Quantitative production of macrophages or neutrophils *ex vivo* using conditional Hoxb8

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Differentiation mechanisms and inflammatory functions of neutrophils and macrophages are usually studied by genetic and biochemical approaches that require costly breeding and time-consuming purification to obtain phagocytes for functional analysis. Because Hox oncoproteins enforce self-renewal of factor-dependent myeloid progenitors, we queried whether estrogen-regulated Hoxb8 (ER-Hoxb8) could immortalize macrophage or neutrophil progenitors that would execute normal differentiation and normal innate immune function upon ER-Hoxb8 inactivation. Here we describe methods to derive unlimited quantities of mouse macrophages or neutrophils by immortalizing their respective progenitors with ER-Hoxb8 using different cytokines to target expansion of different committed progenitors. ER-Hoxb8 neutrophils and macrophages are functionally superior to those produced by many other *ex vivo* differentiation models, have strong inflammatory responses and can be derived easily from embryonic day 13 (e13) fetal liver of mice exhibiting embryonic-lethal phenotypes. Using knockout or small interfering RNA (siRNA) technologies, this ER-Hoxb8 phagocyte maturation system represents a rapid analytical tool for studying macrophage and neutrophil biology.

Phagocytic cells and inflammation are important in immunologic regulation as well as in the pathology of chronic inflammatory diseases (for example, multiple sclerosis, liver cirrhosis, arthritis, atherosclerosis, diabetes, vascular diseases and inflammatory bowel disease) and acute inflammatory disease (for example, toxic shock syndrome). Normal inflammatory processes have also been linked to complex diseases such as the proliferation and metastasis of cancer[1] and the development of obesity[2]. Understanding the molecular mechanisms by which a phagocyte responds to chemokines and cytokines, activates a proinflammatory cascade, modulates lymphocyte expansion and function, and effects microbial killing will ultimately reveal mechanisms of chronic inflammation, whereas identification of the genetic pathways that control phagocyte differentiation is important for understanding myeloid leukemogenesis. Although the function of innate immune proteins can be studied by knockout technologies that reveal their importance in phagocyte functions, there is no simple protocol to generate large numbers of neutrophils or monocytes from these mutant mice to characterize the impact of a genetic mutation on their differentiation, signal transduction or effector functions.

Class I Hox home domain transcription factors promote the expansion of hemopoietic progenitors, and their expression is deregulated in both human and mouse myeloid leukemia. The abundance of Hoxa9 and Hoxa7 is high in CD34+, Sca-1+, lineage-negative (Lin–) bone marrow populations that are enriched in hematopoietic stem cells (HSC) and in lineage-committed progenitors, and both are downregulated coincident with transition to the CD34– stage of early progenitor differentiation[3,4]. *Hoxa9*$^{-/-}$ mice have five- to tenfold fewer marrow HSC and substantial reductions in myeloid and pre–B cell progenitors[4], whereas retroviral expression of Hoxa9 produces a tenfold increase in the number of long-term repopulating HSC (LT-HSC)[5]. Enforced production of Hoxb8 or Hoxa9 blocks differentiation of stem cell factor (SCF)- or granulocyte-macrophage colony-stimulating factor (GM-CSF)–dependent myeloid progenitors[6–9].

Based on the ability of Hox proteins to arrest myeloid differentiation and permit infinite progenitor expansion, we asked whether factor-dependent hematopoietic progenitors immortalized by estrogen receptor fusions (conditional forms) of Hoxb8 or Hoxa9 could yield cell models of normal neutrophil- or macrophage-restricted differentiation. By testing combinations of progenitor isolation protocols, cytokines used for pre-stimulation and long-term culture, and oncoprotein identity, we developed a simple and reliable protocol to derive macrophage-committed progenitor lines and a second protocol to derive neutrophil-committed progenitor lines.

Based on immunologic, functional and genetic criteria, the macrophages and neutrophils produced from these lines model normal exit from the cell cycle, and acquisition of phagocytic and inflammatory functions. In response to Toll-receptor agonists, macrophages derived from ER-Hoxb8 progenitors upregulated components of NF-κB signaling and downstream effectors genes such as *Il1*, *Il6*, *Ptgs2* and *Tgm2*. The ability of ER-Hoxb8 to drive progenitor expansion and permit their subsequent differentiation

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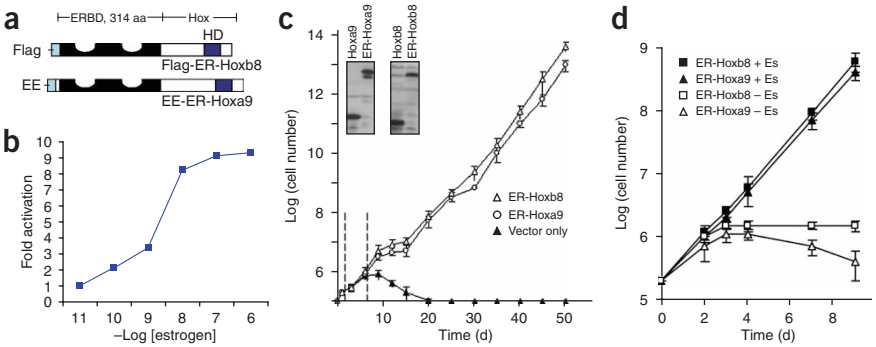

Figure 1 | ER-Hoxb8 and ER-Hoxa9 function conditionally at the biochemical and cellular levels. (**a**) Estrogen-binding domain (ERBD) of the estrogen receptor fused to Hoxa9 or Hoxb8. Epitope tags, Flag and Glu-Glu (EE) are indicated on the left. HD represents the home domain. (**b**) Concentration-dependent transcriptional activity of ER-Hoxa9, measured as coactivation through TGAT-TTAT motifs in cooperation with E2a-Pbx1 in 293T cells. ER-Hoxb8 yielded a similar concentration-dependent activation curve. (**c**) Immortalization kinetics of GM-CSF–dependent progenitors after infection by ER-Hoxa9 or ER-Hoxb8 retrovirus in the presence of estrogen. Retroviral infection was performed at day 0, and dashed lines signify duration of selection in G418. Inserted panel represents a western blot using anti-Hoxa9 (left) and anti-Flag (right) on G418-selected, immortalized progenitors. Immortalization kinetics and doubling times were somewhat faster for progenitors derived in SCF. (**d**) Proliferation of GM-CSF–dependent progenitors immortalized by ER-Hoxa9 or ER-Hoxb8 in continued presence or after withdrawal of estrogen (ES) on day 0.

to neutrophils or macrophages makes this model system an ideal tool to study myeloid differentiation as well as cell biology and inflammatory functions of macrophages and neutrophils.

RESULTS

ER-Hoxb8 and ER-Hoxa9 exhibit estrogen-stimulated function

We fused the estrogen-binding domain of the estrogen receptor (ER) to the N terminus of Hoxb8 and Hoxa9, added N-terminal epitope tags to facilitate subsequent identification, and expressed the fusion cDNAs using a murine stem cell virus (MSCV) retroviral expression vector (**Fig. 1a**). We measured estrogen-regulated transcriptional function of ER-Hoxb8 and ER-Hoxa9 by virtue of their ability to cooperate with activated forms of Pbx to activate transcription of a luciferase reporter driven by TGAT-TTAT Pbx-Hox motifs. Using this assay, ER-Hoxa9 and ER-Hoxb8 exhibited estrogen-dependent transcriptional activation of ten- and threefold, respectively, with half-maximal activation occurring at 10 nM estrogen (**Fig. 1b**).

