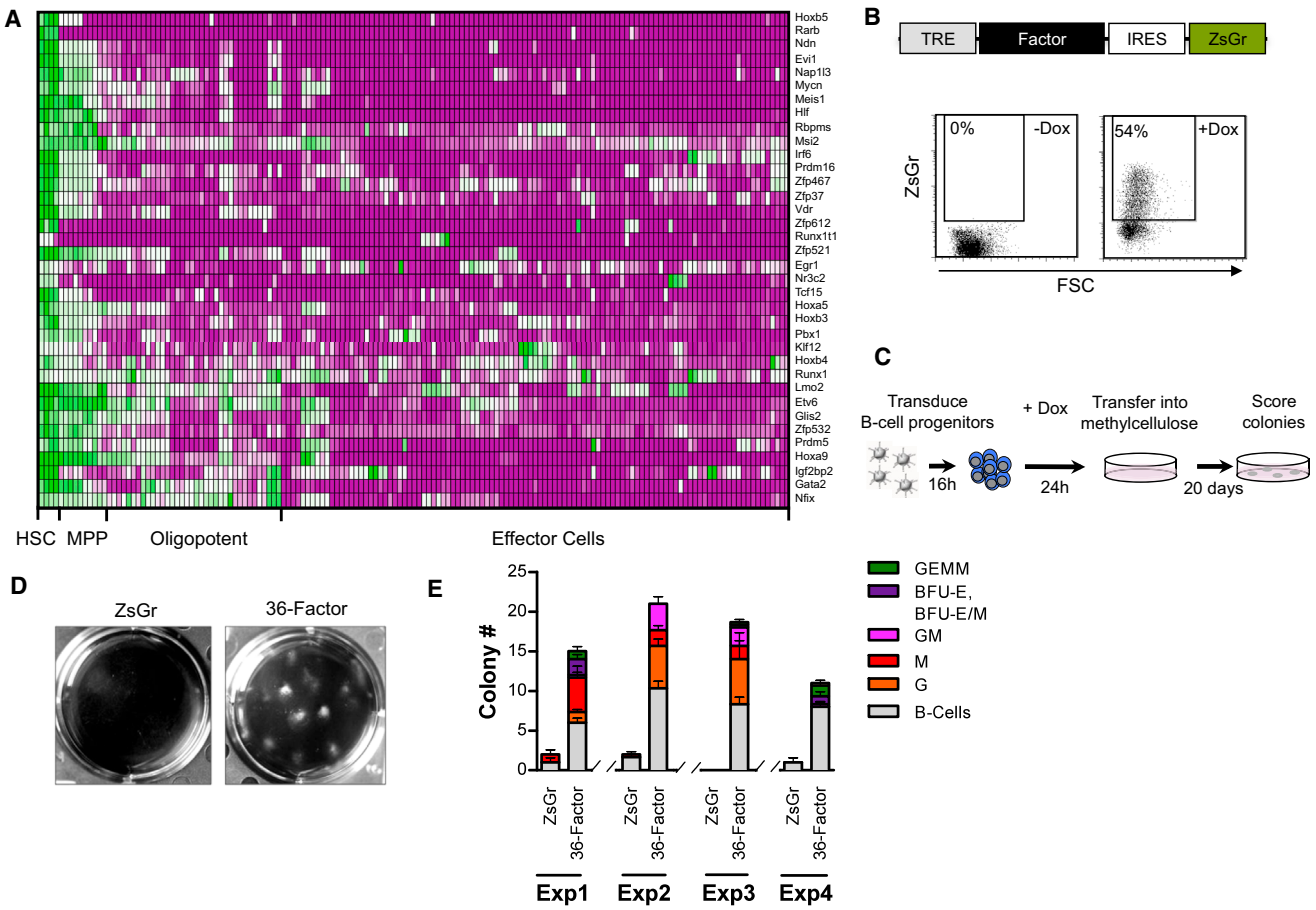

Figure 1. Identification of Factors Capable of Imparting Alternative Lineage Potential In Vitro

(A) Heat map showing relative expression (green;high, to purple;low) of 36 regulatory genes identified as HSC-specific in the indicated cell types (see also Table S1).

(B) Schematic representation of lentivirus transgene expression cassette (top), and flow cytometry plots showing reporter cassette (ZsGr) expression in pro-/pre-B cells ± doxycycline induction (48 hr post).

(C) Schematic representation of in vitro screening strategy for cell fate conversion.

(D) Representative images of wells showing colonies arising in methylcellulose from pro-/pre-B cells transduced with ZsGr or 36-factor cocktail.

(E) Colony number and type arising in methylcellulose from pro-/pre-B cells transduced with ZsGr or 36-factor cocktail. Four independent experiments are shown and each condition performed in triplicate. Error bars represent SD.

See also Table S1.

colonies as expected, cells transduced with the 36-factor cocktail readily gave rise to colonies containing diverse myeloid lineages including granulocytes, erythrocytes, megakaryocytes, and macrophages (Figures 1D and 1E).

We next determined if transient ectopic expression of the 36-factor cocktail imparted HSC-like potential onto lineage-restricted lymphoid or myeloid progenitors in vivo. We took advantage of the fact that HSCs are the only hematopoietic cells capable of long-term multilineage reconstitution in myeloablated recipients upon transplantation, and progenitors transduced with a combination of factors able to instill them with long-term reconstitution potential would be readily detected in these assays (Figure 2A). Moreover, the sensitivity of transplantation is such that the activity of even a single HSC can read out, suggesting that even rare reprogramming events might be evidenced. Moreover, we reasoned that an in vivo setting could provide access to cues present in the microenvironment of the hematopoietic system that might facilitate or even be required for reprogramming. For these experiments we purified pro-/pre-B cells or common myeloid progenitors (CMPs: lin$^-$c-kit$^+$Sca1$^-$Fcγr^{low}CD34$^+$) from *Rosa26*^{rtTA} mice (CD45.2) and following a 2 day transduction protocol with control (Zs-green) or viruses bearing the 36 factors in the presence of doxycycline, we competitively transplanted them along with whole-bone marrow cells (CD45.1) into lethally irradiated congenic recipients (CD45.1) (Figure 2A). Doxycycline was maintained in the drinking water for 2 weeks posttransplant to maintain ectopic expression of the introduced factors, followed by doxycycline withdrawal. Peripheral blood analysis of the reconstituted mice over the 16 week course of the experiment revealed that, as expected, control-transduced pro-/pre-B cells or CMPs did not give rise to long-term engraftment (Figures 2B and 2C). By contrast, a