ER-Hoxb8 immortalizes neutrophil or macrophage progenitors

In the presence of 1 μM estrogen, infection of primary marrow progenitors cultured in interleukin 3 (IL-3), SCF or GM-CSF with retrovirus expressing genes encoding ER-Hoxb8 or ER-Hoxa9 produced immortalized factor-dependent progenitors (**Fig. 1c**), which ceased proliferation (**Fig. 1d**) and exhibited terminal morphologic differentiation upon estrogen withdrawal (**Fig. 2a**). Progenitors were not immortalized when infections were performed in the presence of granulocyte colony stimulating factor (G-CSF) or macrophage colony stimulating factor (M-CSF). By testing different progenitor isolation protocols, cell culture conditions and ER-Hox fusion oncoproteins, we determined conditions for derivation of cell lines demonstrating quantitative neutrophil differentiation or quantitative macrophage differentiation. By using negative selection (removing MacI+, B220+, Thy1.2+ cells) of total bone marrow progenitors followed by a 2-d pre-expansion in SCF, IL-3 and IL-6, and immortalization with *ER-Hoxb8* retrovirus in medium containing SCF as the only cytokine, immortalized

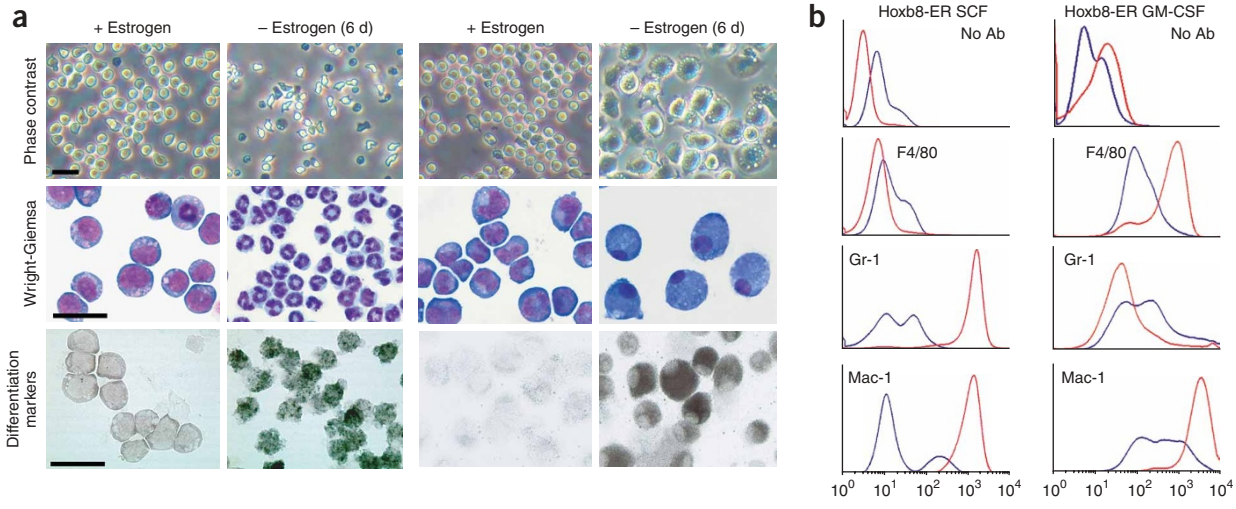

Figure 2 | SCF ER-Hoxb8 progenitors execute neutrophil differentiation and GM-CSF ER-Hoxb8 progenitors execute macrophage differentiation. (**a**) Morphological changes of SCF ER-Hoxb8 neutrophil progenitors (left six images) and GM-CSF ER-Hoxb8 macrophage progenitors (right six images) after 6 d of cell culture in the absence of estrogen. Identical magnifications were used within the context of the phase contrast, Wright-Giemsa and differentiation marker images. Scale bars, 30 μm. (**b**) FACS analysis of neutrophil and macrophage surface markers present on ER-Hoxb8 progenitors in the presences of estrogen (blue) or after the removal of estrogen for 6 d (red). Analyzed are Gr-1 (neutrophil differentiation antigen), F4/80 (macrophage differentiation antigen) and Mac1 (general myeloid differentiation antigen).

progenitors differentiated into 98–99% neutrophils after estrogen withdrawal (1–2% mast cells with an occasional eosinophilic granulocyte; 4 of 4 clones; **Fig. 2a**). We named progenitors derived in this manner 'SCF ER-Hoxb8 progenitors'. SCF ER-Hoxb8 progenitors have proliferated over 9 months, maintaining both a normal 40-XX karyotype (19 of 20 chromosome spreads) and quantitative neutrophil differentiation. By contrast, when we purified total marrow progenitors by centrifugation onto Ficoll (no negative selection for MacI+ committed myeloid progenitors) and immortalized them with *ER-Hoxb8* retrovirus in medium containing GM-CSF as the only cytokine, the progenitors are committed to macrophage differentiation at levels >99% (**Fig. 2a**; 10 of 10 clones). We named progenitors derived in this manner 'GM-CSF ER-Hoxb8 progenitors'. These progenitors have proliferated over two years (over 850 generations), maintaining a normal 40-XX karyotype (in 17 of 20 chromosome spreads; **Supplementary Fig. 1** online) and quantitative macrophage differentiation. Therefore, ER-Hoxb8 progenitors do not become aneuploid as a requirement for immortalization, they do not become aneuploid at a substantial rate over long durations of passage, and they maintain stable commitment to lineage-restricted differentiation pathways.

We characterized neutrophil or macrophage progenitors immortalized by ER-Hoxb8 for surface antigens and enzyme activities characteristic of neutrophils or macrophages. Neutrophils produced by differentiation of SCF ER-Hoxb8 progenitors upregulated NADPH oxidase (**Fig. 2a**), the neutrophil surface antigen Gr-1 and the myeloid integrin Mac1, and downregulated the macrophage marker F4/80 (**Fig. 2b**). By contrast, macrophages produced by differentiation of GM-CSF ER-Hoxb8 macrophage progenitors exhibited activation of macrophage-nonspecific esterase (**Fig. 2a**), upregulation of F4/80 and Mac1 and downregulation of Gr-1 (**Fig. 2b**).

Contrasting the behavior of ER-Hoxb8, ER-Hoxa9 and ER-Hoxa7 progenitors exhibited principally biphenotypic neutrophil and macrophage differentiation regardless of the cytokine used during their derivation. Among 23 clones of GM-CSF ER-Hoxa9 progenitors, 4 exhibited strict neutrophil differentiation, 1 exhibited exclusive macrophage differentiation and 18 exhibited biphenotypic differentiation that favored production of neutrophils over macrophages. We continued characterization of ER–immortalized progenitors because they did not require cloning to select progenitors that differentiated into pure populations of neutrophils or macrophages, and because a simple change in experimental protocol allowed for derivation of either macrophage- or neutrophil-committed progenitors.

Genomic arrays demonstrate normal specific differentiation

We interrogated Affymetrix genome arrays (430 2.0 Array; over 34,000 mouse genes) with RNA from SCF ER-Hoxb8 progenitors undergoing neutrophil differentiation and from GM-CSF ER-Hoxb8 progenitors undergoing macrophage differentiation (**Supplementary Tables 1–4** online) and compared expression of 130 macrophage-specific, neutrophil-specific or general myeloid genes (**Fig. 3**). *Il8rb*, *Ltf*, *Lrg1*, *CD177*, *Camp*, *Lcn2* and other neutrophil marker genes were upregulated selectively during differentiation of SCF ER-Hoxb8 neutrophil progenitors whereas *Mmp12*, *Cd68*, *Msr1*, *Msr2*, *Mgl2*, *Itgax* (CD11c), *Clec4n* and other macrophage-specific marker genes were upregulated selectively with differentiation of GM-CSF ER-Hoxb8 macrophage progenitors (**Supplementary Tables 1** and **2**). The monocytic lineage markers *Irf8*, *Emr1 (F4/80)* and *Tcfec* (also known as *Tfec*) were expressed at high levels in undifferentiated GM-CSF ER-Hoxb8 progenitors, demonstrating their commitment to

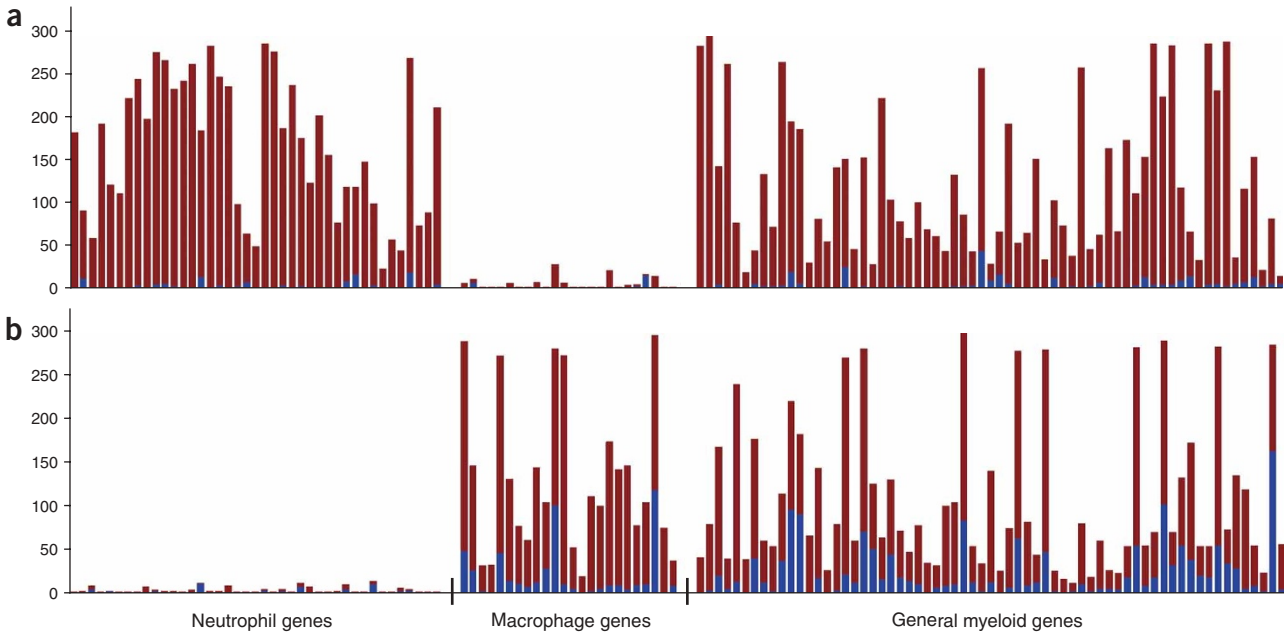

Figure 3 | Lineage-specific gene expression in SCF ER-Hoxb8 neutrophil progenitors and GM-CSF ER-Hoxb8 macrophage progenitors. (**a**,**b**) Affymetrix gene arrays were used to quantify the expression levels of 130 myeloid genes in a ER-Hoxb8 SCF neutrophil cell line (**a**) and a GM-CSF ER-Hoxb8 macrophage cell line (**b**) in the presence of estrogen (blue) and at 6 d of differentiation after estrogen withdrawal (red). Genes plotted are underlined with the categories in **Supplementary Table 3**. The *y*-axis plots the relative abundance of RNA, with blue designating basal levels in undifferentiated progenitors and red designating levels in differentiated cells after 6 d of estrogen withdrawal.

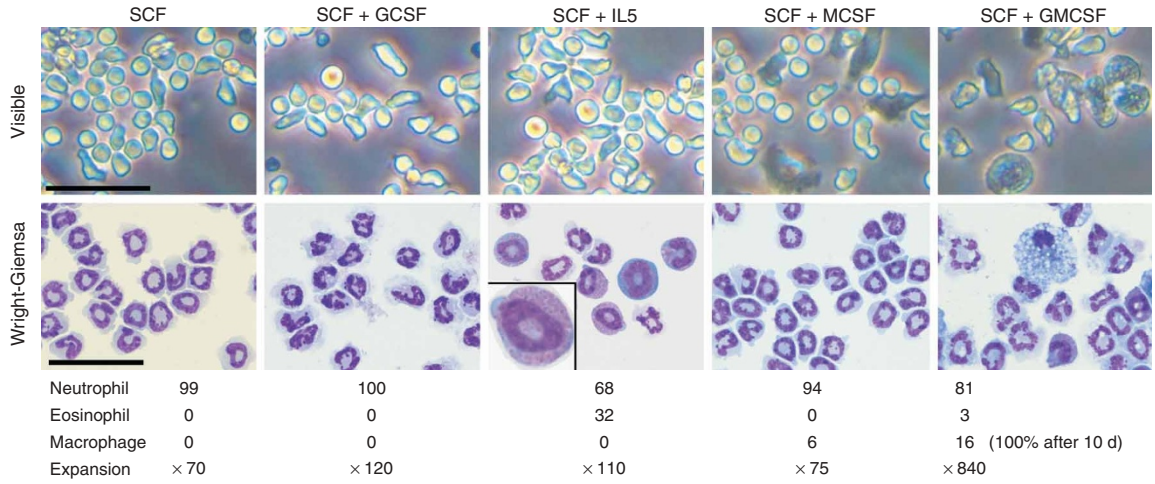

Figure 4 | SCF ER-Hoxb8 progenitors behave as GMP, retaining an ability to differentiate into eosinophils and macrophages. SCF ER-Hoxb8 progenitors were permitted to differentiate in SCF medium supplemented with the lineage-specific cytokines GCSF, IL5, MCSF and GM-CSF, as indicated above the images. Cells were photographed 6 d after differentiation was initiated by removal of estrogen. In the column labeled 'SCF+IL5', a developing eosinophil is magnified at lower left. All photographs within the "Visible" and "Wright-Giemsa" series are presented at identical magnifications. Proportions of mature cell types within cell cultures after 6 d of differentiation are indicated below each column. Scale bars, 30 μm.

macrophage differentiation (**Supplementary Table 1**). Other common myeloid differentiation marker genes were upregulated during maturation of both progenitor cell types (*Fpr1*, *Fpr-rs2*, *Mrc1*, *Tlr2*, *Mmp8*, *Itgam*, *Fgr*, *Lgmn*), whereas common promyelocytic genes, including *Mpo*, *Prt3*, *Ela2* and *Cnn3* were strongly downregulated (**Supplementary Table 1**). Dynamic regulation of neutrophil, macrophage and general myeloid markers demonstrates that both the SCF and GM-CSF ER-Hoxb8 progenitors execute normal differentiation programs. We verified changes in gene expression predicted by Affymetrix arrays for the macrophage scavenger receptor (Msr1) and the transcription factors Rel-B and Jun using immunoblotting, for CD11c using fluorescence-activated cell sorting (FACS) analysis, and for *Gfi1*, *Myb*, *Nolc1*, *Ela2* and *Fos* by northern blotting (**Supplementary Fig. 2** online).

GM-CSF ER-Hoxb8 macrophage progenitors were positioned at a later stage of myeloid differentiation than were SCF ER-Hoxb8 neutrophil progenitors. They exhibited high basal levels of a subset of late differentiation genes, such as *Lyzs*, *Gsn*, *Cd14*, *Lilrb4*, *Pira1*, *Pira6*, *Pilrb*, *Gp49b1* and *Gpnmb* that were not expressed in SCF ER-Hoxb8 neutrophil progenitors, but were strongly upregulated SCF ER-Hoxb8 neutrophil progenitors during their subsequent differentiation (**Fig. 3**). SCF ER-Hoxb8 neutrophil progenitors expressed the stem cell genes *Cd34*, *Flt3*, *Calcr1*, *C1qr1*, *Sox4*, *Hmgn1*, *Hmga2*, *Meis1*, *Erg* and *Nrip1*, which were not expressed in GM-CSF ER-Hoxb8 macrophage progenitors and were shut off during differentiation of SCF ER-Hoxb8 neutrophil progenitors (**Supplementary Table 1**). The more committed differentiation stage of GM-CSF ER-Hoxb8 progenitors was mirrored by their lower expansion potential; inactivation of ER-Hoxb8 in GM-CSF progenitors resulted in cell cycle arrest after only a fourfold expansion, whereas inactivation of ER-Hoxb8 in SCF ER-Hoxb8 neutrophil progenitors was followed by a 90-fold expansion.

Regardless of their striking difference in expansion potential, promyelocytic genes, cell cycle genes and genes encoding cell cycle regulators were downregulated coincident with upregulation of differentiation genes in both progenitor types (**Supplementary Fig. 3** online). Cell cycle genes (for example, *Ccnb1* (also known as *CycB1*) and *Mcm2*), as well as Myc target genes (for example, *Nolc1* and *Shmt2*) fell in parallel with expression of *Myc*, *Myb*, *Ruvbl1* and *Ruvbl2*. Expression of the Myb targets, *Ela2*, *Ctsg*, *Prtn3* and *Mpo* fell in parallel with *Myb*. Synchronous expression of terminal differentiation genes *Fpr1*, *Fpr-rs2*, *Clec7A*, *Mrc1* and *Fgr* paralleled that of the leucine zipper transcription factor genes *Atf3*, *JunB*, *Fos* and *Jund1*. This suggests that a broad program of cell cycle gene down-regulation and differentiation gene upregulation (**Supplementary Table 2**) is driven by downregulation of *Myc* and *Myb* and upregulation of genes encoding bZIP transcription factors[10–12].

Genes controlling divergent aspects of neutrophil and macrophage biology were regulated normally in this cell differentiation model (**Supplementary Tables 1–3**). Maturing phagocytes upregulated genes encoding proteins involved in adhesion (*Itgax*, *Itgam*, *Gpnmb*, *Bst1*), migration (*Cd74*, *Ccr1*, *Ccr5*), phagocytosis (*Sirpb1*, *Clec4b*, *Clec4a3*), activation (*Clec2i*), pathogen pattern recognition (*Mgl2*, *Clec4d*, *Mrc1*, *Clec7a*, *Fpr1*, *Fpr-r2*, *Bst1*), recognition of necrotic cell debris (*Msr1*, *Msr2*, *Cd68*), T-cell activation (*Il18* and genes encoding major histocompatibility complex (MHC) class II antigens Eα, Aβ1, Aα, Eα, Eβ1, DM loci α and β2), MHC class I recognition (*Pira1*, *Pilra*, *Pira6*, *Pilrb1*, *Pirb5*), migration (*Cxcl2*, *Ccr2*), bacterial killing (*Ngp*, *Camp*), opsinophagocytosis (*C3*, *C3ar1*), proteolysis and MHC class II peptide generation (*Lgmn*, *Mmp9*), protease inhibition (*Stfa1*, *Stfa2l1*, *StfA3*, *Timp2*, *Expi1*), nitric oxide biosynthesis (*Arg2*, *Pdi4*), metal ion transport (*Slc11a1*, *Ltf*) and receptor signaling via tyrosine kinases (*Hck*, *Fgr*).