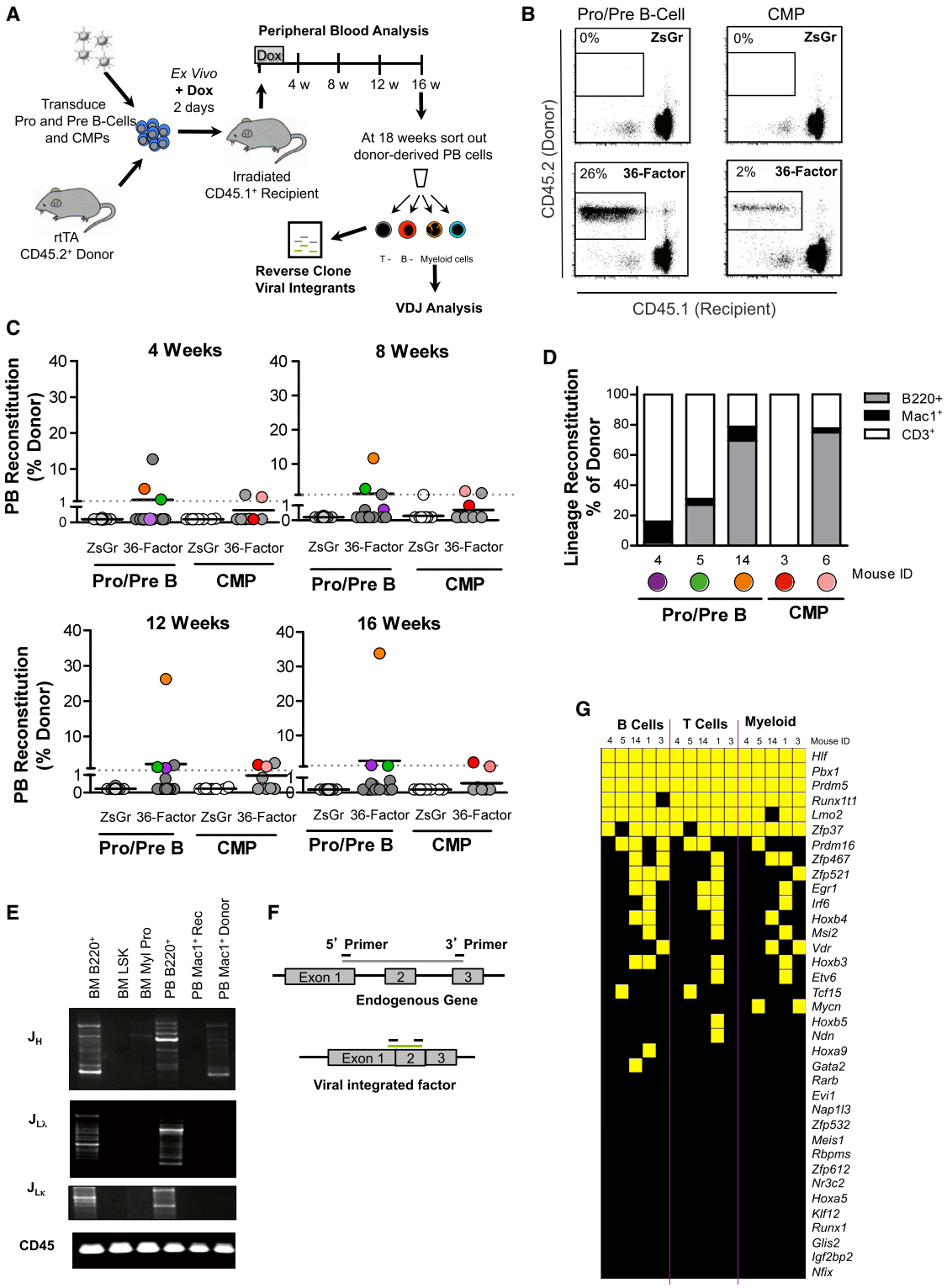

Figure 2. Identification of Factors Capable of Imparting Multilineage Engraftment Potential onto Committed Progenitors In Vivo

(A) Schematic of experimental strategy to identify factors capable of imparting multilineage engraftment potential on committed progenitors in vivo.

(B) Representative flow cytometry plots showing donor (CD45.2) reconstitution of mice transplanted with control (ZsGr) or 36-factor transduced pro-/pre-B cells or CMPs 16 weeks posttransplant.

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few of the recipients transplanted with the 36-factor transduced B cell progenitors (3/15) or CMPs (2/8) exhibited long-term donor-derived reconstitution (Figures 2B and 2C, S2A). All but one of the reconstituted mice showed multilineage engraftment of B, T and myeloid cells though the degree of engraftment of each lineage varied among the different recipients (Figure 2D). Donor-derived cells were found to be negative for expression of the reporter gene present in all viruses indicating an absence of continued factor expression post-dox-induction (Figure S2A). Analysis of V(D)J recombination in sorted donor-derived myeloid cells from the pro-/pre-B cell arm of the experiment confirmed the B-lineage origin of the reconstituting cells as evidenced by recombination of the heavy chain of the *Ig* locus (Figure 2E). The observation of multiple bands in the gel indicated that the reconstituting cells were polyclonal.

These experiments indicated that one or more factors from the 36-factor cocktail could imbue multilineage reconstituting potential onto otherwise committed lymphoid and myeloid progenitors. To determine which factors might be involved in conferring this potential, we sorted donor-derived myeloid, B and T cells to test for the presence of each of the 36 factors using a PCR-based strategy (Figure 2F, Table S2). This analysis revealed that whereas multiple factors could be identified in the donor-derived cells from each of the reconstituted mice, six transcription factors, *Hlf*, *Runx1t1*, *Pbx1*, *Lmo2*, *Zfp37*, and *Prdm5* were consistently detected in the reconstituted recipients in multiple lineages (Figure 2G).

Transient Ectopic Expression of Six Transcription Factors in Committed Progenitors Is Sufficient to Alter Lineage Potential In Vitro and Impart Long-Term Multilineage Engraftment Potential In Vivo

We next assessed if the six transcription factors we had identified in our in vivo screen were sufficient to confer myeloid colony forming potential onto pro-/pre-B cells in methylcellulose. As we had observed with the 36-factor cocktail (Figures 1D and 1E), transduction with the viral combination of *Hlf*, *Runx1t1*, *Pbx1*, *Lmo2*, *Zfp37*, and *Prdm5* was able to imbue lineage-restricted B cell progenitors with myeloid lineage potential in these assays while also augmenting the ability of the cells to give rise to B cell colonies (Figures 3A and 3B). To test the requirement for each of the six transcription factors (6-TF), we performed "*N* minus 1" experiments in which each of the factors was sequentially omitted from the transduction cocktail (Figure 3C). These experiments revealed that whereas *Hlf*, *Runx1t1*, *Pbx1*, *Lmo2*, and *Zfp37* were all required for instilling myeloid colony forming potential onto pro-/pre-B cells in vitro, the 5-factor cocktail minus *Prdm5* gave rise to myeloid colonies albeit at lower numbers than the 6-TF combination (Figure 3C).

We next tested whether transient expression of these six transcription factors was sufficient to impart long-term reconstituting potential onto committed myeloid or B cell progenitors in competitive transplantation assays. Purified pro-/pre-B cells or CMPs were transduced with control (Zs-Green) virus or the 6-TF cocktail followed by transplantation into congenic recipients (CD45.1). In contrast to control-transduced cells, long-term multilineage reconstitution was observed in 1/13 and 2/12 recipients transplanted with 6-TF transduced pro-/pre-B cells or CMPs cells, respectively (Figures 3D). Peripheral blood analysis of recipient mice throughout the course of the experiment revealed that in all cases, donor-derived cells from the reconstituted recipients showed multilineage engraftment (Figures 3D–3F and S2B). *Ig* heavy chain rearrangement was observed in donor-derived myeloid cells sorted from the pro-/pre-B cell reconstituted mouse confirming the B cell origin of the reconstituting cells (Figure 3G). These results indicate that transient ectopic expression of *Hlf*, *Runx1t1*, *Pbx1*, *Lmo2*, and *Zfp37*, and *Prdm5* is sufficient to impart long-term, multilineage transplantation potential onto otherwise committed myeloid and lymphoid progenitors.

Inclusion of *Meis1* and *Mycn* and Use of Polycistronic Viruses Improves In Vivo Reprogramming Efficiency

The absence of donor-derived reconstitution in many of the recipient mice in our 6-TF transplantation experiments (Figure 3D) suggested that the efficiency of conferring long-term multilineage potential onto committed progenitors was low. In order to increase the probability that target cells would be cotransduced with all the factors we developed polycistronic doxycycline-inducible lentiviruses bearing three transcription factors each separated by 2A peptide sequences (*Runx1T1•Hlf•Lmo2* [RHL], *Pbx1•Zfp37•Prdm5* [PZP]). We also included two additional transcription factors (*Mycn and Meis1*) that we had repeatedly identified from primitive colonies generated in in vitro colony forming experiments (Figures 4A and S3, and data not shown). To test the utility of these strategies, we transduced purified pro-/pre-B cells (CD45.1) with control virus, or the 8-transcription factor cocktail as individual viruses (8-TF), or using the RHL and PZP polycistronic viruses along with viruses bearing *Mycn* and *Meis1* (8-TF^{Poly}), and transplanted them along with Sca1-depleted (CD45.2) marrow into irradiated congenic recipients (CD45.2). Peripheral blood analysis of transplanted mice over the course of 16 weeks revealed that in contrast to the control-transduced cells that showed no donor-derived chimerism (0/12), multiple

(C) Donor reconstitution of mice transplanted with ZsGr or 36-factor transduced pro-/pre-B cells or CMPs at indicated time points posttransplantation. Only mice with >1% donor chimerism (dotted line) were considered reconstituted. Recipients transplanted; Pro/PreB;ZsGr n = 15, Pro/PreB;36 factor n = 15, CMP;ZsGr n = 8, and CMP;36 factor n = 8.

(D) Reconstitution of indicated peripheral blood cell lineages of individual recipients showing >1% donor chimerism presented as percentage (%) of donor.

(E) PCR analysis of immunoglobulin rearrangement showing heavy (J_H), and light chain ($J_{L\lambda}$, $J_{L\kappa}$) in bone marrow (BM) cells including B cells (B220+), stem/progenitor (LSK) cells, myeloid progenitors (Myl Pro), and peripheral blood (PB) cells including B cells (B220+), recipient myeloid cells (Mac1+ Rec), and donor myeloid cells (Mac1+ Donor) originating from pro-/pre-B cell;36-factor experiment. Loading control; genomic PCR for CD45.

(F) PCR-based strategy to identify virally integrated factors and discriminate from endogenous genes.

(G) Summary of data showing presence (yellow) or absence (black) of each of the indicated factors in donor B, T, and myeloid cells in each of the reconstituted mice shown in (C).

See also Figures S1 and S2.