SCF ER-Hoxb8 progenitors mimic the GMP stem cell

Although expression of promyelocytic genes (for example, *Mpo* and *Prt3*) established SCF ER-Hoxb8 progenitors as myeloid, their expression of multipotent stem cell genes *Flt3*, *CD34*, *Meis1* and *Hmgn1*, coupled with their negligible basal expression of any terminal differentiation gene and their low level of differentiation to mast cells and eosinophils (<2%), suggested they might retain the ability to execute alternative differentiation fates in response to

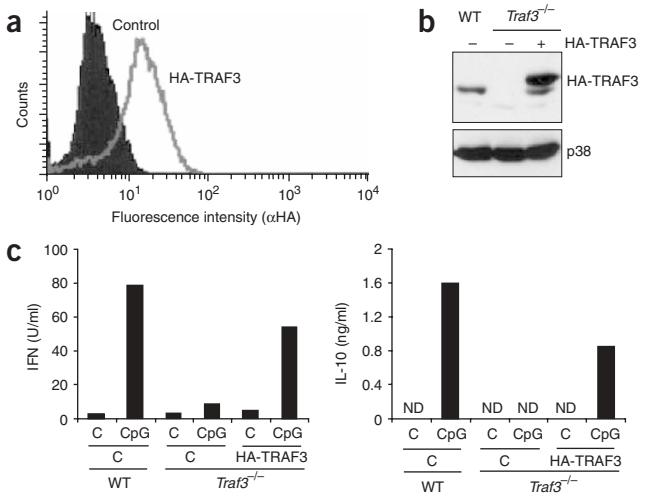

Figure 5 | Re-expression of TRAF3 restores the signaling defect in CpG-induced transactivation of *Ifn* and *IL-10* genes in *Traf3*$^{-/-}$ macrophages produced by ER-Hoxb8. Wild-type (WT) and *Traf3*$^{-/-}$ ER-Hoxb8-immortalized macrophage progenitor cells (cultured in GM-CSF) were transduced with MSCV-Puro retroviral vectors encoding HA-tagged TRAF3 or a control empty vector and selected with puromycin[13]. (**a**) TRAF3 expression was measured by intracellular staining with anti-HA antibodies using flow cytometry, and is presented as fluorescence intensity vs. cell number. (**b**) HA-TRAF3 expression in *Traf3*$^{-/-}$ progenitors transduced with control vector or the HA-TRAF3 expression vector after differentiation for 6 d in the absence of estrogen. Cell lysates were analyzed by immunoblotting, using antibodies to HA and p38. (**c**) Differentiated ER-Hoxb8 macrophages were stimulated with CpG-DNA and analyzed for interferon and IL-10 production by bioassay and ELISA, respectively. ND, not detectable.

other lineage-specific cytokines. To test this hypothesis, we allowed SCF ER-Hoxb8 progenitors to differentiate in SCF medium supplemented with G-CSF, IL-5, M-CSF, GM-CSF or erythropoietin (**Fig. 4**). Inclusion of G-CSF augmented chromatin condensation and increased expansion from 70- to 120-fold. Inclusion of IL-5 induced eosinophilic granules in one-third of maturing granulocytes. Inclusion of GM-CSF increased expansion to 830-fold and produced 16% macrophages. Southern blots demonstrated the same unique retroviral integration site in macrophages derived from differentiation in GM-CSF as for neutrophils derived by differentiation in SCF, proving the multipotent nature of this progenitor (data not shown). M-CSF did not alter expansion, but induced 6% of progenitors to mature as macrophages. Erythropoietin had no impact on neutrophil-committed differentiation. Thus, SCF ER-Hoxb8 progenitors are similar to granulocyte-macrophage progenitors (GMP), which retain the ability to differentiate into eosinophils, neutrophils or macrophages.

ER-Hoxb8 macrophages exhibit robust inflammatory responses

We evaluated the inflammatory response of ER-Hoxb8 macrophages by measuring gene activation in response to lipopolysaccharide, an activator of toll-like receptor 4 (TLR4) and bacterial lipoprotein (BLP), an activator of toll-like receptor 2 (TLR2). Lipopolysaccharide activated strong transcription of the genes encoding members of the NF-κB, Stat, Jun and Egr families of transcription factors (**Supplementary Fig. 4** online), as well as over 50 genes encoding mediators of inflammation (**Supplementary Table 4**), including coactivators of T-cell migration and proliferation (Tnfsf9, Il12b, Il23), monocyte chemokines (Ccl2, Ccl3, Ccl5, Ccl7) and pleotropic cytokines (Ifnb, Tnfa, IL6, IL1a, IL1b, LIF).

ER-Hoxb8 immortalizes e13 fetal liver progenitors

A useful application of the ER-Hoxb8 system would be to immortalize progenitors from mice carrying transgenic or knockout alleles designed to address immunologic and inflammatory functions, particularly those from mice that are difficult to breed or have poor survival rates. To address this issue, we tested whether macrophages produced by maturation of ER-Hoxb8 GM-CSF progenitors from *Traf3*$^{-/-}$ mice would exhibit a signaling defect that could be corrected by expression of wild-type *Traf3* in *trans*. TRAF3 is essential for induction of type I interferons and the anti-inflammatory cytokine IL-10, but is dispensable for induction of pro-inflammatory cytokines[13]. *Traf3*$^{-/-}$ mice die shortly after birth from hypoglycemia and a runting disease that prevents the generation of substantial numbers of macrophages from *Traf3*$^{-/-}$ mice. We infected *Traf3*$^{-/-}$ e13 fetal liver progenitors with ER-Hoxb8 retrovirus and observed progenitor immortalization. To reconstitute Traf3, we expressed hemagglutinin (HA)-tagged Traf3 by infection with an MSCV-based retroviral vector (**Fig. 5a**). Expression of the gene encoding HA-TRAF3 persisted in differentiated monocytes produced by estrogen withdrawal (**Fig. 5b**). We stimulated the differentiated ER-Hoxb8 *Traf3*$^{-/-}$ monocytes and their siblings expressing HA-TRAF3 with CpG-DNA (TLR9 agonist) and measured interferon and IL-10 production by bioassay and enzyme-linked immunosorbent assay (ELISA), respectively. Whereas *Traf3*$^{-/-}$ macrophages produced no interferon or IL-10, we detected robust expression in wild-type cells and in *Traf3*$^{-/-}$ macrophages reconstituted with HA-TRAF3 (**Fig. 5c**). Thus, ER-Hoxb8–immortalized e13 fetal liver progenitors can be prepared from knockout mice whose lethal genetic phenotype would preclude extensive *ex vivo* studies. Then function encoded by the genetic knockout can be reconstituted in ER-Hoxb8 progenitors, and the abundant source of phagocytes generated by the system provides a convenient means to study functional properties of the knockout protein in mature phagocytes.

DISCUSSION

Here we describe a rapid and convenient method to produce unlimited macrophages or neutrophils from mice surviving past e13, a method that overcomes the considerable time, cost and animal mortality involved in using mice as a source of mature phagocytes. Macrophage differentiation of ER-Hoxb8 GM-CSF progenitors is at least as good as that produced by tetradecanoyl phorbol myristyl acetate (TPA) treatment of HL60 cells, IL6 treatment of M1 acute myelocytic leukemia (AML) cells, or M-CSF treatment of Hoxa9 progenitors. Although each of these other models of macrophage differentiation is accompanied by upregulation of *Egr1*, *Egr2*, *Atf3*, *Fos*, *Jun* and *RelB* and downregulation of *Myb* and *Myc* transcription[10–12], they also yield heterogeneous morphologies accompanied by considerable apoptosis, likely to be the result of an incomplete differentiation program that is counteracted by the presence of active oncoproteins. The functional maturation of neutrophils derived from ER-Hoxb8 SCF progenitors is stronger than that produced by G-CSF–induced differentiation of either 32D progenitors or Hoxa9 progenitors. The 32D progenitors fail to upregulate secondary granule genes such as *Ltf*,

and Hoxa9 progenitors fail to downregulate *Myb* and the promyelocytic genes *Ela2* and *Mpo*, and fail to upregulate the secondary granule gene *Ltf*. The incomplete transcriptional modeling of these inducible cell lines is likely due to the persistent oncoprotein activity during differentiation induction, which contrasts the complete inactivation in oncoprotein-ER fusions. All-*trans* retinoic acid (ATRA)-induced differentiation of GM-CSF–dependent EPRO promyelocytes[14] yields results comparable to those observed with ER-Hoxb8, and notably, also inactivates the intrinsic oncoprotein (a dominant-negative retinoic acid receptor α), in the presence of supra-physiologic levels of ATRA.

Other conditional oncoproteins described to date have not evidenced reproducible derivation of lineage-specific progenitors that execute normal differentiation. Avian v-Myb–ER immortalizes primary chicken monocyte progenitors that differentiated, unexpectedly, into multinucleated giant cells similar to bone-marrow–derived osteoclasts[15]. Their expansion in the presence of estrogen is also limited to 10^7 cells. Mll-Enl-ER (tamoxifen-regulated) immortalizes a biphenotypic progenitor that requires 14 d to exit the cell cycle after removal of tamoxifen, and to differentiate into neutrophils and monocytes[16]. Terminal differentiation of progenitors immortalized by E2a-ER-Pbx1 is variable (5–12 d), and although derivation of neutrophil-committed progenitors is frequently observed using the Δ1 E2a-Pbx1 mutant, derivation of macrophage-committed progenitors is rare[17], an observation somewhat akin to the behavior of ER-Hoxa9 in this study, which yielded mostly biphenotypic progenitors, a lower number of neutrophil-committed progenitors and rare monocyte-committed progenitors. Therefore, oncoprotein-specific functions dictate the expansion potential, differentiation stage and differentiation fate of immortalized progenitors. ER-Hoxb8 has the advantage of targeting or programming progenitors so that its subsequent inactivation results in their predictable differentiation into neutrophils or macrophages.

ER-Hoxb8 is unique in being able to target two different types of progenitors, one producing macrophages and another producing neutrophils under defined cell-culture conditions. These properties will be useful for investigations into the differentiation, signaling and effector mechanisms of phagocytes, and having an infinite supply of cells will permit functional analysis by classical biochemistry or molecular biology approaches. One clear application of this system is to understand how Hox oncoproteins maintain the undifferentiated state in human myeloid leukemia, a goal that can now be approached by determining the biochemical link between Hox activity and maintaining transcription of other proto-oncogenes involved in cancer and stem cell expansion, such as *Myb*[18–21], *Myc*[22], *Ruvbl1* (ref. 23), *Gfi1* (ref. 24) and *Hmgb3* (ref. 25). A second application is to understand transcriptional mechanisms governing terminal phagocyte differentiation, such as those controlling activation of *Lfn*, the pattern recognition gene *Fpr*, or the antimicrobial gene *Camp*. Studies focused on macrophage differentiation lack easily derived model systems[14]. A third application is to identify new genes controlling phagocyte differentiation, an example of which may be 30-fold upregulation of the dual-specificity phosphatase *Dusp1* (cell cycle regulation[26]) and strong downregulation of the tyrosine phosphatase *Ptpn1*.

The accuracy of ER-Hoxb8 progenitors in modeling normal differentiation suggests it should be possible to derive hematopoietic progenitors that model erythroid, megakaryocytic or lymphoid differentiation, or even epithelial progenitors that model breast ductal formation or colonic microvillar differentiation using conditional oncogenes that are native to tumors within these progenitors coulpled with cytokines that drive their normal expansion. Such models of tissue differentiation would be useful tools in understanding the biochemistry and genetics of how oncogenes enforce the stem-cell phenotype, as well as in understanding the functions of differentiated cell types that are difficult to culture from native sources.

METHODS

General methods. We performed spectral karyotype analysis[27] and northern blots[28] as described previously. For western blotting, we used antibodies to Hoxa9, Flag, HA, RelB, Jun, RelA and Msr1 as described previously[6,17,29,30]. FACS analysis, Wright-Giemsa staining, the nitroblue tetrazolium reduction (NBT) assay and the nonspecific esterase (NSE) assay were performed as described previously[17].

Derivation of SCF-dependent neutrophil progenitors using negative selection of progenitors followed by cytokine pre-stimulation and cultivation in SCF. We harvested bone marrow from the femur and tibia of female Balb/c mice and obtained lineage-negative progenitors by negative selection using an antibody cocktail reactive against MacI, B220 and Thy1.2 followed by removal of lin+ cells on a magnetic column (Stemcell Technologies). We pre-stimulated progenitors for 48 h in Iscoves modified Dulbecco medium (IMDM) containing 15% fetal bovine serum (FBS), 1% penecillin-streptomycin-glutamine (PSE; Gibco), 50 ng/ml stem cell factor (SCF), 25 ng/ml IL-3 and 25 ng/ml IL-6. We infected 25,000 marrow progenitors with 1 ml *ER-Hoxb8* retrovirus by spinoculation (2,500*g*, 2 h, 22 °C) in the presence of lipofectamine (1:1,000; Gibco), as described in **Supplementary Methods**. We culture the infected progenitors in OptiMem medium containing 10% FBS, 1% PSE, 10 ng/ml SCF, 30 μM β-mercaptoethanol (1 μl into 500 ml medium), and 1 μM β-estradiol (estrogen; Sigma). We approximated an infection efficiency of 10% based on comparison of the initial rates of progenitor outgrowth in the presence or absence of G418 selection. We enriched immortalized myeloid progenitors by the serial passage of nonadherent cells every 3 d into new 12-well tissue culture plates. Immortalized progenitors predominated cultures infected by Hox-ER retroviruses by day 14 (cell generation time 18–20 h), whereas control cultures had reduced proliferation and stopped dividing by day 21. If GM-CSF was substituted for SCF during selection of immortalized progenitors in this protocol, clones yielding biphenotypic differentiation to neutrophils and macrophages arose. A detailed protocol is available in **Supplementary Methods**.

Derivation of macrophage-committed progenitors immortalized by ER-Hoxb8 or of biphenotypic, neutrophil or macrophage progenitors immortalized by Hoxa9-ER. We isolated bone marrow from the femurs of mice after ammonium sulfate lysis of red blood cells and centrifugation onto a cushion of Ficoll-Paque (Pharmacia). Ficoll-purified progenitors can be used directly or pre-stimulated 48 h in 50 ng/ml SCF, 25 ng/ml IL3 and 25 ng/ml IL6. We subjected 100,000 Ficoll-purified mononuclear cells to spinoculation with 1 ml of *ER-Hoxb8* retrovirus. We cultured infected progenitors in 'myeloid cell medium' (RPMI 1640 with

10% FBS, 1% PSQ, 1% GM-CSF–conditioned medium from B16 melanoma expressing the *Csf2* cDNA (approximately 10 ng/ml GM-CSF) and 1 μM estrogen). We stored estrogen as 1,000× (1 mM) or 10,000× (10 mM) stocks in 100% ethanol at –20 °C. We approximated an infection efficiency of 10% based on comparison of rates of progenitor outgrowth in the presence of G418, which selects for the coexpressed neomycine phosphotransferase gene encoded by MSCV. We selected immortalized myeloid progenitors by removal of nonadherent progenitor cells every 3 d to a new well in a six-well culture plate. We continued this procedure over 3 weeks to produce immortalized macrophage progenitor lines. Selection for G418 resistance permitted derivation of immortalized progenitors in 10–14 d. Differentiation to macrophages was performed in the same base medium. Detailed protocols are available in **Supplementary Methods**.

Derivation of neutrophil, macrophage and biphenotypic progenitors, using ER-Hoxa9 or Hoxa7. These progenitors were derived using the same culture system as described for GM-CSF ER-Hoxb8 macrophage progenitors. Derivation of progenitors committed exclusively to neutrophil or monocyte differentiation required cloning, which we performed using 96-well microtiter plates.

Institutional committee approval for animal use. Experimental protocols were approved by the University of California, San Diego animal subjects committee.

Additional methods. contains experimental details on construction of ER-Hox proteins, cell culture and freezing techniques, luciferase reporter assays, Affymetrix array analysis, and in-depth descriptions of the derivation of GM-CSF ER-Hoxb8 and SCF ER-Hoxb8 progenitor lines are available in **Supplementary Methods**.

Note: Supplementary information is available on the Nature Methods website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Methods* website for details).