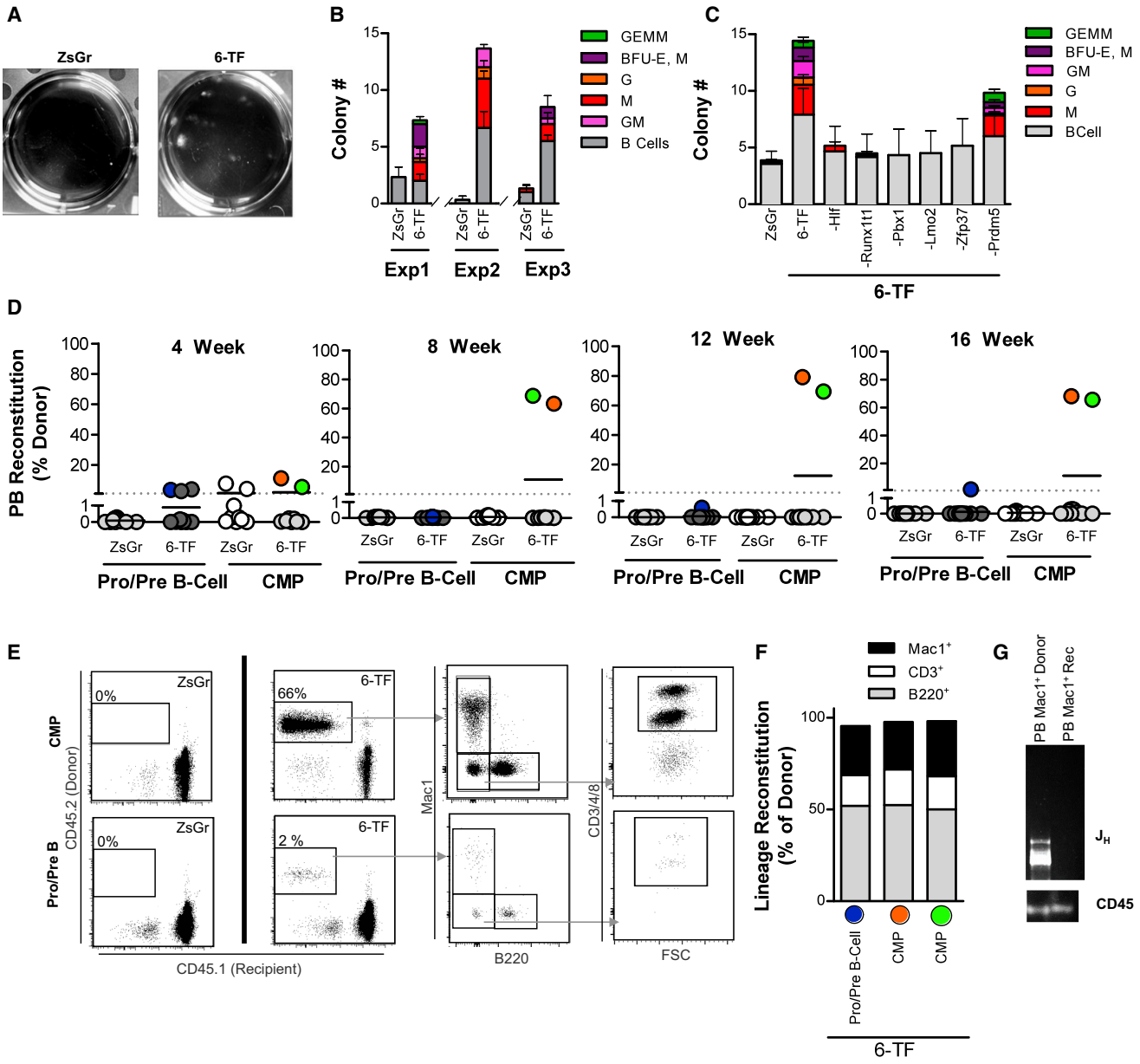

Figure 3. Transient Ectopic Expression of Six Transcription Factors in Committed Progenitors Is Sufficient to Alter Lineage Potential In Vitro and Impart Long-Term Engraftment Potential on Committed Progenitors In Vivo

(A) Representative images of wells showing colonies arising in methylcellulose from pro-/pre-B cells transduced with ZsGr or 6-TF cocktail.

(B) Colony number and indicated colony type arising in methylcellulose from pro-/pre-B cells transduced with ZsGr or 6-TF cocktail. Three independent experiments are shown with each condition performed in triplicate.

(C) Colony number and type arising in methylcellulose from pro-/pre-B cells transduced with ZsGr, 6-TF cocktail, or 6-TF minus the indicated factor. Each condition performed in triplicate.

(D) Donor reconstitution of mice transplanted with ZsGr or 6-TF transduced pro-/pre-B cells or CMPs at indicated time points posttransplantation. Only mice with >1% donor chimerism (dotted line) were considered reconstituted. Recipients transplanted; Pro/PreB;ZsGr n = 10, Pro/PreB;6-TF n = 12, CMP;ZsGr n = 9, and CMP;6-TF n = 9.

(E) Representative flow cytometry plots showing donor reconstitution and lineage composition of mice transplanted with control (ZsGr) or 6-TF transduced pro-/pre-B cells or CMPs 16 weeks posttransplant. Lineage contribution to Mac1+ myeloid cells, B220+ B cells, and CD3/4/8+ T cells is shown.

(F) Reconstitution of indicated peripheral blood cell lineages of individual recipients showing >1% donor chimerism presented as percentage (%) of donor.

(G) PCR analysis of immunoglobulin heavy (J_H) chain rearrangement in recipient myeloid cells (Mac1+ Rec), and donor myeloid cells (Mac1+ Donor) originating from pro-/pre-B cell;6-TF experiment. Loading control; genomic PCR for CD45.

Error bars in panels (B) and (C) represent SD. See also Figure S2.

recipients transplanted with either the 8-TF (3/6) or the 8-TF^{Poly} (9/14) transduced cells exhibited donor-derived chimerism (Figure 4B). All recipients showed multilineage reconstitution 18–22 weeks posttransplant though again the degree of B cell, T cell, and myeloid chimerism varied among recipients (Figures 4C and 4D and S2C). The B cell origin of the reconstituting cells was confirmed through evidence of *Ig* heavy chain rearrangement in donor-derived myeloid cells (Figure 4E).

Reprogrammed Cells Engraft Bone Marrow Progenitor Compartments and Can Reconstitute Secondary Recipients

In addition to reconstituting the peripheral blood, HSCs efficiently repopulate secondary hematopoietic organs and bone marrow progenitor cell compartments upon transplantation. To determine if the B cell progenitors transduced with the 8-TF or 8-TF^{Poly} cocktails possessed this ability, reconstituted mice were sacrificed and analyzed 18–20 weeks posttransplant, showing that all the mice had donor-derived chimerism of the bone marrow, spleen and thymus though the level of chimerism varied between recipients as we had observed in the peripheral blood (Figures 5A). The pro-/pre-B cell origin of the engrafting cells was confirmed through analysis of *Ig* rearrangement from DNA isolated from granulocytes and myeloid cells purified from the bone marrow and spleen, and T cells derived from the thymus (Figure 5B). Immunophenotyping of bone marrow cells revealed donor contribution to common lymphoid progenitors (CLPs: lin$^-$Flk2$^+$IL7Rα^+ckit^{low}Sca1^{low}), CMPs, granulocyte/monocyte progenitors (GMPs: lin$^-$ckit$^+$Sca1$^-$FcγR^{high}CD34$^+$), megarkaryocyte/erythrocyte progenitors (MEPs: lin$^-$ckit$^+$Sca1$^-$FcγR$^-$CD34$^-$), and primitive LSK progenitors (lin$^-$Sca1$^+$ckit$^+$) (Figures 5C–5F), which were comparable to host-derived progenitors analyzed from the same mice (Figures S4A and S4B). Importantly, we also observed donor contribution to megakaryocyte progenitors (MkPs: lin$^-$c-kit$^+$Sca1$^-$CD41$^+$) and erythroid progenitors (EPs: lin$^-$ckit$^+$Sca1$^-$Endoglin$^+$), demonstrating that the reconstituting cells were able of give rise to precursor cells of platelets and erythrocytes. Radioprotection transplantation assays performed using donor-derived MEPs (Na Nakorn et al., 2002) confirmed that the reprogrammed cells possessed a robust ability to generate platelets and red blood cells in vivo (Figures S4C and S4D). Importantly, subfractionation of the LSK compartment revealed donor-derived reconstitution of the multipotent progenitor (MPP1: lin$^-$ckit$^+$Sca1$^+$CD34$^+$Flk2$^-$, MPP2: lin$^-$c-kit$^+$Sca1$^+$CD34$^+$Flk2$^+$) and HSC (lin$^-$c-kit$^+$Sca1$^+$CD34$^-$Flk2$^-$) compartments (Figures 5C, 5D, and 5F). Donor-marked progenitors and HSCs were subsequently analyzed for V(D)J recombination, which revealed *Ig* heavy chain rearrangement confirming their B cell origin (Figure 5G).

A defining property of HSCs is their ability to self-renew, a potential that can be evidenced by an ability to reconstitute secondary recipients upon serial transplantation. To test if the cells generated in our experiments possessed this potential we sacrificed primary recipient mice 18 weeks posttransplant and transplanted unsorted whole-bone marrow or c-kit$^+$ cells into irradiated secondary congenic recipients. Peripheral blood analysis at long-term time points posttransplant (16–22 weeks), revealed donor reconstitution of B, T, and myeloid cells in all secondary recipients (Figures 5H and 5I).

In addition to sustained self-renewal potential, a hallmark property of HSCs is their ability to give rise to multilineage differentiation at the clonal level. Although we had observed clonal multilineage differentiation potential in vitro after induction of our factors (Figures 3B and 3C), our in vivo transplantation experiments, which were done at the population level, precluded us from concluding clonal differentiation potential in vivo. We reasoned that *Ig* heavy chain rearrangements arising in pro-/pre-B cells could be used as a bar code that could be used to trace the clonal origin of donor-derived cells. We therefore isolated DNA from sorted donor-derived B and T cells, granulocytes, and macrophage/monocytes from primary recipients transplanted with pro-/pre-B cells transduced with either 8-TF single or polycistronic viral cocktails (8-TF, 8-TF^{Poly}). *Ig* heavy chain-specific PCR spanning the V(D)J junction was then performed and products common in size to all lineages analyzed were gel purified, cloned and sequenced. Analysis of these sequences revealed unique V(D)J junction sequences common to all four donor-derived lineages analyzed from three independent donors (Figure S5A). These results provide strong evidence that a single reprogrammed cell possessed multilineage differentiation potential, though there is an extremely remote possibility that these results could reflect the contribution of cells bearing identical V(D)J junctions. Using the same approach, we next analyzed DNA isolated from FACS purified donor-derived Mac1+ myeloid cells, B cells, and T cells from secondary recipients that had been transplanted with the bone marrow of one of the mice we had analyzed during primary transplant. We identified the same V(D)J junction in the donor-derived myeloid, B and T cells of the secondary recipient, that had been previously identified in the primary recipient (Figure S5B). From these experiments we conclude that reprogrammed cells possess self-renewal and multilineage differentiation potential at the clonal level.

Taken together, these results demonstrate that transient ectopic expression of eight transcription factors in lineage-restricted B cell progenitors imparts them with the functional hallmarks of HSCs including clonal multilineage reconstituting potential, capacity to reconstitute bone marrow stem and progenitor cell compartments, and long-term self-renewal potential.

Transient Expression of Defined Transcription Factors in Myeloid Effector Cells Is Sufficient to Instill Them Long-Term Multilineage Transplantation Potential In Vivo

Eventual clinical translation of blood cell reprogramming to derive HSCs would benefit from an ability to reprogram cell types that can be readily and noninvasively obtained from the peripheral blood. We therefore sought to determine if multilineage progenitor activity could be conferred onto terminally differentiated blood cells using the transcription factors we identified. To test this, recipient and donor-derived peripheral blood was sorted from mice engrafted with pro-/pre-B cells transduced with the 8-factor cocktail (8-TF or 8TF^{Poly}) 16–22 weeks posttransplant. Sorted cells were then cultured in the absence or presence of doxycycline—with the latter condition expected to reinduce

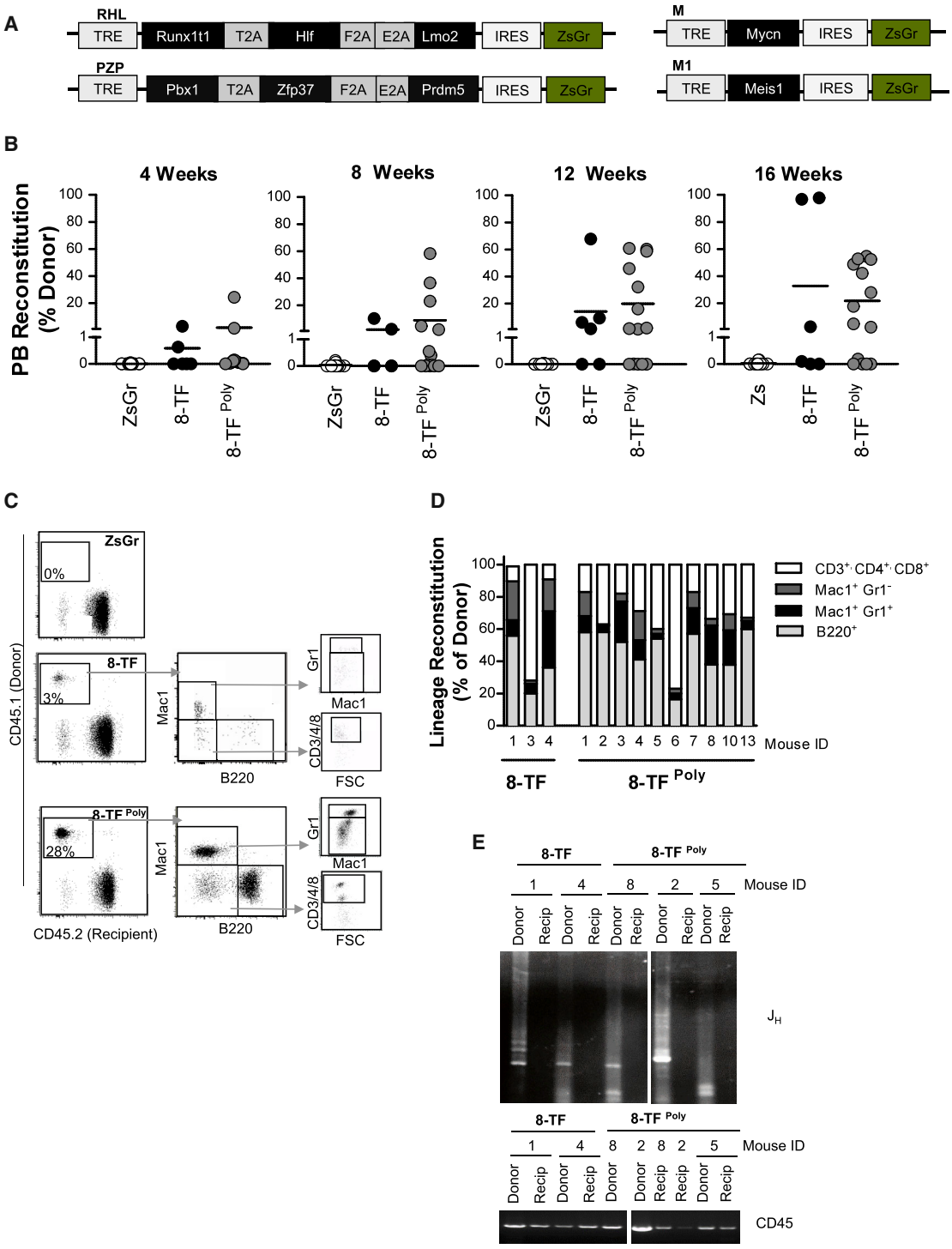

Figure 4. Inclusion of *Meis1* and *Mycn* and Use of Polycistronic Viruses Improves In Vivo Reprogramming Efficiency

(A) Schematic representation of RHL (*Runxt1t1*, *Hlf*, *Lmo2*) and PZP (*Pbx1*, *Zfp37*, *Prdm5*) polycistronic, and *Meis1* and *Mycn* single factor viral constructs.

(B) Donor reconstitution of mice transplanted with ZsGr, 8-TF (eight single factor viruses), or 8-TF^{Poly} (RHL, PZP polycistronic viruses plus *Meis1* and *Mycn* viruses), transduced pro-/pre-B cells at indicated time points posttransplantation. Only mice with >1% donor chimerism were considered reconstituted. Recipients transplanted; ZsGr; n = 12, 8-TF; n = 6, 8TF^{Poly}; n = 14.

(C) Representative flow cytometry plots showing donor reconstitution and lineage contribution of mice transplanted with control (ZsGr), 8-TF, or 8TF^{Poly} transduced pro-/pre-B cells 16 weeks posttransplant. Lineage contribution to Mac1+GR1- myeloid cells, Mac+GR1+ granulocytes, B220+ B cells, and CD3/4/8+ T cells is shown.