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Supplementary Methods

I. Construction of tagged, estrogen receptor fusions of Hoxb8 and Hoxa9. A murine stem cell provirus (Mscv) expressing Hoxb8-ER or Hoxa9-ER was generated by inserting estrogen-binding domain (ERBD) of estrogen receptor (ER) at an N-terminal MluI restriction site engineered into epitope-tagged murine Hoxb8 or Hoxa9 proteins. Tagged Hoxb8 was generated by PCR using the 5' primers (FLAG tag: g **gaa ttc** gcc acc ATG *GAC TAC AAG GAC GAC GAT GAC AAA GGA* **ACG CGT** GGA AGC TCT TAT TTC GTC AAC TCA C; HA tag: g gaa ttc gcc acc ATG GGA *TAC CCA TAC GAT GTT CCG GAT TAC GCT* **ACG CGT** GGA AGC TCT TAT TTC GTC AAC TCA C) and the common 3' primer: ccg **ctc gag** tta **CTA** CTT CTT GTC ACC CTT CTG CG. Underlined sequences complement the 5' sense strand sequences for amino acid positions 2 to 7 and the 3' antisense strand sequences encoding the last 7 amino acids. Sequences encoding the Flag (DYKDDDDKG) or HA (YPYDVPDYA) tags are in italics. There is a unique *EcoRI* site (bold lower case) followed by canonical *Kozac* sequence (gcc acc) preceding the initiating ATG (first capitalized codon), and a unique *XhoI* site (bold lower case) after the stop codon (bold upper case). Following sequences encoding the tag (italics), there is a unique in-frame *MluI* site in each 5' PCR primer (bold upper case) as well as an additional GGA codon encoding glycine which could facilitate flexibility between the tag and Hox protein domains. This is an important consideration, because we have demonstrated that the N-terminal domain of Hox proteins is critical for their immortalizing function. In preparation for cloning, the PCR product was digested by *EcoRI* and *XhoI*, and ligated into the pMscvNeo proviral vector (Clontech). To generate ER fusions of Hoxb8, in-frame sequences encoding the estrogen-binding domain of the human estrogen receptor (residues 282 to 595; ERBD) containing a Gly400Val mutation were produced by PCR using primers containing in-frame *Mlu*I sequences at their 5' ends followed by digestion with MluI and ligation into the MluI site of each tagged Hoxb8 construct. The Gly400Val mutant ER was used because this point mutation renders the receptor insensitive to the low levels of estrogen found in fetal bovine serum (FBS) as well as to the estrogenic effects of other compounds, such as phenol red.

To generate conditional Hoxa9 proteins, codons encoding amino acids 4 to 5 of Hoxa9 were mutated into an *MluI* site in pGEM3zf-EE-Hoxa9, and the same fragment encoding the mutant human ERBD described above was ligated into the Hoxa9 *MluI* site. The EE-ER-Hoxa9 coding sequence was excised by *EcoRI* and inserted into pMscvPuro (Clontech). All plasmids were sequenced over their cloning junctions to verify integrity.

II. Luciferase reporter assay: Estradiol-responsiveness was evaluated by measuring the ability of Hox-ER and an activated form of E2a-Pbx1 (or Vp16-Pbx1) to induce coopertiave activation of a luciferase reporter gene driven by tandem repeats of TGAT-TTAT motifs in Nalm6 (for Hoxa9-ER) or 293T (for Hoxb8-ER) cells cultured in medium supplemented with a wide range of concentration of □-estradiol (10^{-11} M to 10^{-5} M). Transcription activation was calculated as the value of relative light units for firefly luciferase versus that for a control renilla luciferase construct that was cotransfected in all samples.

III. Cell culture: Myeloid progenitor lines were maintained in a 37°C humidified incubator with 5% CO_2. Progenitors were frozen as described below, and stored in liquid nitrogen.

IV. Affymetrix array analysis: Gene expression profiling analysis was performed and analyzed using affymetrix mouse total genome array. Hybridization and quantitation of array signals were performed by the UCSD gene chip core laboratory, using the GeneChip Scanner 3000, enabled for high-resolution scanning, coupled with GeneChip operating software (GCOS). Array data was normalized to internal controls, and the overall chip signal intensity was normalized to the mean signal intensity across a group of 12 chips used to evaluate RNA levels in Hoxb8-ER progenitors undergoing neutrophil or macrophage differentiation, and in Hoxb8-ER macrophages stimulated with LPS or BLP. Data was analyzed using "perfect match minus mismatch" algorithms, and signature genes identified exhibited a minimum 5-fold difference in signal intensity between the averages of each groups of three samples. Normalization and processing of GCOS data was performed using dCHIP software. All data is normalized to a basal level of "1", which was selected as a moderately low level of expression.

V. Producing Retrovirus by CaPO4 transfection of 293T cells

1. Helper-free retrovirus is produced in 293T cells by $CaPO_4$ co-transfection of the retroviral construct with an ecotropic or amphotropic packaging construct (CellGenesys).
2. We use Invitrogen's $CaPO_4$ Transfection Kit (#44-0052)
   a. Day 0
      - Seed 2 x 106 293T cells into a 10cm dish with 10ml DMEM (High glucose) + 10% FBS + penicillin/streptomycin/glutamine
   b. Day 1
      - Remove media and replace with 10ml of fresh, pre-warmed media. Cells should be at ~60-70% confluence
      - Transfect 10µg of retroviral construct + 10µg of packaging construct as per protocol
        (remember to use ecotropic packaging construct for murine marrow, amphotropic for human cells)
      - Incubate overnight
   c. Day 2
      - Remove media and replace with 6ml of fresh, pre-warmed media.
   d. Day 3
      - Harvest 6ml virus to a 15ml conical tube.
      - Centrifuge briefly to pellet all cell debris. Freeze in 1-2ml aliquots in 2ml freezing tubes and store at –80°C. Alternatively, filter the virus supernatant and use immediately.
      - Add another 6ml of fresh, pre-warmed media to transfected cells.
   e. Day 4
      - Harvest an additional 6ml virus.

- At this point, it is a good idea to thaw a small (~250µl) aliquot for the purpose of determining the viral titer

3. Depending on the size of the insert, we usually attain viral titers between 10^5 – 10^6/ml

VI. Titering Retrovirus

If this is the first time working with retrovirus, you should definitely determine the titer by infecting an "easily-infectable" cell line. Having determined that your protocol generates a good titer of virus, it is rarely necessary to titer each additional batch. Generally speaking, NIH3T3 cells are ideal for determining the titer of ecotropic retrovirus, while 293/293T (human embryonic kidney) cells are good for amphotropic virus.

1. Day 1

A. Set up 150,000 NIH3T3 cells onto 6 60mm dishes

B. NIH3T3 cells grow best in DMEM (high glucose) + 10% FBS + P/S/Glutamine

2. Day 2

A. The cells should be ~20-30% confluent

B. Prepare ~25ml of media. Add Lipofectamine 1:1000
(Can alternatively use polybrene at a final 8µg/ml)

C. Thaw an aliquot of virus and prepare a series of 5 tubes of consecutive 1:10 dilutions, serially diluting 100µl of virus with an additional 900µl of media containing Lipofectamine

D. Aspirate the media on the NIH3T3 cells and add 1.5ml of media with Lipofectamine (no virus yet)

E. Add 500µl of each viral dilution and use 500µl of plain media for the control plate

F. Allow the infection to proceed 4 hours at 37°C.

G. Dilute with 5ml of fresh media <u>without</u> Lipofectamine

3. Day 4

A. Aspirate the media

B. Add fresh media with G418 (final concentration of active drug 1mg/ml)

i. We use Gibco's Geneticin (liquid, dissolved at 50mg/ml active drug, cat # 10131-035)

ii. Of course, Puromycin selection (1µg/ml) works with vectors carrying the puro^R cassette

4. Days 6+

A. Change media every 2-3 days, maintaining G418 selection

B. Don't be deceived! After 5 or so days in G418, the cells are not yet dead! Don't assume that those adherent cells are G418 resistant! Continue changing the medium on the cells for the full 10 to 14 days!

C. By day 10-14, the cells on the control plate should be completely dead and detached and individual colonies should be visible on the other plates. If your virus is of good titer, the plates with the 1:10 and 1:100 dilutions of

virus should be completely confluent. These can be discarded. Count the # of colonies on the other plates to determine the viral titer

D. Counting the colonies can be done by staining with Giemsa stain (Sigma)
   i. Aspirate the media. Rinse 1x with PBS
   ii. Flood the dish gently with 80% methanol. Sit for 5 minutes at RT
   iii. Aspirate the methanol. Flood the dish gently with 20% Giemsa. Sit for 10' at RT
   iv. Aspirate the Giemsa and wash the cells 1-2x with PBS
   v. The colonies should be easily visible and easy to count

dilution	*actual virus (in 500µl)*	*expected colonies (viral titer of 10^5/ml)*	*expected colonies (viral titer of 10^6/ml)*
1:10	50µl	5000	50,000
1:100	5µl	500	5000
1:1000	0.5µl	50	500
1:10,000	0.05µl	5	50
1:100,000	0.005µl	0.5	5