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expression of the transduced factors—followed by plating the cells in methylcellulose (Figure 6A). As expected, neither the recipient-marked cells, nor the donor-derived cells cultured and plated in the absence of doxycycline gave rise to colonies (Figure 6B). By contrast, plates seeded with donor cells that had seen reactivation of the eight transcription factors by exposure to doxycycline gave rise to mixed myeloid lineage colonies (Figure 6B). To determine which lineage(s) in the peripheral blood had the potential to give rise to these colonies upon re-expression of the transduced transcription factors, we purified B cells, T cells, myeloid cells, and granulocytes from 8-TF^{Poly} reconstituted mice, and tested their colony forming potential following culturing and plating in the absence or presence of doxycycline. Strikingly, plates seeded with each of the four peripheral blood cell types all developed colonies in the presence of dox, indicating that progenitor activity had been instilled into single cells upon reinduction of the factors. Of these, granulocytes gave rise to the fewest colonies whereas Mac1+ macrophages/monocytes yielded the largest number of colonies and the greatest number of primitive GEMM colonies (Figures 6C and 6D).

Encouraged by these results, we next sought to determine if the transcription factors we identified could impart multilineage reconstituting potential onto terminally differentiated cells in transplantation assays. We focused on differentiated myeloid cells because unlike differentiated lymphoid cells that have rearranged *TCR* (T cells) or *Ig* (B cells) loci, multilineage reconstituting cells derived via reprogramming of myeloid cells would be expected to have the potential to give rise to full repertoires of lymphoid effector cells upon differentiation. We therefore sorted Mac1^{+}c-kit^{-} myeloid effector cells, which include monocytes, macrophages, and granulocytes, from *Rosa26*^{rtTA} mice and transduced them with either 6-factor (6-TF^{Poly}), or 8-factor cocktails (8-TF and 8-TF^{Poly}) and transplanted them into irradiated congenic recipients. Peripheral blood analysis at monthly intervals revealed that, whereas none of mice transplanted with cells transduced with control virus were reconstituted, multiple recipients transplanted with cells transduced with 6-TF^{Poly} (4/7), 8-TF (3/6), and 8-TF^{Poly} (7/8) exhibited long-term donor-derived engraftment (Figures 6F and S6A). Lineage analysis of the reconstituted mice showed donor-derived contribution to B cell, T cell, myeloid, and granulocyte lineages with contribution to each lineage varying between recipients (Figures 6F and S2D). Donor-derived contribution to secondary hematopoietic organs, and bone marrow progenitor cell compartments was also observed in mice sacrificed and analyzed 20 weeks posttransplant (Figures S6B and S6C). Serial transplantation of sorted donor-derived bone marrow cells demonstrated that the 6-TF or 8-TF transduced myeloid effectors could engraft secondary recipients in all lineages to 16 weeks posttransplant (Figures 6G and 6H).

Based on the functional data presented in Figures 1, 2, 3, 4, 5, 6 we conclude that transient ectopic expression of six (Hlf, Runx1t1, Pbx1, Lmo2, Zfp37, and Prdm5) or eight (Hlf, Runx1t1, Pbx1, Lmo2, and Zfp37, Prdm5, Mycn, and Meis1) transcription factors is sufficient to reprogram differentiated hematopoietic progenitors and effector cells to cells that possess the functional properties of HSCs. We term these reprogrammed cells induced-HSCs (iHSCs).

Single-Cell Expression Profiling of iHSCs Reveals Evidence of Partial and Full Reprogramming

To assess the extent to which reprogrammed iHSCs recapitulate the molecular properties of endogenous HSCs, we employed a recently developed single cell gene expression profiling methodology that accurately defines hematopoietic stem and progenitor identity through the simultaneous quantification of expression of 151 lineage-specific transcription factors, epigenetic modifiers, cell surface molecules, and cell-cycle regulators (Guo et al., 2013). We sorted and analyzed donor-derived iHSCs by immunophenotype (CD45.2^{+}lineage^{-}ckit^{+}Sca1^{+}Fk2^{-}CD34$^{-/low}$CD150^{+}) 18 weeks posttransplantation from two different experiments in which pro-/pre-B cells had been transduced with the 8-TF cocktail as single viruses (8-TF), or with polycistronic viruses (8-TF^{Poly}) (Figure 4). We also sorted and analyzed host-derived HSCs (CD45.1^{+}lineage^{-}ckit^{+}Sca1^{+}Fk2^{-}CD34$^{-/low}$CD150^{+}) from the same mice to serve as controls. Single-cell expression data generated from iHSCs and host HSCs was then analyzed in comparison to data generated from pro-/pre-B cells (the starting cell type), and also to data previously generated from HSCs, MPPs, CLPs, CMPs, GMPs, and MEPs purified at steady-state (Guo et al., 2013). Analysis of the raw data revealed high correlation between gene expression for the vast majority of control and test cell types (Figure S7, Table S3). To interrogate the transcriptional relationships among all the cell types analyzed, we performed principal component analysis (PCA) to define the transcriptional distances between the cells. As expected, steady-state HSCs and progenitor cells were largely positioned in agreement with established lineal relationships with HSCs forming a clearly defined cluster, and with MPPs positioned proximal, and oligopotent progenitors (MEPs, GMPs, CLPs) positioned more distal to HSCs (Figure 7A). pro-/pre-B cells positioned closely to CLPs consistent with the lineal relationship between these cell types, while the host-derived HSCs were positioned within the steady-state HSC cluster as expected (Figure 7A). Interestingly, iHSCs derived from the two experiments (8-TF or 8-TF^{Poly}) exhibited very distinct patterns of expression with the iHSCs derived from the 8-TF single virus experiment being more heterogeneous than the iHSCs derived from the 8-TF^{Poly} transduced cells (Figures 7A and S7 and Table S3). Whereas some of the iHSCs 8-TF positioned closely or within the HSC cluster, others mapped closer to MPPs while others positioned closely to the pro-/pre-B cluster (Figure 7A). By contrast, all of the iHSCs derived using the polycistronic virsues (iHSC 8-TF^{Poly}) clustered within the HSC node (Figure 7A). Unsupervised hierarchical clustering analysis showed that whereas approximately equal numbers of iHSCs derived using single 8-TF viruses mapped closely to HSCs (6/23), others

(D) Reconstitution of indicated peripheral blood cell lineages of individual recipients showing >1% donor chimerism presented as percentage (%) of donor.

(E) PCR analysis of immunoglobulin heavy (J_H) chain rearrangement in recipient (Recip), and donor (Donor) myeloid cells. Loading control; genomic PCR for CD45. See also Figures S2 and S3 and Table S2.

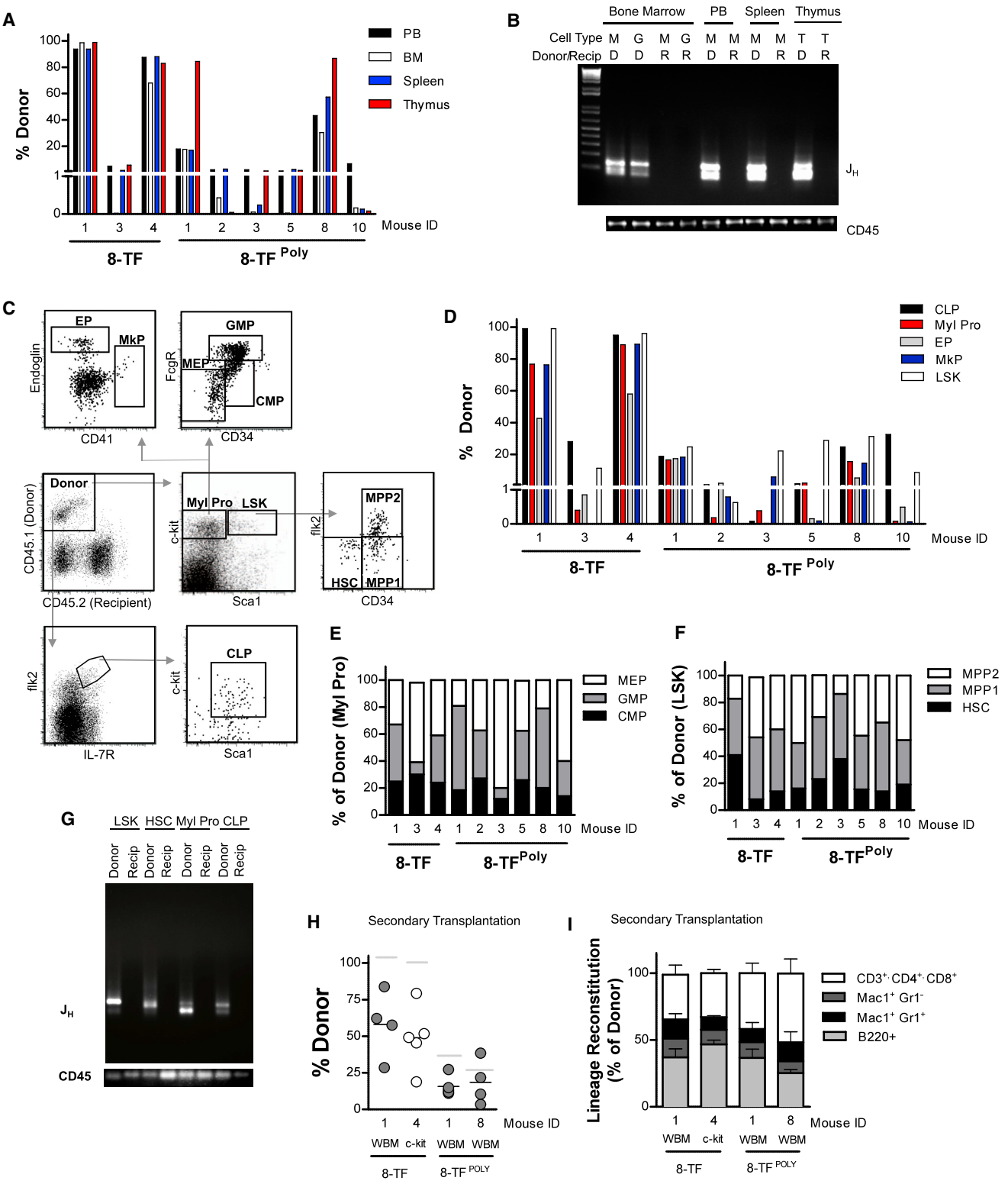

Figure 5. Reprogrammed Cells Engraft Secondary Hematopoietic Organs, Bone Marrow Progenitor Compartments and Reconstitute Secondary Recipients

(A) Donor reconstitution of peripheral blood (PB), bone marrow (BM), spleen, and thymus of mice transplanted with 8-TF or 8-TF^{Poly} transduced pro-/pre-B cells 18–20 weeks posttransplantation.

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mapped closely to MPPs (7/23), while the remainder mapped more closely to pro-/pre-B cells (10/23) (Figure 7B). In contrast, all of the iHSCs derived using polycistronic viruses clustered very closely to host and control HSCs (35/35).

The inclusion of five (*Mycn*, *Hlf*, *Lmo2*, *Meis1*, and *Pbx1*) of the eight reprogramming factors among the 151 genes analyzed in these experiments allowed us to address how endogenous levels of these factors was re-established in iHSCs postreprogramming. Consistent with their known roles in regulating HSCs, high levels of each of *Mycn*, *Hlf*, *Lmo2*, and *Meis1* were observed in steady-state HSCs, which contrasted the low levels observed in pro-/pre-B cells (Figure 7C). *Pbx1* expression was lower in the majority of HSCs and absent in pro-/pre-B cells. Conversely, *Ebf1* and *Pax5*, which are critical transcription factors for B cell development were expressed at high levels in pro-/pre-B cells and negligible levels in HSCs. Analysis of the expression of these genes in iHSCs again revealed distinct differences depending upon whether or not single or polycistronic viruses were used for their derivation. Whereas high levels of endogenous *Mycn*, *Hlf*, *Lmo2*, *Meis1*, and moderate levels of *Pbx1* was reestablished in many of the iHSCs derived using single viruses, low levels of these genes and high levels of *Ebf1* and *Pax5* were still observed in a significant fraction of the cells (Figure 7C). By contrast, the expression of each of these genes in iHSCs derived using the polycistronic viruses closely recapitulated the expression patterns observed in the control HSCs (Figure 7C), as did the expression of all other genes analyzed known to be critical for HSCs function including the transcription factors *Gfi1b*, *Gata2*, and *Ndn*, and the cytokine receptors *Mpl*, and *c-kit* (Figure 7C and Table S3). Taken together, these results demonstrate that 8-TF reprogramming of pro-/pre-B using single viruses generates iHSCs with transcriptional properties consistent with either full or partial reprogramming, whereas iHSCs derived under optimal polycistronic viral conditions exhibit an expression profile highly similar with HSCs.

DISCUSSION

The de novo generation of transplantable HSCs has been a long sought goal in regenerative medicine. Here we report the generation of induced-HSCs via reprogramming from committed hematopoietic progenitor and effector cells. Through functional screening of 36 HSC-enriched factors, we identified six transcription factors *Hlf*, *Runx1t1*, *Pbx1*, *Lmo2*, *Zfp37*, and *Prdm5* whose transient ectopic expression was sufficient to impart HSC functional potential onto committed blood cells in vivo. Inclusion of two additional transcription factors, *Mycn* and *Meis1*, and the use of polycistronic viruses increased reprogramming efficiency. Reprogrammed cells showed multilineage differentiation and extensive self-renewal at the clonal level, and continued ectopic expression of the virally transduced reprogramming factors was not required to sustain the functional potential of the reprogrammed cells in vivo (Figure S2). These findings demonstrate that ectopic expression of a set of defined transcription factors in committed blood cells is sufficient to stably activate the gene regulatory networks governing HSC functional identity.

HSCs are the most well-characterized tissue-specific stem cells yet surprisingly little is known about the molecular mechanisms involved in regulating their central properties. The identification of a defined set of transcription factors capable of stably imparting HSC potential onto otherwise non-self-renewing, lineage-restricted cells, implies that these factors may be critically involved in regulating the transcriptional networks underlying HSC functional identity. Consistent with this, several of the factors that we identified have previously been established to regulate diverse aspects of HSC biology. For example, Pbx1 and Meis1, which interact and can form heterodimeric and heterotrimeric complexes with HOX proteins, regulate HSC self-renewal by maintaining HSC quiescence (Ficara et al., 2008; Kocabas et al., 2012; Unnisa et al., 2012). *Lmo2* is required for hematopoiesis, and in its absence, neither primitive or definitive blood cells form (Warren et al., 1994; Yamada et al., 1998). And while *Mycn* is dispensable for HSC activity due to functional redundancy with *Myc*, combined ablation of both severely disrupts HSC self-renewal and differentiation potential (Laurenti et al., 2008). In contrast to these well-characterized genes, *Prdm5* and *Zfp37* remain unstudied in HSC biology, and although the role of *RUNX1T1* (as known as ETO) as a fusion partner with *RUNX1* in acute myeloid leukemia is well established, its role in normal hematopoiesis remains unclear. Defining the roles that each of the reprogramming factors play in normal HSC biology will be critical for understanding their function in blood cell reprogramming.

Going forward it will also be important to elucidate how the reprogramming factors activate and maintain HSC transcriptional networks. The fact that six of the eight factors we identified

(B) PCR analysis of immunoglobulin heavy (J_H) chain rearrangement in recipient (R), and donor (D) cells. Cell types analyzed include Mac1+ myeloid cells (M), Mac1+GR1+ granulocytes (G), and T cells (T). Loading control; genomic PCR for CD45.
(C) Representative bone marrow stem and progenitor analysis of a recipient transplanted with 8-TF^{Poly} transduced pro-/pre-B cells 18 weeks posttransplantation showing donor-reconstitution of myeloid progenitors (Myl Pro), megarkaryocyte/erythrocyte progenitors (MEP), granulocyte/monocyte progenitors (GMP), common myeloid progenitors (CMP), megakaryocyte progenitors (MkP), erythroid progenitors (EP), common lymphoid progenitors (CLP), Lineage-negative Sca1+ckit+ multipotent progenitors (LSK), multipotent progenitors (MPP1, MPP2), and hematopoietic stem cells (HSC). All cells were pregated through doublet-discriminated, live (propidium-iodide-negative), and lineage-negative cells.
(D) Total donor reconstitution of the indicated populations in mice analyzed in (A).
(E and F) Reconstitution of the indicated myeloid progenitor (E) and primitive multipotent and stem cell (F) populations in mice analyzed in (A) presented as percentage of donor.
(G) PCR analysis of immunoglobulin heavy (J_H) chain rearrangement in the indicated recipient and donor populations. Loading control; genomic PCR for CD45.
(H) Donor reconstitution of secondary recipient mice transplanted with whole-bone marrow (WBM) or c-Kit-positive bone marrow cells derived from primary transplants of 8-TF transduced pro-/pre-B cells analyzed at 16–22 weeks. Number of recipients transplanted; WBM; n = 5, c-Kit+; n = 4. Grey bar indicates donor reconstitution level of primary recipient.
(I) Reconstitution of indicated peripheral blood cell lineages of individual recipients presented as percentage (%) of donor. Error bars represent SEM.
See also Figures S2, S4, S5.