VII. Harvesting Marrow

1. Sacrifice female Balb/c (or other strain, see FAQs) mouse (generally 8-12 weeks, though older is OK)
2. Remove intact femurs and tibia into sterile dishes of PBS on ice
3. Cut off the ends of the bones
4. Use 10ml syringes (filled with RPMI 10% FBS) and 25G needles to shoot the marrow into a 50ml conical tube
   a. the bones should become translucent
   b. Use 1 x 50ml conical tube for each mouse
5. Top up to 50ml with PBS and pellet the cells
6. Resuspend well in 10ml ACK red blood cell lysis buffer. Incubate 5 min at RT.
   a. ACK RBC lysis buffer
      150mM NH_4Cl
      10mM $KHCO_3$
      0.1mM Na_2EDTA
      Adjust to pH 7.2-7.4 with 1N HCl
      Filter sterilize and store at 4°C
7. Top up to 50ml with PBS and pellet the cells.
8. Resuspend the cells in 4ml of PBS

Using 5-Flurouracil prior to isolation of marrow and progenitors

One can inject mice with 5-Flurouracil (5-FU) 3-5 days prior to harvesting the bone marrow. Injections are done at 100-150mg/kg I.P. The 5-FU will greatly reduce the total

cellularity of the marrow with an increased % of progenitors (though we do not find a dramatic increase in total # myeloid CFU). The advantage of the 5-FU is that the marrow from more mice can be processed on the same Ficoll gradient and on the same StemCell Technology column (using less reagent). As mentioned above, if your retroviral infection/immortalization is of high efficiency (as we have observed for Hoxa9-ER, Hoxb8-ER, and E2a-ER-Pbx construct EPSΔ578ER), you do not NEED to treat with 5-FU or isolate marrow progenitors by negative selection. Infections can be done on "crude" marrow simply by removing the bone marrow cells and passing them over a Ficoll gradient before plating them under cytokine pre-stimulating conditions.

NOTES:

A. Cell numbers following purification with no 5-FU pre-treatment
   1. One generally gets 15-30 million cells (post-Ficoll) per mouse.
   2. Of these, ~2-3% are progenitors as isolated from the StemCell Technologies column.
   3. After a 3 day stimulation in cytokine(IL3, IL6, SCF), these cells will usually double in number.

B. Cell numbers following purification WITH 5-FU pre-treatment.
   1. One generally gets 2-5 million cells (post-Ficoll) per mouse
   2. Of these, 5-20% are progenitors, base on the fact that this fraction is isolated from the negative-selection column
   3. In our experience, we do not see much of an increase in the total # of progenitors following 5-FU treatment (i.e. while the total cellularity decreases, the % of progenitors increases but the total # of progenitors does not increase all that much). However, it is very convenient to have fewer cells for the manipulations with the column and thus the use of 5-FU is recommended in my opinion.

VIII. Harvesting Fetal Liver Cells

1. Sacrifice pregnant mouse
2. Remove embryos (we've used them as early as day 11)
3. Harvest fetal liver
4. Using the plunger from a 5ml syringe, disperse the cells through a 70μ cell strainer (Falcon #352350)
5. Pellet cells
6. Resuspend well in 10ml of ACK red blood cell lysis buffer (use recipe in **VII**). Incubate 5 min at RT.
7. Top up to 50ml with PBS and pellet the cells.
8. Pellet cells and rinse 1x in PBS
9. Resuspend the cells in 4ml of PBS

IX. Negative selection using Stem Cell Technologies Murine Progenitor Isolation Cocktail

1. Wash cells derived from marrow or fetal liver 1x in PBS

2. Resuspend in PBS+FBS+Rat Serum at 2-8x10^7cells/ml (~1-2mls is all)
3. Incubate in fridge at 4° for 15minutes
4. Proceed with Progenitor Cell Column Isolation as described in the Stem Cell Technology manual

NOTES:
1. Using no treatment with 5-FU:
    a. One generally gets 15-30 million cells (post-Ficoll) per mouse (tibias and femers).
    b. Of these, ~2-3% are progenitors as isolated from the StemCell Technologies column.
    c. After a 3 day stimulation in cytokine (IL3, IL6, SCF), progenitors will double in number.

2. Using pre-treatment with 5-FU (see "5-FU pre-treatment" protocol in section III)
    a. One generally gets 2-5 million cells (post-Ficoll) per mouse
    b. Of these, 5-20% are progenitors, based on negative-selection by the StemCell Technologies column
    c. In our experience, we do not see much of an increase in the total # of progenitors following 5-FU treatment (i.e. while the total cellularity decreases, the % of progenitors increases but the total # of progenitors does not increase all that much). However, it is very convenient to have fewer cells for the manipulations with the column and thus the use of 5-FU is recommended in my opinion.

Cytokine pre-stimulation of the cells
For a good retroviral infection, progenitors subjected to negative selection are stimulated to enter the cell cycle by transferring them to a cytokine-rich media for 2 days. The media described below is very effective though others that include G-CSF, Flt3-ligand, etc. are likely to be equally effective. Cytokines are purchased from Peprotech as their activity is consistent and they are reasonably priced.

Stem Cell Media
IMDM (Iscove's) + 15%FBS + 1%pen/strep/glutamine
10ng/ml murine IL-3 (5 ng/ml is probably sufficient)
20ng/ml murine IL-6
25ng/ml murine SCF (some protocols use up to 100 ng/ml)

Notes on the cytokine stimulation*:*
A. RPMI with 10-15% FBS can be substituted for Iscove's media
B. This is a "low concentration" cytokine cocktail. Many progenitor cocktails call for up to 100ng/ml as well as the addition of other cytokines such as Flt3 ligand or G-CSF. At the same time, we have gone as low as 10ng/ml for all three cytokines (IL-3, IL-6, and SCF) and for a 3 day culture, this seems sufficient.
C. The cells begin to divide actively after approximately 2 days in culture. Therefore, for a 2-3 day incubation, one expects about one cell doubling. After 3

days, however, the cells are actively dividing and can be expanded should one require more cells. We have not assayed or infected the cells past 4 days in culture, however, and are not sure for how long the cells retain their immature phenotype. During the culture, some mature monocytes will adhere to the dish and will not be harvested with the non-adherent cells used for the retroviral infection.

X. Spin Infection Protocol – for infection in 12-well plates

1. Coat a non-TC treated plate with Fibronectin

a. Falcon sells these plates (12-well #351153 or 6-well #351146)
b. Human fibronectin is supplied as a 1mg/ml solution from Sigma (F-0895)
c. Dilute the fibronectin 1:100 in PBS to a final 10µg/ml solution
d. Aliquot 1ml into each well of a 12-well non-tissue treated plate (or 2ml per well in a 6-well plate). Apparently the Fibronectin coats non-TC treated plates more efficiently, though we have used both types of plates with success
e. incubate at 37° for 1-4hrs or at 4° overnight

2. Set up cells

a. Count cells and resuspend at 10^5-10^6/ml in "Progenitor Outgrowth Medium" Use this medium for the Hoxb8-ER virus. Estradiol is only necessary for use with ER-fusion oncoproteins). We have not tested whether the BME is necessary for derivation of neutrophil progenitors described in the Nature Methods manuscript.
b. Add 1µl of Lipofectamine per ml of cells. Or, add 1µl per ml of expected final volume if you are **NOT** going to add Lipofectamine separately to the virus. This shortcut is useful if one is using 250µl of cells and 1ml of virus in a 12-well plate
c. Aspirate the fibronectin.
d. Aliquot 250µl (~25,000 to 250,000 cells) into each well. Usually I try not to exceed the titer of the virus
e. Place the plate at 37°C while thawing and preparing the virus

3. Virus

a. Thaw the virus rapidly in a 37°C water bath
b. Add 1-2ml of virus to each well of the 12-well plate. Make sure that the final Lipofectamine concentration is 1x (1:1000)

4. Spinoculation

a. Wrap the plate(s) in Saran Wrap with an equivalent balance plate
b. Spin the plate in plate carriers at 1500g for 60-90 minutes at 22°-32°. Forces up to 4500g have been tested with good results. This is easiest in Gernot Walter's Beckman JS5.2 rotor at 2800rpm (r=20cm, ~1300g)
c. You do not need to remove the cells/virus from the 12-well plate.
d. Dilute the Lipofectamine/Polybrene with 3ml of fresh "Progenitor Outgrowth Media" and incubate the cells at 37°C.