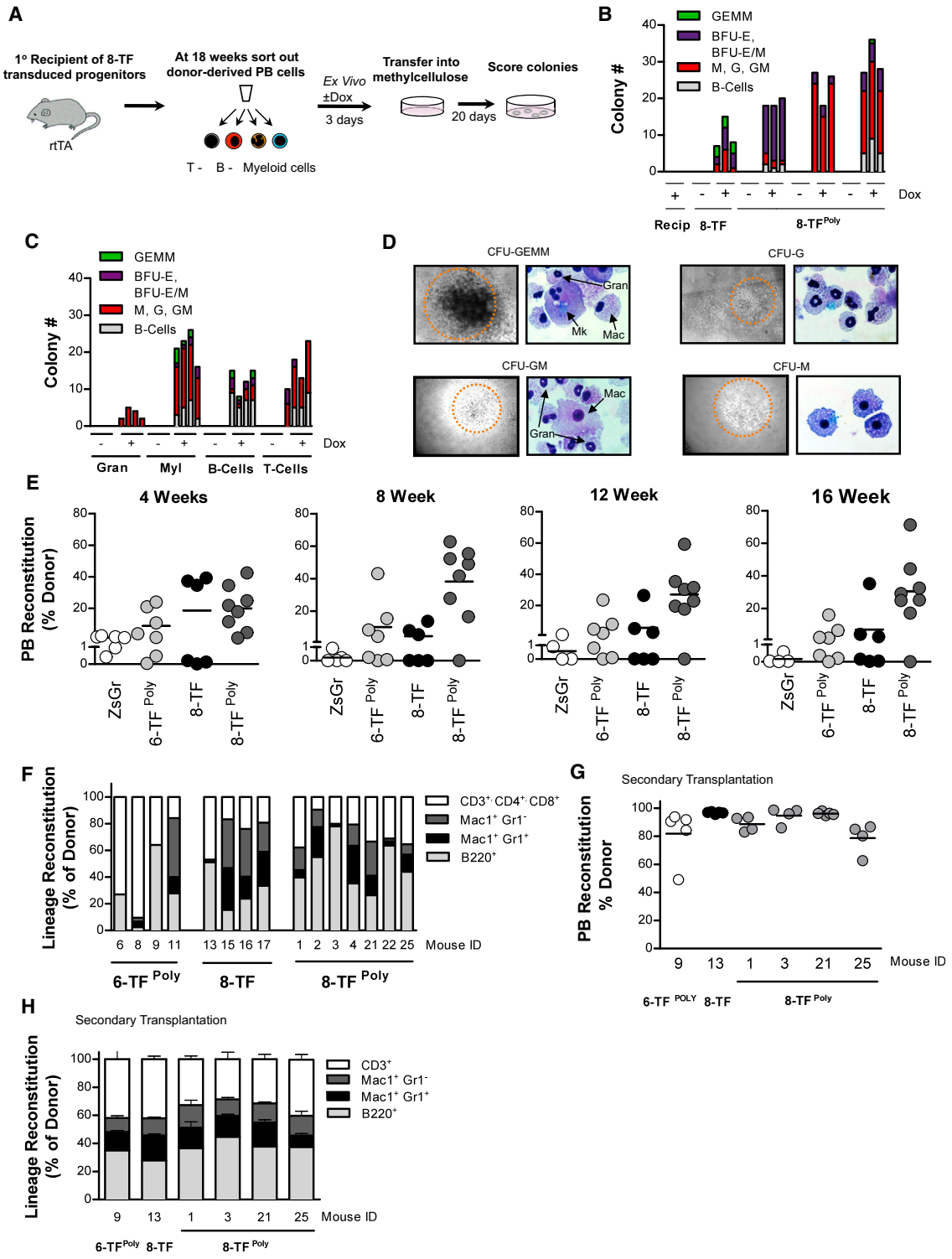

Figure 6. Transient Expression of Defined Transcription Factors in Myeloid Effector Cells Is Sufficient Instill Them with Progenitor Activity In Vitro and Long-Term Multilineage Transplantation Potential In Vivo

(A) Schematic representation of experimental strategy for assaying the colony forming potential of 8-TF transduced peripheral blood cells.

(B) Colony number and type arising in methylcellulose from peripheral blood cells from recipient (left-most lanes) or donor-derived cells from a recipient transplanted with pro-/pre-B cells transduced with 8-TF or 8-TF^{Poly} cocktail, plus (+) or minus (–) exposure to doxycycline. Results from individual mouse performed in triplicate are shown.

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(*Hlf*, *Meis1*, *Lmo2*, *Mycn*, *Pbx1*, and *Runx1t1*) are proto-oncogenes suggests that iHSC reprogramming likely involves the activation and/or repression of gene networks common to stem cells and cancer cells (Reya et al., 2001). This is also consistent with the finding that virtually all the transcription factors required for HSC formation, maintenance, or lineage commitment are targeted by somatic mutation or translocation in hematologic malignancy (Orkin and Zon, 2008). Insights into how individual reprogramming factors may mediate their activity has been provided by recent studies. In one study, Lmo2 overexpression in T cell progenitors led to a preleukemic state with sustained self-renewal yet without blocking T cell differentiation potential (McCormack et al., 2010). Similarly, ectopic expression of Hlf in committed progenitors imbued them with sustained self-renewal activity ex vivo without blocking differentiation potential (Gazit et al., 2013), although this activity was insufficient to impart HSC potential onto downstream progenitors in vivo (R.G., B.S.G., D.J.R. unpublished data). These studies show that while expression of Hlf or Lmo2 can instill some of the functional and molecular properties of HSCs onto committed blood cells, by themselves they cannot activate the full repertoire of programs needed to establish HSC identity. In these regards, it is interesting that whereas iHSCs generated using polycistronic viruses exhibited expression profiles that were indistinguishable from control HSCs, iHSCs generated using monocistronic viruses were heterogeneous at the molecular level, possibly as a result of not receiving the full complement of reprogramming factors.

Although the transcriptional properties of iHSCs derived under optimal 8-TF polycistronic conditions were indistinguishable from endogenous HSCs, further analysis will be required to determine if the epigenetic landscape of these cells is fully reset. In this regard, it was interesting that the donor reconstitution observed in our experiments sometimes, though not always, evolved over time posttransplantation, with donor-derived chimerism and lineage potential changing over time. These results suggest that some reprogrammed iHSCs may need time to fully reset their epigenetic landscape to achieve balanced HSC potential, in a manner similar to the erasure of epigenetic memory observed with continued passage of iPS cells (Polo et al., 2010). Whether or not cell passage influences epigenetic resetting during iHSC derivation is at this point unclear. It is plausible that iHSCs may require a period of ''maturation'' in the stem cell niche to achieve full HSC potential. In these regards it is noteworthy that some of the partially reprogrammed iHSCs we identified had failed to appropriately upregulate the *Mpl* or *cKit* receptors suggesting that an inability to transduce signals in response to TPO or SCF emanating from the niche may have been a factor in the incomplete resetting of their transcriptional state.

Transcription factors play a critical role in the specification of different lineages during development, and as such the discovery of a set of transcription factors capable of activating the gene regulatory networks underlying HSC functional identity suggests that it may be possible to use these factors on cells derived from pluripotent stem cells to facilitate the generation of definitive HSCs. It will also be interesting to test if the reprogramming factors we identified can be used to convert cell types outside of the hematopoietic system to an iHSC fate in a manner similar to the ability of the Yamanaka factors to bestow pluripotency onto cells of diverse lineages. It remains possible, however, that iHSCs derivation using the factors we defined will be limited to the blood system. In these regards it is important to note that although our approach relied on delivery of the reprogramming factors to the target cells ex vivo, it is possible that the reintroduction of the target cells into the hematopoietic microenvironment via transplantation may have provided cues that were critical for iHSC generation. Whether or not derivation of iHSCs from blood cells or other cell types can be achieved ex vivo without input from the hematopoietic microenvironment is unknown, though clearly the lack of suitable culture conditions for ex vivo HSC maintenance may preclude this. Nonetheless, the generation of iHSCs via blood cell reprogramming represents a powerful new experimental paradigm for studying the fundamental mechanisms underlying HSC identity that might eventually be lead to the derivation of transplantable stem cells with clinical potential.

EXPERIMENTAL PROCEDURES

Identification of HSC-Enriched Factors

HSC-enriched factors were identified using microarray data (Affymetrix 430 2.0) of 40 FACs purified populations curated from the Gene Expression Omnibus. For complete list of populations used and accession numbers see Table S1. All data sets were subjected to quality control (QC) measures provided in the ArrayQualityMetrics package of R/Bioconductor. Data sets were normalized (gcRMA) using R. We applied a filter in which the ratio of expression in HSCs to all others was greater than 2.5-fold to define a list of potential HSC regulators that was further defined by cross-referencing the literature.

Colony Forming Assays

pro-/pre-B cells and CMPs sorted from *Rosa26*^{rtTA} mice were plated in 200 μL of S-clone SF-03 media (IWAI North America) supplemented with 10 ng/ml

(C) Colony number and type arising in methylcellulose from plated granulocytes, macrophages/monocytes (Myl), B cells, and T cells purified from the peripheral blood of cells pooled recipients transplanted with pro-/pre-B cells transduced with 8-TF^{Poly} cocktail plus (+) or minus (−) exposure to doxycycline. n = 4 biological replicates per cell type, per condition.

(D) Representative colony types and cytospins stained with May Grunwald of colonies derived in (C).

(E) Donor reconstitution of mice transplanted with ZsGr, 6-TF^{Poly}, 8-TF or 8-TF^{Poly} transduced Mac1+cKit- myeloid effector cells at indicated time points posttransplantation. Only mice with >1% donor chimerism were considered reconstituted. Recipients transplanted; ZsGr; n = 6, 6-TF^{Poly}; n = 7, 8-TF; n = 6, and 8-TF^{Poly}; n = 8.

(F) Reconstitution of indicated peripheral blood cell lineages of mice showing >1% donor chimerism presented as percentage (%) of donor.

(G) Donor reconstitution 16 weeks posttransplant of secondary recipient mice transplanted noncompetitively with 5 × 10^6 donor-derived (CD45.2+) bone marrow cells derived from primary recipients of 6-TF^{Poly}, 8-TF or 8-TF^{Poly} transduced Mac1+cKit- myeloid effector cells. Cells from individual primary donor mice (indicated by ID) were transplanted into n = 5 secondary recipients each.

(H) Average reconstitution of indicated peripheral blood cell lineages presented as percentage (%) of donor. n = 5 recipients per group. Error bars represent SEM. See also Figures S2 and S6.

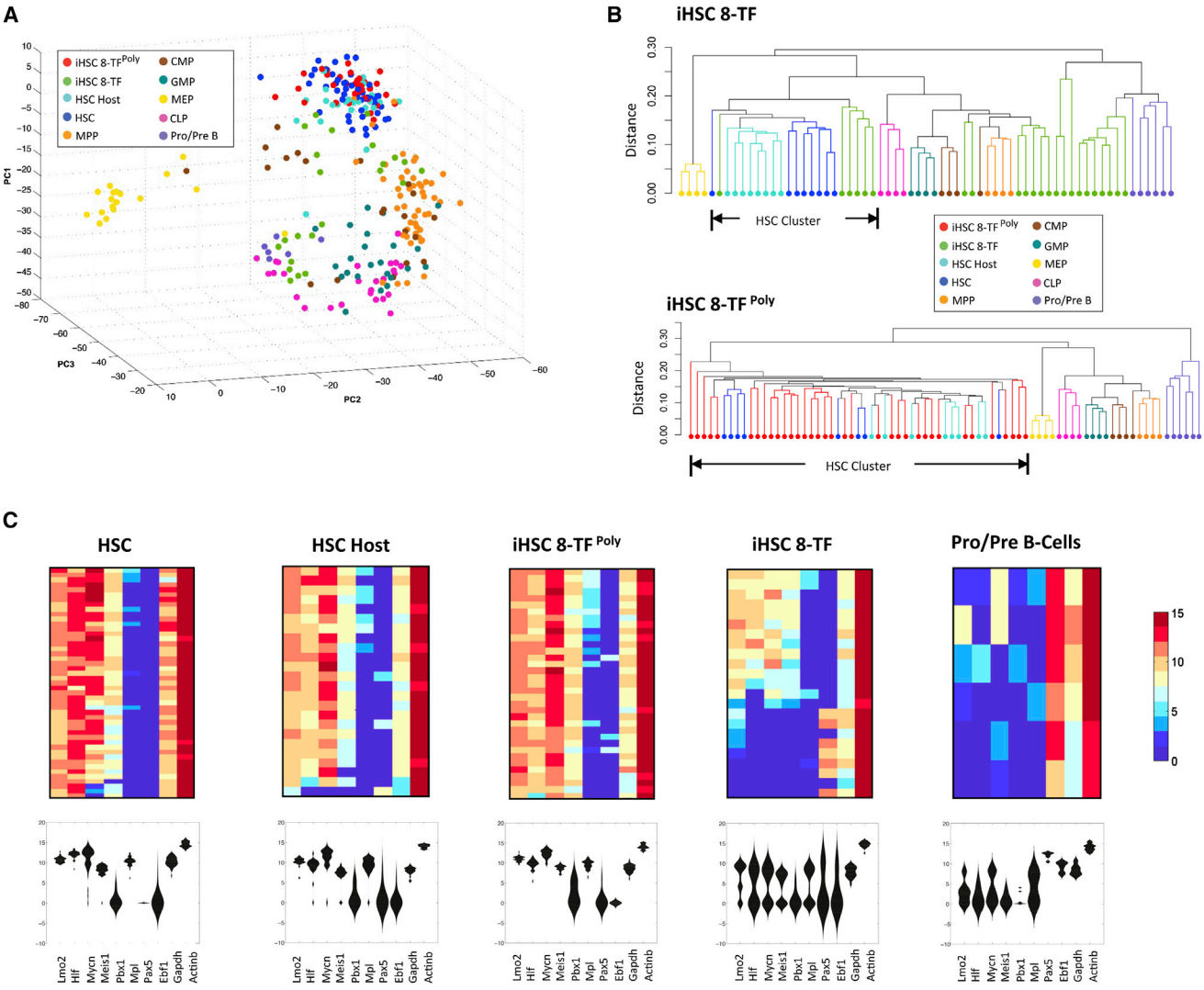

Figure 7. Single-Cell Expression Profiling of iHSCs Reveals Evidence of Partial and Full Reprogramming

(A) Principal component analysis of single-cell expression data of iHSCs and the indicated control cell types. iHSCs were derived from experiments in which pro-/pre-B cells were transduced with the eight identified transcription factors via single (iHSC 8-TF) or polycistronic viruses (iHSC 8-TF^{Poly}). Data for individual cells of given type indicated in the legend.

(B) Dendrograms showing unsupervised hierarchical clustering of single-cell expression data of representative control cells, and all iHSCs generated using single viruses (top), or polycistronic viruses (bottom) as described in (A). Dendrogram branches are color-coded according to cell types indicated in the legend.

(C) Violin plots and the correlation heatmaps showing single cell expression data of the indicated genes. Expression levels are shown with high expression in red, and low expression in blue.

See also Figure S7, Table S3.

SCF, IL-12, and TPO, with 5 ng/ml Flk-3, and 5 ng/ml IL-7 included for pro-/pre-B cells. Viruses were added for 16 hr, followed by addition of 1.0 mg/ml doxycycline for 24–48 hr. Cells were seeded at 10,000 cells/well (pro-/pre-B cells) and 1,000 cells/well (CMPs), into 6-well culture plates, by transferring the contents of the transduction wells including doxycycline into 1.75 ml/well of M3434 methylcellulose (Stem Cell Technologies). For experiments using pro-/pre-B cells, M3434 was supplemented with 5 ng/ml Flk-3 and 5 ng/ml IL-7. For Colony forming cell (CFC) assays involving peripheral blood of transplanted mice, donor-derived blood cells were sorted and cultured at 10,000 cells/well in F12 media supplemented with 10 ng/ml SCF, IL-12, and TPO and 5 ng/ml Flk-3 and IL-7 in the presence or absence of 1.0 mg/ml doxycycline for 3 days followed by transferring the contents of the transduction wells including doxycycline into 1.75 ml/well of M3434 methylcellulose supplemented with IL7 and Flt3L as above. Colonies arising in these assays emerged later than typically observed (10–12 days) when primary HSPCs are plated (Gazit et al., 2013), and were thus scored at 20–22 days postplating.

Transplantation Assays

For experiments with 36-factor and 6-factor single viruses, pro-/pre-B cells or CMPS were transduced with viruses for 2 days with doxycycline added on day 2. On day 3, transduced cells (ZsGr+) were sorted and transplanted at 1×10^4 cells/recipient into irradiated congenic recipients along with 2×10^5 marrow cells. For experiments in which polycistronic viruses were used, sorted pro-/pre-B cells or Mac1+ckit- myeloid effector cells were transduced and

transplanted without re-sorting at 2×10^6 or 5×10^6 cells per recipient into irradiated recipients along with 2×10^5 Sca1-depleted radio-protective bone marrow cells. Due to markedly reduced transduction efficiency of the polycistronic viruses, recipients in experiments involving polycistronic viruses were transplanted at greater cell numbers without presorting transduced cells. Doxycycline was maintained in the drinking water for 2 weeks posttransplant. Serial transplantation from pro-/pre-B cell experiments were performed by transplanting 1×10^7 unfractionated bone marrow cells, or 1×10^4 c-kit-enriched bone marrow along with 2×10^5 Sca1-depleted radio-protective bone marrow cells. Serial transplantation in myeloid effector experiments was performed by transplanting 5×10^6 sorted donor-derived cells per recipient. In all transplant experiments, peripheral blood analysis was performed at 4 week intervals with antibodies against Ter-119, B220, Mac1, Gr1, CD3, CD45.1, CD45.2, and propidium iodide to discriminate dead cells.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2014.04.006.

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