Progenitor Outgrowth Medium

OptiMem
10% FBS
1%PSG,
10ng/ml stem cell factor or 1% culture supernatant from an SCF-producing cell line
30uM beta mercapto ethanol (1ul neat into 500mls medium)
1uM estradiol

XI. Progenitor purification using Ficoll-Hypaque centrifugation (For derivation of macrophage-committed progenitors immortalized by Hoxb8-ER or biphenotypic, neutrophil, or macrophage progenitors immortalizated by Hoxa9-ER**).**

1. In a 15ml conical tube, add 3ml of room-temperature Ficoll-Paque (Pharmacia, Piscataway, NJ) and layer 4ml of total marrow cells in PBS gently on top.
    a. try not to exceed 50 million cells per gradient
    b. usually this means one layer per mouse unless you used 5-FU (see 5-FU protocol and "Harvesting Total Progenitors" protocol)
2. Spin 30 minutes at 1500rpm @ 20° in a Sorvall 6000B rotor (450g)
3. Harvest cells from the interface and collect all supernatant to within ~0.5ml of the pellet
    c. Marrow progenitors will be scattered throughout the Ficoll along with lymphocytes
    d. The pellet will contain left-over RBCs and mature granulocytes
4. Dilute to 50ml in Myeloid Medium. Pellet and count cells. Proceed to spin infection protocol. I like to use around 100,000 to 200,000 cells per spin infection. One gets reliable immortalization at this level. Use myeloid medium throughout derivation of these progenitors.

Note: In this protocol, the Ficoll-buoyant progenitors can be infected directly with retrovirus or pre-expanded in IL-3, IL-6, and SCF 48 hours prior to infection.

Myeloid medium
RPMI1640
10% FBS
1%Pen-Strep-Glut (PSQ, Gibco BRL, Rockville, MD)
20ng/ml GMCSF or 1% culture supernatant from a GM-CSF-producing cell line
1 uM β-estradiol (Sigma).

β-estradiol was kept as 1,000x (1mM) or 10,000x (10mM) stocks in 100% ethanol and stored at -20°C.

XII. Myeoid cell line derivation following retroviral infection

A. All culture following the infection is done in RPMI + 10% FBS + GM-CSF + pen/strep/glutamine

1. When using ER-oncoprotein fusions, be sure to include 1μM ß-estradiol (Sigma E-2758). ß-estradiol can be purchased from Sigma (E-2758). It can be conveniently dissolved at 10mM in 100% ethanol (takes ~30 minutes of end over end at RT). This provides a 10,000x stock solution to be used at a final 1μM in cell culture. When rinsing the cells of ß-estradiol, it is a good idea to rinse 2x in 10ml PBS.

2. We used GM-CSF-conditioned media that contains 1-2ug GM-CSF/ml. We use this conditioned medium at a 1:100 dilution in our final medium, yielding a final GM-CSF concentration of 10-20ng/ml.

B. Derivation procedure:

1. It is a good idea to keep a few wells of "uninfected" or "vector alone" infected cells maintained under the same conditions. Over the course of 2-4 weeks in GM-CSF (or IL-3) containing media, these cells will divide, mature, terminally differentiate to neutrophils and macrophages, and finally die. In 100's of infections, GM-CSF cultures of uninfected or vector alone infected cells have never produced immortalized cells. In the presence of IL-3, however, one can sometimes find the emergence of a very slow growing mast cell population. This is easily differentiated from the rapid emergence of immortalized myeloblasts.

2. Cells should be split to a **new** dish (usually to a 6-well) with fresh media every 3-4 days. All non-adherent cells can be transferred by rather vigorously pipetting the lightly adherent cells off the bottom of the dish. The truly adherent macrophages will not come off no matter how vigorous the pipetting! The cells should <u>not</u> be allowed to become too confluent. This is important as the differentiated monocytes stuck on the bottom of the dish seem to have a pro-differentiative effect on all the other cells in the culture. Furthermore, the presence of fresh GM-CSF is critical in these early stages.

3. The emergence of immortalized cells will appear after about 2-3 weeks as clumps of round and refractile blasts, like little clusters of grapes. It will then become easier to transfer these cells off the differentiated layer of monocytes.

4. Interestingly, in the early stages of culture, the transfer of the immortalized cells to a new dish also seems to have a transient, pro-differentiative effect, maybe due to the mechanical activation of the cells by the pipetting. Thus, the day after the transfer, one often notices many fewer blasts (refractile round morphology) than one actually transferred, and more cells adhering to the plastic. This is normal, and after 24hrs, the cells tend to revert back to their rounded, progenitor-like morphology.

5. Eventually, after 3-4 weeks, all that will be left are the immortalized myeloblasts. These grow very robustly and rapidly and can be cloned by limiting dilution in 96-well plates. Usually, I plate out one 96-well plate at 3 cells/well and one plate at 0.3 cells/well in 200μl of media. After 10-14 days, clones are easily identified and transferred to 24-well plates for characterization.

C. Removing ß-estradiol from progenitors immortalized by ER-Hox oncoproteins

Cells should be rinsed 2x in PBS to get rid of residual ß-estradiol before plating in GM-CSF media without estrogen. Remember, because the "muted" G400V mutant version of the estrogen receptor has been used in all cases within our work published to date (2005), the estrogenic effects of phenol red or residual estrogens in the FBS do not have any effect on the ER domains used in our fusion oncoproteins.

In the absence of estrogen, the cells will undergo between 1-3 cell divisions for ER-Hox progenitors and then terminally differentiate to macrophages (for Hoxb8-ER) or both granulocytes and monocytes (for polyclonal populations of Hoxa9-ER). This usually takes between 2-7 days, depending on the clone and also depending on the cell density. Generally, the less dense the culture, the faster the process of differentiation.

XIII. Freezing & Thawing Myeloid Progenitors

Cells to be frozen should be split the day before and should be very happy. Adherent cells should be about 75-80% confluent while suspension cells should be medium density.

Some general rules apply to freezing cells. Cells should be resuspended gently and thoroughly in cold freezing media and placed on ice for 5-10'. They should then be cooled slowly to -80° (an isopropanol freezing chamber can accomplish this) before transferring to liquid nitrogen.

The freezing media can vary in the concentration of FBS and the concentration of DMSO. Remember, DMSO is the cryopreservative but can also osmotically shock the cells. Some cells are more sensitive than others to its toxic effects. Generally FBS makes up 20-90% and DMSO makes up 5-10% of the freezing media. Most cell lines can be resuspended directly in the FBS/DMSO mixture though some sensitive cells should be resuspended in FBS before the DMSO is added slowly and drop-wise to the cells while mixing.

Protocol

1. Prepare or thaw a mixture of sterile-filtered FBS/20% DMSO. Keep on ice.
2. Pellet cells at 1000rpm (~200g) for 10'. Aspirate media.
3. Resuspend cells in 1/2 volume of media with 10% serum → chill on ice
   i.e. whatever media in which the cells are normally grown
4. Slowly add 1/2 volume of sterile ice-cold 100%FBS/20% DMSO and mix the cells thoroughly and gently.
   note: for some cells, one can lower the final conc. of DMSO to 7.5%
5. Aliquot cells into cryotubes and place on ice for 5'.
6. Place tubes in isopropanol freezing chamber pre-cooled to 4° and place the chamber at -80°.
7. 24-48hrs later, transfer cells to liquid nitrogen.

XIV. Frequently Asked Questions

What mouse strains have been used in this infection protocol? Balb/c and C57Bl/6 mouse strains have all been used with good success. Immortalization of other strains has not been attempted.

Has the number of progenitors used as targets for infection been tittered, and if so, how many are required for reliable immortalization? With a good-titer retrovirus of $2x10^5$-$2x10^6$/ml, which is reproducibly produced from standard transfection conditions, 10,000 progenitors will consistently yield immortalized cultures.

Has retronectin been used in these assays? Is it significantly better than fibronectin? Fibronectin is a good substitute for the much more expensive commercially available Retronectin. Retronectin is merely the fragment of fibronectin which contains the viral binding domain. In side by side comparisons, retronectin does not improve the efficiency of immortalization by more than 2-fold.

My medium turns purple during spinoculation. Is that normal? Yes, during the spinoculation, the media will get quite marginally basic—enough to turn its color purple. This does not affect progenitor viability. Media containing HEPES can be used to maintain a more physiological pH if necessary. HEPES-media can be purchased from Gibco.

Is RPM a significant parameter in the spinoculation procedure? The spinoculation can be done at 1000g up to 4500g without any loss of viability in my hands. The higher the G-force the better the transduction though this is a minimal effect.

During spinoculation, the temperature in the rotor rose to 36 degrees Centigrade. Should I be worried? Although we usually set the temperature parameter for constant temperature of 23 degrees, we have had rotor temperature rise to 36 degrees and have found no ill-effect.

What is the most important parameter during spin-infection? The most important predictor of good transduction efficiency is still the viral titer.

What is the difference between the use of Lipofectamine verses the old standard of polybrene? Lipofectamine is used as a poly-cationic compound at a final 1:1000 dilution. However, we do not believe that there is any significant advantage over using a final 8μg/ml polybrene. Both of these compounds are designed to eliminate the repulsion between the negatively charged virus and the negatively charged cell membrane. Many protocols using fibronectin/retronectin do not also include a poly-cationic compound though we have continued to do so.